antibiotic three residues of tryptophan have replaced three phenylalanines. The influence of controlled levels of environmental amino acids on the biosynthesis of gramicidin A, B, C, and D is still to be tested.

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The Synthesis of *erythro*-γ-Hydroxy-L-lysine and Its Nonoccurrence in Collagen*

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ABSTRACT: *threo*-γ-Hydroxy-L-lysine (II), prepared *via* γ-chloro-L-lysine (I) by photochlorination of L-lysine, was converted to the dicarbobenzyloxy lactone IV, opened to the amide V, and oxidized to the γ-keto derivative VI. Catalytic hydrogenation and debenzylation yielded, after hydrolysis of the amide, 72% of the *erythro* acid–lactone mixture III ⇄ VIII and 28% of the *threo* pair II ⇄ VII, which were separated by ion-exchange chromatography.

Catalytic hydrogenation of ε-diazo-δ-oxo-L-norleucine (DON) gave a mixture of 25% *erythro*- and 75% *threo*-δ-hydroxy-L-lysine. By reaction with *S*-methylisothiourea, *erythro*-γ-hydroxy-L-homoarginine lactone (IX), the diastereoisomer of the natural *threo* amino acid from *Lathyrus*, was prepared. Unlike *trans*-3-hydroxy-L-proline, the position isomer of natural 4-hydroxy-L-proline, neither *erythro*-γ-hydroxy-L-lysine, the position isomer of natural *erythro*-δ-hydroxy-L-lysine, nor its *threo* isomer are regular building stones of collagen.

The photochlorination of L-lysine in strong sulfuric acid (Kollonitsch *et al.*, 1964) makes *threo*-γ-hydroxy-L-lysine (II) easily accessible *via* γ-chloro-L-lysine (I) (Chart I) (Fujita *et al.*, 1965). The γ-hydroxy-L-lysine obtained directly from γ-chloro-L-lysine by treatment with 4 equiv of silver acetate at 90° consisted of 97% *threo*- and 3% *erythro*-γ-hydroxy-L-lysine (III). Pure *threo*, II, is easily obtained by recrystallization of the monohydrochloride from aqueous ethanol. The use of base and silver oxide failed to raise the yield of *erythro* isomer III.

Most of γ- and δ-hydroxyamino acids undergo acid-catalyzed epimerization at the α-carbon (Hamilton and

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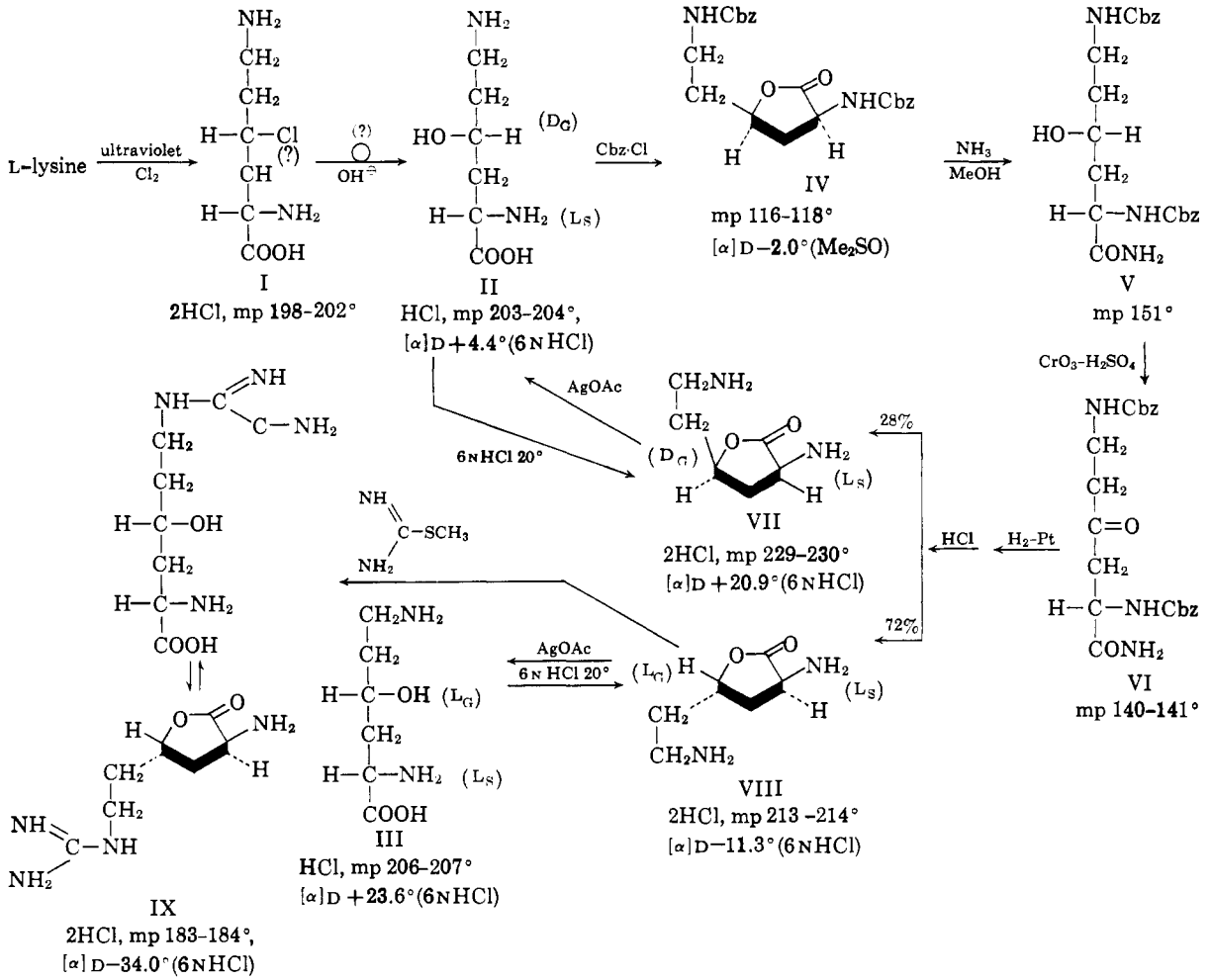
CHART I

Epimerization and Interconversions of the Diastereoisomers of γ-Hydroxy-L-lysine and γ-Hydroxy-L-homoarginine



Anderson, 1955; Witkop and Beiler, 1956; Witkop, 1955). This epimerization can be easily followed by column chromatography of the open hydroxyamino acids (see Experimental Section) on an automatic amino acid analyzer. Figure 1 shows the separation of natural *erythro*- (A) and (allo-) *threo*-δ-hydroxy-L-lysine (B) as well as of *threo*- (C) and *erythro*-γ-hydroxy-L-lysine (D). Like γ-hydroxy-L-ornithine (Witkop and Beiler, 1956) the *threo* isomer of γ-hydroxy-L-lysine is eluted before the *erythro* isomer.

Prolonged refluxing (144 hr) of II in 6.0 N hydrochloric acid gave a mixture of 70% II and 30% *erythro* isomer III. When this acid-catalyzed epimerization was carried out with chlorolysine I, up to 30% of III was formed. However, in this case, epimerization may also have involved the γ-carbon atom. The possibility of epimerization of two asymmetric centers made the preparation of III from *threo*-γ-hydroxy-D-lysine or γ-chloro-D-lysine unfeasible.

The well-known racemization or epimerization of α-amino acids in hot acetic acid with excess acetic anhydride (Greenstein and Winitz, 1961) was then studied with I and II. No epimerization of II was observed. Less than 10% epimerization of I occurred, but epimerization at the γ-position could not be excluded, since acid hydrolysis was used after the acetylation. Sodium acetate (du Vigneaud and Meyer, 1932) gave essentially the same results.

It has been shown previously that sodium borohydride reduction of *N*-carbobenzyloxy-4-keto-L-proline yields exclusively *N*-carbobenzyloxy-4-allohydroxy-L-proline (Patchett and Witkop, 1957). The ring nitrogen has a marked effect on the stereochemistry of reduction by complex metal hydrides. Typical examples are the reduction of free 4-keto-L-proline (Robertson *et al.*, 1962), *N*-carbobenzyloxy-5-keto-L-pipecolic (Witkop and Foltz, 1957), 5-keto-DL-pipecolic (Beyerman and Boekee, 1959), and 4-keto-L-pipecolic acid (Clark-Lewis and Mortimer, 1961). In the present study, *N,N'*-dicarbobenzyloxy-*threo*-γ-hydroxy-L-lysine lactone (IV) was chosen as the starting material. Since the free hydroxyl group was necessary for subsequent oxidation,

alcoholysis of the lactone IV to the ester was first attempted. However, under several conditions of alcoholysis the equilibrium between lactone and ester was so favorable to lactone that no ester was formed. In contrast, ammonolysis of lactone IV proceeded smoothly and *N,N'*-dicarbobenzyloxy-*threo*-γ-hydroxy-L-lysineamide (V) was obtained. Oxidation of the amide V with chromic acid in sulfuric acid gave crystalline dicarbobenzyloxy-4-keto-L-lysineamide (VI).

Previously a pronounced solvent effect on the ratio of products has been observed in the sodium borohydride reduction of *N*-carbobenzyloxy-3-keto-DL-proline methyl ester (Irreverre *et al.*, 1963). The analogous sodium borohydride reduction of VI was carried out in two different solvent systems, namely, in 1,2-dimethoxyethane–water, 2:1 and 100:1. However, under both conditions identical ratios of the reduction products, *viz.*, 60% *threo* and 40% *erythro*, were obtained.

A better yield of the *erythro* isomer III was finally obtained by catalytic hydrogenation and hydrolysis of the amide VI in acetic acid with platinum oxide which gave 72% *erythro* (III) and 28% *threo* (II) isomer. Palladium catalyst instead of platinum offered no advantage; on the contrary, hydrogenation was incomplete.

Reduction (Pt, HOAc) of ε-diazo-δ-oxo-L-norleucine (DON)[1] gave 75% *threo*- and 25% *erythro*-δ-hydroxy-L-lysine. This is the first direct conversion of DON to δ-hydroxylysine (Dion *et al.*, 1956). The ratio differs significantly from that of γ-ketolysineamide in which both amino groups and the carboxyl are neutralized by substituents.[2]

On a preparative scale, the mixture of *threo*- and *erythro*-γ-hydroxy-L-lysineamides obtained after catalytic reduction and decarbobenzylation was hydrolyzed with acid. The resulting lactones were opened to the mixture of hydroxy acids and separated by preparative ion-exchange column chromatography. Both *threo* and *erythro* isomers were crystallized as their lactone dihydrochlorides VII and VIII, respectively. Opening of the *erythro*-lactone VIII with silver acetate, removal of excess silver ion, and acidification to pH 3.5 with hydrochloric acid gave *erythro*-γ-hydroxy-L-lysine (III) as the crystalline monohydrochloride.

Both *threo*- and *erythro*-γ-hydroxy-L-lysine lactonized easily in 6.0 N hydrochloric acid at 20° within 2 hr, as measured by mutarotation. The completion of lactonization was followed by paper electrophoresis and by comparison of the optical rotations of the mutarotated compounds and the authentic lactones. Hudson's extended lactone rule (Witkop, 1956) applies in this case: $[\alpha]_D$(lactone VII·2HCl) +20.9° − $[\alpha]_D$(II·2HCl) +4.4° = +16.5°; $[\alpha]_D$(lactone VIII·2HCl) −11.3° − $[\alpha]_D$(III·2HCl) +23.6° = −34.9°. The positive difference assigns the D_G configuration to the γ-position of the *threo* pair, II and VII; the negative difference assigns the L_G configuration to that of the *erythro* pair, III and VIII.

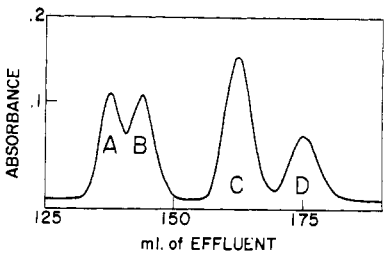


FIGURE 1: Position of the peaks (A–D) of the four hydroxylysines on the automatic amino acid analyzer. The δ-hydroxylysines and γ-hydroxylysines were eluted from a 150-cm column of Amberlite IR-120 at 30°, with 0.38 N sodium citrate, pH 4.26. The ninhydrin values of the amino acids were determined at λ 570 mμ as follows: natural *erythro*-δ-hydroxy-L-lysine, A (0.16 μmole); (allo) *threo*-δ-hydroxy-D-lysine, B (0.16 μmole); *threo*-γ-hydroxy-L-lysine, C (0.27 μmole), *erythro* γ-hydroxy-L-lysine, D (0.27 μmole).

Recently, a new natural amino acid, *threo*-γ-hydroxy-L-homarginine, was isolated from *Lathyrus* species (Bell, 1963, 1964a,b; Ramachandran and Rao, 1963). Its stereoconfiguration was established by synthesis from *threo*-γ-hydroxy-L-lysine (Fujita *et al.*, 1965). For the purpose of comparison the *erythro* isomer of this natural amino acid has now been synthesized. Guanidination of *erythro*-γ-hydroxy-L-lysine gave *erythro*-γ-hydroxy-L-homoarginine as the crystalline lactone dihydrochloride (IX).

The recent discovery of 3-hydroxy-L-proline (Irreverre *et al.*, 1962, 1963) as one of the building stones of collagen prompted us to study the possible occurrence of the comparable analog of *erythro*-δ-hydroxy-L-lysine, *viz.*, of *erythro*-γ-hydroxy-L-lysine or of its *threo* isomer in collagens of various origin.

Under the usual conditions of protein hydrolysis, γ-hydroxy-L-lysine lactonized almost quantitatively. Since the lactone would not be detected by the customary procedure on the amino acid analyzer, each hydrolysate was treated with silver acetate under conditions which quantitatively converted the lactone to the open hydroxy acid.

Two collagen hydrolysates, one from bovine connective tissue and the other from Mediterranean sponge, were analyzed by this method on the automatic amino acid analyzer. Neither *erythro*- nor *threo*-hydroxylysine were detected even with samples large enough to permit the detection of one residue of the amino acid/100,000 g of collagen. It may therefore be concluded that γ-hydroxylysine unlike 3-hydroxyproline is not a regular building stone of collagen.

Experimental Section

Epimerization of γ-Chloro- and threo-γ-Hydroxy-L-lysine (I and II). The results of various epimerization

[1] Abbreviation used: DON, ε-diazo-δ-oxo-L-norleucine.

[2] If alazopeptin (De Voe *et al.*, 1956–1957) were indeed a peptide derivative of 6-diazo-5-oxo-L-norleucine this reductive conversion to a hydroxylysine peptide would be the method for sequence determination by enzymatic or nonenzymatic methods.

TABLE I: Analysis of Ratios of *threo* and *erythro* Isomers in the Epimerizations of γ-Chloro-L-lysine and *threo*-γ-Hydroxy-L-lysine and the Reduction of Dicarbobenzyloxy-γ-keto-L-lysineamide, as well as of Diazo-δ-oxo-L-norleucine (DON).

Starting Material	Method	Ratio of Hydroxylysines *threo*	*erythro*	Remarks
erythro(?)-γ-Chloro-L-lysine·2HCl or *threo*-γ-hydroxy-L-lysine lactone·2HCl	Heated with 4 equiv of CH_3-COOAg to 90° for 30 min	97 96	3 4	Separation effected on a column (50 × 0.9 cm) of Dowex 50, citrate buffer, pH 4.26
erythro(?)-γ-Chloro-L-lysine·2HCl	Ag_2O (5 equiv), 25°, 12 hr	100	0	
erythro(?)-γ-Chloro-L-lysine·2HCl	NaOH (5 equiv), 25°, 12 hr	100	0	
erythro(?)-γ-Chloro-L-lysine·2HCl	2.0 N HCl, 115°, 2 hr 6.0 N HCl, 115°, 72 hr	87 70	13 30	
threo-γ-Hydroxy-L-lysine lactone·2HCl	2.0 N HCl, 115°, 2 hr 6.0 N HCl, 115°, 24 hr 6.0 N HCl, 115°, 48 hr 6.0 N HCl, 115°, 96 hr 6.0 N HCl, 115°, 144 hr	96 77 72 71 70	4 23 28 29 30	
threo-δ-Hydroxy-DL-lysine·HBr	6.0 N HCl, 125°, 72 hr	57	43	Izumiya *et al.*, 1962; Hamilton and Anderson, 1955
erythro(?)-γ-Chloro-L-lysine·2HCl	After acetylation (Ac_2O, 0.2 N NaOH, 1 hr, 0°) + 15 equiv of Ac_2O + 1 equiv of NaOAc, 37°, 20 hr; 2.0 N HCl, 115°, 2 hr	77	23	du Vigneaud and Meyer, 1932
threo-γ-Hydroxy-L-lysine lactone·2HCl	Same as above	93	7	The control experiment: *N,N'*-diacetyl-*threo*-γ-hydroxy-L-lysine lactone with 2.0 N HCl at 115° for 2 hr gave the same ratio of 93:7
N,N'-Diacetyl-*erythro*(?)-γ-chloro-L-lysine	5 equiv of Ac_2O, 130°, 10 min then 2.0 N HCl, 115°, 2 hr	53	47	The control experiment with HOAc instead of Ac_2O gave a ratio of 81:19
N,N'-Diacetyl-*threo*-γ-hydroxy-L-lysine lactone	Same as above	94	6	The control as above gave a ratio of 94:6
ε-Diazo-δ-oxo-L-norleucine (DON)	Hydrogenation in HOAc with Pt	75	25	
N,N'-Dicarbobenzyloxy-γ-keto-L-lysineamide	Hydrogenation in HOAc with Pd	66	34	Hydrogenation incomplete. In all hydrogenation experiments the amide was hydrolyzed with 2.0 N HCl, 115° for 1 hr. Hydrogenolysis of $NaBH_4$ reduction products was carried out in HOAc with palladium black.
N,N'-Dicarbobenzyloxy-γ-keto-L-lysineamide	H_2 + Pt in HOAc	34	66	
N,N'-Dicarbobenzyloxy-γ-keto-L-lysineamide	$NaBH_4$ in dimethoxyethane–water (2:1)	60	40	
N,N'-Dicarbobenzyloxy-γ-keto-L-lysineamide	$NaBH_4$ in dimethoxyethane–water (100:1)	60	40	

attempts and the analyses of the ratio of products are summarized in Table I. Since the lactone was not detected on the usual column of the amino acid analyzer, all lactone samples were opened with silver acetate to the hydroxy acid.

*threo-N,N'-Dicarbobenzyloxy-γ-hydroxy-*L*-lysineamide (V).* *threo*-Dicarbobenzyloxy-γ-hydroxy-L-lysine lactone (IV) (Fujita *et al.*, 1965) (3.76 g, 9.1 mmoles) was dissolved in 150 ml of methanol and saturated with ammonia at 0°. The flask was tightly stoppered and left at room temperature for 20 hr. The solvent was evaporated by a stream of dry air. The crystalline residue was recrystallized from a mixture of acetone, ether, and petroleum ether (bp 30–60°) (50:50:10). The yield of colorless crystals was 3.09 g (79%), mp 151°.

Anal. Calcd for $C_{22}H_{27}N_3O_6$: C, 61.52; H, 6.34; N, 9.79. Found: C, 61.54; H, 6.63; N, 9.51.

*N,N'-Dicarbobenzyloxy-γ-keto-*L*-lysineamide (VI).* To a solution of the amide V (3.09 g, 7.2 mmoles) in 400 ml of acetone was added 7.2 ml of 8.0 N chromic acid in sulfuric acid (Bladon *et al.*, 1951) during 5 min and the mixture was stirred at room temperature for 20 min. Triethylamine (8.4 ml, 60 mmoles) was added and the mixture was concentrated *in vacuo* to a small volume. The concentrate was diluted with water and extracted with chloroform (150 ml). The extract was dried over sodium sulfate and evaporated *in vacuo*. The crystalline residue was collected with the aid of ether and petroleum ether to give 2.70 g (88%) of colorless crystals, mp 137–140°. The analytical sample, recrystallized from ethyl acetate–ether, had mp 140–141°.

Anal. Calcd for $C_{22}H_{25}N_3O_6$: C, 61.81; H, 5.90; N, 9.83. Found: C, 61.64; H, 5.73; N, 9.93.

Catalytic Hydrogenation of the Ketoamide VI. The ketoamide VI (1.62 g, 3.8 mmoles) was dissolved in 50 ml of acetic acid and hydrogenated in the presence of 160 mg of platinum oxide at room temperature for 20 hr. The catalyst was filtered off, the filtrate was evaporated *in vacuo*, and the residue was dissolved in 50 ml of 2.0 N hydrochloric acid. The solution was refluxed for 1 hr. The solvent was evaporated *in vacuo* and the residue dissolved in 100 ml of water. Silver acetate (3.18 g, 19 mmoles) was added to the solution and the mixture was heated at 90° for 40 min with stirring. Silver chloride was removed by filtration, and excess silver ion was removed as silver sulfide. The filtrate was evaporated *in vacuo* and the residue was dissolved in 10 ml of citrate buffer, pH 2.28, used directly for column chromatography. An aliquot of the above solution was analyzed by the automatic amino acid analyzer and the results are shown below.

γ-Hydroxy-L-lysine	Composition: μmoles/10 ml	Composition: %
threo-	660	28
erythro-	1640	72

Preparative Column Chromatography. An Amberlite IR-120 column (particle size 47–65 μ, 1725 ml) was adjusted to pH 4.26 with 0.38 N sodium citrate buffer (Spackman *et al.*, 1958). The above sample solution was put on the column and eluted with the same buffer solution. The flow rate was 140 ml/hr and 11-ml fractions were collected.

*threo-γ-Hydroxy-*L*-lysine Lactone Dihydrochloride (VII).* Tubes 729–772 contained *threo* isomer. These fractions were combined, desalted by filtration over a column of Dowex 50-X8, and eluted with 7.0 N ammonium hydroxide. The eluate was evaporated *in vacuo*, and the residue was dried for several days in a vacuum desiccator over sulfuric acid, then for complete lactonization dissolved in concentrated hydrochloric acid, and evaporated *in vacuo*. The crystalline residue was filtered with the aid of ethanol to yield 92 mg of colorless crystals, mp 225–230°; recrystallized from 90% ethanol, mp 229–230°, lit (Fujita *et al.*, 1965) 229–230°.

*erythro-γ-Hydroxy-*L*-lysine Lactone Dihydrochloride (VIII).* Tubes 776–833 contained *erythro* isomer which was worked up in the same way as described above to yield 231 mg of colorless crystals, mp 212–214° dec. The compound was twice recrystallized from 90% ethanol and then had mp 213–214° dec, $[\alpha]^{20}D$ −11.3° (*c* 1, 6.0 N hydrochloric acid).

Anal. Calcd for $C_6H_{12}N_2O_2 \cdot 2HCl$: C, 33.19; H, 6.50; N, 12.90. Found: C, 33.30; H, 6.51; N, 13.13.

*erythro-γ-Hydroxy-*L*-lysine Hydrochloride (III).* To a solution of the *erythro* lactone VIII (153 mg, 0.71 mmole) in 20 ml of water was added silver acetate (625 mg, 3.75 mmoles) and the mixture was stirred at 90° for 40 min. The solution, freed from silver chloride and excess silver ions, was evaporated to dryness *in vacuo*, and the residue was dissolved in water. The solution was brought to pH 3.5 with hydrochloric acid and evaporated to a syrup. Ethanol was added and the mixture was left in a refrigerator overnight. The yield of colorless crystals was 120 mg (86%), mp 205–207° dec. The compound was recrystallized from 90% ethanol, mp 206–207° dec, $[\alpha]^{20}D$ +23.6° (*c* 1, 6.0 N hydrochloric acid).

Anal. Calcd for $C_6H_{14}N_2O_3 \cdot HCl$: C, 36.27; H, 7.61; N, 14.10. Found: C, 36.31; H, 7.80; N, 13.81.

*erythro-γ-Hydroxy-*L*-homoarginine Lactone Dihydrochloride (IX).* *erythro*-γ-Hydroxy-L-lysine lactone dihydrochloride (94 mg, 0.43 mmole) was dissolved in 2.0 N sodium hydroxide (0.65 ml, 1.3 mmoles), and the solution was left at room temperature for 1 hr. To the solution was added *S*-methylisothiouronium sulfate (60 mg, 0.43 mmole) and the mixture was left at room temperature for 24 hr. After the addition of 2.0 N hydrochloric acid, the solvent was evaporated *in vacuo* and the residue was dissolved in water. The solution was put on a column of Dowex 50-X8 (200–400 mesh, 0.9 × 40 cm). The column was washed with water and, by a gradient elution system of 4.0 N hydrochloric acid running into a mixing chamber containing 200 ml of 1.0 N hydrochloric acid, 7-ml fractions were collected. Fractions 46–60 which were Sakaguchi-positive were

pooled and evaporated *in vacuo*. The addition of concentrated hydrochloric acid and evaporation *in vacuo* was repeated several times to ensure complete lactonization. The final crystalline residue was collected with the aid of ethanol to yield 64 mg (57%) of colorless crystals, mp 182–183° dec. The compound was recrystallized from 90% ethanol, mp 183–184° dec, $[\alpha]^{20}D$ −34.0° (*c* 1, 6.0 N hydrochloric acid).

Anal. Calcd for $C_7H_{14}N_4O_2 \cdot 2HCl$: C, 32.44; H, 6.18; N, 21.62. Found: C, 32.49; H, 6.07; N, 21.73.

Mutarotations. The lactonization of *threo-* and *erythro*-γ-hydroxy-L-lysine (II and III) in 6.0 N hydrochloric acid at 20° was followed by mutarotation. The value for zero time was extrapolated from time *vs.* rotation plots (Table II).

TABLE II: Rate of Lactonization of *threo-* and *erythro*-γ-Hydroxy-L-lysine Hydrochloride in 6.0 N HCl at 20° as Followed by Mutarotation.

Time (min)	γ-Hydroxy-L-lysine *threo*	γ-Hydroxy-L-lysine *erythro*	
0	+4.4	+23.6	Acid
3	+7.0	+21.1	↓
18	+11.0	+11.3	↓
33	+16.0	+2.7	↓
48	+17.8	−5.1	↓
63	+19.6	−6.5	↓
78		−8.6	↓
93	+20.2		↓
108	+20.9	−10.4	↓
∞	+20.9	−11.3	Lactone

Analysis of Hydrolysates of Bovine and Sponge Collagen for γ-Hydroxylysine. LACTONIZATION OF γ-HYDROXY-L-LYSINE UNDER CONDITIONS OF PROTEIN HYDROLYSIS. A sample containing 3.2 μmoles of *threo* and 1.4 μmoles of *erythro*-γ-hydroxy-L-lysine in 3 ml of constant-boiling hydrochloric acid was hydrolyzed in a sealed tube at 110° for 24 hr. After removal of the acid, the samples were analyzed in the automatic amino acid analyzer and showed the presence of 92% lactone for the *threo* and 71% of the *erythro* isomer.

HYDROLYSIS OF BOVINE COLLAGEN. Bovine collagen (Worthington, 200 mg) was hydrolyzed with 5 ml of constant-boiling hydrochloric acid for 24 hr at 110°. After removal of the acid the sample was dissolved in 10.0 ml of water.

OPENING OF THE LACTONE RING. Of this solution 2.5 ml, representing 50 mg of hydrolyzed protein was adjusted to pH 4.0 with Ag_2O. There was then added 170 mg of silver acetate and the mixture was heated to 90° for 30 min. The solids were removed by filtration, the silver ion was recovered by H_2S, and the solution was evaporated to dryness. The residue was dissolved in 5.0 ml of water. Aliquots were analyzed on the amino acid analyzer. There was no detectable amount of γ-hydroxylysine, even when the column was overloaded.

HYDROLYSIS OF SPONGE COLLAGEN. When 200 mg of sponge collagen was hydrolyzed and analyzed in the same way as above, there was no detectable amount of γ-hydroxylysine. The sensitivity of the assay method would have led to the detection of one residue of γ-hydroxylysine in 10^5 g of protein.

Catalytic Hydrogenation of Diazo-δ-oxo-L-norleucine (DON). Diazo-δ-oxo-L-norleucine (Dion *et al.*, 1956) (17.1 mg, 0.1 mmole) dissolved in 1 ml of acetic acid was hydrogenated in the presence of 3.5 mg of platinum oxide at room temperature for 4 hr. The reduction products were analyzed by the automatic amino acid analyzer.

Compound	Composition μmoles	Composition %
δ-Hydroxy-L-lysine	7.0	25
allo-δ-Hydroxy-L-lysine	21.0	75

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Incorporation of *cis*- and *trans*-4-Fluoro-L-prolines into Proteins and Hydroxylation of the *trans* Isomer During Collagen Biosynthesis*

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ABSTRACT: *cis*- and *trans*-4-fluoro-L-prolines (IV and XI), synthesized *via* the tosyloxy-L-proline derivatives I and VIII by SN2 displacement with fluoride ion, were tritiated catalytically to the ^3H derivatives V and XII. Both tritiated fluoroprolines V and XII were incorporated into protein and unhydroxylated precursor of collagen. The latter was degradable by collagenase. Enzymatic hydroxylation of bound tritiated *trans*-4-fluoro-L-proline led to bound 4-hydroxy-L-proline which after hydrolysis was isolated and identified.

Substituted amino acids have frequently been useful in elucidating mechanisms of amino acid metabolism. *p*-Fluorophenylalanine was shown to serve as a substrate of phenylalanine hydroxylase (Kaufman, 1962) and halogen-substituted tyrosines have recently proved useful as inhibitors of tyrosine hydroxylase (Udenfriend *et al.*, 1965). The hydroxylation of proline was shown to take place after incorporation into protein (Peterkofsky and Udenfriend, 1963). Two questions of interest emerged: is fluoroproline incorporated into proteins and, if so, is the fluoroproline bound in the collagen precursor oxidatively defluorinated to yield bound hydroxyproline?

The present report describes the preparation of the tritium-labeled *cis*- and *trans*-4-fluoro-L-prolines, their use as substrates for protein synthesis in *Escherichia coli* extracts and guinea pig granuloma minces, and as substrates for hydroxylation in the latter system.

Experimental Section

The ascorbic acid deficient diet came from Nutritional Biochemical Corp. Carrageenan was obtained from Marine Colloids Inc. Commercial L-proline-3,4-^3H (320 μc/μmole) (New England Nuclear Corp., Boston, Mass.) was purified by chromatography on a Dowex 50-X8 column (H^+ form) with 1.0 N HCl as the eluent. Purified collagenase was obtained from the Worthington Biochemical Corp. This material gave no evidence of proteolysis with the following substrates (1 mg/ml): albumin, casein, fibrin, and elastin. Optimal digestion of hot trichloroacetic acid extractable material from microsomes was achieved by treatment of 15.0-μg quantities of collagenase substrate with 10 μg of purified collagenase for 90 min in a 1-ml volume containing 0.025 M Tris–HCl buffer (pH 7.2) and 0.33 M calcium acetate.

Induction of Granuloma. Male guinea pigs were maintained on regular diets, which included cabbage, or on an ascorbic acid deficient diet for 10 days prior to injection. Granulomata were induced by subcutaneous injection of 8 ml of a 1% solution of carrageenan. The granulomata were excised 6 days after injection, placed in ice-cold 0.01 M glucose, washed once with cold 0.01 M glucose, and then minced thoroughly. Five grams of tissue was incubated in 10 ml of medium containing 25 μc of 3,4-tritiated L-proline-^3H or 100–140 μc of *cis*- or *trans*-4-fluoro-L-proline-^3H for 2 hr at 37° under O_2–CO_2 (95:5). The medium also contained NaCl (0.022 M), KCl (0.003 M), $MgSO_4$ (0.012 M), $CaCl_2$ (0.0013 M), KH_2PO_4 (0.0004 M), $NaHCO_3$ (0.025 M), and D-glucose (0.01 M). At the end of the incubation the mixture was centrifuged at 3°, the supernatant was discarded, and the tissue washed once with 5 ml of cold 0.25 M sucrose solution. Another 5 ml of 0.25 M sucrose

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