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CYCLOSERINE INHIBITION OF GAMMA-AMINOBUTYRIC-ALPHA-KETOGLUTARIC TRANSAMINASE

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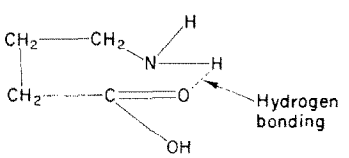
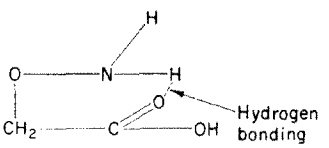
Abstract—Cyloserine was found to inhibit γ-aminobutyric-α-ketoglutaric transaminase of *Escherichia coli* and of cat and monkey brain *in vitro*. The inhibition was characterized by two phases: one was rapid, reversible, and competive with α-aminobutyric acid; the other was slow, progressive, and not reversible. The inhibition was reversed by γ-aminobutyric acid in the initial phase of the reaction but was not reversed by pyridoxal phosphate.

Rats and guinea pigs treated acutely or chronically with cycloserine exhibited high concentrations of γ-aminobutyric acid in the brain, a finding consistent with γ-aminobutyric-α-ketoglutaric transaminase inhibition by cycloserine *in vivo*.

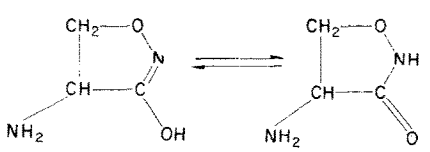
THE ANTIBIOTIC cycloserine has been reported to inhibit several transaminases, especially those involving glutamic acid and asparagine.[1, 2] Braunstein *et al.* found that the amino acid substrate competitively reversed cycloserine inhibition in the initial phase of the reaction but became progressively less effective in reactivating the enzyme as the reaction proceeded.[2] Wallach demonstrated that aminooxyacetic acid competitively inhibited γ-aminobutyric-α-ketoglutaric transaminase.[3] The inhibition of some transaminases by cycloserine, and the similarity in structure of



γ-Aminobutyric acid



Aminooxyacetic acid



Cycloserine (tautometric forms)

γ-aminobutyric acid, aminooxyacetic acid, and cycloserine suggested that the last might inhibit γ-aminobutyric-α-ketoglutaric transaminase.

The present studies demonstrate that such inhibition does occur *in vitro*. Through what is undoubtedly a similar inhibitory mechanism, the administration of cycloserine to animals is shown to increase the brain concentration of γ-aminobutyric acid. The latter substance has been implicated in energy-yielding reactions and in regulatory phenomena in the central nervous system.[4-9]

MATERIALS AND METHODS

DL-Cycloserine (Seromycin) was generously supplied by Eli Lilly and Co. Succinic semialdehyde ethyl ester was a gift of Dr. D. P. Wallach. All other chemicals were obtained from commercial sources.

Gamma-aminobutyric-α-ketoglutaric transaminase coupled with succinic semialdehyde dehydrogenase was prepared from *Escherichia coli*, strain ATCC-26, by a modification of the method of Wallach.[3] The cells were grown with aeration for 24 hr in 8-liter batches of nutrient broth. The cells were disrupted by grinding with twice their wet weight of alumina A-303 and were extracted with 1 mM Tris buffer, pH 7·2, which contained 1 mM magnesium acetate. Nucleic acids were removed by precipitation with 0·25 vol. of 5% streptomycin, and the extract was diluted with an equal volume of 0·1 M potassium phosphate buffer, pH 7·4, which contained 0·01% 2-mercaptoethanol. Solid ammonium sulfate was added slowly to the extract to 55% saturation at 4°, and the precipitate was discarded. The protein fraction that precipitated between 55 and 65% saturation with ammonium sulfate was collected by centrifugation. The precipitate was dissolved in approximately 3 volumes of the mercaptoethanol–phosphate buffer for every gram of cells employed in the preparation. The enzyme solution was dialyzed against 4 l. of this buffer for 24 hr at 4°. The preparation contained approximately 6 mg protein/ml, and represented a purification of about 80-fold. It could be stored at −15° for several weeks without significant loss of activity.

The coupled enzyme system was also prepared from acetone powders of cat and rhesus monkey brains by an adaptation of the method of Baxter and Roberts for steer brain.[10] The acetone powder was blended in 10 volumes of 0·1 M Tris buffer, pH 8·2, and was centrifuged at 34,000 *g* for 30 min. The supernatant portion was brought to 40% saturation by the slow addition of solid ammonium sulfate. The preparation was centrifuged at 105,000 *g* for 30 min, and the precipitate was discarded. The clear supernatant fraction was brought to 50% saturation with ammonium sulfate, and was then centrifuged for 30 min at 34,000 *g*. The precipitate was dissolved in 1 ml of 0·1 M Tris buffer, pH 8·2, for every gram of acetone powder employed in the procedure. The solution was dialyzed for 3 hr against 1 l. of the Tris buffer with six changes of buffer. This preparation was used immediately, since the γ-aminobutyric-α-ketoglutaric transaminase activity was lost in a few hours or when frozen. The succinic semialdehyde dehydrogenase activity was stable at 4° and at −15°. The brain enzyme preparation contained approximately 8 mg protein/ml and represented a purification of about 150-fold over the crude acetone powder extract. No significant differences in the kinetics and inhibition were observed between the cat and monkey preparations.

Assays of γ-aminobutyric acid were performed by measuring spectrophotometrically the rate of reduction of NADP by the coupled γ-aminobutyric-α-ketoglutaric transaminase–succinic semialdehyde dehydrogenase system prepared from *E. colli*.[3, 11, 12] The reactions proceed as follows:

$$1\ \gamma\text{-aminobutyric acid} + \alpha\text{-ketoglutaric acid} \rightarrow \text{succinic semialdehyde} + \text{glutamic acid}$$

$$2\ \text{succinic semialdehyde} + \text{NADP} + H_2O \rightarrow \text{succinic acid} + \text{NADPH} + H^+$$

The latter reaction is not rate limiting, since it has a very small Km.[13] The experimental enzyme kinetic and inhibition studies on the bacterial and brain enzymes were conducted with the same system except that NAD was used with the brain enzyme.

Experiments *in vivo* were conducted with adult male white rats and 350-g male and female guinea pigs. The brains were extracted with 75% ethanol for assay of γ-aminobutyric acid according to the method of Baxter and Roberts.[14] One ml of distilled water was added to the dried extract for every gram of fresh weight of brain. The suspension was centrifuged at 105,000 *g* for 30 min to clarify the preparation. Aliquots of the supernatant portion derived from extracts of individual brains were assayed enzymatically.[3, 11, 12] In some experiments the enzymatic reaction was further characterized by determining the production of glutamic acid by undimensional paper chromatography in butanol–acetic acid–water (2 : 1 : 1). Reaction mixtures were also assayed for the disappearance of α-ketoglutaric acid by the semicarbazide method of MacGee and Doudoroff.[15]

Free succinic semialdehyde was prepared from the ethyl ester according to the method of Bessman *et al.*[16] Solutions of γ-ketoglutaric acid were made before each use.

RESULTS

Cyloserine effects observed in vitro

Cycloserine inhibited γ-aminobutyric-α-ketoglutaric transaminase of *E. coli* when the reaction was started by the addition of enzyme to the complete reaction mixture (Fig. 1). The inhibition was greater when cycloserine was incubated with the bacterial enzyme for various intervals of time before the addition of γ-aminobutyric acid (Fig. 1, Table 1). If the amino acid was present during the exposure of enzyme to inhibitor before adding α-ketoglutaric acid to start the reaction, the increase in inhibition was much less marked, and the kinetics followed curve II, Fig. 1, for the first 5 min before leveling off. The presence of α-ketoglutaric acid in the incubation mixture did not influence the degree of inhibition. Hence γ-aminobutyric acid, but not α-ketoglutaric acid, appeared to protect the enzyme from cycloserine inhibition. The inhibition of the transaminase by cycloserine was competitive with respect to γ-aminobutyric acid and noncompetitive with respect to α-ketoglutaric acid (Fig. 2, 3). Since the transaminase reaction was determined by following the coupled succinic semialdehyde dehydrogenase reduction of NADP, a control was included in which succinic semialdehyde was the substrate, and cycloserine had no effect on the dehydrogenase component.

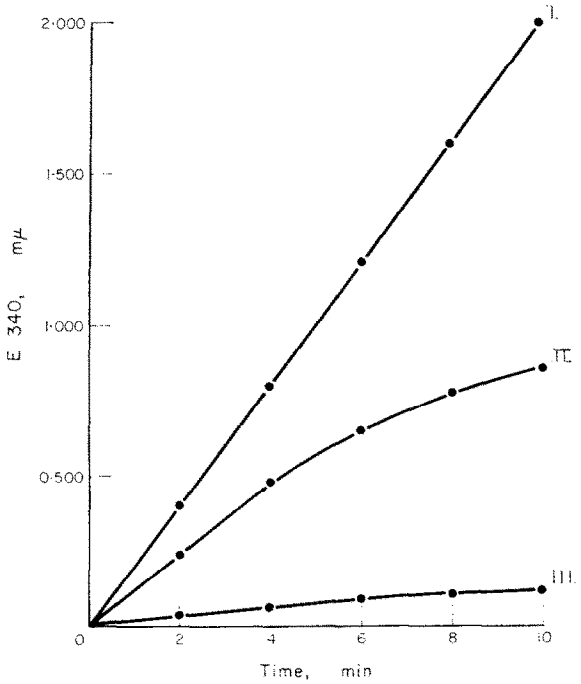


FIG. 1. Cycloserine inhibition of bacterial γ-aminobutyric-α-ketoglutaric transaminase. The reaction mixture contained 6 μmoles each of γ-aminobutyric acid and α-ketoglutaric acid. I, no cycloserine; II, 1·6 mM cycloserine, no incubation; III, 1·6 mM cycloserine, 5-min incubation of enzyme and inhibitor in the absence of γ-aminobutyric acid.

TABLE 1. INHIBITION OF BACTERIAL γ-AMINOBUTYRIC-α-KETOGLUTARIC TRANSAMINASE*

Time of incubation (min)	Inhibition (%)
0	20
1	20
2	33
5	75
10	90
15	95
30	100

* As a function of time of incubation of enzyme and cycloserine ($6{\cdot}5 \times 10^{-1}$ mM) prior to the addition of γ-aminobutyric acid.

Although cycloserine inhibition of the bacterial transaminase was competitive with respect to γ-aminobutyric acid (Fig. 2), the inhibition was not completely reversed by the addition of excess amino acid substrate. When bacterial enzyme and cycloserine were incubated for various intervals of time prior to the addition of γ-aminobutyric acid, the inhibition became mixed competitive and noncompetitive, according to Lineweaver–Burk plots,[17] and the calculated K_i values were somewhat smaller. Very little or no reversal of the inhibition was effected by the addition of excess γ-aminobutyric acid under these conditions.

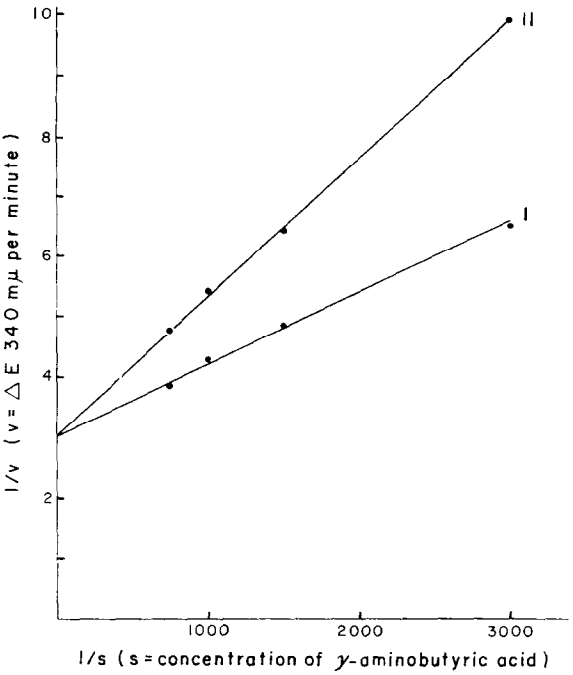


FIG. 2. Reciprocal of velocity of the reaction, catalyzed by bacterial enzyme, as a function of reciprocal of γ-aminobutyric acid concentration (I), and inhibition by cycloserine, $3{\cdot}3 \times 10^{-1}$ mM (II). The reaction was initiated by the addition of enzyme to the complete reaction mixture. The inhibition is competitive with respect to γ-aminobutyric acid.

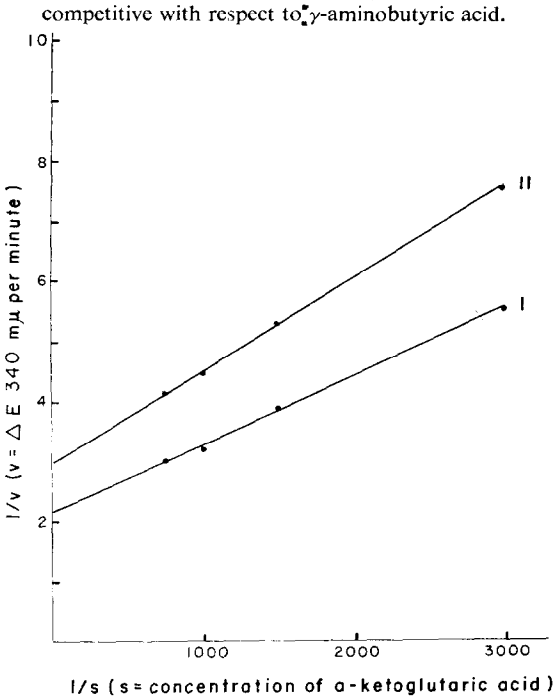


FIG. 3. Same as Fig. 2, but with reciprocals of α-ketoglutaric acid concentration on the abscissa. I, no cycloserine; II, $3{\cdot}3 \times 10^{-1}$ mM cycloserine. The inhibition is noncompetitive with respect to α-ketoglutaric acid.

Experiments performed with the enzymes prepared from cat and monkey brain produced results similar to those obtained with the bacterial enzyme, but the brain transaminase was about twice as sensitive to the inhibitory effects of cycloserine (Table 2). Incubation of brain enzyme and cycloserine before the addition of γ-aminobutyric acid produced markedly increased inhibition, as with the *E. coli* enzyme. The kinetics of inhibition of the brain transaminase was of the type characterized as mixed competitive and noncompetitive with respect to γ-aminobutyric acid,[18] whether or not enzyme and cycloserine were incubated prior to the addition of the amino acid. The inhibition was noncompetitive with respect to α-ketoglutaric acid.

TABLE 2. MICHAELIS–MENTEON CONSTANTS (K_m) AND INHIBITOR CONSTANTS (K_i) FOR BACTERIAL AND BRAIN TRANSAMINASES, AS OBTAINED FROM LINEWEAVER–BURK PLOTS*

	Substrate	K_m	K_i
Bacterial transaminase	γ-Aminobutyric acid	$4{\cdot}2 \times 10^{-4}$	$4{\cdot}0 \times 10^{-4}$
	α-Ketoglutaric acid	$5{\cdot}0 \times 10^{-4}$	$7{\cdot}7 \times 10^{-4}$
Brain transaminase	γ-Aminobutyric acid	$4{\cdot}5 \times 10^{-4}$	$2{\cdot}3 \times 10^{-4}$
	α-Ketoglutaric acid	$6{\cdot}7 \times 10^{-4}$	$2{\cdot}7 \times 10^{-4}$

* Substrates were γ-aminobutyric acid (with 6 μmoles α-ketoglutaric acid) or α-ketoglutaric acid (with 6 μmoles γ-aminobutyric acid). Reactions were carried out at pH 8·4 and NADP (bacterial enzyme) or NAD (brain enzyme) concentrations of 4×10^{-4} mM. Cycloserine, $3{\cdot}3 \times 10^{-4}$ mM, was not incubated with the enzymes prior to the initiation of the reactions.

TABLE 3. EFFECT OF CYCLOSERINE OBSERVED *in vitro* ON BRAIN γ-AMINOBUTYRIC ACID CONCENTRATIONS IN RAT AND GUINEA PIG

Animal	No. of animals per group	Dose of cycloserine (mg/100 g body wt)	Interval before sacrifice	γ-Aminobutyric acid in brain (μmoles/g)*
Rat	2	Saline	3 hr; 2 days	2·5
	2	80	3 hr	2·8
	2	600†	2 days	7·3
Guinea pig	2	Saline	3 hr; 7 days	1·3
	1	300	3 hr	1·5
	1	450	3 hr	1·6
	2	700	3 hr	2·4
	1	1,000‡	7 days	3·4

* The actual values do not differ by more than 0·1 from the averages given here.
† Total amount per 100 g body weight given in 125-mg doses four times a day.
‡ Total amount per 100 g body weight given in 250-mg doses twice a day.

The inhibition of bacterial and brain enzymes was not reversed by the addition of pyridoxal phosphate, glutathione, cysteine, Mg^{2+}, Mn^{2+}, or Zn^{2+} at concentrations of 5 mM. Isoniazid, which inhibits some pyridoxal phosphate-dependent enzymes,[19, 20] had no effect on the reaction at a concentration of 1 mM. Cycloserine inhibition of both the bacterial and the brain transaminases was increased with greater enzyme purification and was diminished with greater age of the preparations. This may be due to nonspecific binding of cycloserine by sites other than those of the active enzyme or to inactivation of the drug by the crude or aged preparations.

Cycloserine effects observed in vivo

Cycloserine injected intraperitoneally into rats and guinea pigs resulted in an increase in brain γ-aminobutyric acid concentration which was dependent on dosage (Table 3). This effect occurred whether the drug was given in a single dose or over a period of several days. Prolonged administration produced higher brain γ-aminobutyric acid concentrations and more marked toxic effects.

Physical manifestations seen after cycloserine administration were similar in both species. Animals appeared moderately lethargic and sedated but exhibited generalized twitchings at intervals. There was an accentuated startle response. All animals performed peculiar, rapid, face-washing movements. With prolonged administration there was hyperventilation, decreased tone, ataxia, and more pronounced twitchings. None of these effects was observed in the saline-injected controls.

DISCUSSION

Cycloserine inhibition of bacterial γ-aminobutyric-α-ketoglutaric transaminase is competitive with respect to γ-aminobutyric acid, whereas the brain enzyme shows mixed competitive and noncompetitive characteristics. Netither system shows reversal of inhibition by pyridoxal phosphate. Hence the antibiotic does not appear to react with a dissociated pyridoxal phosphate to form an intermediate complex which inhibits the action of the transaminase. Although the observed cycloserine inhibition is similar to that seen with various carbonyl reagents acting on pyridoxal phosphate-dependent enzymes,[19, 21] isoniazid and semicarbazide do not inhibit γ-aminobutyric-α-ketoglutaric transaminase.[1, 10] Cycloserine also is not a strong antagonist of pyridoxal *in vivo*. Administration of pyridoxal reduces but does not eliminate cycloserine toxicity clinically, and animal experiments have not shown that the antibiotic produces or enhances vitamin-B_6 depletion.[22]

Incubation of cycloserine with purified γ-aminobutyric-α-ketoglutaric transaminase in the absence of γ-aminobutyric acid leads to an inhibition of the enzyme, which greatly exceeds that observed when the amino acid is present in the mixture. This suggests that the antibiotic is tightly bound to a reactive site on the enzyme when the latter is not occupied by γ-aminobutyric acid.

The fact that the degree of inhibition progressively increases with incubation of enzyme and cycloserine before the addition of γ-aminobutyric acid suggests that a slow, perhaps irreversible, association of inhibitor and enzyme occurs. The decrease in the rate of reaction with time (Fig. 1, curve II), obtained when the reaction is begun by the addition of enzyme, is probably also due to this slow, progressive inhibition. As the reaction proceeds and γ-aminobutyric acid is transaminated, it is no longer available to protect the enzyme. The longer this reaction continues the less reversibility can be achieved by the addition of excess γ-aminobutyric acid. The mixed competitive and noncompetitive curves seen in some of the Lineweaver–Burk plots are probably produced by the combined effect of competitive inhibition and this slow, irreversible component.

Khomutov *et al.* have evidence that cycloserine competitively inhibits transaminases by rapid formation of an azomethine derivative with pyridoxal phosphate.[23] Amino acid substrates can displace the drug in this initial stage, with reactivation of the enzyme. The isoxazolidone ring is then opened, and an amino group of the active center of the enzyme is acylated. Thus the aldehyde group of the coenzyme is joined by

covalent bonds to an amino group of the protein moiety by β-amino-hydroxyalanine in a slow, progressive, irreversible reaction.

The finding that cycloserine increases brain γ-aminobutyric acid concentrations in the rat and guinea pig indicates that *in vivo* the drug inhibits γ-aminobutyric-α-ketoglutaric transaminase. It may be speculated that this antagonism occurs to a greater extent than any inhibition which cycloserine has on glutamic acid decarboxylase. Both of these statements are consistent with the effects observed with the purified enzyme preparations *in vitro*.[23]

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