γ-Hydroxyarginine, a New Guanidino Compound from a Sea-cucumber II. Determination of the Configuration

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A new guanidino compound was first discovered in a sea-cucumber, *polycheira rufescence*[1], and then isolated purely and identified as γ-hydroxy-L-arginine (I)[2]. The compound contains two asymmetric carbon atoms (α and γ) and should, therefore, exist theoretically in four stereoisomeric forms. In the preceding paper[2] it was concluded that the configuration on the α-carbon is the L$_S$-form[3] in view of the susceptibility to arginase[4] and L-amino acid oxidase[5], whereas that on the γ-carbon was not yet decided. The conclusion was further supported by Makisumi[6] in our laboratory. He observed that I was quantitatively decarboxylated by the action of L-arginine decarboxylase from *E. coli* 7020[7].

In contrast with γ-hydroxyarginine, γ-hydroxyornithine was studied in detail with regard to its stereoisomers by Witkop et al.[8,9]. They proved that *erythro*- and *threo*-γ-hydroxy-L-ornithine were stereochemically equivalent to hydroxy-L-proline and allohydroxy-L-proline, respectively.

In the present study, the configuration on the γ-carbon was established according to the method of Witkop et al.[8] with a slight modification. γ-Hydroxy-L-ornithine (II) produced from I by the action of arginase was treated first with nitrosyl chloride, and then with barium hydroxide. From this reaction mixture, a product which gave a yellow spot with ninhydrin reagent on a paper chromatogram was isolated by fractionation on a column of Amberlite CG-120. The product was identified

1) H. Sasaki, Y. Fujita, S. Makisumi and S. Shibuya, *J. Japanese Biochem. Soc.* (*Seikagaku*), **30**, 642 (1958).
2) Y. Fujita, This Bulletin, **32**, 439 (1959).
3) E. J. Crane, *Chem. Eng. News*, **25**, 1363 (1947).
4) D. M. Greenberg, "The Enzymes", Vol. I, edited by J. B. Sumner and K. Myrbäck, Academic Press Inc., New York (1951), p. 894.
5) Y. Robin, *Bull. Soc. Chim. Biol.*, **35**, 285 (1953). H. Blashko and D. B. Hope, *Biochem. J.*, **62**, 335 (1956).
6) S. Makisumi, unpublished data.
7) E. F. Gale, *Biochem. J.*, **41**, vii (1947). S. M. Birnbaum and J. P. Greenstein, *Arch. Biochem. Biophys.*, **39**, 108 (1952).
8) B. Witkop and T. Beiler, *J. Am. Chem. Soc.*, **78**, 2882 (1956).
9) B. Witkop, *Special Publication No. 3.*, The Chemical Society, Burlington House, W. 1, London, (1955), p. 60.

as allohydroxy-D-proline (III). The optical purity of III was assured with the aid of column chromatography (Fig. 2).

The fact that a compound having the L_S configuration as to the α-carbon atom converts by the action of a certain reagent into a product having the D_S configuration, suggests that it is a result of the Walden inversion. According to the extensive studies by Izumiya et al.[10] on the Walden inversion of α-amino acid, it is generally accepted that in an α-amino acid possessing a secondary carbon atom at β-position the inversion does not occur by the halogenation with nitrosyl halide, but takes place on treating the resulting α-halogeno acid with basic reagents such as ammonia.

When, as in the case of the present experiment, nitrosyl chloride acts on II, there is a possibility that three chlorinated acids (α-chloro-δ-amino-γ-hydroxyvaleric, δ-chloro-α-amino-γ-hydroxyvaleric, and α,δ-dichloro-γ-hydroxyvaleric acids) are produced therefrom. Of the three acids, α-chloroacid and δ-chloroacid are concerned in the formation of hydroxyproline after cyclization. The composition of the reaction mixture was, therefore, examined by means of determination of the total nitrogen, carbon dioxide liberated with Chloramine T and chloride ions (Table I). The results indicated that the amount of α-chloroacid was much grater than that of δ-chloroacid, showing a preferential reactivity of the α-amino group of II. It is reasonable, as in the cases of chain compound, that the inversion should also occur in the stage of a base-catalyzed cyclization of α-chloro-δ-amino-γ-hydroxyvaleric acid. Accordingly, the allohydroxy-D-proline obtained by the inversion can be established as originating from *erythro*-γ-hydroxy-L-ornithine. The latter compound, if the inversion did not occur, would convert into hydroxy-L-proline (IV). Therefore, the whole configuration of I should, as a matter of course, be concluded to be *erythro*-γ-hydroxy-L-arginine.

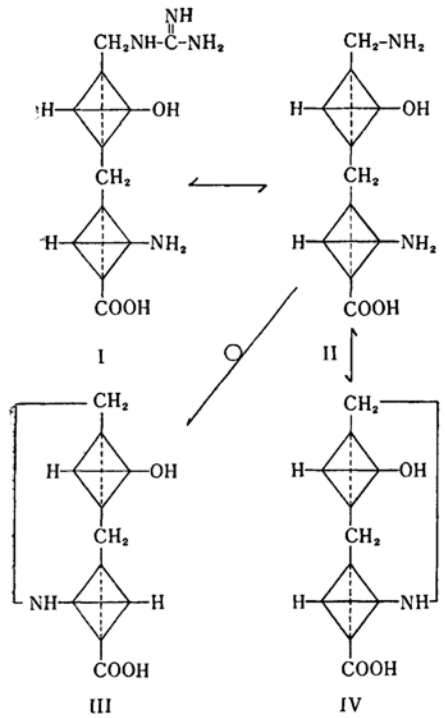

Fig. 1. Stereochemical correlation between γ-hydroxyarginine, γ-hydroxyornithine and (allo)hydroxyproline.
⟷ Stereochemical equivalence
⟲ Walden inversion

Experimental

Material. — The γ-hydroxy-L-ornithine hydrochloride used in the present study was prepared from γ-hydroxy-L-arginine hydrochloride by the action of arginase[2]: m. p. 182~183°C (decomp.), $[\alpha]_D^{20}$ +10.6° (*c* 5, in water)[11].

According to Witkop et al.[8], in the conversion of γ-hydroxyornithine to (allo)hydroxyproline different results are obtained by using either the open acid or the lactone as the starting material. Therefore, whether or not contaminated with lactonized γ-hydroxyornithine, the material was checked by determining the carbon dioxide evolved with Chloramine T at pH 4.7 and 25°C. On mixing γ-hydroxyornithine with the reagent, a brisk evolution of the gas was observed. This amounted to 90% after 5 min. and to 100% after 15 min. The observation was in good accordance with that of Witkop on the open acid.

Conversion of γ-Hydroxy-L-ornithine to Allohydroxy-D-proline. — A nitrosyl chloride solution (6 ml.) prepared according to Witkop et al.[8] was added drop by drop to an ice-cold solution of γ-hydroxy-L-ornithine hydrochloride (185 mg.) dissolved in 9 N hydrochloric acid (10 ml.). The reaction mixture was stirred for 10 min. in the cold and then heated at 55°C for 20 min., followed by evaporation to dryness in vacuo. The residue was dissolved in water (2 ml.), and heated with 0.2 N barium hydroxide solution (10 ml.) in a boiling water bath for 10 min. The solution was freed from barium by neutralizing with sulfuric acid and centrifugation. The supernatant solution was passed through a column of Amberlite CG-120 (H-form, 1×18 cm.). The adsorbed substances were eluted with 0.2 N hydrochloric acid (15 ml./hr.), and collected in 5 ml. fractions. Each fraction was analyzed by paper chromatography and only the fractions (31st to 40th tubes) which gave a single yellow spot with ninhydrin were united. The resulting solution was evaporated up to dryness in

10) N. Izumiya et al., *J. Chem. Soc. Japan, Pure Chem. Sec.* (*Nippon Kagaku Zasshi*), **72**, 26, 149, 445, 550, 1050 (1951); This Bulletin, **25**, 265 (1952); **26**, 53 (1953).

11) The author wishes to correct the erroneous figure for the specific rotation described in Ref. 2.

vacuo. The crystalline residue was dissolved in a small volume of water and the solution was passed through a column of Amberlite IR-4B (OH-form). The effluent freed from chloride by this treatment was again concentrated in vacuo and the crystalline residue was triturated with methanol (2 ml.), filtration and washing with methanol and ether being followed. The product was recrystallized from water-ethanol. Yield 31 mg.; m. p. 237~239°C (decomp.); $[\alpha]_D^{15}$ +57.0° (*c* 1, in water).

Found: C, 45.87; H, 6.78; N, 10.86. Calcd. for $C_5H_9NO_3$: C, 45.80; H, 6.92; N, 10.68%.

The above product (1 mg.) dissolved in citrate buffer pH 3.20 (1 ml.) was passed through a column of Dowex 50-X8 (0.9×50 cm.)[12,13] maintained at 37°C, and eluted (4 ml./hr.) with the same buffer. The effluent was collected in 1 ml. fractions and each fraction was analyzed by ninhydrin colorimetry[14]. The elution pattern of the product had a single peak which corresponded to allohydroxyproline and no other peak was observed, while the authentic mixture of hydroxyproline and allohydroxyproline[15] was completely resolved into two peaks under the same condition (Fig. 2).

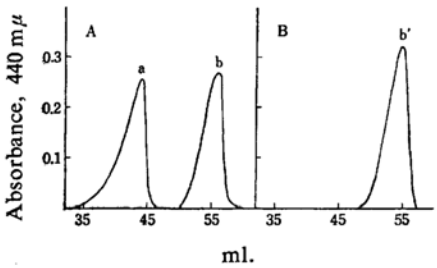

Fig. 2. The elution pattern of hydroxyproline (HP) and allohydroxyproline (AHP) from a column of Dowex-50 (0.9×50 cm.) at pH 3.20 and 37°C.
A: Authentic mixture of L-HP (a) and D-AHP (b)
B: D-AHP (b') prepared by cyclization from γ-hydroxy-L-ornithine

12) S. Moore and W. H. Stein, *J. Biol. Chem.*, **192**, 663 (1951).
13) K. A. Piez, *J. Biol. Chem.*, **207**, 77 (1954).
14) E. W. Yemm and E. C. Cocking, *Analyst*, **80**, 209 (1955); *Biochem. J.*, **58**, xii (1954).
15) The author is indebted to Dr. S. M. Birnbaum and Dr. M. Winitz for a generous supply of the sample of hydroxy-L-proline and allohydroxy-D-proline, respectively.

Composition of the Reaction Mixture with Nitrosyl Chloride. — A solution of γ-hydroxy-L-ornithine hydrochloride (100 mg.) was treated with a nitrosyl chloride solution (3 ml.) as described above. The reaction mixture was evaporated up to dryness in vacuo and further dried in a vacuum desiccator on potassium hydroxide until it reached a constant weight. It was analyzed for total nitrogen by the micro Kieldahl method, for the liberation of carbon dioxide with Chloramine T at 25°C in a Warburg manometer and for chloride by the titration with silver nitrate[16]. The following values were obtained: 5.32% total nitrogen, 4.12% CO_2 and 13.43% Cl^-. The results were summarized in Table I.

Table I. Composition of the reaction mixture with nitrosyl chloride

Symbol*	Mol. wt.	Theory, % N_2	CO_2	Cl^-	Composition of the reaction mixture, %
W	221.09	12.7	19.9	32.1	20
X	204.06	6.9	0	17.4	39
Y	204.06	6.9	21.6	17.4	1
Z	187.03	0	0	0	40

* W: γ-Hydroxyornithine·2HCl
X: α-Chloro-δ-amino acid·HCl
Y: δ-Chloro-α-amino acid·HCl
Z: α,δ-Dichloro acid

The figures for W, X, Y and Z in Table I were calculated from the following equations: 12.7W+6.9X+6.9Y=5.32; 19.9W+21.6Y=4.12; 32.1W+17.4X+17.4Y=13.43; W+X+Y+Z=1.

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16) S. Ueo and S. Sakamoto, *J. Pharm. Soc. Japan* (*Yakugaku Zasshi*), **58**, 711 (1938).