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# Cycloserine inhibition of gamma-aminobutyric-alpha-ketoglutaric transaminase

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CYCLOSERINE INHIBITION

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OF GAMMA-AMINOBUTYRIC-ALPHA-KETOGLUTARIC TRANSAMINASE


by


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A. B. Columbia University, 1958


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I want to thank Dr. Charles E. Carter--a teacher.

I want to thank Dr. Charles L. Carter---a teacher.

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## ABBREVIATIONS

GABA: $\gamma$-aminobutyric acid; $\alpha$-KA: $\alpha$-ketoglutaric acid; SS: succinic semialdehyde; GAD: glutamic acid decarboxylase; GABA-T: $\gamma$-aminobutyric-$\alpha$-ketoglutaric transaminase; SS-D: succinic semialdehyde dehydrogenase; PalP: pyridoxal phosphate; DPN: diphosphopyridine nucleotide; TPN: triphosphopyridine nucleotide; ADP: adenosine diphosphate; ATP: adenosine triphosphate; GDP: guanosine diphosphate; UDP: uridine diphosphate; Tris buffer: tris(hydroxymethyl)aminomethane buffer; EEG: electro-encephalogram.

INTRODUCTION

## Metabolism of Gamma-aminobutyric Acid in Brain

The discovery of high concentrations of $\gamma$-aminobutyric acid in mammalian brain (1-4) focused interest on the possible functions of this amine in the central nervous system.  Gamma-aminobutyric acid is formed in brain from glutamic acid through the action of a specific enzyme, glutamic acid decarboxylase (1, 3, 5), and is reversibly transaminated with $\alpha$-ketoglutaric acid, yielding glutamic acid and succinic semialdehyde, by means of another specific enzyme, $\gamma$-aminobutyric-$\alpha$-ketoglutaric transaminase (6, 7).  Both of these enzymes are pyridoxal phosphate dependent, although the coenzyme is apparently more firmly bound to the apoenzyme in the transaminase than in the decarboxylase (8-10).  Succinic semialdehyde, which is formed in the transamination reaction, is oxidized in brain to succinic acid (8, 11), by a DPN dependent enzyme, succinic semialdehyde dehydrogenase (12, 13).

Evidence for the participation of metabolites of C-14 labeled $\gamma$-aminobutyric acid in the citric acid cycle has been obtained by several workers (11, 14, 15).  The reactions catalyzed by the above mentioned enzymes provide an alternative pathway for $\gamma$-aminobutyric acid around succinyl-coenzyme A in the citric acid cycle, as shown in fig. 1.  The operation of this system has been demonstrated only in the mammalian central nervous system, although the enzymes glutamic
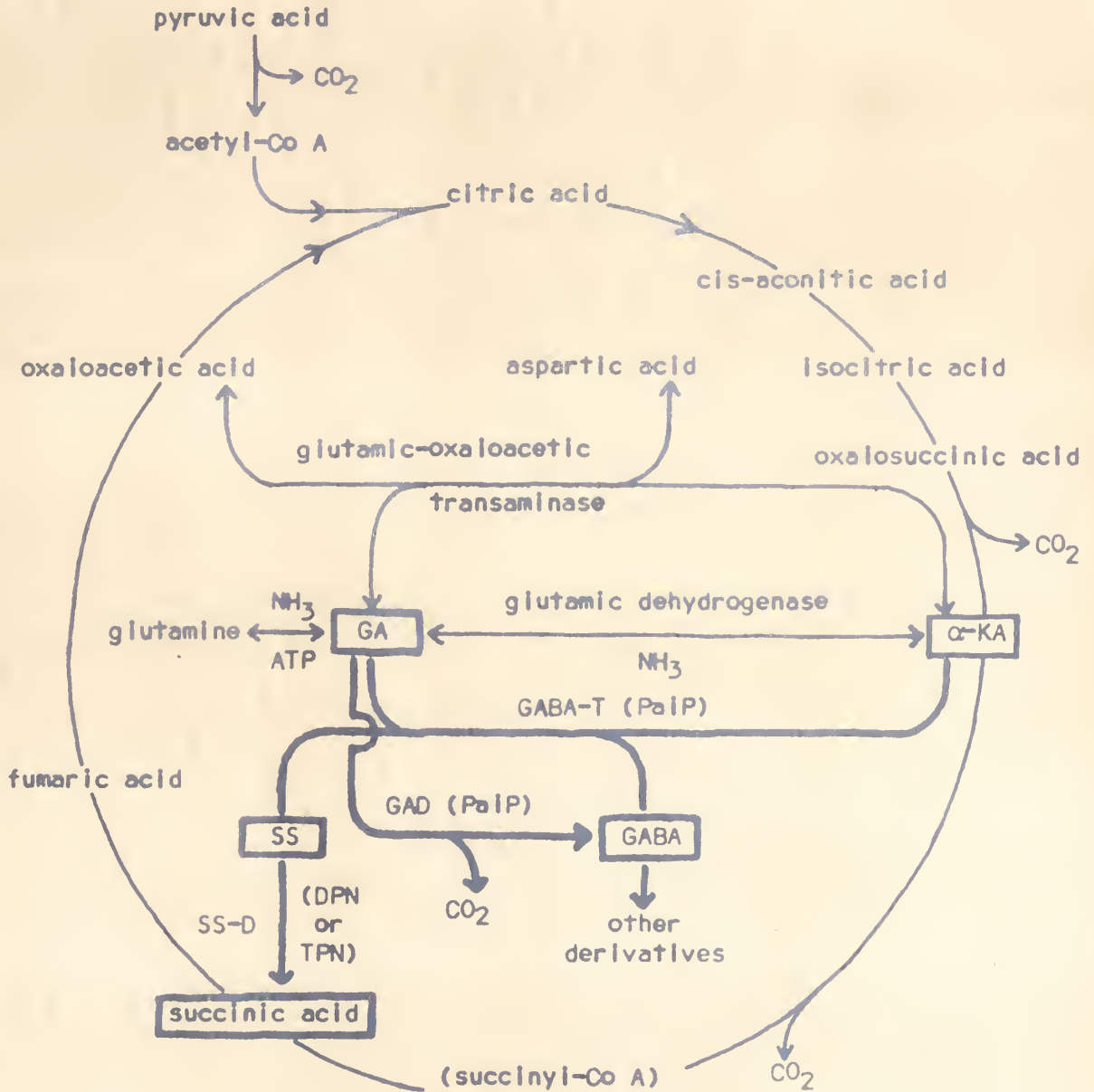
Fig. I. Metabolism of GABA in brain and its relationship to the citric acid cycle. Pathways shown are only those which have been definitely established. Note that for each mole of GABA metabolized to succinic acid, a mole of GA is formed by transamination with α-KA. GA is then decarboxylated to GABA.

acid decarboxylase, γ-aminobutyric-α-ketoglutaric transaminase, and succinic semialdehyde dehydrogenase (TPN dependent) have been found in microorganisms (16-21). Gamma-aminobutyric acid and glutamic acid decarboxylase have been isolated from a wide variety of plants (22, 23). Among the various tissues of higher animals, γ-aminobutyric acid and glutamic acid decarboxylase have been reported to occur naturally only in the central nervous system, and mainly in the gray matter. The decarboxylase, the transaminase, and the dehydrogenase are associated with the mitochondrial cell fractions.

## Function of Gamma-aminobutyric Acid

Several theories have been advanced to explain the function of γ-aminobutyric acid and the metabolic pathway in which it is involved. Evidence has been obtained that the amine may serve as a neurohumoral inhibitory mediator, or that it may release such a substance (cf. ref. 24). Gamma-aminobutyric acid perfusion slows and stops the crayfish heart, and opposes the action of acetylcholine in various invertebrate and vertebrate preparations (25-27). It inhibits the crayfish stretch receptor, and this characteristic is used in a biologic assay for the amino acid (28-30). It blocks excitatory synapes in the cat cerebral cortex (31, 32), and depresses various excitatory electrical responses in the dog and rat cortices (33, 34), when applied locally to the exposed brain surfaces. Hydrazides, such as semicarbazide, inhibit the decarboxylase, decrease brain γ-aminobutyric acid, and produce convulsions when administered to mammals; convulsions are terminated

or prevented and EEG seizure patterns are diminished or obliterated by hydroxylamine, which increases brain γ-aminobutyric acid through inhibition of the transaminase (35-37). However, many data are reported, at least on a mammalian level, which make it difficult to ascribe any simple and direct neural impulse regulator function to this amino acid itself. No consistent correlation exists between γ-aminobutyric acid concentrations in particular areas of the brain and seizure susceptibility of these areas (38). Hydroxylamine fails to counteract thiosemicarbazide convulsions, yet it produces increased brain γ-aminobutyric acid levels (39). Central nervous system stimulation caused by various non-hydrazine agents, such as metrazole and strychnine, is not consistently accompanied by decreased γ-aminobutyric acid concentration, nor is such stimulation necessarily counteracted by agents which raise its concentration (40). Topical application of γ-aminobutyric acid does not block spike potentials produced by strychnine or picrotoxin (41; cf. refs. 25, 140).

A second line of evidence suggests that γ-aminobutyric acid functions as a precursor of several compounds and reactions which may play an important role in the nervous system (cf. ref. 42). Gamma-aminobutyrylcholine, which has characteristics of an inhibitory neurotransmitter and antagonizes many actions of acetylcholine, is probably formed from γ-aminobutyric acid in dog brain (43). Gamma-amino-β-hydroxybutyric acid has been isolated as an oxidation product in guinea pig brain homogenate upon addition of ATP (44). This substance has an inhibitory action in the central nervous system

(27, 45, 46, 141), and blocks contractions of isolated mammalian
intestine induced by acetylcholine (26). Its metabolism via oxidation
and transamination has recently been investigated (47). Gamma-amino-
β-hydroxybutyryl-coenzyme A and γ-aminobutyryl-coenzyme A have been
reported to occur in brain extracts (48). An unidentified methylation
product of γ-aminobutyric acid, formed in the presence of ATP, has a
central nervous system excitatory action (45). The methyl ester of
γ-aminobutyric acid stimulates isolated mammalian intestine (49).
Gamma-aminobutyryllysine, γ-aminobutyrylarginine, and γ-aminobutyryl-
histidine (homocarnosine) are formed in chicken muscle from the
corresponding amino acids in the presence of ATP (50). The latter
dipeptide has recently been isolated from steer brain (51). Gamma-
guanidinobutyric acid, a substance with marked sedative effects (52),
is synthesized from γ-aminobutyric acid and arginine in mammalian
brain (53-55), and represents a potential mechanism for ammonia
metabolism in the central nervous system (56, 57). Gamma-aminobutyric
acid has been reported to react with inosine monophosphate to form
adenylic acid in rat brain homogenate (58). Gamma-aminobutyric acid
is formed from γ-butyrolactam (2-pyrrolidinone) in cat cerebral
cortex (59). The latter compound has anticonvulsant activity in
mice (60).

The importance of these various substances and reactions, and their
relationship to the established γ-aminobutyric acid metabolic pathway
shown in fig. I, is not understood. If γ-aminobutyric acid is a
physiologically active substance, it is not surprising that it can

exist in a combined form in brain.

Several investigations suggest that $\gamma$-aminobutyric acid participates in an energy yielding metabolic pathway (fig. I). This pathway may regulate a part of the available energy in the central nervous system, and hence may control levels of functional neuronal activity. Malfunction in the nervous system might result from disturbances in the rates of turnover of substances in this pathway, even if the levels of $\gamma$-aminobutyric acid or a product were not significantly altered. Gamma-aminobutyric acid and succinic semialdehyde are reported to support respiration and oxidative phosphorylation in brain tissue preparations (II, 44, 6I, 62) to an extent comparable to glutamic acid and glucose (63). Thus, all the $\gamma$-aminobutyric acid pathway metabolites apparently support oxidative metabolism. Both human (64) and animal (65) studies indicate that cerebral metabolic rate is reduced during pyridoxine deficient seizure activity, and emphasize the importance of pyridoxal phosphate in maintaining normal rates of oxygen consumption in brain (cf. ref. 66). The portion of $\alpha$-ketoglutaric acid which is metabolized through the $\gamma$-aminobutyric acid alternative to succinic acid, rather than directly via succinyl-coenzyme A, has been determined by various investigators to be between 20 and 50 per cent (67-69). Presumably, the portion which passes via the $\gamma$-aminobutyric acid pathway, and the rate of metabolism through this system, are important for central nervous system energy and neuronal activity, but the factors which determine these quantities are incompletely known (68, 70, 71).

It has been proposed that the $\gamma$-aminobutyric acid pathway may serve

a regulatory function for various brain biochemical reactions which are mediated through succinyl-coenzyme A by modifying the available concentration of the latter intermediate (72). The alternative γ-aminobutyric acid pathway, which apparently does not produce succinyl-coenzyme A, may be a more economical one under some circumstances. This may spare succinyl-coenzyme A for use in other brain reactions.

Much work is currently being done to elucidate the significance of γ-aminobutyric acid and its metabolism. Several recent reviews and symposia discuss the biochemical and physiological investigations related to γ-aminobutyric acid (73-79, 139).

## Purpose of the Present Work

The antibiotic cycloserine has been reported to inhibit various transaminases obtained from bacterial (80-82) and animal (83-86) sources. The greatest effects have been demonstrated with glutamic-pyruvic transaminase of rat brain (86), and with glutamic-oxaloacetic transaminase of human brain (83); inhibitions of 50% were obtained with $10^{-6}$ to $10^{-5}$ $\underline{M}$ cycloserine. A structural similarity between γ-aminobutyric acid acid and cycloserine (see p. 37) suggested the possibility of a competitive antagonism between the two substances. In view of these considerations, investigations were carried out on the effects of cycloserine observed <u>in vitro</u> on γ-aminobutyric-α-ketoglutaric transaminase.

It has been reported that $10^{-4}$ $\underline{M}$ D-cycloserine will produce up to

50% inhibition of glutamic acid decarboxylase obtained from E. coli, although the concentration must be increased to $10^{-2}$ M to obtain this degree of inhibition of the enzyme prepared from mouse brain (87). As has been indicated, some substances which inhibit the decarboxylase and lower brain $\gamma$-aminobutyric acid, including isonicotinic acid hydrazide (isoniazid) (88, 89), cause central nervous system excitation. Substances which counteract this excitatory effect inhibit the transaminase and raise brain $\gamma$-aminobutyric acid. Cycloserine exerts effects in the central nervous system, consisting of sedative and excitatory manifestations, observed clinically in man (90-92) and experimentally in animals (93-96). Hence, studies were conducted on the effects of cycloserine observed in vivo on $\gamma$-aminobutyric acid metabolism, as measured by changes in brain concentrations of the amino acid following administration of cycloserine.

## MATERIALS

DL-cycloserine (aminoisoxazolidone) used in these experiments was kindly supplied by K. K. Chen of the Eli Lilly and Company, Indianapolis 6, Indiana. Succinic semialdehyde ethyl ester was a gift of D. P. Wallach of the Upjohn Company, Kalamazoo, Michigan. This may also be obtained from Chemicals Procurement Company, 550 Fifth Avenue, New York 36, New York. All other chemicals were supplied by commercial sources.

METHODS

## Preparation of Bacterial Enzyme

Jakoby and Scott found high activities of $\gamma$-aminobutyric-$\alpha$-ketoglutaric transaminase coupled with succinic semialdehyde dehydrogenase in a strain of Pseudomonas fluorescens, and used this system in an enzymatic assay of $\gamma$-aminobutyric acid (19, 20), by following the reduction of TPN spectrophotometrically (97). Wallach also isolated these enzymes from Escherichia coli, strain ATCC-26, and utilized them for the same purpose (21).

The E. coli organism was maintained on 10 ml slants of nutrient agar at $4^{\circ}$. Each month an innoculum from a slant was grown in nutrient broth at room temperature for 24 hours, and was then transferred to a fresh slant.

For bacterial enzyme preparation, a 100 ml innoculum of cells grown in nutrient broth at room temperature was transferred into eight liters of the same medium. Air at 5 to 7 pounds pressure was bubbled through the medium for 24 hours. Several eight-liter cultures could be prepared at one time.

The cells were harvested in the continuous flow apparatus of a Lourdes centrifuge. The yield was about 3 grams per liter. The cells were washed once in approximately 10 volumes of cold isotonic saline. They were then frozen, and could be kept for months at $-20^{\circ}$ without loss of enzyme activity.

To isolate bacterial enzyme, the cells were thawed and ground in twice their weight of cold alumina A-303. Alumina grinding gave better results than cracking the cells with glass beads, sonication, or protoplast formation with lysozyme and subsequent lysis in distilled water. Three volumes of 0.001 $\underline{M}$ magnesium acetate in 0.001 $\underline{M}$ Tris buffer, pH 7.2, were added in small aliquots during grinding, and the preparation was centrifuged for 10 minutes at about 5000 g to remove alumina and cellular debris. 0.25 volumes of 5% streptomycin were added slowly to the supernatant. The mixture was stirred for 30 minutes, and then centrifuged for 15 minutes at approximately 20,000 g to remove the precipitate of nucleic acid. An equal volume of 0.1 $\underline{M}$ potassium phosphate buffer, pH 7.4, containing 0.01% 2-mercaptoethanol, was then added to the supernatant. The solution was brought to 55% saturation of ammonium sulfate by the slow addition of the solid compound, and the preparation was stirred for one hour. It was then centrifuged for 30 minutes at 34,000 g. This fraction contains some of the desired enzymes, but the procedure removes alcohol dehydrogenase from the subsequent fraction. The latter enzyme might interfere slightly in the assay of brain γ-aminobutyric acid.

Solid ammonium sulfate was slowly added to the supernatant to give a final saturation of 65%. The mixture was stirred for 30 minutes, and then centrifuged for 30 minutes at 34,000 g. The precipitate was dissolved in one volume of 0.1 $\underline{M}$ potassium phosphate buffer, pH 7.4, containing 0.01% 2-mercaptoethanol. This solution, the 55 to 65% ammonium sulfate fraction, contains the needed γ-aminobutyric-α-ketoglutaric

transaminase and succinic semialdehyde dehydrogenase. It was dialyzed

against four liters of phosphate buffer for 24 hours, with one change

of buffer. The dialyzed enzyme preparation was kept at $-20^{o}$. A

purification of the enzyme of about 80 fold is acheived by this method.

All steps in both the bacterial and the brain enzyme extraction

procedures were performed at approximately $4^{o}$.

## Preparation of Brain Enzyme

Brain enzyme was prepared from acetone powders of whole brains

of cats (Felis domesticus) and rhesus monkeys (Macaca rhesus), as

modified from the procedure of Baxter and Roberts for steer brain (10).

Cats were sacrificed by intravenous injection of pentobarbital sodium;

monkeys were given a blow on the head, the great vessels of the neck

were severed, and the animals were exsanguinated. The brains were

rapidly removed, and were either frozen and used within two days, or

were blended immediately in 10 volumes of acetone at $-20^{o}$ in a glass

cup of a Waring blendor. The preparation was filtered in a Buechner

funnel under suction, and the resulting cake was twice blended in 5

volumes of cold acetone and filtered. The powder was stored in a

vacuum desiccator at $4^{o}$ in the presence of silica gel and paraffin

shavings. The crude brain preparation could be stored for several

months in this state without loss of activity.

To isolate brain enzyme, the powder was blended in 10 volumes of

0.1 $\underline{M}$ Tris buffer, pH 8.2. The preparation was centrifuged at 34,000 g

for 30 minutes. The supernatant was brought to 40% saturation by the

slow addition of solid ammonium sulfate, and the mixture was stirred
for one hour. It was then centrifuged--first at 34,000 g for 30
minutes in a Lourdes centrifuge, and then at 105,000 g for 30 minutes
in a Spinco ultracentrifuge--and the precipitate was discarded. The
supernatant was brought to 50% saturation with ammonium sulfate, and
was then centrifuged for 30 minutes at 34,000 g. The precipitate was
dissolved in one volume of 0.1 M Tris, pH 8.2. The solution was
dialyzed for 3 hours against 1000 ml of the Tris buffer with 6 changes
of buffer, and was used on the same day.

Brain enzyme was also prepared directly from fresh tissue. The
brains were removed and blended immediately in 10 volumes of 0.1 M Tris,
pH 8.2. The mixture was centrifuged for 30 minutes at 34,000 g, and
ammonium sulfate fractionations were carried out in the same manner as
were those of the acetone powder preparation. The bulk of the enzyme
activity was contained in a 50 to 60% fraction. Activity was lost with
dialysis. No significant differences were observed in the kinetics and
inhibition of the acetone powder and fresh preparations.

A highly active TPN dependent isocitric dehydrogenase was present
in the partially purified preparations from cat and monkey brain.

## Animal Procedures

Experiments in vivo were conducted with adult male white rats
(Sprague-Dawley strain) and 350 gram male and female guinea pigs (short-
haired, mixed English type). These animals were given varying doses of
cycloserine intraperitoneally. The brains were extracted with ethanol

for γ-aminobutyric acid analysis in a manner similar to that of Baxter and Roberts (35). Each animal was decapitated and the brain was quickly removed and weighed. The whole brain was homogenized in a glass homogenizer with Teflon pestle in 5 volumes of 70% ice cold ethanol. The homogenizer and pestle were rinsed in one volume of ethanol, and the rinsing were added to the homogenate. The homogenate was centrifuged at 5000 g for 15 minutes, and the supernatant was decanted. The precipitate was washed in 4 volumes of ethanol, and the washings were added to the supernatant. The extracts were then evaporated to dryness in a flash evaporator. One ml of distilled water was added to the dried extract for every gram of fresh weight of brain. The resuspended extract was then centrifuged at 105,000 g for 30 minutes in a Spinco ultracentrifuge to remove lipoprotein. Aliquots of supernatant (0.05 to 0.3 ml) were then assayed by the enzymatic method.

Negligible losses of γ-aminobutyric acid are incurred during the procedure (98). Total brain γ-aminobutyric acid is determined, including both free and occluded forms, as described in the biologic assay using the crayfish stretch receptor (28-30).

## Preparation of Reagents

Alpha-ketoglutaric acid was prepared in 0.1 $\underline{M}$ Tris, pH 8.5. This solution was made daily and kept cold to avoid slow degradation. Gamma-aminobutyric acid was dissolved in water, and could be stored indefinitely at refrigerator temperatures. Free succinic semialdehyde was prepared from the ethyl ester by hydrolysis in 1 $\underline{M}$ hydrochloric

acid in a boiling water bath for one hour. The solution was then

cooled to $4^O$, buffered with potassium monohydrogen phosphate, and

brought to pH 6.7 with sodium hydroxide (99, 6). The slightly acid

solution was stable for several weeks at refrigerator temperatures.

Cycloserine was prepared as a 0.01 M solution, and adjusted to pH 8.5

with concentrated ammonium hydroxide. At this pH it can be stored at $4^O$

without loss of activity for three to four days (100). However, it was

prepared daily in order to avoid any possible effects of degradation

products.

## Assay of Gamma-aminobutyric Acid

The mixture added to a 3 ml Beckman cuvette for the determination

of enzyme activity was as follows (21): 6 μmoles each of $\gamma$-aminobutyric

acid and $\alpha$-ketoglutaric acid (or 6 μmoles of succinic semialdehyde);

approximately 600 μmoles of Tris buffer, pH 8.5; 5 μmoles of

2-mercaptoethanol; I mg of TPN or DPN; 0.1 ml of enzyme; water to a

final volume of 3 ml. Appropriate changes in amounts of substrate and

enzyme, and additions of cycloserine, were made when needed in the

various kinetic and inhibition studies, in the preparation of standard

curves for the assay of $\gamma$-aminobutyric acid, and in the assay of unknown

samples. A new standard curve, a typical example of which is shown

in fig. 2, was constructed whenever an assay was to be carried out, in

order to compensate for slight changes in enzyme activity with

different preparations and with freezing and thawing of the enzyme.

Unknown samples contained 0.05 to 1.0 μmoles of $\gamma$-aminobutyric acid.

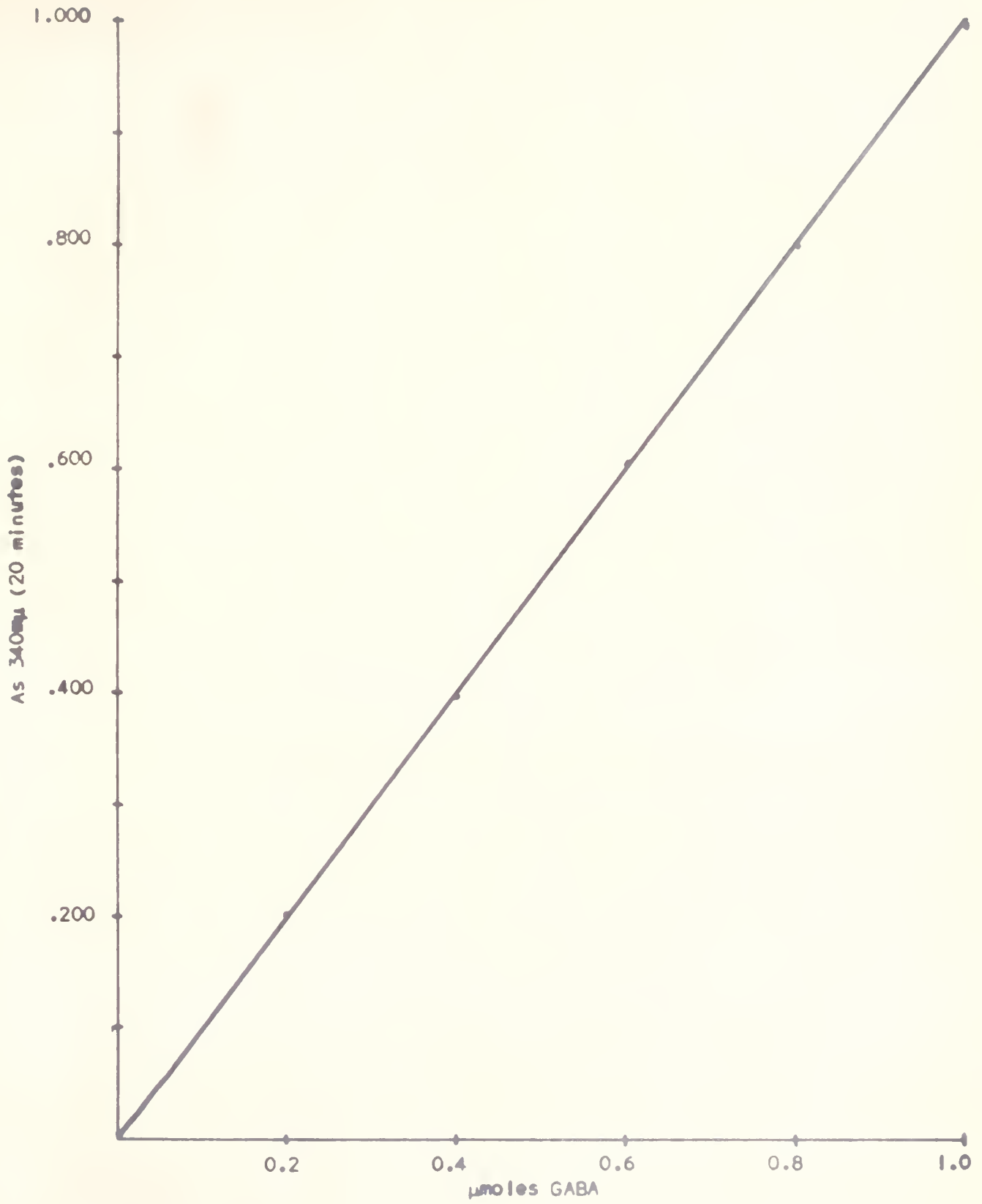Fig. 2. Standard curve for assay of GABA.

All reactions were carried out at room temperature.

The production of glutamic acid was determined by unidimensional paper chromatography using N-butanol-acetic acid-water (2:1:1) as solvent and ninhydrin as developer. Reaction mixtures were also assayed for the disappearance of $\alpha$-ketoglutaric acid by the semicarbazide method of MacGee and Doudoroff (101).

RESULTS

## Properties of the Enzyme System Observed in vitro

### pH Range and Stability

(a) Bacterial:  The enzyme system prepared from E. coli was most
stable in the pH range of 7.2 to 7.5.  At slightly acid pH's it was
rapidly inactivated.  If the enzyme was not dialyzed it lost its
activity in a few days.  After dialysis, which removed excess ammonium
sulfate and adjusted the pH to the optimum range, the enzyme system
was stable at $-20^{\circ}$ for two to three months, providing excessive tnawing
and refreezing of the preparation was avoided.

The bacterial enzyme system was most active in a reaction mixture
at a pH of 8.2 to 8.5.

(b) Brain:  The enzyme system obtained from cat and monkey brain
had an optimum pH range of 8.2 to 8.5 for both stability and activity.
No significant differences were observed between the cat and the monkey
preparations, nor between the acetone powder and the fresh preparations,
in any of the studies.

Succinic semialdehyde dehydrogenase was far more stable under the
conditions of isolation employed than was $\gamma$-aminobutyric-$\alpha$-ketoglutaric
transaminase.  The purified enzyme could be kept at $-20^{\circ}$ for at least two
months without loss of the dehydrogenase activity, whereas it could not
be frozen and had to be used on the day of preparation in order to

retain the transaminase activity. Perhaps the addition of small
amounts of $\gamma$-aminobutyric acid during the purification procedure would
increase the stability of the brain enzyme.

### Specificity

(a) Bacterial: The enzyme system isolated from E. coli did not
reduce TPN in the presence of D-alanine, L-alanine, $\beta$-alanine, or
2,4-diaminobutyric acid. TPN was reduced about five times more rapidly
than was DPN with either $\gamma$-aminobutyric acid and $\alpha$-ketoglutaric acid,
or with succinic semialdehyde, as substrates.

(b) Brain: In contrast to the findings of Albers et. al. (12, 13),
TPN was reduced by the enzyme prepared from cat and monkey brain with
succinic semialdehyde as substrate; however, DPN was reduced twice as
rapidly. With $\gamma$-aminobutyric acid and $\alpha$-ketoglutaric acid as substrates,
DPN was reduced about ten times as rapidly as TPN. These observations
might be explained by the presence of small amounts of pyridine
nucleotide transhydrogenase in the partially purified enzyme preparations
(102, 103), along with traces of DPN in the commercial TPN used.

### Metal and Cofactor Requirements

(a) Bacterial: No effect on the velocity of TPN reduction by the
bacterial enzyme system was observed with the addition of $10^{-3}$ $\underline{M}$ $Mg^{++}$
or $Mn^{++}$. Slight inhibition was seen in the presence of $10^{-3}$ $\underline{M}$ $Zn^{++}$.
It was unnecessary to add pyridoxal phosphate for the transamination
reaction to proceed.

(b) Brain:  The addition of pyridoxal phosphate was not required

with the crude brain enzyme, but added coenzyme increased the activity

about twofold as the preparation was further purified.  The optimum

pyridoxal phosphate concentration was approximately $4 \times 10^{-4}$ $\underline{M}$ (cf.

ref. 10).  The transamination reaction was inhibited slightly by excess

pyridoxal phosphate.  No effect was observed with the addition of ADP,

GDP, or UDP and inorganic phosphate at concentrations of up to $3.3 \times 10^{-3}$ $\underline{M}$.

Inhibition by Cycloserine

(a) Bacterial:  Cycloserine inhibited $\gamma$-aminobutyric-$\alpha$-ketoglutaric

transaminase of $\underline{E.\ coli}$ when the reaction was started by the addition

of enzyme (table I, fig. 3).  When cycloserine was allowed to remain in

contact with the enzyme for 5 minutes in the absence of $\gamma$-aminobutyric

acid, and the reaction was then started by the addition of the latter

substrate, the inhibition was greatly enhanced (table I, fig. 3).  The

degree of inhibition was proportional to the length of time of incubation

of enzyme and cycloserine prior to the addition of $\gamma$-aminobutyric acid

at constant cycloserine concentration (table 2, fig. 4).  When enzyme

and cycloserine were incubated in the presence of $\gamma$-aminobutyric acid,

and the reaction was then started after several minutes by the addition

of $\alpha$-ketoglutaric acid, the inhibition was only slightly greater than

that without prior incubation.  More pronounced inhibition was

observed with low concentrations of $\gamma$-aminobutyric acid (excess

$\alpha$-ketoglutaric acid) than with low concentrations of $\alpha$-ketoglutaric acid

(excess $\gamma$-aminobutyric acid), as may be seen by comparing the slopes

Table 1.  DL-cycloserine inhibition[*] of bacterial $\gamma$-aminobutyric-$\alpha$-ketoglutaric transaminase without incubation and with five minute incubation of enzyme and cycloserine in the absence of $\gamma$-aminobutyric acid.  The reaction mixture contained 6 $\mu$moles each of $\gamma$-aminobutyric acid and $\alpha$-ketoglutaric acid.

| Concentration of cycloserine | Per cent inhibition with no incubation | Per cent inhibition with five minute incubation |
|---|---|---|
| $3.3 \times 10^{-5}$ M | ——— | 25% |
| $3.3 \times 10^{-4}$ M | ——— | 50% |
| $6.5 \times 10^{-4}$ M | < 20% | 75% |
| $1.6 \times 10^{-3}$ M | 40% | > 95% |
| $3.3 \times 10^{-3}$ M | 60% | ——— |

[*]Per cent initial velocity with inhibitor present as compared to that with no inhibitor present, minus 100%.

Table 2.  Per cent inhibition of bacterial $\gamma$-aminobutyric-$\alpha$-ketoglutaric transaminase as a function of time of incubation of enzyme and cycloserine ($6.5 \times 10^{-4}$ M) in the absence of $\gamma$-aminobutyric acid.

| Time of incubation in minutes | Per cent inhibition |
|---|---|
| 0 | < 20% |
| 1 | 20% |
| 2 | 33% |
| 5 | 75% |
| 10 | 90% |
| 15 | 95% |
| 30 | 100% |
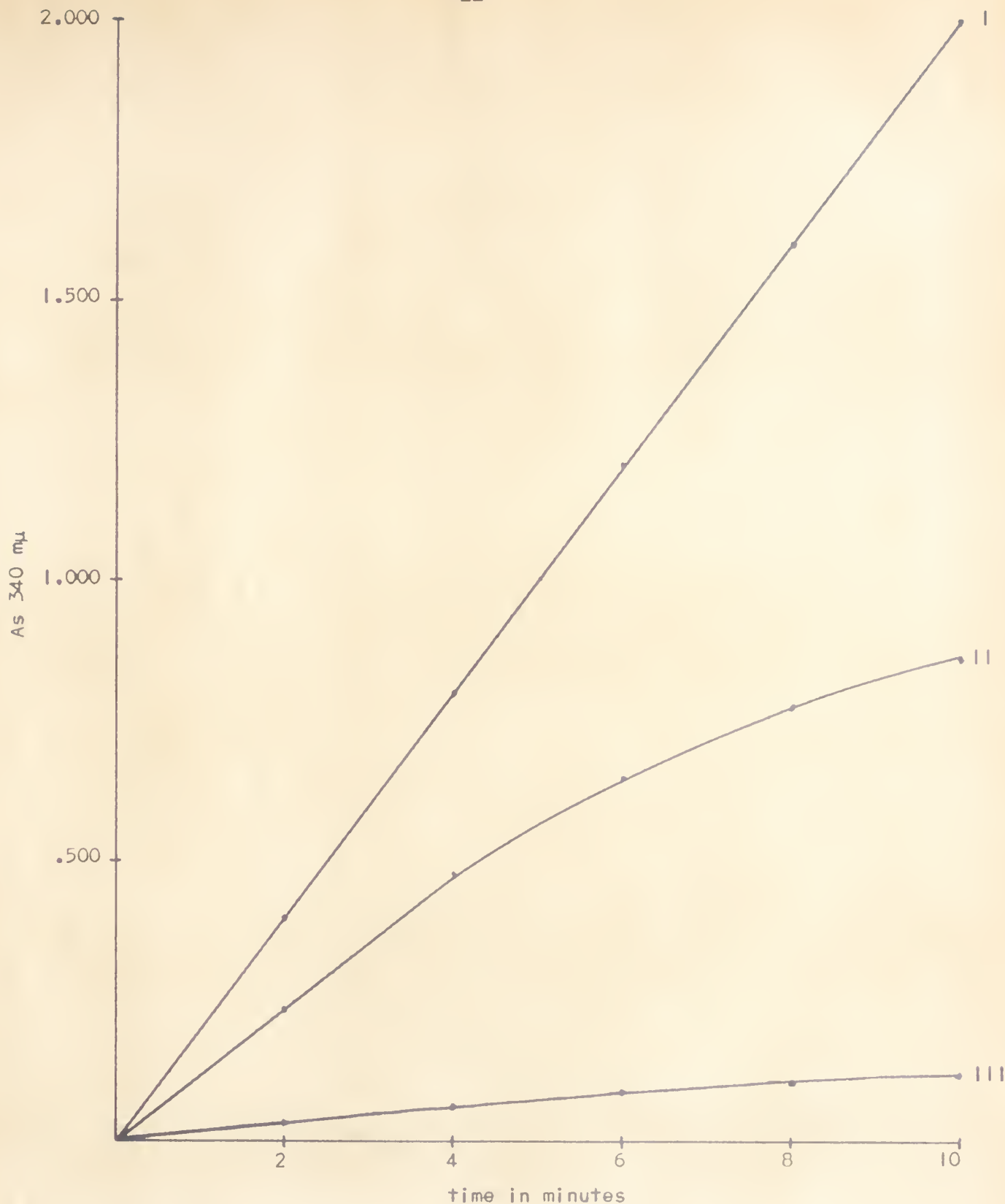
Fig. 3. Cycloserine inhibition of bacterial $\gamma$-aminobutyric-$\alpha$-ketoglutaric tramsaminase. The reaction mixture contained 6 $\mu$moles each of GABA and $\alpha$-KA. I: no cycloserine; II: $1.6 \times 10^{-3}$ M cycloserine, no incubation (40% inhibition); III: $1.6 \times 10^{-3}$ M cycloserine, five minute incubation of enzyme and inhibitor in the absence of GABA ( > 95% inhibition).
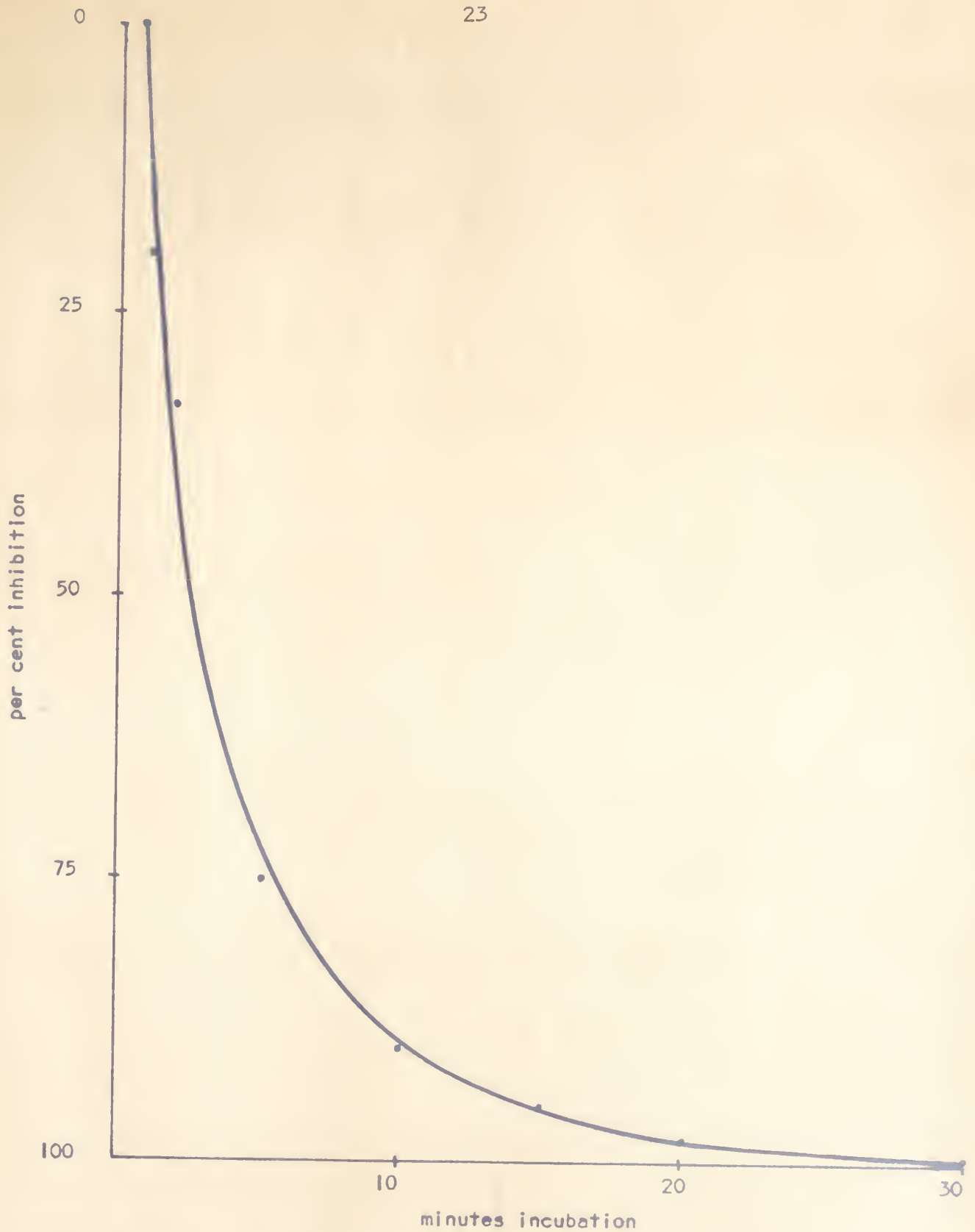
Fig. 4. Per cent inhibition as a function of time of incubation of bacterial enzyme and inhibitor in the absence of GABA. The reaction mixture contained 6 μmoles each of GABA and α-KA, with a cycloserine concentration of $6.5 \times 10^{-4}$ M. The relationship approximates the equation of a rectangular hyperbola.

with and without inhibitor in the Lineweaver-Burk plots. Thus, the
presence of $\gamma$-aminobutyric acid, but not $\alpha$-ketoglutaric acid, partially
protects the enzyme from cycloserine inhibition.

Enzyme concentrations were plotted against initial velocity of the
reaction (Ackermann and Potter, 104), with 5 minute incubation of
enzyme and cycloserine prior to starting the reaction by the addition
of $\gamma$-aminobutyric acid. The intercepts of the slopes remained constant
at the origin, but the slopes decreased with increasing concentrations
of cycloserine (fig. 5). This demonstrated that the inhibition was
apparently reversible under these conditions.

A Lineweaver-Burk plot (105) revealed that the bacterial transaminase
inhibition by cycloserine was competitive with respect to $\gamma$-aminobutyric
acid (fig. 6). The Michaelis-Menton constant (Km) for $\gamma$-aminobutyric
acid (excess $\alpha$-ketoglutaric acid) and the inhibitor constant (Ki) were
of nearly the same value (table 3). Hence, with competitive inhibition
it was anticipated that approximately 50% inhibition would be produced
with equal concentrations of $\gamma$-aminobutyric acid and cycloserine. It
may be observed that this is so by reference to table I, in which it
can be seen that a concentration of $2 \times 10^{-3}$ $\underline{M}$ cycloserine (6 $\mu$moles)
could be expected to produce an inhibition of about 50% in the presence
of the same concentration of $\gamma$-aminobutyric acid. However, when
excess $\gamma$-aminobutyric acid was added, the inhibition was not completely
reversed.

The measurements for the Lineweaver-Burk plot were obtained with
reactions which were started by the addition of enzyme. When enzyme
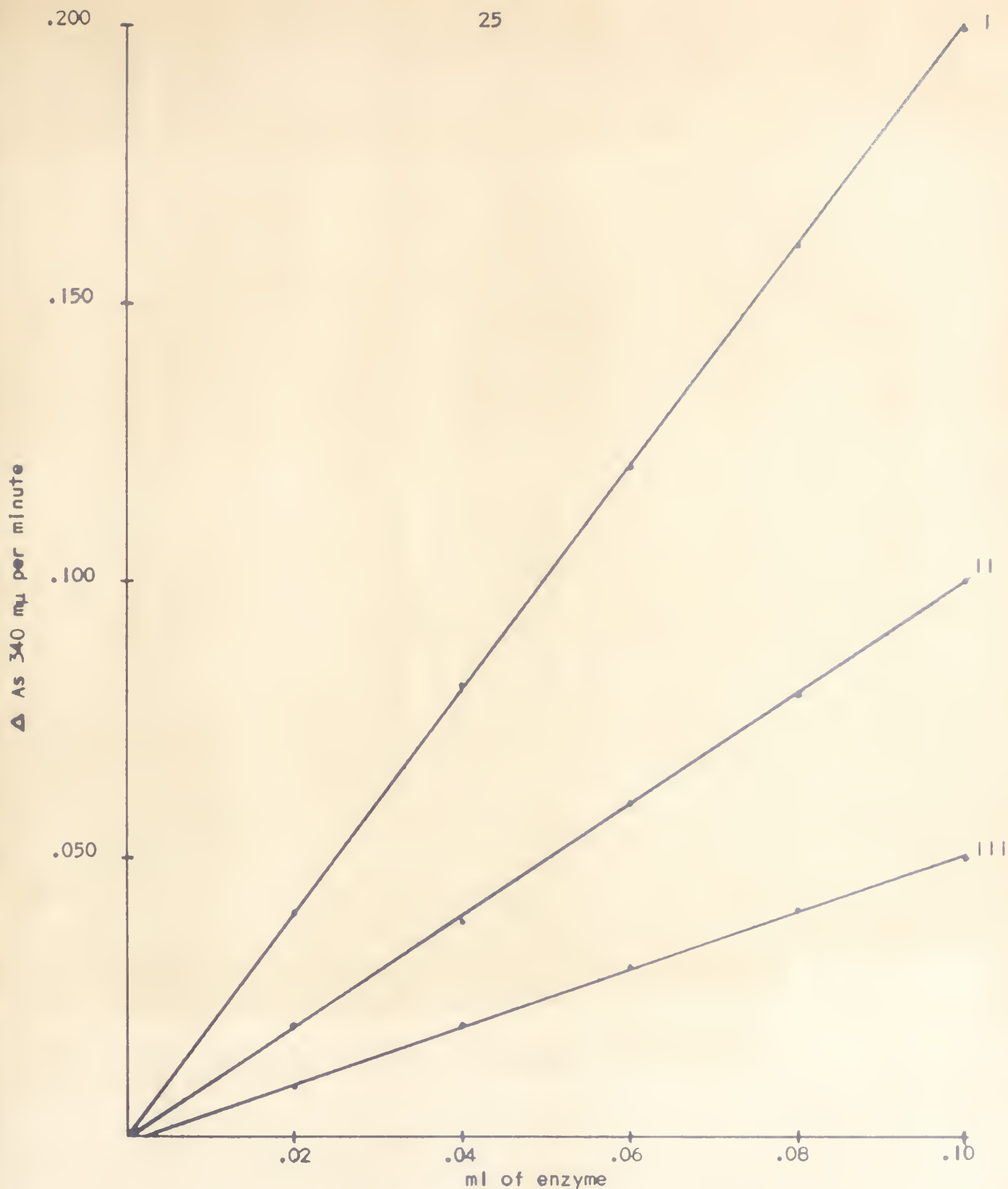
Fig. 5. Ackermann-Potter graph, showing reversible inhibition of bacterial
γ-aminobutyric-α-ketoglutaric transaminase by cycloserine. Enzyme and
inhibitor were incubated in the absence of GABA for five minutes before the
reaction was started. 6 μmoles each of GABA and α-KA were present in the
reaction mixture. I: no cycloserine; II: $3.3 \times 10^{-4}$ $\underline{M}$ cycloserine (50%
inhibition); III: $6.5 \times 10^{-4}$ $\underline{M}$ cycloserine (75% inhibition).
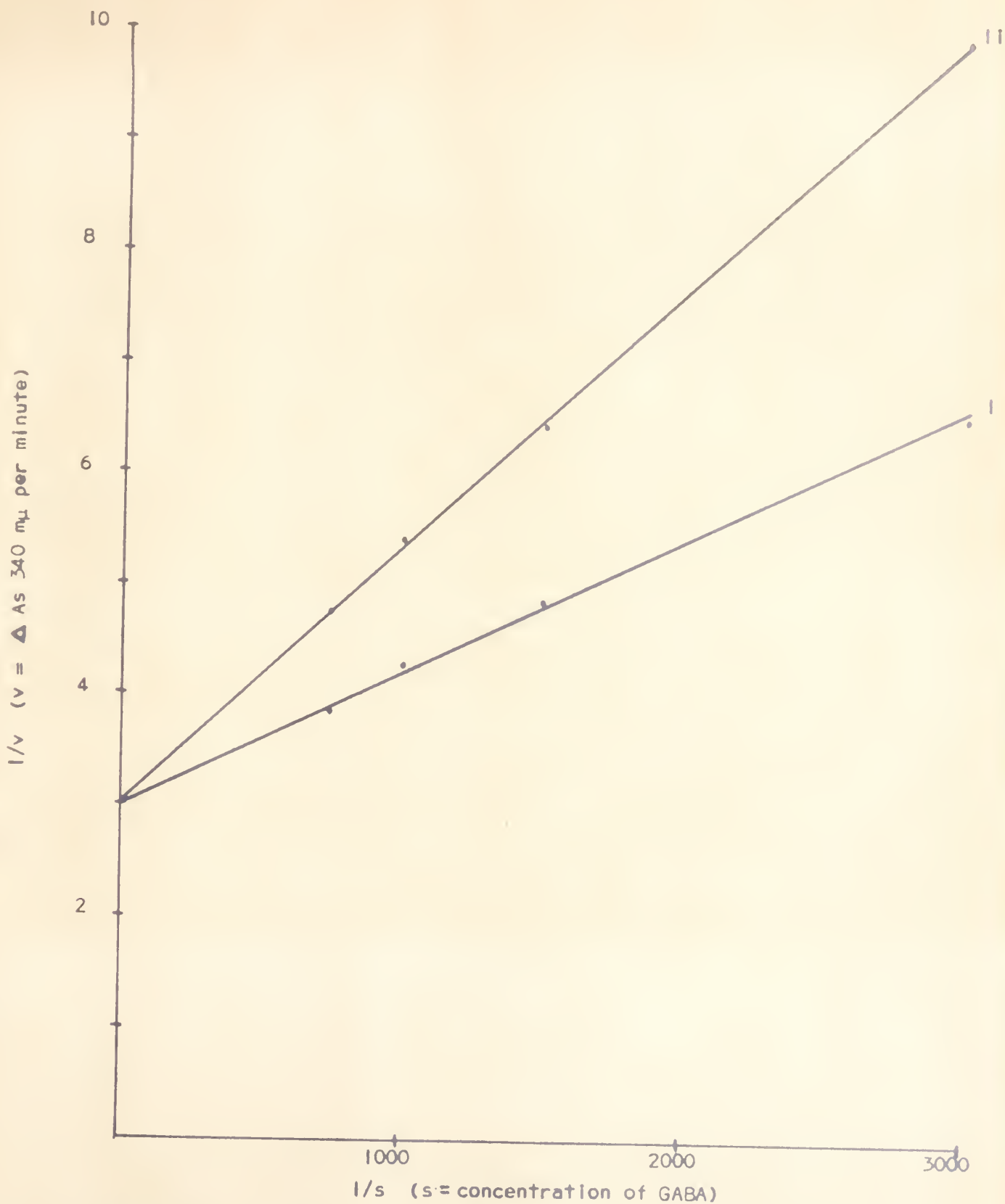
Fig. 6. Reciprocal of velocity of the reaction, catalyzed by bacterial enzyme, as a function of reciprocal of GABA concentration (I), and inhibition by cycloserine, $3.3 \times 10^{-4}$ M (II). Enzyme and cycloserine were not incubated before the reaction was initiated. The inhibition is competitive with respect to GABA.

Table 3.  Michaelis-Menton constants (Km) and inhibitor constants (Ki)
for bacterial and brain transaminases, as obtained from Lineweaver-
Burk plots (figs. 6, 7, 9, 10).  Substrates were $\gamma$-aminobutyric acid
(with 6 $\mu$moles of $\alpha$-ketoglutaric acid) or $\alpha$-ketoglutaric acid (with
6 $\mu$moles of $\gamma$-aminoglutaric acid).  Reactions were carried out at pH
8.4 and TPN (bacterial enzyme) or DPN (brain enzyme) concentrations
of $4 \times 10^{-4}$ $\underline{M}$.  Cycloserine, $3.3 \times 10^{-4}$ $\underline{M}$, was not incubated with the
enzymes prior to the initiation of the reactions.

|  | Substrate | Km | Ki |
|---|---|---|---|
| Bacterial transaminase | GABA | $4.2 \times 10^{-4}$ | $4.0 \times 10^{-4}$ |
|  | $\alpha$-KA | $5.0 \times 10^{-4}$ | $7.7 \times 10^{-4}$ |
| Brain transaminase | GABA | $4.5 \times 10^{-4}$ | $2.3 \times 10^{-4}$ |
|  | $\alpha$-KA | $6.7 \times 10^{-4}$ | $2.7 \times 10^{-4}$ |

and cycloserine were allowed to remain in contact for various intervals of time prior to the addition of $\gamma$-aminobutyric acid, the inhibition became mixed competitive and non-competitive, according to Lineweaver-Burk plots, and the calculated Ki values were somewhat smaller. Very little or no reversal of the inhibition was effected by the addition of excess $\gamma$-aminobutyric acid (12 $\mu$moles) under these conditions.

Cycloserine inhibition was non-competitive with respect to $\alpha$-ketoglutaric acid when the reaction was started by the addition of enzyme (fig. 7, table 3). When enzyme and cycloserine were incubated for several minutes prior to the initiation of the reaction by the addition of $\gamma$-aminobutyric acid, the inhibition was of a much greater degree, as in the previous experiments, but remained non-competitive with respect to $\alpha$-ketoglutaric acid, according to Lineweaver-Burk plots.

The inhibition produced by cycloserine was not reversed by the addition of pyridoxal phosphate, glutathione, or cysteine at concentrations of $5 \times 10^{-3}$ $\underline{M}$, nor by $10^{-3}$ $\underline{M}$ $Mg^{++}$, $Mn^{++}$, or $Zn^{++}$. Cycloserine at concentrations of $10^{-4}$ to $10^{-2}$ $\underline{M}$ had no effect on the bacterial succinic semialdehyde dehydrogenase, as shown by the absence of inhibition when succinic semialdehyde was the substrate. The presence of hydroxylamine, an acid hydrolysis product of cycloserine (106, 107), and a potent inhibitor of $\gamma$-aminobutyric-$\alpha$-ketoglutaric transaminase (35-37), was not revealed by the acetic anhydride-ferric chloride test (108), even when cycloserine had been stored at $4^{\circ}$ for two months. DL-serine, the other acid hydrolysis product (106, 107), produced no inhibitory effect on either the transaminase or the
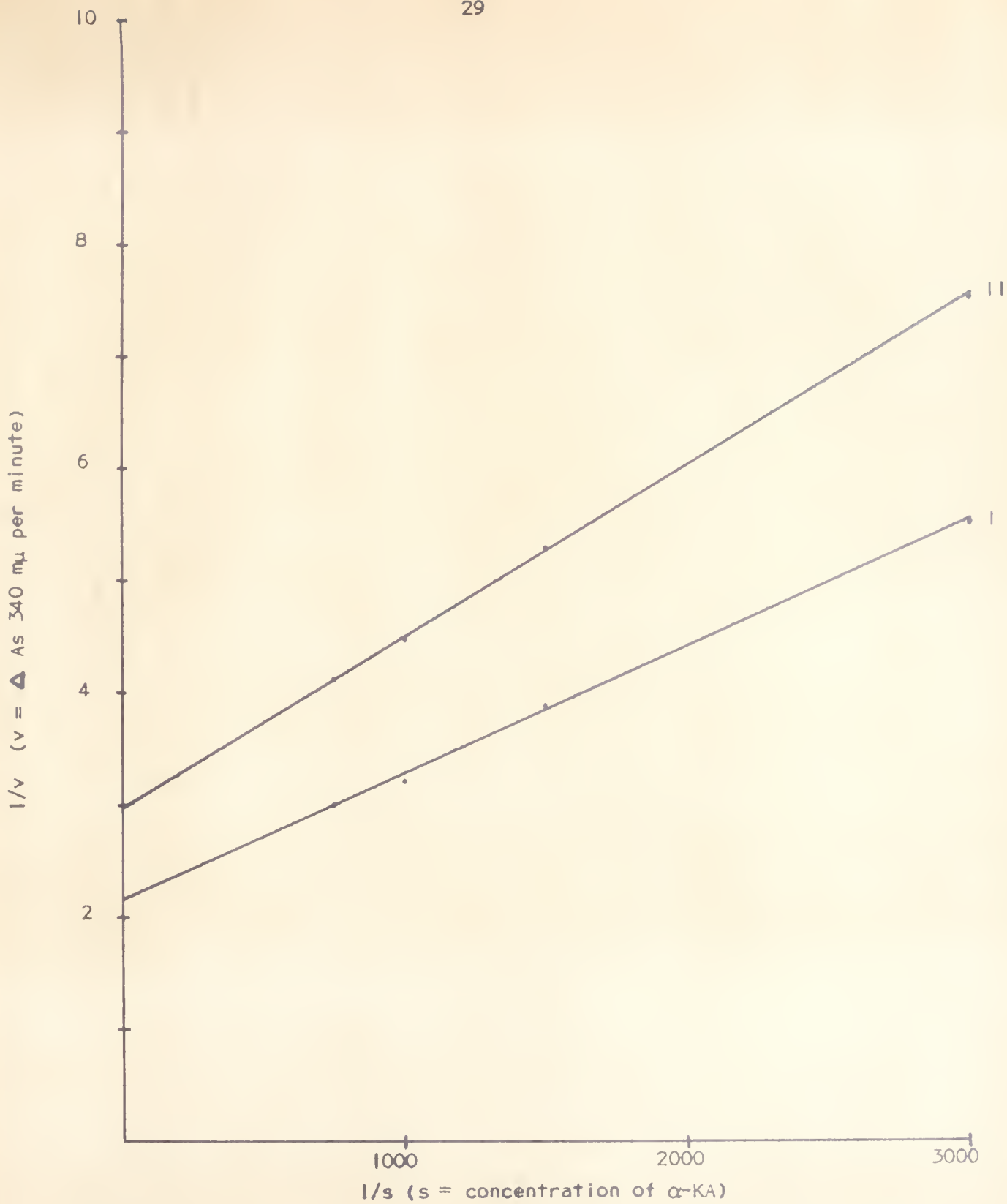
Fig. 7. Same as figure 6, but with reciprocals of α-KA concentrations on the abscissa. I: no cycloserine; II: $3.3 \times 10^{-4}$ $\underline{M}$ cycloserine. The inhibition is non-competitive with respect to α-KA.

dehydrogenase at concentrations of $10^{-2}$ M.

(b) Brain: Cycloserine inhibited γ-aminobutyric-α-ketoglutaric transaminase of cat and monkey brain to a greater extent than the bacterial preparation when the reaction was started by the addition of enzyme. The inhibition was 50% at 6.5 × $10^{-4}$ M and 90% at 1.6 × $10^{-3}$ M cycloserine concentrations (cf. table I). Incubation of enzyme and cycloserine prior to the addition of γ-aminobutyric acid produced increased inhibition, although not to as marked a degree as with the E. coli enzyme. Equimolar amounts of cycloserine and pyridoxal phosphate were premixed and allowed to remain in contact for various intervals of time before being added to the reaction mixture. This procedure resulted in slightly increased inhibition. Inhibition was increased to a similar slight extent when cycloserine and pyridoxal phosphate, plus enzyme and γ-aminobutyric acid, were premixed, and then the reaction was started after several minutes by the addition of DPN and α-ketoglutaric acid. Isoniazid, which inhibits some pyridoxal phosphate dependent enzymes (109-112), had no effect on the reaction at concentrations of $10^{-3}$ M.

Cycloserine inhibition of the brain transaminase was of the reversible type, under the conditions recorded in fig. 8, as shown by an Ackermann-Potter plot (104). The inhibition was mixed competitive and non-competitive with respect to γ-aminobutyric acid, whether or not enzyme and cycloserine were allowed to remain in contact prior to the addition of the amino acid (fig. 9). Cycloserine inhibition was non-competitive with respect to α-ketoglutaric acid (fig. 10). The Km
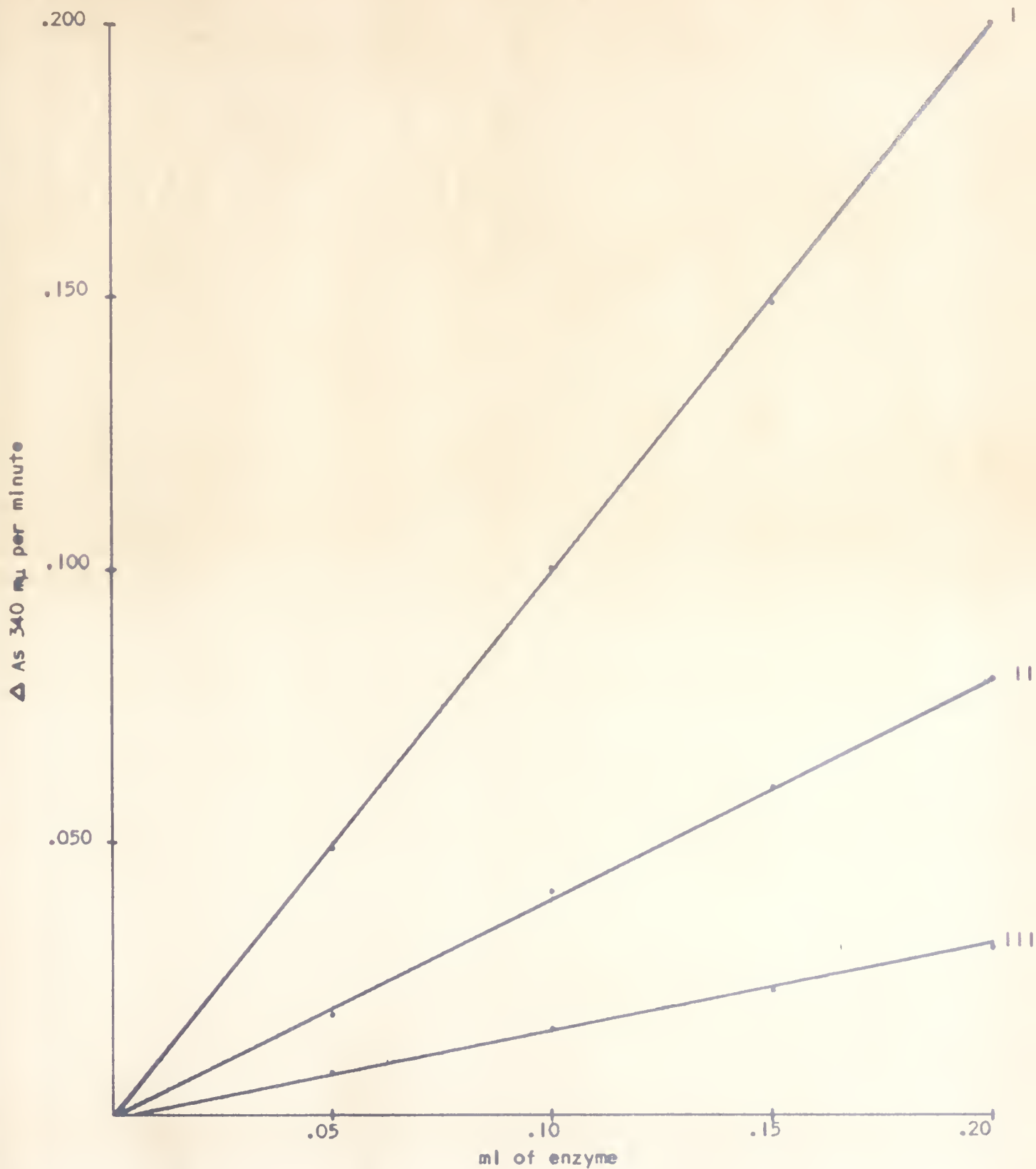
Fig. 8. Ackermann-Potter graph, showing reversible inhibition of brain
γ-aminobutyric-α-ketoglutaric transaminase by cycloserine. Five minute incubation
of enzyme and cycloserine in the absence of GABA was carried out before the
reaction was initiated. 6 μmoles each of GABA and α-KA were present in the
reaction mixture. I: no cycloserine; II: 3.3x10⁻⁴ M cycloserine (60% inhibition);
III: 6.5x10⁻⁴ M cycloserine (85% inhibition).
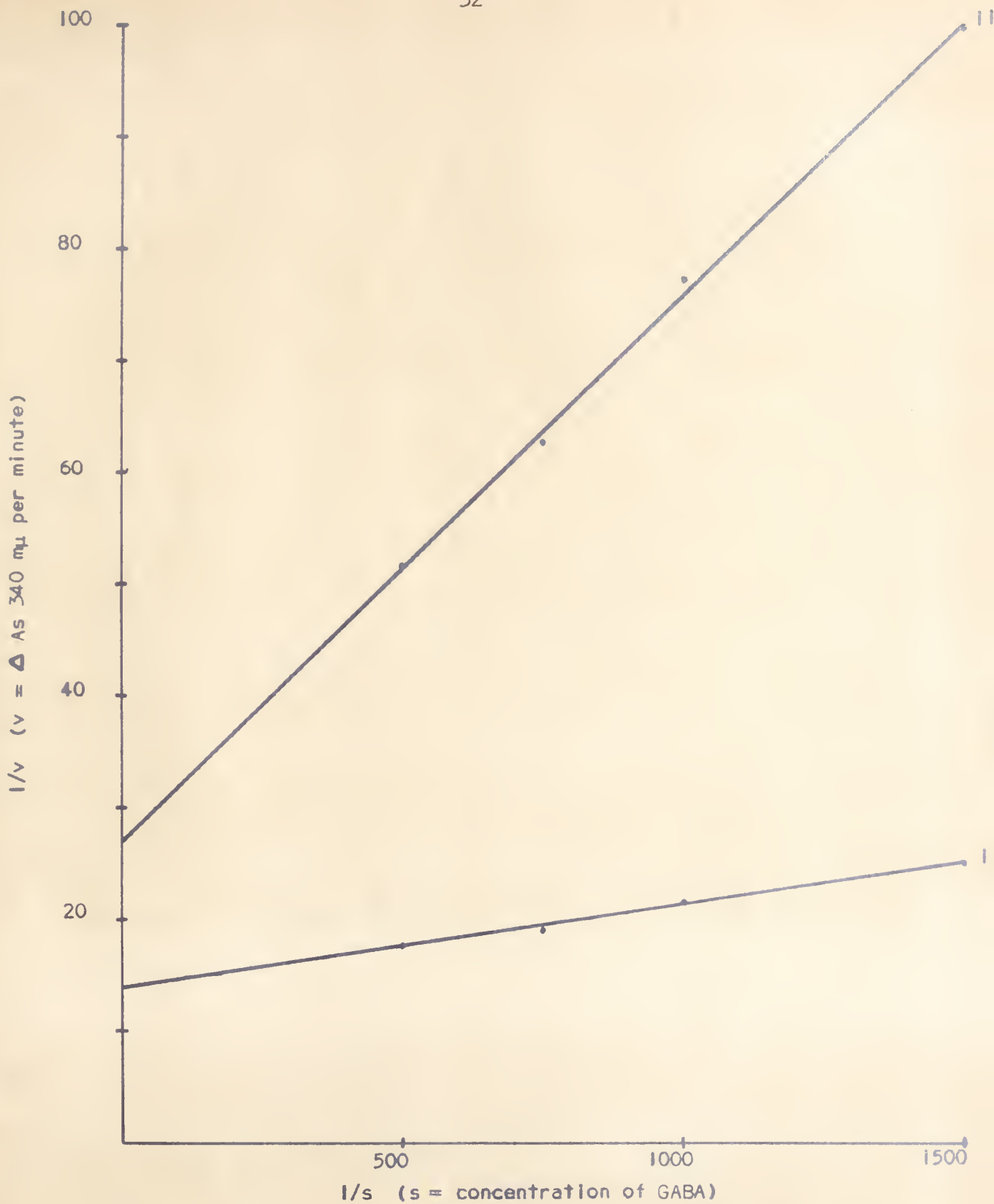
Fig. 9. Lineweaver-Burk plot with brain enzyme, using GABA as the independent variable. I: no cycloserine; II: $3.3 \times 10^{-4}$ $\underline{M}$ cycloserine. The inhibition is mixed competitive and non-competitive.
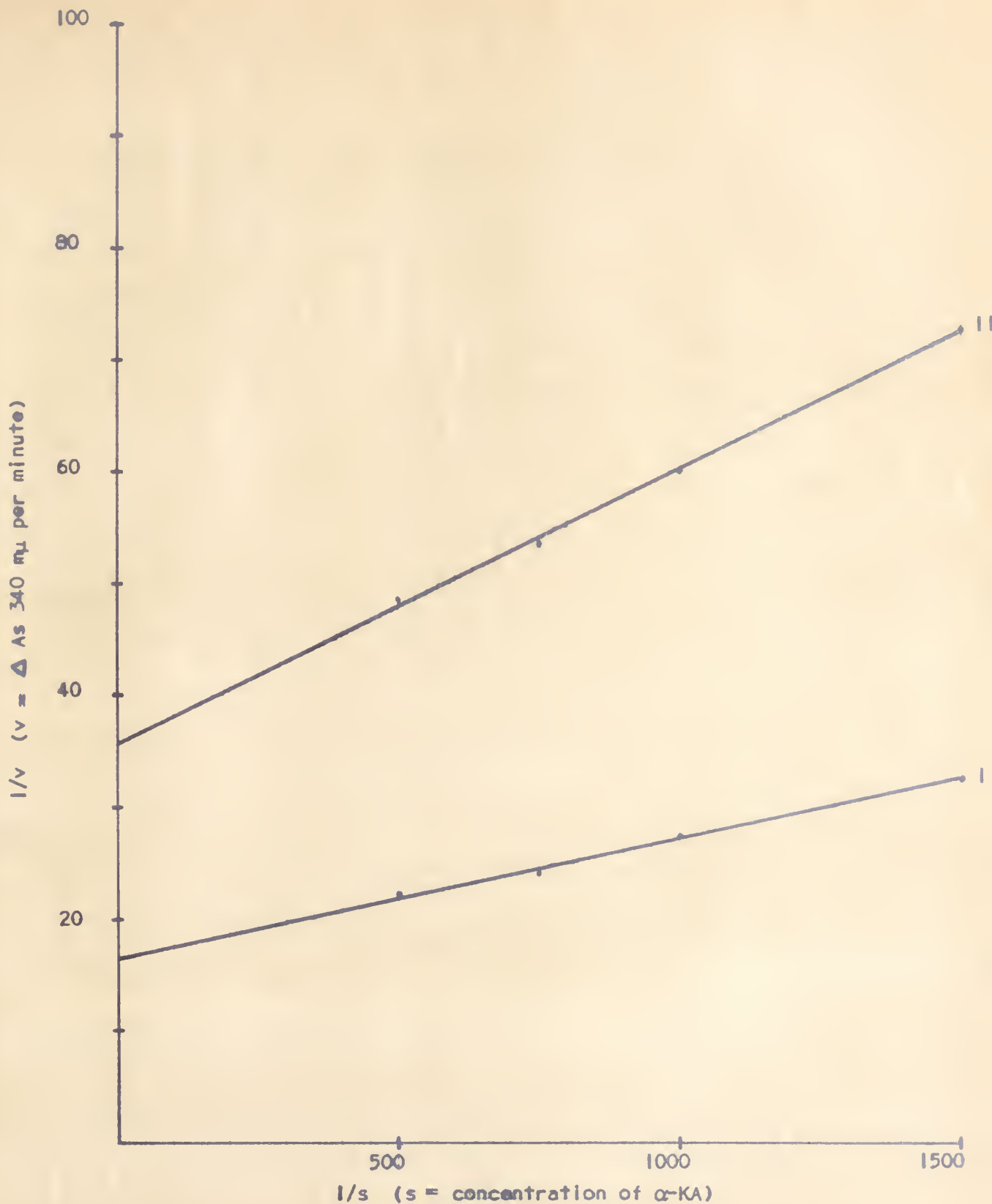
Fig. 10. Same as figure 9, but with α-KA as the independent variable. I: no cycloserine; II: 3.3x10$^{-4}$ M cycloserine. The inhibition is non-competitive.

and Ki values for each of these reactants is shown in table 3.

The inhibition produced by cycloserine could not be reversed by excess pyridoxal phosphate. Cycloserine at $10^{-2}$ $\underline{M}$ concentration had no effect on the brain succinic semialdehyde dehydrogenase.

Cycloserine inhibition of both the bacterial and the brain transaminases was enhanced with greater enzyme purification, and diminished with greater age of the preparations (cf. ref. 86). This may be due to non-specific binding of cycloserine by sites other than those of the active enzyme, or to inactivation of the drug by the crude or aged preparations.

The enzyme kinetics and inhibition studies on both the bacterial and the brain preparations are thought to be relevant to the transaminase, even though the rates were actually measured by the dehydrogenase reaction, because (a) the dehydrogenase is not rate limiting since it has a very small Km (13, 72), and (b) cycloserine has no inhibitory effect on the dehydrogenase. However, it is possible that some differences would appear if the rate of the transaminase reaction were determined directly.

## Cycloserine Effects Observed in vivo

Cycloserine injected intraperitoneally in rats and guinea pigs resulted in an increase in brain $\gamma$-aminobutyric acid concentration which was proportional to dosage (table 4). This occurred whether the drug was given in a single dose or over a period of several days. Prolonged administration produced higher brain $\gamma$-aminobutyric acid

Table 4.  The effect of cycloserine observed <u>in vivo</u> on brain γ-aminobutyric acid concentrations in the rat and the guinea pig.

| Animal | No. of animals per group | Dose of cycloserine (mg/100gm body wt.) | Interval before sacrifice | GABA in brain ($\mu$moles/gm) |
|---|---|---|---|---|
| Rat | 2 | saline | 3 hr., 2 da. | 2.5 |
| | 1 | 80 | 3 hr. | 2.8 |
| | 2 | 600* | 2 da. | 7.3 |
| Guinea pig | 2 | saline | 3 hr., 7 da. | 1.3 |
| | 1 | 300 | 3 hr. | 1.5 |
| | 1 | 450 | 3 hr. | 1.8 |
| | 1 | 700 | 3 hr. | 2.4 |
| | 1 | 1000† | 7 da. | 3.4 |

*Total amount per 100gm body weight given in 125 mg doses four times a day.

†Total amount per 100gm body weight given in 250 mg doses twice a day.

concentrations and more marked toxic side effects. This is probably attributable to a larger total amount of drug given, and to the effect of more sustained blood levels.

Physical manifestations seen following cycloserine administration were similar in both species. Animals appeared moderately lethargic and sedated, but exhibited generalized twitchings at variable 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 (cf. refs. 93, 94). None of these effects was observed in the saline injected controls.

DISCUSSION

Studies in vitro

Structural similarities of cycloserine and γ-aminobutyric acid suggest a basis for the competitive inhibition of the transaminase by the antibiotic.



γ-aminobutyric acid

cycloserine (tautometric forms)
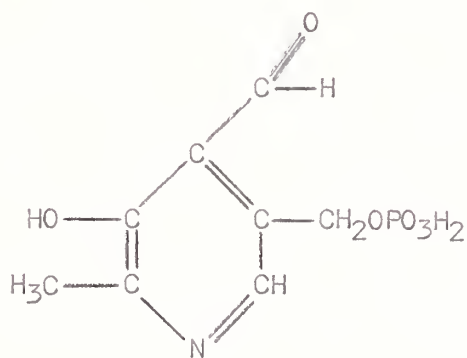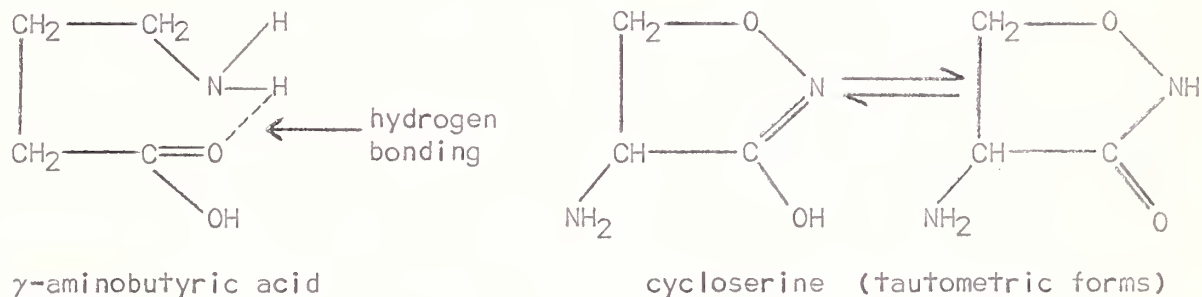


pyridoxal phosphate

The inhibition depended on the length of time of incubation of cycloserine and enzyme prior to the addition of γ-aminobutyric acid, and on the concentration of the latter substance in the reaction

mixture. Also, the inhibition was not completely reversed with excess $\gamma$-aminobutyric acid, even when the reaction was started by the addition of enzyme. These findings indicate that competitive inhibition with respect to $\gamma$-aminobutyric acid does not completely explain the interaction of cycloserine and $\gamma$-aminobutyric-$\alpha$-ketoglutaric transaminase, and suggest that a second type of inhibition is occurring.

Pyridoxal phosphate and cycloserine are reported to form a Schiff base in vitro (82). It has been demonstrated that non-enzymatic indole formation from tryptophan, in the presence of pyridoxal, is inhibited by high concentrations of cycloserine ($10^{-3}$ to $10^{-2}$ $\underline{M}$) (113, 80), and that cycloserine inhibition of tryptophanase, a pyridoxal phosphate dependent enzyme, varies with the length of time of incubation of the drug and the coenzyme prior to the initiation of the reaction (82, 113). Several investigators have shown that other pyridoxal phosphate dependent enzymes are inhibited by high concentrations of cycloserine, and have suggested that the inhibition is due to Schiff base formation between the antibiotic and pyridoxal phosphate (114, 80). It is unlikely that this is an important mechanism in the inhibition of $\gamma$-aminobutyric-$\alpha$-ketoglutaric transaminase, since premixing of cycloserine and pyridoxal phosphate gave only slightly increased inhibition of the brain transaminase (cf. refs. 115-117).

It has also been suggested that Schiff base formation may play a significant role in cycloserine inhibition of pyridoxal phosphate dependent enzymes through a stabilization by the apoenzyme of the

otherwise readily dissociable Schiff complex (118, 119). In the present study, however, the inhibition was again only slightly increased when all components except α-ketoglutaric acid were mixed and allowed to remain in contact for several minutes, and then the reaction was begun by the addition of the latter substrate. Hence, Schiff base formation between cycloserine and coenzyme is apparently not promoted by the presence of the apoenzyme. Moreover, potent carbonyl reagents which might be expected to form Schiff bases, such as isoniazid and semicarbazid, do not inhibit the transaminase (10)[*].

The addition of pyridoxal phosphate partially reactivates hydroxylamine inhibited brain γ-aminobutyric-α-ketoglutaric transaminase, in which apoenzyme and coenzyme are loosely associated, but has no effect on the inhibited E. coli transaminase, in which apoenzyme and coenzyme are tightly bound (120). This suggests that hydroxylamine is reacting with the coenzyme. But in the present study, added pyridoxal phosphate did not reactivate either the brain or the bacterial transaminase after cycloserine inhibition. Also, incubation of enzyme and cycloserine prior to the addition of γ-aminobutyric acid produced a greater increase in inhibition, as compared to that with no incubation, in the bacterial than in the brain transaminase, whereas the reverse

[*]Isoniazid at concentrations of $10^{-3}$ $\underline{M}$ was found to have no effect on glutamic-oxaloacetic transaminase from human brain, whereas $10^{-5}$ $\underline{M}$ D-cycloserine produced 50% inhibition of this enzyme (83). In another study, significant inhibition of glutamic-oxaloacetic transaminase from pig heart by carbonyl reagents, including isoniazid, was achieved only at high concentrations of inhibitor ($10^{-2}$ $\underline{M}$) (110).

would be expected if the drug were exerting a primary effect on the coenzyme.

It has been proposed that the effect of high concentrations of cycloserine on transaminases may be due to a resemblance between the carbonyl group of the antibiotic and the aldehyde group of pyridoxal phosphate (83, 86). In this respect it should again be noted that in the present study the effect of the inhibitor was not reversed by excess coenzyme. Administration of vitamin $B_6$ reduces but does not eliminate cycloserine toxicity clinically, and animal experiments have not shown that the antibiotic produces or enhances vitamin $B_6$ depletion (91). However, cycloserine, at least in high concentrations, inhibits glutamic acid decarboxylase and many transaminases (80-86), all of which are pyridoxal phosphate dependent, and it is probable that this agent may exert some effect on pyridoxal phosphate in these enzymes.

Cycloserine is known to chelate several divalent metals, most strongly copper and zinc (121). It has been shown that the drug inhibits non-enzymatic indole formation from tryptophan, with copper rather than pyridoxal as cofactor (82). Cycloserine inhibits the copper containing enzyme ascorbic acid oxidase (82), although there is some question as to whether copper activates or inhibits this enzyme (122, 123). Of several dehydrogenases examined in tubercle bacilli, cycloserine inhibited only malic dehydrogenase (124). It has been reported that this enzyme contains copper and zinc in mammalian preparations (125). Various catalases prepared from both bacterial and mammalian sources are inhibited by cycloserine (124, 126, 127).

These enzymes are said to contain or be activated by copper (128, 129). No evidence was obtained in the present study, however, that the drug acts by chelating copper or some other divalent metal necessary for the activation of $\gamma$-aminobutyric-$\alpha$-ketoglutaric transaminase (cf. ref. 130). This could be investigated further by studying the effect of chelating agents, such as ethylenediaminetetraacetic acid, on a more purified preparation of the enzyme.

The fact that the degree of inhibition progressively increased and eventually became complete after 30 minutes of incubation of enzyme and cycloserine in the absence of $\gamma$-aminobutyric acid (table 2, fig. 4), suggests that a slow, perhaps irreversible inhibition was occurring. The flattening out of curve II, fig. 3, is indicative of this slow, progressive inhibition, which is also manifested when the reaction is begun by the addition of enzyme, for as the reaction proceeds and $\gamma$-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 $\gamma$-aminobutyric acid. The inhibition of the enzyme when incubated with cycloserine for several minutes was not reversed by the addition of substrate, coenzyme, or any of various possible cofactors or activators.

This apparent irreversibility, however, is not consistent with the Ackermann-Potter plots (figs. 5 and 8), which indicate that the inhibition is reversible. Incubation of enzyme and cycloserine for 10 minutes prior to the addition of $\gamma$-aminobutyric acid produced slopes which were less steep than those of figs. 5 and 8, but which similarly

passed through the origin. It is possible, however, if incubation were carried out for a longer time than this before the addition of $\gamma$-aminobutyric acid, that an Ackermann-Potter plot would show some titration of enzyme with inhibitor in an irreversible complex. However, it is equally possible that other methods, such as extensive dialysis or dilution (131, 132), would bring about at least a partial reversal of the cycloserine inhibited enzyme.

It is concluded (a) that cycloserine is competitive with respect to $\gamma$-aminobutyric acid, non-competitive with respect to $\alpha$-ketoglutaric acid, and produces a rapid, reversible inhibition, and (b) that cycloserine may react with the apoenzyme, the active center of which is protected by $\gamma$-aminobutyric acid, in a slowly progressive inhibition, which is not readily reversible (cf. refs. 110, 133-135). The lack of complete reversibility of the competitive aspect of inhibition is undoubtedly due to this slow, second type of inhibition. The chemical nature of this latter inhibition is unknown, although it may be due to strong hydrogen bonding or to chelation with a metal group, or possibly to the formation of a covalent bond, in a truly irreversible inhibition.

In view of the demonstrated complex nature of the interaction of cycloserine and $\gamma$-aminobutyric-$\alpha$-ketoglutaric transaminase, it is thought that a more detailed study of the inhibition can profitably be undertaken only with a more highly purified enzyme preparation.

Studies in vivo

The finding that cycloserine increased brain $\gamma$-aminobutyric acid

concentrations in the rat and guinea pig is evidence that _in vivo_ the
drug inhibited the transaminase to a greater extent than the decarboxylase.
This is consistent with the effects observed with the purified enzyme
preparations _in vitro_ (87). Cycloserine is an agent which both raises
brain γ-aminobutyric acid and produces central nervous system excitation,
comparable to the combined effects of hydroxylamine and thiosemicarbazide.
This constitutes further evidence that a simple increase in
γ-aminobutyric acid does not protect against heightened central nervous
system activity, although it does not rule out the possibility that
seizures may be related to a lowered rate of γ-aminobutyric acid
turnover (136).


With the present state of investigation it is not possible to
advance a unified theory for the role of the γ-aminobutyric acid pathway.
Studies on the properties of succinic semialdehyde and its associated
dehydrogenase, both of which are important participants in this pathway,
have only recently been initiated (12, 13, 137). Further elucidation
of the significance of the γ-aminobutyric acid system may be anticipated
with greater knowledge of the properties of the enzymes involved and
of their intracellular localization, and with correlation of these
studies with membrane potentials and electrical events measured
intracellularly (138). The use of other substances with an inhibitory
effect on the γ-aminobutyric acid system could provide valuable information.
One such compound might be the new anti-neoplastic agent, hadacidin (142),
which is structurally similar to cycloserine and γ-aminobutyric acid.

## SUMMARY

Experimental evidence relating to the function of $\gamma$-aminobutyric acid and its metabolism have indicated: (1) that $\gamma$-aminobutyric acid may serve as a neurohumoral inhibitory mediator, (2) that it may be a precursor for various other physiologically and biochemically active substances, and (3) that the rate of its metabolism may be important for central nervous system activity, possibly by regulating available energy.

The fact that several transaminases have been shown to be strongly inhibited by cycloserine, and the structural similarities between the antibiotic and $\gamma$-aminobutyric acid, led to the present study _in vitro_ on the interaction of $\gamma$-aminobutyric-$\alpha$-ketoglutaric transaminase and cycloserine. The reported inhibition of glutamic acid decarboxylase by the drug, and its known central nervous system toxicity, were preliminary to a study of the _in vivo_ effects of cycloserine on brain $\gamma$-aminobutyric acid concentrations.

A method was developed, as modified from Wallach, for the extraction of $\gamma$-aminobutyric-$\alpha$-ketoglutaric transaminase and succinic semialdehyde dehydrogenase from _E. coli_, and this enzyme system was used in an assay of $\gamma$-aminobutyric acid.

The transaminase, as prepared from _E. coli_ and from cat and monkey brain, was inhibited by cycloserine. The initial inhibition was reversible, competitive with respect to $\gamma$-aminobutyric acid, and non-competitive with respect to $\alpha$-ketoglutaric acid. The degree of

inhibition depended on the length of time of incubation of cycloserine and enzyme prior to the addition of $\gamma$-aminobutyric acid. The subsequent addition of the latter substance did not reverse the inhibition of the enzyme when so incubated, although it did partially protect the enzyme from the inhibition if present initially. Hence, two types of inhibition were thought to occur: one was rapid and reversible; the other was slow, progressive, and not readily reversible.

It was believed that cycloserine may have been interacting with the apoenzyme rather than with the coenzyme in the inhibition mechanism.

Cycloserine raised brain $\gamma$-aminobutyric acid concentrations when injected intraperitoneally in rats and guinea pigs. This was attributed to an inhibition of the transaminase in vivo. At the same time the drug produced central nervous system excitatory effects. This was further evidence in disagreement with the theory that $\gamma$-aminobutyric acid acts as an inhibitor of excitatory impulses.

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