Biochemical Pharmacology, Vol. 25, pp. 253–258. Pergamon Press, 1976. Printed in Great Britain.

INHIBITION OF METABOLISM OF β-ALANINE AND D-β-AMINOISOBUTYRIC ACID BY D-CYCLOSERINE

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(*Received* 7 *April* 1975; *accepted* 24 *July* 1975)

Abstract—Significant increases in the concentrations of β-aminoisobutyric acid and β-alanine in the urine of patients under D-cycloserine treatment for tuberculosis were found during a study of biochemical changes caused by surgical operation. β-Alanine increased in all cases on the administration of cycloserine at a clinical dose. β-Aminoisobutyric acid excretion was increased by cycloserine treatment only in the genetic low excretors of this amino acid, but not in the high excretors who lack the degradative enzyme, D-β-aminoisobutyrate:pyruvate aminotransferase. Injection of cycloserine increased the concentrations of the amino acids in the liver of rats, and inhibited strongly D-β-aminoisobutyrate: pyruvate and β-alanine:α-ketoglutarate aminotransferases. The increase in the amino acids and the enzyme inhibition occurred after D-cycloserine disappeared from the liver. The inhibition was not caused by D-cycloserine itself, but by its metabolite, D-aminoxyalanine, which was isolated and identified from urine of rats after injection of D-cycloserine. The toxicity of D-aminoxyalanine was also determined using mice, and the relation with clinical toxic effects of D-cycloserine is discussed.

D-β-Aminoisobutyric acid (D-BAIB) and β-alanine are metabolites of thymine and uracil, respectively. The concentrations of these amino acids rise in general after surgical operation regardless of the organ involved or type of operation [1]. This was not observed after surgery on patients with tuberculosis. It was found that patients with tuberculosis under treatment with cycloserine excrete higher concentrations of the amino acids in urine and that the concentrations fall for a few days after the operation during which period the drug was withdrawn. It was established that D-cycloserine is metabolized to D-β-aminoxyalanine which inhibits D-BAIB:pyruvate aminotransferase and β-alanine : α-ketoglutarate aminotransferase effectively *in vivo*.

METHODS AND MATERIALS

Determination of BAIB and β-alanine in urine and rat liver. Urine or a trichloroacetic acid extract of liver was desalted using ion exchange resin and was subjected to high voltage electrophoresis. Amino acids were stained by ninhydrin reaction; coloured bands corresponding to BAIB and β-alanine were extracted and their optical densities were measured by the method of Yanai *et al.* [2] and Takao *et al.* [3], respectively.

Determination of cycloserine in rat liver. Cycloserine was determined as BAIB. It was extracted from the liver with trichloroacetic acid. The extract was desalted and subjected to high voltage electrophoresis. The concentration was determined colourimetrically with ninhydrin. The drug was resolved well from tissue amino acids.

Assay of D-BAIB:pyruvate aminotransferase. Rat liver was homogenized in 5 volumes of 0·01 M phosphate buffer, pH 7·5, and the supernatant fraction obtained by centrifugation at 12,000 *g* for 20 min was used as the enzyme source. The activity was measured by the method of Taniguchi *et al.* [4] using D-BAIB-methyl^{14}C.

Assay of β-alanine:α-ketoglutarate aminotransferase. To the rat liver extract prepared as above, solid ammonium sulfate was added to 55% saturation. The supernatant after centrifugation was brought to 80% saturation with ammonium sulfate and the precipitate was dissolved in 0·01 M sodium borate buffer, pH 8·8 and used as the enzyme source. The activity was measured by the method of Kakimoto *et al.* [5].

β-Aminoxyalanine. β-Aminoxyalanine was prepared by the method of Stammer [6].

Schiff's base of β-aminoxyalanine with pyridoxal phosphate. 387 mg of β-aminoxyalanine was mixed with 750 mg of pyridoxal phosphate in a small volume of water, and the mixture was passed through a 2 × 20 cm column of Amberlite IR-120, pyridine form. The column was washed with 200 ml of water and the compound was eluted with 245 ml of pyridine–acetic acid–water (2:8:190). The eluate was evaporated to dryness under reduced pressure. The residue was crystallized from hot water to obtain 710 mg of crystalline $C_{11}H_{16}N_3O_8P \cdot H_2O$.

Found

C:36·34, H:5·20, N:11·15, P:8·45

Calculated

C:36·18, H:4·97, N:11·51, P:8·48

RESULTS

Concentrations of BAIB and β-alanine in urine of patients with tuberculosis. Concentrations of BAIB and β-alanine in the urine of patients with tuberculosis fell after surgical operations, and then returned

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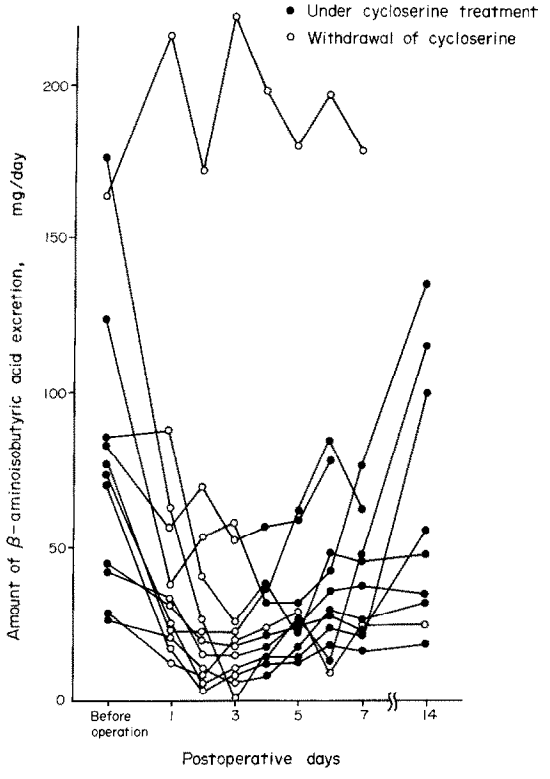

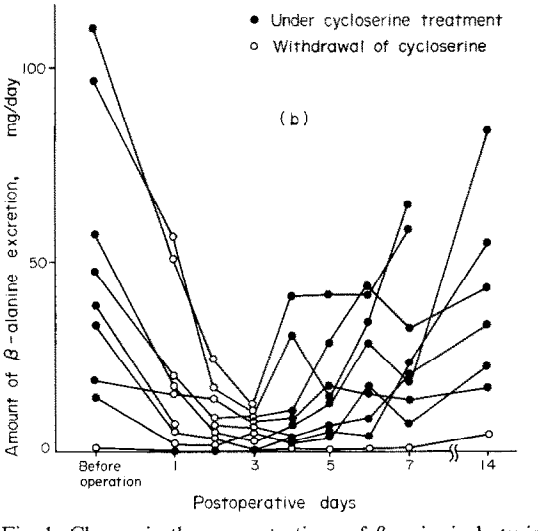

Fig. 1. Change in the concentrations of β-aminoisobutyric acid (1a) and β-alanine (1b) in urine of patients with pulmonary tuberculosis before and after surgical operation.

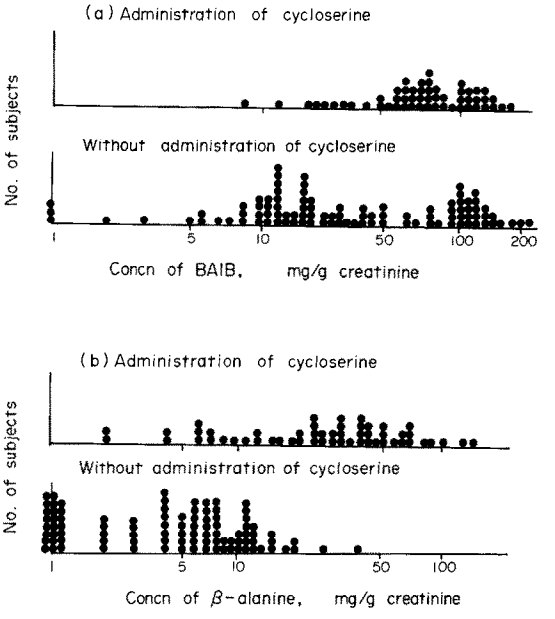

Fig. 2. The concentrations of β-aminoisobutyric acid (2a) and β-alanine (2b) in urine of patients with pulmonary tuberculosis. The concentrations in the urine of the patients receiving cycloserine and not receiving cycloserine were compared. Each dot represents the amount of the amino acids for each subject.

to the preoperative level. This was observed in patients receiving cycloserine treatment as shown in Fig. 1. Preoperative levels of the amino acids were higher than normal controls. The curve at the top of Fig. 1a which shows the highest concentration of BAIB represents the urinary level of the genetic high excretor of BAIB not under cycloserine treatment. The concentrations of BAIB and β-alanine in urine from two groups of patients with pulmonary tuberculosis, one under cycloserine treatment and the other without cycloserine, were determined and are shown in Fig. 2. Patients who were receiving cycloserine excreted significantly higher concentrations of BAIB and β-alanine. It should be mentioned here that the distribution of concentrations of BAIB in a Japanese population is bimodal as shown in the lower figure of Fig. 2a. Japanese are classified into high and low excretors [2], the concentration of BAIB in urine being controlled genetically. The high excretor is lacking in D-BAIB: pyruvate aminotransferase activity [4, 7]. When cycloserine was administered to the patients, urinary concentrations of BAIB reached the level of the high excretors who lack the above enzyme genetically, as shown in Fig. 2. This was verified by observation of the change in the concentration of urinary BAIB after the administration of cycloserine to patients and healthy human subjects (Fig. 3). The concentration rose in the low excretors but not in the high excretors. β-Alanine in urine increased concomitantly.

Changes in the concentration of BAIB and β-alanine in rat liver after cycloserine injection. D-Cycloserine (200 mg/kg) was injected i.p. into rats weighing 150 g, and the rats were killed after various times. The concentrations of BAIB, β-alanine and cycloserine and the activities of BAIB: pyruvate and β-alanine: α-ketoglutarate aminotransferases were measured using 5–10 rats for each time. As shown in Fig. 4, cycloserine disappeared rapidly from liver falling to an undetectable concentration within 4 hr after the injection, while the concentrations of BAIB and β-alanine gradually increased for 4 hr; the increased level was sustained for 12 hr and then fell slowly. The enzyme activities dropped rapidly within 30 min and the inhibition of D-BAIB: pyruvate aminotransferase lasted for more than 24 hr as shown in Fig. 5. Inhibition

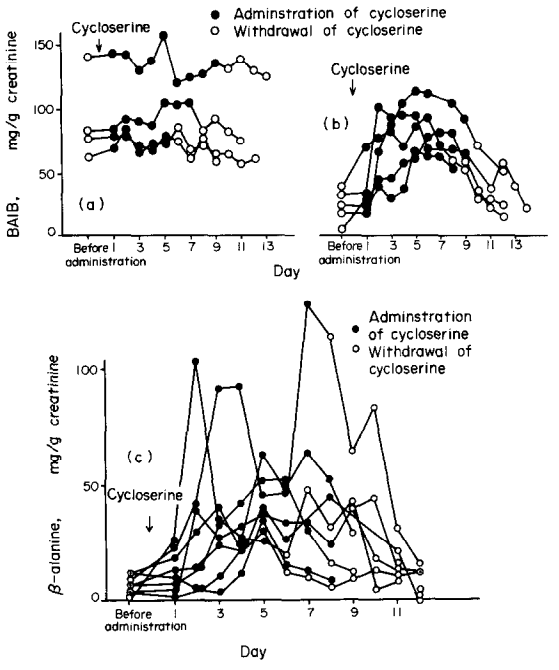

Fig. 3. Effect of cycloserine administration on the concentrations of β-aminoisobutyric acid (3a, b) and β-alanine (3c) in human urine. Figure 3a and 3b are the difference in β-aminoisobutyric acid concentration in the urine of genetic high and low excretors of β-aminoisobutyric acid, respectively.

was more than 90% for the first 4 hr and it was about 80% even 24 hr after the injection.

Inhibition of D-*BAIB: pyruvate and β-alanine: α-ketoglutarate aminotransferases by* D-*cycloserine* in vitro. Various concentrations of cycloserine were added to the reaction mixtures. A low concentrations cycloserine did not inhibit the enzyme activities as shown in Table 1; about 2×10^{-3} M was required to attain 50% inhibition, and this concentration was far higher than the tissue level of the drug found in rat liver, 3×10^{-4} M. Combining this finding with the observation that the time course of inhibition of the enzyme was not parallel to that of the concentration of cycloserine as shown in Fig. 5, it was considered that enzyme inhibition is not due to cycloserine itself, but probably to its metabolite.

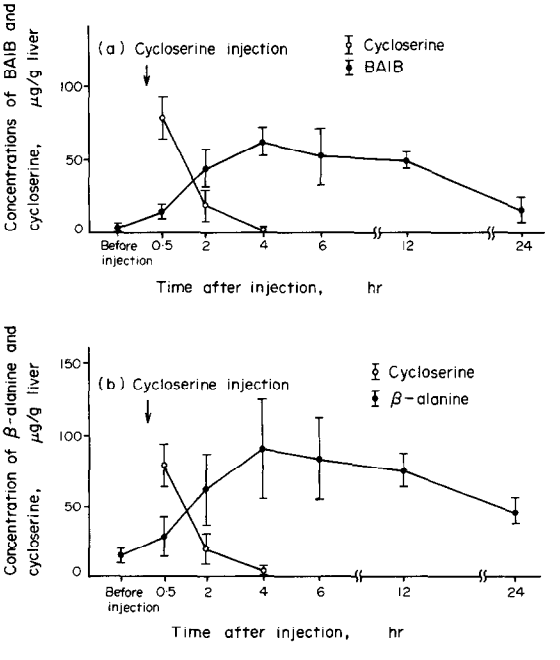

Fig. 4. The concentrations of β-aminoisobutyric acid (4a), β-alanine (4b) and cycloserine in the rat liver after the intraperitoneal injection of cycloserine.

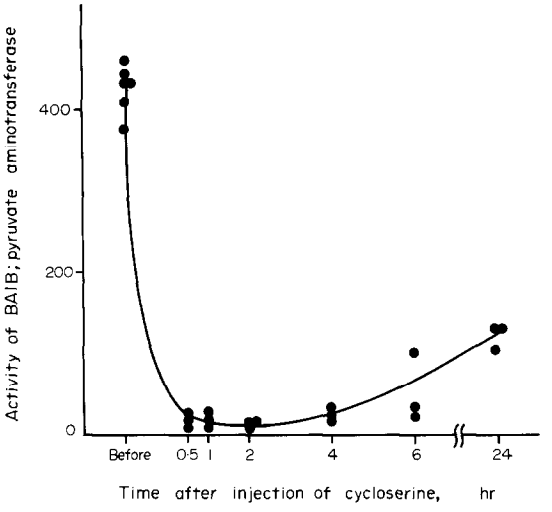

Fig. 5. Activity of D-β-aminoisobutyrate: pyruvate aminotransferase in rat liver after the intraperitoneal injection of cycloserine. The activity of the enzyme is expressed as the amount of decreased β-aminoisobutyric acid (mμ moles) in 20 min per g liver.

Presence of an enzyme inhibitor in the liver of rat after injection of D-*cycloserine.* Five grams of rat liver obtained 2 hr after the injection of cycloserine were homogenized in 5 vol of 0·01 M potassium phosphate buffer, pH 7·5. The homogenate in a cellophane bag was dialyzed against 100 ml of the same buffer. The concentration of cycloserine in the solution outside the bag was $4{\cdot}9 \times 10^{-6}$ M. While this concentration of cycloserine did not show any detectable inhibition of BAIB: pyruvate aminotransferase, the addition of 0·1 ml of the above solution to 0·5 ml of the reaction mixture inhibited the enzyme activity by 35%. The extract obtained from normal rat liver did not show any inhibitory effect.

Presence of the inhibitory substance in urine of rats after injection of cycloserine. D-Cycloserine was injected into 2 rats weighing 150 g at the dose of 500 mg per kg body wt. Urine was collected for 24 hr after the injection. The urine was found to contain a substance inhibiting D-BAIB: pyruvate aminotransferase, and was purified using the enzyme as an indicator. Urine was passed through a 2 × 10 cm column of Amberlite IR-120, H^+ form, 100–200 mesh. The resin was washed with water and the compound was eluted with 120 ml of 1 M pyridine. The eluate was evaporated to dryness under reduced pressure and the residue, dissolved in a small volume of water, was passed through a 1 × 10 cm column of Dowex 1 × 10, acetate form, and the column was washed with

Table 1. Effect of D-cycloserine on D-β-aminoisobutyrate:pyruvate and β-alanine:α-ketoglutarate aminotransferases

Concentration of cycloserine (M)	% Inhibition of D-β-aminoisobutyrate:pyruvate aminotransferase
$1/6 \times 10^{-3}$	12
$1/6 \times 10^{-2}$	61
$1/6 \times 10^{-1}$	96
Concentration of cycloserine (M)	**% Inhibition of β-alanine:α-ketoglutarate aminotransferase**
$1/7 \times 10^{-3}$	40
$1/7 \times 10^{-2}$	64
$1/7 \times 10^{-1}$	99

An aqueous solution of D-cycloserine was added to the enzyme reaction mixtures to obtain the final concentrations given in the table.

30 ml of water. The effluent was evaporated to dryness. The dried residue was applied to a 2 × 20 cm column of Amberlite IR-120, pyridine form, and the compound was eluted with 2 M pyridine. Five ml fractions were collected. An aliquot of each fraction was subjected to paper electrophoresis to locate cycloserine and another aliquot was used to determine the inhibition of BAIB:pyruvate aminotransferase. Cycloserine was eluted in the 11th tube, but an inhibitory substance was eluted earlier, the maximal inhibitory activity being in tube 7 as shown in Fig. 6. This peak was not observed when normal rat urine was processed in the same manner. Judging from its behaviour in ion exchange chromatography and paper electrophoresis, the inhibitory compound was neutral, and from the chemical structure of D-cycloserine, D-β-aminoxyalanine was considered to be a probable candidate.

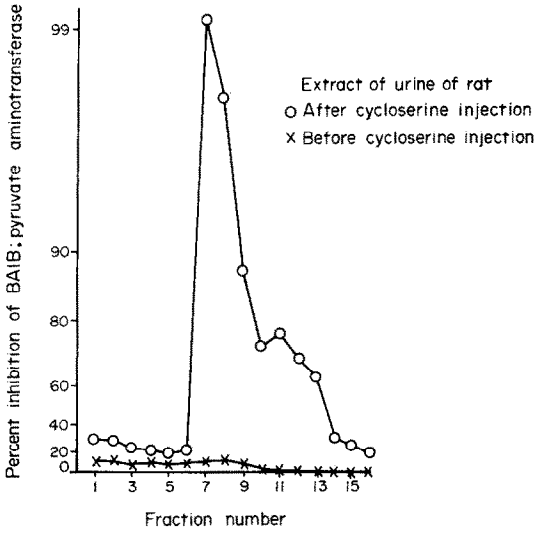

Fig. 6. Ion exchange chromatography of an inhibitory substance extracted from urine of rats injected with cycloserine. Inhibition was examined by the inhibition of D-β-aminoisobutyrate:pyruvate aminotransferase. Chromatography was carried out on Amberlite IR-120, pyridine form, with 2 M pyridine. The small peak corresponding to tube 11 is cycloserine.

Identification of the inhibitory substance in urine of rats treated with cycloserine. The fraction containing the inhibitory substance was purified further by chromatography on a 2 × 18 cm column of Dowex 50 × 2 equilibrated with pyridine–acetic acid–water (1:9:190), and eluted with pyridine–acetic acid–water (3:7:190). A fraction of the eluate between 77 and 132 ml was evaporated to dryness under reduced pressure. Aliquots of this fraction were examined by paper chromatography and paper electrophoresis. The purified solution contained a substance with properties the same as those of β-aminoxyalanine. The chromatographic pattern of the purified fraction on Amberlite IR-120, pyridine form, eluted with 2 M pyridine was the same as that of β-aminoxyalanine. Because β-aminoxyalanine was too unstable to purify extensively, the inhibitory substance was stabilized by making a complex with pyridoxal phosphate to obtain a final proof of identification. 190 mg of cycloserine was injected into two rats and 48 hr urine was collected. The urine was treated with Amberlite IR-120, H^+ form, and Dowex 1, acetate form, as described above. To the effluent 50 mg of pyridoxal phosphate were added, and the solution was evaporated to dryness. The residue was dissolved in 5 ml of water. The solution was applied to a 1 × 8 cm column of Dowex 1 × 10, acetate form, and the resin was washed with 100 ml of water. The compound was eluted with 300 ml of 1 M acetic acid. The eluate was dried and applied to a 2 × 20 cm column of Amberlite IR-120, equilibrated with pyridine–acetic acid–water (0·5:9·5:190). The compound was eluted with pyridine–acetic acid–water (2:8:190). The fraction of the eluate between 15 and 50 ml was evaporated to dryness. The purified complex was identical with authentic Schiff's base of β-aminoxyalanine with pyridoxal phosphate by paper chromatography and electrophoresis. The compounds were located by fluorescence. U.V. spectra of both the compounds matched completely as shown in Fig. 7.

Inhibition of D-BAIB:pyruvate and β-alanine:α-ketoglutarate by β-aminoxyalanine. The inhibitory effect of D-β-aminoxyalanine on both enzymes is shown in

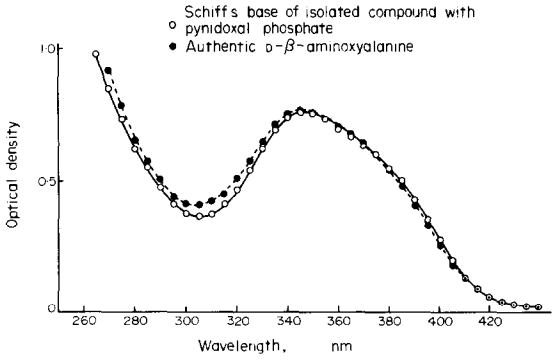

Fig. 7. Absorption spectra of Schiff's bases of the isolated compound and of authentic D-β-aminoxyalanine.

Table 2. The concentration of D-β-aminoxyalanine required for 50% inhibition of D-BAIB:pyruvate and β-alanine:α-ketoglutarate aminotransferase was less than 2×10^{-6} M. The inhibition was abolished by the addition of pyridoxal phosphate as shown in Table 3.

Toxicity of β-aminoxyalanine. Various amounts of D-β-aminoxyalanine were injected into mice and the lethal dose was calculated from data in Table 4. The LD_{50} for the compound was 2·57 ± 0·02 g per kg, which is a little lower than the reported LD_{50} value of D-cycloserine, 4·30 ± 0·35 g [8]. Generalized seizures were observed in all mice injected with the lethal doses of β-aminoxyalanine, but were not observed with D-cycloserine. The toxic effect of this amino acid was not protected by the injection of pyridoxal phosphate, and Schiff's base of the amino acid with pyridoxal phosphate had the same toxicity as β-aminoxyalanine.

Table 4. Toxicity of β-aminoxyalanine to mice

Amount of β-aminoxyalanine injected (mg/g body weight)	Number of mice	Mortality (%)
4·5	10	100
4·0	10	90
3·5	10	50
3·0	10	30
2·5	10	0

DISCUSSION

Administration of D-cycloserine to pateints led to a significant increase in the excretion of BAIB and β-alanine in urine. This is due to the inhibition of enzymes metabolizing the two amino acids by D-β-aminoxyalanine, a metabolite of cycloserine. D-BAIB:

Table 2. Effect of β-D-aminoxyalanine on D-β-aminoisobutyrate:pyruvate and β-alanine:α-ketoglutarate aminotransferases

Concentration of β-aminoxyalanine (M)	% Inhibition of D-β-aminoisobutyrate:pyruvate aminotransferase
$1/6 \times 10^{-6}$	13
$1/6 \times 10^{-5}$	66
$1/6 \times 10^{-4}$	94
$1/6 \times 10^{-3}$	98
Concentration of β-aminoxyalanine (M)	**% Inhibition of β-alanine:α-ketoglutarate aminotransferase**
$1/7 \times 10^{-5}$	63
$1/7 \times 10^{-4}$	93
$1/7 \times 10^{-3}$	99

An aqueous solution of D-cycloserine was added to the enzyme reaction mixture to obtain the final concentrations given in the table.

Table 3. Inhibitory rate of D-β-aminoisobutyrate:pyruvate aminotransferase by β-aminoxyalanine in the absence and presence of pyridoxal phosphate

Final Concentration (M)		Inhibition of the enzyme activity (%)
β-Aminoxyalanine	Pyridoxalphosphate	
$1·3 \times 10^{-5}$	0	90
	$3·4 \times 10^{-5}$	0
$2·6 \times 10^{-5}$	0	92
	$3·4 \times 10^{-5}$	4
$3·9 \times 10^{-5}$	0	94
	$3·4 \times 10^{-5}$	89
$5·2 \times 10^{-5}$	0	96
	$3·4 \times 10^{-5}$	96

pyruvate aminotransferase in the patients under treatment with the drug is considered to be inhibited almost completely. The amount of BAIB in the urine of pateints reached the level of that of the genetic high excretors of BAIB whose BAIB: pyruvate aminotransferase activity is almost absent. The possibility of the increased amounts of BAIB and β-alanine in urine being due to an increase in the turnover rate of nucleotides or breakdown of nucleic acids by D-cycloserine is unlikely since the administration of D-cycloserine to the genetic high excretors of BAIB did not cause any increase in BAIB concentration.

Inhibition of various transaminases by cycloserine has been reported. Aoki [8] and Barbieri [9] reported the inhibition of glutamate: asparate aminotransferase and Barbieri [10] and Porfirieva [11] the inhibition of glutamate: alanine aminotransferase. We injected 200 mg per kg of D-cycloserine intraperitoneally into rats. No significant inhibition of the activities of either enzyme was found in the liver 6 hr after the injection, when BAIB: pyruvate aminotransferase was almost completely inhibited. This enzyme seems to be selectively inhibited by D-aminoxyalanine. Dann and Garter [12] reported the inhibition of γ-aminobutyrate: glutamate aminotransferase by cycloserine, but injection of 6 g per kg of the drug was required to attain an observable increase in the concentration of γ-aminobutyrate in rat brain.

Correlation between the enzyme inhibition, which is estimated by the increase of urinary concentrations of BAIB and β-alanine, and side effects such as dizziness, headache and convulsion was not found. The toxicity of D-aminoxyalanine was similar to that of D-cycloserine. It was interesting that D-β-aminoxyalanine induced convulsive seizure prior to death without exception while cycloserine did not. Convulsive seizure is a common clinical side effect of cycloserine. The antituberculous activity of D-β-aminoxyalanine was a little less than D-cycloserine when added to a culture medium [13]. Metabolism of D-cycloserine to β-aminoxyalanine therefore seems to be an undesirable reaction which increases the side effects such as convulsions. A derivative of D-cycloserine which is resistant to enzymic hydrolysis or a nontoxic inhibitor of the hydrolase of cycloserine may be clinically a desirable development.

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