Poly-L-histidine and the Copolymer of L-Histidine with L-Glutamic Acid. Their Synthesis and Catalytic Activity on the Hydrolysis of *p*-Nitrophenyl Acetate*1

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It has previously been established that the histidine residue plays an important role in certain proteolytic enzymes. Poly-L-histidine is, therefore, an interesting material to use as a model compound in order to study the catalytic behavior of the histidine residue.

Poly-L-histidine has been prepared by Patchornik et al.[1] However, better results have been achieved in the present experiments, where the synthesis of homo- and co-polymers of L-histidine by the carbothiophenyl method[2] will be described. An attempt to polymerize 1-benzyl-α, *N*-carbothiophenyl-L-histidine with γ-benzyl-L-glutamate *N*-carboxy anhydride (NCA) and their catalytic activities will also be described.

The Synthesis and Properties of Poly-L-histidine.—Two series of reactions are outlined in scheme A. In one process, the free imidazole group was used, and in the other, the imidazole imino group, which was protected by a benzyl group.

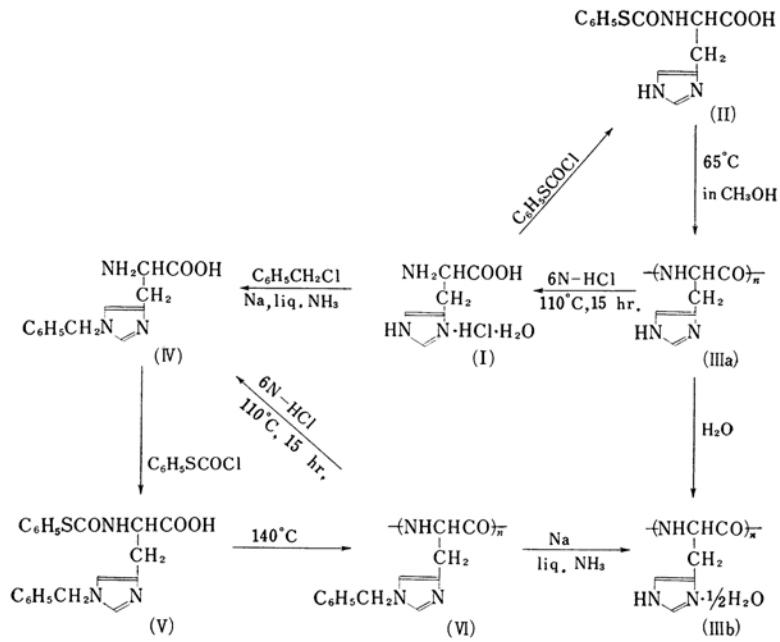
L-Histidine hydrochloride (I) was treated with sodium methylate to give free L-histidine, from which α, *N*-carbothiophenyl-L-histidine (II) was obtained in a 43% yield by the action of carbothiophenyl chloride. II was polymerized in methanol at 65°C for 3 weeks to yield poly-L-histidine (mol. wt.$\fallingdotseq$2040, $n\fallingdotseq$15)*2 (IIIa). 1-Benzyl-α, *N*-carbothiophenyl-L-histidine (V) was obtained from 1-benzyl-L-histidine (IV) in a 81% yield by a reaction with carbothiophenyl chloride in an anhydrous basic medium. In order to obtaine poly-1-benzyl-L-histidine (mol. wt.$\fallingdotseq$2830, $n\fallingdotseq$12.5)*2 (VI), V was polymerized at 140°C in vacuo for ten hours. The reduction of VI with metallic sodium in liquid ammonia gave poly-L-histidine (IIIb). Since the average degree of the polymerization of IIIb was shown to be ca. 13.3 (mol. wt.$\fallingdotseq$1940),*2 it was concluded that the average chain length of VI was unaffected by the reduction. The insolubility of

*1 T. Saito and J. Noguchi, Partially presented at the 15th Annual Meeting of the Chemical Society of Japan, Kyoto, April, 1962.

1) A. Patchornik, A. Berger and E. Katchalski, *J. Am. Chem. Soc.*, **79**, 5227 (1957).

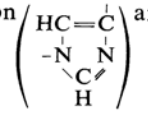
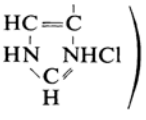
2) J. Noguchi, *J. Chem. Soc. Japan, Pure Chem. Sec. (Nippon Kagaku Zassi)*, **74**, 961 (1953).

*2 The molecular weight was determined by a terminal end group titration with anhydrous sodium methylate.



Scheme A

VI in an alkaline solution can perhaps be explained by the absence of any free imidazole group in VI, and the solubilities of IIIa and IIIb in a dilute acid and in a strongly alkaline solution (above 3 N) showed that they behaved as polyampholytes. IIIb contained half a molecule of crystalline water per histidine residue; this could not be removed by drying at 100°C for 5 hr. in vacuo on phosphorus pentoxide.[1] However, neither water nor methanol was contained in IIIa. When IIIa, ($[\alpha]_D = -19.5°$),[1] was hydrolyzed with acid, L-histidine hydrochloride monohydrate resulted; this was proved to be optically pure ($[\alpha]_D = +13.0°$).[3] The configuration of the histidine residue in IIIa was, therefore, unaffected in the process of the synthesis.

The ultraviolet absorption spectra of IIIa showed the absorption maximum at 294 mμ ($\varepsilon = 29.8$) in 1 N sodium hydroxide, and at 275 mμ ($\varepsilon = 12.8$) in 1 N hydrochloric acid respectively. The former absorption band may be attributed to the imidazolium ion  and the latter, to the imino group 

The Synthesis of the Copolymer of L-Histidine with L-Glutamic Acid.—The synthetic process of the copolymer is shown in scheme B.

V + γ-Bz-L-GluNCA $\xrightarrow{140°C}$
-(-1-Bz-L-His, γ-Bz-L-Glu-)$_n$- (VII) $\xrightarrow[\text{liq. NH}_3]{\text{Na}}$
[-(-L-His, L-Glu(NH$_2$)-)$_n$-] (VIII) $\longrightarrow$
-(-L-His, L-Glu-)$_n$- (IX)

Bz; benzyl

Scheme B

The bulk copolymerization of the equi-molar V and γ-benzyl-L-glutamate NCA gave the copoly(1-benzyl-L-histidine, γ-benzyl-L-glutamate)$_n$ (1 : 1) (VII) with an average degree of polymerization of ca. 25 (mol. wt. ≒ 11300)*2 and in a 86% yield. Copoly(L-histidine, L-glutamic acid)$_n$ (1 : 1) (IX) was obtained from VII by reduction with metallic sodium in liquid ammonia in order to remove the protected groups. Van Slyke terminal nitrogen analysis for IX indicated that neither a remarkable decrease in its chain length nor the cyclization of the *N*-terminal residue had taken place and that the average degree of the polymerization of IX was ca. 20 (mol. wt. ≒ 5320). Poly-β-benzyl-L-aspartate was converted to the β-amide of the polymer (polyasparagine) by treatment with liquid ammonia,[4] analogously,

3) L-Histidine HCl H_2O: $[\alpha]_D^{24} = +13.15°$ (in 6 N hydrochloric acid), S. Akabori and S. Mizushima, "Protein Chemistry" (Tanpakushitu Kagaku), Vol. I, Kyoritsu Shuppan K. K., Tokyo (1952), p. 112.

4) J. Noguchi, T. Saito and M. Asai, *J. Chem. Soc. Japan, Pure Chem. Sec.* (*Nippon Kagaku Zasshi*), **81**, 620 (1960).

poly-γ-methyl-L-glutamate was converted to the γ-hydrazide of the polymer by treatment with hydrazine.[5] The elementary analysis for IX, however, proved that the glutamate residues were reduced to free glutamic acid residues. The discrepancy mentioned above might be due to the fact that a possible intermediate, VIII, was hydrolyzed to give IX in an alkaline treatment during the isolation of IX.

Catalytic Activity on the Hydrolysis of *p*-Nitrophenyl Acetate. — In a previous work,[6] catalytic activity on the hydrolysis of *p*-nitrophenyl acetate was examined in many histidine-containing oligopeptides and some polypeptides, and it was found that amino acids which were adjacent to the *N*- or the *C*-terminal of histidine through the peptide linkage increased the activity of histidine. However, the activity was not increased by the polymerization of these oligomers. For example, the activity of carnosine (β-alanyl-L-histidine) was 2.40 times that of histidine, while that of ε-aminocaproylhistidine was much the same as that of histidine. Each of the polymers prepared from carnosine and ε-aminocaproylhistidine (i.e., polycarnosine (poly-β-alanyl-L-histidine) and poly ε-aminocaproylhistidine), had the same activity as its monomer.

The activities of IIIa and IX on the hydrolysis of *p*-nitrophenyl acetate were determined by on the basis of the rate of appearance of *p*-nitrophenylate ions. It was observed that the catalytic activities of IIIa and IX were, respectively, 1.43 and 1.45 times that of L-histidine. The ratio, 1.43 of IIIa which has an average degree of polymerization ($n \fallingdotseq 15$), agreed with that (1.40) of the low molecular weight preparation ($n \fallingdotseq 3.3$).[6],*3 The ratio of IX, 1.45, agreed approximately with the average of the ratios, 1.64 in L-glutamyl-L-histidine and 1.12 in L-histidyl-L-glutamic acid. The activation of the hydrolytic effect for the substrate was, therefore, not affected by the increase in the degree of polymerization. The results concerning IX, however, seemed to suggest that L-histidine and L-glutamic acid were arranged irregularly in the copolymer chain.

Experimental

All melting points are uncorrected.

***α*, *N*-Carbothiophenyl-L-histidine (II).** — L-Histidine hydrochloride monohydrate (10 g.) was added to 2 N sodium methylate in methanol (47.8 ml.) at 0—5°C, and then carbothiophenyl chloride (9.0 g.) was vigorously stirred into the solution at −5°C. The resulting precipitates were filtered, and from the filtrate, II (m. p. 165—167°C (decomp.)) was obtained in a 48% (6.0 g.) yield.

Found: C, 53.5; H, 4.50; N, 14.4; S, 11.3; equiv. wt. 286 and 149 (determined by anhydrous perchloric acid and sodium methylate titration respectively). Calcd. for $C_{13}H_{13}O_3N_3S$: C, 53.6; H, 4.48; N, 14.4; S, 11.0%; equiv. wt. 291.3, 145.6.

Poly-L-histidine (IIIa). — II (1.0 g.) was dissolved in anhydrous methanol (15 ml.), and the atmosphere was replaced with carbon dioxide. The polymerization was carried out in a sealed tube at 65°C for 3 weeks. The resulting precipitate was collected by centrifugation, washed with methanol, and dried to yield 0.3 g. (64%) of IIIa, which gave positive biuret and Pauli reactions, $[\alpha]_D^{32} = -19.5°$ (*c* 0.77, glacial acetic acid). IIIa was soluble in glacial acetic acid and dichloroacetic acid, but insoluble in alcohol, acetone, and ether. It also dissolved in dilute hydrochloric acid and 3 N sodium hydroxide, but it was insoluble in water.

Found: C, 52.1; H, 5.17; N, 30.2; equiv. wt. 135 (determined by perchloric acid titration). Calcd. for $C_6H_7ON_3$: C, 52.5; H, 5.15; N, 30.6%; equiv. wt. 137.

1-Benzyl-*α*, *N*-carbothiophenyl-L-histidine (V). — 1-Benzyl-L-histidine[7] (10 g.) was treated with carbothiophenyl chloride (7.7 g.) in the presence of 2 N sodium methylate in methanol (20.4 ml.) at −5°C. The precipitate was collected by filtration, washed with warm water and then with distilled water, and dried to yield 12.5 g. (81%) of V (m. p. 143°C).

Found: C, 62.5; H, 5.01; N, 11.1; S, 8.42; equiv. wt. 378 and 189 (determined by perchloric acid and sodium methylate titration respectively). Calcd. for $C_{20}H_{19}O_3N_3S$: C, 63.0; H, 4.99; N, 11.0; S, 8.40%; equiv. wt. 381.5, 190.7.

Poly-1-benzyl-L-histidine (VI). — V (2 g.) was added to dioxane (2 ml.), and the atmosphere was replaced with nitrogen. Polymerization was carried out at 140°C for 30 min., and then it was continued in vacuo at the same temperature for 10 hr. The solidified reaction mixture was triturated with acetone and ether. The precipitate was contrifugated, washed with ether, and dried to yield 1.1 g. (93%) of VI, which gave a negative Pauli reaction, $[\alpha]_D^{32} = +43.5°$ (*c* 1.86, benzyl alcohol), $[\alpha]_D^{25} = -20.3°$C (*c* 0.94, glacial acetic acid). VI was soluble in chloroform, *N*, *N*-dimethylformamide, benzyl alcohol, glacial acetic acid, and dichloroacetic acid. VI was dissolved in 0.1 N hydrochloric acid and precipitated from this solution by neutralization with alkali, but it was insoluble in concentrated hydrochloric acid and caustic alkali.

Found: C, 68.3; H, 5.83; N, 18.4; equiv. wt. 230 (determined by perchloric acid titration). Calcd. for $C_{13}H_{13}ON_3$: C, 68.7; H, 5.77; N, 18.5%; equiv. wt. 227.3.

Poly-L-histidine Hemi-hydrate (IIIb). — VI ($n \fallingdotseq 12.5$) (1.0 g.) was suspended in anhydrous liquid

5) V. Bruckner, J. Kovacs and K. Kovacs, *J. Chem. Soc.*, **1953**, 1512.

6) J. Noguchi and T. Saito, "Polyamino Acid, Polypeptides, and Proteins," Ed. by M. A. Stahmann, University of Wisconsin Press, Madison (1962), p. 313.

*3 In this case, each adjoining histidine residue is considered to be activated up from 1.00 to 1.40.

7) V. du Vigneaud and O. K. Behrens, *J. Biol. Chem.*, **117**, 27 (1937).

ammonia (50 ml.); finely divided metallic sodium (ca. 2 g.) was added to the mixture over a period of 15 min. until a blue color persisted for at least 5 min. The excess sodium was then discharged with ammonium chloride, and the ammonia was allowed to evaporate spontaneously. The residue was dissolved in 1 N hydrochloric acid (10 ml.). After the solution had been extracted with 20 ml. of ether, the aqueous layer was filtered and neutralized with 1 N sodium hydroxide. The precipitates were collected by centrifugation and washed with water. The product was dried in vacuo on phosphorus pentoxide at 100°C for 5 hr. to yield 0.5 g. (83%) of IIIb. which gave positive biuret and Pauli reactions, $[\alpha]_D^{32} = -13.3°$ (*c* 1.24, glacial acetic acid). The solubilities of IIIb were the same as those of IIIa.

Found: C, 48.8; H, 5.59; N, 28.9. Calcd. for $C_6H_7ON_3\ 1/2\ H_2O$: C, 49.3; H, 5.50; N, 28.8%.

The Hydrolysis of Poly-1-benzyl-L-histidine.—A suspension of VI (0.1 g.) in 6 N hydrochloric acid (2.0 ml.) was heated at 110°C for 15 hr. in a sealed tube. From the hydrolysate, 0.08 g. of 1-benzyl-L-histidine melting at 241°C was obtained by neutralization with 6 N sodium hydroxide and recrystallization from water.

Found: Equiv. wt. 240 and 119 (determined by sodium methylate and perchloric acid titration respectively). Calcd. for $C_{13}H_{15}O_2N_3$: Equiv. wt. 245, 122.5.

The Hydrolysis of Poly-L-histidine.—IIIa (0.1 g.) was hydrolyzed under the same conditions as VI. When the hydrolysate was worked up in the usual manner, it gave L-histidine hydrochloride monohydrate (0.07 g., m. p. 160°C, $[\alpha]_D^{25} = +13.0°$ (*c* 1.15, 6 N hydrochloric acid)).

Poly-L-histidine Hemi-hydrate (IIIb) from Poly-L-histidine (IIIa).—IIIa (0.3 g.) was dissolved in 1 N hydrochloric acid (2.0 ml.) and the solution was filtered, then 1 N sodium hydroxide (2.0 ml.) was added to the filtrate. The precipitates were collected by centrifugation and washed with cold water. The analytical sample was dried on phosphorus pentoxide in vacuo at 100°C for 5 hr.

Found: C, 48.9; H, 5.54; N, 28.3; equiv. wt. 143 (determined by perchloric acid titration). Calcd. for $C_6H_7ON_3\ 1/2\ H_2O$: C, 49.3; H, 5.50%; N, 28.8%; equiv. wt. 146.

Copoly(1-Benzyl-L-histidine, γ-Benzyl-L-glutamate)$_n$ (1:1) (VII).—A mixture of V (1.00 g.), γ-benzyl-L-glutamate NCA[8] (0.69 g.), and 2.0 ml. of dioxane was heated under a nitrogen atmosphere at 140°C for 24 hr. The product was triturated with acetone and ether, and collected by centrifugation to yield 1.0 g. (86%) of VII.

Found: C, 67.0; H, 5.93; N, 12.3; equiv. wt. 427 (determined by perchloric acid titration). Calcd. for $C_{25}H_{26}O_4N_4$: C, 67.3; H, 5.87; N, 12.6%; equiv. wt. 446.5.

Copoly(L-Histidine, L-Glutamic Acid)$_n$ (1:1) (IX).—To a suspension of VII (0.9 g.) in liquid ammonia (ca. 50 ml.), finely divided metallic sodium was added over a period of 15 min. until a blue color persisted for at least 5 min. The excess sodium was discharged with ammonium chloride, and the ammonia was allowed to evaporate spontaneously. The residue was then dissolved in water (20 ml.) and extracted twice with 20 ml. portions of ether. The aqueous layer was filtered and neutralized with 6 N hydrochloric acid to pH 6 and concentrated to dryness in vacuo. The residue was redissolved in water (20 ml.) and dialyzed. Then the solution was lyophilized to yield 0.4 g. (67%) of IX.

Found: C, 47.7; H, 5.51; N, 20.6; equiv. wt. 267 and 260 (determined by perchloric acid and sodium methylate titration respectively). Calcd. for $C_{11}H_{14}O_4N_4\ 1/2\ H_2O$: C, 48.0; H, 5.45; N, 20.4%; equiv. wt. 275.3.

The Determination of the Amino Acid Contents of IX.—The histidine content of IX was determined by the reaction of the histidine residue with diazotized sulfanilic acid, followed by colorimetric analysis. After the hydrolysis of IX with 6 N hydrochloric acid at 110°C for 10 hr., both histidine and glutamic acid were assayed by the colorimetric determination of paper chromatography, using ninhydrine as a developer. It was demonstrated that they were in the ratio of ca. 1:1.

The Determination of Catalytic Activity.—The catalyst, IIIa or IX, in the concentration of 100 mg./l. and the substrate, *p*-nitrophenyl acetate, in the concentration of 3.311 mmol./l. were incubated for 30 min. at 25°C in a 1/30 mol. phosphate buffer solution of pH 6.4 containing 10% (V/V) acetone in order to dissolve the *p*-nitrophenyl acetate. The liberated *p*-nitrophenylate ions were determined by measuring the increases in optical density at 400 mμ using a Hitachi EPU type 2 spectrometer.

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8) W. E. Hanby, S. G. Waley and J. Watson, *J. Chem. Soc.*, 1950, 3239.