phoryl moieties. The neighboring carboxyl group appears to have no demonstrable function.

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L-Lysine–α-Ketoglutarate Aminotransferase. I. Identification of a Product, Δ^1-Piperideine-6-carboxylic Acid*

Kenji Soda, Haruo Misono, and Tatsuo Yamamoto

ABSTRACT: The product derived from L-lysine was isolated from the L-lysine–α-ketoglutarate aminotransferase system of *Flavobacterium fuscum* and its properties were studied. Radioactivity from DL-[1-^{14}C]lysine was incorporated exclusively into a product which reacted with *o*-aminobenzaldehyde. Chromatographic and electrophoretic studies, characterization of the bisulfite and *o*-aminobenzaldehyde adducts and the condensation product, and comparison with authentic Δ^1-piperideine-2-carboxylic acid offer evidence that the product is Δ^1-piperideine-6-carboxylic acid. In this aminotransferase reaction, ε-amino group of L-lysine is transferred to α-ketoglutarate to yield glutamate and α-aminoadipate-δ-semialdehyde which is immediately converted into the intramolecularly dehydrated form, Δ^1-piperideine-6-carboxylic acid.

Although the metabolism of lysine has been extensively studied as reviewed by Meister (1965) and Broquist and Trupin (1966), the mechanism of the enzymatic deamination of lysine has, until recently, remained unsolved. Both D and L isomers of lysine are known to be scarcely deaminated by the respective amino acid oxidases (Bender and Krebs, 1950; Greenstein *et al.*, 1953; Scannone *et al.*, 1964). In 1960, the occurrence of a bacterial enzyme which catalyzes the conversion of L-lysine into δ-aminonorvalerate, ammonia, and carbon dioxide was reported (Hagihira *et al.*, 1960). This enzyme was later demonstrated to be an oxygenase (Itada *et al.*, 1961), which has been crystallized (Takeda and Hayaishi, 1966). This oxygenase reaction involves initially the oxygenative de-

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carboxylation of L-lysine producing δ-aminonorvaleramide, which is deaminated to δ-aminonorvalerate by an amidase. Aspen and Meister (1962a) have shown that the nonenzymatic transamination of lysine and glyoxylate at pH 5 and 100° gives α-keto-ε-aminocaproate rather than α-aminoadipate-δ-semialdehyde. They (Aspen and Meister, 1962b) reported also that Δ^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.

Unambiguous evidence has been obtained for the occurrence of L-lysine–α-ketoglutarate aminotransferase in bacterial extracts and some properties of the partially purified enzyme have been demonstrated (Soda *et al.*, 1961). 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.*, Δ^1-piperideine-2-carboxylic acid or Δ^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 Δ^1-piperideine-6-carboxylic acid derived from L-lysine would yield L-pipecolate, while nonenzymatic reduction of Δ^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 on the pathway of L-lysine transamination.

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

Experimental Section

Materials. *o*-Aminobenzaldehyde was prepared by reduction of *o*-nitrobenzaldehyde according to the procedure of Smith and Opie (1955). Δ^1-Piperideine-2-carboxylic acid was synthesized from ε-*N*-carbobenzoxy-L-lysine as described by Meister (1954). 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 Conditions of Culture. *Flavobacterium fuscum* AKU 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. The pH was adjusted to 7.2 with sodium hydroxide. The cultures were grown at 30° under aeration for 18 hr. The cells harvested by centrifugation were washed twice with 0.85% sodium chloride solution and stored at −20° 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)/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°. The enzyme was purified approximately sevenfold by these procedures, although this enzyme has been purified to be homogeneous from *Achromobacter liquidum* as reported by Soda and Misono (1968).

Analytical Methods. Glutamate formed in the reaction mixture was determined as previously described (Soda *et al.*, 1961). Amino acids in the fractions eluted from Dowex 50 column were assayed by the method of Rosen (1957). Δ^1-Piperideine-2-carboxylic acids, X and

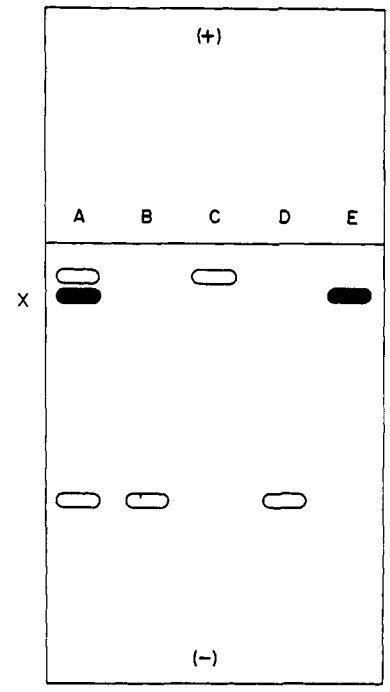


FIGURE 1: Paper electrophoresis of reaction mixture. Electrophoresis was conducted at pH 3.5 in 0.2 M pyridine acetate buffer at 2000 V for 90 min. (A) Complete system, (B) potassium α-ketoglutarate omitted, (C) L-glutamate, (D) L-lysine, and (E) Δ^1-piperideine-2-carboxylic acid. Development with *o*-aminobenzaldehyde 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.

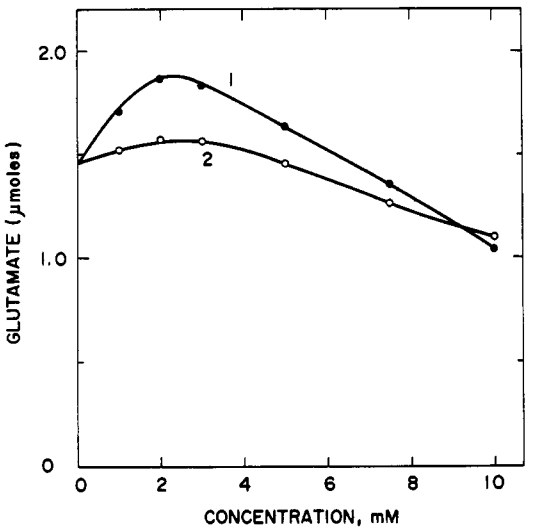


FIGURE 2: Effect of bisulfite and dimedon on the enzyme activity. The reaction mixture containing various concentrations of sodium bisulfite (in curve 1) or dimedon (in curve 2) was incubated at 37° for 10 min. The activity was determined according to procedure A.

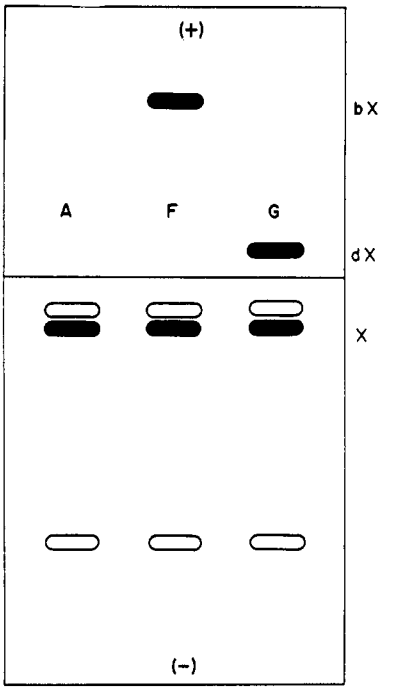


FIGURE 3: Paper electrophoresis of reaction mixture incubated in the presence of sodium bisulfite or dimedon. Electrophoresis was conducted at pH 3.5 in 0.2 M pyridine acetate buffer. (A) Complete system, (F) sodium bisulfite (10^{-3} M) added, and (G) dimedon (10^{-3} M) added. The other conditions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

bX, which were identified as Δ^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 Δ^1-piperideine-2-carboxylic acid was used, the reaction mixture was incubated at 37° for 8 hr and the color intensity was measured against a blank at 450 mμ using a Shimadzu QV-50 spectrophotometer. In the case of X and bX, after the incubation at 37° for 1 hr, the absorbance was determined at 465 mμ. Protein was determined by the method of Lowry *et al.* (1951). 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 μmoles of L-lysine, 20 μmoles of potassium α-ketoglutarate, 1 μmole of pyridoxal 5′-phosphate, 90 μmoles of potassium phosphate buffer (pH 8.0), and enzyme in a final volume of 2.0 ml. The mixture was incubated at 37° 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 mμ. 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° 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 Δ^1-piperideine-6-carboxylic acid was not prepared. The specific activity was expressed as micromoles of glutamate or Δ^1-piperideine-6-carboxylic acid formed per milligram 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° for 15 min and examined for the appearance of the colored spots.

Results

Paper Electrophoresis of Reaction Products. The reaction mixture described above was incubated at 37° 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 Figure 1. Glutamate and X were not produced in the reaction system lacking any of L-lysine, α-ketoglutarate, and

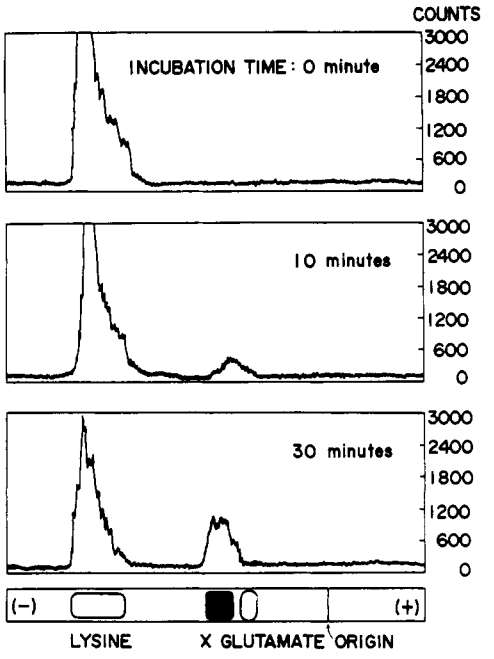


FIGURE 4: Incorporation of the radioactivity into X from DL-[1-^{14}C]lysine. The reaction mixture containing 0.08 μmole (0.86 μCi) of DL-[1-^{14}C]lysine, 5.0 μmoles of L-lysine, 5.0 μmoles of potassium α-ketoglutarate, 0.75 μmole of pyridoxal 5′-phosphate, 5.0 μmoles of potassium phosphate buffer (pH 8.0), and 75 μg of enzyme in a final volume of 0.5 ml was incubated at 37° 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*-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.

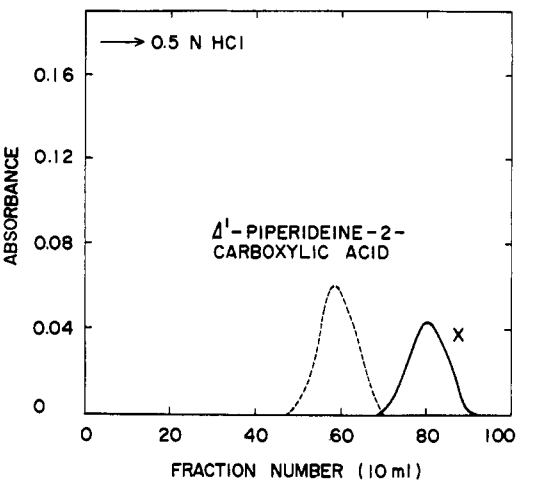


FIGURE 5: Chromatography of the mixture of Δ^1-piperideine-2-carboxylic acid and the reaction products on Dowex 50-X8 (H^+) column. The absorbance caused by the reaction of *o*-aminobenzaldehyde with Δ^1-piperideine-2-carboxylic acid and compound X was measured at 450 mμ (- - - -) and 465 mμ (——), respectively. Other conditions are shown in text.

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 Δ^1-piperideine-2-carboxylic acid under the experimental conditions employed in Figure 1, it was electrophoretically separated from Δ^1-piperideine-2-carboxylic acid in 1 N formic acid at 1000 V as described later (in Figure 6). 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.*, Δ^1-piperideine-2-carboxylic acid or Δ^1-piperideine-6-carboxylic acid.

Effect of Bisulfite and Dimedon. Addition of sodium bisulfite (final concentration 2×10^{-3} M) to the reaction system resulted in an increase in the aminotransferase reaction rate by about 30%, as shown in Figure 2. 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 (Figure 3). In the reaction system containing dimedon, dX, which migrated scarcely 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 Figure 4. 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 dipping. As shown in Figure 4, 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 α-ketoglutarate, 0.5 mmole of pyridoxal 5′-phosphate, 1.5 mmoles of sodium bisulfite, and partially purified enzyme (4 g of protein) in a final volume of 1 l. The pH of the mixture was adjusted to 8.0 with ammonium hydroxide solution. After incubation at 37°

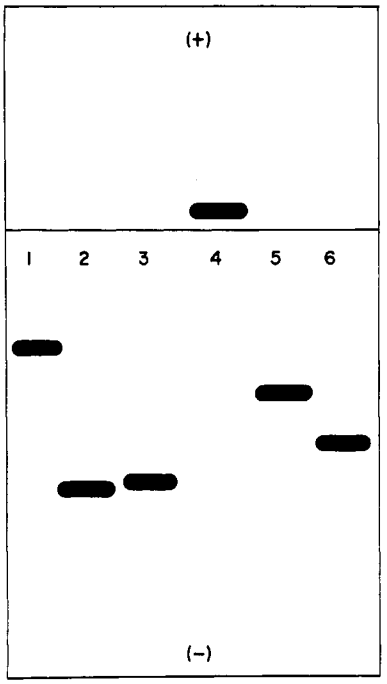


FIGURE 6: Paper electrophoresis. Electrophoresis was conducted in 1 N formic acid at 1000 V for 2 hr. (1) Δ^1-Piperideine-2-carboxylic acid, (2) compound X, (3) compound $(X)_2$, (4) compound bX, (5) dihydroquinazolinium complex formed from Δ^1-piperideine-2-carboxylic acid and *o*-aminobenzaldehyde,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

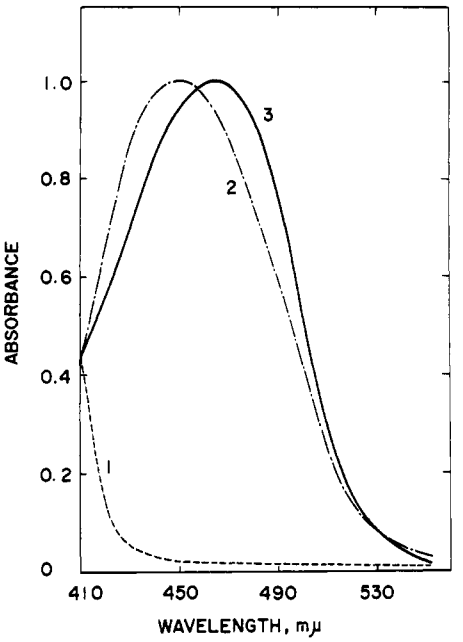


FIGURE 7: Absorption spectra of colored products of Δ^1-piperideine-2-carboxylic acid and compound X with *o*-aminobenzaldehyde. Δ^1-Piperideine-2-carboxylic acid and compound X were incubated with *o*-aminobenzaldehyde (0.004 M) in 0.2 M acetate buffer (pH 5.0) at 37° for 5 hr. Curve 1: spectrum of the *o*-aminobenzaldehyde; curve 2: spectrum of the colored solution of Δ^1-piperideine-2-carboxylic acid with *o*-aminobenzaldehyde; curve 3: spectrum of the colored solution of compound X with *o*-aminobenzaldehyde.

for 8 hr, the pH was brought to 4.0 with concentrated hydrochloric acid and 1 l. of 99% ethanol was added. The precipitated protein was removed by centrifugation and ethanol in the supernatant was evaporated out at 40° under reduced pressure. The solution was continuously treated with ether for 12 hr to free it from α-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 × 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 × 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 × 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 Δ^1-piperideine-2-carboxylic acid was chromatographed on Dowex 50 (H^+) in the aforementioned way. This compound was eluted just ahead of X under the same conditions. The cochromatography of X with Δ^1-piperideine-2-carboxylic acid on Dowex 50 (H^+) confirmed that they were not identical (Figure 5).

TABLE I: R_F Values in Paper Chromatography.[a]

Compound	Solvent			
	a	b	c	d
Δ^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		

[a] 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.

Properties of X. Purified compound X was satisfactorily separated from Δ^1-piperideine-2-carboxylic acid by paper electrophoresis conducted in 1 N formic acid

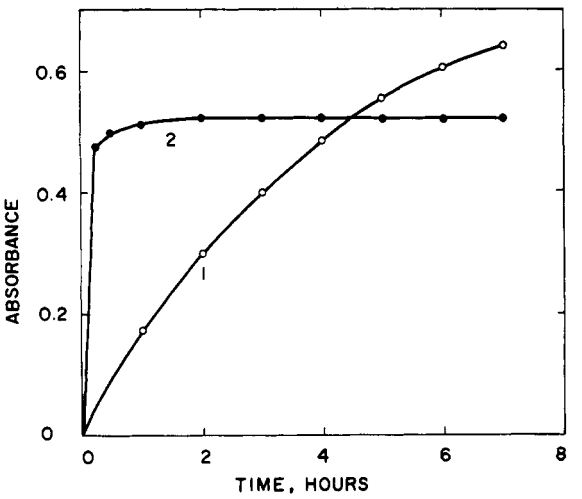


FIGURE 8: Reaction rate of Δ^1-piperideine-2-carboxylic acid and X with *o*-aminobenzaldehyde. Δ^1-Piperideine-2-carboxylic acid (in curve 1) and X (in curve 2) were incubated with 0.004 M *o*-aminobenzaldehyde in 0.2 M acetate buffer (pH 5.0) at 37°.

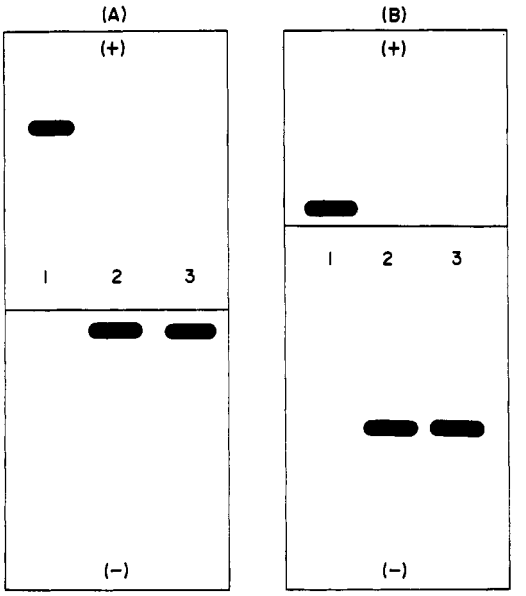


FIGURE 9: Paper electrophoresis of bX treated with hydrochloric acid. Paper electrophoresis was conducted at pH 3.5 in 0.2 M pyridine acetate buffer at 3000 V for 1 hr (A) and in 1 N formic acid at 1000 V for 2 hr (B). 1: compound bX; 2: bX treated with hydrochloric acid; 3: compound X.

at 1000 V for 2 hr (Figure 6), although the paper chromatographic behaviors of both the compounds were similar in the several kinds of solvents as demonstrated in Table I. As mentioned above, Δ^1-piperideine-2-carboxylic acid and X react with *o*-aminobenzaldehyde to produce the orange compounds, which appear to be the dihydroquinazolinium compounds (Vogel and Davis, 1952). The reaction products of X and Δ^1-piperideine-2-carboxylic acid with *o*-aminobenzaldehyde were submitted to paper electrophoresis. The *o*-aminobenzaldehyde adduct of X was distinguished from that of Δ^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 (Figure 7). The adduct of Δ^1-piperideine-2-carboxylic acid produced in the same way has an absorption maximum at approximately 450 mμ. Difference between the absorption spectra of these *o*-aminobenzaldehyde addition compounds seem not to be large, but to be distinct.

As shown in Figure 8, the maximal development of the color was obtained at 37° within 30 min, when X reacted with *o*-aminobenzaldehyde. On the other hand, the maximal color intensity of the *o*-aminobenzaldehyde adduct of Δ^1-piperideine-2-carboxylic acid required the incubation for more than 8 hr under the same conditions. Many attempts were made to determine optical activity of compound X without success. The definite value could not be obtained, because compound X exists as a mixture with its dimer in a solution as mentioned below.

Hydrogenation of X. When the solution of X in 0.5 N hydrochloric acid was hydrogenated with platinum oxide catalyst at about 15° for 30 min, X was converted into pipecolate, which was identified by a comparison of the R_F 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 (1962b), 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 Figure 6, 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.* (1962) on an interconversion reaction of Δ^1-piperideine-6-carboxylic acid and its dimer, tetrahydroanabasinedicarboxylic acid. If X is identical with Δ^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° 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 (Figure 9). Similar conversion of dX 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 Δ^1*-Piperideine-2-carboxylic Acid in to X.* When authentic Δ^1-piperideine-2-carboxylic acid was added to the reaction mixture omitting L-lysine or α-ketoglutarate and incubation was performed under the standard conditions, Δ^1-piperideine-2-carboxylic acid was shown to be unchanged and the formation of X was not observed. This result excludes the possibility that Δ^1-piperideine-2-carboxylic acid produced initially is converted into X enzymatically or nonenzymatically.

Effect of NADH.[1] 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 μmoles). When the reaction mixture containing 0.5 μmole of NADH was incubated in the silica cuvets at room temperature (approximately 15°), the decrease in absorbance at 340 mμ was not observed. These results show that it is highly unlikely for Δ^1-piperideine-6-carboxylic acid and glutamate to be formed from L-lysine and α-ketoglutarate through saccharopine as an intermediate.

When the crystalline enzyme from *A. liquidum* (Soda and Misono, 1968) and the partially purified enzyme from *F. flavescens* were used, the same results on the product from L-lysine as described here were obtained.

Discussion

The previous studies have shown that L-lysine–α-ketoglutarate aminotransferase occurs in the extracts of *Flavobacterium fuscum*, *F. flavescens*, and *A. liquidum* and that glutamate and a cyclic form of the keto analog of lysine are enzymatically formed from the substrates in the reaction mixture (Soda *et al.*, 1961). 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.

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 Figure 4. The properties of X are similar to those of authentic Δ^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 the low concentration of sodium bisulfite in the reaction mixture led to the enhancement of the activity and 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 enzyme activity was diminished by addition of the higher concentration of the aldehyde trapping agents, but further addition of pyridoxal 5′-phosphate partially released such inhibition. These facts suggest that the inhibition by excess bisulfite results from the reaction of pyridoxal 5′-phosphate with bisulfite.

The isolated X is clearly distinguishable from Δ^1-piperideine-2-carboxylic acid chromatographically and electrophoretically in 1 N formic acid as shown in Figures 5 and 6. The R_F values in paper chromatography and the mobilities in paper electrophoresis of X are in agreement with those of Δ^1-piperideine-6-carboxylic acid previously reported (Aspen and Meister, 1962a; Hasse *et al.*, 1962).

It was reported that both Δ^1-piperideine-2-carboxylic acid and Δ^1-piperideine-6-carboxylic acid react with *o*-aminobenzaldehyde to form dihydroquinazolinium salts (Basso *et al.*, 1962; Larson *et al.*, 1963). The absorption spectrum of the *o*-aminobenzaldehyde derivative of X is slightly but distinctly different from that of Δ^1-piperideine-2-carboxylic acid, although such derivatives were not isolated because of their instability. The reaction rate of Δ^1-piperideine-2-carboxylic acid with *o*-aminobenzaldehyde is extremely lower than that of X. This finding is essentially consistent with the results obtained by Aspen and Meister (1962a), 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 Δ^1-piperideine-2-carboxylic acid by the incubation of the aqueous solution (pH 7.0) with *o*-aminobenzaldehyde at 37° for 5 hr was reported (Macholán, 1962).

Pipecolate produced from X by the catalytic reduction was not oxidized by D-amino acid oxidase. If X is Δ^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 (1962b) with synthetic DL-pipecolate.

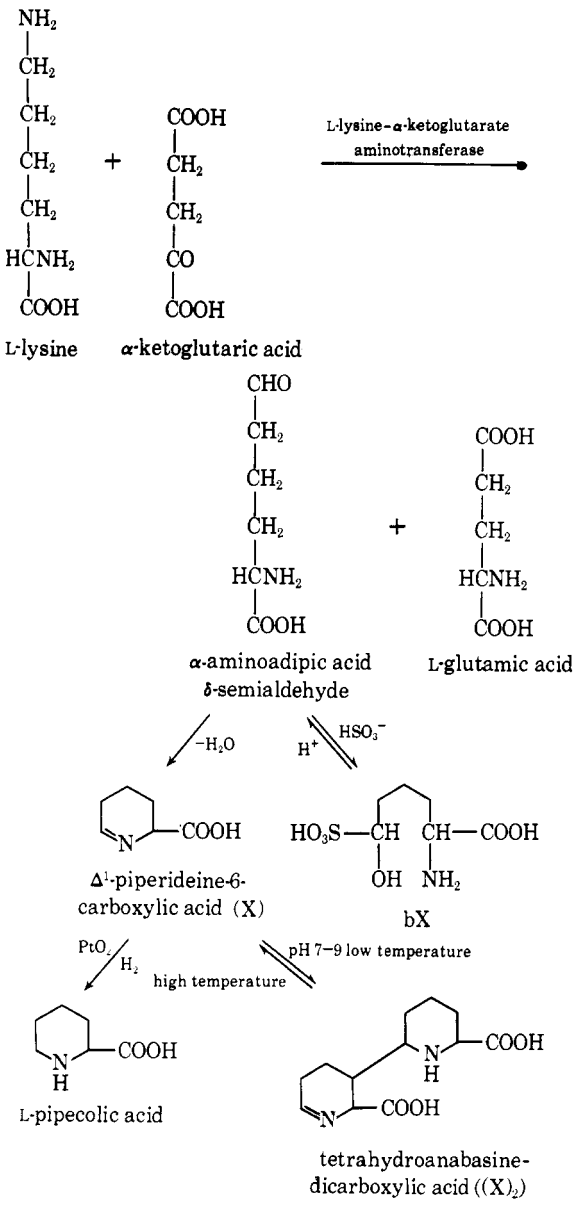
The interconversion of X and $(X)_2$ by standing at the neutral pH and at room temperature and heating at the acid pH is consistent with the observation of Hasse *et al.* (1962) on the reversible condensation of Δ^1-piperideine-6-carboxylic acid to its dimer, tetrahydroanabasinedicarboxylic acid, by the similar treatment.

Attempts to get the crystalline, X, were unsuccessful because of its instability and tendency to undergo dimerization. The possibility that Δ^1-piperideine-2-carboxylic acid initially formed through α-keto-ϵ-aminocaproate is converted into X was excluded. Broquist and his coworkers reported the reversible formation of L-lysine through saccharopine by the two combined dehydrogenase reactions in the biosynthesis of L-lysine in yeasts and *Neurospora crassa* (Kuo *et al.*, 1964; Trupin and Broquist, 1965; Jones and Broquist, 1965). But, the investigation on the effect of NADH upon the aminotransferase reaction and the homogeneity of this enzyme (Soda and Misono, 1968) 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 Δ^1-piperideine-

[1] See *Biochemistry 5*, 1445 (1966).

SCHEME I



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.

The physiological function of L-lysine-α-ketoglutarate aminotransferase and Δ^1-piperideine-6-carboxylic acid and its metabolism in the bacteria need further investigation.

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