γ-Hydroxyarginine, a New Guanidino Compound from a Sea-cucumber.
I. Isolation and Identification

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In the course of studies[1] on the extractable naturally occurring guanidino compounds by means of paper chromatography using Makisumi's method[2], the author found an unknown guanidino compound from the extract of a sea-cucumber, *Polycheira rufescens*, and isolated it as a hydrochloride. It was hydrolyzed, though somewhat more slowly than arginine, but completely by the action of arginase, into urea and an unknown amino compound. In 1953, Robin[3] oxidatively prepared γ-guanidinobutyric acid from L-arginine with the action of a homogenate obtained from the hepatopancreas of *Mytilus edulis*. The homogenate from this species appears to contain L-amino acid oxidase only as amino acid oxidase[4]. The isolated compound could also be converted enzymically and chemically into its oxidation products whcih were positive for the Sakaguchi reaction. Further characterizations of the isolated compound and its degradation products led the author to assume the unknown guanidino compound to be γ-hydroxyarginine. Consequently, the unknown amino compound produced from the isolated compound by the action of arginase should be γ-hydroxyornithine.

On the other hand, γ-hydroxyornithine was synthesized according to Tomita's method[5] which was somewhat modified by the author and then transformed into γ-hydroxyarginine by guanidization with S-methylisothiourea. The provisional structures of the isolated guanidino compound and the amino compound produced therefrom were proved to be correct by comparing them on a paper chromatogram with the synthetic γ-hydroxyarginine and γ-hydroxyornithine, respectively.

Experimental

Isolation of the Unknown Guanidino Compound.—The sea-cucumbers used as the material were collected at the tidal zone near the Amakusa Marine Laboratory, the Faculty of Science, Kyushu University. The washed materials (43.5 kg.) removed from their internal organs were chopped in a meat-chopper and introduced into hot water (45 l., 70∼80°C) for the purpose of extraction for 2 hr. with stirring. The residue separated from the extract was re-extracted in the same manner. The combined extract, deproteinized with a hot saturated basic lead acetate solution and freed from the lead, was passed through a column of Amberlite IR-120 (H-form). After washing the column with water, the adsorbed fraction was eluted with 2 N aqueous ammonia. The eluate, after removal of the ammonia by evaporation in vacuo, was again passed through a column of Amberlite IR-120 (ammonium form) to adsorb basic substances only. The elution from the washed column was carried out by using the same aqueous ammonia. The eluate freed from the ammonia was made up to 300 ml. with water and neutralized with 6 N hydrochloric acid. The solution, after addition of flavianic acid (50 g.), was heated for 30 min. on a boiling water bath. When the resulting solution was allowed to stand overnight in an

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[1] H. Sasaki, Y. Fujita, S. Makisumi and S. Shibuya, *J. Jap. Biochem. Soc.* (*Seikagaku*), **30**, 642 (1958).

[2] S. Makisumi, *J. Chem. Soc. Japan, Pure Chem. Sec.* (*Nippon Kagaku Zasshi*), **73**, 737 (1952).

[3] Y. Robin, *Bull. Soc. Chim. Biol.*, **35**, 285 (1953).

[4] H. Blaschko and D. B. Hope, *Biochem. J.*, **62**, 335 (1956).

[5] M. Tomita, *Z. physiol. Chem.*, **158**, 58 (1926).

ice-box, a flavianate crystallized out. The flavianate was separated and washed with a small amount of cold water. It consisted almost entirely of the unknown guanidino compound, except for slight contamination with arginine. The product was recrystallized from hot water, until its paper chromatogram showed only one spot with the Sakaguchi or ninhydrin reaction. The purified flavianate was dissolved in hot water (100 ml.), and it was treated with a barium hydroxide solution to remove the flavianic acid moiety in the usual manner. The filtrate freed from the excessive barium was evaporated up to dryness in vacuo. The residue was extracted repeatedly with 50% hot ethanol. The extract, after evaporation to dryness, was dissolved in water (50 ml.), and the resulting solution was brought to pH 6~7 with 1 N hydrochloric acid. It was decolorized with charcoal and concentrated to a syrup in vacuo. The syrup was dissolved in 50% hot ethanol (10 ml.), and then absolute ethanol was added to it until the solution became opalescent. When the solution was kept overnight in a refrigerator, colorless plate crystals of the guanidino compound appeared. The compound was dissolved in a minute amount of 70% hot ethanol and then recrystallized with the addition of absolute ethanol. The yield was about 2.7 g.; m.p. 190~191°C (decomp.); $[\alpha]_{D}^{23}$ +5.4° (c 2, in 5 N hydrochloric acid).

Anal. Found: C, 31.75; H, 6.95; N, 24.65; Cl, 15.68. Calcd. for $C_6H_{14}N_4O_3 \cdot HCl$: C, 31.79; H, 6.67; N, 24.72; Cl, 15.64%.

It gave the Sakaguchi, the Lieben (iodoform), and ninhydrin reaction, indicating the presence of guanidino, alcoholic hydroxy (on the basis of the hydrogen content, alcoholic hydroxy group is superior to ketonic), and amino groups, respectively.

Enzyme Actions on the Guanidino Compound.—*1. Arginase.*—The arginase used in this experiment was a partially purified beef liver preparation of Step D according to Greenberg[6]. The guanidino compound was incubated to hydrolyze with arginase at pH 9.5 and 35°C for 24 hr. The incubate, after deproteinization with basic lead acetate, was fractionally purified by using Amberlite IR-120 (H- and ammonium forms). Since the purified fraction obtained here showed only one spot on a paper chromatogram with ninhydrin reaction, it was not necessary to carry out further purification. The fraction was decolorized with charcoal and crystallized similarly as in the case of isolation of the guanidino compound. The yield of the recrystallized compound was 280 mg. from 500 mg. of the guanidino compound; m. p. 183°C (decomp.); $[\alpha]_{D}^{32}$ +21.0° (c 2, in water).

Anal. Found: C, 32.65; H, 6.86; N, 15.2.1 Calcd. for $C_5H_{12}N_2O_3 \cdot HCl$: C, 32.58; H, 7.10; N, 15.17%.

This gave ninhydrin- and iodoform reaction but not the Sakaguchi reaction. The effluent passed through a column of Amberlite IR-120 (H^+ form) was again passed through a column of Amberlite (OH^- form), and from this effluent urea was identified as dixanthylurea and urea nitrate.

2. L-Amino acid oxidase.—The guanidino compound was incubated with the enzyme preparation obtained from the hepatopancreas of *Mytilus edulis* according to Robin's method[3] at pH 6.2 and 35°C for 24 hr. under aeration. The incubate acidified with acetic acid to pH 3~4 was boiled for 10 min. and filtered. To the filtrate (200 ml.) was added 30% hydrogen peroxide (1 ml.) for further oxidation of the keto acid to be produced by oxidative deamination from the guanidino compound, and it was allowed to stand for 15 hr. at room temperature. In this condition the guanidino compound itself is not oxidized at all with hydrogen peroxide. The resulting solution was passed through a column of Amberlite IR-120 (H-form), the column was washed with water, and then the adsorbed oxidation product was eluted from it by using 2 N aqueous ammonia. The eluate was evaporated up to dryness, and the residue was dissolved in 50% hot ethanol. When the solution was cooled in an ice-box, colorless needle crystals of the product appeared. The yield was 100 mg. from 300 mg. of the material; m. p. 238~240°C (decomp.); $[\alpha]_{D}^{22}$ −15.5° (c 2, in 5 N hydrochloric acid).

Anal. Found: C, 37.26; H, 6.88; N, 26.08. Calcd. for $C_5H_{11}N_3O_3$: C, 37.25; H, 7.11; N, 26.25%.

This compound gave the Sakaguchi and iodoform reaction, but not ninhydrin reaction.

Identification of the Oxidation Product.—*1. Reduction with hydroiodic acid.*—The oxidation product (10 mg.) was heated with hydroiodic acid (d=1.7, 0.5 ml.) for 2 hr. at 140°C in a sealed tube. After removal of the hydroiodic acid, the solution was tested by paper chromatography. On the basis of the result, it was found that the product was reduced to a compound which was identified as γ-guanidinobutyric acid (Table I).

TABLE I. R_f VALUES OF REDUCTION PRODUCT WITH HYDROIODIC ACID AND γ-GUANIDINOBUTYRIC ACID

	Solvent			
	I	II	III	IV
Reduction product	0.55	0.52	0.62	0.43
γ-Guanidinobutyric acid	0.55	0.52	0.62	0.43

One dimensional ascending method. Toyo-Roshi No. 52. Solvent: I, n-Butanol : Acetic Acid : Pyridine : Water = 40 : 10 : 10 : 20 (vol. %); II, n-Butanol : Acetic Acid : Pyridine : Water=30 : 6 : 24 : 20 (vol. %); III, n-Butanol : Acetic Acid : Water=40 : 10 : 20 (vol. %); IV, n-Propanol : Water=70 : 30 (vol. %).
The R_f values shown in Tables II and III were also obtained by using the same procedures.

2. Comparison with the synthetic γ-guanidino-β-hydroxybutyric acid.—In a preliminary experiment, it was observed paper-chromatographically

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[6] D. M. Greenberg, "The Enzymes", vol. I, edited by J. B. Sumner and K. Myrbäck, Academic Press Inc., New York (1951), p. 905.

that the naturally occurring guanidino compound was oxidatively degraded with barium permanganate to guanidine and glycocyamine by way of an intermediate which was later proved to be identical with the oxidation product formed by the action of L-amino acid oxidase. The production of glycocyamine led to the conclusion that the alcoholic hydroxy group should situate in β- or γ-, probably γ-position, but not δ-position of the guanidino compound; accordingly, the structure of the enzymatic oxidation product before reduction by hydroiodic acid is to be γ-guanidino-β-hydroxybutyric acid. The conclusion was supported satisfactorily by comparing the paper chromatographic behaviors of the oxidation product with those of the synthetic γ-guanidino-β-hydroxybutyric acid[7,8] (Table II).

TABLE II. R_f VALUES OF OXIDATION PRODUCT WITH OXIDASE AND γ-GUANIDINO-β-HYDROXYBUTYRIC ACID

	Solvent			
	I	II	III	IV
Oxidation product	0.40	0.38	0.48	0.35
γ-Guanidino-β-hydroxybutyric acid	0.40	0.38	0.48	0.35

Synthesis of γ-Hydroxyornithine.—Finely powdered sodium ethyl acetylaminomalonate (24 g.) and γ-phthalimido-β-hydroxypropyl chloride (24 g.) were thoroughly mixed, and the mixture was heated at 140∼150°C for 5 hr. with vigorous stirring. To the extremely viscous reaction mixture was added concentrated hydrochloric acid (100 ml.), and it was refluxed for 10 hr. and then allowed to stand overnight. The filtrate removed from the phthalic acid was concentrated in vacuo. Dilution with water and evaporation in vacuo were repeated three times to remove the excess of hydrochloric acid as much as possible. The syrup obtained thus was dissolved in water (200 ml.), and from this solution a basic fraction was separated by using Amberlite IR-120 (H- and ammonium form) in the same manner as described in the isolation procedures for the guanidino compound. The basic fraction, after being passed through a column of finely powdered Amberlite IR-120 (ammonium form, 100∼200 mesh, 3 cm. × 40 cm.), was fractionally eluted and collected as follows. The column was washed with water, and the adsorbed substances were developed and eluted with 0.2 N aqueous ammonia. The eluate collected between 350 and 650 ml. was concentrated to a syrup in vacuo. The syrup was dissolved in a small amount of water, and the solution was adjusted to pH 6∼7 with 1 N hydrochloric acid, decolorized with charcoal, and again concentrated to a syrup in vacuo. The syrup dissolved in a small amount of 50% hot ethanol was subjected to crystallization in an ice-box with the addition of absolute ethanol. After 2 days, the crystals of γ-hydroxyornithine hydrochloride were obtained. Yield, 8%; m. p. 181∼183°C (decomp.).

Anal. Found: C, 32.72; H, 7.30; N, 15.65. Calcd. for $C_5H_{12}N_2O_3 \cdot HCl$: C, 32.53; H, 7.10; N, 15.17%.

Synthesis of γ-Hydroxyarginine.—γ-Hydroxyornithine hydrochloride (370 mg.) and S-methylisothiourea (280 mg.) were dissolved in 1 N sodium hydroxide (5 ml.). The solution which had been left to stand for 5 days at room temperature was passed through a column of Amberlite IR-120 (H- form). After washing the column with water, the adsorbed compounds were eluted with 2 N ammonia. The eluate was evaporated up to dryness in vacuo and the residue was dissolved in water (10 ml.). The solution was heated on a boiling water bath for 30 min. with the addition of flavianic acid (2 g.). On keeping the solution for 2 days in an ice-box, a flavianate of γ-hydroxyarginine crystallized out. The flavianate, after removal of the flavianic acid moiety in the usual manner, was converted into γ-hydroxyarginine hydrochloride. Yield, 57%; m. p. 189∼190°C (decomp.).

Anal. Found: C, 31.88; H, 6.76; N, 24.83. Calcd. for $C_6H_{14}N_4O_3 \cdot HCl$: C, 31.79; H, 6.67; N, 24.72%.

Comparison of the Naturally Occurring Guanidino and Amino Compounds with the Synthetic Ones. — The synthetic γ-hydroxyarginine and γ-hydroxyornithine showed the same paper chromatographic behaviors as those of the naturally occurring guanidino compound and the amino compound produced therefrom by the action of arginase as shown in Table III.

TABLE III. R_f VALUES OF NATURALLY OCCURRING GUANIDINO AND AMINO COMPOUNDS AND SYNTHETIC ONES

	Solvent			
	I	II	III	IV
Guanidino compound	0.16	0.19	0.20	0.17
γ-Hydroxyarginine	0.16	0.19	0.20	0.17
Amino compound	0.12	0.15	0.17	0.13
γ-Hydroxyornithine	0.12	0.15	0.18	0.13

Summary

A new guanidino compound was isolated from a sea-cucumber, *Polycheira rufescens*, and identified as γ-hydroxyarginine. γ-Hydroxyornithine and γ-guanidino-β-hydroxybutyric acid produced enzymically from the guanidino compound were also isolated and identified. It was, further, concluded that the new guanidino compound susceptible to arginase and L-amino acid oxidase should be γ-hydroxy-L-arginine, although the configuration of the γ-hydroxy group was not yet decided.

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7) M. Tomita, *Z. physiol. Chem.*, **124**, 253 (1923).

8) T. Fukagawa, ibid., **231**, 203 (1935).

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