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Biosynthesis of the Polyoxins, Nucleoside Peptide Antibiotics: Biosynthetic Pathway for 5-*O*-Carbamoyl-2-amino-2-deoxy-L-xylonic Acid (Carbamoylpolyoxamic Acid)†

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ABSTRACT: The biosynthetic pathway for 5-*O*-carbamoyl-2-amino-2-deoxy-L-xylonic acid (carbamoylpolyoxamic acid) was studied. This unusual amino acid is an N terminus of the nucleoside peptide antibiotics, the polyoxins, produced by *Streptomyces cacaoi* var. *asoensis*. The pathway, glutamate → glutamate-γ-semialdehyde → α-amino-δ-hydroxyvalerate → α-amino-δ-carbamoyloxyvalerate → 5-*O*-carbamoyl-2-amino-2-deoxy-L-xylonate → polyoxins, was established by the following experimental evidence: (1) Two intermediate amino acids, DL-[5-^{14}C;2-^{3}H]-α-amino-δ-hydroxyvaleric acid and DL-[*carbamoyl*-^{14}C]-α-amino-δ-carbamoyloxyvaleric acid, were synthesized and fed to growing cells of *S. cacaoi*. Both amino acids were incorporated into carbamoylpolyoxamic acid with high efficiency without randomization. However, tritium was lost almost completely. (2) DL-[5-^{14}C;2-^{3}H; *amino*-^{15}N]-α-Amino-δ-hydroxyvaleric acid was synthesized and fed to washed cells. Dilution factors for ^{14}C, ^{3}H, and ^{15}N were 10, 52, and 22, respectively. This result can be reasonably explained by functioning of transaminase(s), and the incorporation of the intact α-amino acid structure of α-amino-δ-hydroxyvaleric acid into that of carbamoylpolyoxamic acid is indicated. (3) In vivo experiments showed that [^{14}C]carbonate was incorporated into C-1 and the carbamoyl carbon of carbamoylpolyoxamic acid. Further, a cell-free extract of *S. cacaoi* was able to synthesize [*carbamoyl*-^{14}C]-α-amino-δ-carbamoyloxyvaleric acid from α-amino-δ-hydroxyvaleric acid and [^{14}C]carbamoyl phosphate. Partially purified ornithine carbamoyltransferase of this organism was shown not to be able to use α-amino-δ-hydroxyvalerate as a substrate. (4) Biosynthetically prepared [1, *carbamoyl*-^{14}C]carbamoylpolyoxamic acid was incorporated intact into the polyoxins with the incorporation ratio of 48%, whereas [^{14}C]polyoxamic acid was not appreciably incorporated.

The nucleoside peptide antibiotics, the polyoxins, are the metabolites of *Streptomyces cacaoi* var. *asoensis* (Isono et al., 1969). The general structure is shown in Scheme I. In our biosynthetic study of this group of antibiotics, we have shown that the 5-substituted uracil base of the polyoxins is biosynthesized from uracil and C-3 of serine (Isono and Suhadolnik, 1976; Funayama and Isono, 1976). 5-Fluorouracil can replace uracil to form the aberrant 5-fluoropolyoxins (Isono et al., 1973; Isono and Suhadolnik, 1976). It was also shown that the intact carbon skeleton of L-isoleucine is incorporated into 3-ethylidene-L-azetidine-2-carboxylic acid, a C-terminal amino acid of the polyoxins (Isono et al., 1975).

5-*O*-Carbamoyl-2-amino-2-deoxy-L-xylonic acid (carbamoylpolyoxamic acid, CPOAA[1]) is an N-terminal amino acid common to all the biologically active polyoxins. In our

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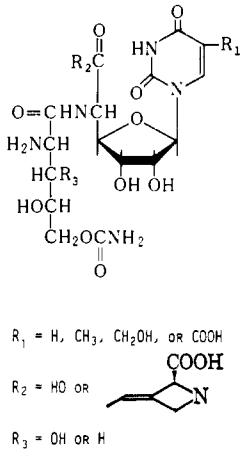
[1] Abbreviations used are: POAA, 2-amino-2-deoxy-L-xylonic acid (polyoxamic acid); CPOAA, 5-*O*-carbamoyl-2-amino-2-deoxy-L-xylonic acid (carbamoylpolyoxamic acid); AHV, α-amino-δ-hydroxyvaleric acid; ACV, α-amino-δ-carbamoyloxyvaleric acid; DEAE, diethylaminoethyl.

SCHEME I: The Structure of the Polyoxins.



previous paper (Funayama and Isono, 1975), we reported metabolic roles of glutamate and the Krebs cycle for the biosynthesis of 2-amino-2-deoxy-L-xylonic acid (polyoxamic acid, POAA). It was shown that, in spite of extensive randomization by an equilibrium between glutamate and α-ketoglutarate followed by recycling through the Krebs cycle, the carbon skeleton of glutamate serves as an origin for this amino acid. Finding the enzyme, ω-hydroxy-α-amino acid dehydrogenase from *Neurospora crassa* (Yura and Vogel, 1957, 1959) and the occurrence of α-amino-δ-hydroxyvaleric acid in a nonprotein fraction of some plants (Thompson et al., 1964; Dunnill and Fowden, 1967) prompted us to prove intermediacy of this amino acid for the biosynthesis of CPOAA. This paper concerns the synthesis and the in vivo incorporation of intermediate amino acids. The results establish the biosynthetic sequence for CPOAA. An in vitro study utilizing radioactive carbamoyl phosphate as a carbamoyl donor is also described.

Materials and Methods

DL-[5-^{14}C]Glutamic acid, DL-[5-^{14}C]ornithine monohydrate, [^{14}C]phosgene, and [^{14}C]carbamoyl phosphate (dilithium salt) were purchased from New England Nuclear Corp.; DL-[2-^{3}H]glutamic acid, DL-[1-^{14}C]ornithine monohydrochloride, DL-[5-^{3}H]ornithine dihydrochloride, and [^{14}C]barium carbonate were purchased from the Radiochemical Centre, Amersham; DL-[1-^{14}C]proline was from CEA, France; DL-[*amino*-^{15}N]glutamic acid monohydrate (95.5 atom %) was a product of Prochem (British Oxygen Co. Ltd.). Radioactivity measurement was made in a Packard TriCarb Model 3330 liquid scintillation spectrometer using Bray's solution (Bray, 1960) with the addition of 2% 0.6 N toluene solution of hyamine (NCS tissue solubilizer, Amersham/Searle). Emission spectrographic analysis of ^{15}N was run on a JASCO NIA-I N-15 analyzer with a N_2 sample after being processed by the Dumas' method. Preparative paper chromatography was done by the descending method using Whatman 3 MM paper. Solvent systems used were: (A) 1-butanol–acetic acid–water (4:1:2), (B) 75% phenol. Microcrystalline Avicel was employed for cellulose chromatography and Whatman DE52 was used for DEAE-cellulose chromatography. Fuji x-ray film Ix-150 was employed for autoradiography.

The maintenance and fermentation of *S. cacaoi* and the procedure for the feeding experiments with growing cells were the same as described before (Isono et al., 1975). Five-hundred-milliliter flasks containing 60 mL of the culture medium were used for fermentation. The polyoxin complex was purified from the culture filtrates as described before (Isono et al., 1967; Funayama and Isono, 1976). Acid and alkaline hydrolyses of the polyoxin complex and isolation of polyoxamic acid were described (Funayama and Isono, 1975). Procedures for ninhydrin and periodate oxidation were also described in the same paper.

Synthesis of DL-[5-^{14}*C;2-*^{3}*H]AHV.* The procedure is essentially the same as the synthesis of L-α-amino-δ-hydroxyvaleric acid described by Goodman and Felix (1964), except utilizing DL-[5-^{14}C;2-^{3}H]glutamic acid as a starting material. From DL-[5-^{14}C;2-^{3}H]glutamic acid (1.47 g, 10 mmol, 1 mCi of ^{14}C and 5 mCi of ^{3}H), 2.2 g of a *N-p*-toluenesulfonyl derivative was obtained, mp 169–172 °C. Cyclization with phosphorus trichloride followed by azeotropic dehydration afforded anhydrous 1-*p*-toluenesulfonyl-DL-pyrrolid-5-one-2-carboxylic acid (1.89 g), which was then converted to the salt with an equal molar amount of lithium in methanol. The anhydrous lithium salt was reduced with lithium borohydride in tetrahydrofuran to give δ-hydroxy-α-tosylaminovaleric acid (400 mg), mp 156–159 °C. Detosylation by sodium in liquid ammonia followed by work-up with Dowex 50W (H^+) yielded crystalline α-amino-δ-hydroxyvaleric acid (130 mg): mp 233–234 °C (dec); specific activity ^{14}C, 0.11 Ci/mol; ^{3}H, 0.37 Ci/mol.

Synthesis of DL-[^{15}*N]AHV.* The synthetic procedure is the same as that of DL-[5-^{14}C;2-^{3}H]AHV. Starting from 2 g of DL-[^{15}N]glutamic acid monohydrate (95.5 atom %), 300 mg of crystals of DL-[^{15}N]-α-amino-δ-hydroxyvaleric acid was obtained, mp 225 °C. Anal. Calcd for $C_5H_{11}{}^{15}NO_3$: C, 44.67; H, 8.25; N, 11.19. Found: C, 44.76; H, 8.20; N, 10.49. Mass spectrum (pentatrimethylsilyl derivative) M^+ 526, $M^+ - CH_3$ 511.

Synthesis of DL-[*carbamoyl-*^{14}*C]ACV.* The synthesis was achieved in three steps from δ-hydroxy-α-tosylaminovaleric acid, namely, benzyl esterification, carbamoylation, and Birch reduction. δ-Hydroxy-DL-α-tosylaminovaleric acid (1.3 g) was suspended in 100 mL of benzene. To this solution, 2 mL of benzyl alcohol and 100 mg of *p*-toluenesulfonic acid were added, and the resulting solution was refluxed azeotropically for 4 h. The reaction solution was washed with 5% sodium bicarbonate, followed by water. The solution was dehydrated with anhydrous sodium sulfate and concentrated in vacuo to dryness. The resulting syrup was a mixture of benzyl alcohol, δ-lactone, benzyl ester, and dimeric ester. It was subjected to silica gel column chromatography with the solvent benzene–acetone (10:1), and fractions were collected (each 12 mL). Fractions 43–73 were combined and concentrated in vacuo to dryness, giving an oil of benzyl DL-α-amino-δ-hydroxyvalerate (725 mg), which was dried at 100 °C over P_2O_5: ^{1}H NMR ($CDCl_3$) 1.4–2.1 (m, 4 H, 3,4-CH_2), 2.38 (s, 3 H, CH_3), 3.59 (t, 2 H, 5-CH_2), 3.99 (m, 1 H, 2-CH), 4.91 (s, 2 H, benzyl-CH_2), 5.58 (d, 1 H, SO_2NH), 7.1–7.9 (m, 9 H, aromatic H).

The ester (600 mg) was dissolved in 5 mL of dry toluene, to which was added 0.12 mL of dry pyridine. The solution was cooled in an ice bath. [^{14}C]Phosgene in 5 mL of toluene (565 mg, 75 μCi, specific activity, 13 mCi/mol) was added at once under vigorous stirring. Stirring was continued for 30 min in an ice bath, and then for 2 h at room temperature. The reaction mixture was concentrated in vacuo to dryness to give a syrup, which was ice cooled. A cold ammonium hydroxide solution (28%, 10 mL) was added with vigorous agitation. After stirring for 1 h in an ice bath, crystalline benzyl DL-α-tosylamino-δ-carbamoyloxyvalerate was collected by filtration and washed with cold water; yield, 690 mg. It was recrystallized from

ethanol, mp 115–125 °C: ^{1}H NMR ($CDCl_3$) 1.54–2.00 (m, 4 H, 3,4-CH_2), 2.39 (s, 3 H, CH_3), 4.02 (m, 3 H, 2-CH and 5-CH_2), 4.74 (broad s, 2 H, $CONH_2$), 4.90 (s, 2 H, benzyl-CH_2), 5.46 (d, 1 H, 2-CH), 7.1–7.8 (m, 9 H, aromatic H).

Benzyl DL-δ-carbamoyloxy-α-tosylaminovalerate (325 mg) was reduced in 50 mL of liquid ammonia by the addition of sodium until the blue color lasted for a few minutes. This color was discharged by adding ammonium acetate. The ammonia was evaporated off and the residue was dissolved in water and passed thrugh a column of Dowex 50W X-8 (H^+, 15 mL). After the column was washed with water, amino acids were eluted with 1 N NH_4OH. Ninhydrin-positive fractions were collected and concentrated to a small volume. It was then applied to a carbon column (8 g). The column was eluted with water (200 mL) and then with 20% aqueous acetone. From the second ninhydrin-positive fraction eluted with 20% aqueous acetone, 33 mg of DL-ACV was obtained from aqueous ethanol: mp 248–250 °C (dec); specific activity, 13 mCi/mol. Anal. Calcd for $C_6H_{12}N_2O_4$: C, 40.90; H, 6.87; N, 15.90. Found: C, 40.43; H, 6.70; N, 15.42. From the first fraction eluted with water, 40 mg of a nonradioactive DL-AHV was recovered, mp 225–230 °C.

Acid Hydrolysis of the Polyoxin Complex and Isolation of CPOAA. The polyoxin complex (1 g) was heated in 16 mL of 3 N hydrochloric acid on a steam bath for 2 h. The hydrolysate was passed through a column of Amberlite IR-45 (OH^-, 30 mL), then through Dowex 50W X8 (H^+, 60 mL), from which amino acids were eluted with 0.6 N NH_4OH. The eluate was concentrated in vacuo to dryness. The residue was purified by cellulose chromatography with the solvent 1-butanol–acetic acid–water (4:1:1)–(4:1:1.5). Fractions containing CPOAA were collected and concentrated in vacuo to dryness. The residue was dissolved in 5 mL of water and applied to a carbon column (20 g). After washing with water (100 mL), the column was eluted with a linear gradient, water (200 mL)–20% aqueous acetone (200 mL). Fractions containing CPOAA were combined and concentrated in vacuo to dryness. The residue was further purified by paper chromatography with the solvent B, followed by recrystallization from aqueous ethanol, yielding 10 mg of crystals of CPOAA, mp 226–232 °C.

Degradation of CPOAA. CPOAA (8 μmol) was dissolved in water. To this was added 1.5 mL of 5% ninhydrin solution in 0.5 M citrate buffer (pH 2.5). The resulting solution was heated on a steam bath for 45 min. Nitrogen was bubbled through the reaction mixture and the CO_2 evolved was trapped in 1 mL of hyamine solution (0 °C). To the oxidized solution was added 3 mL of 2 N sodium hydroxide. The solution was heated for 2 h on a steam bath. After cooling, the hydrolysate was acidified by adding 4 mL of 4 N sulfuric acid. Nitrogen was bubbled through the reaction solution and CO_2 was trapped in 1 mL of hyamine solution. When synthetic [*carbamoyl*-^{14}C]ACV was reacted in the same way, 86% of the radioactivity was recovered in CO_2 evolved by alkaline hydrolysis, whereas only 0.1% of the radioactivity was found in CO_2 evolved by ninhydrin oxidation. To isolate crystalline POAA, CPOAA (20 mg including carrier CPOAA) was hydrolyzed in 2 mL of 0.5 N NaOH on a steam bath for 2 h. The hydrolysate was passed through a column of Dowex 50W X-8 (4 mL). An eluate (0.6 N NH_4OH) was concentrated to a small volume and put on a carbon column (10 mL), followed by elution with water. Ninhydrin-positive fractions were combined and concentrated to dryness. The residue was crystallized from a small volume of aqueous ethanol to give crystals of POAA.

Biosynthesis of the Polyoxin Complex from DL-[5-^{14}*C;2-*^{3}*H;amino-*^{15}*N]AHV in a Washed Cell System. Streptomyces cacaoi* var. *asoensis* was cultured as described before (Isono et al., 1975) except using a medium in which the concentration of soluble starch was reduced to 3%. After 48 h of fermentation (twenty-five 500-mL flasks), cells were collected by centrifugation, and washed with a cold 0.5% sodium chloride solution. The cells were suspended in 1.5 L of a sterile solution of sodium chloride (0.5%) and glucose (1%), to which a sterile solution of DL-[5-^{14}C;2-^{3}H;*amino*-^{15}N]AHV (admixture of 100 mg of DL-[*amino*-^{15}N]AHV and 6.0 mg of DL-[1-^{14}C,2-^{3}H]AHV) was added. The cell suspension was divided into 25 flasks and incubated at 27 °C for 24 h on a rotatory shaker. The incubation mixture (final pH 7.3) was acidified to pH 2.0 with 2 N hydrochloric acid and centrifuged. The isolation procedure of the polyoxin complex (Isono et al., 1967; Funayama and Isono, 1976) and the procedure for acid hydrolysis to isolate POAA (Funayama and Isono, 1975) were already described. POAA was finally recrystallized from aqueous ethanol to the constant specific activity; yield 15 mg.

Biosynthesis of [*1, carbamoyl*-^{14}*C]CPOAA.* Sterile powder of [^{14}C]barium carbonate was added to the culture medium 45 h after inoculation (25 flasks). Seventy-two hours later, the polyoxin complex was isolated from the culture filtrate. It was then hydrolyzed with 3 N hydrochloric acid and CPOAA was isolated (Funayama and Isono, 1975); yield 30 mg. It was hydrolyzed with 0.5 N sodium hydroxide to collect the CO_2 evolved. From the hydrolysate, crystalline POAA was isolated (Isono et al., 1969). POAA was oxidized with ninhydrin and the CO_2 evolved was collected in hyamine (Funayama and Isono, 1975).

Incorporation of [^{14}*C]CPOAA and* [^{14}*C]POAA into the Polyoxins.* A sterile solution of either [1, *carbamoyl*-^{14}C]-CPOAA or [5-^{14}C]POAA was added to the culture medium 45 h after inoculation (each five flasks). Seventy-two hours later, the polyoxin complex was purified from the culture filtrate; 155 mg. To prove the absence of contamination with CPOAA or POAA, cochromatography was performed as follows. The polyoxin complex (60 mg) was dissolved in 2 mL of water together with carrier CPOAA (10 mg). The solution was applied to a carbon column (5 g). Elution was made by a linear gradient: water (600 mL)–20% acetone (600 mL) and then 20% aqueous acetone (600 mL)–50% aqueous acetone (600 mL). Each 8-mL fraction was collected. CPOAA appeared in the fraction 5–23 in the first gradient, whereas the polyoxins appeared in the fraction 41–60 in the second gradient.

Preparation of Crude Extract and Fractionation with Ammonium Sulfate. A 4-day fermented broth of *S. cacaoi* was centrifuged (7000*g*, 20 min) and the cells were washed two times with 0.5% sodium chloride and then with 0.1 M phosphate buffer (pH 6.5). The washed cells were suspended in 250 mL of 0.05 M phosphate buffer and sonicated. The homogenate was centrifuged (27 000*g*, 30 min) and the supernatant was regarded as a crude extract (enzyme solution I). To 200 mL of the supernatant, 62.5 g of ammonium sulfate was added to give 50% of saturation. After 1 h at 4 °C, the precipitates were collected by centrifugation (12 000*g*, 20 min). The precipitates, after washed with 50% saturated ammonium sulfate solution, were dissolved in 50 mL of 0.05 M phosphate buffer and dialyzed against 2 L of the same buffer at 4 °C, affording 70 mL of the enzyme solution II. To the supernatant of 50% saturation solution (178 mL) was added 53.8 g of ammonium sulfate. After 1 h at 4 °C, the precipitates were collected by centrifugation, dissolved in 40 mL of 0.05 M phosphate buffer,

TABLE I: Incorporation and Distribution of Labeled Amino Acids into POAA.

Compounds added					POAA isolated							
	Total μCi added (^{14}C)	Sp act (μCi/μmol) ^{14}C	Sp act (μCi/μmol) ^{3}H	$^{3}H/^{14}C$	Sp act. (nCi/μmol) ^{14}C	Sp act. (nCi/μmol) ^{3}H	$^{3}H/^{14}C$	% Retention of ^{3}H	Incorp[a] of ^{14}C (%)	Dilution[b] of ^{14}C ($\times 10^{-3}$)	% ^{14}C distribution[c] C-1	% ^{14}C distribution[c] C-5
DL-[1-^{14}C;5-^{3}H]Ornithine	31	1.24	4.23	3.41	0.012	0.011	0.92	27	0.4	103		
DL-[5-^{14}C;5-^{3}H]Ornithine	38	1.50	4.03	2.69	0.012	0.006	0.50	19	0.5	125	18	67
DL-[1-^{14}C,2-^{3}H]Glutamate	89	0.440	1.83	4.16	0.084	0	0	0	2.0	10.6	96	1
DL-[5-^{14}C,2-^{3}H]Glutamate	89	0.435	1.92	4.41	0.12	0	0	0	2.4	7.42	27	73
DL-[1-^{14}C]Proline	35	1.40			0.01				0.5	140	89	2
DL-[5-^{14}C;2-^{3}H]AHV	26	0.11	0.37	3.36	0.47	0	0	0	28	0.23	0	100

[a] The incorporation value was expressed on the basis of total radioactivity of POAA present in the polyoxin complex. Total micromoles of the polyoxin complex was calculated from A_{262} (ε 8000). Calculation is as follows. Total μCi = sp act. of POAA (nCi/μmol) × (total A_{262} of the polyoxin complex/8000). The incorporation value was expressed on the basis of L isomer added. [b] Specific activity of the compound added/specific activity of POAA isolated. [c] Periodate oxidation was performed as described before (Funayama and Isono, 1975). C-1 was obtained from the total disintegration per minute of CO_2 evolved. C-5 was obtained from the specific activity of formaldimedone.

and dialyzed overnight against 2 L of 0.05 M phosphate buffer at 4 °C. The resulting solution was regarded as enzyme solution III.

In Vitro Synthesis of ACV from AHV and [^{14}C]Carbamoyl Phosphate. The reagents were mixed in the following order: (1) 0.1 mL of 0.2 M phosphate buffer (pH 6.5), (2) 0.2 mL of 0.01 M AHV, (3) 0.1 mL of 0.2 M magnesium sulfate, (4) 0.2 mL of 0.04 M ATP (disodium), (5) 0.1 mL of 0.04 M dithiothreitol, (6) 0.2 mL of 0.01 M carbamoyl phosphate (dilithium), (7) 0.5 μCi of [^{14}C]carbamoyl phosphate, (8) 0.8 mL of the enzyme solution. The mixture was incubated at 37 °C for 5 h. The reaction solution was centrifuged (3000 rpm, 15 min) and the supernatant was passed through a column of Dowex 50W X8 (H^+) (10 mL). After washing with water, the column was eluted with 100 mL of ammonium hydroxide. The eluate was concentrated in vacuo to a small volume and passed through a carbon column (20 mL). The column was washed with water (50 mL) and then eluted with a linear gradient: water (100 mL)–10% aqueous acetone (100 mL). Nonradioactive AHV appeared in fractions eluted with water, whereas radioactive ACV appeared in fractions eluted with aqueous acetone. The latter was further purified by paper chromatography in solvent B and then in solvent A. To the eluate from the paper, 5 mg of carrier ACV was added. Recrystallization was repeated to the constant specific activity. Before the addition of carrier ACV, a portion was subjected to two-dimensional thin-layer chromatography, which was developed by autoradiography.

Determination of Ornithine Carbamoyltransferase Activity. The procedure is practically the same as the method described by Davies et al. (1969) for the assay of aspartate transcarbamoylase. L-Ornithine, instead of L-aspartate, and [^{14}C]-carbamoyl phosphate were used as substrates. Reaction was conducted in Tris-HCl buffer (0.02 M, pH 7.2).

Results

Incorporation and Distribution of DL-[1-^{14}C;5-^{3}H]- and DL-[5-^{14}C;5-^{3}H]Ornithine into POAA. In our last paper, we showed that the carbon skeleton of glutamate is a precursor of POAA. However, in the case of the [5-^{14}C]glutamate experiment, there was observed a considerable randomization of ^{14}C from C-5 to C-1 due to an equilibrium between glutamate and α-ketoglutarate followed by recycling through the Krebs cycle. To confirm this finding and to elucidate the pathway from glutamate to POAA, ornithine was first examined. [1-^{14}C;5-^{3}H]- and [5-^{14}C;5-^{3}H]ornithine was utilized to examine the randomization of the carbon skeleton and the fate of two protons on C-5. As shown in Table I, ^{14}C of both ornithines was incorporated into POAA, but the dilution factors were about ten times greater than that of glutamate. In the DL-[5-^{14}C;5-^{3}H]ornithine experiments, the distribution of ^{14}C was 18% on C-1 and 67% on C-5, which is similar to the [5-^{14}C]glutamate experiment (Funayama and Isono, 1975). This randomization of ^{14}C could be explained by the following two pathways: (A) ornithine → glutamate-γ-semialdehyde → glutamate → α-ketoglutarate → Krebs cycle, and (B) ornithine → putrescine → α-aminobutyrate → succinate semialdehyde → succinate → Krebs cycle. The pathway A would yield [1,5-^{14}C]glutamate from [5-^{14}C;5-^{3}H]ornithine. Operation of the pathway B would result in the formation of [1-^{14}C]glutamate. Thus, if ornithine was incorporated into POAA exclusively via glutamate, two protons on C-5 must be lost. Experimentally, percent retention of ^{3}H was 27 and 19% for the [1-^{14}C;5-^{3}H]- and [5-^{14}C;5-^{3}H]ornithine experiments, respectively (Table I). Clearly, this result indicates that at least one of the two protons on C-5 of ornithine was utilized for the biosynthesis of POAA, which is consistent with the intermediacy of glutamate-γ-semialdehyde. The actual ^{3}H retention was lower than the theoretical value, which can be explained by the partial randomization through either the pathway A or B.

Incorporation of DL-[1-^{14}C]Proline into POAA. Proline is also directly related to glutamate semialdehyde catalyzed by proline oxidase. DL-[1-^{14}C]Proline was shown to be incorporated into POAA with a large dilution factor comparable to that of ornithine (Table I).

Incorporation and Distribution of DL-[5-^{14}C;2-^{3}H]AHV into POAA. Since the above experiments strongly support the intermediacy of glutamate-γ-semialdehyde, the next logical intermediate may be α-amino-δ-hydroxyvalerate. The presence of this amino acid in some plants was reported (Thompson et al., 1964; Dunnil and Fowden, 1967). Further, ω-hydroxy-α-amino acid dehydrogenase was extracted from *Neurospora crassa* (Yura and Vogel, 1957, 1959). Therefore, DL-[5-^{14}C;2-^{3}H]AHV was synthesized from the corresponding DL-glutamic acid, and fed to growing cells of *S. cacaoi*. The

TABLE II: Incorporation of DL-[5-^{14}C;2-^{3}H;*amino*-^{15}N]AHV into POAA.

AHV added						POAA Isolated							
^{14}C		^{3}H				^{14}C		^{3}H					
Total μCi	Sp act. (nCi/μmol)	Total μCi	Sp act. (nCi/μmol)	^{3}H/^{14}C	^{15}N atom %	Sp act. nCi/μmol)	Dilution	Sp act. (nCi/) μmol)	Dilution	^{3}H/^{14}C	% Retention of ^{3}H	^{15}N Atom %	^{15}N Dilution
4.63	5.81	16.7	20.9	3.6	90.1	0.583	10	0.405	52	0.69	19.3	4.03 ± 0.05	22.5

TABLE III: Incorporation of [^{14}C]CPOAA and [^{14}C]POAA into the Polyoxin Complex.

Compounds Added			Polyoxin Complex Isolated			
Type of Compd	Total dpm[a] (× 10^{-3})	Sp act. (μCi/mmol)	Total dpm[a] (× 10^{-3})	Sp act.[b] (nCi/mmol)	Incorp (%)	Dilution
[1,*carbamoyl*-^{14}C]CPOAA	142	0.778	67.9	16	48.0	48.6
[5-^{14}C]POAA	121	0.471	3.6	0.4	3.0	1180

[a] Disintegrations per minute. [b] Total micromoles of the polyoxin complex was calculated from A_{262} (ε 8000).

incorporation ratio was very high (28% on the basis of L isomer) and the dilution factor was 1/540 of ornithine and 1/32 of glutamate. As shown in Table I, 100% of ^{14}C resided on C-5, indicating that no randomization occurred. This is in sharp contrast with those of the glutamate and ornithine experiments, in which 18–20% of ^{14}C was distributed on C-1.

Incorporation of DL-[5-^{14}C;2-^{3}H,*amino*-^{15}N]*AHV in a Washed Cell System.* In spite of the efficient incorporation of ^{14}C, almost all of ^{3}H was lost during the biosynthesis of CPOAA from AHV. To elucidate if the L-α-amino acid structure of AHV is incorporated intact into POAA, DL-[amino-^{15}N]AHV was synthesized from the corresponding glutamate. To make dilution minimum, the experiment was performed in a washed cell system, to which triple-labeled [5-^{14}C;2-^{3}H,*amino*-^{15}N]AHV was fed. Analysis of ^{15}N of POAA isolated was performed by emission spectrography. Nitrogen gas evolved by the Dumas method was analyzed (^{14}N^{15}N vs. ^{14}N^{14}N), giving a ^{15}N atom % of 4.03 ± 0.05 (Table II).[2] This is a direct proof that the amino acid structure was incorporated intact into that of POAA. In this experiment, 2-^{3}H was incorporated into POAA in contrast to the experiments using growing cells. The dilution factors for ^{14}C, ^{3}H, and ^{15}N were 10, 52, and 22.5, respectively (Table II). A reasonable explanation for these different values is the reversible action of transaminase(s). Because deprotonation of the α-hydrogen precedes the hydrolysis of a Schiff base, the exchange of the α hydrogen would predominate to that of the α nitrogen. In the case of the experiments using growing cells, transamination is considered to be too extensive to lose all of ^{3}H from C-2.

Incorporation of [^{14}C]*Carbonate into CPOAA.* Carbamoyl phosphate was presumed to be a carbamoyl donor in the CPOAA biosynthesis. An in vivo experiment was performed employing [^{14}C]barium carbonate. A good incorporation into CPOAA was observed. ^{14}C was distributed 87% on the carbamoyl carbon (calculated from the difference between the specific activities of CPOAA and POAA) and 11% on C-1 (obtained by ninhydrin oxidation). The latter distribution can be explained by operation of pyruvate carboxylase.

[2] Professor J. A. McCloskey of the University of Utah performed mass-spectrometric analysis of this sample and obtained a value of 4.1 ± 0.7% (unpublished data).

Incorporation of [^{14}C]*POAA and* [^{14}C]*CPOAA into the Polyoxin Complex.* Biosynthetically prepared [1, *carbamoyl*-^{14}C]CPOAA (from [^{14}C]barium carbonate) and [5-^{14}C]POAA (from [5-^{14}C]AHV) were utilized for the incorporation experiments using growing cells of *S. cacaoi*. The polyoxin complex was purified from the culture filtrate. As shown in Table III, [1, *carbamoyl*-^{14}C]CPOAA was very efficiently incorporated (incorporation ratio of 48%), whereas only a negligible amount of ^{14}C was incorporated from [5-^{14}C]POAA. A proof for the direct incorporation of CPOAA into the polyoxins was obtained from the alkaline hydrolysis of the polyoxins. Hydrolysis of the polyoxins in 0.5 N sodium hydroxide liberates an equimolar amount of CO_2 from the carbamoyl carbon (Isono et al., 1969). Seventy-eight percent of ^{14}C was found to reside in CO_2 evolved by alkaline hydrolysis. This value is close to the percentage of ^{14}C on the carbamoyl carbon of CPOAA added (87%). The data indicate that CPOAA is a direct precursor for the polyoxins and POAA is not a precursor.

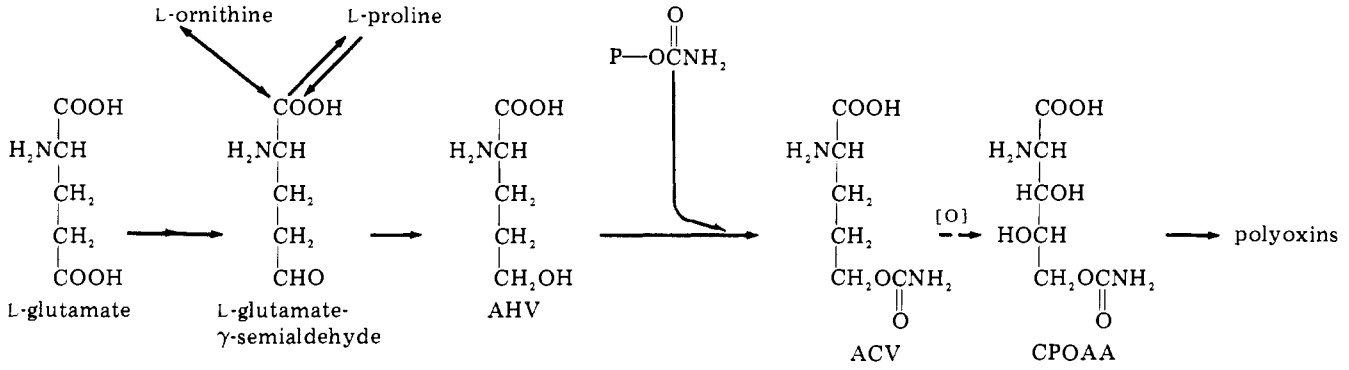
Incorporation of DL-[*carbamoyl*-^{14}C]*ACV into CPOAA.* The above experiments indicated clearly that the carbamoylation of the C-5 hydroxyl of AHV precedes the oxidation at C-3 and C-4. Therefore, [*carbamoyl*-^{14}C]ACV was synthesized (specific activity, 0.013 Ci/mol). It was incorporated very efficiently into CPOAA (specific activity, 0.035 mCi/mol). Incorporation ratio (40%) was higher than that of AHV. The degradation showed that there was no randomization. CO_2 evolved by ninhydrin oxidation (C-1) showed no radioactivity. On the other hand, 80% of the total radioactivity was found in CO_2 evolved by alkaline hydrolysis. Synthetic [*carbamoyl*-^{14}C]ACV gave the 86% recovery of ^{14}C as CO_2 by the same alkaline hydrolysis procedure.

Transcarbamoylation with Cell-Free Extract. Because the in vivo experiments have shown that both AHV and ACV are very efficient precursors for the CPOAA moiety of the polyoxins, we attempted to find a transcarbamoylase activity in the cell-free extract. AHV and [^{14}C]carbamoyl phosphate were used as substrates. Three preparations were used: (1) crude cell-free extract (obtained by ultrasonic disintegration), (2) ammonium sulfate precipitates (0–50% saturation), and (3) ammonium sulfate precipitates (50–90% saturation). From the reaction mixture, an amino acid fraction was purified by

TABLE IV: Specific Activity of Enzymatically Prepared ACV.

Enzyme	Total nCi present in the eluate from paper chromatogram	Total μmol of carrier ACV added	Sp act. (nCi/μmol) Theor	Sp act. (nCi/μmol) Expt
Crude extract	24.9	32.5	0.77	0.60
50% $(NH_4)_2SO_4$ fraction	23.3	32.0	0.73	0.72

SCHEME II: Biosynthetic Pathway for CPOAA.



Dowex 50W followed by carbon chromatography. This sample was subjected to two-dimensional thin-layer chromatography with the solvent A/B. The chromatogram was developed by autoradiography and ninhydrin. A reaction product with the crude extract showed two radioactive spots: a main spot corresponding to R_f values of ACV, and a weaker spot corresponding to citrulline. Since the crude extract is considered to contain ornithine carbamoyltransferase and pooled ornithine, this result was expected. On the other hand, reaction with a 0–50% saturation fraction gave only one radioactive spot which corresponds to ACV. Apparently, ornithine is considered to be removed by the salt precipitation and the following dialysis. The same reaction with a 50–90% saturation fraction yielded neither radioactive ACV nor citrulline. Each amino acid from the reactions using a crude extract and a 0–50% saturation fraction was purified further by paper chromatography in the solvents A and B. Addition of carrier ACV followed by recrystallization afforded radioactive crystals of ACV with a constant specific activity. The experimental values were close to the approximate calculation (total μCi present/μmol of carrier ACV added), as shown in Table IV.

Discussion

The present study has established the biosynthetic pathway for the CPOAA moiety of the polyoxins, as illustrated in Scheme II. The presence of free AHV was demonstrated in soybean seeds (Thompson et al., 1964) and in a number of *Astragalus* species (Dunnill and Fowden, 1967). Also, ω-hydroxy-α-amino acid dehydrogenase, which catalyzes the reversible conversion of α-amino-δ-hydroxyvaleric acid and α-amino-ε-hydroxycaproic acid to the respective ω-semialdehyde was partially purified (Yura and Vogel, 1957, 1959). However, the metabolic function of this amino acid has never been reported. The precursor for the biosynthesis of the polyoxins described in this paper is the first demonstration regarding the metabolic role of this amino acid.

It is worth noting that in the experiments performed with growing cells of *S. cacaoi*, ^3H on C-2 of glutamate was lost completely. It was even the case with AHV which was a much more efficient precursor than glutamate. However, the present study utilizing triple-labeled AHV in a washed-cell system has shown some retention of ^3H and ^{15}N, indicating the incorporation of the intact L-α-amino acid structure into that of CPOAA. Clearly, this is to exclude the possibility of α-keto acid as an intermediate. In addition, any other mechanism which involves abstraction of an α hydrogen must be abandoned. The only valid explanation is the operation of transminase(s), which could function in every step between AHV and CPOAA.

The in vitro study showed that there was AHV carbamoyltransferase activity in the ammonium sulfate precipitates (0–50%). A carbamoyl donor was shown to be carbamoyl phosphate. This is apparently an analogous reaction to ornithine carbamoyltransferase. To confirm that ornithine carbamoyltransferase of this organism is not responsible for the carbamoylation of AHV, ornithine carbamoyltransferase was purified by DEAE-cellulose chromatography (0.03 M Tris-HCl, 0–0.5 M KCl). A peak fraction of ornithine carbamoyltransferase activity was shown not to be able to synthesize [^{14}C]ACV from AHV and [^{14}C]carbamoyl phosphate. Because of instability of AHV carbamoyltransferase activity, further purification of this enzyme has been unsuccessful.

Oxidation at C-3 and C-4 of ACV to give CPOAA remains to be clarified. Polyoxins E and G have a 3-deoxy derivative of CPOAA (Isono et al., 1969), which may be to suggest that this is an intermediate amino acid between ACV and CPOAA. It should be noted that 2-amino-4,5-dihydroxyvaleric acid (Larsen, 1967), 4-hydroxyglutamic acid (Virtanen and Ettala, 1957; Hatanaka, 1962), and 3,4-dihydoxyglutamic acid (Brandner and Virtanen, 1963) were isolated from plants. More recently, a peptide antibiotic, longicatenamycin produced by *Streptomyces diastaticus* was shown to contain *threo*-β-hydroxy-L-glutamic acid (Shoji and Sakazaki, 1970). Configuration at C-2 and C-3 is same as that of POAA.

Efficient incorporation of preformed CPOAA into the N terminus of the polyoxins is significant. It was also observed that the biosynthetically prepared C-terminal amino acid, [1-^{14}C]polyoximic acid (Isono et al., 1975), was efficiently incorporated into the polyoxins (unpublished data). Since both the N-terminal and C-terminal amino acids of the polyoxins were incorporated after completion of their biosynthesis, the final step of the biosynthesis of the polyoxins may be the for-

mation of the peptide bonds. This is similar to the biosynthesis of typical peptide antibiotics (Kurahashi, 1974) and in contrast to that of penicillins and cephalosporins (Abraham, 1974), which are believed to be formed by modification of a precursor peptide.

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Conformation of DNA Modified with a Dihydrodiol Epoxide Derivative of Benzo[*a*]pyrene†

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ABSTRACT: The conformation of calf thymus DNA modified by reaction with (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene, which binds covalently mainly to the 2-amino group of guanosine residues, was studied. With samples in which 1.5 or 2.2% of the bases were modified, there was a slight decrease in T_m during heat denaturation and a slight increase in susceptibility to the single strand specific nuclease S_1. In a DNA sample in which 4.5% of the bases were modified, there was an appreciable decrease in T_m and a marked increase in susceptibility to S_1 nuclease. The kinetics of the reaction of the modified DNAs with formaldehyde provided evidence for locally destabilized regions ranging from 1 to 7 base plates, depending on the extent of modification. Alkaline and neutral sucrose gradient analyses revealed no evidence for strand breakage in the 1.5 and 2.2% modified samples, although single-strand breaks were found in the 4.5% modified sample. Taken together, these results suggest that DNA molecules containing a covalently bound benzo[*a*]pyrene derivative have an altered conformation characterized by small localized regions which are destabilized and easily denatured. The conformational changes associated with the covalent binding of the benzo[*a*]pyrene derivative to native DNA appear to be different from, and less marked, than those associated with the covalent binding of *N*-2-acetylaminofluorene to native DNA.

There is strong evidence that the covalent binding of chemical carcinogens to cellular macromolecules, and in particular to nucleic acids, appears to be a prerequisite for their action (Brookes and Lawley, 1964; Goshman and Heidelberger, 1967; Brookes and Heidelberger, 1969; Duncan et al., 1969; Miller, 1970; Gelboin et al., 1972). Most carcinogens, including polycyclic aromatic hydrocarbons (PAH[1]), require metabolic

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[1] Abbreviations used: PAH, polycyclic aromatic hydrocarbons; BP, benzo[*a*]pyrene; BPDE, (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; AAF, *N*-2-acetylaminofluorene; BP-DNA and BP-G, designate DNA or G modified by reaction with BPDE; T_m, the temperature at which the DNA has attained 50% of its maximal hyperchromicity during heat denaturation; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.