

Different Modes of Action of Inhibitors of Bacterial D-Amino Acid Transaminase

A TARGET ENZYME FOR THE DESIGN OF NEW ANTIBACTERIAL AGENTS*

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D-Amino acid transaminase from *Bacillus sphaericus* shows a deuterium kinetic isotope effect (V_H/V_D) between 2 and 3 in the transamination of α -protio- or α -deuterio-D-alanine and α -ketoglutarate, suggesting that α -proton abstraction is at least partially rate-limiting for this reaction. This transaminase also catalyzes a β -elimination reaction with substrates such as β -fluoroalanine with no detectable deuterium isotope effect ($V_H/V_D = 1$). These results, taken together with previous work (Soper, T. S., and Manning, J. M. (1978) *Biochemistry* 17, 3377-3384) suggest that the rate-limiting step in the β -elimination reaction is solvolysis of an α -aminoacrylate-pyridoxal-P Schiff's base intermediate.

D-Cycloserine is an active site titrant of D-amino acid transaminase. Inactivation by cycloserine can be completely reversed by dialysis against pyridoxal phosphate at neutral pH. Gabaculine is also an efficient inhibitor of this enzyme and possesses some antibacterial activity. The latter two inhibitors probably act by sequestration of the coenzyme rather than by alkylation of the protein as with the β -halo derivatives of D-alanine.

A number of antibacterial agents are thought to act by inhibiting specifically certain key enzymes involved in the biosynthesis of the peptidoglycan layer of the bacterial cell wall. This layer, which consists of long carbohydrate chains of alternating N-acetylglucosamine and N-acetylmuramic acid residues cross-linked by short peptide bridges, is the cells' primary defense against osmotic lysis. The penicillins and the cephalosporins prevent cross-linking of the peptide bridges (1). D-Cycloserine has been shown to inhibit alanine racemase (EC 5.1.1.1), which catalyzes the synthesis of the D-alanine found in the peptide cross-links (2). In addition, D-cycloserine also inhibits D-alanyl-D-alanine ligase (3) (EC 6.3.2.4). More recently, β -chloro-D-alanine has been shown to inhibit bacterial growth. This compound acts, in part, by inhibiting alanine racemase (4-6) thus creating a conditional D-alanine auxotrophy (4).

The peptide bridges that cross-link the carbohydrate chains

also contain D-glutamate (1) in addition to D-alanine. In *Bacillus*, D-glutamate is not formed by racemization of L-glutamate, since glutamate racemase is apparently absent in this genus (7, 8), although this enzyme has been described in one species of *Lactobacillus* (9). D-Glutamate is usually formed by the transamination of D-alanine and α -ketoglutarate catalyzed by D-amino acid transaminase (EC 2.6.1.21). This enzyme was first described by Thorne *et al.* (7) in extracts of *B. subtilis*. β -Chloro-D-alanine is an effective inactivator of this transaminase and we have suggested that the antibacterial action of this β -haloamino acid is due to the dual inhibition of both D-amino acid transaminase and alanine racemase (4, 10, 11).

Since D-amino acid transaminase appears to be another key enzyme in the biosynthesis of the peptidoglycan layer of the bacterial cell wall, we have investigated the effects of a number of different inhibitors of this enzyme; potent inhibitors could perhaps be developed into a clinically useful antibacterial agents. Of the compounds studied here, D-cycloserine is a known antibacterial agent, the β -halo-D-amino acids are currently at the developmental stage, and gabaculine has not been previously considered as an antibacterial agent.

EXPERIMENTAL PROCEDURES

Materials—Lactate dehydrogenase, D-amino acid oxidase, D-alanine, D-aspartate, L-aspartate, α -ketoglutarate, pyridoxal-P, D-cysteine, β -fluoropyruvate, D-alanine methyl ester, D-cycloserine, and α -aminoethylphosphonic acid were obtained from Sigma. L-Cysteine and DL-gabaculine were from Calbiochem. D-Glutamate was from Vega and alaninol was from Aldrich. L-Alanine and L-glutamate had been prepared previously in this laboratory and were pure by elemental analysis.

The protio- and deuterio-derivatives of β -fluoroalanine were synthesized essentially as described by Dolling *et al.* (12); β -fluoropyruvate was dissolved in concentrated NH_4OH and reduced with sodium borohydride or sodium borodeuteride to prepare the desired products. The fluoroamino acids were purified on AG50-X8 (Bio-Rad) with a linear gradient from 0 to 1 M HCl. The amount of fluoroamino acid was estimated by amino acid analysis on a column (0.9 \times 60 cm) of AA-15 resin; β -fluoroalanine was eluted by 50 ml of 0.2 N sodium citrate, pH 3.25. The protio and deuterio derivatives of alanine were prepared from pyruvate by the same procedure.

Bacillus sphaericus strain ATCC 14577 was obtained from the American Type Culture Collection. *Staphylococcus epidermidis* was obtained from Ward's Natural Science Establishment, Inc., Rochester, NY, and *Salmonella typhimurium* strain LT-2 was a gift of Dr. G. Kohlhaw, Purdue University.

D-Amino acid transaminase, purified as described previously (10), was assayed by the production of pyruvate from D-alanine and α -ketoglutarate with an assay system coupled to lactate dehydrogenase described previously (10).

Inactivation Kinetics—Enzyme was incubated with inhibitor at room temperature. When the inactivation required 20 to 30 min to obtain significant absorbance changes, aliquots were removed every 5 to 10 min and diluted at least 1:20 into the assay mixture. The assay with lactate dehydrogenase was followed for at least 3 min to ensure

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This manuscript is dedicated to Professor A. Rossi Fanelli on the occasion of his 75th birthday.

linearity; less than 0.4% of available substrate was consumed. When inactivation was rapid and short time periods were required, aliquots were removed and diluted with assay mixture without one substrate, usually α -ketoglutarate. The excess of D-alanine prevented further inactivation for at least 1 h. At the time of assay, α -ketoglutarate was added and assayed as described above. The data were plotted as a semilog function of time. The slope of this plot yields the pseudo-first order rate constant for inactivation.

Bacterial Extracts—*B. sphaericus* was grown to early stationary phase in the rich medium described by Soda *et al.* (13) and harvested by centrifugation; the cells were washed with physiological saline and the cell paste was stored frozen. Extracts were prepared by suspending the cells in 50 mM potassium phosphate buffer, pH 8.5, 1 mM in dithiothreitol (1 g wet weight cells/4 ml buffer). Cells were broken by sonication with a Branson Sonifier Cell Disruptor model 125 and cell debris was removed by centrifugation at $34,800 \times g$ for 20 min. The crude extract contained about 20 mg/ml of protein as determined by A_{280} .

Enzyme Assays in Crude Extracts—To test the effects of an inhibitor on some of the enzymes of *B. sphaericus* that metabolize alanine, a single set of experimental conditions was selected to assay the following pyridoxal-P enzymes: D-amino acid transaminase, alanine racemase, and L-alanine transaminase. The flavin-dependent enzymes, D-amino acid oxidase, and L-amino acid oxidase, were also assayed. The assay measured the production of pyruvate with salicylaldehyde from either D- or L-alanine. All assays were performed in 0.4 M potassium phosphate buffer, pH 8.5, 1 mM in dithiothreitol and 1 mM in pyridoxal-P. Because α -ketoglutarate reacts weakly with salicylaldehyde, the transaminase assays required a separate blank containing α -ketoglutarate, but the blank for the remaining enzyme assays did not. For all assays, the final substrate concentrations were 100 mM. The substrates used and the enzymes assayed under each set of conditions were: D-alanine and α -ketoglutarate for both D-amino acid transaminase and D-amino acid oxidase, L-alanine and α -ketoglutarate for both L-alanine transaminase and L-amino acid oxidase, L-alanine, and D-amino acid oxidase (10 μ g/ml) for alanine racemase, D-alanine for D-amino acid oxidase, and L-alanine for L-amino acid oxidase. Approximately 1 mg of protein from the crude extract was added and incubated at 37 °C for 20 min to get reproducible values for the pyridoxal-P enzymes. The activities of D- and L-amino acid oxidase were low.

Inhibition of Bacterial Growth—Tubes with 5 ml of culture media (nutrient broth, Difco) were inoculated with 0.05 ml of an overnight culture of bacteria and incubated. Upon entering the early log phase of growth, inhibitor was added. Bacterial growth was followed by measuring turbidity at either A_{660} or A_{540} .

Minimal inhibitory concentrations were measured in a standard 2-fold dilution series of from 256 μ g/ml to 4 μ g/ml or from 64 μ g/ml to 0.125 μ g/ml (14). Overnight cultures of the organism to be tested were diluted to A_{540} of 0.3 with the same medium. The diluted culture was used to inoculate the dilution series (0.05 ml/ml) and the tubes read 6 to 10 h after inoculation. Bacteria that grew in the highest concentration of inhibitor used were judged to be resistant.

RESULTS AND DISCUSSION

Reversible Inhibition by Analogs of D-Alanine—Perhaps the simplest type of inhibitor results from minor modifications of a substrate which alters the ability of the enzyme to interact efficiently with the substrate and thus reduce catalytic turnover. For transaminases the carboxyl group of the substrate falls into this category. Thus, α -aminoethylphosphonic acid is a good competitive inhibitor of D-amino acid transaminase ($K_i = 1$ mM).

Blockage of the charge on the carboxyl group with a methyl group results in a less potent inhibitor ($K_i = 65$ mM). There will still be some ionic character to the ester group which is consistent with the fact that alanine methyl ester is a better inhibitor than alaninol ($K_i > 90$ mM). These results suggest that a charged carboxyl group is important in the transamination of D-amino acids catalyzed by this enzyme. Similar conclusions have been made in related studies on glutamate-aspartate transaminase (15).

Martinez-Carrion and Jenkins (16) indicated that D-amino acid transaminase purified from *B. subtilis* was inhibited by

the D- and L-isomers of cysteine. However, Yonaha *et al.* (17) found that the transaminase from *B. sphaericus* strain IFO 3525 was not inhibited by either isomer of cysteine. We find that the transaminase purified from *B. sphaericus* strain ATCC 14577, is inhibited by both isomers of cysteine. D-Cysteine is a very potent competitive inhibitor ($K_i = 20$ μ M) with respect to D-alanine. L-Cysteine is also a good inhibitor with very tight binding ($K_i = 4$ mM) especially for an L-amino acid. In comparison we have estimated that the L-isomer of alanine has a K_i of 300 mM (10). We observed this inhibition by cysteine only in the presence of a vast excess of dithiothreitol and for relatively short times. After about 5 to 10 min, the inhibition with either D- or L-cysteine was no longer present due to the nonenzymatic disappearance of cysteine; cystine was not inhibitory. The reason that Yonaha *et al.* (17) were not able to detect inhibition by cysteine was probably due to the fact that cysteine rapidly disappears from solution under the basic conditions employed to assay this enzyme.

For a competitive inhibitor to be successful as an antibacterial agent, a sustained high concentration of the compound must be present. Since D-cysteine is readily oxidized, it is unlikely that enough of it would be present to inhibit bacterial growth effectively. Indeed, we were not able to detect any inhibition of bacterial growth by D-cysteine. α -Aminoethylphosphonic acid is an effective competitive inhibitor with a K_i (1 mM) below the K_m values of good D-amino acid substrates for the enzyme (10). For effective inhibition of D-glutamate synthesis, the required amount of α -aminoethylphosphonic acid must be 5- to 10-fold greater than the K_i to achieve 83% and 91% inhibition, respectively, assuming saturation of the enzyme by substrates (18); such concentrations are not likely attainable within the bacterial cell. Indeed, this compound is not an antibacterial agent (19).

Deuterium Kinetic Isotope Effects in the Transamination and β -Elimination Reactions Catalyzed by D-Amino Acid Transaminase—D-Amino acid transaminase shows a V_{max} kinetic isotope effect, V_H/V_D , between 2 and 3 for the α -protio and α -deuterio derivatives of D-alanine (Fig. 1). This result suggests that the strength of the carbon-deuterium bond compared with the carbon-proton bond results in alteration of the overall rate-limiting step(s) in the transamination of D-amino acids. There is no apparent effect on the K_m for D-alanine. Theoretical calculations indicate that a kinetic isotope effect greater than 8 is required for a given reaction in a pathway to be the sole rate-limiting step (20). Therefore, some other steps in the reaction must be as slow as α -proton removal. It should be noted that several other L-amino acid-specific, pyridoxal-P-requiring enzymes that catalyze α -proton abstraction also show similar kinetic isotope effects (21–25).

D-Amino acid transaminase catalyzes a β -elimination reaction with substrates having halo substituents at the β -carbon (10). This enzyme catalyzes the β -elimination of α -deuterio- β -fluoroalanine at a rate nearly identical with that of the α -protio derivative (Fig. 2), a result which indicates that there is no detectable kinetic isotope effect for the β -elimination reaction. Thus, while α -proton abstraction is at least partially rate-limiting for transamination, this step must be fast relative to the slowest step in the β -elimination reaction catalyzed by this enzyme.

Inactivation during β -Elimination of β -Substituted Alanine Derivatives—In previous studies we have shown that D-amino acid transaminase catalyzes a unimolecular β -elimination of β -chloro-D-alanine (10) in which an α -aminoacrylate Schiff's base with pyridoxal-P is a key intermediate (26). Concomitant with the hydrolysis of this intermediate to form pyruvate and ammonia, the enzyme is progressively inactivated; about 1 molecule of transaminase is inactivated for

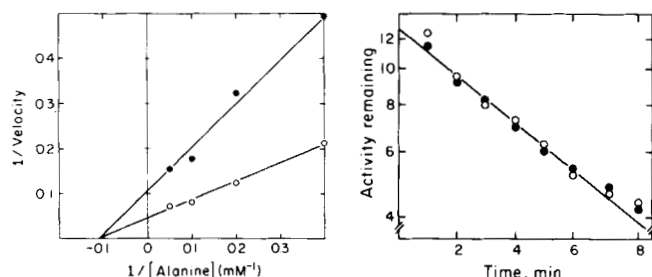


FIG. 1 (left). Deuterium isotope effect on the transamination of D-alanine and α -ketoglutarate. Pyruvate production from α -protio-DL-alanine (○) or α -deuterio-DL-alanine (●) with 33 mM α -ketoglutarate was measured with an assay coupled to lactate dehydrogenase. Each assay contained 2 μ g/ml of D-amino acid transaminase in 0.3 M potassium phosphate, pH 8.5. The data are presented as double reciprocal plots. The units for reciprocal velocity are (nanomoles of pyruvate produced)⁻¹, min.

FIG. 2 (right). Deuterium isotope effect on the β -elimination of β -fluoro-DL-alanine. Pyruvate production from 8 mM α -protio- β -fluoro-DL-alanine (○) or from 7.5 mM α -deuterio- β -fluoro-DL-alanine (●) was followed with the lactate dehydrogenase assay. At the indicated times, a tangent to the curve was drawn; this value represents the rate of production of pyruvate at that time. Both samples contained 18 μ g of D-amino acid transaminase in 0.3 M potassium phosphate, pH 8.5. The data are presented as a semilog plot of the residual activity against time. The units of activity are (nanomoles of pyruvate produced), min⁻¹.

every 1500 turnovers at pH 8.5 (10). This intermediate may be trapped by adding low molecular weight thiols, which diffuse into the active site and stereospecifically add to the double bond of this intermediate preventing inactivation (26).

β -Bromo-D-alanine is also a substrate for this β -elimination reaction with nearly identical kinetic behavior as for the β -chloro derivative (26). β -Fluoro-D-alanine is also a potent inhibitor of D-amino acid transaminase. This substrate undergoes the same β -elimination reaction as does the β -chloro (10) and β -bromo derivatives (26) of D-alanine. The initial rates of pyruvate production from β -fluoro- and β -chloroalanine are identical, as are the respective partition ratio of turnover to inactivation. Fig. 3A shows a Dixon plot of the competitive inhibition between β -fluoroalanine and D-alanine. The K_i for β -fluoroalanine is approximately 20 μ M, very similar to that for chloroalanine (10 μ M) (10) and for β -bromoalanine (35 μ M) (26). Fig. 3B shows weak uncompetitive inhibition between β -fluoroalanine and α -ketoglutarate, which is also very similar to that observed with chloroalanine (10).

Unlike β -chloro-D-alanine (10), β -fluoro-D-alanine inhibits bacterial growth only under certain conditions. The fluoro derivative displays a phenomenon termed "self reversal" (27) in which growth inhibition is observed only at a low concentration of the compound; at higher concentrations β -fluoroalanine is apparently incorporated into the bacterial cell wall in place of D-alanine. Nevertheless, β -fluoroalanine acts as a broad spectrum antibacterial agent in a synergistic manner in combination with cycloserine (27).

Since the initial rate of β -elimination, the partition ratio of turnover to inactivation, and the relative binding (as measured by the K_i) are nearly identical for β -fluoro-, β -chloro-, and β -bromo-D-alanine, the nucleophilicity of the leaving group is probably not important in determining the overall rate-limiting step in the β -elimination of these β -halo derivatives of D-alanine.

Earlier, we demonstrated that the β -elimination and transamination reactions of this enzyme have distinct pH dependent rate-limiting steps (28). The maximum velocities for these reactions also differ greatly (10) since the rate of pyruvate formation from β -chloro-D-alanine is only 10% the rate of

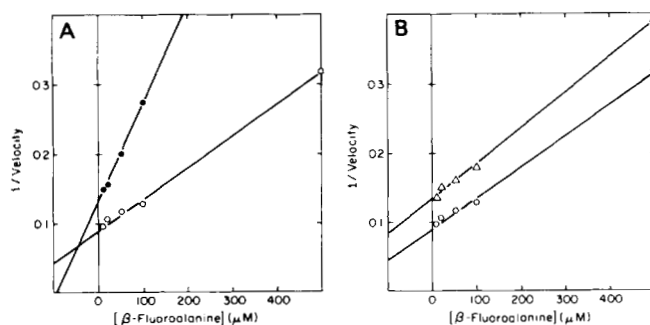


FIG. 3. Inhibition of D-amino acid transaminase by β -fluoro-DL-alanine. A, Competitive inhibition between β -fluoroalanine and D-alanine. D-Amino acid transaminase (1.5 μ g) was mixed with 0.3 M potassium phosphate pH 8.5, 25 mM α -ketoglutarate, 5 mM (●) or 25 mM (○) D-alanine and the indicated concentrations of β -fluoroalanine in a total volume of 1 ml. The production of pyruvate from D-alanine was continuously monitored with the lactate dehydrogenase-coupled assay. All assays were linear for 3 to 5 min. The data are presented as a Dixon plot. The units for reciprocal velocity are (nanomoles of pyruvate produced)⁻¹, min. B, uncompetitive inhibition between β -fluoroalanine and α -ketoglutarate. D-Amino acid transaminase (1.5 μ g) was mixed with 0.3 M potassium phosphate, pH 8.5, 25 mM D-alanine, 5 mM (Δ) or 25 mM (○) α -ketoglutarate and the indicated concentrations of fluoroalanine in a total volume of 1 ml. Assays were performed as described above. The data are presented as a Dixon plot. The units for reciprocal velocity are (nanomoles of pyruvate produced)⁻¹, min.

pyruvate formation from D-alanine and α -ketoglutarate (normal transamination).

Since both transamination and β -elimination reaction require removal of the α -proton, the same catalytic residues are probably involved in both reactions. Therefore, to explain the difference in the kinetic isotope effects for these two catalytic activities of D-amino acid transaminase, one would have to postulate that there is a different rate-limiting step for the β -elimination reaction. The mechanism proposed for the β -elimination reaction invoked: 1) substrate binding, 2) α -proton abstraction, 3) elimination of halide, and 4) solvolysis of the α -aminoacrylate-pyridoxal-P Schiff's base or alkylation of the enzyme (10, 26). On the basis of the results above, we can predict the rate-limiting step of the β -elimination reaction of D-amino acid transaminase. Since the K_i values for the three β -halo derivatives of alanine are all much lower than the K_m for D-alanine, it is unlikely that substrate binding is rate-limiting. As discussed above, α -proton abstraction and β -elimination of halide are also unlikely to be rate-limiting steps. Thus, solvolysis of the Schiff's base intermediate remains as the probable rate-limiting step for this reaction. This conclusion is also supported by our earlier experiments in which we trapped the intermediate by diffusing small thiols into the active site to add to the double bond of the α -aminoacrylate moiety (26). The steady state theory of enzyme catalysis would predict that the concentration of any intermediate in the reaction should be vanishingly small since, as soon as the intermediate were formed, it would be rapidly converted to the next intermediate. This would apply for all intermediate except the one which immediately precedes the overall rate-limiting step of the reaction. The concentration of the intermediate would increase until most of the enzyme molecules were present in that form.

Thus, the finding that the α -aminoacrylate intermediate can be trapped and isolated as a derivative in substantial amount is consistent with the proposal that it precedes the rate-limiting step in the β -elimination pathway.

Active Site Titration of D-Amino Acid Transaminase by D-Cycloserine—D-Cycloserine has been previously reported to

inhibit D-amino acid transaminase; the K_i with a partially purified enzyme from *B. subtilis* is 33 nM (29) and 25 nM with the pure enzyme from the same source (16), values considerably lower than the inhibition constants for several other pyridoxal-P enzymes for L-amino acids that are inhibited by cycloserine (30). Such low K_i values are close to the concentrations of transaminase that we routinely use to assay this enzyme. We have reinvestigated the inhibition of D-amino acid transaminase by D-cycloserine. For mole ratios of cycloserine to enzyme of less than 0.8, the inactivation is very rapid and complete within 15 s; the inactivation shows a clear linear titration (Fig. 4). The extrapolated end point of this titration indicates that 1 molecule of D-cycloserine completely inhibits 1 molecule of dimeric transaminase. Indeed, when equimolar amounts of cycloserine and enzyme are mixed, there is complete inactivation but the time required is 30 to 60 min. There is no detectable transamination between α - ^{14}C ketoglutarate and D-cycloserine.

In the presence of excess pyridoxal-P at pH 8.5, cycloserine still inactivates the enzyme efficiently. Even after overnight dialysis against excess pyridoxal-P at pH 8.5, inactivation remains complete (Table I). However, at lower pH values, activity is restored and complete activity is regained by dialysis against pyridoxal-P at pH 6.5 or 7 (Table I).

The stoichiometry of the inactivation is very unusual since the dimeric transaminase has been shown to contain two pyridoxal-P cofactors (17) and inactivation by β -chloro- ^{14}C alanine has a stoichiometry of 2 suggesting that the enzyme has two active sites.¹ Therefore, the finding that only 1 mol of cycloserine is required to inactivate this enzyme may indicate that this inhibitor acts via a half-of-the-sites reactivity mechanism, although cooperativity had not been previously reported for D-amino acid transaminase. However, although D-amino acid transaminase contains two pyridoxal-P cofactors (17), attempts to resolve the enzyme lead to the removal of only one of the two coenzymes as for some other pyridoxal-P enzymes (32). Thus, there may be a functional nonidentity to the subunits. Similar observations on such stoichiometry have been made by John *et al.* (33) with a related inhibitor, aminooxyacetate and γ -aminobutyric acid transaminase.

The mechanism by which cycloserine inactivates this enzyme is not readily apparent. Earlier work on the inhibition of aspartate transaminase by cycloserine suggested that the enzyme was alkylated by the inhibitor (34). However, more recently, Churchill demonstrated that aspartate transaminase that had been inactivated by cycloserine at neutral pH, could be significantly reactivated at pH 8.2 in the presence of pyridoxal-P (35). He proposed that cycloserine acted by breaking the Schiff's base between pyridoxal-P and the active site lysine residue. These results suggest that it is unlikely that there are strong covalent bonds between aspartate transaminase and the cycloserine-pyridoxal-P Schiff's base, as would be expected if alkylation of the enzyme had occurred. Our finding that D-amino acid transaminase inactivated by cycloserine could also be reactivated by dialysis against neutral or mildly acidic phosphate buffer containing pyridoxal-P suggests that the interactions between this D-specific transaminase and the cycloserine-pyridoxal-P adduct might be related to those observed by Churchill (35). However, we did not observe reactivation at pH 8 or higher, a finding which is different than that with aspartate transaminase.

Pashkina demonstrated that D-cycloserine was a competitive inhibitor of D-amino acid transaminase (29). We show here that this enzyme can undergo apparent irreversible in-

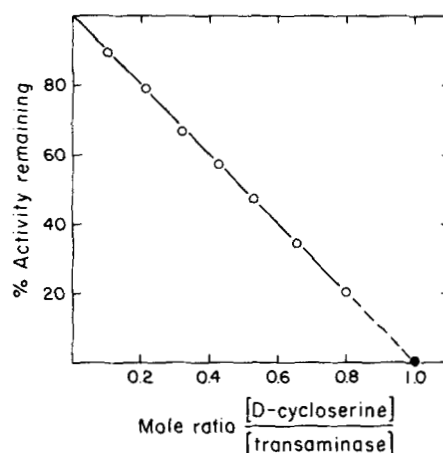


FIG. 4. Active site titration of D-amino acid transaminase by D-cycloserine. D-Amino acid transaminase (10 $\mu\text{g}/\text{ml}$) was mixed with 0 to 0.8 mol of D-cycloserine/mol of enzyme (○) in 0.3 M potassium phosphate buffer, pH 8.5. Aliquots were removed over a period of 15 s to 30 min and diluted into assay mixture. For mole ratios of cycloserine to enzyme of less than 0.8, inactivation by cycloserine is complete within 15 s. At a mole ratio of 1 (●), inactivation was not complete within 15 s but required 60 min.

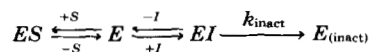
TABLE I
Reactivation of D-amino acid transaminase inactivated by D-cycloserine

Conditions for reversal ^a	Reactivation
pH	%
8.5	0
8.0	0
7.5	28
7.0	99 ^b
6.5	83

^a Enzyme was inactivated >99% with excess D-cycloserine. The inactive samples were dialyzed against two changes of 1000 volumes of 1 M potassium phosphate buffer plus 1 mM pyridoxal-P for 16 h at 4°C.

^b Average of three determinations.

activation at pH 8.5. Similarly, Lambert and Neuhaus (2) have shown that cycloserine is a reversible competitive inhibitor of alanine racemase. However, Wang and Walsh (6) found that alanine racemase was irreversibly inactivated by cycloserine. We suggest that these two sets of apparently contradictory results may simply be due to differences in experimental conditions. Both Lambert and Neuhaus (2) and Pashkina (29) did their studies in the presence of appropriate substrates, while in our work and that of Wang and Walsh (6) the enzymes were treated with inhibitor in the absence of substrates. The basic kinetic model to explain these results is:



If the on and off rate constants for both S and I are rapid relative to k_{inact} then in the presence of both I and S , there will be a rapid equilibrium between ES , E , and EI , with few molecules remaining as EI long enough for significant inactivation to be seen at early times, i.e. apparent reversible competitive inhibition. In the absence of S , the enzyme will be saturated by I . The same phenomenon was apparent when we showed that β -chloro-D-alanine could behave either as a reversible competitive inhibitor or as an irreversible inactivator of D-amino acid transaminase depending upon the choice of experimental conditions (10).

Inactivation of D-Amino Acid Transaminase by

¹ T. S. Soper and J. M. Manning, unpublished results.

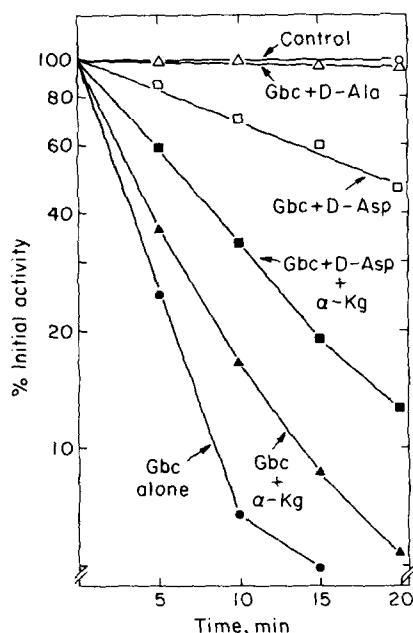


FIG. 5. Effects of substrates on the inactivation of D-amino acid transaminase by DL-gabaculine. D-Amino acid transaminase (1.6 μ g) was incubated for the indicated times with 80 μ M gabaculine (Gbc, \bullet), 80 μ M gabaculine and 20 mM D-alanine (D-Ala, Δ), 80 μ M gabaculine and 20 mM α -ketoglutarate (α -Kg, \blacktriangle), 80 μ M gabaculine and D-aspartate (D-Asp, \square), or 80 μ M gabaculine and 20 mM D-aspartate and 20 mM α -ketoglutarate (\blacksquare). Control activities (\circ) did not change significantly over time period tested. The data are presented as semilog plots of relative activity versus time. The concentration of gabaculine was determined by amino acid analysis; this compound is eluted by 30 ml of 0.35 M sodium citrate, pH 5.26, from a column (0.6 \times 20 cm) of Spinco AA-27 resin with a color value 60% that of leucine.

Gabaculine—L-Gabaculine (5-amino-1,3-cyclohexyldienyl carboxylic acid) is a natural product isolated from a strain of *Streptomyces toyocaensis* by Kobayashi *et al.* (36, 37). Both the L-isomer and synthetic DL-gabaculine are potent inhibitors of γ -aminobutyrate transaminases (36–39). Jung and Seiler (40) reported that gabaculine also inhibits ω -ornithine transaminase. The specificity of this inhibitor for such enzymes was suggested to be due to the relative positions of the carboxyl and amino groups, *meta* to each other on the cyclohexadienyl ring. Thus, Rando reported that alanine transaminase, aspartate transaminase, and several other pyridoxal-P requiring enzymes which act on the α -amino group of substrates, were not inhibited by gabaculine in the millimolar range (41). Thus, the inactivation of D-amino acid transaminase, an α -transaminase, found in the present studies was not anticipated. However, Wood *et al.* (42) recently found that alanine transaminase was inactivated in extracts of both mouse brain and liver treated with gabaculine. Lower concentrations of gabaculine were effective in inhibiting γ -aminobutyrate transaminase than were needed for inhibition of L-alanine transaminase. Aspartate transaminase was not inhibited. When mice were treated with gabaculine, the activities of γ -aminobutyrate transaminase, L-alanine transaminase and L-aspartate transaminase were reduced in liver but the latter two enzymes were not affected in brain. We have extended the *in vitro* observations of Wood *et al.* (42), and we have determined that the difference between partially purified alanine and aspartate transaminase is in the relative affinity of these enzymes for gabaculine.²

² T. S. Soper and J. M. Manning, manuscript in preparation.

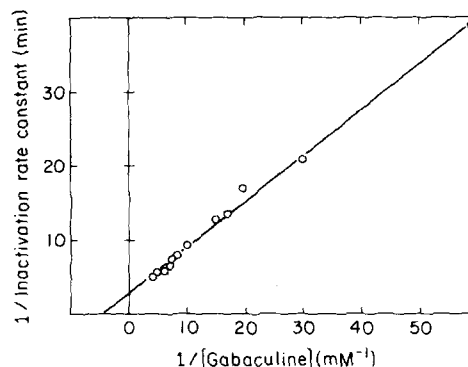


FIG. 6. Concentration dependence of the inactivation of D-amino acid transaminase by DL-gabaculine. D-Amino acid transaminase (1.5 μ g) was incubated with the indicated concentrations of gabaculine in 0.3 M potassium phosphate, pH 8.5, and the inactivation rate constant was calculated as described in "Experimental Procedures." The data are presented as a double reciprocal plot of the inactivation rate constant versus the concentration of gabaculine.

TABLE II
Effects of DL-gabaculine on enzyme activities in *B. sphaericus*

Enzyme	Source	
	Crude extract ^a	Whole cells ^b
	% inhibition	
D-Amino acid transaminase	93	49
L-Alanine transaminase	87	68
Alanine racemase	14	0

^a *B. sphaericus* was grown to early stationary phase of growth (13), cells were harvested and washed with physiological saline. Extracts were prepared, treated with 1 mM gabaculine (30 min at 37°C), and assayed as described under "Experimental Procedures." Inhibition is expressed relative to a control extract similarly treated but without gabaculine.

^b *B. sphaericus* was grown to mid-log phase of growth at 37°C (13), gabaculine was added (15 μ g/ml) and the cells were incubated for 60 min. After the cells were harvested and washed, extracts were prepared and the enzymes assayed as described under "Experimental Procedures." Inhibition is expressed relative to a control culture similarly treated but without gabaculine.

We find that purified D-amino acid transaminase is rapidly inactivated by relatively low amounts of gabaculine (Fig. 5); a good substrate such as D-alanine protects the enzyme from inactivation while a poorer substrate such as D-aspartate is less effective. α -Ketoglutarate has only a small effect on the inactivation but can overcome the protective effect of D-aspartate. The concentration dependence of the inactivation of D-amino acid transaminase by gabaculine also shows saturation kinetics; the K_i is 0.1 mM (Fig. 6).

Antibacterial Effects of Gabaculine—The effects of DL-gabaculine on the inactivation of some of the enzymes concerned with the metabolism of D- and L-alanine were tested in crude extracts of *B. sphaericus*. Table II shows that both D-amino acid transaminase and L-alanine transaminase are inhibited by gabaculine but that alanine racemase is not significantly affected by this inhibitor. There is also little change in the activity of either D- or L-amino acid oxidase but, because of the low activities of these two enzymes in the extract, we cannot rule out small effects on these enzymes.

If gabaculine were to be an effective antimicrobial agent, it must first penetrate the cell wall and then inactivate specific target enzymes. When intact cells of *B. sphaericus* were treated with DL-gabaculine (15 μ g/ml), about 50% of the D-amino acid transaminase activity was lost (Table II). L-Alanine transaminase was also inhibited but alanine racemase

was not affected. Thus, both isomers of gabaculine are apparently able to penetrate the bacteria membrane.

Because D-amino acid transaminase produces the D-glutamate which is important in cell wall biosynthesis, inhibition of the enzyme by D-gabaculine should result in a conditional D-glutamate auxotrophy. Although the optically pure D-isomer is not yet available, we decided to test the antibacterial activity of the racemic compound. DL-Gabaculine inhibits the growth of some bacteria for 6 to 8 h; for both *B. sphaericus* and *Staphylococcus epidermidis*, the minimal inhibitory concentration of gabaculine is 16 µg/ml when grown in Difco nutrient broth. *Salmonella typhimurium*, strain LT-2, is apparently resistant to DL-gabaculine since this bacteria grew in the presence of 256 µg/ml of DL-gabaculine. Further bacteriological tests await the preparation of the pure D-isomer of gabaculine.

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REFERENCES

- Blumberg, P. M., and Strominger, J. L. (1974) *Bacteriol. Rev.* **38**, 291-335
- Lambert, M. P., and Neuhaus, F. C. (1972) *J. Bacteriol.* **110**, 978-987
- Neuhaus, F. C. (1967) *Antimicrob. Agents Chemother.* 304-313
- Manning, J. M., Merrifield, N. E., Jones, W. M., and Gotschlich, E. C. (1974) *Proc. Natl. Acad. Sci. U. S. A.* **71**, 417-421
- Henderson, L. L., and Johnston, R. B. (1976) *Biochem. Biophys. Res. Commun.* **68**, 793-798
- Wang, E., and Walsh, C. (1978) *Biochemistry* **17**, 1313-1321
- Thorne, C. B., Gómez, C. G., and Housewright, R. D. (1955) *J. Bacteriol.* **69**, 357-362
- Troy, F. A. (1973) *J. Biol. Chem.* **248**, 305-315
- Glaser, L. (1960) *J. Biol. Chem.* **235**, 2095-2098
- Soper, T. S., Jones, W. M., Lerner, B., Trop, M., and Manning, J. M. (1977) *J. Biol. Chem.* **252**, 3170-3175
- Soper, T. S., Jones, W. M., and Manning, J. M. (1979) *J. Biol. Chem.* **254**, 10901-10905
- Dolling, U. H., Douglas, A. W., Grabowski, E. J. J., Schoenewaldt, E. F., Sohar, P., and Slettinger, M. (1978) *J. Org. Chem.* **43**, 1634-1640
- Soda, K., Yonaha, K., Misono, H., and Osugi, M. (1974) *FEBS Lett.* **46**, 359-363
- Washington, J. A., II, and Barry, A. L. (1974) in *Manual of Clinical Microbiology*, (Lennette, F. H., Spaulding, E. H., and Traut, J. P., eds) 2nd Ed., pp. 410-417, American Society for Microbiology, Washington, D.C.
- Fasella, P., and Turano, C. (1970) in *Vitamin and Hormones* (Harris, R. S., Munson, P. L., and Diczfalussy, E., eds) pp. 157-194, Academic Press, New York
- Martinez-Carrion, M., and Jenkins, W. T. (1965) *J. Biol. Chem.* **240**, 3547-3552
- Yonaha, K., Misono, H., Yamamoto, T., and Soda, K. (1975) *J. Biol. Chem.* **250**, 6983-6989
- Segel, I. H. (1975) *Enzyme Kinetics*, p. 105, Wiley-Interscience, New York
- Atherton, F. R., Hall, M. J., Hassaell, C. H., Lambert, R. W., and Ringrose, P. S. (1979) *Antimicrob. Agents. Chemother.* **15**, 677-683
- Northrop, D. B. (1975) *Biochemistry* **14**, 2644-2651
- Bank, B. E. C., Bell, M. P., Lawrence, A. J., and Vernon, C. A. (1966) in *Pyridoxal Catalysis: Enzymes and Model Systems* (Snell, E. E., Braunstein, A. E., Severin, E. S., and Torchinsky, Yu. M., eds) pp. 191-202, John Wiley and Sons, New York
- Dunathan, H. C., Davis, L., Kury, P. G., and Kaplan, M. (1968) *Biochemistry* **7**, 4532-4542
- Walsh, C. T., Schonbrunn, A., and Abeles, R. H. (1971) *J. Biol. Chem.* **246**, 6855-6866
- Miles, E. W., and McPhie, P. (1974) *J. Biol. Chem.* **249**, 2852-2857
- Cooper, A. J. L. (1976) *J. Biol. Chem.* **251**, 1088-1096
- Soper, T. S., and Manning, J. M. (1978) *Biochemistry* **17**, 3377-3384
- Kollonitsch, J., and Barash, L. (1976) *J. Amer. Chem. Soc.* **98**, 5591-5593
- Manning, J. M., and Soper, T. S. (1978) in *Enzyme-activated Irreversible Inhibitors* (Seiler, N., Jung, M. J., and Koch-Weser, J., eds) pp. 163-176, Elsevier, Amsterdam
- Paskhina, T. S. (1964) *Vopr. Med. Khim.* **10**, 526-533
- Wang, D. T., Fuller, R. W., and Molloy, B. B. (1973) *Adv. Enzyme Regul.* **11**, 139-166
- Yonaha, K., Misono, H., and Soda, K. (1975) *FEBS Lett.* **46**, 265-267
- Misono, H., and Soda, K. (1977) *J. Biochem.* **82**, 535-543
- John, R. A., Charteris, A., and Fowler, L. J. (1978) *Biochem. J.* **171**, 771-779
- Khomutov, R. M., Severin, E. S., Kovalera, G. K., Gulyaev, N. N., Gnuchev, N. V., and Sastchenko, L. P. (1968) in *Pyridoxal Catalysis: Enzymes and Model Systems* (Snell, E. E., Braunstein, A. E., Severin, E. S., and Torchinsky, Yu. M., eds) pp. 631-650, John Wiley and Sons, New York
- Churchich, J. E. (1967) *J. Biol. Chem.* **242**, 4414-4417
- Kobayashi, K., Miyazawa, S., Terahara, A., Mishima, H., and Kurihara, H. (1976) *Tetrahedron Lett.* **7**, 537-540
- Kobayashi, K., Miyazawa, S., and Endo, A. (1977) *FEBS Lett.* **76**, 207-210
- Rando, R. R., and Bangerter, F. W. (1976) *J. Amer. Chem. Soc.* **98**, 6762-6764
- Burnett, G., Yonaha, K., Toyama, S., Soda, K., and Walsh, C. (1980) *J. Biol. Chem.* **255**, 428-432
- Jung, M. J., and Seiler, N. (1978) *J. Biol. Chem.* **253**, 7431-7439
- Rando, R. R., and Bangerter, F. W. (1977) *Biochem. Biophys. Res. Commun.* **76**, 1276-1281
- Wood, J. D., Kurylo, E., and Tsui, D. S.-Y. (1979) *Neurosci. Lett.* **14**, 327-331