Microbial Synthesis and Degradation of Indole-3-acetic Acid. III. The Isolation and Characterization of Indole-3-acetyl-ε-L-lysine*

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ABSTRACT: A water-soluble metabolite of indole-3-acetic acid is formed by certain strains of *Pseudomonas savastanoi*. This metabolite has been isolated from culture filtrates of this organism and purified by adsorption onto charcoal and by ion-exchange chromatography on sulfonic acid and quaternary ammonium resins.

Hydrolysis of this isolated material by cation-exchange resin and barium hydroxide yielded indole-3-acetic acid and lysine. Acid hydrolysis yielded lysine which was decarboxylated by L-lysine decarboxylase. The specific rotation of the isolated lysine indicates that the compound is the L isomer. Acid hydrolysis of the dinitrophenyl derivative of the unknown compound yielded α-dinitrophenyllysine. These results suggested that the unknown compound was indole-3-acetyl-ε-L-lysine. An authentic sample of the latter compound was prepared by reaction of indole-3-acetic carbonic half-ester anhydride with the copper complex of L-lysine and decomposition of the resulting copper derivative with hydrogen sulfide. The synthetic compound and the natural material showed identical infrared and ultraviolet spectra, melting points, and gave identical dinitrophenyl derivatives. Thus, the unknown compound was conclusively identified as indole-3-acetyl-ε-L-lysine.

It has been shown that indole-3-acetic acid (IAA)[1] is formed from L-tryptophan *via* indole-3-acetamide by *Pseudomonas savastanoi* (Magie *et al.*, 1963; Kosuge *et al.*, 1966). IAA is not an end-product in this biosynthetic scheme but is further converted to water-soluble metabolites (Kosuge *et al.*, 1966). The present paper describes the isolation, structure, synthesis, and some of the properties of a water-soluble metabolite of IAA from *P. savastanoi*.

Experimental and Results Section

Paper and Thin-Layer Chromatography. Solvents used for paper (Whatman No. 1 filter paper) and thin-layer chromatography (Eastman chromagram K301R2) were (A) 1-butanol–concentrated ammonium hydroxide–water (10:1:1), (B) 1-propanol–concentrated ammonium hydroxide (7:3), (C) 1-butanol–glacial acetic acid–water (4:1:2), (D) chloroform–methanol–9 N ammonium hydroxide (1:2:1), (E) benzene–pyridine–glacial acetic acid (30:10:1), and (F) chloroform–*t*-pentyl alcohol–glacial acetic acid (70:30:3). Amino acids were detected on the chromatograms by a ninhydrin spray (Stahl, 1965a). The Ehrlich reagent (Stahl, 1965b) was used to detect indole derivatives on the chromatograms.

Isolation and Purification of the Unknown Compound. The media used in these studies were (A) 0.5% D-glucose, 0.25% Difco Casamino Acid, 0.03% $MgSO_4 \cdot 7H_2O$, 0.17% K_2HPO_4, 0.2% NaH_2PO_4, and 0.7% Difco Bacto agar; and (B) as medium A except Difco Bacto agar was omitted and 0.04% IAA was added.

P. savastanoi isolate 2009A was used in these studies. Cells from a 2-day-old culture on a medium A slant were suspended in 10 ml of sterile, distilled water. This suspension was used to inoculate 500 ml of medium B in a 1-l. erlenmeyer flask. The flask was agitated at 200 rpm on a gyrorotary shaker (New Brunswick Model G-26) for 24 hr at 25°. Then the entire contents of the flask were used to inoculate a carboy containing 12 l. of medium B. An additional 260 μg of IAA-2-^{14}C (specific activity, 3.25 mc/mmoles; New England Nuclear) was added/l. of the latter medium to facilitate detection and isolation of the IAA metabolite. The medium was vigorously aerated during the growth period.

After 2–3-days incubation at 23°, the bacterial suspension was centrifuged at 10,000*g* for 20 min. The precipitate of bacterial cells was discarded and the supernatant liquid was cooled to 2°. Partially deactivated charcoal (10 g), prepared from 40 g of activated charcoal Darco G-60 (Matheson Coleman and Bell) and 1.6 g of stearic acid by the method of Dalgliesh (1955), was added and the suspension was mechanically stirred. After 1 hr and again after 2 hr, additional 10-g portions of charcoal were added. Stirring was continued for 1 hr after addition of the third lot of charcoal. The temperature was kept at

* From the Department of Plant Pathology, University of California, Davis, California 95616. *Received October 2, 1967.* This investigation was supported by a research grant (AI-06323) from the National Institutes of Health, U. S. Public Health Service.

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[1] Abbreviations used: IAA, indole-3-acetic acid; IAA-LYS, indole-3-acetyl-ε-L-lysine; ATP, adenosine triphosphate.

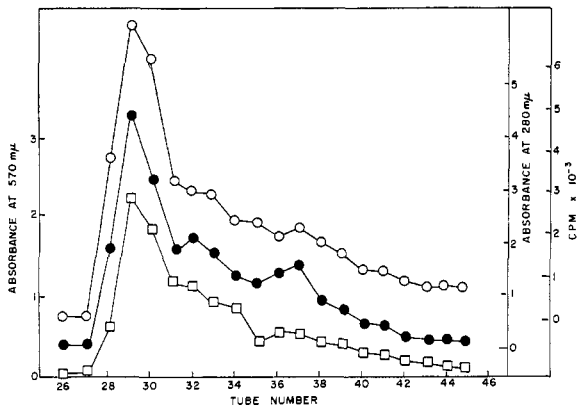

FIGURE 1: Elution, in 5-ml fractions, of the unknown metabolite of IAA from an anion-exchange resin (Bio Rad AG 1-X2, formate form). (□——□) Absorbance at 570 mμ of 5-ml aliqots of each fraction analyzed by the procedure of Yemm and Cocking (1955). (●——●) Absorbance at 280 mμ. (○——○) Total counts per minute in each fraction.

2–5° during this procedure. The charcoal was allowed to settle and the upper charcoal-free layer was decanted. Samples (0.5 ml) of the decanted liquid were dried on planchets and counted in a gas-flow counter. Such determinations revealed that the charcoal had adsorbed over 90% of the radioactive material initially present in the culture filtrate. The charcoal then was collected by filtration, washed with 200 ml of water, and suspended in 250 ml of pyridine–methanol–water (25:25:50). The suspension was shaken for 15 min and the charcoal was sedimented by centrifugation. The supernatant liquid was decanted and the suspension of the charcoal in pyridine–methanol–water solvent and centrifugation process were repeated twice. The supernatant extracts were combined and evaporated to dryness under 10 mm at 55°. The brown residue was dissolved in hot water (50 ml) and this solution was filtered to remove residual amounts of charcoal and stearic acid. This aqueous solution was passed through a sulfonic acid ion-exchange column (Dowex 50W-X8, 20 × 2.5 cm) in the ammonium form. Washing the column with 250 ml of water removed all unreacted IAA and colored impurities. Elution with 0.2 M NH_4OH removed a radioactive material which absorbed maximally at 280 mμ. Thin-layer chromatography of the eluates in solvents B and C revealed the presence of a compound which fluoresced a weak blue color under shortwave ultraviolet light (maximum emission 257 mμ) and produced a purple color with ninhydrin. Passage of the chromatogram through a strip counter confirmed that the compound was radioactive. Fractions which contained radioactivity were pooled and concentrated to 50 ml on a rotary evaporator at 55° and 10 mm. The concentrated material was passed through a column (2.5 × 20 cm) of an anion-exchange resin (Bio-Rad AG 1-X2, formate form). The column was eluted with water and the eluates were collected in 5-ml fractions. Figure 1 shows that the unknown compound was eluted by water and indicates that color yield with ninhydrin, radioactivity, and absorbance at 280 mμ of the eluted material was in close agreement. The contents of tubes 28 through 38 (Figure 1) were pooled, taken to dryness, and the residue was twice crystallized from water to yield 80 mg of small, colorless prisms which melted at 259–261° with decomposition. The compound was insoluble in ethyl acetate and diethyl ether, slightly soluble in methanol and ethanol, and moderately soluble in water. The ultraviolet absorption spectrum of the compound in water recorded on a Beckman DK-2 spectrophotometer showed maxima at 218, 278, and 287 mμ. Log ε at these maxima were, respectively, 4.48, 3.73, and 3.66. The units of ε (molar absorptivity) are M^{-1} cm^{-1}. The values for ε which are characteristic for indole-3-acetic acid and color production with ninhydrin suggested that the compound might be a basic amino acid conjugate of indoleacetic acid.

Hydrolysis of the Unknown Compound. A mixture of the unknown compound (20 mg), Dowex 50W-X8 (1 g), and 0.02 M HCl (3 ml) was heated in a sealed, evacuated glass vial at 100–105° for 5 hr. The mixture was cooled and filtered, and the resin was washed twice with 5 ml of water and then with 10 ml of 17% ammonia solution. The solutions were evaporated separately, the residues were dissolved in 6 ml of water, and the two samples were combined, adjusted to pH 2.5, and extracted with ethyl acetate (2 × 5 ml). The ethyl acetate extracts were dried (Na_2SO_4) and evaporated to dryness. The residue was sublimed *in vacuo* and recrystallized from ethyl acetate–hexane to give a product (2.7 mg) which melted at 166–167°. A mixture melting point with authentic IAA showed no depression.

The aqueous phase was reduced in volume to 1 ml, sodium bicarbonate (40 mg) and 1 ml of a solution of 1-fluoro-2,4-dinitrobenzene (100 mg) in acetone (3 ml) were added, and the mixture was stirred for 1.5 hr at 30–35° in the dark. Colorless crystals which formed during the reaction were removed by filtration and discarded. Water (2 ml) was added to the filtrate and the solution was extracted with ether (2 × 5 ml). The aqueous phase was acidified to pH 1.5 with 6 N HCl. Yellow crystals which formed were filtered, washed with water, and twice recrystallized from aqueous methanol. The small yellow prisms (2 mg) melted at 171–172°. Melting points for the DNP-LYS derivative previously reported are 170–172 (Rao and Sober, 1954) and 173–174.5° (Green and Kay, 1952). A mixture melting point with an authentic sample of di-DNP-L-lysine prepared by the method of Levy and Chung (1955) showed no depression.

The unknown compound (1 mg) and a saturated solution of barium hydroxide in water (0.4 ml) were heated in a sealed, evacuated glass vial at 100–105° for 16 hr. The solution was cooled, diluted with 1 ml of water, and adjusted to pH 3 with 0.1 M sulfuric acid. The precipitate of barium sulfate was removed by filtration and the filtrate was extracted with ethyl acetate (2 × 2 ml). The ethyl acetate extract was

reduced in volume by evaporation and spotted on thin-layer sheets. The compound in the ethyl acetate fraction and an authentic sample of IAA indicated identical behavior by chromatography in solvent A. The aqueous phase was concentrated to 0.1 ml by evaporation under reduced pressure (20 mm). The product in the concentrate and authentic L-lysine showed identical behavior by thin-layer chromatography in solvents B–D.

By alkaline hydrolysis and extraction with ethyl acetate as described above, 0.36 mg of the unknown compound yielded 0.18 mg (1.1 μmoles) of IAA in the ethyl acetate fraction as determined by spectrophotometric determination at 280 mμ (ϵ 5.2 $\times$ 10^3). The aqueous phase contained 0.15 mg (1.06 μmoles) of lysine as determined by the procedure of Yemm and Cocking (1955). Thus it appeared that IAA and lysine occurred in equimolar amounts and that the unknown compound was a lysine conjugate of IAA. Cleavage by alkali and by the resin suggested that the bond linking the two involved the carboxyl group of IAA and an amino group of lysine.

Anal. Calcd for lysine conjugate of IAA, $C_{16}H_{21}N_3O_3$: C, 63.34; H, 6.97; N, 13.85. Found: C, 63.5; H, 6.98; N, 13.97.

Preparation and Acid Hydrolysis of the 2,4-Dinitrophenyl Derivative of IAA-lysine. A mixture of the unknown compound (49 mg), sodium carbonate (100 mg), water (4 ml), acetone (3 ml), and 0.32 ml of a solution of 1-fluoro-2,4-dinitrobenzene (200 mg) in acetone (2 ml) was stirred in the dark at 30–35° for 1.5 hr. Water (5 ml) was added and the mixture was extracted with ether (2 $\times$ 10 ml). The ether fraction was discarded and the aqueous phase was acidified with 6 N HCl to pH 1.5. The orange-yellow crystals that separated out were washed with water and recrystallized twice from aqueous methanol–acetone to yield fine orange-yellow plates of IAA-LYS DNP (37 mg), melting at 224–225°.

Anal. Calcd for the dinitrophenyl derivative for indole-3-acetyl-lysine, $C_{22}H_{23}N_5O_7$: C, 56.28; H, 4.93; N, 14.91%. Found: C, 56.43; H, 4.97; N, 15.02%.

The dinitrophenyl (DNP) derivative (5 mg) and 6 N HCl (2 ml) were heated *in vacuo* in a sealed glass vial at 110° for 24 hr. The behavior of the yellow product in the hydrolysate on thin-layer chromatograms (solvent systems B–D) was identical with that of an authentic sample of α-DNP-L-lysine prepared from ϵ-acetyl-L-lysine (Neuberger and Sanger, 1943; Folk, 1956) and not with ϵ-DNP-L-lysine (Porter and Sanger, 1948). The results of these and the experiments described above indicated that the unknown compound was indole-3-acetyl-ϵ-lysine.

Reaction of Isolated Lysine with L-Lysine Decarboxylase. The isolated lysine conjugate (6 mg) and 6 N HCl (1 ml) were heated in a sealed, evacuated glass tube for 16 hr at 105°. Then the tube was cooled and its contents were diluted to 3 ml with water. The diluted solution was treated with activated charcoal to remove unreacted conjugate and a dark pigment which formed during treatment with acid. The charcoal was removed by filtration and the pH of the filtrate was adjusted to 6.0 by addition of 2 N NaOH. The filtrate was freeze dried and the residue was taken up in 1 ml of water. Analysis of the sample by the procedure of Yemm and Cocking (1955) showed that it contained 21.1 μmoles of lysine. A portion (0.5 ml) of the sample containing 10.55 μmoles of lysine was reacted with 10 units of L-lysine decarboxylase (Sigma Chemical Co.) by the procedure of Najjar (1955). A yield of 10.1 μmoles of CO_2 was obtained after 60-min reaction at 30°. A yield of 14.5 μmoles of CO_2 was obtained from 15 μmoles of authentic L-lysine under the same conditions. An authentic sample of D-lysine was not decarboxylated by this preparation. These results indicated that the lysine in the conjugate was the L isomer.

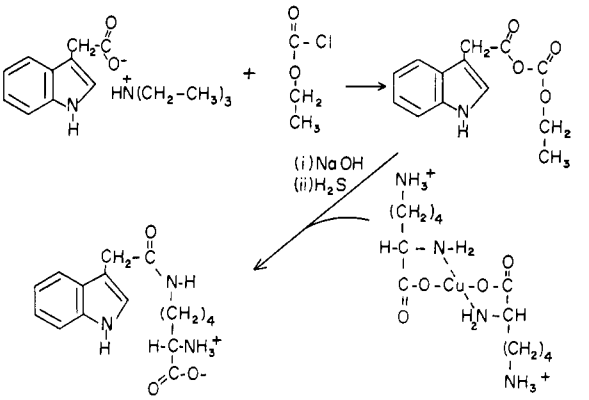

FIGURE 2: Reactions involved in the chemical synthesis of indole-3-acetyl-ϵ-L-lysine. Indole-3-acetic acid as the triethylamine salt is reacted with ethyl chlorocarbonate to yield the carbonic acid half-ester anhydride of indole-3-acetic acid. The latter is reacted under alkaline conditions with the copper complex of L-lysine to yield the copper complex of indole-3-acetyl-ϵ-L-lysine. The copper complex is then decomposed by H_2S to yield indole-3-acetyl ϵ-L-lysine.

Optical Rotation. The specific rotation, $[\alpha]^{23}$, in 2 N HCl was 22° for the isolated lysine conjugate and 20.7° for the lysine sample obtained by acid hydrolysis of the isolated conjugate. Under identical conditions $[\alpha]^{23}$ for an authentic sample of L-lysine was determined to be 20.8°. These results further indicated that the lysine in the conjugate was the L isomer.

Synthesis of Indole-3-acetyl-ϵ-L-lysine. A solution of IAA (350 mg) and triethylamine (0.28 ml) in dry dioxane (6 ml) was cooled to 0° and ethyl chlorocarbonate (0.2 ml) was added with vigorous stirring. The reaction was allowed to proceed for 5 min and then a mixture of 1 N sodium hydroxide (2 ml) and a solution (5 ml) of the copper chelate of L-lysine (prepared from 380 mg of L-lysine-HCl; Neuberger and Sanger, 1943) was added under further cooling. The reaction mixture was then allowed to come to room temperature and was stirred for 10 more min. The light blue precipitate was removed by filtration, washed with water, and

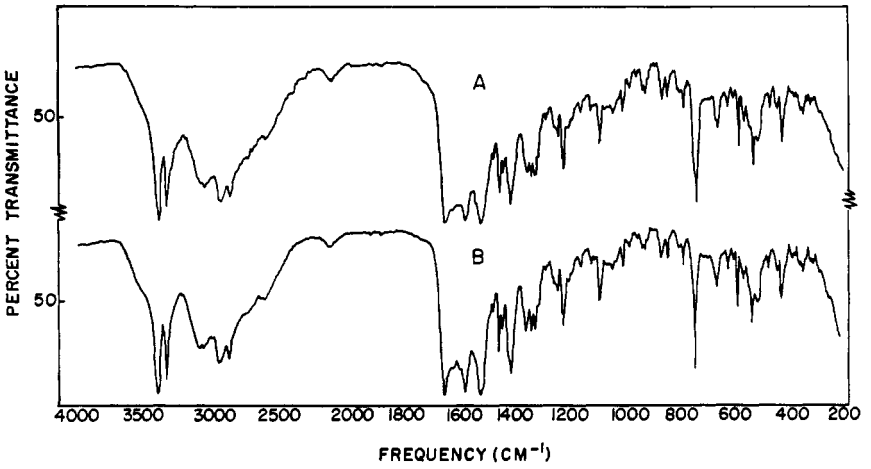

FIGURE 3: Infrared spectra (KBr pellet) of synthetic (A) and isolated (B) indole-3-acetyl-ε-L-lysine.

added to 20 ml of 2 N HCl. This suspension was heated to 60° and hydrogen sulfide was passed through until no further precipitate appeared (Figure 2). This mixture was filtered and the water and hydrochloric acid were removed in a vacuum desiccator over sodium hydroxide. The crude material (580 mg) was further purified by a column of Bio-Rad AG1-X2 ion-exchange resin in the formate form as previously described and recrystallized. The resulting IAA-LYS (310 mg) showed an infrared spectrum identical with that of the natural material (Figure 3). In addition, a mixture melting point showed no depression. The infrared spectra of the dinitrophenyl derivatives of the natural and synthetic lysine conjugates were identical (Figure 4).

Production. When. *P. savastanoi* was grown in liquid culture (5 l.) without added IAA, 4.7 mg of IAA-LYS was isolated. Allowing for losses during the isolation procedure as judged from experiments with radioactive products, the concentration of IAA-LYS after 2 days in the medium was approximately 10^{-5} M.

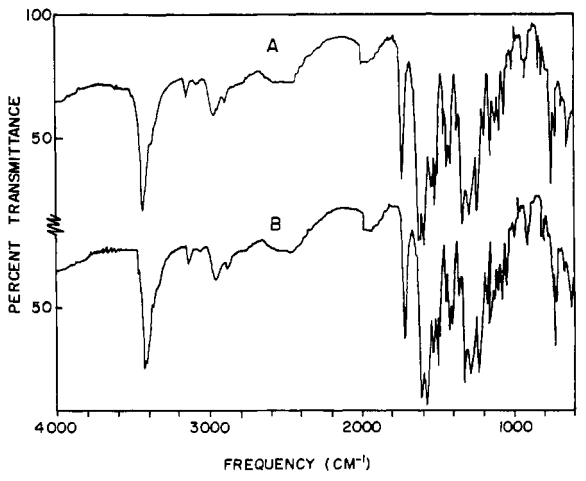

FIGURE 4: Infrared spectra (KBr pellet) of the dinitrophenyl derivatives of synthetic (A) and isolated (B) indole-3-acetyl-ε-L-lysine.

Discussion

When considering the metabolism of IAA to a water-soluble compound, the following well-known chemical reactions of indoles and 3-substituted indoles have to be considered: (a) opening of the pyrrole ring and further reactions, (b) hydroxylation of the aromatic moiety, (c) peroxidation and further reactions, (d) alteration of the side chain at position 3 (breakdown, oxidation), and (e) conjugation (*i.e.*, "coupling" with amino acids, carbohydrates, or other small molecules).

Because the formation of the new metabolite by the intact bacterium requires oxygen (Hutzinger and Kosuge, 1967) it was first assumed that the compound was formed by an oxidative degradation of IAA. However, experiments with differentially labeled IAA (Hutzinger and Kosuge, 1967) and the ultraviolet spectrum of a partially purified sample indicated that the IAA moiety was unchanged. Conjugation of IAA with lysine was suggested by results obtained from hydrolysis and chromatography of the hydrolysate. The oxygen requirement by the intact bacterium for the conjugate synthesis appears to be associated with the generation of ATP. The latter is required for the enzymic synthesis of the conjugate (Hutzinger and Kosuge, 1967). Deactivated charcoal proved to be singly the most effective step in the isolation and purification of the metabolite from culture filtrates since aromatic compounds are readily adsorbed by this system (*cf.* Synge and Tiselius, 1949). Retention by sulfonic acid ion-exchange resin (in the ammonium form) and elution with ammonia under conditions similar to those used by Thompson *et al.* (1959) for

the separation of aromatic amino acids gave further purification. Ion-exchange chromatography of this material on a quaternary ammonium-type resin in the formate form (Figure 1) gave a single, ninhydrin-reactive compound on thin-layer chromatography.

Acid hydrolysis of the new metabolite (IAA-LYS) yielded L-lysine but destroyed the indole moiety. The most successful way to preserve both IAA and lysine proved to be the hydrolysis with $Ba(OH)_2$ or sulfonic acid type ion-exchange resin (*cf.* Paulson *et al.*, 1953). By this procedure, IAA could be extracted and L-lysine converted to its didinitrophenyl derivative. However, the IAA-LYS DNP derivative was difficult to hydrolyze in acid or alkali at a variety of concentrations and temperatures. Several attempts to synthesize IAA-LYS by the methods of peptide synthesis were not satisfactory because either low yields or several products were obtained. Wolf *et al.* (1952) synthesized ϵ-*N*-biotinyl-L-lysine (biocytin) by the action of biotin acid chloride on the copper complex of L-lysine using a two-phase water–chloroform system at low temperatures. This procedure, when used for the preparation of IAA-LYS, resulted in low yields presumably due to the instability of indole-3-acetic acid chloride. The use of ethyl half-ester anhydride of carbonic and indoleacetic acids (Boissonnas, 1951; Wieland and Hörlein, 1955) proved to be successful in our experiments. Indole-3-acetic carbonic acid half-ester anhydride was allowed to react with the copper complex of L-lysine in an aqueous–dioxane phase. The resulting insoluble copper complex was decomposed by H_2S to give IAA-LYS in high yield (Figure 2). This procedure apparently is not adapted for the synthesis of indole-3-acetylaspartic acid (Good, 1956; Klämbt, 1961).

The lysine conjugate is approximately 40% as effective as indole-3-acetic acid in the *Avena* first internode straight growth test (Hutzinger and Kosuge, 1967). Although the conversion could be considered a mechanism for reducing the biological activity of indole-3-acetic acid, the function of the conjugate in the bacterium is unknown. Such derivatives of IAA are interesting from the point of view of plant physiology, since various conjugates of IAA have been isolated as products of the metabolism of IAA in higher plants (Shantz, 1966).

Acknowledgment

The authors are indebted to Dr. Jack Preiss of the Department of Biochemistry and Biophysics for the optical rotation determinations.

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