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Bacterial Metabolism of Lysine Analogues

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Aerobacter aerogenes was found capable of growing well in the medium containing *S*-(β-aminoethyl)-L-cysteine as a sole nitrogen source. The main metabolic products from *S*-(β-aminoethyl)-L-cysteine have been isolated, purified and identified as *S*-(β-*N*-acetyl-aminoethyl)-L-cysteine and *S*-(β-*N*-acetyl-aminoethyl)-α-keto-mercaptopropionate. The terminal amino group of *S*-(β-aminoethyl)-L-cysteine is acetylated by acetyltransferase to yield *S*-(β-*N*-acetyl-aminoethyl)-L-cysteine, and then the acetylated compound is readily deaminated by L-amino acid oxidase to form the corresponding α-keto acid, *S*-(β-*N*-acetylaminoethyl)-α-keto-mercaptopropionate.

The enzyme catalyzing the first step, *S*-(β-aminoethyl)-L-cysteine ω-*N*-acetyltransferase has been purified about 450-fold from the cell-free extract of *A. aerogenes* grown in the *S*-(β-aminoethyl)-L-cysteine-peptone medium. The purified enzyme is homogeneous by the criterion of disc gel electrophoresis, and has an approximate molecular weight of 100,000. The optimum pH is at about 8.0. The following lysine analogues were prepared to investigate substrate specificity of the enzyme; *S*-(β-aminoethyl)-L-cysteine hydroxamate, *S*-(β-aminoethyl)-L-cysteine-*dl*-sulfoxide and sulfone, *S*-(β-aminoethyl)-mercaptopropionate and *S*-β-(2-pyridylethyl)-L-cysteine. In addition to *S*-(β-aminoethyl)-L-cysteine, its D-enantiomer, sulfoxide, sulfone and a higher homologue, and an oxygen analogue of lysine, *O*-(β-aminoethyl)-DL-serine act as acetyl acceptors. L- or D-Lysine and L- or D-ornithine also can accept an acetyl group to a certain extent, but α- or ω-acetylated derivatives of both *S*-(β-aminoethyl)-L-cysteine and L-lysine are not active. Acetyl-CoA is the exclusive acyl donor in the transfer reaction. The Michaelis constants were determined as follows; *S*-(β-aminoethyl)-L-cysteine, 2.1×10^{-3} M, *S*-(β-aminoethyl)-D-cysteine, 1.6×10^{-3} M, *O*-(β-aminoethyl)-DL-serine, 1.7×10^{-3} M, *S*-(β-aminoethyl)-L-homocysteine, 7.1×10^{-4} M, and acetyl-CoA, 4.5×10^{-3} M. The enzyme activity is inhibited competitively by propionyl-CoA, butyryl-CoA and benzoyl-CoA against acetyl-CoA. *S*-(β-aminoethyl)-mercaptopropionate and *S*-(β-aminoethyl)-α-*N*-acetyl-L-cysteine are strong competitive inhibitors of *S*-(β-aminoethyl)-L-cysteine acetylation. The enzyme catalyzing the second step has been shown to be an L-amino acid oxidase, which occurs widely in bacteria.

INTRODUCTION

The compounds which have a close structural similarity with naturally occurring amino acids are generally known to possess inhibitory effects on growth of certain organisms.[1~4] Richmond[1] proposed that such compounds should be named isosteres to stress their stereochemical similarity. In practice, however, they are commonly known as amino acid analogues. Some of these amino acid analogues have proven of interest in the biochemical and therapeutical aspects.

Relationship between the structure and physiological activity of the amino acid analogues has not yet been established. The accumulated experimental results suggest that to show the metabolic antagonism the analogues must not only have an overall shape similar

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to those of a protein amino acids, but also they must exist as a similarly charged ionic species at all points within the normal physiological pH range. Richmond has listed the types of structural variation for the effective amino acid analogues.[1] These include (a) the replacement of a residue in the skeleton of the amino acid by another of similar size and shape, *e.g.*, an –H in phenylalanine or tyrosine by –F (*p*-fluorophenylalanine, 3-fluorotyrosine), the –S in methionine by $-CH_2-$ (norleucine), or a $-CH_2-$ in isoleucine or glutamine by –O– (*O*-methylthreonine, *O*-carbamylserine); (b) the replacement of phenyl by another ring system possessing similar resonance, *e.g.*, by a pyridine ring as in β-(5–hydroxy-2-pyridyl)-alanine which acts as a tyrosine analogue; and (c) the replacement of one type of heterocyclic ring by another, *e.g.*, as in 2-thiazolealanine or 1,2,4-thiazolealanine which acts as an effective histidine analogue. In addition, homologues of protein amino acids sometimes act as metabolic antagonists. Ethionine is inhibitory to the growth of various organisms,[5~9] and homoarginine also inhibits the growth of *E. coli*.[10] Many amino acid analogues have been prepared chemically, and some of them occur in nature. These compounds are mainly found in higher plants.[11] Inhibitory effects of amino acid analogues are generally reversed by administration of the corresponding natural amino acids.

Some of amino acid analogues function as a false corepressor or a false feedback inhibitor of an enzyme participating in biosynthesis of the corresponding natural amino acids, and also inhibit incorporation of the amino acids into protein.[12] Amino acid analogues are enzymically transformed in the same manner as found in the natural amino acids, and some of them inhibit various enzyme reactions. For example, ethionine is converted by which participates in transethylation reaction with adenosine triphosphate into *S*-adenosylethionine.[13,14] Amino acid analogues are transaminated as reviewed by Meister.[3,15] Certain amino acid analogues are decarboxylated by amino acid decarboxylases. γ-Hydroxyarginine and canavanine, and β-hydroxyglutamic acid are decarboxylated by arginine decarboxylase from *E. coli*[16] and glutamic acid decarboxylase of *P. fischeri*,[17] respectively. The analogues also serve as a substrate of amino acid oxidases as reviewed by Shive *et al.*[2] and Meister.[3] It has been also shown that certain microorganisms are not susceptible to the metabolic antagonists, but rather are capable of utilizing them. This phenomenon may be regarded as a kind of detoxication.

Lysine is one of the most important amino acids. Much attention has been devoted to the interesting characteristic of lysine analogues from the chemical and biological viewpoints. Many of them act as metabolic antagonists against lysine.

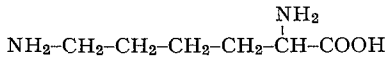
1. Structures of Lysine Analogues and their Biological Effects

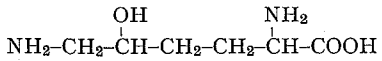
δ-Hydroxylysine was shown to inhibit growth of *Saccharomyces* and *Streptococcus* as an antimetabolite of lysine.[19,20] The inhibition is dependent on concentration of the analogue and reversed by a small amount of lysine added to the medium. ε-*C*-Methyllysine (2,6-diaminoheptanoic acid) suppresses utilization of lysine by *Leuconostoc mesenteroides* and *Streptococcus faecalis*, whereas ornithine and homolysine are not toxic.[21,22] α-Amino-ε-hydroxyhexanoic acid is toxic to rats[23] and certain bacteria,[24] but can replace lysine for growth of some lysine auxotrophs of *Neurospora*.[25] A *trans*-like conformation at the β- and ε-carbons in lysine analogues has been reported to be essential to inhibit growth of organisms. For example, 3-aminocyclohexane glycine,[25] 3-aminocyclohexane alanine[26] and *trans*-4,5-dehydrolysine,[27] which have β- and ε-carbons in the

trans-like configulation, antagonize lysine in several bacteria. On the other hand, 4–aminocyclohexane glycine[26] and *cis*-4,5-dehydrolysine,[27] which have β- and ε-carbons in a *cis*-like configuration are inactive.

2,6-Diamino-4-hexynoic acid, which has a linear arrangement of the β-, γ-, δ-, and ε-carbons, strongly inhibits bacterial utilization of lysine.[28] *O*-(β-Aminoethyl)-serine (oxalysine), *S*-(β-aminoethyl)-cysteine (thialysine) and α-amino-β-(2-aminoethylamino)–propionic acid (azalysine), in which 4-methylene group of lysine is replaced by an oxygen, sulfur or nitrogen atom are potent lysine antagonists. Either oxalysine or azalysine antagonizes lysine to inhibit growth of *E. coli* and various lactobacilli.[29~31] The inhibition of growth of *L. dextranicum* by azalysine is competitively reversed by lysine over a 30-fold range of concentration with an inhibition index of about 10. *S*-(β-Aminoethyl)–L-cysteine and some of its peptides also inhibit growth of *Leuconostoc mesenteroides*, *Lactobacillus arabinosus*,[32,33] *E. coli* and *Brevibacterium flavum*.[34] The inhibition is also competitively reversed by L-lysine, L-lysylglycine and glycyl-L-lysine in lactobacilli.

L-Lysine Analogues


L-Lysine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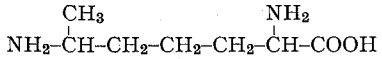
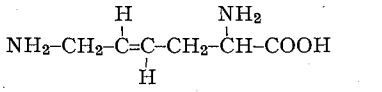
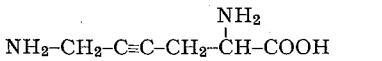
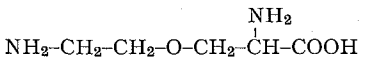
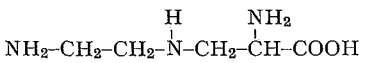
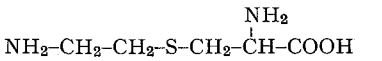

ε-*C*-Methyl-lysine

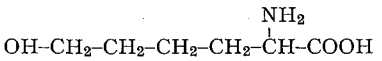

trans-4,5-Dehydrolysine

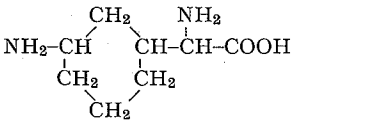

2,6-Diamino-4-hexynoic acid

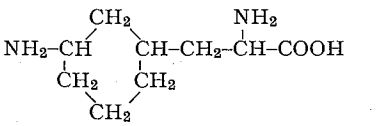

O-(β-Aminoethyl)-serine

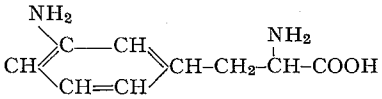

α-Amino-β-(2-aminoethyl)-propionic acid


S-(β-Aminoethyl)-cysteine


α-Amino-ε-hydroxyhexanoic acid


3-Aminocyclohexane glycine


3-Aminocyclohexane alanine


m-Amino-phenylalanine

Growth of *B. flavum* is weakly inhibited by *S*-(β-aminoethyl)-L-cysteine alone, and the inhibition is markedly enhanced in the presence of threonine. *m*-Amino-phenylalanine inhibits utilization of lysine by *Saccharomyces cerevisiae,* but is antagonistic to phenylalanine in *E. coli*.[35] Thus, distance between the two amino groups in the analogue is probably as important as the molecular shape and the ionic behavior.

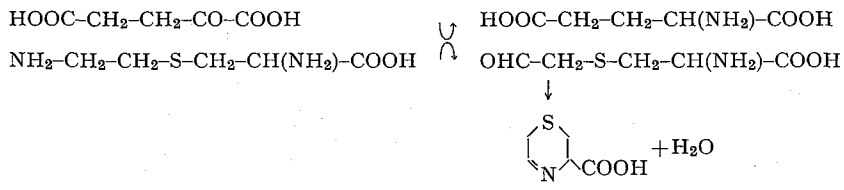
2. Control of Lysine Biosynthesis by Analogue

L-Lysine is biosynthesized through two distinct pathways. One of them (α,ε-diaminopimelic acid pathway) is a biosynthetic sequence that includes α, ε-diaminopimelic acid as an important intermediate, and occurs in bacteria, low fungi, algae, and higher plants. The other pathway (α-aminoadipic acid pathway) have been found in yeasts and molds. In this pathway lysine is synthesized *via* homocitric acid and α-aminoadipic acid from α-ketoglutarate and acetyl-CoA. A number of studies have been devoted to elucidate the biochemical and genetic aspects of the former pathway, while the enzymology and metabolic regulation in the α-aminoadipate pathway have not been studied in detail. It is well known that an important control point in α,ε-diaminopimelic acid pathway is aspartokinase catalyzing the first step of lysine synthesis, *i.e.,* conversion of aspartic acid and adenosine triphosphate to β-phosphoaspartic acid. This enzyme is susceptible to lysine feedback control. Three different forms of aspartokinase are operative in the feedback control of lysine biosynthesis in *E. coli*.[36] *S*-(β-Aminoethyl)-L-cysteine inhibits strongly the activity. Aspartokinase is also subjected to a false concerted feedback inhibition by *S*-(β-aminoethyl)-L-cysteine plus threonine in *Brevibacterium flavum*.[34] Recently, Rosner[37] reported that L-lysine inhibits diaminopimerate decarboxylase from *B. subtilis,* and indicated that L-lysine ultimately regulates its own biosynthesis through the sequential feedback inhibition. At high ionic strength the decarboxylase is inhibited also by the L-lysine sulfur analogue. Maragoudakis *et al.*[38] showed that in a yeast lysine synthesis through the pathway is controlled by lysine, and that accumulation of radioactive homocitric acid decreases in the presence of lysine. Therefore, it is likely that homocitric acid condensing enzyme, the first enzyme of the pathway is a control site. Shinha *et al.*[39] studied the effect of δ-hydroxylysine on biosynthesis of lysine in *Saccharomyces* to find out the inhibition of homocitrate synthesis by δ-hydroxylysine.

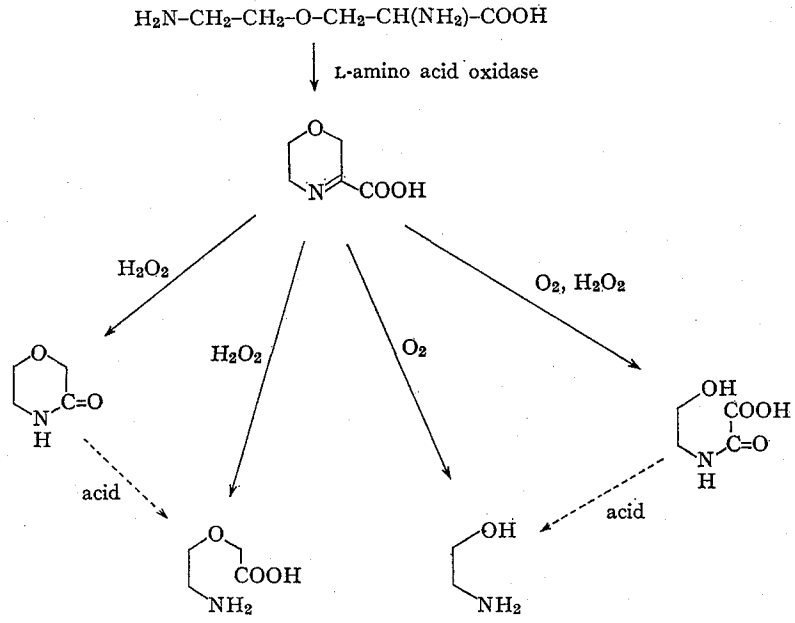
Since the studies by Adelberg and Cohen,[40,41] use of amino acid analogues and mutant resistants to them has increased to investigate the regulatory process in the bacterial cells. Sano and Shiio[34] found that some of *S*-(β-aminoethyl)-L-cysteine-resistant mutant of *B. flavum* produce high concentration of lysine in a minimal medium, and is not effected by threonine. The mutant site in the *S*-(β-aminoethyl)-L-cysteine-resistant mutants producing lysine effectively is suggested to be aspartokinase.

3. Enzymic Transformation of Lysine Analogues

δ-Hydroxylysine has been reported to be decarboxylated by L-lysine decarboxylase of *E. coli*[42] and *Bacterium cadaveris*.[43] The enzyme also attacks *S*-(β-aminoethyl)-L-cysteine to yield bis-(β-aminoethyl)-sulfide. Lysine-α-ketoglutarate ε-aminotransferase from *Achromobacter liquidum* catalyzes transamination between *S*-(β-aminoethyl)-L-cysteine and α-ketoglutarate to yield 4-thia-Δ^1-piperidine-6-carboxylic acid and glutamate, as follows.[44]



δ-Hydroxylysine and *O*-(β-aminoethyl)-DL-serine also are transaminated, though less effectively, by this aminotransferase than the sulfur analogue. Hope[45,95] demonstrated that *S*-(β-aminoethyl)-L-cysteine and *O*-(β-aminoethyl)-DL-serine are oxidized by the L-amino acid oxidase of *Mytilus edulis* and that the final oxygen consumption is three times as great with L-lysine as substrate. The products of oxidation of the oxygen analogue are ethanolamine, *N*-oxalylethanolamine and 3-morpholone, although the mechanism of further oxidation of the sulfur analogue has not been established.



δ-Hydroxylysine has been reported to be oxidatively deaminated by turkey liver L-amino acid oxidase.[46]

Incubation of labeled δ-hydroxylysine with homogenetes of rat liver and kidney leads to formation of radioactive δ-hydroxypipecolic acid.[47] Little knowledge of the metabolism of the other lysine analogues is currently available.

In the present paper, the bacterial metabolism of some lysine sulfur analogues, particularly *S*-(β-aminoethyl)-L-cysteine by *Aerobacter aerogenes* is described from the enzymological point of view.

I BACTERIAL UTILIZATION OF *S*-(β-AMINOETHYL)-L-CYSTEINE AND FORMATION OF NEW INTERMEDIATES

S-(β-Aminoethyl)-L-cysteine was reported to inhibit growth of lactic acid bacteria.[33,34] Cavallini *et al.*[48] investigated metabolism of this compound *in vivo* by the rat, and identified

the metabolites in the excretion as *S*-(β-aminoethyl)-α-*N*-acetyl-L-cysteine, cystamine and lanthionamine. *S*-(β-Aminoethyl)-L-cysteine is decarboxylated by L-lysine decarboxylase of *E. coli*[42] and *Bacterium cadaveris*,[43] and also oxidized by L-amino acid oxidase of *Mytilus edulis*.[45] The present investigation was undertaken to elucidate the metabolic fate of this lysine antimetabolite in bacteria. In this chapter, isolation and identification of the bacterial metabolic products from *S*-(β-aminoethyl)-L-cysteine are described.

Experimental Procedures

Materials. *S*-(β-Aminoethyl)-L-cysteine·HCl was synthesized from L-cysteine and ethylenimine by a modification[49] of the method of Cavallini *et al.*[50] *S*-(β-Aminoethyl)-L-[^{35}S]-cysteine and *S*-(β-aminoethyl)-α-*N*-acetyl-L-cysteine were also synthesized in the same manner from L-[^{35}S]-cysteine (specific activity 53 mc/mmole, The Radiochemical Center, England) and *N*-acetyl-L-cysteine, respectively. *N*-Acetyl-L-cysteine was obtained by reduction of *N*-acetyl-cysteine prepared by the method of Hollander and Du Vigneaud.[51,52] *S*-(β-*N*-Acetyl-aminoethyl)-L-cysteine was prepared essentially according to the procedure of synthesis of ε-*N*-acetyl-L-lysine.[53] The other chemicals were analytical grade reagents.

Microorganism and Conditions of Culture. *Aerobacter aerogenes* IFO 3320 was grown in the medium containing 0.5% glucose, 0.3% *S*-(β-aminoethyl)-L-cysteine·HCl, 0.1% NaCl, 0.1% KH_2PO_4, 0.1% K_2HPO_4, 0.01% $MgSO_4 \cdot 7H_2O$, 0.01% $MnSO_4 \cdot H_2O$, and 0.01% yeast extract. The pH was adjusted to 7.2 with sodium hydroxide. The cultures were carried out with 100 ml of the medium placed in a 500 ml flask and kept on a reciprocating shaker at 30° for various periods.

Analytical Methods. Amino acids in the culture fluid were paper-chromatographically separated and determined with ninhydrin according to the method of Soda *et al.*[54] Amino acids in the fractions eluted from a Dowex-50 and Dowex-1 column were assayed by the method of Rosen.[55] Sulfur amino acids on paper chromatogram were visualized with a platinum reagent.[56] Electrophoresis was carried out using a high-voltage paper electrophoresis apparatus (Handex HC-HEP, Shiraimatsu, Osaka) and Toyo filter paper strips (No. 53, 15×54 cm). Nuclear magnetic resonance spectrum was measured with a Varian associates recording spectrometer at 60 MHz in a deuterium oxide with tetramethylsilane as an internal standard. Chemical shifts are reported in δ values (parts per million). Radioactivity was measured with a Tri-Carb Liquid Scintillation Counter 3320.

Results

1. Bacterial Utilization of *S*-(β-Aminoethyl)-L-cysteine

Growth of about 30 strains of bacteria in a medium containing *S*-(β-aminoethyl)-L-cysteine as a sole nitrogen source was investigated. Several strains of *Aerobacter aerogenes* were found capable to utilize the lysine sulfur analogue, and *A. aerogenes* IFO 3320 was employed throughout because of its rapid growth and the high yield of cells.

2. Formation of New Sulfur Compounds Containing Intermediates

After organisms were grown for 48 hr in a medium containing *S*-(β-aminoethyl)-L-[^{35}S]-cysteine, followed by centrifugation, aliquot samples of the supernatant fraction of the culture fluid were chromatographed, and the radioactivities were followed by a paper

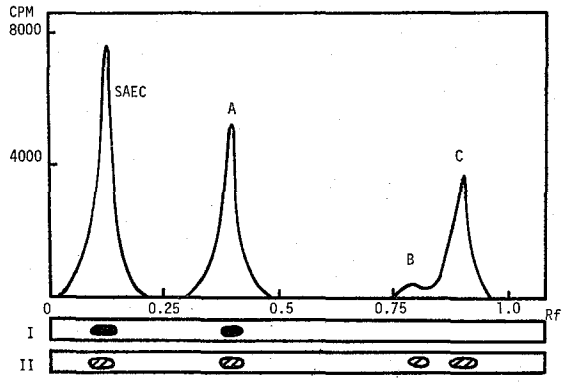


Fig. 1. Paper chromatography of the culture fluid.
Solvent system: n-butanol-acetic acid-water (4 : 1 : 1). The spots were visualized with ninhydrin (I) and a platinum reagent (II). SAEC: *S*-(β-aminoethyl)-L-cysteine.

chromatogram scanner (Aloka, JPC-102). Also the spots were visualized with ninhydrin and a platinum reagent. In addition to *S*-(β-aminoethyl)-L-cysteine, two distinct and one small radioactive peaks were observed as shown in Fig. 1. These three peaks are termed tentatively peak A, B, and C in the order of their increasing Rf values. Peak A was positive to ninhydrin and platinum reagent tests, while peaks B and C were not visualized with ninhydrin but with a platinum reagent. The bacterial growth and changes in peaks A, B, and C were determined at various times of cultivation. After the short lag phase, the organisms grew rapidly and attained the stationary phase about 50 hr after the initiation of cultivation (Fig. 2). As the cultures were prolonged, the radioactivity of *S*-(β-aminoethyl)-L-cysteine decreased, and those of peaks A and C increased up to approximately 50 hr and 100 hr, respectively. The final radioactivity of peak B was very low, though it

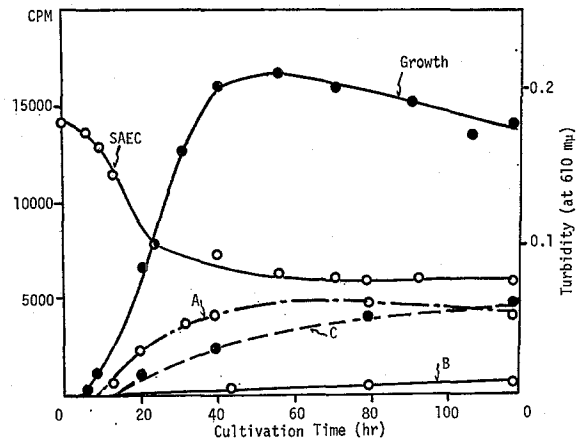


Fig. 2. Incorporation of the radioactivity into the products from *S*-(β-aminoethyl)-L-[^{35}S]-cysteine during bacterial growth.
Growth was determined by measurement of turbidity of the culture fluid at 610 nm at designated times. SAEC: *S*-(β-aminoethyl)-L-cysteine.

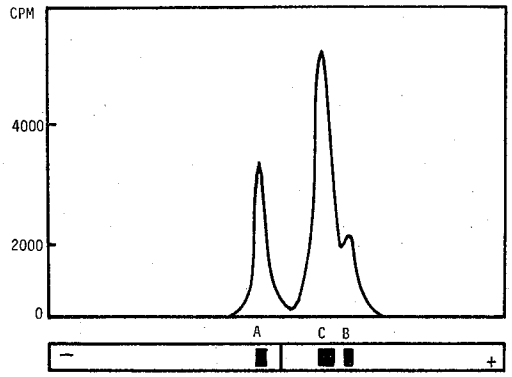


Fig. 3. Conversion of Compound A to Compounds B and C.
The reaction mixture contained 5 μmoles of [^{35}S]-Compound A, 100 μmoles of Tris-HCl buffer pH 7.5 and the resting cells (9.87 mg dry weight) in a final volume of 0.9 ml. After incubation at 30°, the reaction was terminated by addition of 0.1 ml of 50% trichloroacetic acid. The clear supernatant (20 μl) obtained by centrifugation was applied to a paper electrophoresis strip and electrophoresis was conducted in 0.25 M pyridine–acetate buffer, pH 4.8 at 3000 V for 20 min. The radioactivity was followed by a paper chromatogram scanner.

increased continuously over 100 hr. When the resting cells of *A. aerogenes* was incubated with the radioactive Compound A corresponding to peak A, the oxygen consumption and the formation of Compounds B and C were observed (Fig. 3). To the reaction mixture was added 100 μmoles of semicarbazide and the mixture was incubated. All of the radioactivities were found in peak C, but the formation of any other compound was not observed. Thus, *S*-(β-aminoethyl)-L-cysteine was suggested to be converted to Compounds A, C, and B successively under the conditions employed.

3. Isolation of Products

After *A. aerogenes* was grown for 100 hr, the culture fluid (3 liters) was collected by centrifugation and evaporated to approximately 100 ml under reduced pressure at 45°. The insoluble materials formed were removed by centrifugation. The clear supernatant

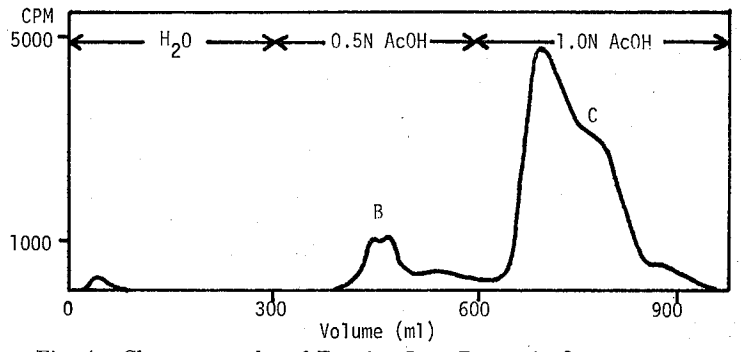


Fig. 4. Chromatography of Fraction I on Dowex-1×8.
The Compounds B and C were eluted with acetic acid whose concentration was varied as indicated in the figure.

fraction was applied to a column of Dowex -50×8 (H^+ form; 3.5×70 cm), and eluted thoroughly with water (Fraction I), and then with 2.5 N hydrochloric acid (Fraction II). The effluent of Fraction I was condensed as described above. The solution was chromatographed on a column of Dowex-1×8 (acetate form) and aliquots of each fraction were used to determine the radioactivity by the scintillation counter. The materials of Fraction I were found to contain Compounds B and C (Fig. 4). Each fraction was evaporated to dryness under reduced pressure at 40°. The effluent of Fraction II was collected in 10-ml fractions, each of which was examined with ninhydrin and a platinum reagent, and fractions being positive to both the tests were combined and condensed as described above. The compound from the Fraction II was demonstrated to be identical with Compound A by paper chromatography. The solution was applied to a column of Dowex-50×8 (pyridinum form, 1.0×70 cm), washed with water, and eluted with 0.2 M pyridine-formate buffer, pH 3.1. The fractions containing Compound A were pooled and condensed to a small volume. This chromatography was repeated three times to remove trace amounts of impurities. The solution was evaporated to dryness under reduced pressure at 40°. The residue was dissolved in a minimum volume of water and crystallized by addition of absolute ethanol. Recrystallization was performed in the same way.

4. Determination of Structure

Compound A,.

The nuclear magnetic resonance spectrum of the Compound A gave signals at δ 1.98 (3 H, singlet, >ω-N-COCH$_3$), at δ 2.74 and 3.41 (2H, splitted triplet and 2H, triplet, -S-CH$_2$-CH$_2$-N<), at δ 3.09 (2H, splitted doublet, C-3-H$_2$), and 3.93 (1H, doublets of doublet, >C-2-H). The last was shifted to the lower field by 26 Hz in the presence of trifluoroacetic acid. These results suggest that the Compound A is *S*-(β-*N*-acetyl-aminoethyl)-cysteine (Fig. 5). Compound A was analyzed with an automatic amino acid analyzer (Yamaginoto LC-5S). It emerged between aspartic acid and threonine. *S*-(β-Aminoethyl)-α-*N*-acetyl-L-cysteine was eluted between methionine and isoleucine under the same

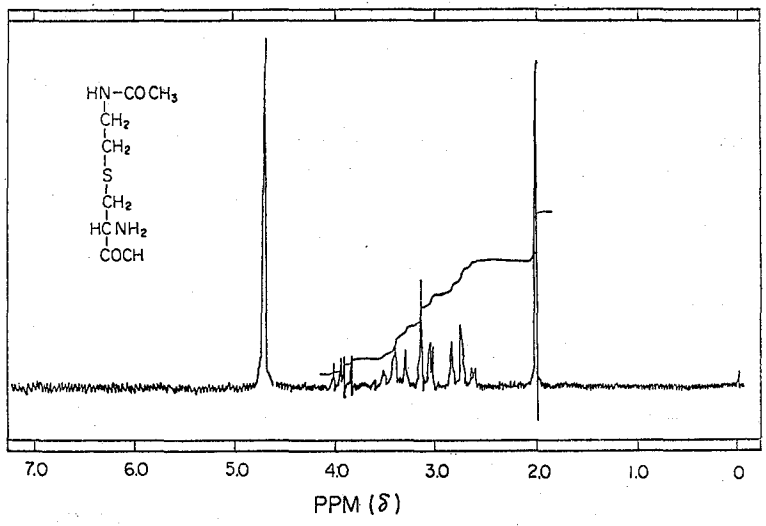


Fig. 5. Nuclear magnetic resonance of Compound A.

conditions. Cochromatography of the Compound A with synthetic *S*-(β-*N*-acetyl-aminoethyl)-L-cysteine confirmed their identity, which was also shown by the infrared spectra and X-ray diffraction patterns of the Compound A and the synthetic sample. Compound A and the synthetic product (20 μmoles) were hydrolyzed by boiling under reflux with 0.5 ml of 6 N HCl for 8 hr. The pH was adjusted to 5.8 by addition of 6 N KOH. The hydrolyzate of both the compounds, and authentic *S*-(β-aminoethyl)-L-cysteine were all quantitatively decarboxylated with L-lysine decarboxylase as reported by Work.[42] These results indicate that Compound A is the L-isomer. Thus, structure of Compound A was established to be *S*-(β-*N*-acetyl-aminoethyl)-L-cysteine.

Compound C

The solution of Compound C was examined by high-voltage paper electrophoresis conducted in 0.25 M pyridine-acetate buffer. Compound C, which was negative to a ninhydrin test, moved toward anode. To the solution of Compound C was added 0.5% 2,4-dinitrophenylhydrazine solution dissolved in 2 N HCl to yield the 2,4-dinitrophenylhydrazones. The results suggest that Compound C may be a keto analogue of ω-*N*-acetylated *S*-(β-aminoethyl)-L-cysteine. To examine the possibility, *S*-(β-*N*-acetyl-aminoethyl)-α-keto-mercaptopropionate was prepared by enzymic oxidation of *S*-(β-*N*-acetyl-aminoethyl)-L-cysteine using L-amino acid oxidase according to a modification of the procedure of Meister,[57] as follows. Dialyzed catalase (100 units) and dialyzed L-amino acid oxidase (100 mg) from snake venom (Sigma Biochemical Co., St. Louis, Mo., U. S. A.) were added successively to a suspension of 1 g of *S*-(β-*N*-acetyl-aminoethyl)-L-cysteine in 50 ml of water. The pH was adjusted to 7.4 with 2 N sodium hydroxide, and water was added to a final volume of 100 ml. A continuous stream of oxygen was bubbled into the flask. After 12 hr, the mixture was filtered using Dia flow (Amicon, U. S. A). The filtrate was condensed *in vacuo,* acidified with hydrochloride, treated with charcoal, filtered and extracted with an equal volume of ethyl acetate. The ethyl acetate fractions were evaporated to dryness at 45°. The residue was crystallized and recrystallized from acetone-ether; m.p. 184°–185° (decomp). On elemental analysis, the following result was obtained. Calculated for $C_7H_{11}O_4NS$: C, 40.96; H, 5.40; N, 6.82%. Found: C, 41.51; H, 5.43; N, 6.78%. The solution of Compound C and synthetic compound was subjected to paper chromatography with n-butanol-acetic acid-water (4 : 1 : 1) as a solvent. The paper developed was sprayed with a solution of 0.05% 2,4-dinitrophenylhydrazine in 2 N HCl, and dipped in 2% NaOH in 90% ethanol, after drying. The Rf value of Compound A was demonstrated to be identical with that of synthetic compound (Rf 0.84). To the solution of Compound C was added 0.5% 2,4 dinitrophenylhydrazine solution dissolved in 2 N HCl to yield the 2,4-dinitrophenylhydrazones. After incubation at 37° for 20 min, 10 ml of ethyl acetate was added to the solution and air was bubbled through the mixture for 3 min. The aqueous layer was washed twice with ethyl acetate in the same manner. The combined ethyl acetate layers were washed twice with water and mixed with 1.0 ml of 10% sodium carbonate solution; air was then bubbled through the mixture for 3 min. Some portion of the sodium carbonate layer was chromatographed. The hydrazone of synthetic sample was also prepared from *S*-(β-*N*-acetyl-aminoethyl)-α-keto-mercaptopropionate in a similar way. Both the two specimens yield two spots on paper chromatography using n-butanol, ethanol, water (4 : 1 : 4 upper layer) as a solvent. It seems that these 2,4-dinitrophenylhydrazones exist in *syn*- and *anti*-forms due to the isomerism around

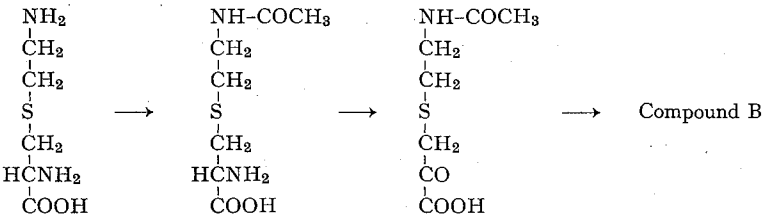
the double bond. The Rf values of 2,4-dinitrophenylhydrazone of Compound C were the same as those of 2,4-dinitrophenylhydrazone of the synthetic compound (Rf; 0.32, 0.42). Thus, Compound C was identified as *S*-(β-*N*-acetyl-aminoethyl)-α-keto-mercaptopropionate, an α-keto analogue of ω-*N*-acetylated *S*-(β-aminoethyl)-L-cysteine.

Compound B

When the solution of Compound B was examined by paper electrophoresis, this compound moved toward anode under the conditions employed. Compound B did not react with ninhydrin and 2,4-dinitrophenylhydrazine, but with a platinum reagent. Further studies on the structure of Compound B have not been carried out.

Discussion

S-(β-Aminoethyl)-L-cysteine was reported to inhibit the growth of lactic acid bacteria,[32,33] but *A. aerogenes* was found capable of growing in the medium containing as a sole nitrogen source as described here, although the growth was much slower than in the synthetic medium containing a usual nitrogen source *e.g.*, ammonium sulfate, L-asparagine and sodium L-glutamate. Three different compounds were formed as metabolites in the culture fluid. Among them, two compounds (A and C) were identified by the chemical and spectroscopic studies as *S*-(β-*N*-acetyl-aminoethyl)-L-cysteine and *S*-(β-*N*-acetyl-aminoethyl)-α-keto-mercaptopropionate, respectively. When the resting cells of *A. aerogenes* were incubated with the radioactive *S*-(β-*N*-acetyl-aminoethyl)-[^{35}S]-cysteine and semicarbazide, only semicarbazone of *S*-(β-*N*-acetyl-aminoethyl)-α-keto-[^{35}S]-mercaptopropionate was formed. These findings suggest that at first the terminal amino group of *S*-(β-aminoethyl)-L-cysteine is acetylated to yield *S*-(β-*N*-acetyl-aminoethyl)-L-cysteine, and then the acetylated compound is deaminated by either L-amino acid oxidase or transaminase to form the corresponding α-keto compound, *S*-(β-*N*-acetyl-aminoethyl)-α-keto-mercaptopropionate. The metabolic scheme is shown as follows.



Although the further fate of *S*-(β-*N*-acetyl-aminoethyl)-α-keto-mercaptopropionate has not been established, it seems that Compound B is formed *via* *S*-(β-*N*-acetyl-aminoethyl)-α-keto-mercaptopropionate as described above. Compound B did not react with ninhydrin and 2,4-dinitrophenylhydrazine, but did with a platinum reagent, and moved toward anode. Thus, Compound B may be *S*-(β-*N*-acetyl-aminoethyl)-α-hydroxy-mercaptopropionate as posturated in lysine metabolism. Paik and Kim[58] suggested that in mammalian tissues L-lysine is first acetylated to give ε-*N*-acetyl-L-lysine, although the acetylation of the sulfur analogue was not investigated. ε-*N*-Acetyl-L-lysine can be oxidatively deaminated either by L-amino acid oxidase[57] or by transaminase,[59] and the resultant α-keto-ε-acetamido-caproic acid is converted into α-keto-ε-amino-caproic acid by the action of ε-lysine acylase,[60] and further into α-hydroxy-ε-acetamido-caproic acid

by a dehydrogenase.[57] Compound B may arise from *S*-(β-*N*-acetyl-aminoethyl)-α-keto-mercaptopropionate through a dehydrogenase reaction, too. The metabolic products formed from L-lysine by *Hansenula saturnus* which is able to utilize lysine as a nitrogen source, were found to be ε-acetamido-α-hydroxy-caproic acid, α-hydroxy-valeric acid and glutaric acid.[61] ε-*N*-Acetyl-L-lysine was also isolated in the ethanolic cell extracts. *S*-(β-*N*-Acetyl-aminoethyl)-L-cysteine was also found as a main product from *S*-(β-aminoethyl)-L-cysteine in the growing cultures of *Neurospora crassa*.[62] The *N*-acetylation of *S*-(β-aminoethyl)-L-cysteine described in this chapter may function as a detoxication mechanism to eliminate its inhibitory effect on lysine metabolism. It is conceivable that some other naturally occurring *N*-acetylated amino acids also result from a detoxification. For example, acyl derivatives of glycine, glutamine, ornithine, and cysteine are involved in a detoxication reaction in various animal species.[63] *S*-(β-Aminoethyl)-L-cysteine is probably attacked very slowly by L- and D-amino acid oxidase and common aminotransferases except L-lysine-α-ketoglutarate aminotransferase of *Achromobacter liquidom*.[44] However, the sulfur analogue, by analogy with lysine, appears to be readily metabolized through oxidative deamination, when the terminal amino group is acetylated.

II OCCURRENCE, PURIFICATION AND PROPERTIES OF *S*-(β-AMINOETHYL)-L-CYSTEINE ω-*N*-ACETYLTRANSFERASE

A number of *N*-acyl amino acids are known to occur in animals, plants, and microorganisms. Several reports about the enzymic formation of some *N*-acetyl amino acids have appeared.

It has been described in the chapter I that *A. aerogenes* is capable of utilizing *S*-(β-aminoethyl)-L-cysteine as a sole nitrogen source and the main metabolic products have been isolated, purified, and identified as *S*-(β-N-acetyl-aminoethyl)-L-cysteine and *S*-(β-*N*-acetyl-aminoethyl)-α-keto-mercaptopropionate. *S*-(β-*N*-acetyl-aminoethyl)-L-cysteine is formed first and subsequently metabolized to *S*-(β-*N*-acetyl-aminoethyl)-α-keto-mercaptopropionate.

The present chapter describes evidence for the occurrence of an enzyme in the cell-free extract of *A. aerogenes* which catalyzes the transfer of acetyl group from acetyl-CoA to the terminal amino group of *S*-(β-aminoethyl)-L-cysteine. Purification and properties of the enzyme are also presented.

Experimental Procedures

Materials

S-(β-Aminoethyl)-L-cysteine, *S*-(β-*N*-acetyl-aminoethyl)-L-cysteine *S*-(β-aminoethyl)-α-*N*-acetyl-L-cysteine were prepared as described in the previous chapter. *O*-(β-Aminoethyl)-DL-serine·2HCl was prepared by the catalytic hydrogenation from ε-*N*-benzoyloxycarbonyl-*O*-(β-aminoethyl)-DL-serine which was kindly supplied by Dr. G. I. Tesser of Katholieke Universiteit, The Netherlands. *S*-(β-Aminoethyl)-L-homocysteine,[64] *S*-β-(4-pyridylethyl)-L-cysteine,[65] α-*N*-acetyl-L-lysine and ε-*N*-acetyl-lysine[53] were synthesized according to the methods given in the literatures. Acetyl phosphate was prepared according to the procedure of Avison.[66] Acetyl-CoA, propionyl-CoA, butyryl-CoA and benzoyl-CoA were prepared by treatment of CoA (Boehringer Mannheim GmbH)

with the corresponding acid anhydrides.[67] 1-[^{14}C]-Acetyl-CoA was also prepared from 1-[^{14}C]-acetic anhydride (Dai-ichi Chemicals, Tokyo). The residual acid anhydride was either removed by ether extractions or decomposed by boiling for 5 min at pH 3.0. The preparation was further purified by chromatography on Toyo No. 51 filter paper with ethanol-0.1 M sodium acetate buffer of pH 4.5 (1 : 1, v/v) as a solvent, if necessary. Phosphotransacetylase (*Clostridium kluyveri*) was purchased from Boehringer Mannheim GmbH. The specific activity was approximately 1,000 international units per mg of protein.

Synthesis of *S*-(β-aminoethyl)-L-cysteine-*dl*-sulfoxide

S-(β-Aminoethyl)-L-cysteine (0.05 mole) was dissolved in 20 ml of water. To this solution was added with cooling 7 ml of 30% hydrogen peroxide and the resulted solution allowed to stand for 2 days at room temperature. The clear solution was evaporated in vacuum to oil-like residue. The residue was adjusted to pH 6.0 with ammonium chloride solution, and ethanol was added to precipitate the sulfoxide. The oily residue was converted to solid by scratching the flask. Dissolution of the compound in water and the reprecipitation with ethanol were repeated several times until the sulfoxide became free from chloride, which was followed by test with silver nitric acid solution. The yield was about 9.3 mg. The Rf value of this compound (m.p., 157°–159° with decomposition) in n-butanol-acetic acid-water (4 : 1 : 1) was 0.13 (Rf value of *S*-(β-aminoethyl)-L-cysteine; 0.38). Anal. Calcd. for $C_5H_{13}O_3N_2SCl$, C, 27.71, H, 6.04, N, 12.93%; Found; C, 27.54, H, 6.32, N, 12.43%.

Synthesis of *S*-(β-aminoethyl)-L-cysteine-sulfone

S-(β-Aminoethyl)-L-cysteine (1 mole) was mixed with 0.025 mole of ammonium molybdate and 40 ml (0.36 mole) of 60% perchloric acid. To this mixture was added with 380 ml of 30% hydrogen peroxide (in 10-ml portions with cooling). After standing for several hr the reaction mixture was adjusted to pH 5.5 with ethanolamine, and ethanol was added to precipitate the solution. After washing with ethanol, the compound was recrystallized from ethanol-water. Anal. Calcd. for $C_5H_{13}O_4N_2SCl$, C, 25.81, H, 5.63, N, 12.0%; Found; C, 26.21, H, 5.60, N, 12.58%.

Synthesis of *S*-(β-Aminoethyl)-L-cysteine Hydroxamate Hydrochloride

S-(β-Aminoethyl)-L-cysteine hydroxamate hydrochloride was synthesized from *S*-(β-aminoethyl)-L-cysteine ethylester hydrochloride by a modification of the procedure of Ropper and McIlwain for the synthesis of *N*-γ-glutamyl hydroxamate.[68] *S*-(β-Aminoethyl)-L-cysteine ethyl ester hydrochloride was prepared essentially according to the synthesis of L-lysine ethylester.[69] Hydroxylamine was prepared by dissolving 7g hydroxylamine hydrochloride in 140 ml of ethanol and the pH was adjusted to about 8.0 with 10 N NaOH. NaCl formed was removed by filtration. After solution of *S*-(β-aminoethyl)-L-cysteine ethylester hydrochloride (5 g) in 30 ml ethanol was brought to pH 8.0 with 10 N NaOH and NaCl precipitated was removed, these two ethanol solutions were mixed and allowed to stand at room temperature for 20 hr followed by 48 hr at 5°. The reaction mixture was filtered and the filtrate was evaporated to 10 ml. The pH of the solution was adjusted to about 1 with concentrated HCl, and approximately 3 volumes of ethanol were added. After standing at room temperature for 30 min, the precipitate was

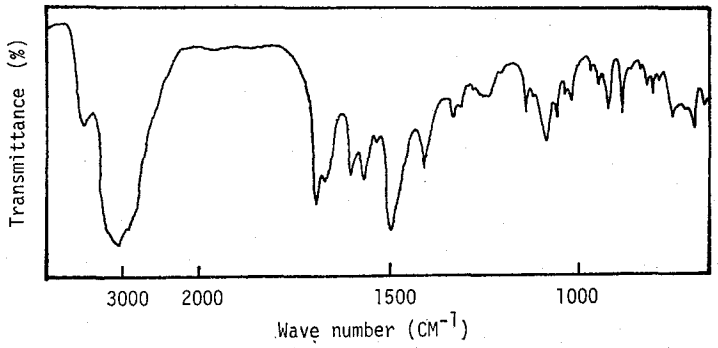


Fig. 1. Infrared absorption spectrum of *S*-(β-aminoethyl)-L-cysteine hydroxamate.

removed by filtration and the reaction mixture was then kept at 4° overnight to precipitate the product. The materials were dissolved in 3 ml of water and heated with active carbon on a boiling water bath. The filtrate was evaporated in vacuum to dryness. The residue was suspended in 40 ml of ethanol. Under boiling, a small volume of water was added until a complete solution was obtained, followed by the slow addition of cold ethanol. After chilling for 24 hr, the crystals of *S*-(β-aminoethyl)-L-cysteine hydroxamate hydrochloride was filtered, washed with ethanol and dried. The compound was recrystallized from a little hot water by the addition of an excess of boiling ethanol with yield of about 23%. The obtained crystals, which were positive to a $FeCl_3$ test, had a melting point of 175° to 177° (decomposition). The nuclear magnetic resonance spectrum of this compound was demonstrated to be almost identical with that of *S*-(β-aminoethyl)-L-cysteine except chemical shift of α-proton. The chemical shift of α-proton of *S*-(β-aminoethyl)-L-cysteine hydroxamate gave at δ 4.13 (1 H, triplet, C-2-H). This value shifts slightly to the lower field than that of *S*-(β-aminoethyl)-L-cystein. This is probably due to anisotropic effect. The infrared spectrum is given in Fig. 1. A band which is seen at 1685 cm^{-1} arises from a carbonyl bond of R-CO-NHOH. Anal. Calcd. for $C_5H_{14}O_2N_3SCl$; C, 27.84, H, 6.50, N, 19.48%, Found; C, 27.17, H, 6.32, N, 19.98%.

Synthesis of *S*-(β-aminoethyl)-mercaptopropionate

S-(β-Aminoethyl)-mercaptopropionate was synthesized from β-mercaptopropionate and ethylenimine according to the procedure of synthesis of *S*-(β-aminoethyl)-L-cysteine.[49] β-Mercaptopropionate (10.6 g) in 200 ml of boiled water was taken to pH 8.5 with concentrated NH_3 and immediately reacted with 6.2 ml of ethylenimine. The pH was maintained at about 8.5 by bubbling CO_2 into the solution and the reaction was allowed to proceed for 1 hr with stirring at room temperature. The pH was adjusted to 5.0 with HCl and then the reaction mixture was concentrated to 50 ml. Dilution of the concentrated reaction mixture with 55 ml of ethanol led to precipitation of crude *S*-(β-aminoethyl)-mercaptopropionate (7.5 g), which was separated by filtration and washed with cold 50% ethanol and then with acetone. The combined precipitates were recrystallized from ethanol-water to yield 5.8 g of ninhydrin positive crystalline materials (m.p., 170°–172° (decomp)).

Synthesis of *S*-β-(2-pyridylethyl)-L-cysteine

S-β-(2-Pyridylethyl)-L-cysteine was prepared essentially according to the method of Cavins.[65] To a solution of 14 g of L-cysteine in 300 ml of distilled and deionized water were added 16 ml of triethylamine and 12.5 ml of 2-vinylpyridine. The reaction mixture was stirred under an atmosphere of nitrogen for 24 hr and then evaporated to dryness at 40°. A crystalline material precipitated from the solution during evaporation. The compound was recrystallized three times from 99% ethanol, (m. p. 165°). The structure of *S*-β-(2-pyridylethyl)-L-cysteine was confirmed by the infrared and nuclear magnetic resonance analyses.

Microorganisms and Conditions of Culture

A. aerogenes was cultured in a medium containing 0.5% (w/v) peptone, 0.1% meat extract, 0.1% glucose, 0.1% KH_2PO_4, 0.1% K_2HPO_4, 0.1% NaCl, 0.02% *S*-(β-aminoethyl)-L-cysteine·HCl, 0.01% $MgSO_4 \cdot 7H_2O$ and 0.01% yeast extract (pH 7.2). The cultures were grown in a 30–1 jar fermentor at 28° for 17 to 20 hr under aeration. The cells harvested by centrifugation were washed with 0.85% (w/v) NaCl, and then with 0.01 M Tris-HCl buffer, pH 7.4 containing 0.01% (w/v) 2-mercaptoethanol.

Enzyme Assay

The standard assay system consisted of 5 μmoles of *S*-(β-aminoethyl)-L-cysteine, 10 μmoles of acetyl phosphate, 0.5 μmole of CoA, 2 μg of phosphotransacetylase as protein, 100 μmoles of Tris-HCl buffer (pH 7.8) and enzyme in a final volume of 0.9 ml. The reaction was initiated by addition of acetyl phosphate. After incubation at 37° for 20 min, the reaction was terminated by addition of 0.1 ml of 50% (w/v) trichloroacetic acid solution followed by centrifugation at 17,000 g for 10 min. The ω-N-acetyltransferase was assayed by determining *S*-(β-N-acetyl-aminoethyl)-L-cysteine formed with ninhydrin after separation by paper chromatography, or by measuring incorporation of radioactive *S*-(β-aminoethyl)-L-[^{35}S]-cysteine into *S*-(β-N-acetyl-aminoethyl)-L-cysteine. The radioactivity of the latter compound separated by paper-chromatography was determined with a Tri-Carb Liquid Scintillation 3320 spectrometer. Since non-enzymic acetylation of the amino acid with acetyl phosphate occurred, a blank in which enzyme was replaced by water was run for every assay of the enzyme. One unit of *S*-(β-aminoethyl)-L-cysteine ω-*N*-acetyltransferase is defined as the amount of enzyme that catalyzes the formation of 1.0 μmole of *S*-(β-*N*-acetyl-aminoethyl)-L-cysteine per min. The specific activity is expressed as units per mg of protein. Protein was determined by the method of Lowry *et al.*[70] using crystalline egg albumin as a standard; with most column fractions, protein elution patterns were estimated by the 280 nm absorption.

Results

1. Occurrence of *S*-(β-aminoethyl)-L-cysteine ω-*N*-acetyltransferase.

The washed cells of *A. aerogenes* were suspended in 0.01 M Tris-HCl buffer, pH 7.4, containing 0.01% (w/v) 2-mercaptoethanol, and subjected to sonication in a 19-kc Kaijo Denki oscillator at 0–5° for 15 min. After centrifugation at 17,000 g for 30 min, the supernatant was dialyzed against the same buffer, and used as a crude enzyme. When *S*-(β-aminoethyl)-L-cysteine was incubated with acetyl phosphate at pH 7.8 in the presence

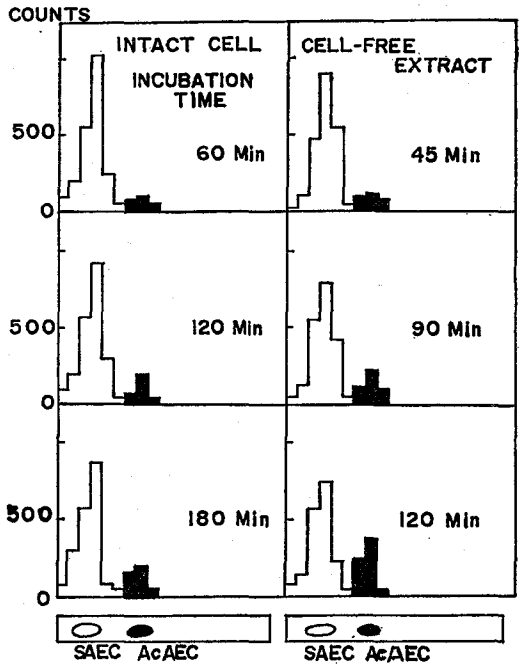


Fig. 2. Incorporation of the radioactivity into *S*-(β-N-acetyl-aminoethyl)-L-cysteine from *S*-(β-aminoethyl)-L-cysteine.

The reaction mixture contained 10 μmoles of *S*-(β-aminoethyl)-L-cysteine, 0.025 μmole of *S*-(β-aminoethyl)-L-[^{35}S]-cysteine, 20 μmoles of acetyl phosphate, 1 μmole of CoA, 500 μmoles of Tris-HCl buffer, pH 7.4 and the intact cells (6.03 mg dry weight) or the crude enzyme (1.2 mg protein) in a final volume of 0.9 ml. After incubation at 37° for various periods, the reaction was terminated by addition of 1.0 ml of 50% trichloroacetic acid. The clear supernatant (20 μl) obtained by centrifugation was chromatographed on Toyo No. 53 filter paper, using ethanol, ammonia and water (18 : 1 : 1) as a solvent. The paper strip (3×40 cm) was cut into serial 1.5-cm sections. They were transferred to scintillation vials containing the toluene system, and the radioactivity was measured with a Tri-Carb Liquid Scintillation 3320 spectrometer. SAEC: *S*-(β-aminoethyl)-L-cysteine, AcAEC: *S*-(β-*N*-acetyl-aminoethyl)-L-cysteine.

of washed cells or crude enzyme, *S*-(β-*N*-acetyl-aminoethyl)-L-cysteine was formed and identified as described previously. The bacterial and enzymic incorporation of radioactivity of *S*-(β-aminoethyl)-L-[^{35}S]-cysteine into *S*(-β-*N*-acetyl-aminoethyl)-L-cysteine was investigated. Two radioactive spots corresponding to the starting material and its *N*-acetylated product are shown in Fig. 2. The radioactivity of *S*-(β-*N*-acetyl-aminoethyl)-L-cysteine increased and that of the substrate decreased as the incubation was prolonged in both the reaction systems. It is clear that *S*-(β-*N*-acetyl-aminoethyl)-L-cysteine is formed as the exclusive product from the substrate.

Attempts were made to elucidate the acetyl donor (Table I). The possibility that *S*-(β-aminoethyl)-L-cysteine is acetylated enzymically in the presence of sodium acetate, as in the case of enzymic synthesis of *N*-acetyl amino acids by acylase I,[71] was excluded. Acetyl-CoA and acetyl phosphate were shown to be effective acetyl donors in Tris-HCl

Table I. Acetyl Donor Specificity

Acetyl donor	Formation of AcAEC (μmoles)*	
	I	II
Sodium acetate	0	0
Acetyl phosphate	4.37	1.98
Acetyl phosphate + CoA	9.54	4.82
Acetyl phosphate + CoA**	10.02	5.92
Acetyl CoA	9.38	9.40
Acetyl phosphate***	0.93	0.93

* The reaction mixture contained 20 μmoles of *S*-(β-aminoethyl)-L-cysteine, 20 μmoles of acetyl donor with or without 1 μmole of CoA, 500 μmoles of Tris-HCl (I) or potassium phosphate (II) buffer, pH 7.4 and 2 mg of enzyme protein in a final volume of 0.9 ml. AcAEC; *S*-(β-*N*-acetyl-aminoethyl)-L-cysteine

** Phosphotransacetylase (2 μg) was added.

*** The enzyme was omitted.

buffer, but the latter required the presence of CoA for the full activity. When the incubation was carried out in the presence of potassium phosphate buffer, which inhibited the phosphotransacetylase activity approximately 40%, *N*-acetylation was diminished in the reaction systems containing either acetyl phosphate alone or acetyl phosphate and CoA, although acetyl-CoA was utilized as well as in Tris-HCl buffer. This fact suggests that acetyl-CoA is the direct donor of acetyl group. The non-enzymic *N*-acetylation with acetyl phosphate was observed to a certain extent under the conditions employed. This enzyme preparation contained considerably high activity of phosphotransacetylase, because its addition to the reaction mixture resulted in only a slight activation. The findings described above present good evidence for the occurrence of an enzyme catalyzing the transfer of acetyl group from acetyl-CoA to the terminal amino group of *S*-(β-aminoethyl)-L-cysteine, (acetyl-CoA: *S*-(β-aminoethyl)-L-cysteine ω-*N*-acetyltransferase). The enzymic acetylation with acetyl phosphate alone implies that the crude enzyme preparation must contain a small amount of CoA.

2. Purification of *S*-(β-aminoethyl)-L-cysteine ω-*N*-acetyltransferase.

1) Effect of *S*-(β-aminoethyl)-L-cysteine added in the growth medium on enzyme activity

The effect of concentrations of *S*-(β-aminoethyl)-L-cysteine in a growth medium on the activity of *S*-(β-aminoethyl)-L-cysteine ω-*N*-acetyltransferase was investigated. The cell-free extract from the cells grown in an *S*-(β-aminoethyl)-L-cysteine-free medium had only a slight activity, while the activity increased with increasing the concentration of *S*-(β-aminoethyl)-L-cysteine in a medium (Fig. 3). These results suggest that this enzyme is inducibly formed. When *S*-(β-aminoethyl)-L-cysteine was substituted for L-lysine, L-ornithine or L-cysteine, the enzyme activity increased only slightly.

2) Purification of enzyme

All operations, unless otherwise stated, were carried out at 0–5°.

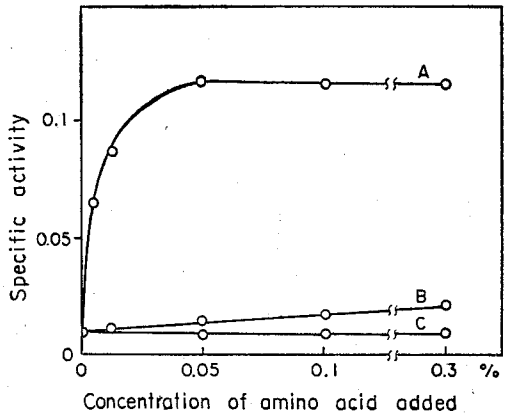


Fig. 3. Effect of amino acids added in the growth medium on the enzyme activity.
The growth medium contained 0.5% (w/v) peptone, 0.1% meat extract, 0.1% glucose, 0.1% KH_2PO_4, 0.1% K_2HPO_4, 0.1% NaCl, 0.01% $MgSO_4 \cdot 7H_2O$, 0.01% yeast extract and the indicated concentrations of amino acids (pH 7.2). The activity of the dialyzed crude enzyme was determined as described in the text. Curve A: *S*-(β-aminoethyl)-L-cysteine, Curve B; L-lysine or L-ornithine, Curve C; L-cysteine.

Step 1. The washed cells were ground thoroughly with levigated aluminum oxide in a mortar. To the mortar was added the small volume of 0.01 M Tris-HCl buffer, pH 7.4, containing 0.01% (w/v) 2-mercaptoethanol and mixed well with the paste. The supernatant solution obtained by centrifugation was dialyzed overnight against 200 volumes of 1 mM Tris-HCl buffer, pH 7.4 containing 0.01% 2-mercaptoethanol. The precipitate formed during dialysis was discarded.

Step 2. The cell-free extract was brought to 30% saturation with solid ammonium sulfate. After 30 min, the precipitate was removed by centrifugation at 17,000 g for 30 min. Ammonium sulfate was added to the supernatant to 65% saturation. The precipitate collected by centrifugation was dissolved in a small volume of the same buffer.

Step 3. The dialyzed enzyme solution was placed on a DEAE-cellulose column (100×5 cm) which was previously equilibrated with 1 mM Tris-HCl buffer, pH 7.4. After the column was thoroughly washed with the buffer containing 0.2 M NaCl, the active protein was eluted with the buffer containing 0.35 M NaCl at the flow rate of 100 ml per hr and 20-ml fractions were collected. The active fractions were combined and precipitated by addition of ammonium sulfate (70% saturation). The precipitate was dissolved in 1 mM Tris-HCl buffer, pH 7.4 and dialyzed overnight against 100 volumes of the same buffer.

Step 4. The enzyme solution was applied to a Sephadex G–150 column (2.8×70 cm) buffered with 1 mM Tris-HCl buffer, pH 7.4 and eluted by the same buffer. The active fractions were pooled and concentrated by addition of ammonium sulfate (70% saturation). The precipitate was dissolved in 1 mM Tris-HCl buffer, pH 7.4, and dialyzed against 100 volumes of the same buffer.

Step 5. The enzyme solution was chromatographed on a DEAE-cellulose column (2×20 cm) equilibrated as described in Step 3. After application of the enzyme, the column was washed with 1 mM Tris-HCl buffer, pH 7.4 containing 0.1 M NaCl. The

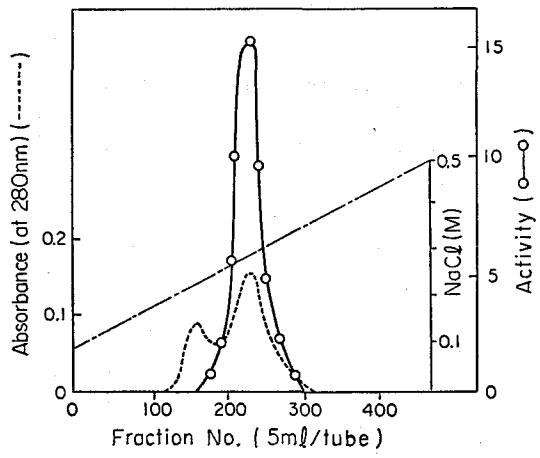


Fig. 4. Second DEAE-cellulose column chromatography.

The dialyzed enzyme solution was placed on a DEAE-cellulose column (2×20 cm) previously equilibrated with 0.01 M Tris-HCl buffer, pH 7.4 containing 0.1 M NaCl. The elution was carried out with a linear gradient between this buffer and the buffer containing 0.5 M NaCl. Five-ml aliquots of the eluate were collected.

elution was carried out with a linear gradient between 500 ml of this buffer in the mixing chamber and 500 ml of the buffer containing 0.5 M NaCl in the reservoir at the flow rate of 50 ml per hr. A typical elution pattern is given in Fig. 4. The active fractions were combined and brought to 70% saturation with ammonium sulfate. The precipitate was dissolved in a small volume of 1 mM Tris-HCl buffer, pH 7.4 and dialyzed against 100 volumes of the same buffer. A summary of the purification procedure is presented in Table II. The enzyme was purified approximately 450-fold with an over-all yield of 21.5%.

Table II. Purification of *S*-(β-Aminoethyl)-L-cysteine ω-*N*-Acetyltransferase

Step	Fraction	Total protein	Total units	Specific activity	Yield	L-Lys/L-SAEC*
		mg			%	
1	Crude extract	35,000	5900	0.17	100	0.59
2	Ammonium sulfate fractionation	5,800	5600	0.97	96	0.17
3	First DEAE-cellulose chromatography	380	3500	9.3	60	0.11
4	Sephadex G–150 chromatography	100	2800	27.1	47	0.11
5	Second DEAE-cellulose chromatography	18	1260	69.7	21	0.10

* Ratio of ω-*N*-acetyltransferase activity of L-lysine (L-Lys) to that of *S*-(β-aminoethyl)-L-cysteine (L-SAEC).

3. Properties of *S*-(β-aminoethyl)-L-cysteine ω-N-acetyltransferase.

1) Disc gel electrophoresis

Disc gel electrophoresis in 7.5% polyacrylamide gel was performed by a modification of the procedure of Davis.[72] The enzyme (20 μg) was applied on the top of spacer gel in 1 M sucrose. After the run, protein was stained with 1.0% Amind-Schwarz in 7% acetic acid. As shown in Fig. 5, *S*-(β-aminoethyl)-L-cysteine ω-*N*-acetyltransferase migrates toward the anode as a single band.

2) Molecular weight

The molecular weight of the enzyme was determined by chromatography on Sephadex G–150 column (1×95 cm) at 4° according to the method of Andrews,[73] and Determann and Michel.[74] The column was standardized with RNase I, serum albumin, asparaginase of *E. coli*,[75] catalase and blue dextran. From plots of the elution positions *versus* logarithm of the molecular weight, a molecular weight of the enzyme was determined to be approximately 100,000 (Fig. 6).

3) Stability of enzyme

The purified enzyme can be stored at 4° as a suspension in 1 mM Tris-HCl buffer, pH 7.4 containing 70% saturated ammonium sulfate for periods of over 3 months without loss of activity. The enzyme was found stable between 6.5 and 9.0, when the enzyme solution (5 mg per ml) was heated at 45° for 5 min at various values of pH (Fig. 7).

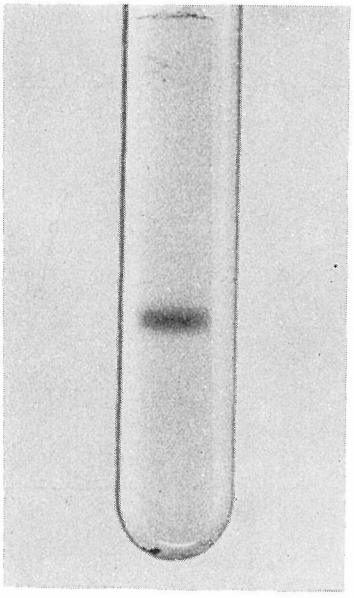


Fig. 5. Disc gel electrophoresis of the enzyme.
The enzyme (20 μg) was submitted to electrophoresis under the conditions of Davis, using 7.5% polyacrylamide gel and Tris-glycine buffer (pH 9.0). After the run, protein was stained with 1.0% Amido-Schwarz in 7% acetic acid.

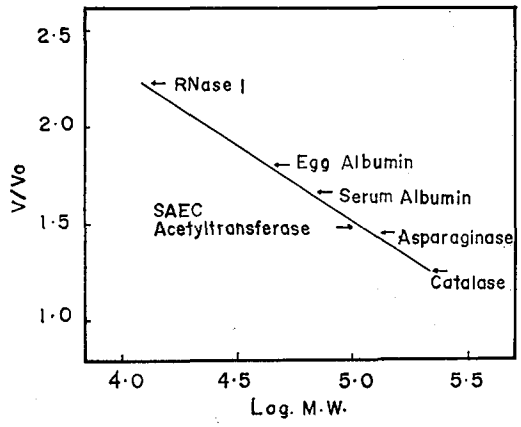


Fig. 6. Molecular weight determination for *S*-(β-aminoethyl)-L-cysteine ω-*N*-acetyltransferase by Sephadex G–150 gel chromatography.

The ratio of the protein elution volume (Ve) to the column void volume (Vo) against logarithm of molecular weight of protein was plotted. The void volume (Vo) of the column was determined by the elution of a 0.1 ml sample of blue dextrane. A 0.4 ml sample containing 5 mg each of the indicated references protein was allowed to run into the column and was eluted with 1 mM Tris-HCl buffer, pH 7.4 containing 0.1 M sodium chloride. The flow rate was 3 ml per hr and 2.0 ml aliquots of eluate were collected.

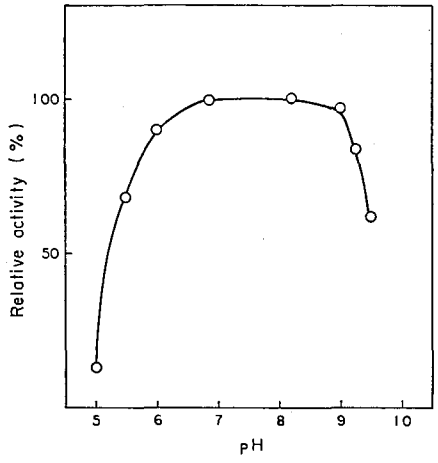


Fig. 7. Effect of pH on the enzyme stability.

The enzyme was heated at 45° for 5 min in the following buffers (final concentration, 0.01 M), and diluted 6 times with water. The enzyme activity was then determined. Sodium acetate buffer, pH 4.5–5.5; potassium phosphate buffer, pH 5.5–7.2; Tris-HCl buffer, pH 7.2–9.0; borate buffer, pH 9.5–10.5.

4) Acyl donor specificity

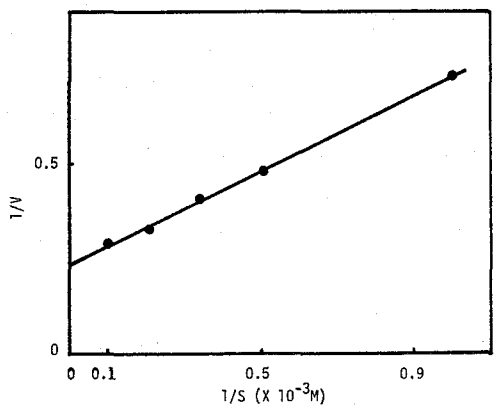
Acetyl-CoA is required as the direct acetyl donor in this enzymic N-acetylation. To define acyl donor specificity several acyl-CoA compounds were tested with 10 μg of enzyme under the standard conditions. The *N*-acylation of *S*-(β-aminoethyl)-L-cysteine was followed by determining the amount of remaining *S*-(β-aminoethyl)-L-cysteine with ninhydrin after separation by paper chromatography. None of propionyl-CoA, butyryl-CoA and benzoyl-CoA acted as an acyl donor in the reaction: acetyl-CoA is the exclusive acylating agent.

5) Acetyl acceptor specificity

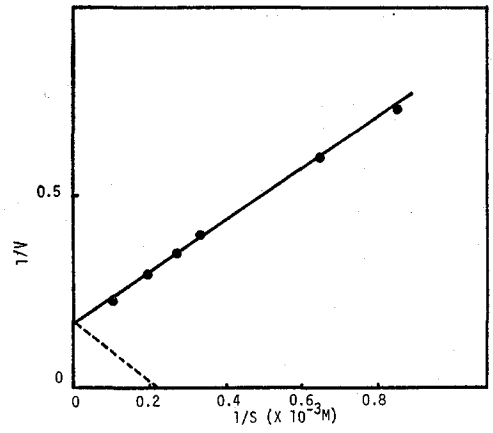
The ability of this acetyltransferase to catalyze the transfer of acetyl group from acetyl-CoA to various acceptors is presented in Table III. In addition to *S*-(β-aminoethyl)-L-cysteine, several other lysine analogues serve as the acetyl acceptor. *O*-(β-Aminoethyl)-DL-serine, an oxygen analogue of lysine, and *S*-(β-aminoethyl)-L-cysteine-*dl*-sulfoxide are acetylated by the enzyme as well as *S*-(β-aminoethyl)-L-cysteine. *S*(β-Aminoethyl)-D-

Table III. Acetyl Acceptor Specificity

Acetyl acceptor	Relative activity
	%
S-(β-Aminoethyl)-L-cysteine	100
S-(β-Aminoethyl)-D-cysteine	81
S-(β-Aminoethyl)-L-cysteine *dl*-sulfoxide	100
S-(β-Aminoethyl)-L-cysteine sulfone	56
S-(β-Aminoethyl)-L-cysteine hydroxamate	0
S-(β-Aminoethyl)-L-cysteine methyl ester	0
S-(β-Aminoethyl)-L-homocysteine	71
S-(β-Aminoethyl)-α-*N*-acetyl-L-cysteine	0
S-(β-*N*-Acetyl-aminoethyl)-L-cysteine	0
S-β-(2-Pyridylethyl)-L-cysteine	0
S-β-(4-Pyridylethyl)-L-cysteine	0
S-(β-Aminoethyl)-mercaptopropionate	0
O-(β-Aminoethyl)-DL-serine	104
L-Lysine	12
D-Lysine	9
α-*N*-Acetyl-L-lysine	9
ϵ-*N*-Acetyl-L-lysine	0
DL-δ-Hydroxylysine	7
L-Ornithine	12
D-Ornithine	10
Cadaverine	0
α-Amino-n-caproate	0
δ-Amino-n-valerate	0
α-Amino-n-butyrate	0
β-Amino-n-butyrate	0
α,γ-Diamino-n-butyrate	0
DL-Lanthionine	0



(a)



(b)

Fig. 8-a. Effect of *S*-(β-aminoethyl)-L-cysteine concentration on ω-*N*-acetyltransferase activity.

The activity was determined under the standard assay conditions except the indicated concentration of *S*-(β-aminoethyl)-L-cysteine and 20 μmoles of acetyl phosphate. The reciprocal velocity was plotted against the reciprocal concentration of *S*-(β-aminoethyl)-L-cysteine.

Fig. 8-b. Effect of acetyl-CoA concentration on ω-*N*-acetyltransferase activity.

The reaction mixture contained 100 μmoles of Tris-HCl buffer, pH 7.8, 10 μmoles of *S*-(β-aminoethyl)-L-cysteine, the indicated concentration of acetyl-CoA and enzyme in a final volume of 0.9 ml. Other conditions were given in the experimental procedures.

cysteine, *S*-(β-aminoethyl)-L-homocysteine and *S*-(β-aminoethyl)-L-cysteine sulfone are also good substrate, whereas hydroxamate and methyl ester of *S*-(β-aminoethyl)-L-cysteine, *S*-(β-aminoethyl)-α-*N*-acetyl-L-cysteine, *S*-(β-aminoethyl)-mercaptopropionate and α-*N*-acetyl-L-lysine are inert. L- and D-Lysine and ornithine, and δ-hydroxylysine (a mixture of DL and DL-allo) can be acetylated though slowly, while cadaverine, α-amino acids and ω-amino acids, *e.g.*, α-amino-butyrate and ε-amino-caproate are all inactive. The ratio of ω-*N*-acetylation activity of L-lysine to that of *S*-(β-aminoethyl)-L-cysteine decreased with increasing the specific activity of the enzyme during the course of purification as shown in Table II. This finding suggests that there may be another enzyme that catalyzes predominantly *N*-acetylation of L-lysine in the cell-free extract.

6) Kinetics

The Michaelis constants for *S*-(β-aminoethyl)-L-cysteine and acetyl-CoA were determined under the standard assay conditions (Figs. 8a and 8b). They were calculated to be 2.1×10^{-3} M for *S*-(β-aminoethyl)-L-cysteine and 4.5×10^{-3} M for acetyl-CoA. When *S*-(β-aminoethyl)-D-cysteine, *O*-(β-aminoethyl)-DL-serine and *S*-(β-aminoethyl)-L-homocysteine were employed as an acetyl acceptors, the Km values were found to be 1.6×10^{-3} M, 1.7×10^{-3} M and 7.1×10^{-4} M, respectively.

7) Effect of pH

The enzyme is optimally active at about pH 8.0, when examined in the presence of Tris-HCl buffer and sodium pyrophosphate (Fig. 9).

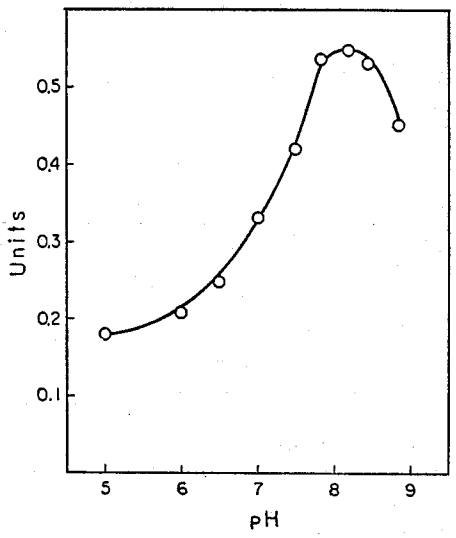


Fig. 9. Effect of pH on *S*-(β-aminoethyl)-L-cysteine ω-*N*-acetyltransferase activity.
The reaction mixture contained 5 μmoles of *S*-(β-aminoethyl)-L-cysteine, 5 μmoles of acetyl-CoA and 50 μmoles of the following buffers in a final volume of 0.45 ml. Tris-maleate-NaOH buffer, pH 5.0–6.0; potassium phosphate buffer, pH 6.0–7.2; Tris-HCl buffer, pH 7.2–9.0; sodium pyrophosphate buffer, pH 7.5–8.5. The enzyme activity was determined as described in the text.

8) Inhibitors

The effects of various acyl-CoA and amino acids on the ω-*N*-acetyltransferase activity were tested. Propionyl-CoA, butyryl-CoA and benzoyl-CoA which are inert as an acyl donor inhibit markedly this enzyme. These inhibitions result from the competition between them and acetyl-CoA for enzyme. Ki values for propionyl-CoA, butyryl-CoA and benzoyl-CoA were calculated to be 3.2×10^{-4} M, 2.2×10^{-4} M and 5.7×10^{-4} M, respectively (Fig. 10). Among amino acids tested *S*-(β-aminoethyl)-mercaptopropionate and *S*-(β-aminoethyl)-α-*N*-acetyl-L-cysteine inhibit strongly *S*-(β-aminoethyl)-L-cysteine ω-*N*-acetyl-transferase reaction, but L-lysine and L-ornithine which are very poor acyl acceptors have only negligible influence on the activity. Lineweaver and Burk plots of the inhibitions with *S*-(β-aminoethyl)-mercaptopropionate and *S*-(β-aminoethyl)-α-*N*-acetyl-L-cysteine show a competition between them and *S*-(β-aminoethyl)-L-cysteine (Ki: 1.1×10^{-3} M for *S*-(β-aminoethyl)-mercaptopropionate and 2.6×10^{-2} M for *S*-(β-amino-

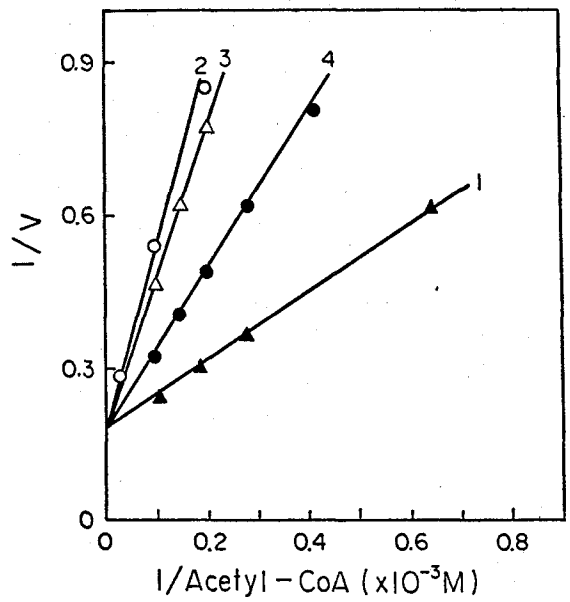


Fig. 10. Inhibition of *S*-(β-aminoethyl)-L-cysteine ω-*N*-acetyltransferase by propionyl-CoA, butyryl-CoA and benzoyl-CoA.

The enzyme activity was assayed by measuring incorporation of radioactivity of 1-[^{14}C]-acetyl-CoA (36,000 cpm per μmole) into *S*-(β-*N*-acetyl-aminoethyl)-L-cysteine in the absence (1) of, or the presence of propionyl-CoA (2), butyryl-CoA (3) or benzoyl-CoA (4). The reaction mixture contained 5 μmoles of *S*-(β-aminoethyl)-L-cysteine, 50 μmoles of Tris-HCl buffer (pH 7.8), and various amount of 1-[^{14}C]-acetyl-CoA as indicated in a final volume of 0.45 ml. The concentration of propionyl-CoA, butyryl-CoA and benzoyl-CoA was 1.3×10^{-3} M, 0.7×10^{-3} M, and 1.3×10^{-3} M, respectively. After incubation at 37° for 10 min, the reaction was terminated by addition of 0.05 ml of 50% trichloroacetic acid. The clear supernatant (20 μl) obtained by centrifugation was chromatographed with ethanol, ammonia and water (18 : 1 : 1) system as a solvent. The spot corresponding to *S*-(β-*N*-acetyl-aminoethyl)-L-cysteine was cut off, and the radioactivity was measured using toluene system.

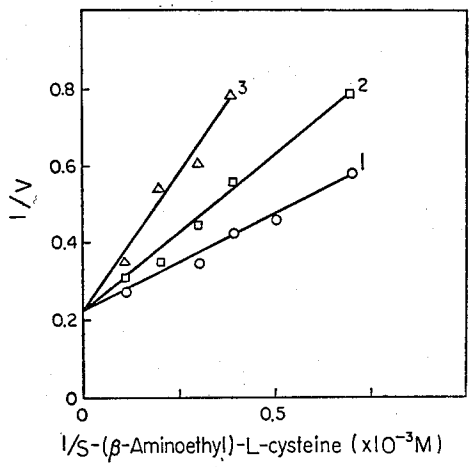


Fig. 11. Inhibition of *S*-(β-aminoethyl)-L-cysteine ω-*N*-acetyltransferase by *S*-(β-aminoethyl)-α-*N*-acetyl-L-cysteine and *S*-(βaminoethyl)-mercaptopropionate.

The enzyme activity was saasyed by measuring incorporation of radioactivity of *S*-(β-aminoethyl)-L-[^{35}S]-cysteine (63,400 cpm per μmole) into *S*-(β-*N*-acetyl-aminoethyl)-L-cysteine in the absence (1) of, or the presence of *S*-(β-aminoethyl)-α-*N*-acetyl-L-cysteine (2) or *S*-(β-aminoethyl)-mercaptopropionate (3). The reaction mixture contained 10 μmoles of acetyl phosphate, 0.5 μmole of CoA, 2 ug of phosphotransacetylase as protein, 100 μmoles of Tris-HCl buffer (pH 7.8) and various amounts of *S*-(β-aminoethyl)-L-[^{35}S]-cysteine in a final volume of 0.9 ml. The concentrations of *S*-(β-aminoethyl)-α-*N*-acetyl-L-cysteine and *S*-(β-aminoethyl)-mercaptopropionate were 1.3×10^{-2} M and 1.3×10^{-3} M, respectively. The other conditions and assay methods are given in Fig. 10.

ethyl)-α-*N*-acetyl-L-cysteine) as shown in Fig. 11. The enzyme is not inhibited by several other compounds, *e.g.*, EDTA and hydroxylamine, though *p*-chloromercuric benzoate and $HgCl_2$ (0.1 mM) are slightly inhibitory (approximately 17%).

Discussion

The occurrence of several enzymes catalyzing the *N*-acylation of L-α-amino acids has so far been reported. L-Aspartate *N*-acyltransferase isolated and purified from the rat and cat brains catalyzes exclusively acetyl-CoA dependent *N*-acetylation of aspartic acid.[76,77] L-Glutamate *N*-acetyltransferase was discovered in *E. coli* and *Clostridium kluyveri*,[78] but has not been highly purified. Stadtman *et al.* reported a cyanide-induced *N*-acetylation of a few amino acids, *e.g.*, glycine, L-leucine and L-serine by the enzyme from *Clostridium kluyveri*.[79] The occurrence of glycine *N*-acyltransferase (acyl-CoA: glycine *N*-acyltransferase) was demonstrated in the mitochondrial fractions of liver and kidney.[80,81] This enzyme catalyzes the conversion of various aliphatic ($(C_2$–C_{10}) and aromatic acyl-thioesters of CoA into the corresponding *N*-acyl derivatives of glycine which is the only acyl acceptor. Recently acetyl-CoA-L-phenylalanine α-*N*-acetyltransferase was demonstrated in the extracts of *E. coli*, and purified 160-fold to investigate the enzymological properties.[82] An enzyme catalyzing the transfer of an acetyl group from acetyl-CoA to an α-amino group of D-amino acids (acetyl-CoA: D-amino acid α-*N*-acetyltransferase)

was also found in baker's yeast,[83] and has been purified to near homogeneity.[84] This enzyme has very broad substrate specificity and strictly high optical specificity. D-Lysine, which is a less potent acceptor, is acetylated by the enzyme only at the α-amino group. Paik and Kim[58] have obtained evidence for the occurrence of an enzyme which catalyzes the synthesis of ε-*N*-acetyl-L-lysine in beef liver, although the acetylation of the sulfur analogue of lysine was not investigated. In this enzymic reaction the acetyl donor is not acetyl-CoA, but acetyl phosphate. The enzymic conversion of L-lysine to ε-*N*-acetyl-L-lysine was found also in the yeast, *Hansenula saturnus,* although the enzymological aspect of acetylation has remained unsettled.[61] Recently, Edmunds and Barker[85] reported the occurrence of an enzyme in the cell-free extracts of *Pseudomonas,* strain B4 isolated from soil, which catalyzes a reaction between acetyl-CoA and L-β-lysine to form ε-*N*-acetyl-L-lysine. Lysine, ornithine and other diamino and monoamino acids are not acetylated by this enzyme.

Acetyl-CoA: *S*-(β-aminoethyl)-L-cysteine ω-*N*-acetyltransferase, an enzyme catalyzing the transfer of an acetyl group from acetyl-CoA to the terminal amino group of *S*-(β-aminoethyl)-L-cysteine has been purified about 450-fold from the cell-free extracts of *A. aerogenes* IFO 3320. The purified enzyme is homogeneous by the criterion of disc gel electrophoresis, and has an approximate molecular weight of 100,000, when examined by gel filtration method. The enzyme has a pH optimum at about 8.0, which is similar to the values for most of the other amino acid *N*-acyltransferases. In addition to *S*-(β-aminoethyl)-L-cysteine, the D-enantiomer, the sulfoxide, the sulfone, a higher homologue of *S*-(β-aminoethyl)-L-cysteine, and *O*-(β-aminoethyl)-DL-serine were effective acetyl acceptors for the enzyme. *O*-(β-aminoethyl)-DL-serine, an oxygen analogue of lysine which is also antagonistic to lysine, was reported to be oxidized to yield 4-oxa-Δ^1-piperideine-2-carboxylic acid by L-amino acid oxidase of *Mytilus edulis*.[45] This finding indicates that the *N*-acetylation is probably the common first step in metabolism (detoxication pathway) of these lysine antagonists in this organism. L- or D-Lysine and L- or D-ornithine also function as acetyl acceptors to a certain extent. However, α- or ω-*N*-acetylated derivatives of *S*-(β-aminoethyl)-L-cysteine and L-lysine are not susceptible to the enzyme. Hydroxamate and methyl ester of *S*-(β-aminoethyl)-L-cysteine also are inactive as an acetyl acceptor. These findings suggest that the occurrence of both α- and ω-amino groups, and α-carboxyl group in the free state is essential for an acetyl acceptor of the enzyme as well as the molecular structure similar to *S*-(β-aminoethyl)-cysteine. It seems of interest that all of the good substrates have either a sulfur or an oxygen atom inserted in their carbon chain, although the relationship between the structure of substrate and enzyme activity has not been elucidated. It is known that most of the enzymes concerned with amino acid metabolism are optically specific. But, this acetyltransferase has very low optical specificity, although the L-enantiomers are generally a little more susceptible to the enzyme than the D-enantiomers. The enzyme activity was inhibited competitively by some other fatty acid-CoA derivatives against acetyl-CoA. These inhibitory effects by their acyl-CoA derivatives also have been reported in the enzymic *N*-acetylation (acetyl-CoA dependent) of L-aspartic acid.[77] *S*-(β-Aminoethyl)-α-*N*-acetyl-L-cysteine and *S*-(β-aminoethyl)-mercaptopropionate, which are not substrates, are in competition with *S*-(β-aminoethyl)-L-cysteine to depress potently the enzyme activity. This fact and the optical nonspecificity of enzyme imply that α-amino group of the acetyl acceptor is not necessary for the substrate to bind

the binding site of enzyme: the lysine analogues probably bind the active site of enzyme through their COOH and terminal NH_2 groups. The α-amino group, however, must play an indispensable role in order to make the terminal amino group of acetyl acceptor bound to the enzyme accessible to the acetyl-CoA bound on the own binding site to accomplish transfer of the acetyl group.

III OCCURRENCE OF L-AMINO ACID OXIDASE IN *AEROBACTER AEROGENES*

Enzymic deamination of amino acids to the corresponding α-keto acids was first reported by Neubauer[86] and Knoop.[87] There are two general mechanisms for the deamination. One of them is transamination, and the other is oxidative deamination. Since the occurrence of L-amino acid oxidase was discovered and characterized by Krebs,[88] a number of reports on microbial and animal L-amino acid oxidases have appeared. Recently L-amino acid oxidase has been highly purified from several sources and its physicochemical and enzymological properties, mechanism of action and some structural features have been considerably clarified.

The present chapter describes evidence for the occurrence of an enzyme in *A. aerogenes*, which catalyzes oxidative deamination of *S*-(β-*N*-acetyl-aminoethyl)-L-cysteine.

Experimental Procedures

Materials. *S*-(β-Aminoethyl)-L-cysteine and *S*-(β-*N*-acetyl-aminoethyl)-L-cysteine were prepared as described in Chapter I. L-Leucine and L-methionine were products of Kyowa Hakko Kogyo Co., Tokyo. The other chemicals were analytical grade reagents.

Microorganisms and Conditions of Culture. *A. aerogenes* was grown in a medium containing 0.5% peptone, 0.1% glycerol, 0.1% KH_2PO_4, 0.1% K_2HPO_4, 0.1% NaCl, 0.01% $MgSO_4 \cdot 7H_2O$, 0.01% yeast extract and 0.01% *S*-(β-aminoethyl)-L-cysteine. The pH of the medium was adjusted to 7.2 with 2 N NaOH. The bacteria were grown aerobically in 2-liter flasks containing 500 ml of the medium, with vigorous shaking on a reciprocating shaker at 28° for 20 hr. The cells harvested by centrifugation were washed twice with 0.85% NaCl solution. The washed cells were suspended in 0.01 M potassium phosphate buffer, pH 7.0 for 3 hr, and used as resting cells.

Enzyme Assay. L-Amino acid oxidase activity was followed by measuring the rate of oxygen uptake by means of the Warburg manometer. The main compartment of a Warburg vessel contained 100 μmoles of potassium phosphate buffer, pH 7.0 and resting cells or cell-free extract in a final volume of 1.9 ml. The center well contained 0.2 ml of 20% KOH. The side arm contained 10 μmoles of *S*-(β-*N*-acetyl-aminoethyl)-L-cysteine or certain other L-amino acids in 0.4 ml. Measurements were made at 30° with air in the gas phase. Since there was a slight endogenous respiration, a blank in which substrate was replaced by water run for every assays of the enzyme.

Results

Occurrence of L-amino Acid Oxidase in *A. aerogenes*.

When *S*-(β-*N*-acetyl-aminoethyl)-L-cysteine was incubated with resting cells or cell-free extract of *A. aerogenes*, oxygen uptake was observed (Fig. 1). After the oxidation of *S*-(β-*N*-acetyl-aminoethyl)-L-cysteine, the reaction mixture was removed from a mano-

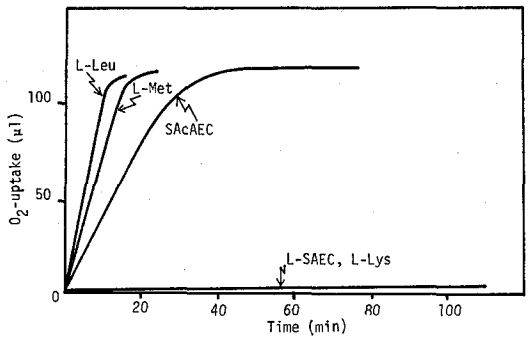


Fig. 1. Oxidation of *S*-(β-*N*-acetyl-aminoethyl)-L-cysteine.
The enzyme activity was determined as described in the text. SAcAEC: *S*-(β-*N*-acetyl-aminoethyl)-L-cysteine.

meter flask, deprotenized and centrifuged at 17,000 g for 20 min. When electrophoresis of the supernatant was carried out in 0.25 M pyridineacetate buffer at pH 4.8, a spot which reacted with 2,4-dinitrophenylhydrazine reagent migrated the same distance as *S*-(β-*N*-acetyl-aminoethyl)-α-keto-mercaptopropionate, as described in the previous I. These findings suggest that an enzyme catalyzing the oxidative deamination of *S*-(β-*N*-acetyl-aminoethyl)-L-cysteine occurs in *A. aerogenes*. Monoamino-monocarboxylic acids are in general most susceptible to L-amino acid oxidases. When L-methionine and L-leucine were used as a substrate, they also serve as a good substrate. On the other hand, L-lysine and *S*-(β-aminoethyl)-L-cysteine which are very poor substrates of L-amino acid oxidase are oxidized slowly.

The results obtained here present good evidence for the occurrence of L-amino acid oxidase in the cells of *A. aerogenes*. Thus, it is clear that *S*-(β-*N*-acetyl-aminoethyl)-L-cysteine is oxidatively deaminated by L-amino acid oxidase to yield *S*-(β-*N*-acetyl-aminoethyl)-α-keto-mercaptopropionate.

Discussion

An α-amino group of lysine undergoes hardly reversible deamination with some exceptions, *e.g.*, L-lysine-oxaloacetate α-transaminase in a bean, *Phaselus aureus*.[89] However, L- or D-amino acid oxidase and transaminase are able to remove the α-amino group efficiently when the ε-amino group is blocked. In microorganisms, L-amino acid oxidase has been found in strains of *Neurospora crassa, Penicillium, Aspergillus niger, Proteus vulgaris* and *Aerobacter aerogenes*.[90~94]

L-Amino acid oxidase of *A. aerogenes* attacks only slowly the basic amino acids, although it has been reported that L-amino acid oxidase of *Mytilus edulis* catalyzes oxidative deamination of *S*-(β-aminoethyl)-L-cysteine, *O*-(β-aminoethyl)-DL-serine, L-lysine and L-arginine.[95] But neutral amino acids are readily oxidized by L-amino acid oxidase of *A. aerogenes*.

S-(β-*N*-Acetyl-aminoethyl)-L-cysteine was found to be deaminated oxidatively by the resting cells or cell-free extract from *A. aerogenes*. This finding strongly suggests that *S*-(β-*N*-acetyl-aminoethyl)-L-cysteine is converted into the corresponding α-keto compound,

S-(β-*N*-acetylaminoethyl)-α-keto-mercaptopropionate by the action of L-amino acid oxidase from *A. aerogenes*.

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