on phosphorus. The isomerization is promoted by the development of the strong acidity, >P(O)OH, and is made possible by the tendency of five-membered cyclic phosphates to form the corresponding cyclic oxyphosphoranes.

Supplementary Material Available: Table III, A. Carbon–Hydrogen Bond Distances (Å) and Angles (deg), B. Calculated Hydrogen Atom Positions, and Table IV, Structure Factors (19 pages). Ordering information is given on any current masthead page.

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Reaction of the Neurotoxin Gabaculine with Pyridoxal Phosphate

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Abstract: The naturally occurring neurotoxin gabaculine (5-amino-1,3-cyclohexadienyl carboxylate) is a potent irreversible inhibitor of pyridoxal phosphate linked γ-aminobutyrate (GABA):α-ketoglutarate transaminase. The mechanism of inhibition requires the catalytic turnover of gabaculine to give an activated intermediate which reacts with the holoenzyme. In this report we show that gabaculine itself reacts with pyridoxal phosphate to afford *N-m*-carboxyphenylpyridoxamine phosphate (CPP$_P$). The limiting pseudo-first-order rate constant for its formation at 70 °C is 3.2×10^{-3} min^{-1} and the activation energy for its formation is 24.8 kcal/mol. The mechanism of CPP$_P$ formation involves Schiff base formation between gabaculine and pyridoxal phosphate followed by a rate-limiting transamination reaction. A deuterium isotope effect of 4.26 is exhibited at this step. The activated transaminated intermediate spontaneously aromatizes to afford the highly fluorescent CPP$_P$. This is the first demonstration of a discrete chemical reaction between a naturally occurring irreversible enzyme inhibitor and pyridoxal phosphate.

Gabaculine is a naturally occurring small molecule neurotoxin isolated from *Streptomyces toyocaenis*.[1] When administered to animals it causes a dramatic increase in brain levels of the inhibitory neurotransmitter γ-aminobutyric acid (GABA). We have found that gabaculine irreversibly inhibits pyridoxal phosphate linked γ-aminobutyric acid (GABA): α-ketoglutaric acid transaminase.[2] This molecule is in a class of highly specific irreversible enzyme inhibitors that require
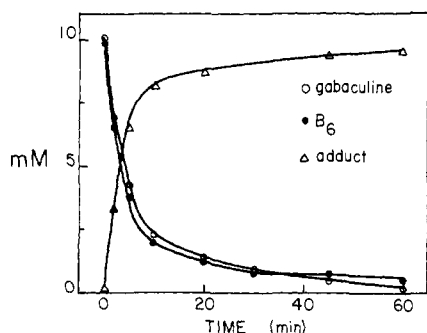
Figure 1. The reaction of gabaculine with equimolar amounts of pyridoxal phosphate. Aliquots (0.1 mL) of a 0.2 M potassium phosphate buffer (pH 7.0 at 25 °C) containing 10 mM gabaculine and 10 mM pyridoxal phosphate were placed in 1-mL screw cap Pyrex test tubes. The individual tubes were saturated with nitrogen, capped, and wrapped in aluminum foil. The tubes were placed in a boiling water bath and at the indicated times the test tubes were removed and the concentrations of gabaculine, pyridoxal phosphate, and CPP$_P$ were determined. B$_6$ refers to pyridoxal phosphate.
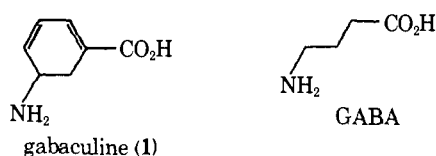
gabaculine (1)   GABA

catalytic activation by the target enzyme prior to the inactivation step.[3] The scheme shown below accounts for the behavior of these inhibitors:

$$E + I \underset{}{\overset{K_1}{\rightleftharpoons}} E \cdot I \xrightarrow{k_{cat}} E \cdot I' \xrightarrow{k_{inh}} E\text{-}I'$$
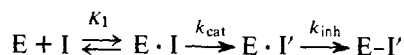
Gabaculine inhibits mammalian GABA transaminase with a K_1 of 5.87×10^{-7} M and $k_{cat} = 1.15 \times 10^{-3}$ s^{-1} at 15 °C.[2] The conclusion that catalytic conversion of this inhibitor necessarily precedes the observed inhibition is based on the following kind of evidence. (1) Gabaculine is only an irreversible inhibitor of the enzyme when the cofactor is in the pyridoxal form. (2) There is a deuterium isotope effect on the rate of the inhibition when a deuterium is incorporated into the 5 position of gabaculine. (3) The pH vs. rate of inhibition profile is similar to that of substrate turnover and (4) external nucleophilic trapping agents such as mercaptoethanol have no effect on the rate of inactivation. Mechanistic considerations led us to propose that the mode of action of this inhibitor involves one of the following choices shown in Scheme I. To further elucidate the mechanism of action of gabaculine we have studied its reaction with pyridoxal phosphate, the natural cofactor of GABA transaminase. In this article we show that a smooth reaction ensues between pyridoxal phosphate and gabaculine to afford a single major product. This product is 5 which is identical with the borohydride reduction product of the Schiff base formed between *m*-anthranilic acid and pyridoxal phosphate.

Experimental Section

Gabaculine was synthesized by the synthetic scheme outlined by Mishima et al.[1] We are indebted to Dr. Mishima for providing us with details of the synthesis. 4,5-Dideuteriogabaculine was synthesized by this route starting with 4,5-dideuteriobutadiene.[4] The product melted at 194-195 °C and NMR analysis showed that there was 90% deuterium at the 5 position. The ultraviolet spectrum of this material exhibited the same λ_{max} and log ϵ as that of gabaculine.[1] 4,5-Dideuteriogabaculine was used in the isotope studies instead of 5-deuteriogabaculine because there is no easy way to synthesize this latter material. Pyridoxal phosphate was a product of the Sigma Chemical Co. and *m*-anthranilic acid was from the Aldrich Co. *N*-*m*-Carboxyphenylpyridoxamine phosphate (CPP$_P$) was prepared by reducing the Schiff base formed between *m*-anthranilic acid and pyridoxal

Scheme I
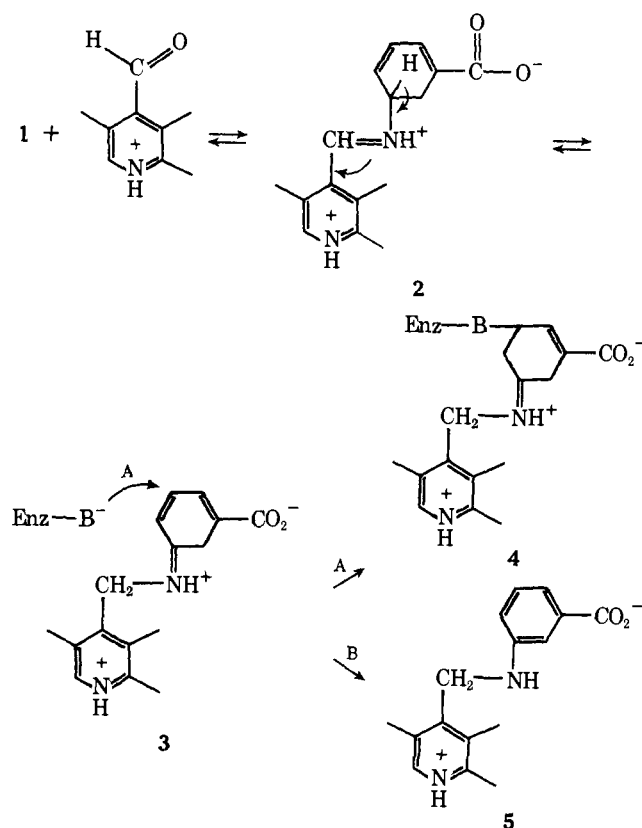
phosphate. The method used was identical with that published for the general synthesis of reduced Schiff bases between amino acids and pyridoxal phosphate.[5] The yield of CPP$_P$ was 30% after purification. It behaved as a single spot on silica gel thin-layer plates developed with BuOH-H$_2$O-HOAc (2:2:1) and when analyzed by paper electrophoresis using either formic-acetic acid buffer (pH 1.8) (25 mL of formic acid + 80 mL of acetic acid per L) or pyridine-acetic acid buffer (pH 3.1) (5 mL of pyridine + 100 mL of acetic acid per L). The ultraviolet and fluorescence excitation and emission spectra for CPP$_P$ are given in the figure legends. Anal. Calcd for C$_{15}$H$_{17}$N$_2$O$_7$·H$_2$O: C, 50.70; H, 5.35; N, 4.88. Found: C, 50.78; H, 5.30; N, 7.73. Thin-layer chromatographic separations were done on EM-HP-254-silica gel plates. Electrophoretic separations were achieved in a Durrum cell (Beckman Instruments Inc.). Ultraviolet spectra were recorded on a Cary 118 recording spectrophotometer and fluorescence measurements were made on a Hitachi-Perkin-Elmer Model 512 fluorometer.

Transamination reactions were carried out in a manner similar to those described by Metzler and Snell.[6] Incubations were done in 0.1 M potassium phosphate (pH 7.0). The mixtures of gabaculine and pyridoxal phosphate in the buffer were mixed at room temperature, flushed thoroughly with N$_2$, capped, and covered with aluminum foil before heating for the indicated times in a boiling water bath (100 °C) or in a Dubnoff metabolic shaking bath. Electrophoretic separation of the reaction mixture at different times indicated that a single new major compound formed which was highly fluorescent. Minor decomposition products of gabaculine also formed accounting for ~10% of the starting concentrations. The decomposition products proved to be benzoic acid and *m*-anthranilic acid. The major new product that formed was identical with CPP$_P$ by various analytical tests (chromatographic behavior, TLC, paper electrophoresis) and spectral correlates (UV and fluorescence). During the kinetic runs the remaining pyridoxal concentration was determined by the phenylhydrazine technique.[7] None of the other components in the reaction mixture interfered with this determination. The remaining gabaculine was determined by the ninhydrin assay and the remaining optical density at 275 nm (log ϵ = 3.93[1]). None of the other components interfered with the ninhydrin assay. The amount of CPP$_P$ forming with time was determined by removing aliquots from the reaction mixture and measuring the optical density at 320 nm. In 0.1 M potassium phosphate at pH 7.0 CPP$_P$ shows λ_{max} at 320 nm (log ϵ = 3.74) and a shoulder at 288 nm.
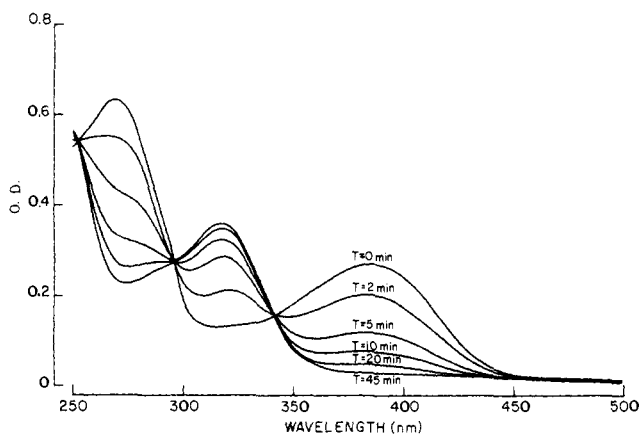
Figure 2. Ultraviolet spectral changes accompanying the reaction of gabaculine and pyridoxal phosphate. This experiment was run identically with the one above; at the indicated times the ultraviolet spectrum of the content of each test tube was determined. Gabaculine itself has λ_{max} at 275 nm and log ϵ = 3.93.

Results

(1) The Reaction of an Equimolar Amount of Gabaculine with Pyridoxal Phosphate. When gabaculine and pyridoxal phosphate are heated together (100 °C) at pH 7.0 in a 0.1 M potassium phosphate buffer the starting materials were rapidly consumed and a single new major fluorescent compound was formed (Figure 1). Both gabaculine and pyridoxal phosphate are relatively stable to the conditions of the incubations when treated independently. After a 1-h incubation pyridoxal phosphate and gabaculine were destroyed to an extent of 10 and 20%, respectively. Benzoic acid was the major decomposition product of gabaculine.

(2) The Ultraviolet Spectral Changes Accompanying the Reaction. The ultraviolet spectra of the incubation mixture described in Figure 1 were recorded with time upon incubation at 100 °C. As can be seen in Figure 2, the gabaculine (λ_{max} at 275 nm) and the pyridoxal phosphate-anthranilic acid Schiff base (λ_{max} at 390 nm) were destroyed and a new peak was produced with a λ_{max} at 320 nm and a shoulder at 288 nm. These latter peaks represent the formation of the adduct.

(3) The Identification of the Gabaculine-Pyridoxal Phosphate Adduct. When m-anthranilic acid and pyridoxal phosphate were mixed together and then reduced with sodium borohydride, a single new highly fluorescent compound was formed. This compound cochromatographed with the gabaculine-pyridoxal phosphate adduct. The R_f values on silica gel thin-layer plates in BuOH-H₂O-HOAC at 2:1:1 and 4:1:1
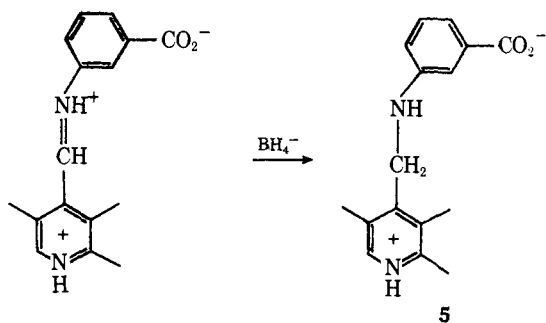
were identical. In addition, the two compounds chromatographed identically on paper electrophoresis in an HOAc-formic acid buffer. More importantly, however, is the fact that the ultraviolet, fluorescence, and NMR spectra of the two compounds were also identical. Figure 3 shows the ultraviolet spectra of the adduct and Figure 4 shows its fluorescence excitation and emission spectra.
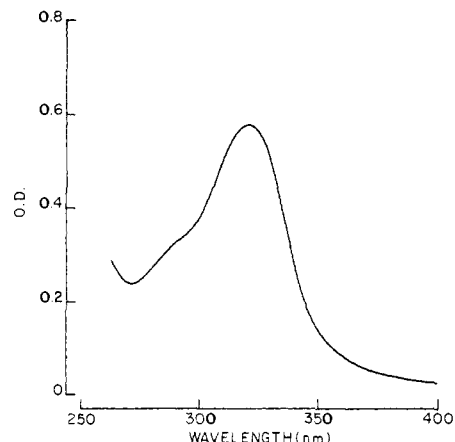

Figure 3. The ultraviolet spectrum of CPP$_P$. The ultraviolet spectrum of a 1.07 × 10⁻⁴ M solution of CPP$_P$ in 0.2 M potassium phosphate (pH 7.0) was recorded. The λ_{max} is at 320 nm with log ϵ = 3.74.
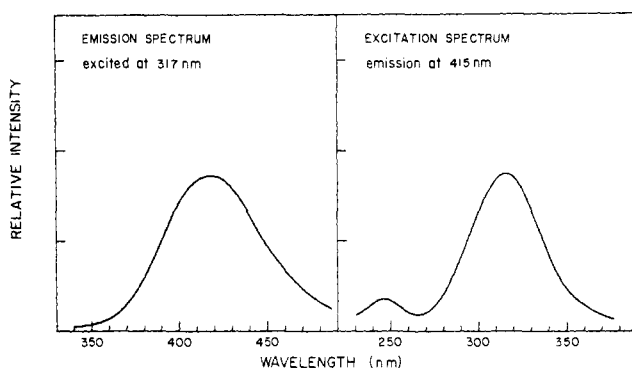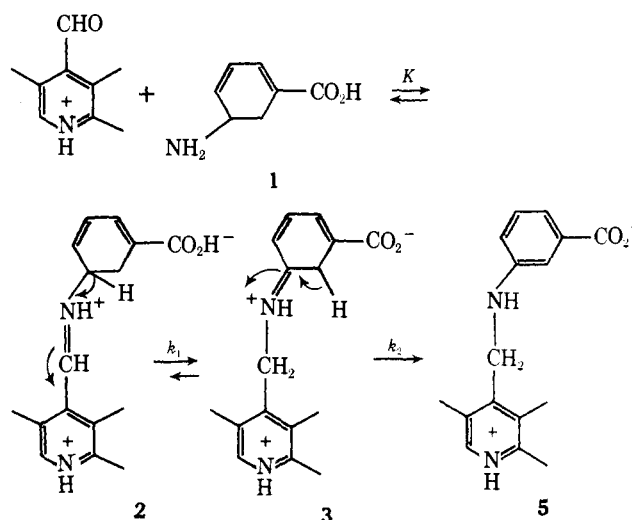

Figure 4. The fluorescence excitation and emission spectra of CPP$_P$. A 5 × 10⁻⁶ M solution of CPP$_P$ on the above buffer was made up. The excitation and emission spectra were determined 1-cm pathlength cells. The spectra are given as recorded and are uncorrected.

Scheme II

(4) The Effect of the Gabaculine Concentrations on the Rate of CPP$_P$ Formation. If the CPP$_P$ is formed from gabaculine and pyridoxal phosphate by the mechanism shown in Scheme II, then the rate of CPP$_P$ formation should level off at high concentrations of gabaculine relative to pyridoxal phosphate. That is, Michaelis–Menten kinetics should be observed. This occurs because a Schiff base complex between gabaculine and pyridoxal phosphate must occur prior to further chemical reactions. This expectation was realized, as shown in Figure 5.
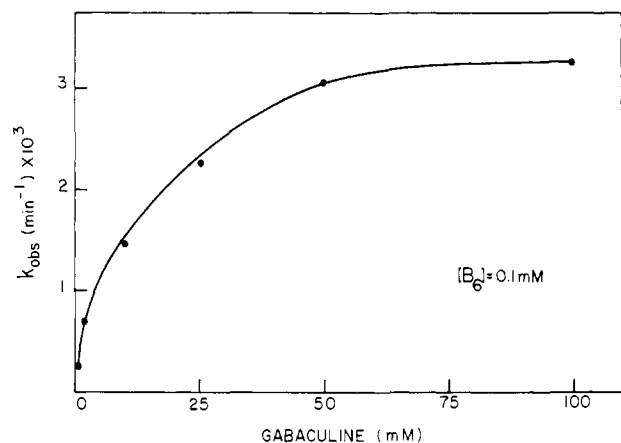
Figure 5. The pseudo-first-order rates of reaction of gabaculine with pyridoxal phosphate. These experiments were run similarly to those run in Figure 1 except that the starting concentration of pyridoxal phosphate was 0.1 mM and the concentrations of gabaculine used were 1, 3, 10, 25, 50, and 100 mM. In addition the temperature was maintained at 70 °C. The kinetics of the reactions were followed by measuring the amount of pyridoxal phosphate remaining by the phenylhydrazine method.[7] Rates of the reactions were determined at each concentration and the $t_{1/2}$ values determined. The pseudo-first-order rate constants were determined from $k_{obsd} = 0.693/t_{1/2}$. In this figure pyridoxal phosphate is referred to as B_6.
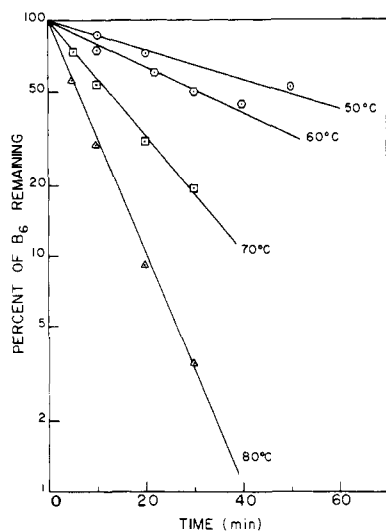

Figure 6. The effect of temperature on the pseudo-first-order rate of the reaction of gabaculine with pyridoxal phosphate. These experiments were conducted with pyridoxal phosphate at 0.1 mM and gabaculine at 100 mM. For each temperature the rates of disappearance of pyridoxal phosphate were measured. The reactions were conducted at 50, 60, 70, and 80 °C.

Pseudo-first-order rate constants for the rate of disappearance of pyridoxal phosphate at 70 °C have been plotted vs. gabaculine concentration. The lowest gabaculine concentration plotted is 10 times that of pyridoxal phosphate and the highest is 10^3 times. At 70 °C the limiting $k_{obsd} = 3.2 \times 10^{-3}$ min^{-1}.

(5) Temperature Effect on Pseudo-First-Order Rate of Adduct Formation. The pseudo-first-order rate of decline of pyridoxal phosphate measured at different temperatures is shown in Figure 6. The Arrhenius plot from these data is shown in Figure 7. The activation energy for the conversion is calculated to be 24.8 kcal/mol.

(6) Deuterium Isotope Effect on the Rate of Adduct Formation. If the rate-limiting step is the abstraction of the C-5-H bond, then there ought to be a deuterium isotope effect on the
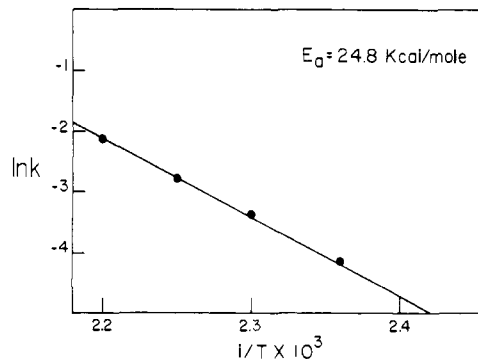

Figure 7. Arrhenius plot for the pseudo-first-order reaction of pyridoxal phosphate and gabaculine. From the previous experiment the k_{obsd} values were calculated. These values were plotted in the usual way to give the plot shown in this figure. The slope of the line gives $-E_a/R$ and E_a calculates out to equal 24.8 kcal/mol.
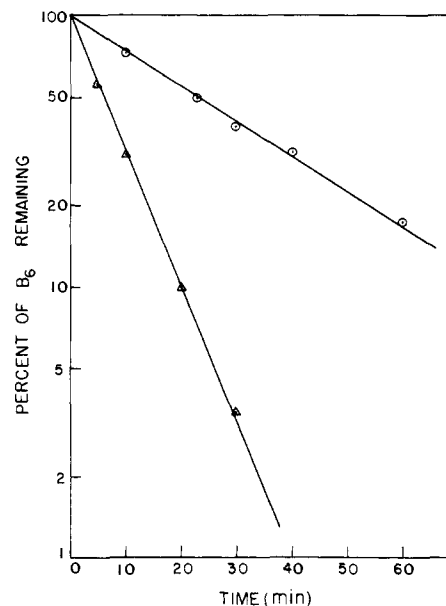

Figure 8. Deuterium isotope effect on the rate of adduct formation. Gabaculine and 4,5-dideuteriogabaculine (both at 10 mM) were heated together (80%) with pyridoxal phosphate at 0.1 mM. At the indicated times the pseudo-first-order rates of pyridoxal phosphate consumption were determined in the presence of gabaculine (△) and dideuteriogabaculine (○). Knowing that 90% of the 5 position of dideuteriogabaculine contains deuterium the k_H/k_D calculates out to be 4.26.

rate of adduct formation with 5-deuteriogabaculine. 4,5-Dideuteriogabaculine was reacted with pyridoxal phosphate under pseudo-first-order conditions and the rate of adduct formation was measured. As can be seen in Figure 8, this rate is depressed by a factor of 4.26 compared to the rate with gabaculine.

(7) The Effect of Phosphate Ion Concentration on the Rate of Adduct Formation. The rate-limiting abstraction of the γ-C-H bond should be buffer catalyzed. A twofold rate enhancement is observed when the phosphate concentration is increased from 0.1 to 1 M at constant ionic strength (Figure 9).

Discussion

Snell and coworkers have shown that many pyridoxal phosphate cocatalyzed reactions can be reproduced in the test tube under appropriate conditions without the enzyme.[6] For example, several α-amino acids are transaminated when heated to 100 °C with pyridoxal phosphate and catalytic amounts of metal ion (Cu^{2+}, Al^{3+}) are added. We have used this model
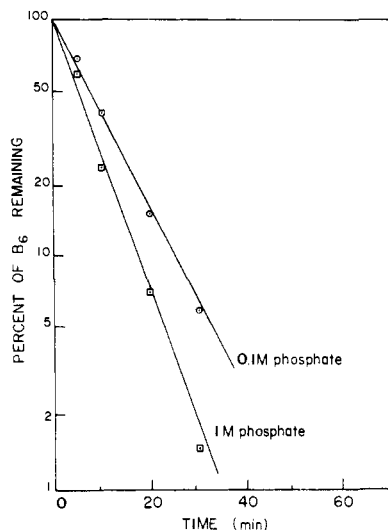
Figure 9. Buffer catalysis on the rate of the reaction. At 70 °C the rates of the reaction between pyridoxal phosphate at 0.1 mM and gabaculine at 100 mM were measured in 0.1 and 1 M potassium phosphate buffer at pH 7.0. The ionic strength was maintained the same in both cases by the addition of potassium chloride. The rates of the reactions were determined by following the rate of disappearance of pyridoxal phosphate with phenylhydrazine.[7]

system approach to study the interaction of gabaculine with pyridoxal phosphate in the expectation that these studies would shed light on the mechanism of the inhibition of GABA transaminase by this molecule.

Upon heating gabaculine with pyridoxal phosphate in 0.1 M potassium phosphate at pH 7.0, a single major product is formed. Metal ions are not required for this conversion and, in fact, they retard the reaction. Minor amounts of anthranilic acid and benzoic acid are also found (~10% of total) but they most probably arise via the decomposition of gabaculine. The major product is highly fluorescent and is identical with the reduction product of the Schiff base formed between anthranilic acid and pyridoxal phosphate. The structure of this adduct is clearly that of CPP$_P$ 5. This compound reasonably arises via a direct transamination reaction followed by tautomerization to yield the fully aromatic product. Our evidence for this sequence of events is as follows: The pseudo-first-order rate of CPP$_P$ formation using excess gabaculine levels off at higher concentrations of the amino acid. The rate-limiting step itself is the cleavage of the C-5-H bond. The deuterium isotope effect (k_H/k_D) for this cleavage proved to be 4.26. This step is sensitive to buffer catalysis as shown by the twofold rate enhancement observed with increasing P$_i$ at constant ionic strength. The measured activation energy for the transamination reaction is 24.8 kcal/mol. It should be noted that GABA itself will not undergo transamination at all under the conditions used in the gabaculine experiments. The increased activity of the C-5-H bond in gabaculine relative to GABA must be the determining factor here. Gabaculine can be viewed as being a vinylogue of an α-amino acid. The aromatization of 3 to afford CPP$_P$ must be a facile process according to our scheme. This reaction would be expected to be highly exothermic, as it gives rise to an aromatic product. To the extent that the stability of the product is reflected in the transition state the activation energy for this conversion should be relatively small. A full mechanistic analysis of the overall formation of 5 will be undertaken with a simplified and more stable analogue of pyridoxal phosphate, such as 3-hydroxypyridine-4-aldehyde.[8]

The relatively large energy of activation for the reaction of

gabaculine and pyridoxal phosphate means, of course, that gabaculine is not a general pyridoxal phosphate antagonist. In solution at 37 °C the reaction between the drug and pyridoxal phosphate is not observable. Along these lines, we have not found gabaculine to inhibit any other pyridoxal phosphate containing enzymes studied.[9] Among those tested were glutamate decarboxylase, ornithine decarboxylase, aspartate aminotransferase, and alanine aminotransferase.

Although the reaction of gabaculine with pyridoxal phosphate is of interest in its own right because it represents the first example of a naturally occurring toxin reacting with this cofactor in a discrete way, it might be queried whether this process is at all relevant to the observed inhibition of GABA transaminase. Although experiments to definitely decide this point are still in progress, preliminary experiments suggest that this may be the case. For example, although gabaculine is an exceedingly potent irreversible inhibitor of bacterial GABA transaminase, 1,2-dehydrogabaculine 6 is inert.[9] This molecule
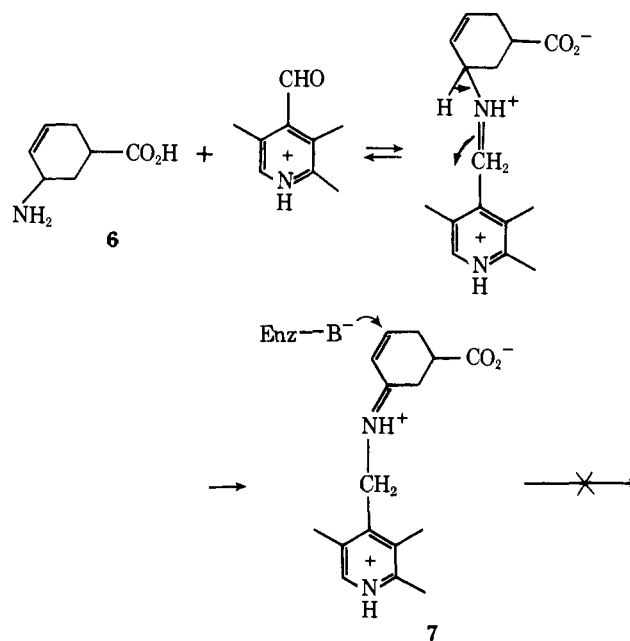
can form an activated Michael acceptor 7 but, of course, cannot aromatize. If a Michael reaction were important in the gabaculine case, we would have expected 6 to be a potent irreversible inhibitor of the enzyme.[10]

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(10) NOTE ADDED IN PROOF. We have now found that the inactivation of bacterial GABA transaminase by gabaculine can be fully accounted for by the enzyme catalyzed formation of CPP$_P$ (R. R. Rando, submitted for publication).