the subsequent Edman degradation step as described above. The results are summarized in Table I and Figure 1.

Cleavage of Gramicidin B by N-Bromosuccinimide. A 1.4-mg. (0.76 μmole) sample of gramicidin B was dissolved in 0.5 ml. of 60% aqueous ethanol containing 1 drop of 0.10 *N* sulfuric acid and allowed to react with 0.8 mg. (4.6 μmoles) of N-bromosuccinimide at room temperature. Similarly, 1.1 mg. (0.58 μmole) of gramicidin A was oxidatively cleaved with 0.8 mg. (4.6 μmoles) of N-bromosuccinimide. Aliquots of the reaction mixtures were examined by paper electrophoresis. In both cases a ninhydrin-positive substance, migrating as fast as 2-aminoethanol, was liberated. To the remainder of the reaction mixture was added 100 μl. of an aqueous solution containing 200 μg. of 1-amino-2-hydroxypropane per ml. After the addition of 2 drops of 2.0 *N* hydrochloric acid the samples were evaporated and dried. The residues were dissolved in 500 μl. of 1,2-dimethoxyethane, acylated with 10 μl. of trifluoroacetic anhydride, and analyzed by gas chromatography on a column containing 4% neopentyl glycol succinate on Chromosorb W (6 ft. × $^1/_6$ in., flame ionization detector). At a column temperature of 145° with 36 ml./min. of nitrogen the bistrifluoroacetylated 1-amino-2-hydroxypropane and 2-aminoethanol were eluted after 2.9 and 4.4 min., respectively. By comparison of the peak areas[9] a release of 0.18 mole of 2-aminoethanol per 1882 g. of gramicidin A and of 0.19 mole of 2-aminoethanol per 1845 g. of gramicidin B was calculated.

Determination of the Optical Configuration of the Amino Acids in Gramicidin B. A 2.1-mg. (1.15 μmoles) sample of gramicidin B was hydrolyzed in an evacuated and sealed tube with 0.45 ml. of acetic acid and 2.25 ml. of constant boiling hydrochloric acid at 110° for 24 hr. The hydrolysate was evaporated and kept in a vacuum desiccator over phosphorus pentoxide and potassium hydroxide. The residue was dissolved in 3.3 ml. of water, and 1-ml. aliquots of this solution were used for the enzymatic incubation reactions and for the amino acid analysis. The incubation with L-amino acid oxidase from *Crotalus adamanteus* was carried out at 38° in a Warburg apparatus with oxygen as the gas phase. The main compartment contained 1 ml. of 0.1 *M* 2-amino-2-hydroxymethyl-1,3-dihydroxypropane HCl buffer (pH 7.66 at 24°), 1 ml. of 0.1 *M* KCl solution, and 1 ml. of the hydrolysate. A control flask contained 1 ml. of water instead of the hydrolysate. The side arm contained 1 mg., in a second experiment 10 mg., of the enzyme in 0.5 ml. of 0.1 *M* KCl. The center well contained 0.1 ml. of 5 *N* KOH. After a 10-min. equilibration period the flasks were tipped and readings were taken at 10-min., later 60-min., intervals for 10 hr. until the oxygen uptake had virtually ceased. The reaction mixture was evaporated and analyzed on an amino acid analyzer. The results are given in Table II.

The Stereospecific Synthesis of *threo*-γ-Hydroxyhomo-L-arginine from *Lathyrus* Species

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γ-Hydroxy-L-lysine (III) prepared from L-lysine (I) by photochemical chlorination and subsequent reaction with silver acetate was cyclized with nitrosyl chloride to a mixture of 80% cis-4-hydroxy-L-pipecolic acid (VI) and 20% trans-4-hydroxy-D-pipecolic acid (VII). This establishes the threo configuration (III) for γ-hydroxy-L-lysine (III) which was converted to threo-γ-hydroxy-L-homoarginine (VIII ⇌ IX), identical in all respects with the naturally occurring amino acid from Lathyrus seeds.

The photochlorination of amino acids in strong acid permitted an easy synthesis of several biochemically interesting C-substituted amino acids.[3] The photochlorination of L-lysine (I) is specific both with regard to position and configuration. γ-Hydroxy-L-lysine (III) prepared from the γ-chloro-L-lysine (II) showed a single sharp peak by ion-exchange column chromatography under conditions which easily resolve the diastereoisomers of δ-hydroxylysine as well as of γ-hydroxyornithine.[4]

The configuration of γ-hydroxy-L-lysine is of interest for the mechanism of the photochlorination and for the related γ-hydroxyhomoarginine, a new natural amino acid of unknown configuration recently isolated from *Lathyrus* species.[5,6] In this investigation the configuration of γ-hydroxylysine is shown to be *threo*. It is assumed that γ-chlorolysine has the *erythro* configuration and that its conversion to hydroxylysine involves 100% inversion.

The configuration of the asymmetric centers of γ-hydroxy-L-lysine was determined in the same way as that of γ-hydroxyornithine, the next lower homolog,[4] in which the *erythro* diastereoisomer is sterically

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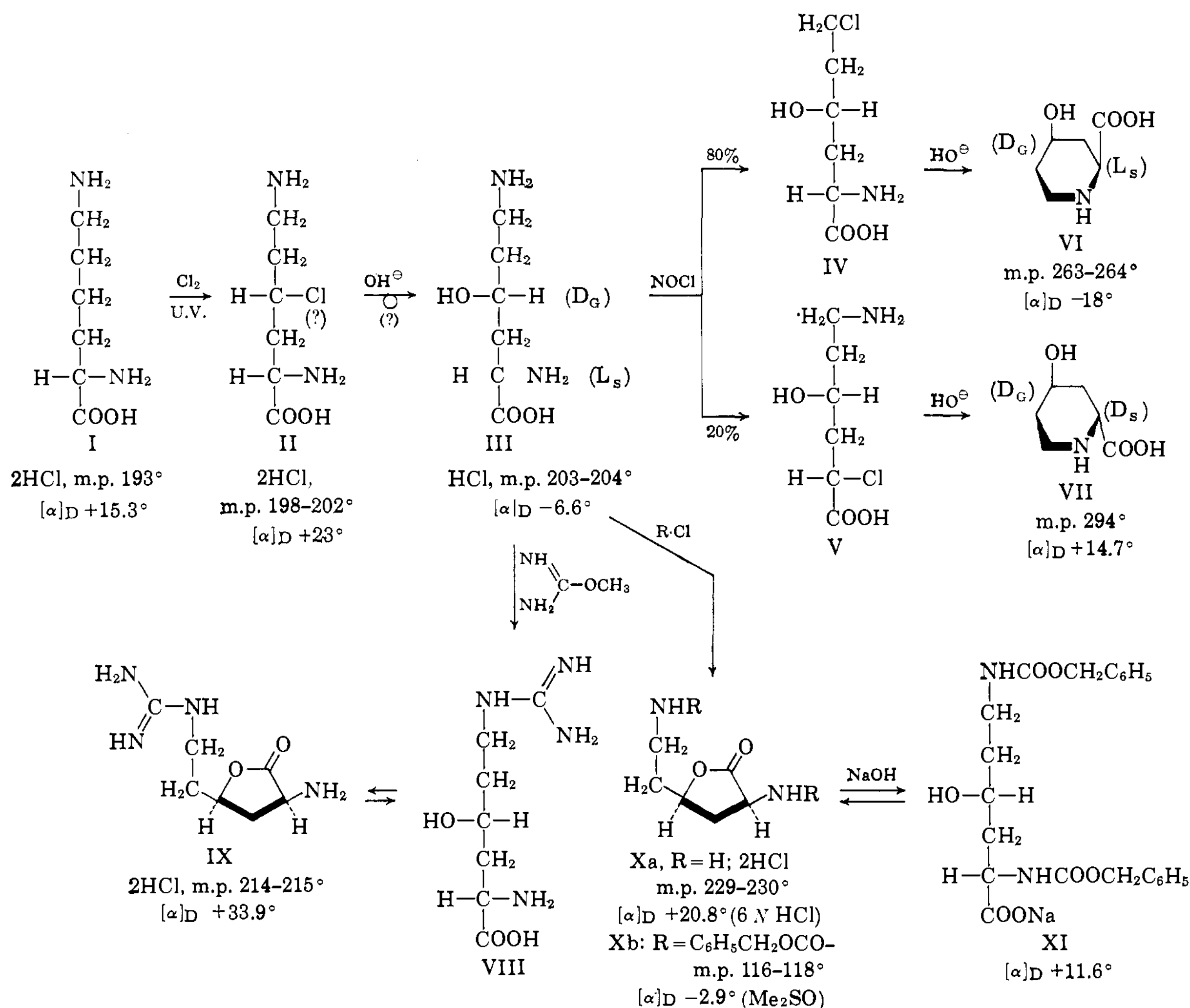

related to hydroxy-L-proline, and *threo*-γ-hydroxy-L-ornithine to *allo*-hydroxy-L-proline.

In the present study the cyclization of γ-hydroxy-L-lysine and the separation of the products presented no difficulties when the following procedure[7] was used. The open amino acid, γ-hydroxy-L-lysine (III), was treated first with nitrosyl chloride and then with barium hydroxide. From this reaction mixture a product which gave a greenish-yellow color with ninhydrin reagent was isolated by fractionation on a column of Dowex 50-X8. A special solvent system for paper chromatography which resolves the diastereoisomers of cyclic imino acids[8] showed the product to be a mixture of *cis*- and *trans*-4-hydroxypipecolic acid. The isomers were separated by preparative column chromatography following the same procedure used for the separation of *cis*- and *trans*-3-hydroxyprolines.[9] Both isomers were isolated and crystallized. The major isomer (80%) was identified as *cis*-4-hydroxy-L-pipecolic acid (VI) and the minor one (20%) was *trans*-4-hydroxy-D-pipecolic acid (VII). This shows that nitrosyl chloride reacted predominantly with the ε-amino group to give the ε-chloro-γ-hydroxy-α-aminocaproic acid (IV) which undergoes base-catalyzed intramolecular cyclization with intact asymmetry of the two active centers.

It may be recalled that by contrast the cyclization of *erythro*-γ-hydroxy-L-ornithine gave *allo*-hydroxy-D-proline as the major product.[7] Further investigations are necessary to find a theoretical basis for the differential reactivity of α- and ω-amino groups toward nitrosyl chloride in open diaminohydroxy acids and their lactones.

The fact that the major product of the conversion was *cis*-4-hydroxy-L-pipecolic acid (VI) establishes the configuration of γ-hydroxylysine as *threo*-γ-hydroxy-L-lysine (III). In addition, the configuration of the α-carbon atom of γ-hydroxy-L-lysine (III) was shown to be L by the positive shift in rotation on acidification (Lutz–Jirgensen rule)[10]: $[\alpha]^{20}\text{D}$ γ-hydroxylysine (*c* 2%, H_2O) 0°; dihydrochloride in 6.0 *N* HCl +12.8°; $[\alpha]^{6N\ HCl}\text{D} - [\alpha]^{H_2O}\text{D} = +12.8°$. This proof of the L-configuration for the α-carbon eliminates the need to consider the product of photochlorination and hydrolysis to be *erythro*-γ-hydroxy-D-lysine which could also yield the mixture of the hydroxypipecolic acids VI and VII.

When hydroxylysine dihydrochloride is repeatedly evaporated in the presence of excess concentrated HCl the lactone dihydrochloride Xa is obtained. Hudson's extended rule[11] applies in this case: $[\alpha]^{\text{lactone (Xa)}\cdot 2\text{CHl}}\text{D} + 20.8 - [\alpha]^{\text{III}\cdot 2\text{HCl}}\text{D} + 12.8 = +8.0.$

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This positive difference correctly assigns the D$_G$ configuration to the γ-carbon atom. Natural γ-hydroxy-L-ornithine,[12] γ-hydroxy-L-arginine,[12-14] and δ-hydroxylysine[11,15] from collagen all have *erythro* configuration.

However, this rule is not applicable to N,N'-dicarbobenzyloxy-γ-hydroxylysine lactone, $[\alpha]^{25}$D lactone $-2.9°$; $[\alpha]^{25}$D (after addition of 1 equiv. of KOH) $+11.6°$. The rotational difference between the lactone and the salt of the open acid is $-14.5°$, suggestive of the L$_G$ configuration for the γ-carbon atom. This is inconsistent with the results of the stereochemical interconversions which provide rigid and conclusive evidence for the configurations at the α- and γ-carbon atoms. It has been emphasized previously[11] that no assignments of configuration should be based on the lactone rule alone, and this restriction has recently been borne out by several exceptions to Hudson's rule.[16,17]

Guanidination of γ-hydroxylysine gave *threo*-γ-hydroxy-L-homoarginine lactone dihydrochloride which was identical with the naturally occurring γ-hydroxyhomoarginine lactone dihydrochloride[18] with regard to melting point, mixture melting point, optical rotation, and infrared spectrum. This establishes the configuration of the naturally occurring amino acid as *threo*-γ-hydroxy-L-homoarginine.

Whether the principles of nonenzymatic cleavage of peptide bonds[19] may now be extended to peptides of photohalogenated amino acids is under investigation.

Experimental

*Conversion of threo-γ-Hydroxy-*L*-lysine to cis- (VI) and trans-4-Hydroxypipecolic Acid (VII).* A solution of nitrosyl chloride (6 ml.), prepared according to Witkop, *et al.*, was added dropwise to an ice-cold solution of γ-hydroxy-L-lysine hydrochloride (199 mg., 1 mmole) dissolved in 9.0 *N* hydrochloric acid (10 ml.). The reaction mixture was stirred for 10 min. in the cold and then heated at 55° for 20 min., followed by evaporation *in vacuo* to dryness. The residue was dissolved in water (2 ml.) and heated with 0.2 *N* barium hydroxide (10 ml.) in a boiling water bath for 10 min. The solution was neutralized with dilute sulfuric acid and centrifuged. The supernatant was put on a column of Dowex 50-X8 (H^+, 1.2 $\times$ 40 cm.) and eluted with 0.2 *N* hydrochloric acid. The effluent was collected in 5-ml. fractions.

Fractions 91–114, which showed a single greenish-yellow spot on a paper chromatogram, were pooled. The solvent was evaporated to yield 140 mg. of crystalline residue. The crystals were dissolved in water (2 ml.) and diluted with 0.2 *N* citrate buffer, pH 3.25 (2 ml.). A Dowex 50-X8 column (200–400 mesh, 2000 ml.) was prepared according to Moore and Stein[20] and adjusted to pH 3.25 with 0.2 *N* citrate buffer.[21] A solution of the sample in this buffer was put on the column and eluted with the same buffer (100 ml./hr.), and the effluent was collected in 10-ml. fractions. Aliquots (10 μl.) from each tube were spotted on paper and sprayed with ninhydrin reagent.

*cis-4-Hydroxy-*L*-pipecolic Acid (VI).* Tubes 258–298 contained *cis*-4-hydroxypipecolic acid (*cf.* ref. 21). These fractions were combined, desalted by filtration over a column of Dowex 50-X8 (H^+, 80 ml.), and eluted with 7.0 *N* ammonium hydroxide. The effluent was evaporated *in vacuo* to give colorless crystals (81 mg.). They were recrystallized twice from 90% ethanol; yield 40 ml.; after drying *in vacuo* at 100° over phosphorus pentoxide the melting point was 263–264° dec.; $[\alpha]^{20}$D $-18.0 \pm 2.0°$ (*c* 0.86, water) [lit.[22] m.p. 265° dec., $[\alpha]^{23}$D $-17°$ (*c* 1.1, water, as monohydrate)].

Anal. Calcd. for $C_6H_{11}NO_3$: C, 49.64; H, 7.64; N, 9.65. Found: C, 49.82; H, 7.93; N, 9.68.

*trans-4-Hydroxy-*D*-pipecolic Acid (VII).* Tubes 318–338 contained *trans*-4-hydroxypipecolic acid which was worked up in a similar way to give 9 mg. of colorless recrystallized product, m.p. 294° dec., $[\alpha]^{20}$D $+14.7 \pm 2.0°$ (*c* 0.5, water) [lit.[22] for the L-isomer m.p. 294°, $[\alpha]^{20}$D $-13.0 \pm 0.4°$ (*c* 1, water)].

*Guanidination of threo-γ-Hydroxy-*L*-lysine.* γ-Hydroxy-L-lysine hydrochloride (1.00 g., 5 mmoles) was dissolved in 15 ml. of water. To the solution was added basic copper carbonate (0.83 g., 3.75 mmoles) and the mixture was refluxed for 20 min. Excess copper carbonate was collected and the filtrate was cooled to 0°. O-Methylisourea sulfate (1.23 g., 5 mmoles) was added to the solution and the pH was adjusted to 10.5 with 2.0 *N* sodium hydroxide. The mixture was left at room temperature for 7 days, brought to pH 2–3 with concentrated hydrochloric acid, and Cu^{2+} was removed with hydrogen sulfide. The filtrate from the copper sulfide was evaporated *in vacuo* and the residue was dissolved in water, put on a Dowex 50-X8 column (H^+, 200-400 mesh, 3 $\times$ 40 cm.), and eluted with a gradient of hydrochloric acid (4.0 *N* hydrochloric acid running into a mixing chamber containing 500 ml. of 0.5 *N* hydrochloric acid). The eluate was collected in 10-ml. fractions.

*γ-Hydroxy-*L*-lysine Lactone Dihydrochloride (Xa).* Tubes 106–126 contained unchanged starting material. These fractions were combined and evaporated *in vacuo* leaving a crystalline residue which was filtered with the aid of ethanol to yield 371 mg. (34% recovery) of colorless crystals, m.p. 225-230° dec. The compound was recrystallized from 90% ethanol to give 272 mg., m.p. 229–230° dec.; $[\alpha]^{20}$D $+20.8$ (*c* 1, 6.0 *N* hydrochloric acid).

Anal. Calcd. for $C_6H_{14}N_2O_2Cl_2$: C, 33.19; H, 6.50; N, 12.90; Cl, 32.67. Found: C, 33.16; H, 6.70; N, 13.19; Cl, 32.50.

*γ-Hydroxy-*L*-homoarginine Lactone Dihydrochloride (IX).* Tubes 178–220 contained γ-hydroxy-L-homoarginine which was worked up in the same way as described above to yield 434 mg. (36%) of colorless crystals, m.p. 211-213° dec. The compound was

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recrystallized twice from 90% ethanol, and then had m.p. 214-215° dec. (lit.[5] 214° dec.), $[\alpha]^{20}D$ +33.9 ± 1.0° (c 1, 6.0 N hydrochloric acid) [authentic sample isolated from *Lathyrus* seeds (*cf.* ref. 12) $[\alpha]^{20}D$ +32.8 ± 1.0° (c 1, 6.0 N hydrochloric acid)].

Anal. Calcd. for $C_7H_{16}N_4O_2Cl_2$: C, 32.44; H, 6.18; N, 21.62; Cl, 27.41. Found: C, 32.73; H, 6.04; N, 21.86; Cl, 27.41.

N,N'-Dicarbobenzyloxy-γ-hydroxy-L-lysine Lactone (Xb). This derivative was synthesized in the same way as described for the preparation of dicarbobenzyloxy-δ-hydroxylysine lactone.[23] The yield of recrystallized lactone was 78%, m.p. 116–118°, $[\alpha]^{20}D$ −2.9° (c 1, dimethyl sulfoxide).

Anal. Calcd. for $C_{22}H_{23}N_2O_6$: C, 64.06; H, 5.87; N, 6.79. Found: C, 64.16; H, 5.67; N, 6.90.

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The Tautomeric Form of Helical Polyribocytidylic Acid

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In contrast to the pK of 4.3 seen in the titration curve of cytidine, the polymer of cytidylic acid exhibits two pK values in the acid range, one at 5.7 and the other at 3.0. Each of these involves the uptake of one-half proton for each base. At neutral pH the polymer is single stranded, while below pH 5.7 it is believed to exist as a two-stranded helix in which the bases are held together by three hydrogen bonds, one of which can form only when a proton is added to one ring nitrogen for each pair of bases. Between pH 5.7 and 3.0 the helix is stable, while lowering the pH below 3.0 destroys the structure through the addition of more protons. Infrared studies show that both protonated and nonprotonated cytosine rings are present when the helical form of the molecule is stable. Formation of the helical complex can also be seen by changes in the ultraviolet spectrum as well as in the sedimentation properties of the polymer. Thermal denaturation studies show that the addition of the first half proton per base stabilizes the molecule, while addition of the second half proton per base destabilizes it. These properties are all in agreement with the proposed type of hydrogen bonding.

The synthetic polyribonucleotides represent a large class of polymer molecules which have been studied extensively because of their close relation to the naturally occurring nucleic acids. They have often been used as model compounds for understanding the reactivity of the naturally occurring polymers, and a great deal is known about them at the present time. An X-ray diffraction study has been carried out on oriented fibers of polyribocytidylic acid (poly C) which shows that this molecule exists in a helical form with a pitch of 37.3 Å. and a diameter of approximately 14 Å.[1] The molecule is made of two strands of polycytidylic acid which are helically wrapped round each other in a parallel configuration in which they are related by a twofold rotation axis. An unusual type of hydrogen bonding was postulated as responsible for holding the two chains together. In this form the two strands are held together by sets of three hydrogen bonds between the cytosine bases in the center of the molecule. In order to achieve this type of hydrogen bonding, one of the two cytosine rings must be protonated, as shown in Figure 1. This type of hydrogen bonding has also been found in the crystal structure of cytosine-5-acetic acid.[2]

The present studies were undertaken in an attempt to answer the following question: Does the poly C molecule exist in a helical form in solution and does it maintain the same type of hydrogen bonding between the strands which was suggested by the X-ray diffraction study in the solid state? Akinrimisi, *et al.*,[3] have presented some thermal denaturation and optical rotatory data related to this question. Here we present the results of acid–base titration, infrared and ultraviolet spectroscopy, and thermal denaturation studies from which we conclude that the molecule has a structure in solution which is very similar to that seen in the solid state.

Methods and Materials

Samples. The poly C used in these experiments came from two sources. Part of it was polymerized by using a polynucleotide phosphorylase enzyme obtained from *Micrococcus leisodeikticus*, with methods which have already been described.[4] After the cytidine diphosphate and enzymes had been mixed, they were allowed to incubate at 37° for 4 hr. and were then precipitated by adding two volumes of cold 95% ethyl alcohol. The precipitate was redissolved in water and precipitated a second time with alcohol. The polymer was then dissolved in water again and dialyzed exhaustively against 5×10^{-3} M NaCl at neutral pH to rid the preparation of contaminating residual cytidine diphosphate. The polymer solution was frozen, lyophilized, and stored in the cold in the form of dry fibers. In addition, part of the poly C used in these experiments was obtained from Miles Chemical Company, Clifton, N. J. Although the materials from these two sources differed slightly in

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