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# Bacterial L-Lysine-α-Ketoglutarate Aminotransferase

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L-Lysine-α-ketoglutarate aminotransferase from *Flavobacterium fuscum*, *Flavobacterium flavescens* and *Achromobacter liquidum*, catalyzes ε-transamination of L-lysine with α-ketoglutarate. The terminal amino group of L-lysine is transferred to α-ketoglutarate to yield L-glutamate and α-aminoadipate δ-semialdehyde, which is immediately converted into the intramolecularly dehydrated form, $\Delta^1$-piperideine-6-carboxylic acid. L-Lysine-α-ketoglutarate aminotransferase was purified and crystallized from the cell-free extract of *Achromobacter liquidum* grown in the peptone medium. The crystalline enzyme preparation is homogeneous by the criteria of ultracentrifugation and electrophoresis. The sedimentation coefficient ($S^\circ_{20,w}$) and molecular weight were determined to be 6.37 S and 116,000, respectively. The enzyme catalyzes transfer of the terminal amino groups of L-lysine, L-ornithine and S-(β-aminoethyl)-L-cysteine to α-ketoglutarate. α-N-Acetylated derivatives of these amino acids are also transaminated though very slowly, while ω-N-acetylated derivatives and their D isomers are completely inactive. This aminotransferase reaction is strongly inhibited by δ-aminovaleric acid and hydroxylamine.

## INTRODUCTION

L-Lysine, L-α,ε-diaminocaproic acid, is biosynthesized by two distinct pathways,[1] *i.e.*, α,ε-diaminopimelic acid pathway in bacteria and α-aminoadipic acid pathway in yeast and mold, whereas L-lysine, which is an essential dietary amino acid for mammals, is rather metabolically inert in mammal as compared with other amino acids.

The metabolism of lysine has been extensively studied as reviewed by Meister,[2] and Broquist and Trupin.[3] L-Lysine is known to be metabolized to glutaric acid through some intermediates, *e.g.*, pipecolic acid. This compound was before assumed to be derived from α-keto-ε-aminocaproic acid, a deaminated product from lysine, by cyclization and reduction. However, the mechanism of the enzymatic deamination of lysine has, until recently, remained unsolved. Both D and L isomers of lysine are scarcely deaminated by the respective common amino acid oxidases,[4~6] though oxidative deamination of L-lysine was observed with an amino acid oxidase of turkey liver,[7] which exhibits a high specificity for the basic amino acids. There have been several reports of transamination of lysine with α-keto acids in various animal tissue preparations,[8~10] but the rate of reaction was extremely low in all instances and the product of lysine transamination has not been demonstrated.

Both enzymatic transamination and oxidative deamination of lysine have been observed when the α-amino group is acylated.[3] The enzymatic ε-N-acetylation of lysine has been found by Paik and Kim[11] in beef liver. Meister has suggested the

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possibility that acetylation of the ε-amino group of lysine takes place prior to removal of the α-amino group. α-Keto-ε-N-acetylaminocaproic acid, which is the deaminated product of ε-N-acetyllysine, is hydrolyzed to yield α-keto-ε-aminocaproic acid followed by cyclization in the presence of the enzyme of mammalian tissues[12] as well as that of microorganisms.[13] The conversion of $\Delta^1$-piperideine-2-carboxylic acid to pipecolic acid has been demonstrated enzymologically by Meister *et al.*[14] The metabolism of pipecolic acid to α-aminoadipic acid has been elucidated in mammalian tissues[15] and microorganisms.[16]

Recently, Higashino *et al.*[17] have found that L-lysine is metabolized by rat liver mitochondria *via* a pathway including saccharopine (ε-N-(L-glutaryl-2-)-L-lysine) as a first intermediate *in vitro*, which is the reverse of the lysine biosynthetic pathway in yeast and mold, and suggested that in the rat liver, lysine degradation proceeds through formation of saccharopine rather than cyclization to pipecolic acid in the presence of excess of α-ketoglutarate. Ghadimi and Zischka[18] studied the metabolism of lysine with the radioactive compound, and homogenates of dog tissues and human liver in order to elucidate the biochemical anomaly in "hypersinemia associated with retardation". They have shown no trace of pipecolic acid but found the formation of saccharopine from lysine catalyzed an enzyme of the human liver. This finding confirms the saccharopine pathway of lysine degradation by Higashino *et al.*[17]

On the other hand, in microorganisms L-lysine undergoes various degradative reactions, and the respective reactions have been studied enzymatically in details. L-Lysine is decarboxylated to cadaverine in *Escherichia coli* and *Bacterium cadaveris*.[19] L-Lysine decarboxylase has been purified and crystallized from the extract of *Bacterium cadaveris* and some properties of this enzyme were reported.[20] The occurrence of a bacterial enzyme which catalyzes the conversion of L-lysine into δ-aminovaleric acid, ammonia and carbon dioxide was reported by Hagihira *et al.*[21] This enzyme was later demonstrated to be an oxygenase,[22] which has been crystallized.[23] This oxygenase reaction involves initially the oxygenative decarboxylation of L-lysine producing δ-aminovaleramide. The latter compound is deaminated to δ-aminovaleric acid by an amidase. This amidase has been purified from the extract of *Pseudomonas putida* by Reitz and Rodwell.[24]

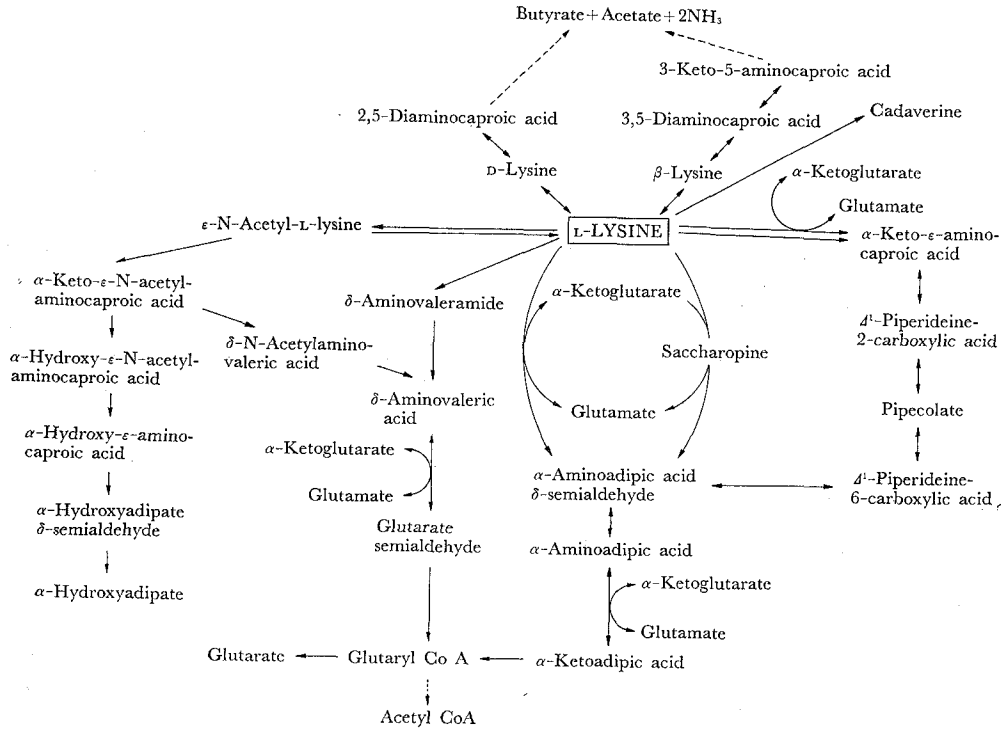
The anaerobic degradation of D- and L-lysine to butyric acid, acetic acid and ammonia has been found in *Clostridium sticklandii* and other clostridia.[25,26] In this reaction, lysine 2,3-mutase, β-lysine mutase and NAD-dependent oxidative deaminase of 3,5-diaminocaproic acid participate. The first intermediate in the reaction is L-β-lysine formed by migration of the α-amino group to the β-position.[26] The next reaction step is a cobamide coenzyme dependent migration of the amino group on carbon 6 of β-lysine to carbon 5, resulting in the formation of 3,5-diaminocaproic acid.[27] Lysine 2,3-mutase has been purified 53-fold from *Clostridium sp.*[28] 3,5-Diaminocaproic acid is oxidatively deaminated to 3-keto-5-aminocaproic acid by a NAD-dependent dehydrogenase.[29] From D-lysine, 2,5-diaminocaproic acid is formed as the first intermediate.[30]

Lysine racemase reaction has been studied by Fuang *et al.*[31] and Ichihara *et al.*[32] in *Pseudomonas sp.*, and amino acid racemase with low substrate specificity of *Pseudomonas striata*[33] and arginine racemase of *Pseudomonas graveolens*,[34] which have been purified to

homogeneity and crystallized, also catalyze the racemization of lysine.

Aminotransferase reaction of lysine was studied by Feldman and Gunsalus[35] in bacteria, but they could not obtain the unequivocal evidence for the L-lysine aminotransferase reaction. The lack or negligible participation of the aminotransferase reaction was regarded as even a feature of lysine metabolism. However, Soda *et al.*[36,37] who studied on the distribution of the aminotransferase reaction in bacteria, have found occurrence of the high activity of aminotransferase reaction between L-lysine and α-ketoglutarate in the cell-free extracts of *Flavobacterium fuscum*, *Flavobacterium flavescens* and *Achromobacter liquidum*, and reported on some properties of the partially purified enzyme. The transamination product from lysine was shown to react with *p*-dimethylaminobenzaldehyde and *o*-aminobenzaldehyde, but not 2,4-dinitrophenylhydrazine. From these facts, they suggested that the product formed from L-lysine by the aminotransferase reaction exists in the form of dehydropipecolic acid which was derived from the keto analog of lysine by the intramolecular cyclization. But it has not been demonstrated which amino group of lysine is transaminated to α-ketoglutarate. Hasse *et al.*[38] studied the transamination of ornithine and lysine in plant, and found that the enzyme preparations from *Lupinus angustifolius* and *Phaseolus aureus* Roxb catalyze their transamination. This aminotransferase was purified 21-fold from *Lupinus angustifolius* and shown to catalyze α-transamination of ornithine and lysine.

As described above (Scheme I), the degradative pathways of lysine are diverse




Scheme I. Degradative pathways of lysine.

in various organisms and such diversity is the most striking characteristics of the lysine metabolism.

This investigation was undertaken to elucidate chemically and enzymologically the mechanism of transamination of lysine as the first step of its metabolism.

The present thesis describes the identification of the transamination product from L-lysine, and purification, crystallization and characterization of the L-lysine-α-ketoglutarate aminotransferase of *Achromobacter liquidum*.

## I. ISOLATION AND IDENTIFICATION OF A PRODUCT FROM L-LYSINE

Aspen and Meister[39] have shown that the nonenzymatic transamination of lysine and glyoxylate at pH 5 and 100°C gives α-keto-ε-aminocaproate ($\Delta^1$-piperideine-2-carboxylate) rather than α-aminoadipic-δ-semialdehyde ($\Delta^1$-piperideine-6-carboxylate). They reported also that $\Delta^1$-piperideine-6-carboxylate was accumulated in the lysine-requiring mutants of *Aspergillus nidulans* and proposed the pathway for the formation of it and the related cyclic compounds.[40] Hasse *et al.*[38] have shown that α-amino group of lysine is transaminated to α-ketoglutarate to yield $\Delta^1$-piperideine-2-carboxylate by the enzyme from *Lupinus angustifolius* and *Phaseolus aureus* Roxb. Soda *et al.*[37] have shown that L-lysine-α-ketoglutarate aminotransferase occurs in the extracts of *Flavobacterium fuscum, Flavobacterium flavescens* and *Achromobacter liquidum* and that glutamate and a cyclic form of the keto analog of lysine are enzymatically formed from the substrates in the reaction mixture. The present investigation has been performed in order to elucidate what compound is produced from lysine, *i.e.*, which amino group of L-lysine is transaminated to α-ketoglutarate in those bacteria.

In the present chapter, the isolation and some properties of the lysine transamination product are described.

### Experimental Procedures

**Materials.** *o*-Aminobenzaldehyde was prepared by reduction of *o*-nitrobenzaldehyde according to the procedure of Smith and Opie.[41] $\Delta^1$-Piperideine-2-carboxylic acid was synthesized from ε-N-carbobenzoxy-L-lysine as described by Meister.[42] DL-[1-$^{14}$C]-Lysine was purchased from New England Nuclear Corp., Boston, Mass. L-Lysine was obtained from Tanabe Seiyaku Co., Osaka; α-ketoglutaric acid from Ajinomoto Co., Tokyo; and pyridoxal 5′-phosphate from Dainippon Seiyaku Co., Osaka. The other chemicals were analytical grade reagents.

**Microorganism and Condition of Culture.** *Flavobacterium fuscum* AKU (Department of Agricultural Chemistry, Faculty of Agriculture, Kyoto University) 0140 was grown in the medium containing 1.5% peptone, 0.01% yeast extract, 0.2% potassium dihydrogen phosphate, 0.2% dipotassium hydrogen phosphate, 0.01% magnesium sulfate, and 0.2% sodium chloride in tap water. The pH of medium was adjusted to 7.2 with sodium hydroxide. The cultures were grown at 30°C under aeration for 18 hours. The cells harvested by centrifugation were washed twice with 0.85% sodium chloride solution and stored at −20°C until use.

**Preparation of Partially Purified Enzyme.** The washed cells were suspended in 0.1 M potassium phosphate buffer (pH 8.0) containing $10^{-5}$ M pyridoxal 5′-phosphate and 0.02% 2-mercaptoethanol, and subjected to sonication in a 19-kc Kaijo Denki oscillator for 10 min. After centrifugation at 17,000 g for 30 min, the supernatant was dialyzed against 0.02 M potassium phosphate buffer (pH 7.2) containing $10^{-5}$ M pyridoxal 5′-phosphate and 0.01% 2-mercaptoethanol. To the dialyzed cell-free extract was added 1.0 ml of 2% protamine sulfate solution (pH 7.2) per 100 mg of protein with stirring. The mixture was centrifuged and the bulky inactive precipitate was discarded. The supernatant was brought to 30% saturation with solid ammonium sulfate and the precipitate was removed by centrifugation. The precipitate obtained by addition of ammonium sulfate to 65% saturation was dissolved in 0.01 M potassium phosphate buffer (pH 7.5) containing $10^{-5}$ M pyridoxal 5′-phosphate and 0.01% 2-mercaptoethanol, and dialyzed against the same buffer. The inactive precipitate formed during dialysis was removed by centrifugation. All operations were carried out at 0–5°C. The enzyme was purified approximately sevenfold by these procedures, although this enzyme has been purified to be homogeneous from *Achromobacter liquidum* as described in Chapter II.

**Analytical Methods.** Glutamate formed in the reaction mixture was determined according to the method of Soda *et al.*[37] Amino acids in the fractions eluted from Dowex 50 column were assayed by the method of Rosen.[43] $\Delta^1$-Piperideine-2-carboxylic acid, X and bX, which were identified as $\Delta^1$-piperideine-6-carboxylic acid and its bisulfite adduct as described below, in the fractions eluted from Dowex 50 column, were determined with *o*-aminobenzaldehyde as follows. To a 2.0-ml aliquot of the sample solution were added 0.2 ml of 5 N sodium hydroxide solution, 1.0 ml of 0.2 M acetate buffer (pH 5.0), and 2.0 ml of 0.04 M *o*-aminobenzaldehyde solution. The sample solution was replaced by water in a blank. When $\Delta^1$-piperidenine-2-carboxylic acid was used, the reaction mixture was incubated at 37°C for 8 hr and the color intensity was measured against a blank at 450 m$\mu$ using a Shimadzu QV-50 spectrophotometer. In the case of X and bX, after the incubation at 37°C for 1 hr, the absorbance was determined at 465 m$\mu$. Protein was determined by the method of Lowery *et al.*[44] Radioactivities of the paper electrophoresis strips were scanned in a paper scanner (Aloka PCS-4).

**Assay of Aminotransferase.** The standard assay system consisted of 20 $\mu$moles of L-lysine, 20 $\mu$moles of potassium $\alpha$-ketoglutarate, 1 $\mu$mole of pyridoxal 5′-phosphate, 90 $\mu$moles of potassium phosphate buffer (pH 8.0), and enzyme in a final volume of 2.0 ml. The mixture was incubated at 37°C for 10 min. The reaction was terminated by addition of 1.0 ml of 5% trichloroacetic acid solution. The aminotransferase was assayed by determining the amount of glutamate formed (procedure A) or by measuring the color intensity of the *o*-aminobenzaldehyde adduct of X at 465 m$\mu$. The adduct was formed by the incubation of 3.0 ml of 4 mM *o*-aminobenzaldehyde solution in 0.2 M potassium phosphate buffer (pH 8.0), with a 2.0 ml aliquot of the deproteinized reaction mixture at 37°C for 1 hr (procedure B). The molecular extinction coefficient of the *o*-aminobenzaldehyde adduct of X was estimated by determining the amount of glutamate formed simultaneously in the same reaction system, because authentic $\Delta^1$-piperideine-6-carboxylic acid was not prepared. The specific activity

was expressed as micromoles of glutamate or $\Delta^1$-piperideine-6-carboxylic acid formed per mg of protein per hour.

**Paper Electrophoresis.** Electrophoresis was carried out using the high-voltage paper electrophoresis apparatus (Handex HC-HEP, Shiraimatsu Co., Osaka) and Toyo filter paper strips (No. 53, 15×54 cm). The electrophorogram was cooled by being immersed in ice-cold hexane. The pH level was maintained using 0.2 M pyridine acetate buffer (pH 3.5) or 1 N formic acid. After electrophoresis, the strips were dried and sprayed with 0.5% ninhydrin solution in a mixture of ethanol and acetone (1 : 1) or 0.1% *o*-aminobenzaldehyde solution in acetone. The strips were heated at about 80°C for 15 min and examined for the appearance of the colored spots.

## Results

### (A) Product from L-Lysine.

**Paper Electrophoresis of Reaction Products.** The reaction mixture described above was incubated at 37°C for 30 min. After deproteinization by addition of 0.1 ml of 50% trichloroacetic acid followed by centrifugation, the supernatant was continuously treated with ether to remove α-ketoglutarate and trichloroacetic acid. Aliquot samples of the ether-treated solution were examined by high voltage paper electrophoresis at pH 3.5. In addition to lysine and glutamate, an unknown compound which was tentatively designated X, was observed in a complete system as shown in Fig. 1. Glutamate and X were not produced in the reaction system lacking any of L-lysine, α-ketoglutarate, and enzyme. Compound X, which reacted with *o*-aminobenzaldehyde and ninhydrin to develop an orange and a yellow color, respectively, migrated slightly faster than glutamate toward the cathods. Although compound X was indistinguishable from authentic $\Delta^1$-piperideine-2-carboxylic acid under the experimental conditions employed in Fig. 1, it was electrophoretically separated from $\Delta^1$-piperideine-2-carboxylic acid in 1 N formic acid at 1000 V as described later (in Fig. 5). Compound X reacted with *p*-dimethylaminobenzaldehyde to develop an orange color, but did not react with 2,4-dinitrophenylhydrazine. These findings suggest that X may be a heterocyclic compound derived from L-lysine, *i.e.*, $\Delta^1$-piperideine-2-carboxylic acid or $\Delta^1$-piperideine-6-carboxylic acid.

**Effect of Bisulfite and Dimedon.** Addition of sodium bisulfite (final concentration $2\times10^{-3}$ M) to the reaction system resulted in an increase in the aminotransferase reaction rate by about 30%. At the higher concentration, both bisulfite and dimedon, which had only a slightly stimulative effect even at the lower concentration, showed an inhibitory effect on the enzyme activity. These inhibitions were partially eliminated by further addition of pyridoxal 5′-phosphate. The reaction mixture incubated in the presence of sodium bisulfite or dimedon (final concentration $10^{-3}$ M) was deproteinized and treated with ether as mentioned above. Some aliquots of the solution were submitted to paper electrophoresis. When sodium bisulfite was involved in the reaction mixture, in addition to lysine, glutamate, and X, another unknown compound, bX, that bore high negative charge even at pH 3.5, was produced (Fig. 2). In the reaction system containing dimedon, dX, which migrated scarcely

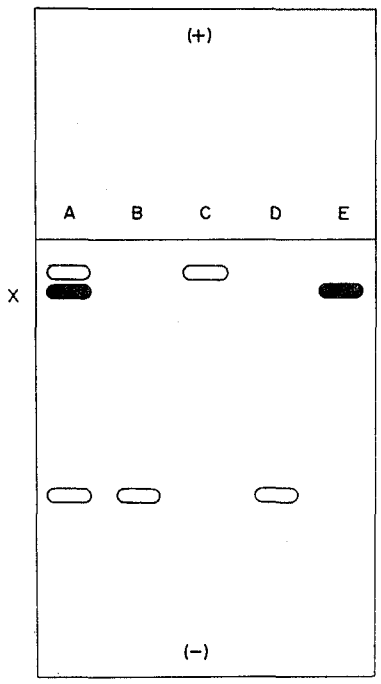



Fig. 1.

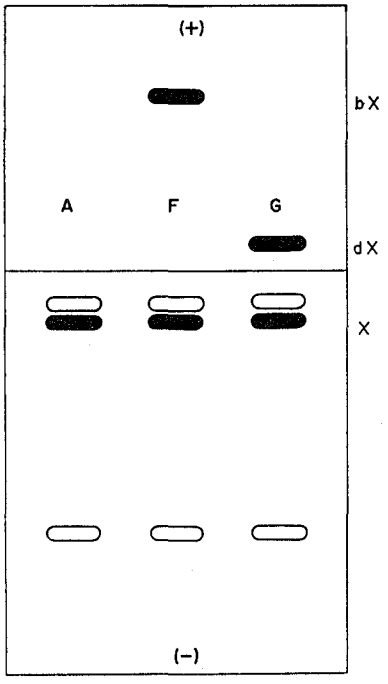



Fig. 2.

Fig. 1. Paper electrophoresis of reaction mixture (*left*). Electrophoresis was conducted at pH 3.5 in 0.2 M pyridine acetate buffer at 2000 V for 90 min. (A) Complete system, (B) potassium $\alpha$-ketoglutarate omitted, (c) L-glutamate, (D) L-lysine, and (E) $\Delta^1$-piperideine-2-carboxylic acid. Development with *o*-aminobenzaldehyde and ninhydrin showed an orange and a yellow color, respectively, in the black areas. On development with ninhydrin, the light areas were violet, but were negative with *o*-aminobenzaldehyde.

Fig. 2. Paper electrophoresis of reaction mixture incubated in the presence of sodium bisulfite or dimedon (*right*). Electrophoresis was conducted at pH 3.5 in 0.2 M pyridine acetate buffer, (A) Complete system, (F) sodium bisulfite ($10^{-3}$ M) added, and (G) dimedon ($10^{-3}$ M) added. The other conditions are shown in Fig. 1.

under the conditions employed, was formed. Both bX and dX gave a yellow color with ninhydrin and an orange color with *o*-aminobenzaldehyde. The formation of bX and dX in the presence of sodium bisulfite and dimedon, respectively, suggests that some intermediate which has an aldehyde group in it to react with such trapping agents may be formed in the reaction.

**Isotopic Study on Formation of X.** The reaction mixture containing DL-[1-$^{14}$C] lysine was incubated as described in Fig. 3. After deproteinization by addition of 0.1 ml of 10% trichloroacetic acid and centrifugation, 0.05 ml aliquots of the supernatant were analyzed by paper electrophoresis and scanned for radioactivity. The spots were visualized with ninhydrin spray and *o*-aminobenzaldehyde

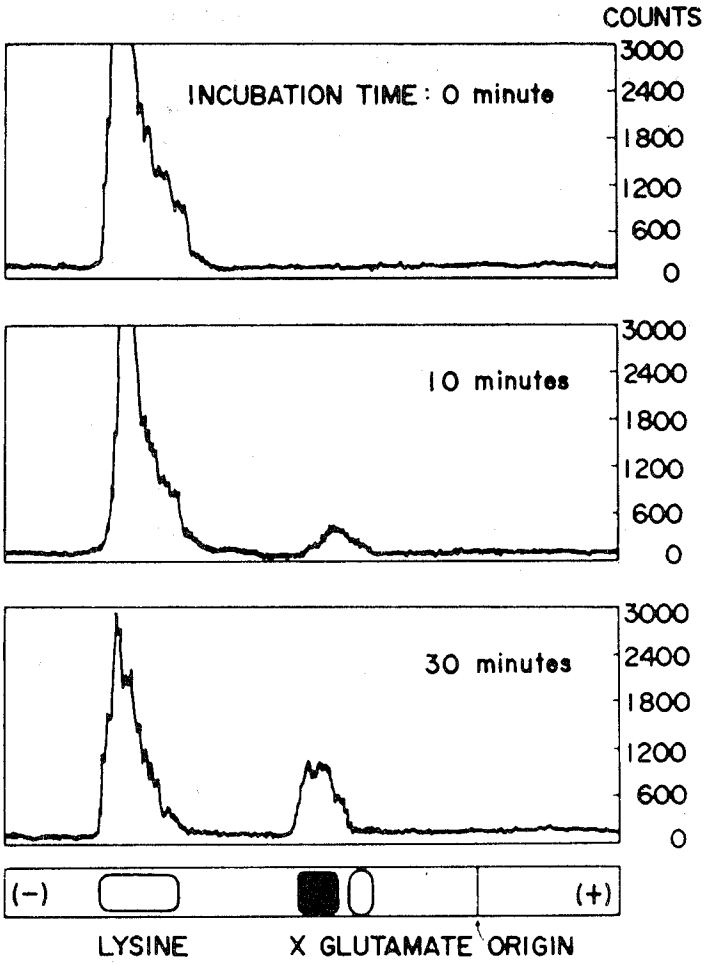



Fig. 3. Incorporation of the radioactivity into X from DL-[1-$^{14}$C] lysine. The reaction mixture containing 0.08 μmole (0.86 μCi) of DL-[1-$^{14}$C] lysine, 5.0 μmoles of L-lysine, 5.0 μmoles of potassium α-ketoglutarate, 0.75 μmole of pyridoxal 5′-phosphate, 5.0 μmoles of potassium phosphate buffer (pH 8.0), and 75 μg of enzyme in a final volume of 0.5 ml was incubated at 37°C for the time indicated. After the reaction was terminated by addition of 0.1 ml of 10% trichloroacetic acid, the reaction mixture was applied in 0.05-ml aliquots to a paper electrophoresis strip and electrophoresis was conducted in 1 N formic acid at 3000 V for 30 min. After thorough drying, the strip was scanned for radioactivity. The spots were visualized with ninhydrin spray and *o*-aminobenzaldehyde dipping. Development with *o*-amino benzaldehyde and ninhydrin showed an orange and a yellow color, respectively, in the black areas. On development with ninhydrin, the light areas were violet but were negative with *o*-aminobenzaldehyde.

dipping. As shown in Fig. 3, two radioactive spots corresponding to lysine and X were seen. The radioactivity of X increased and that of lysine decreased as the incubation was prolonged.

**Isolation of the Products.** The aminotransferase reaction was carried out on a large scale in order to isolate compounds X and bX as follows. The reaction mixture consisted of 75 mmoles of L-lysine, 50 mmoles of potassium $\alpha$-ketoglutarate, 0.5 mmole of pyridoxal 5'-phosphate, 1.5 mmole of sodium bisulfite, and partially purified enzyme (4 g of protein) in a final volume of 1–1. The pH of the mixture was adjusted to 8.0 with ammonium hydroxide solution. After incubation at 37°C for 8 hr, the pH was brought to 4.0 with concentrated hydrochloric acid and 1–1 of 99% ethanol was added. The precipitated protein was removed by centrifugation and ethanol in the supernatant was evaporated out at 40°C under reduced pressure. The solution was continuously treated with ether for 12 hr to free it from $\alpha$-ketoglutarate. Neither X nor bX was extracted with ether. The solution obtained was placed on a column of Dowex 50-X8 ($H^+$ form, $0.9 \times 70$ cm) and eluted with 0.5 N hydrochloric acid at the flow rate of 25 ml/hr and 10 ml/fraction was collected. Each fraction was examined with ninhydrin and *o*-aminobenzaldehyde as described above. Compound bX was unabsorbed by the resin. After glutamate, ammonia, and a small peak originating from the impurities of the enzyme preparation were eluted with 0.5 N hydrochloric acid, compound X emerged. The fractions containing bX were concentrated to a small volume under reduced pressure. After the pH was adjusted to 7.0, the solution was placed on a column of Dowex 1-X4 ($OH^-$ form, $3 \times 60$ cm), washed with water, and eluted with 0.1 N hydrochloric acid. The fractions containing bX were pooled and lyophilized. Compound X eluted was purified by rechromatography on a column of Dowex 50-X8 ($H^+$ form, $0.9 \times 100$ cm) as mentioned above and lyophilized. Both compounds X and bX isolated were electrophoretically and paper chromatographically pure, but attempts to further purify and crystallize them were unsuccessful because of their lability. Authentic $\Delta^1$-piperideine-2-carboxylic acid was chromatographed on

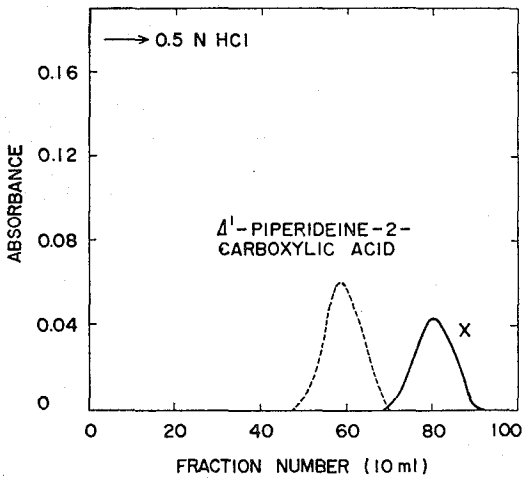



Fig. 4. Chromatography of the mixture of $\Delta^1$-piperideine-2-carboxylic acid and the reaction products on Dowex 50-X8 ($H^+$) column. The abosobance caused by the reaction of *o*-aminobenzaldehyde with $\Delta^1$-piperideine-2-carboxylic acid and compound X was measured at 450 m$\mu$ (----) and 465 m$\mu$ (——), respectively. Other conditions are shown in text.

Dowex 50 ($H^+$) in the aforementioned way. This compound was eluted just ahead of X under the same conditions. The cochromatography of X with $\Delta^1$-piperideine-2-carboxylic acid on Dowex 50 ($H^+$) confirmed that they were not identical (Fig. 4).

**Properties of X.** Purified compound X was satisfactorily separated from $\Delta^1$-piperideine-2-carboxylic acid by paper electrophoresis conducted in 1 N formic acid at 1000 V for 2 hr (Fig. 5), although the paper chromatographic behaviors of both the compounds were similar in the several kinds of solvents as demonstrated in Table 1. As mentioned above, $\Delta^1$-piperideine-2-carboxylic acid and X react with *o*-aminobenzaldehyde to produce the orange compounds, which appear to be the dihydroquinazolinium compounds.[45] The reaction products of X and $\Delta^1$-piperideine-2-carboxylic acid with *o*-aminobenzaldehyde were submitted to paper electrophoresis. The *o*-aminobenzaldehyde adduct of X was distinguished from that of $\Delta^1$-piperideine-2-carboxylic acid. Both the adducts were decomposed during the development on the paper chromatography with the following solvents: *n*-butyl alcohol-acetic acid-water (4 : 1 : 1) and *t*-butyl alcohol-formic acid-water (70 : 15 : 15).

The *o*-aminobenzaldehyde adduct of X (pH 5.0) has an absorption maximum at about 465 mμ (Fig. 6). The adduct of $\Delta^1$-piperideine-2-carboxylic acid produced in the same way has an absorption maximum at approximately 450 mμ. Difference

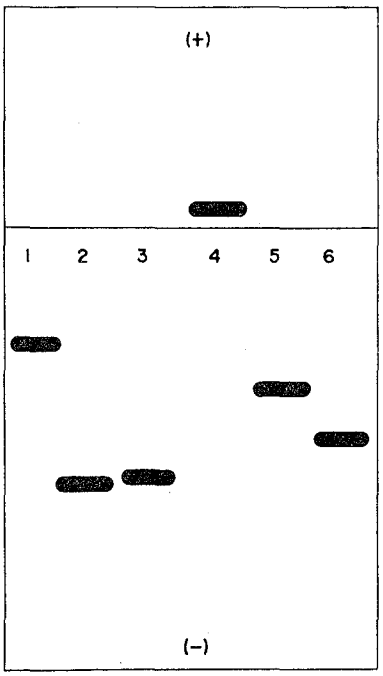



Fig. 5. Paper electrophoresis. Electrophoresis was conducted in 1 N formic acid at 1000 V for 2 hr. (1), $\Delta^1$-Piperideine-2-carboxylic acid; (2), compound X; (3), compound $(X)_2$; (4), compound bX; (5), dihydroquinazolinium complex formed from $\Delta^1$-piperideine-2-carboxylic acid and *o*-aminobenzaldehyde; (6), dihydroquinazolinium complex formed from compound X with *o*-aminobenzaldehyde.

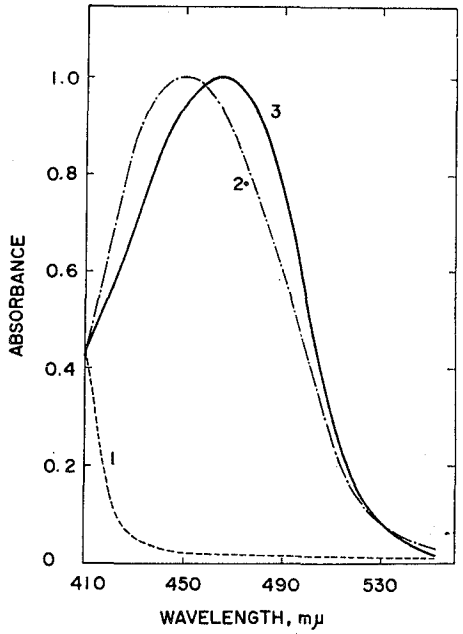



Fig. 6. Absorption spectra of colored products of $\Delta^1$-piperideine-2-carboxylic acid and compound X with *o*-aminobenzaldehyde. $\Delta^1$-Piperideine-2-carboxylic acid and compound X were incubated with *o*-aminobenzaldehyde (0.004 M) in 0.2 M acetate buffer (pH 5.0) at 37°C for 5 hr. Curve 1: spectrum of the *o*-aminobenzaldehyde; curve 2: spectrum of the colored solution of $\Delta^1$-piperideine-2-carboxylic acid with *o*-aminobenzaldehyde; curve 3: spectrum of the colored solution of compound X with *o*-aminobenzaldehyde.

Table 1. Rf Values in Paper Chromatography.

| Compound | Solvent | | | |
|---|---|---|---|---|
| | a | b | c | d |
| $\Delta^1$-Piperideine-2-carboxylic acid | 0.42 | 0.50 | 0.63 | 0.63 |
| X | 0.44 | 0.45 | 0.65 | 0.60 |
| $(X)_2$ | 0.11 | 0.17 | 0.62 | |
| bX | 0.43 | 0.48 | 0.67 | |
| Glutamate | 0.23 | 0.47 | | |
| Lysine | 0.09 | 0.22 | | |

Paper chromatography was performed in the following solvents: (a) *n*-butyl alcohol-acetic acid-water (4 : 1 : 1), (b) *t*-butyl alcohol-formic acid-water (70 : 15 : 15), (c) methanol-water-pyridine (4 : 1 : 1), and (d) ethanol-water (77 : 23). Toyo filter paper No. 50 was used.

between the absorption spectra of these *o*-aminobenzaldehyde addition compounds seem not to be large, but to be distinct. As shown in Figure 7, the maximal development of the color was obtained at 37°C within 30 min, when X reacted with *o*-amino-

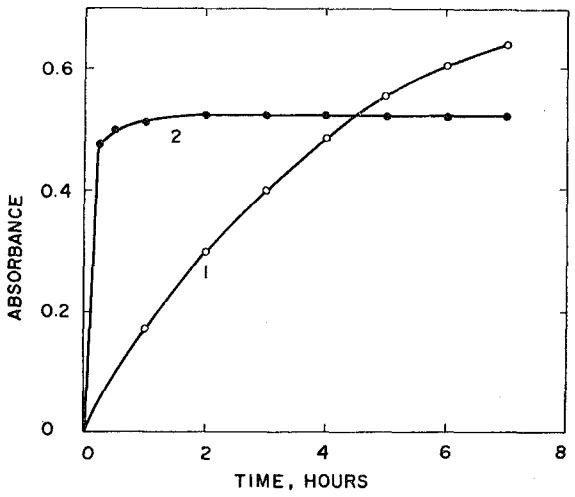



Fig. 7. Reaction rate of $\Delta^1$-piperideine-2-carboxylic acid and X with *o*-aminobenzaldehyde. $\Delta^1$-Piperideine-2-carboxylic acid (in curve 1) and X (in curve 2) were incubated with 0.004 M *o*-aminobenzaldehyde in 0.2 M acetate buffer (pH 5.0) at 37°C.

benzaldehyde. On the other hand, the maximal color intensity of the *o*-aminobenzaldehyde adduct of $\Delta^1$-piperideine-2-carboxylic acid required the incubation for more than 8 hr under the same conditions.

**Hydrogenation of X.** When the solution of X in 0.5 N hydrochloic acid was hydrogenated with platinium oxide catalyst at about 15°C for 30 min, X was converted into pipecolate, which was identified by a comparison of the Rf values with those of authentic sample in the several solvent systems. The reduction product of X was insusceptible to D-amino acid oxidase under the conditions described by Aspen and Meister,[39] although D-pipecolate was quantitatively oxidized by the oxidase under the same conditions. This finding suggests that the reduction product is L-pipecolate. The parent compound, X, also should have the L configuration.

**Dimerization of X.** Compound X was gradually converted into a new compound, $(X)_2$, when the neutral solution of X was allowed to stand at room temperature for a long time, *e.g.*, 24 hr. Compound $(X)_2$ reacts with *o*-aminobenzaldehyde and ninhydrin to give an orange and a yellow color, respectively. Although the paper electrophoretic behavior of $(X)_2$ was similar to that of X as shown in Fig. 5, the separation of these compounds was accomplished by the paper chromatography using *n*-butyl alcohol-acetic acid-water (4 : 1 : 1) and *t*-butyl alcohol-formic acid-water (70 : 15 : 15) as the solvents (Table I). On heating the acidic solution of $(X)_2$ in a boiling-water bath for 30 min, X was produced from $(X)_2$. These facts are in substantial agreement with the results obtained by Hasse *et al.*[46] on an interconversion reaction of $\Delta^1$-piperideine-6-carboxylic acid and its dimer, tetrahydroanabasinedicarboxylic acid. If X is identical with $\Delta^1$-piperideine-6-carboxylic acid, $(X)_2$ is suggested to be the dimer formed by condensation.

**Conversion of bX into X.** The treatment of the purified compound bX with 0.5 N hydrochloric acid at 100°C for 30 min followed by paper electrophoresis

demonstrated the disappearance of bX and the appearance of X formed by the elimination of the anionic group. Similar conversion of dX was into X was observed by the same treatment. These findings suggest that some precursor of X involving a free aldehyde group in it reacts with sodium bisulfite or dimedon to yield the addition product, bX or dX, respectively.

**Possibility of Conversion of $\Delta^1$-Piperideine-2-carboxylic Acid into X.** When authentic $\Delta^1$-piperideine-2-carboxylic acid was added to the reaction mixture omitting L-lysine or $\alpha$-ketoglutarate and incubation was performed under the standard conditions, $\Delta^1$-piperideine-2-carboxylic acid was shown to be unchanged and the formation of X was not observed. This result excludes the possibility that $\Delta^1$-piperideine-2-carboxylic acid produced initially is converted into X enzymatically or non-enzymatically.

**Effect of NADH.** The incubation was carried out in the absence of and in the presence of NADH under the standard conditions. The activity was never influenced by addition of NADH (10 $\mu$moles). When the reaction mixture containing 0.5 $\mu$mole of NADH was incubated in the silica cuvets at room temperature (approximately 15°C), the decrease in absorbance at 340 m$\mu$ was not observed. These results show that it is highly unlikely for $\Delta^1$-piperideine-6-carboxylic acid and glutamate to be formed from L-lysine and $\alpha$-ketoglutarate through saccharopine as an intermediate.

When the crystalline enzyme from *Achromobacter liquidum* and the partially purified enzyme from *Flavobacterium fuscum* were used, the same results on the product from L-lysine as described here were obtained.

### (B) Configuration of Glutamate formed from $\alpha$-Ketoglutarate by L-Lysine-$\alpha$-Ketoglutarate Aminotransferase.

The standard aminotransferase reaction mixture was incubated at 37°C for 60 min. The reaction was terminated by immersing the tube for 5 minutes in a boiling-water bath. The amount of glutamate formed was measured to be 5.4 $\mu$moles per 1.5 ml of the reaction mixture by a Yanagimoto Model LC-5S automatic amino acid analyzer. A 1.5-ml aliquot of the reaction mixture was submitted to the L-glutamate decarboxylase reaction. The reaction mixture consisted of 1.5 ml of the sample, 0.5 ml of 0.2 M pyridine-HCl buffer, pH 4.0, containing 0.1 M sodium chloride, 0.2 ml of 0.01 M semicarbazide-HCl, 0.5 ml of L-glutamate decarboxylase (10 mg of acetone powder of *Escherichia coli* strain Crooks in 0.1 M pyridine-HCl buffer, pH 4.0, containing 0.1 M sodium chloride) and 0.3 ml of water. The incubation was performed at 30°C to reach completion. The evolution of 5.36 $\mu$moles of $CO_2$ was measured manometrically. This value corresponds to that of total amount of glutamate formed. The present finding indicates that glutamate formed from $\alpha$-ketoglutarate by this aminotransferase reaction is completely L configuration. This result was also supported by the substrate specificity as described below.

## Discussion

Unambiguous evidence has been obtained for the occurrence of L-lysine-$\alpha$-ketoglutarate aminotransferase in bacterial extracts and some properties of the partially

purified enzyme have been demonstrated.[37] In this transamination, glutamate was stoichiometrically formed from α-ketoglutarate and reaction product derived from L-lysine was found not to react with 2,4-dinitrophenylhydrazine, but to do so with *o*-aminobenzaldehyde and *p*-dimethylaminobenzaldehyde. This suggests that a cyclized form of the keto analog of lysine, *i.e.*, $\Delta^1$-piperideine-2-carboxylic acid or $\Delta^1$-piperideine-6-carboxylic acid, is produced. Identification of the product formed from L-lysine should provide determination of which amino group of lysine participates in the aminotransferase reaction. Catalytic hydrogenation of $\Delta^1$-piperideine-6-carboxylic acid derived from L-lysine would yield L-pipecolate, while nonenzymatic reduction of $\Delta^1$-piperideine-2-carboxylic acid would be expected to give DL-pipecolate. Determination of optical activity of the hydrogenated product also should give confirmatory information of the pathway of L-lysine transamination.

It is clear that X is the sole product derived from L-lysine in this aminotransferase reaction, because the radioactivity of DL-[1-$^{14}$C] lysine as the amino donor was incorporated into X only, as demonstrated in Fig. 3. The properties of X are similar to those of authentic $\Delta^1$-piperideine-2-carboxylic acid in the following points. Both of them do not react with 2,4-dinitrophenylhydrazine, but do so with *o*-aminobenzaldehyde and ninhydrin to develop an orange and a yellow color, respectively. They also migrate electrophoretically at pH 3.5 in a similar way.

The presence of sodium bisulfite in the reaction mixture led to the formation of an acidic compound, bX, which reacts with *o*-aminobenzaldehyde as well as X to develop an orange color. The isolated bX was converted into X by the treatment with hydrochloric acid. The isolated X is clearly distinguishable from $\Delta^1$-piperideine-2-carboxylic acid chromatographically and electrophoretically in 1 N formic acid as shown in Fig. 4 and 5. The Rf values in paper chromatography and the mobilities in paper electrophoresis of X are in agreement with those of $\Delta^1$-piperideine-6-carboxylic acid previously reported.[39,46]

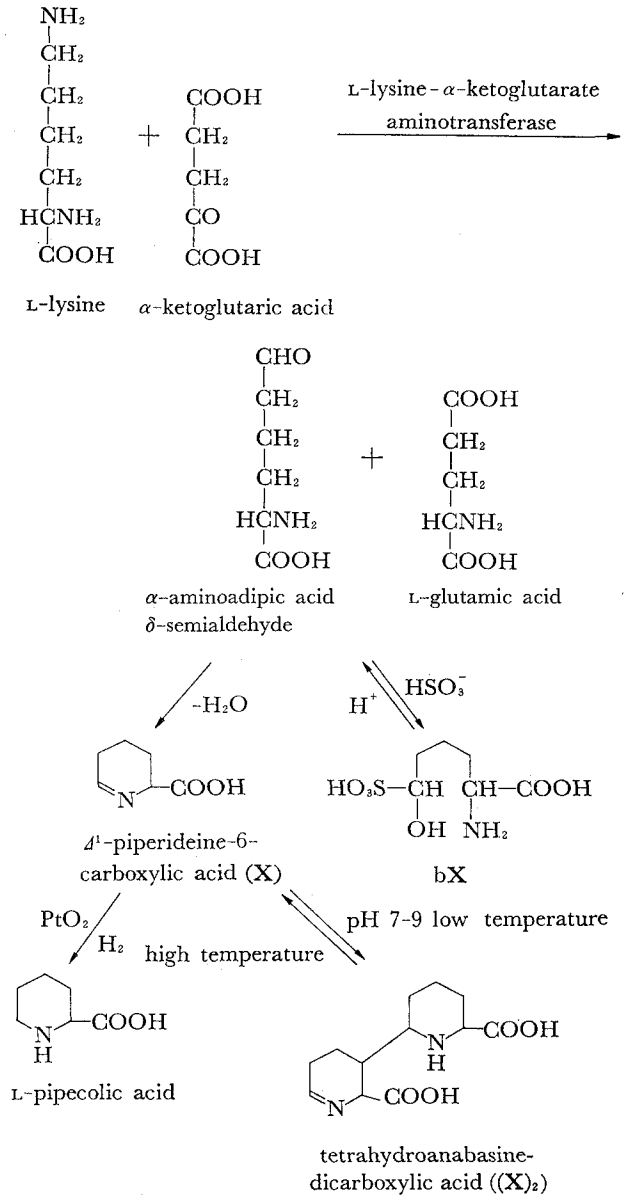
It was reported that both $\Delta^1$-piperideine-2-carboxylic acid and $\Delta^1$-piperideine-6-carboxylic acid react with *o*-aminobenzaldehyde to form dihydroquinazolinium salts.[47,48] The absorption spectrum of the *o*-aminobenzaldehyde derivative of X is slightly but distinctly different from that of $\Delta^1$-piperideine-2-carboxylic acid, although such derivatives were not isolated because of their instability. The reaction rate of $\Delta^1$-piperideine-2-carboxylic acid with *o*-aminobenzaldehyde is extremely lower than of X. This finding is essentially consistent with the results obtained by Aspen and Meister,[39] although the reaction rate observed by them in the alcoholic solution were much higher than that obtained here in the aqueous solution. The spectrophotometric determination of $\Delta^1$-piperideine-2-carboxylic acid by the incubation of the aqueous solution (pH 7.0) with *o*-aminobenzaldehyde at 37°C for 5 hr was reported.[49]

Pipecolate produced from X by the catalytic reduction was not oxidized by D-amino acid oxidase. If X is $\Delta^1$-piperideine-2-carboxylic acid, the catalytic hydrogenation of it would be expected to yield DL-pipecolate, a half of which must be oxidized by D-amino acid oxidase as shown by Aspen and Meister[40] with synthetic DL-pipecolate.

The interconversion of X and $(X)_2$ by standing at the neutral pH and at room temperature and heating at the acid pH is consistent with the observation of Hasse *et al.*[46] on the reversible condensation of $\Delta^1$-piperideine-6-carboxylic acid to its dimer,

tetrahydroanabasinedicarboxylic acid, by the similar treatment. Attempts to get the crystalline X were unsuccessful because of its instability and tendency to undergo dimerization.

α-Amino group of lysine was transaminated in plants as reported by Hasse *et al.*[38] and $\Delta^1$-piperideine-2-carboxylic acid which is intramolecularly cyclized form of α-keto-ε-aminocaproic acid was produced from lysine. But the possibility that $\Delta^1$-piperideine-2-carboxylic acid initially formed through α-keto-ε-aminocaproic acid is converted into X was excluded. Broquist and his coworkers reported the reversible




Scheme I

formation of L-lysine through saccharopine by the two combined dehydrogenase reactions in the biosynthesis of L-lysine in yeasts and *Neurospora crassa*.[50~52] Higashino *et al.*[17] also reported that L-lysine was metabolized to α-aminoadipic acid-δ-semialdehyde ($\Delta^1$-piperideine-6-carboxylic acid) by rat liver mitochondria through saccharopine as an intermediate. But, the investigation on the effect of NADH upon the aminotransferase reaction and the homogeneity of this enzyme as described in the following chapter ruled out the possibility of participation of such coupled reactions.

Thus, the findings stated in the present paper support the conclusion that the compound X is $\Delta^1$-piperideine-6-carboxylic acid, the intramolecularly dehydrated and cyclized form of α-aminoadipate-δ-semialdehyde which is initially derived from L-lysine by L-lysine-α-ketoglutarate aminotransferase reaction. Compounds bX and dX must be the bisulfite adduct and the dimedon adduct of α-aminoadipate-δ-semialdehyde, respectively. The reaction pathways described above are given in Scheme I.

## II. PURIFICATION AND CRYSTALLIZATION OF L-LYSINE-α-KETOGLUTARATE AMINOTRANSFERASE

Although considerable effort has been devoted to the purification and the characterization of α-amino acid aminotransferases in recent years,[53~55] little attention has been paid to the aminotransferase catalyzing the ε-transamination of L-lysine. Evidence was obtained for the occurrence of enzymatic transamination between L-lysine and α-ketoglutarate in *Flavobacterium fuscum, Flavobacterium flavescens, and Achromobacter liquidium* by Soda *et al*,[37] In previous chapter, identification and characterization of the product derived from L-lysine have been undertaken in an effort to obtain information concerning which amino group of the amino donor is transaminated. These investigations have revealed that the terminal amino group of L-lysine is enzymatically transferred to α-ketoglutarate to yield α-aminoadipate-δ-semialdehyde which is immediately converted into the intramolecularly dehydrated form, $\Delta^1$-piperideine-6-carboxylic acid. In the present chapter, the preparation of crystalline L-lysine-α-ketoglutarate aminotransferase from *Achromobacter liquidum* is described.

### Experimental Procedures

**Materials.** *o*-Aminobenzaldehyde[39] and hydroxylapatite[56] were prepared according to the methods given in the literatures. DEAE-cellulose was purchased from Sigma Chemical Co., St. Louis, Mo. L-Lysine and L-arginine were obtained from Tanabe Seiyaku Co., Osaka. L-Ornithine, L-histidine and L-threonine were purchased from Kyowa Hakko Kogyo Co., Tokyo. α-Ketoglutaric acid was a product of Ajinomoto Co., Tokyo. Pyridoxal 5′-phosphate was a product of Dainippon Seiyaku Co., Osaka.

**Microorganisms and Medium.** The bacterial strains used here were obtained from Faculty of Agriculture, Kyoto University (AKU) and Institute for Fermentation, Osaka (IFO). The medium for cultures consisted of 1.5% peptone, 0.2% potassium dihydrogen phosphate, 0.2% dipotassium hydrogen phosphate, 0.01%

magnesium sulfate, 0.01% yeast extract and 0.2% sodium chloride in tap water. The pH of the medium was adjusted to 7.2 with sodium hydroxide.

**Culture.** Various strains were picked from an agar slant of the medium described above and inoculated into the subculture (5.0 ml of medium in a 20-ml test tube). Cultivation was performed at 30°C for 18 hr with shaking. The subcultures were inoculated into a 100 ml of medium in 500-ml flask and cultivation was carried out at 30°C on a reciprocating shaker.

**Assay of Aminotransferase.** The standard assay system consisted of 20 $\mu$moles of L-lysine, 20 $\mu$moles of potassium $\alpha$-ketoglutarate, 1 $\mu$mole of pyridoxal 5'-phosphate, 90 $\mu$moles of potassium phosphate buffer (pH 8.0), and enzyme in a final volume of 2.0 ml. The mixture was incubated at 37°C for 10 min. The reaction was terminated by addition of 1.0 ml of 5% trichloroacetic acid solution and the reaction mixture was deproteinized by centrifugation at 17,000 g for 10 min. The aminotransferase was assayed by determining the amount of glutamate (Procedure A) or $\Delta^1$-piperideine-6-carboxylic acid (Procedure B) formed according to the method given in the previous chapter.

**Definition of Unit and Specific Activity.** One unit of enzyme was defined as the amount that catalyzes the formation of 1.0 $\mu$mole of glutamate or $\Delta^1$-piperideine-6-carboxylic acid per hr. The specific activity is expressed as units per mg of protein.

**Protein Determination.** Protein in the crude and partially purified enzyme preparations was determined by the procedure of Lowry *et al.*[44] using egg albumin as a standard; with most column fractions, protein elution patterns were estimated by the 280 m$\mu$ absorption. Concentrations of purified enzyme were derived from the absorbance at 280 m$\mu$. An $E_{1cm}^{1\%}$ value of 7.35 at 280 m$\mu$ was used throughout which was obtained by absorbance and dry weight determinations.

**Spectrophotometric Determination.** Spectrophotometric determinations were carried out with Shimazdu MPS-50L recording spectrophotometer, Shimadzu QV-50 spectrophotometer and Hitachi Photoelectric photometer EPO-B.

## Results

**Distribution of L-Lysine-$\alpha$-Ketoglutarate Aminotransferase.** Various strains of bacteria were grown in the peptone medium (100 ml) described in experimental procedure. Cultivation was carried out at 30°C for 24 hr on a reciprocating shaker. The cells harvested by centrifugation were washed twice with 0.85% sodium chloride solution, suspended in 10 ml of 0.1 M potassium phosphate buffer, pH 8.0, containing $10^{-4}$ M pyridoxal 5'-phosphate and 0.01% 2-mercaptoethanol, and disrupted by treatment for 2 min in a 19-kc Kaijo Denki ultrasonic disintegrator. The intact cells and cell debris were removed by centrifugation. The supernatant was dialyzed against 0.01 M potassium phosphate buffer, pH 7.4, containing $10^{-5}$ M pyridoxal 5'-phosphate and 0.01% 2-mercaptoethanol. The L-lysine-$\alpha$-ketoglutarate aminotransferase activity was determined with the dialyzed cell extract by measuring the amount of $\Delta^1$-piperideine-6-carboxylic acid formed from L-lysine. The L-lysine-$\alpha$-ketoglutarate aminotransferase activity was found exclusively in the cell extracts of

Table 1. Distribution of L-Lysine-α-Ketoglutarate Aminotransferase in Bacteria.

| Strain | | | Specific Activity |
|---|---|---|---|
| *Escherichia coli Crooks* | AKU | 0001 | 0 |
| *Proteus vulgaris* | IFO | 3045 | 0 |
| // | IFO | 3167 | 0 |
| // | IFO | 3851 | 0 |
| *Pseudomonas fluorescens* | IFO | 3461 | 0 |
| *Achromobacter liquidum* | IFO | 3084 | 2.8 |
| *Achromobacter superficialis* | AKU | 0123 | 0 |
| *Flavobacterium fuscum* | AKU | 0140 | 2.4 |
| *Flavobacterium flavescens* | AKU | 0141 | 1.8 |
| *Flavobacterium aquatile* | IFO | 3772 | 0 |
| *Flavobacterium capsulatum* | IFO | 12533 | 0 |
| *Flavobacterium gasogenes.* | IFO | 12065 | 0 |
| *Flavobacterium esteroaromaticum* | IFO | 3751 | 0 |
| *Flavobacterium heparinum* | IFO | 12017 | 0 |
| // | IFO | 12534 | 0 |
| *Flavobacterium meningesepticum* | IFO | 12535 | 0 |
| *Flavobacterium okeanokoites* | IFO | 12536 | 0 |
| *Flavobacterium suaveolens* | IFO | 3752 | 0 |
| *Brevibacterium acetylicum* | IFO | 12146 | 0 |
| *Brevibacterium ammoniagenes* | IFO | 12071 | 0 |
| // | IFO | 12072 | 0 |
| *Brevibacterium incertum* | IFO | 12145 | 0 |
| *Brevibacterium leucinophagum* | IFO | 12147 | 0 |
| *Brevibacterium lines* | IFO | 12141 | 0 |
| *Brevibacterium protophoriae* | IFO | 12128 | 0 |
| *Brevibacterium saperdae* | IFO | 12129 | 0 |

The cell extract was prepared as described in the text. The measurement of the enzyme activity was carried out under the standard assay conditions.

*Achromobacter liquidum, Flavobacterium fuscum and Flavobacterium flavescens* as shown in Table 1. The highest activity was found in the cell extract of *Achromobacter liquidum.*

**Effect of Added Amino Acids and α-Ketoglutarate in the Medium on the Growth and Formation of L-Lysine-α-Ketoglutarate Aminotransferase.** *Achromobacter liquidum* was used throughout in this experiment. Since *Achromobacter liquidum* grew in the medium containing L-lysine, L-lysine and α-ketoglutarate, or other amino acid in place of peptone, a basal medium containing 0.2% peptone was used. Five ml of cell suspension of *Achromobacter liquidum* grown in the medium containing 0.5% peptone was inoculated into 100 ml of the basal medium supplemented with 2% of various amino acids. Cultivation was carried out at 30°C for 41 hr on a reciprocating shaker. The cell growth was determined by measuring the turbidity at 610 mμ. The cells grew more slowly in the medium containing various amino acids than in the medium containing 1.5% peptone and no added amino acid. The specific activity of L-lysine-α-ketoglutarate aminotransferase, which was determined with the dialyzed cell-free extract, was found to be very high when the cells was grown in the medium

Table 2. Effect of Added Amino Acids in the Medium on the Formation of L-Lysine-α-Ketoglutarate Aminotransferase.

| Additions | concentration (%) | Cell Growth | Total Protein (mg) | Specific Activity (Units/mg) | Total Units |
|---|---|---|---|---|---|
| L-Lysine | 2.0 | 0.965 | 45.6 | 1.37 | 64.5 |
| L-Ornithine | 2.0 | 0.906 | 26.4 | 2.84 | 75.0 |
| L-Glutamate | 2.0 | 1.41 | 31.8 | 2.84 | 91.0 |
| α-Ketoglutarate | 2.0 | 0.415 | 7.76 | 1.14 | 8.8 |
| L-Lysine | 2.0 | | | | |
| + α-Ketoglutarate | 2.0 | 0.618 | 27.7 | 0.73 | 20.1 |
| L-Arginine | 2.0 | 0.76 | 21.2 | 1.30 | 27.0 |
| L-Threonine | 2.0 | 0.79 | 25.1 | 1.00 | 25.1 |
| L-Histidine | 2.0 | 0.513 | 17.1 | 1.45 | 24.8 |
| L-α-Aminoadipate | 2.0 | 0.175 | — | — | — |
| Peptone * | 1.5 | 2.57 | 96.0 | 2.3 | 220.0 |

*Achromobacter liquidum* was grown in the medium containing various amino acids as described in text at 30°C for 41 hr or 24 hr (*). L-Lysine-α-ketoglutarate aminotransferase activity was assayed as described in Experimental Procedure.

containing L-ornithine or L-glutamate (Table 2). The highest total activity was obtained in the cells grown in the medium containing 1.5% peptone. Inducibility of the enzyme by addition of L-lysine, L-glutamate or α-ketoglutarate could not be established because the organism required peptone for growth.

**Change in L-Lysine-α-Ketoglutarate Aminotransferase Activity during the Cell Growth.** Five-ml portions of cell suspension of *Achromobacter liquidum*

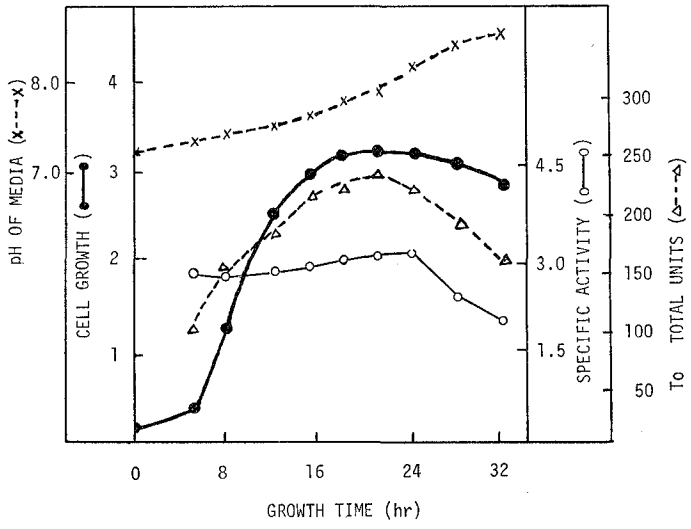



Fig. 1. Change in L-lysine-α-ketoglutarate aminotransferase activity during the cell growth. Cell growth was expressed as turbidity at 610 mμ. The cell-free extract was prepared as described in text and the enzyme activity was assayed as described in Experimental Procedure.

grown at 30°C for 18 hr in the medium containing 1.5% peptone, were inoculated again into 100 ml of the medium containing 1.5% peptone. Cultivation was carried out at 30°C for various times, and cell growth and the enzyme activity were determined. As shown in Fig. 1, the specific activity of the enzyme was not changed remarkably and the maximal total activity of the enzyme was obtained at the early stationary phase of cell growth.

**Purification of Enzyme.** *Achromobacter liquidum* IFO 3084 was employed for purification of the enzyme. Cultivation was performed at 30°C for 18 hr with a 10-l Fermentor Jar containing 7.5 l of the 1.5% peptone medium. The cells harvested by centrifugation were washed twice with 0.85% sodium chloride solution. The usual yield of cells was approximately 1.4 g dry weight per l of the medium. All subsequent operations were carried out at 0–5°C, unless specified otherwise.

*Step I. Preparation of cell-free extract.* The washed cells (80 g, dry weight) are suspended in 1–l of 0.1 M potassium phosphate buffer, pH 7.4, containing $10^{-5}$ M pyridoxal 5′-phosphate and 0.02% 2-mercaptoethanol, and disrupted in 100-ml portions by treatment for 10 min in a 19 kc Kaijo Denki ultrasonic disintegrator. The intact cells and cell debris were removed by centrifugation at 17,000 for 20 min. The supernatant was dialyzed overnight against 0.01 M potassium phosphate buffer, pH 7.4, containing $10^{-5}$ M pyridoxal 5′-phosphate and 0.01% 2-mercaptoethanol.

*Step II. DEAE-cellulose column chromatography.* The dialyzed enzyme solution is placed on a DEAE-cellulose column (6.0×45 cm) equilibrated with the dialysis buffer. After the column was washed thoroughly with 5–l of the same buffer, the enzyme was eluted stepwise with the buffer supplemented with sodium chloride (3–l with a final concentration of 0.04 M NaCl, then 3–l with a final concentration of 0.1 M). The enzyme activity was found only in the fractions eluted with buffer containing

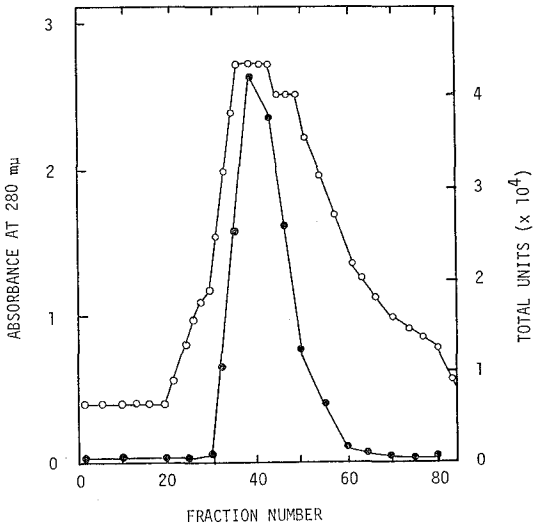



Fig. 2. The elution pattern from a DEAE-cellulose column. The flow rate was approximately 100 ml/hr and 20 ml fractions were collected. (○) Absorbance at 280 mμ; (●) L-lysine-α-ketoglutarate aminotransferase activity determined by procedure B. Other conditions are given in text.

0.1 M sodium chloride (Fig. 2). The active fractions are combined and concentrated by addition of ammonium sulfate (60% saturation). The precipitate was dissolved in a small volume of 0.01 M potassium phosphate buffer, pH 7.4, containing $10^{-4}$ M pyridoxal 5′-phosphate and 0.02% 2-mercaptoethanol.

*Step III. Heat treatment.* The enzyme solution was heated in a water bath (70°C) to bring its temperature to 55°C; this takes approximately 5 min. The enzyme solution was then kept at 55°C for 5 min, cooled rapidly and centrifuged at 17,000 g for 20 min to remove the precipitate.

*Step IV. Ammonium sulfate fractionation.* The supernatant was brought to 55% saturation with ammonium sulfate and centrifuged at 17,000 g for 30 min. The precipitate obtained is dissolved in a small volume of 0.001 M potassium phosphate buffer, pH 7.2, containing $10^{-5}$ M pyridoxal 5′-phosphate and 0.01% 2-mercaptoethanol, dialyzed overnight against the same buffer, and centrifuged.

*Step V. Hydroxylapatite column chromatography.* The supernatant solution of Step IV was applied to a hydroxylapatite column (3×25 cm) equilibrated with 0.001 M potassium phosphate buffer, pH 7.2, containing $10^{-5}$ M pyridoxal 5′-phosphate and

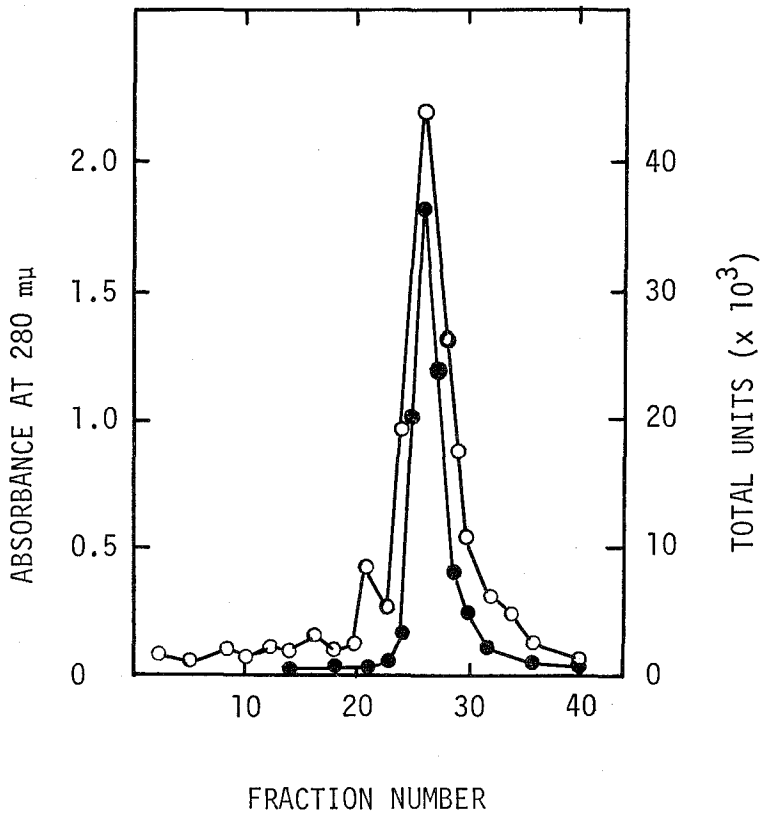



Fig. 3. The elution pattern from a hydroxylapatite column. The flow rate was approximately 10 ml/hr and 10 ml fractions were collected. (○) Absorbance at 280 mμ; (●) L-lysine-α-ketoglutarate aminotransferase activity determined by procedure B. Other conditions are given in text.

0.01% 2-mercaptoethanol. The enzyme was eluted with the same buffer (Fig. 3). The active fractions were collected and concentrated by ammonium sulfate precipitation (60% saturation). The precipitate was dissolved in a small volume of 0.2 M potassium phosphate buffer, pH 7.2, containing $10^{-4}$ M pyridoxal 5′-phosphate and 0.01% 2-mercaptoethanol.

*Step VI. Crystallization.* Ammonium sulfate was added slowly to the enzyme solution until a faint turbidity was obtained. The pH of the solution was kept constant at 7.2 with 14% ammonium hydroxide solution. On standing overnight at about 3°C, crystal formation occurred. The crystals take the form of yellow rods (Fig. 4).

*Step VII. Recrystallization.* The crystalline enzyme was collected by centrifugation

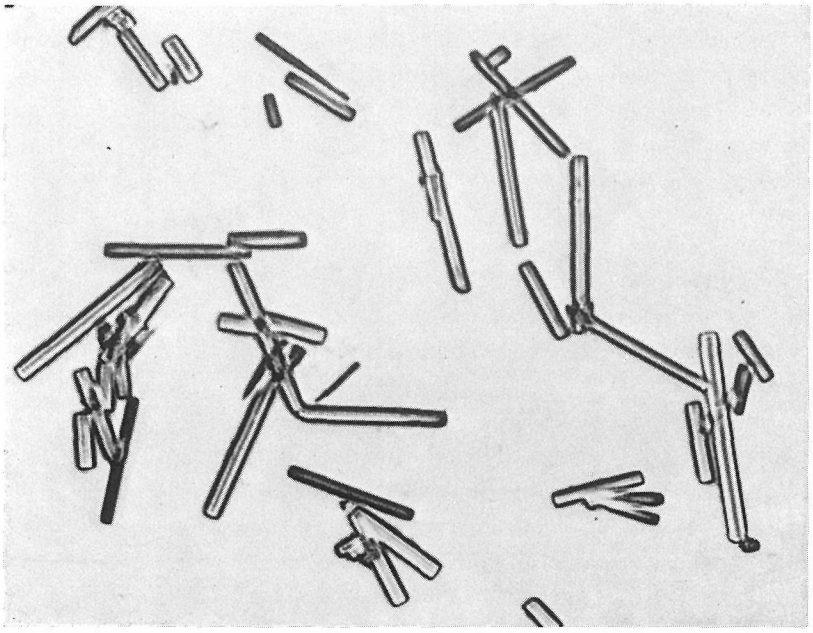


Fig. 4. Crystals of L-lysine-α-ketoglutarate aminotransferase.

Table 3. Purification of L-Lysine-α-Ketoglutarate Aminotransferase.

| Step | Procedure | Total Protein (mg) | Specific Activity (units/mg) | Total Units | Yield (%) |
|---|---|---|---|---|---|
| I. | Cell-free extract | 39,400 | 5.96 | 235,000 | 100 |
| II. | DEAE-cellulose chromatography | 1,594 | 117.2 | 187,000 | 80 |
| III. | Heat treatment | 625 | 395.0 | 247,000 | 105 |
| IV. | Ammonium sulfate, 0–55% saturation | 537 | 354.0 | 190,000 | 81 |
| V. | Hydroxylapatite chromatography | 110 | 1,006 | 111,000 | 47.2 |
| VI. | Crystallization | 85.3 | 1,100 | 93,800 | 40.0 |
| VII. | Recrystallization | 42.4 | 1,120 | 47,500 | 20.2 |

and dissolved in a small volume of 0.01 M potassium phosphate buffer, pH 7.2, containing $10^{-4}$ M pyridoxal 5'-phosphate and 0.01% 2-mercaptoethanol. The enzyme was recrystallized as described in Step VI.

The specific activity of the enzyme was not appreciably enhanced by crystallization or recrystallization. Approximately 180-fold purification was achieved with an overall yield of 20.2%. A summary of the purification procedure is given in Table 3.

## Discussion

Aminotransferase reaction of lysine was studied in bacteria by Feldman and Gunsalus,[35] but unequivocal evidence was not obtained for occurrence of L-lysine-$\alpha$-ketoglutarate aminotransferase. L-Lysine-$\alpha$-ketoglutarate aminotransferase was first found in the cell extracts of *Flavobacterium fuscum, Flavobacterium flavescens* and *Achromobacter liquidum* by Katagiri *et al.*[36] The authors also tested distribution of the L-lysine-$\alpha$-ketoglutarate aminotransferase activity in bacteria, especially *Flavobacterium* family and *Brevibacterium* family by measuring the $\Delta^1$-piperideine-6-carboxylic acid formed from L-lysine. The enzyme activity was exclusively observed in the cell extract of *Achromobacter liquidum, Flavobacterium fuscum* and *Flavobacterium flavescens* as shown previously by Soda *et al.*[37] and was absent in the cell extract of other *Flavobacterium* species and *Brevibacterium* species. The aminotransferase was found most aboundantly in the cell extract of *Achromobacter liquidum*. Occurrence of L-lysine-$\alpha$-ketoglutarate aminotransferase activity may be utilized as a simple tool of the classification of these bacterial strains.

*Achromobacter liquidum* never grew in the medium containing L-lysine as a sole carbon or nitrogen source. When the microorganism was grown in the basal medium supplemented with various amino acids, the growth of cells was slow and the inducibility of the enzyme by addition of substrates was not observed.

L-Lysine-$\alpha$-ketoglutarate aminotransferase has been purified 180-fold and crystallized from the cell-free extracts of *Achromobacter liquidum*. During the purification of the enzyme, it was observed that the enzyme in the crude preparation was less stable than the enzyme purified partially by the DEAE-cellulose column chromatography. This instability may be due to the existence of proteolytic enzyme to destroy the native enzyme in the crude preparation, which is probably removed by DEAE-cellulose column chromatography. Therefore, the dialyzed crude extract of *Achromobacter liquidum* was packed on a DEAE-cellulose column and the proteolytic enzyme which is not adsorbed on DEAE-cellulose column under the conditions was removed. The active fractions eluted with 0.01 M potassium phosphate buffer, pH 7.4, containing 0.1 M sodium chloride from DEAE-cellulose column were concentrated with ammonium sulfate (60% saturation) and was heated. The enzyme was activated by this treatment. The mechanism of such activation is at present unknown. Heat-treated enzyme was further purified by ammonium sulfate fractionation, hydroxylapatite column chromatography, and yellow rod crystals were obtained by addition of ammonium hydroxide. This crystalline enzyme preparation is homogeneous by the criteria of ultracentrifugation, free-boundary electrophoresis and disc gel electrophoresis as described in the following chapter.

## III. PROPERTIES OF L-LYSINE-α-KETOGLUTARATE AMINOTRANSFERASE FROM *ACHROMOBACTER LIQUIDUM*

Preparation of crystalline L-lysine-α-ketoglutarate aminotransferase from the cell-free extract of *Achromobacter liquidum* was described in Chapter II. In this chapter, some of its physicochemical and enzymological properties are presented.

### Experimental Procedures

**Materials.** S-(β-Aminoethyl)-L-cysteine was synthesized through the condensation of L-cysteine and β-bromoethylamine.[57] α-N-Acetyl-L-lysine, ε-N-acetyl-L-lysine, α-N-acetyl-L-ornithine, δ-N-acetyl-L-ornithine, S-(β-aminoethyl)-α-N-acetyl-L-cysteine and S-(β-N-acetylaminoethyl)-L-cysteine were prepared principally according to the methods of Neuberger and Sanger.[58] O-(β-Aminoethyl)-DL-serine was prepared from O-(β-N-benzyloxycarbonyl-aminoethyl)-DL-serine, which was kindly supplied by Dr. G. I. Tesser, Nijmegen, The Netherlands. ε-N-Methyl-L-lysine-HCl was kindly supplied by Dr. N. L. Benoiton, University of Ottawa, Canada. Sodium pyruvate, sodium α-ketobutyrate, oxalacetic acid, α-ketocaproic acid, sodium α-ketovalerate, δ-aminovaleric acid and L-α,γ-diaminobutyric acid were purchased from Sigma Chemical Co., St. Louis, Mo. U. S. A. L-Lysine-HCl and L-arginine-HCl were obtained from Tanabe Seiyaku Co., Osaka. α-Ketoglutaric acid, L-valine, L-phenylalanine, ε-aminocaproic acid and γ-aminobutyric acid were products of Ajinomoto Co., Tokyo. L-Aspartic acid and L-leucine were purchased from Kyowa Hakko Kogyo Co., Tokyo. L-α-Aminoadipic acid was a product of Calibiochem, Los Angeles, Calif., U. S. A. Pyridoxal 5′-phosphate was a product of Dainippon Seiyaku Co., Osaka. Pyridoxal 5′-phosphate was chromatographically purified according to the method of Peterson and Sober.[54] The other chemicals were analytical grade reagents.

**Enzyme and Assay Methods.** Preparation of crystalline L-lysine-α-ketoglutarate aminotransferase and assay methods were described in Chapter II.

**Protein Determination.** Protein determination was performed spectrophotometrically by measuring the absorbance at 280 mμ ($E^{1\%}_{1cm}$ ; 7.35).

**Ultracentrifugal Analysis.** The purity of the crystalline enzyme and its sedimentation coefficient were determined with a Spinco Model E ultracentrifuge equipped with a phase plate as a schlieren diaphragm. The molecular weight of the enzyme was determined by the ultracentrifugal sedimentation equilibrium method according to the procedure of Van Holde and Baldwin.[60] The experiments were carried out in a Spinco Model E ultracentrifuge equipped with Rayleigh interference optics. Multicell operation was employed in order to perform the experiment on five samples of different initial concentration ranging from 0.1 to 0.5% with the use of An-G rotor and double-sector cells of different side-wedge angles. According to the method of Hexner *et al.*[61] to shorten the time to attain the equilibrium, the rotor was centrifuged initially at 11,272 rpm for 2 hr and 30 min and then at 9,341 rpm. The liquid height was 0.15 cm. Interference patterns were photographed with Fuji

panchromatic process plate at intervals of 1 hr to compare and make sure the equilibrium was established. The relation between the concentration of the enzyme and the fringe shift was determined using the synthetic boundary cell. The molecular weight of the enzyme was also determined by the ultracentrifugal sedimentation equilibrium method according to the procedure of Yphantis.[62] The experiment was carried out in a Spinco Model E ultracentrifuge equipped with schlieren optics. In order to perform the experiment on four samples of different initial concentration 0.095 to 0.57%, an 8-channel centerpiece and An-D rotor were used. The rotor was centrifuged at 14,298 rpm at 20°C. Schlieren patterns were photographed with Fuji panchromatic process plate at intervals of 30 min to compare and make sure the equilibrium was established.

**Electrophoresis.** Free-boundary electrophoresis was carried out at 4°C in a Hitachi (HTB-2A) free-boundary electrophoresis apparatus equipped with a 2-ml cell. Disc gel electrophoresis in 7.5% polyacrylamide gel was performed by a modification of the method of Davis.[63] The enzyme (29.3 $\mu$g) was placed on the top of spacer gel in 1 M sucrose. After the run, protein was stained with 1.0% Amino-Schwarz in 7% acetic acid. Destaining was accomplished electrophoretically in 7% acetic acid.

## Results

**Purity and Physicochemical Properties.** The crystalline enzyme preparation gives a single component when examined by free-boundary electrophoresis (Fig. 1) and by disc gel electrophoresis (Fig. 2). The crystalline enzyme was sedimented as a single component in the ultracentrifuge as shown in Fig. 3. The centrifugation studies in which enzyme solutions at different concentrations, 0.1–0.6 mg per ml in 0.01 M potassium phosphate buffer, pH 7.4, were employed, showed that the sedimentation coefficient of the enzyme protein, calculated for water at 20°C and zero protein concentration, is 6.37 S. The molecular weight was determined by the method of Van Holde and Baldwin[60] as described above. Enzyme solutions at five different concentrations were centrifuged at 20°C. The plot of log protein concentration against the square of the distance from the center of the rotor is shown in Fig. 4. Assuming a partial specific volume of 0.74, a molecular weight of 116,000$\pm$3,000 was obtained. The same value of molecular weight was also obtained when the enzyme was analyzed by the sedimentation equilibrium method of Yphantis.[62]

**Stability.** The stability of the enzyme depended upon the degree of purification and the conditions. The enzyme in the dialyzed sonicate was considerably labile to heating at 50°C, but when partially purified by DEAE-cellulose column chromatography, it was stable to heating and was activated by standing at 50–60°C for 5 min as demonstrated in Fig. 5a. When a preparation of this aminotransferase was heated at various values of pH, the enzyme was found to be stable between pH 6.0 and 7.5 (Fig. 5b). Pyridoxal 5′-phosphate (0.1 mM) and 2-mercaptoethanol (0.02%) afforded good protection of the enzyme activity against heat inactivation. The crystalline enzyme can be stored with little loss of activity at 0–5°C in 0.2 M potassium phosphate buffer, pH 7.2, containing 50% saturation of ammonium sulfate,

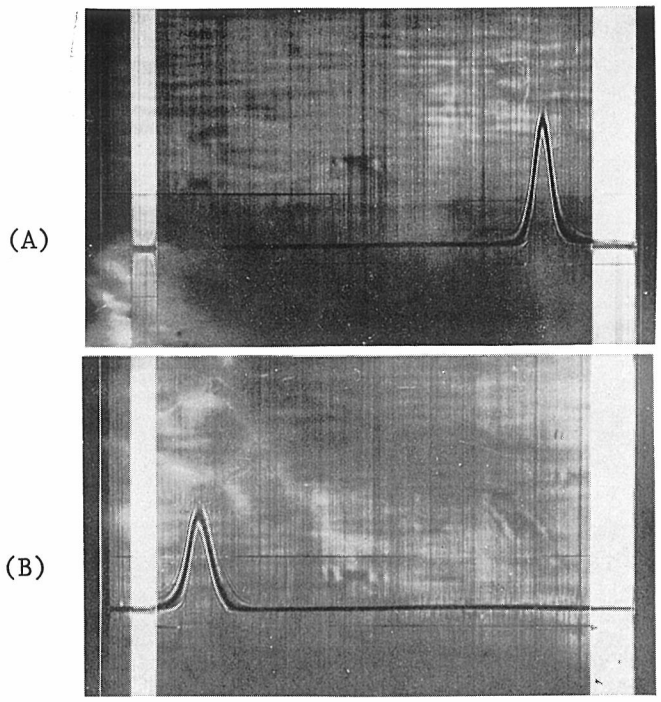



Fig. 1. Free-boundary electrophoresis pattern of L-lysine-α-ketoglutarate aminotransferase: (A) ascending boundary; (B) descending boundary. The enzyme solution (8.9 mg/ml) was subjected to electrophoresis in a Hitachi (HTB-2A) free-boundary electrophoresis apparatus equipped with a 2-ml cell. The temperature was 4° and sodium phosphate buffer (pH 7.0; ionic strength, 0.2) was employed. A current of 10 mA (56 V) was applied. Pictures were taken at bar angle of 30° at 2 hr after initiation of the experiment. Migration is toward the anode on the left (A) and on the right (B).

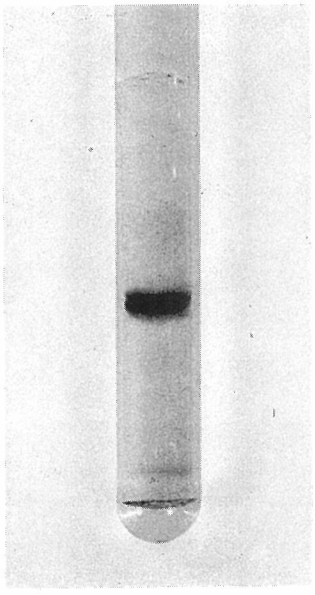


Fig. 2. Disc gel electrophoresis of L-lysine-α-ketoglutarate aminotransferase. Electrophoresis was conducted at a current of 2 mA for 2 hr in Tris-glycine buffer (pH 9.0). The direction of migration is from the cathode (top of photo) to the anode.

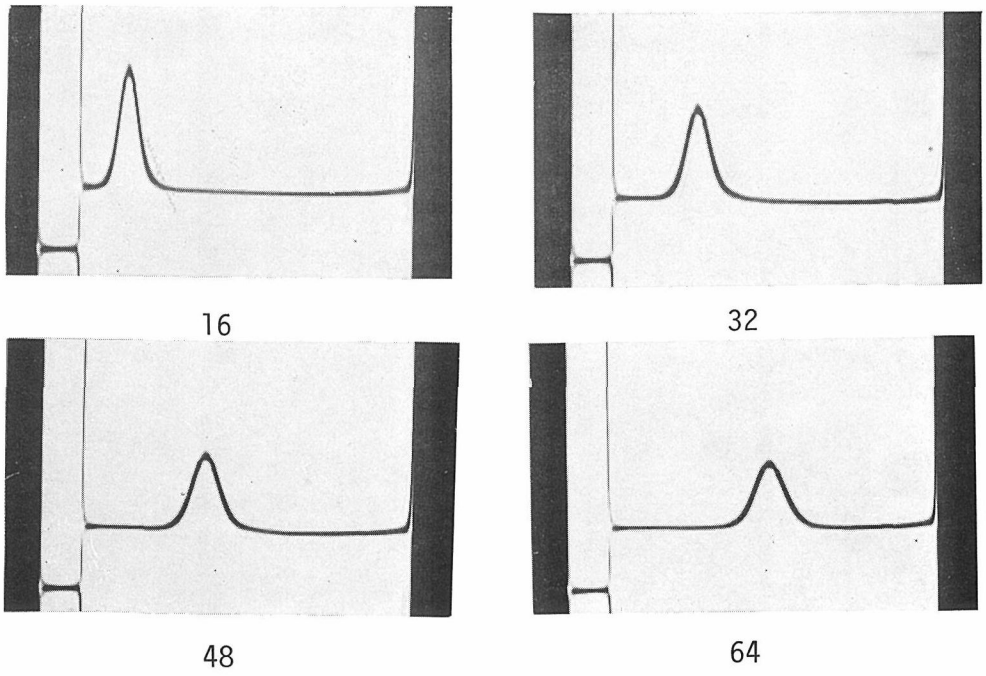



Fig. 3. Sedimentation pattern of crystalline L-lysine-$\alpha$-ketoglutarate aminotransferase. Protein concentration, 8 mg/ml in 0.01 M potassium phosphate buffer (pH 7.0). Pictures were taken at bar angle of 70° at 16, 32, 48 and 64 min after achieving top speed (59, 780 rpm).

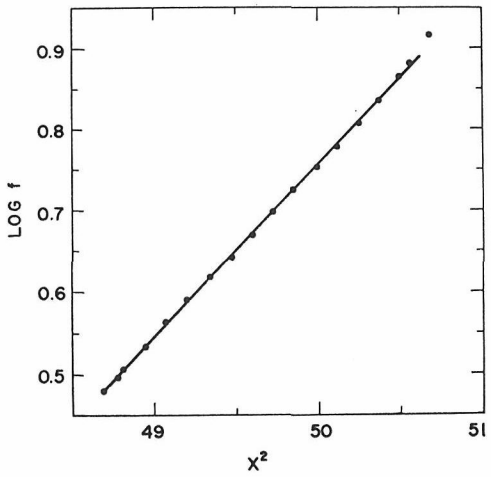



Fig. 4. Sedimentation equilibrium pattern of L-lysine-$\alpha$-ketoglutarate aminotransferase. The sample initially contained 5 mg of protein/ml in 0.01 M potassium phosphate buffer (pH 7.0). Log protein concentration is shown as a function of square of the distance from the center of rotor: f, fringe shift (millimeters); X, the distance from the center of the rotor (centimeters).

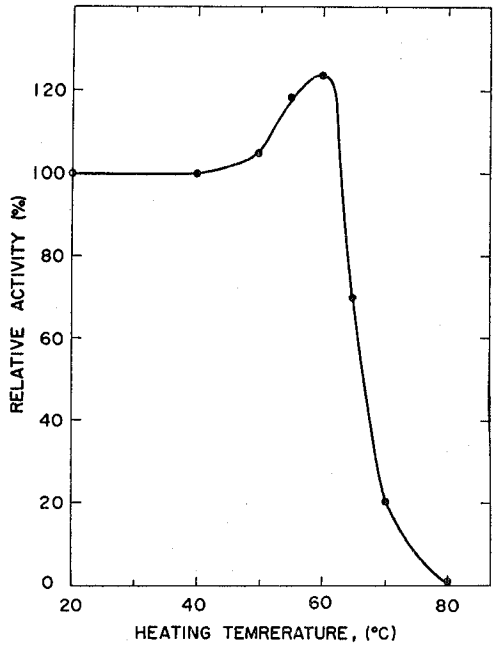



Fig. 5a.

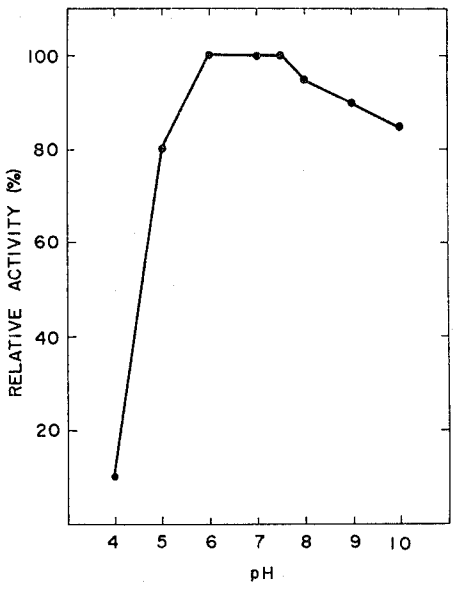



Fig. 5b

Fig. 5a. Effect of temperature on the enzyme stability. The enzyme preparation was heated at the indicated temperature for 5 min. The enzyme activity was assayed by procedure B.

Fig. 5b. Effect of pH on the enzyme stability. The enzyme preparation was heated at 55° for 5 min at the indicated pH. The enzyme activity was assayed by procedure B.

0.1 mM pyridoxal 5′-phosphate and 0.01% 2-mercaptoethanol, for several weeks. When the crystalline enzyme was dissolved in 0.01 M potassium phosphate buffer, pH 7.4, containing $10^{-5}$ M pyridoxal 5′-phosphate and 2-mercaptoethanol, and stored at 0–5°C, the enzyme activity was decreased gradually.

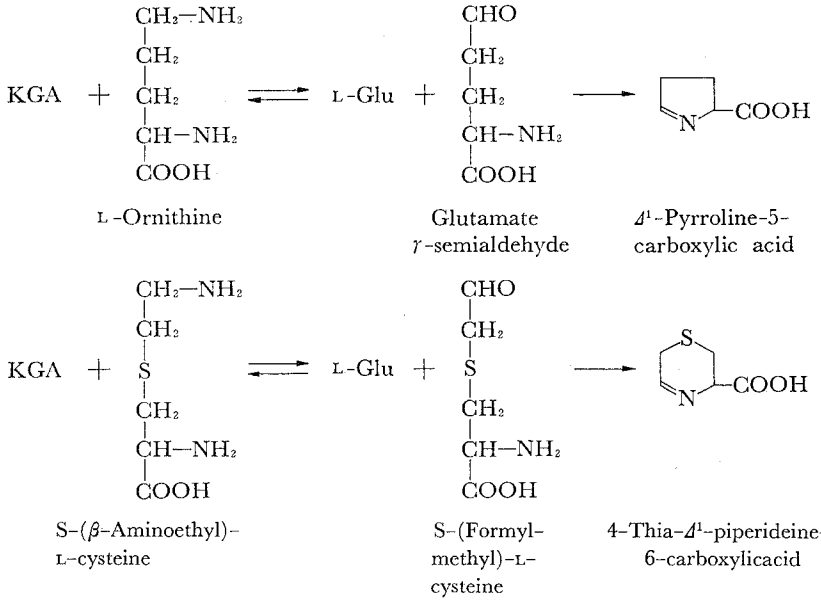
**Substrate Specificity.** In addition to L-lysine, which is the preferred substrate, several other L-amino acids serve as the amino donor for α-ketoglutarate (Table I). The relative activity of the enzyme is 100 for L-lysine, 55 for L-ornithine, 16 for S-(β-aminoethyl)-L-cysteine, 5.7 for ε-N-acetyl-L-lysine, 4.0 for δ-N-acetyl-L-ornithine, 3.3 for S-(β-N-acetylaminoethyl)-L-cysteine, 2.0 for δ-hydroxylysine, 1.9 for ε-aminocaproic acid, 1.8 for O-(β-aminoethyl)-DL-serine and 1.2 for δ-aminovaleric acid under the conditions employed. D-Amino acids, α-N-acetyl-L-lysine, α-N-acetyl-L-ornithine, S-(β-aminoethyl)-α-N-acetyl-L-cysteine, cadaverine and putrescine are inactive as the amino donor for this aminotransferase. α-Ketoglutarate is the most active amino acceptor for these amino donors. The rates of ε-transamination of L-lysine to α-ketoglutarate, pyruvate, oxalacetate, α-ketocaproate, α-ketovalerate, α-ketobutyrate and β-phenylpyruvate relative to α-ketoglutarate are 4.7, 0.64, 0.58, 0.46, 0.46 and 0.27%, respectively.

Table 1. *Amino Donor Specificity*

| Amino Donor [a] | Relative Activity % |
|---|---|
| L-Lysine | 100 |
| D-Lysine | 0.0 |
| δ-Hydroxylysine (DL, DL-allo) [c] | 2.0 |
| α-N-Acetyl-L-lysine | 0.0 |
| ε-N-Methyl-L-lysine | 0.43 |
| ε-N-Acetyl-L-lysine | 5.7 |
| O-(β-Aminoethyl)-DL-serine [b] | 1.8 |
| S-(β-Aminoethyl)-L-cysteine | 16.0 |
| S-(β-Aminoethyl)-D-cysteine | 0.0 |
| S-(β-Aminoethyl)-α-N-acetyl-L-cysteine | 0.0 |
| S-(β-N-Acetylaminoethyl)-L-cysteine | 3.3 |
| L-Ornithine | 54.5 |
| D-Ornithine | 0.0 |
| α-N-Acetyl-L-ornithine | 0.0 |
| δ-N-Acetyl-L-ornithine | 4.0 |
| L-α,γ-Diaminobutyric acid | 0.3 |
| L-α,ε-Diaminopimelic acid | 0.3 |
| ε-Aminocaproic acid | 1.9 |
| δ-Aminovaleric acid | 1.2 |
| γ-Aminobutyric acid | 0.35 |
| β-Alanine | 0.0 |
| Cadaverine | 0.0 |
| Putrescine | 0.0 |
| L-Arginine | 0.76 |
| L-Citrulline | 0.67 |
| L-Valine | 0.49 |
| L-Leucine | 0.35 |
| L-Isoleucine | 0.40 |
| L-Glutamine | 0.92 |
| L-Asparagine | 0.19 |
| L-Tyrosine | 0.30 |
| L-Phenylalanine | 0.30 |
| L-Alanine | 0.30 |
| S-Methyl-L-cysteine | 0.03 |
| Taurine | 0.0 |
| Cysteic acid | 0.0 |
| L-Threonine | 0.0 |
| L-Serine | 0.0 |
| α-Methyl-L-glutamic acid | 0.0 |
| Glycine | 0.0 |
| L-Tryptophan | 0.0 |

[a] The concentration of the amino donor 10 mM, [b], 20 mM, and [c], 40 mM. The enzyme activity was assayed by procedure A.

The transaminated product from L-ornithine and S-(β-aminoethyl)-L-cysteine were reacted with o-aminobenzaldehyde and ninhydrin to develop an orange and a yellow color, respectively, in the same manner as reported on the product from L-lysine (Chapter I). Authentic $\Delta^1$-pyrroline-2-carboxylic acid was prepared by the oxidation of D-proline with D-amino acid oxidase prepared from pig kidney in order to compare it with the product from L-ornithine. Authentic $\Delta^1$-piperideine-2-carboxylic acid was prepared as described by Meister.[42] On paper electrophoresis, the transamination products from L-ornithine and S-(β-aminoethyl)-L-cysteine were separated satisfactorily from $\Delta^1$-pyrroline-2-carboxylic acid and $\Delta^1$-piperideine-2-carboxylic acid and showed properties similar to $\Delta^1$-piperideine-6-carboxylic acid. Although the isolation of these products and their further investigation were not performed, these findings suggest that the terminal amino groups of L-ornithine and S-(β-aminoethyl)-L-cysteine were transaminated to α-ketoglutarate to yield $\Delta^1$-pyrroline-5-carboxylic acid and 4-thia-$\Delta^1$-piperideine-6-carboxylic acid, respectively. $\Delta^1$-Pyrroline-5-carboxylic acid and 4-thia-$\Delta^1$-piperideine-6-carboxylic acid, respectively. $\Delta^1$-Pyrroline-5-carboxylic acid and 4-thia-$\Delta^1$-piperideine-6-carboxylic acid must be formed from glutamate-γ-semialdehyde and S-(formylmethyl)-L-cysteine which are produced directly from L-ornithine and S-(β-aminoethyl)-L-cysteine by the aminotransferase reaction as follows.




**Kinetics.** The Michaelis constants for L-lysine and α-ketoglutarate were determined according to the method of Velick and Vavra.[64] They were calculated to be $2.8\times10^{-3}$ M for L-lysine and $5.0\times10^{-4}$ M for α-ketoglutarate, as shown in Fig. 6. The estimated maximum turnover number is 2200 moles per min per mole of enzyme. When the L-ornithine is employed as an amino donor, the Km values for L-ornithine and α-ketoglutarate are $2.0\times10^{-3}$ M and $1.3\times10^{-4}$ M, respectively. In the reaction between S-(β-aminoethyl)-L-cysteine and α-ketoglutarate, Km value for

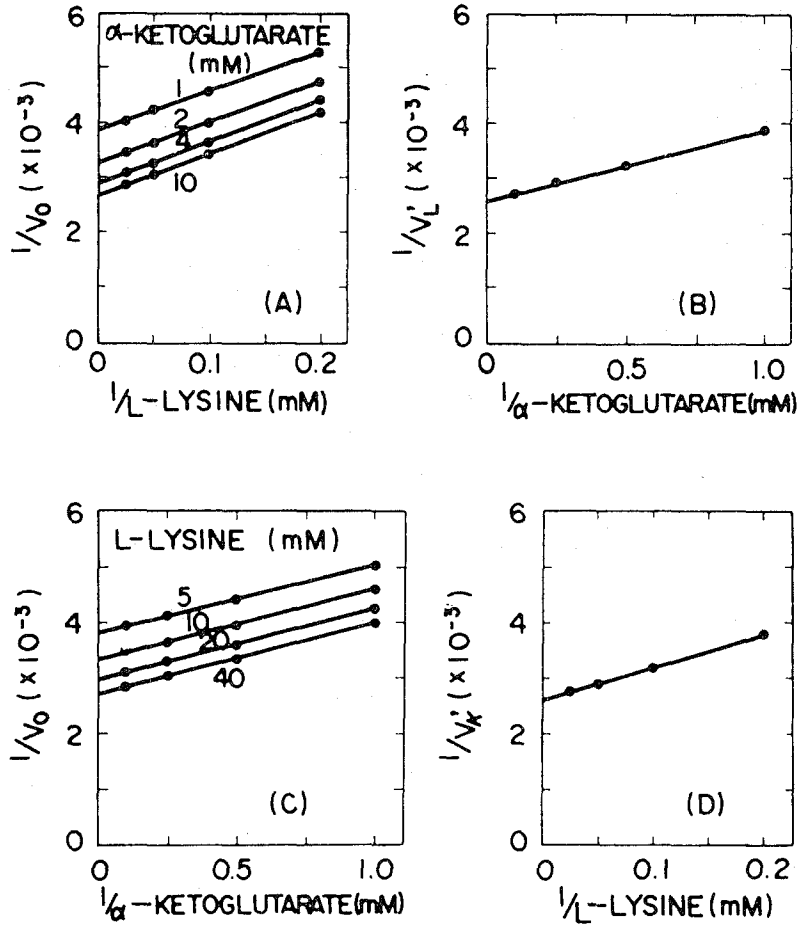



Fig. 6. Determination of the Michaelis constants for L-lysine and $\alpha$-ketoglutarate. The reaction mixture contained 1 $\mu$mole of pyridoxal 5'-phosphate, 90 $\mu$moles of potassium phosphate buffer (pH 8.0), and variable amounts of the substrates as indicated in the figure. The enzyme activity was assayed by procedure B. (A) Double-reciprocal plots of initial velocity, $V_O$, against L-lysine concentration at a series of fixed concentrations of $\alpha$-ketoglutarate; (B) secondary plot from the intercepts, $1/V_L'$, of A; (C) double-reciprocal plots of $V_O$ against $\alpha$-ketoglutarate concentration at a series of fixed concentrations of L-lysine; (D) secondary plot from the intercepts $1/V_K'$ of C.

the amino donor is $2\times10^{-3}$ M and $2\times10^{-3}$ M for the amino acceptor.

**Effect of pH.** The enzyme when examined in the presence of potassium phosphate, Veronal, and borate buffers has an optimum reactivity in the pH range of 8.3–8.5 for L-lysine-$\alpha$-ketoglutarate transamination, at pH 7.5 for L-ornithine-$\alpha$-ketoglutarate transamination and at pH 8.8 for S-($\beta$-aminoethyl)-L-cysteine-$\alpha$-ketoglutartae transamination as shown in Fig. 7. The aminotransferase reaction was inhibited approximately 25%, when Tris-HCl buffer (pH 8.0, 45 mM) was employed.

**Effect of Temperature.** When the aminotransferase reaction was performed at the various temperature, the maximum reaction velocity was obtained at 50–55°C (Fig. 8). The reaction velocity increased linearly when the temperature was raised

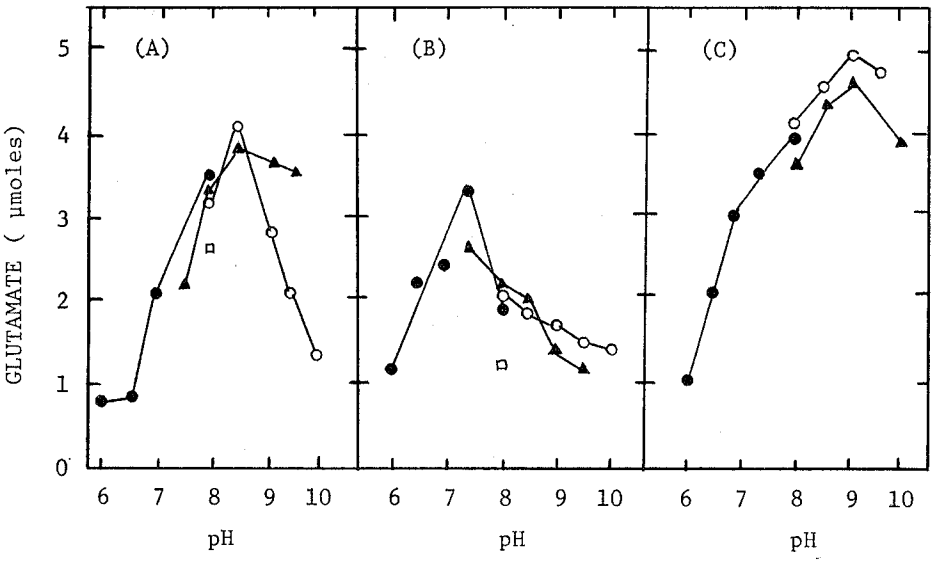



Fig. 7. Effect of pH on L-lysine-α-ketoglutarate transamination (A), L-ornithine-α-ketoglutarate transamination (B) and S-(β-aminoethyl)-L-cysteine-α-ketoglutarate transamination (C). The reaction mixture contained 20 μmoles of L-lysine, L-ornithine or S-(β-aminoethyl)-L-cysteine, 20 μmoles of α-ketoglutarate, 1 μmole of pyridoxal 5′-phosphate, and 90 μmoles of the following buffer in a final volume of 2.0 ml: (●—●) potassium phosphate, pH 6.0–8.0; (▲—▲) Veronal, pH 7.5–9.5; (○—○) borate, pH 8.0–10.0; (□) Tris-HCl, pH 8.0. The amount of enzyme was 20 μg (A, B) or 120 μg (C). The enzyme activities were assayed by procedure A.

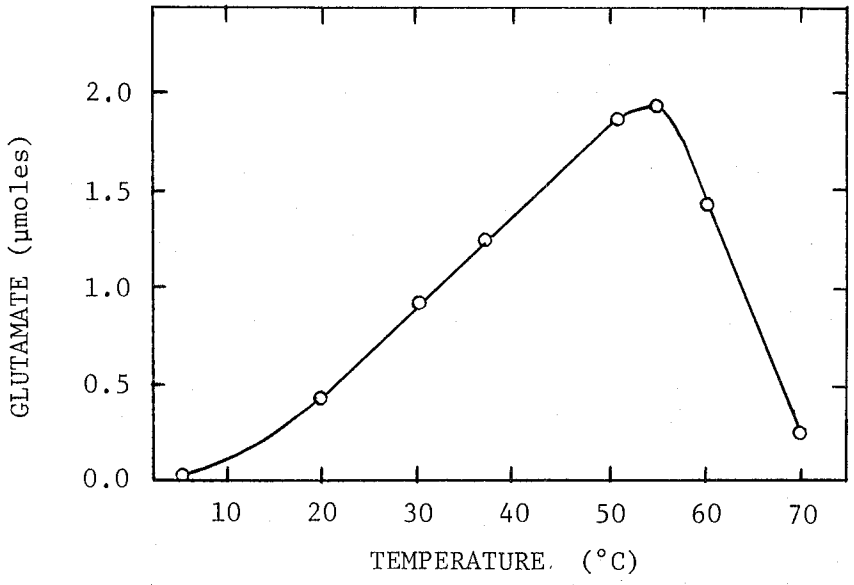



Fig. 8. Effect of temperature on L-lysine-α-ketoglutarate aminotransferase reaction. The standard reaction mixture was incubated at the indicated temperature for 10 min. The enzyme activity was assayed by procedure A.

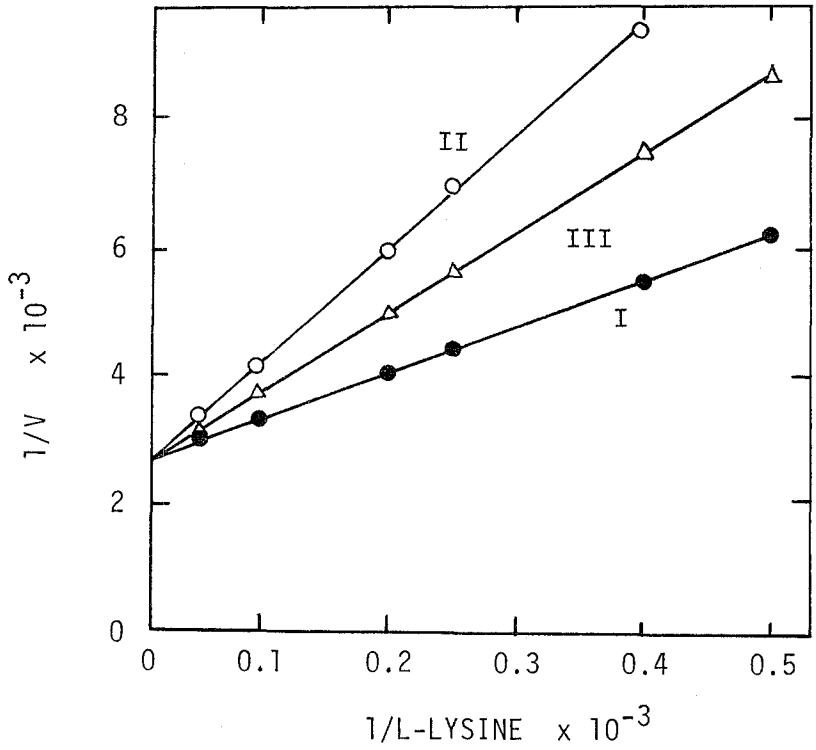



Fig. 9. Inhibition of L-lysine-$\alpha$-ketoglutarate aminotransferase reaction by $\delta$-aminovaleric acid and $\delta$-hydroxylysine. The enzyme activity was assayed in the absence (I) or presence of $\delta$-aminovaleric acid (II) or $\delta$-hydroxylysine (III). The reaction mixture contained 1 $\mu$mole of pyridoxal 5'-phosphate, 90 $\mu$moles of potassium phosphate buffer (pH 8.0), 40 $\mu$moles of potassium $\alpha$-ketoglutarate, and variable amounts of L-lysine as indicated, in a final volume of 2.0 ml. The concentration of $\delta$-aminovaleric acid or $\delta$-hydroxylysine was $1\times10^{-4}$ M or $1\times10^{-2}$ M, respectively. Velocity was expressed as amounts of $\Delta^1$-piperideine-6-carboxylate formed per mg of enzyme per hr.

in the range of 19–45°C but over 55°C, the velocity decreased rapidly.

**Inhibitors.** Effects of the various amino acids and amines on the aminotransferase activity were tested (Table 2). $\delta$-Aminovaleric acid, $\delta$-hydroxylysine (DL, DL-allo) and O-($\beta$-aminoethyl)-DL-serine, which are very poor amino donors, inhibited strongly L-lysine-$\alpha$-ketoglutarate aminotransferase reaction. These inhibitions result from the competition between them and L-lysine. Ki values for $\delta$-aminovaleric acid and $\delta$-hydroxylysine are calculated to be $7.7\times10^{-5}$ M and $1.4\times10^{-2}$ M, respectively, though the latter was obtained using DL and DL-allo mixture (Fig. 9).

Other various compounds were also investigated on their inhibitory effects on the activity of L-lysine-$\alpha$-ketoglutarate aminotransferase (Table 3). Semicarbazide, hydroxylamine, D-cycloserine, L-penicillamine, D-penicillamine and isoniazid, which are known to be inhibitors of vitamin $B_6$ enzymes, were tested. Hydroxylamine inhibited most strongly the aminotransferase reaction. D-Cycloserine and L-penicillamine also showed inhibitory effects.

Effects of *p*-chloromercuribenzoate (PCMB), $HgCl_2$, iodoacetate, iodoacetamide

Table 2. Effects of the Various Amino Acids and Amines on the L-Lysine-α-Ketoglutarate Aminotransferase Activity

| Additions | Concentration (mM) | Relative Activity (%) |
|---|---|---|
| None | | 100 |
| O-(β-Aminoethyl)-DL-serine | 20 | 58 |
| δ-Hydroxylysine (DL, allo) | 40 | 54.5 |
| α-N-Acetyl-L-lysine | 10 | 87 |
| ε-N-Acetyl-L-lysine | 10 | 100 |
| ε-N-Methyl-L-lysine | 10 | 100 |
| DL-Norleucine | 20 | 85 |
| DL-Norvaline | 20 | 67 |
| ε-Aminocaproic acid | 10 | 78 |
| δ-Aminovaleric acid | 10 | 12 |
| γ-Aminobutyric acid | 10 | 100 |
| Cadaverine | 10 | 99 |
| Putrescine | 10 | 100 |
| Canavanine | 10 | 85.8 |
| L-α,γ-Diaminobutyric acid | 10 | 84.6 |
| L-Arginine | 10 | 100 |
| L-Phenylalanine | 10 | 96 |
| L-α-Aminoadipic acid | 10 | 96 |

The reaction mixture containing a compound tested, was incubated under the standard conditions and the enzyme activity was assayed with procedure B.

and N-ethylmaleimide, which are typical inhibitors of thiol-enzymes, were examined. L-Lysine-α-ketoglutarate aminotransferase reaction was inhibited by high concentration of PCMB and $HgCl_2$ but not by iodoacetate, iodoacetamide and N-ethylmaleimide. The enzyme was activated to a certain extent by thiol compounds such as 2-mercaptoethanol and dithiothreitol, and the enzyme activity inhibited by PCMB was recovered by addition of these thiol compounds.

## Discussion

L-Lysine-α-ketoglutarate aminotransferase purified and crystallized from the cell-free extracts from *Achromobacter liquidum* IFO 3084 is homogeneous by the criteria of both disc gel and free-boundary electrophoresis and also by ultracentrifugation. The molecular weight of the enzyme was estimated to be 116,000±3,000 by two different methods of sedimentation equilibrium.

L-Lysine and L-ornithine are the preferred amino donors for this aminotransferase. The transamination product of ornithine was found to be $\Delta^1$-pyrroline-5-carboxylic acid; the terminal amino group of ornithine is transferred to α-ketoglutarate to form L-glutamic-γ-semialdehyde which is spontaneously converted to the cyclic form. Mammalian L-ornithine δ-aminotransferase was purified from rat liver and its properties were investigated by Katsunuma *et al.*,[65,66] Strecker[67] and Vogel *et al.*[68] L-

Table 3. Effects of Various Compounds

| Additions | Concentration (mM) | Relative Activity (%) |
|---|---|---|
| None | — | 100 |
| Semicarbazide-HCl | 1 | 100 |
| Hydroxylamine | 1 | 3.8 |
| D-Cycloserine | 1 | 86.3 |
| L-Penicillamine | 1 | 71.0 |
| D-Penicillamine | 1 | 100 |
| Isoniazid | 1 | 105 |
| *p*-Chloromercuribenzoate | 1 | 46 |
| | 0.1 | 72 |
| $HgCl_2$ | 1 | 6.4 |
| | 0.1 | 36 |
| | 0.01 | 64 |
| Iodoacetate | 1 | 98 |
| Iodoacetamide | 1 | 97 |
| N-Ethylmaleimide | 1 | 90 |
| Ethylenediaminetetraacetic acid | 1 | 105 |
| $\alpha,\alpha$-Dipyridyl | 1 | 100 |
| *o*-Phenanthroline | 1 | 100 |
| 2-Mercaptoethanol | 1 | 125 |
| Dithiothreitol | 1 | 120 |
| 2-Aminoisothiouronate | 1 | 115 |
| Glutathione | 1 | 120 |
| Thioglycolate | 1 | 120 |
| L-Cysteine | 1 | 110 |

The reaction mixture containing a compound tested, was incubated under the standard conditions and the enzyme activity was assayed with procedure B.

Ornithine $\delta$-aminotransferase cannot catalyze the transamination of L-lysine with $\alpha$-ketoglutarate. L-Lysine-$\alpha$-ketoglutarate aminotransferase differs unequivocally from L-ornithine $\delta$-aminotransferase, though the former catalyzes also the reaction of the latter.

S-($\beta$-Aminoethyl)-L-cysteine, thialysine, which is a sulfur analog of lysine is known to function as a metabolic antagonist of L-lysine. It and some of its peptides were reported to inhibit growth of certain lactic acid bacteria.[69,70] Such inhibitions were competitively reversed by L-lysine, L-lysylglycine and glycyl-L-lysine. Cavallini *et al.*[71] investigated the metabolism of S-($\beta$-aminoethyl)-L-cysteine *in vivo* by the rat and identified the metabolic products as S-($\beta$-aminoethyl)-$\alpha$-N-acetylcysteine, lanthionine and cysteamine. L-Lysine decarboxylase of *Escherichia coli* was found to catalyze the decarboxylation of S-($\beta$-aminoethyl)-L-cysteine.[72] Hope[73] demonstrated that S-($\beta$-aminoethyl)-L-cysteine was oxidized by L-amino acid oxidase of *Mytilus edulis*. The acetylation of the terminal amino group of S-($\beta$-aminoethyl)-L-cysteine was demonstrated by Soda *et al.*[74] in *Aerobacter aerogenes*. S-($\beta$-Aminoethyl)-L-cysteine

is also transaminated to α-ketoglutarate by L-lysine-α-ketoglutarate aminotransferase at a rate 16% of that for L-lysine. The transamination product of S-(β-aminoethyl)-L-cysteine is suggested strongly to be 4-thia-$\Delta^1$-piperideine-6-carboxylic acid which is formed *via* the initial product from S-(β-aminoethyl)-L-cysteine, S-(formylmethyl)-L-cysteine. Although an oxygen analog of lysine, O-(β-aminoethyl)-DL-serine which is also an antagonist of lysine, has been found to be oxidized to yield 4-oxa-$\Delta^1$-piperideine-2-carboxylic acid by L-amino acid oxidase of *Mytilus edulis*,[73] this compound is transaminated less effectively by this aminotransferase than the sulfur analog.

The ω-transaminase reactions of β-alanine,[75] γ-aminobutyric acid,[76,77] δ-aminovaleric acid[10,78] and ε-aminocaproic acid[79] have been studied with various enzyme preparations. In this L-lysine-α-ketoglutarate aminotransferase, β-alanine and γ-aminobutyric acid were inactive as an amino donor, but ε-aminocaproic acid and δ-aminovaleric acid were very poor substrates. Strecker[67] reported that L-ornithine δ-aminotransferase was inhibited perfectly by canavanine or δ-aminovaleric acid at the concentration of 10 mM. δ-Aminovaleric acid, which is a very poor amino donor of L-lysine-α-ketoglutarate aminotransferase, inhibited effectively the enzyme because of its strong affinity for the enzyme. δ-Aminovaleric acid which is a decarboxylation product of α-keto-ε-aminocaproic acid is formed from lysine by the coupled actions of two enzymes; lysine oxygenase and δ-aminovaleramide amidase obtained from a pseudomonad.[21~24] However, L-lysine oxygenase activity was not observed in the crude extracts of *Achromobacter liquidum, Flavobacterium fuscum* and *Flavobacterium flavescens*.

On the other hand, Hasse *et al.*[46,80] reported occurrence of aminotransferase reaction between α-N-acetyllysine and pyruvate in the extract of *Pseudomonas fluorescens* and of that between ε-N-acetyllysine and α-ketoglutarate in higher plant and *Neurospora crassa*. By L-lysine-α-ketoglutarate aminotransferase, ω-N-acetylated derivatives of L-lysine, L-ornithine and S-(β-aminoethyl)-L-cysteine are transaminated to α-ketoglutarate though slowly but the α-N-acetylated derivatives are inert. Cadaverine and putrescine are insusceptible to the enzyme as an amino donor, although they have been known to be transaminated by amine aminotransferase from a mutant of *Escherichia coli* B.[81,82]

L-Lysine-α-ketoglutarate aminotransferase reaction is inhibited by carbonyl reagents, especially hydroxylamine, as well as other vitamin $B_6$ enzymes. This aminotransferase reaction is activated by the thiol compounds, whereas it is inhibited either by high concentration of PCMB or by low concentration of $HgCl_2$. The activity inhibited by the SH-reagents in restored by addition of thiol compounds.

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