Helix-Coil Stability Constants for the Naturally Occurring Amino Acids in Water. 22. Histidine Parameters from Random Poly[(hydroxybutyl)glutamine-*co*-L-histidine]

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ABSTRACT: The synthesis and characterization of water-soluble random copolymers containing L-histidine with N^5-(4-hydroxybutyl)-L-glutamine, and the thermally induced helix-coil transitions of these copolymers in water and in aqueous 0.5 N KCl solution, are described. The incorporation of both charged and uncharged L-histidine was found to decrease the helix content of the polymer in water, even in the presence of 0.5 N KCl, which effectively shields the electrostatic repulsions among charged L-histidine residues in the α-helical and coil conformations. The Zimm-Bragg parameters σ and s for the thermally induced helix-coil transition of charged and uncharged poly(L-histidine) in water and in 0.5 N KCl were deduced from an analysis of the melting curves of the copolymers in the manner described in earlier papers. At physiological temperature, 37 °C, the helix-coil stability constants, s, for charged and uncharged L-histidine are similar, viz., 0.66 and 0.70, respectively. This demonstrates that ionization-deionization of histidine residues does not in itself affect the conformational stabilities of proteins. The synthesis of *N*-acetyl-*N'*-methylhistidinamide and its titration, as well as that of the copolymers, in 0.1 N KCl are described. Finally, the values of σ and s, determined for 18 amino acids from experiments on host-guest random copolymers, and those obtained for cystine and proline by extrapolation, are summarized here.

Introduction

Since the pK_a of the imidazole group of histidine residues (His) is around 6.6,[1] both the charged and uncharged forms of His are present in proteins at neutral pH, and their concentration ratio is very sensitive to small changes in pH under physiological conditions. Thus, it is of interest to determine the effect of the state of ionization of His residues on the conformational stability of proteins. Therefore, this paper is devoted to a study of the influence of L-histidine on the thermal stability of the *α-helix*, i.e., on the Zimm-Bragg parameters[2] σ and s for charged and uncharged His. For this purpose, we make use of the "host-guest" technique that was applied previously to evaluate the helix-coil stability constants for 17 other amino acids in water.[3-23]

We have synthesized random copolymers having N^5-(4-hydroxybutyl)-L-glutamine (HBG) as the major component (host residue) and L-histidine as the minor component (guest residue). Such copolymers are water soluble, are α-helical, and undergo a thermally induced helix-coil transition between 0 and 75 °C. The transition curve for the host homopolymer (PHBG), with nonionizable side chains, is influenced by the presence of the guest residues. By studying the thermally induced helix-coil transitions of the copolymers in water and in 0.5 N KCl solution at pH 3.0 and in water at pH 9.0, we can evaluate the Zimm-Bragg parameters[2] σ and s for charged and uncharged L-histidine, respectively.

The synthesis of water-soluble random copolymers of L-His with HBG is described in the Experimental Section, and the experimental characterization of these copolymers and their melting and titration behavior in aqueous solution are presented in the Results section along with the titration curve of *N*-acetyl-*N'*-methylhistidinamide. Finally, the determination of the Zimm-Bragg parameters for charged and uncharged L-His, obtained from an analysis of the melting behavior in aqueous solution, is described in the Discussion section. These results are compared with those from previous conformational studies of L-histidine in polypeptides and proteins.

Experimental Section

Preparation and Characterization of the Copolymers. The protected copolymers were prepared by copolymerization of the *N*-carboxyanhydrides (NCA) of N^{im}-isobutyloxycarbonyl-L-histidine [His(N^{im}-Ioc)] and γ-benzyl L-glutamate in dioxane with sodium methoxide as an initiator; the resulting copolymers were then converted to a water-soluble form by treatment with hydroxybutylamine (4-amino-1-butanol) under conditions that simultaneously deblocked the histidyl imidazole groups.

A. Materials. L-Glutamic acid and N^{α}-benzyloxycarbonyl-L-histidine (N^{α}-Z-L-His) were purchased from Sigma Chemical Co. and Vega Biochemical, respectively. 4-Amino-1-butanol (Tridom) was dried with Linde molecular sieves (4 Å) and distilled at reduced pressure. The isobutyloxycarbonyl chloride (Eastman Organic Chemicals) was used without further purification. N^{α}-Acetyl-L-histidine was purchased from Aldrich Chemical Co. All other reagents and solvents were identical in quality and preparation with those used in paper 9 of this series.[11]

B. Synthesis. *N*-Carboxyanhydrides. γ-Benzyl L-glutamate *N*-carboxyanhydride was prepared by treatment of γ-benzyl L-glutamate [(Glu(OBzl)] with phosgene, using dioxane as a solvent as described by Blout and Karlson.[24] The isobutyloxycarbonyl group (Ioc) was used to block the imidazole side chain of L-histidine, as N^{α}-Z-His(N^{im}-Ioc); the latter (mp 93.5-94.5 °C dec) was prepared from N^{α}-benzyloxycarbonyl-Lhistidine and isobutyloxycarbonyl chloride by the procedure of Gronvald et al.[25] N^{α}-Z-N^{im}-Ioc-L-His (2.91 g, 7.5 mmol) was dissolved in 15 mL of anhydrous dioxane, and 1.5 mL of $SOCl_2$ was added to form the NCA. After 2 h, the yellow gel that formed was collected by filtration and washed with anhydrous dioxane and anhydrous ether. The product, His(N^{im}-Ioc)-NCA·HCl, was recrystallized from glacial acetic acid-ether (twice) and acetonitrile-ether to give white crystals: yield 0.823 g (35%); mp 154-155 °C dec; $[\alpha]_D^{21}$ -4.4° (*c* 1, acetic acid). Anal. Calcd for $C_{12}H_{16}N_3O_5Cl$: C, 45.36; H, 5.08; N, 13.23; Cl, 11.16. Found: C, 45.29; H, 5.04; N, 13.26; Cl, 11.36.

Poly(γ-benzyl L-glutamate-*co*-N^{im}-isobutyloxycarbonyl-L-histidine), Poly[Glu(OBzl),His(N^{im}-Ioc)], Copolymers I and II. The combined total of 10 mmol of Glu(OBzl)-NCA and His(N^{im}-Ioc)-NCA·HCl was polymerized in dioxane (80 mL) (polymer I) or in a mixed solution (80 mL) of acetonitrile and dioxane (1:4) (polymer II) with sodium methoxide as the initiator.[11] Sodium methoxide was used to neutralize the salt of His(N^{im}-Ioc)-NCA·HCl and to give an anhydride-to-initiator ratio of 40/1. The polymerization reaction was monitored by assaying the unreacted *N*-carboxyanhydrides as described in paper 10 of this series.[12]

The randomness of the polymerization was monitored by taking 0.5-mL aliquots from the reaction solution at different times. The reactions in the aliquots were quenched by adding 0.1 M HCl/ethanol. The precipitated polymer was separated from the supernatant by centrifugation and washed three times with ethanol.

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Table I
Compositions and Chain Lengths of the Unfractionated Poly[Glu(OBzl),His(N^{im}-Ioc)]

polymer no.	His(N^{im}-Ioc) content of reaction mixture, mol %	L-His content of polymer, mol %	av mol wt $\times 10^{-3}$ [a]	$\overline{DP}$
I	10	9	160	710
II	17	18	130	670

[a] By viscometry, using the relation of Fujita et al.[26] for polymers in dichloroacetic acid.

Appropriate volumes of the supernatant were dried and hydrolyzed (for amino acid analysis) with 6 N HCl at 110 °C for 20 h.

The polymers were worked up after 2 h by pouring the dioxane solution into 800 mL of stirred absolute ethanol, as usual.[11] The unreacted NCA was less than 2% of the initial NCA. Yields of the polymers were in the range 60–70%. The *approximate* chain lengths of these polymers were determined with the viscosity–molecular weight relationship of Fujita et al.,[26] using dichloroacetic acid as a solvent, and are given in Table I.

Poly[N^5-(4-hydroxybutyl)-L-glutamine-*co*-L-histidine], Poly[HBG,His], Copolymers III and IV. The protected copolymers I and II were treated with 4-amino-1-butanol to convert benzyl-L-glutamate to N^5-(4-hydroxybutyl)-L-glutamate according to the procedure described previously.[6] The aminolysis reaction was monitored by assaying for unexchanged γ-benzyl ester groups as described in paper 10,[12] and the reaction was terminated when no fewer than 99.5% of the γ-benzyl ester groups had been exchanged. In this aminolysis reaction, the protecting (isobutylcarbonyl) groups on the histidine residues were also removed because of their instability in alkaline solution. Less than 0.2% Ioc-His remained in copolymers III and IV after aminolysis, according to their 90-MHz ^{1}H NMR (Brüker) spectra. The reaction mixture was then poured into a stirred, ice-cold, dilute aqueous solution of 1 N acetic acid, kept stirring for 20 min, and then dialyzed against water at room temperature until amines could no longer be detected in the dialyzate by a ninhydrin test.[27] The aqueous poly[HBG,His] solution was filtered through a 0.45-μm Millipore filter, and the copolymer was recovered by lyophilization in 60–83% yield based upon the number of moles of poly[Glu(OBzl),His(N^{im}-Ioc)].

The water-soluble copolymers III and IV were fractionated by precipitation in a methanol–ether solvent system. This procedure, described in paper 2 of this series,[4] produced from seven to ten fractions for each copolymer. Each fractionated copolymer was dissolved in water, lyophilized, dried in vacuo, and stored at about 5 °C.

C. Analytical Methods. Removal of the isobutyloxycarbonyl protecting groups from copolymers I and II was assessed with a Brüker 90-MHz ^{1}H NMR spectrometer. The absence of a peak corresponding to the isobutyloxycarbonyl protecting group in 1% copolymer solutions indicated that less than 0.2%, which is the limit of detection under these conditions, of the histidine side chain was protected.

Determination of Composition. In order to determine the amino acid composition of copolymers I–IV, each of the unfractionated or fractionated copolymers was hydrolyzed in 6 N HCl at 110 °C for 24, 48, and 72 h in evacuated, degassed, and sealed ampules and was worked up according to the procedure of Spitz[28] in order to eliminate formation of artifacts.[15,16] A Technicon TMS amino acid analyzer was used for the amino acid analysis.

Determination of Concentration. The concentrations of all copolymer solutions were determined by micro-Kjeldahl nitrogen analysis, using Lang's digestion procedure[29] and the semi automated method of Noel and Hambleton[30] for detection of ammonia. The average error in the concentrations is estimated to be ±5%.

Optical Purity. Absence of racemization of the monomeric starting material as well as of the acid hydrolyzates of the final copolymers was checked by the L-phenylalanine-NCA dipeptide method of Manning and Moore,[31] Glutamic acid, histidine, and 4-amino-1-butanol were obtained from the hydrolysis of the copolymers and separated with two ion-exchange columns. The first column (Dowex 50W in the hydrogen form, 60 mm × 5 mm, equilibrated with CH_3COOH) separated 4-amino-1-butanol from the two amino acids after elution with 1.0 M pyridine solution. The mixture of amino acids was then added with 0.1 N acetic acid to a second column of Dowex I (60 mm × 10 mm, equilibrated with HCl) and was separated by elution with water.

The separated glutamic acid and histidine were coupled with L-phenylalanine-NCA, and the resulting diastereoisomeric dipeptides were separated by high-performance liquid chromatography (Spectra Physics SP8000) using an RP18 (30 cm × 4.5 mm) column, with detection at 258 nm and elution with 0.01 M aqueous ammonia–acetic acid buffer of pH 6.5 and acetonitrile (volume ratio 97.5:2.5). Appropriate dipeptide standards were prepared from DL-glutamic acid and DL-histidine in order to check for differences in molecular extinction coefficient between L-Phe-L-Glu and L-Phe-D-Glu and also between L-Phe-L-His and L-Phe-D-His. Possible racemization of L-glutamic acid and L-histidine during hydrolysis with 6 N HCl was assessed by hydrolyzing L-glutamic acid and L-histidine under the same conditions as used for hydrolysis of the copolymers. The "hydrolysis" products were coupled with L-phenylalanine-NCA and were separated by the same chromatographic procedure (SP8000) used for the copolymers.

pH Titration. The copolymers were titrated to determine the number and pK_a of the imidazole groups in the copolymer. The model compound *N*-acetyl-*N'*-methylhistidinamide was also titrated. These potentiometric titrations were carried out on the instrument previously described,[18] using combined glass and reference electrodes (no. 6030-02, Ingold Electrode Co). These electrodes showed high stability with negligible drift (0.01 pH unit/h) and were calibrated just before and after each use with phthalate and phosphate buffers made up according to Bates.[32] The titration vessel was equipped with a water jacket and thermostated at 25 ± 0.1 °C, and all titrations were performed under a nitrogen atmosphere.

The copolymer (about 15 mg) was dissolved in 10 mL of 0.1 N KCl in the titration vessel and titrated. The titrant, 0.1 N KOH or 0.1 N HCl was delivered with an Agla Micrometer syringe with a Teflon needle. Each titration was demonstrated to be reversible and carried out twice. The apparent pK at any degree of ionization was calculated by using the well-known equation[33]

$$pK_{app} = pH - \log [\alpha/(1 - \alpha)] \quad (1)$$

where α is the fraction of ionized histidine and is calculated in the usual manner.[34] Net titration curves of the copolymers were obtained by subtracting the titration curve of the solvent from that of the copolymer. The curves of pK_{app} vs. α often curved up or down sharply at high and low degrees of ionization. This problem has been observed before[35] and was attributed to the sensitivity of pK_{app} to small errors in the determination of the end points, composition, and concentration of sample. The procedure for correcting for this error is the same as that used in paper 9 of this series.[11]

Viscosity, ORD, and CD Measurements. Viscosity, ORD, and CD measurements were all carried out as described previously.[4]

Molecular Weight. The molecular weights of the fractionated copolymers were determined by conventional sedimentation equilibrium with a Spinco Model E ultracentrifuge as reported ealier,[4] with the following modification. The copolymers were dissolved in 0.2 N KCl solution and dialyzed against a large volume of 0.2 N KCl solution at neutral pH overnight, in order to eliminate polyelectrolyte effects.[36] The dialysate was used as the solvent in the molecular weight measurements. The concentration dependence of the weight-average molecular weight, $\bar{M}_w$, was determined for each sample, and $\bar{M}_z$ was computed from the run at the lowest concentration for each fraction. The estimated precision in the values of $\overline{DP}_w$ was within ±5%. The partial specific volumes ($\bar{v}$) of poly(HBG,His) required for the calculation of molecular weight, were determined from the amino acid content as described by Cohn and Edsall.[37] A value of $\bar{v}$ = 0.816 for PHBG was used in the calculation of $\bar{v}$ for the copolymers.[4]

Results

A. Characterization of the Copolymers. The randomness of the polymerization was monitored as described

Table II
Characterization of the Water-Soluble Fractionated Copolymers

fraction[a]	L-His content, mol %	$\bar{M}_w \times 10^{-3}$ [b]	$\bar{M}_z/\bar{M}_w$	$\overline{DP}_w$
III-B	8.7	86	1.24	444
III-D	9.3[c]			
III-G	9.7	32	1.14	165
IV-B	18.4	58	1.18	308
IV-H	17.9	46	1.16	243

[a] Samples III-B, III-D, and III-G were obtained from copolymer no. I. Samples IV-B and IV-H were obtained from copolymer no. II. [b] This value was determined for these samples by conventional sedimentation equilibrium (with an extrapolation to zero concentration). [c] This sample was used for the titration experiments.

in the Experimental Section. At various times during the polymerization, the relative amounts of unreacted Glu(OBzl)-NCA and His(N^{im}-Ioc)-NCA·HCl varied from those at the start of the reaction, within allowed limits. Hence, copolymers I and II were sufficiently random[3] for the purpose of the experiments carried out here.

Table I summarizes the composition and average degree of polymerization ($\overline{DP}$) of the unfractionated poly(γ-Bzl-L-Glu$^{m'}$,N^{im}-Ioc-L-His$^{n'}$) copolymers, and Table II shows the data for the fractionated poly(HBG,His) copolymers that were investigated. The usual decrease in $\overline{DP}$ attributed to transaminolysis,[4,38] upon conversion of these polymers to their (hydroxybutyl)glutamine derivatives, can be seen. It is difficult to assess the exact magnitude of this possible side reaction, however, because molecular weights were determined on only those fractions used for the analysis of σ and s.

The His(N^{im}-Ioc) contents (10 and 17 mol %) of the polymerization reaction mixture (Table I) are consistent with the histidine contents of copolymers I (9.2%) and II (18.2%), respectively. Also the composition is essentially independent of chain length for a given parent copolymer (Table II). These analytical results further suggest that there is little depature from randomness in the amino acid sequence of these copolymers. The near-randomness was verified for poly(L-Glu,L-Met) by determining the distribution of methionine from a CNBr digest of the copolymer.[39] In any case, paper I[3] of this series amply demonstrated that even large deviations from randomness do not affect the melting behavior of a copolymer.

Table II also gives information about the molecular weight and the polydispersity ($\bar{M}_z/\bar{M}_w$) of the samples used for the analysis of σ and s. The values of $\bar{M}_z/\bar{M}_w$ do not depart significantly from unity, indicating that the fractions in Table II were relatively homogeneous.

The concentration dependence of the apparent molecular weights of the fractions studied, illustrated in Figure 1, is small in most cases, and the data have been extrapolated to infinite dilution to obtain $\overline{DP}_w$.

Using the Manning–Moore[31] dipeptide procedure, we found the hydrolysates of copolymer I (6 N HCl for 24 h) to contain less than 1 mol % D residues. We consider the amount of racemization found here to be too minor to affect the computed values of σ and s for L-histidine.

B. CD Data for the Copolymers. The CD data for representative fractions of poly(HBG,His) at 25 °C and high and low pH in water and 0.5 N KCl are shown in Figure 2. The CD data clearly indicate the presence of a right-handed α-helical structure mixed with random coil in all of the spectra.[40] Contributions of β-structure to these spectra are not evident. With increasing temperature, the

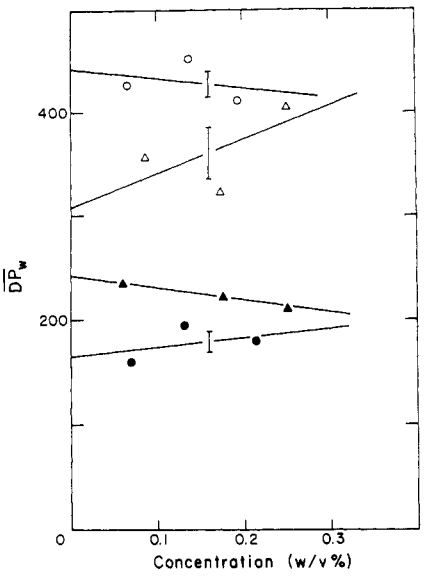

Figure 1. Concentration dependence of molecular weight for fractions used in analysis to obtain σ and s: (○) 8.7% His, $\overline{DP}_w$ = 444 (fraction III-B); (●) 9.7% His, $\overline{DP}_w$ = 165 (fraction III-G); (△) 18.4% His, $\overline{DP}_w$ = 308 (fraction IV-B); (▲) 17.9% His, $\overline{DP}_w$ = 243 (fraction IV-H).

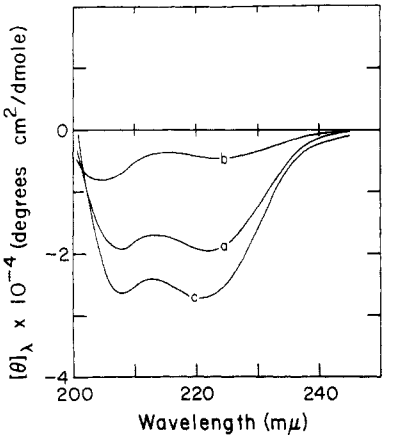

Figure 2. CD data for poly(HBG,His) copolymers at 25 °C: (a) 8.7% His, $\overline{DP}_w$ = 444 (fraction III-B) in 0.5 N KCl at pH 3.0; (b) 17.9% His, $\overline{DP}_w$ = 243 (fraction IV-H) in 0.5 N KCl at pH 3.0; (c) 8.7% His, $\overline{DP}_w$ = 444 (fraction III-B) in water at pH 9.0.

ORD spectra (not shown here) become characteristic of the random coil mixed with small amounts of α-helix,[41] indicating that these copolymers undergo a thermally induced transition from α-helix to random coil in water.

Their thermally induced helix–coil transition curves were obtained by measuring the temperature dependence of b_0 and taking θ_h (fraction helix) to be $-b_0/750$. Measurement of b_0, shown in Figures 3–5, for the four copolymer fractions used to obtain σ and s at pH 3.0 and 9.0 studied over the range of λ 280–450 nm as a function of temperature confirm the above conclusion. The procedures used to obtain these curves were the same as those used previously.[8] The curves exhibited no concentration dependence, and they were demonstrated to be reversible in all cases. The size of the error symbols in Figures 3–5 reflects the experimental error in θ_h arising from errors in concentration (±3%), from possible errors in the choice of b_0 for the full helix and coil (±3%), and from errors in the slope of the Moffitt–Yang plot (±300/b_0%). All the curves were reproducible.

The helix content of fractions IV-B and IV-H with high histidine contents (about 18% His) was influenced by

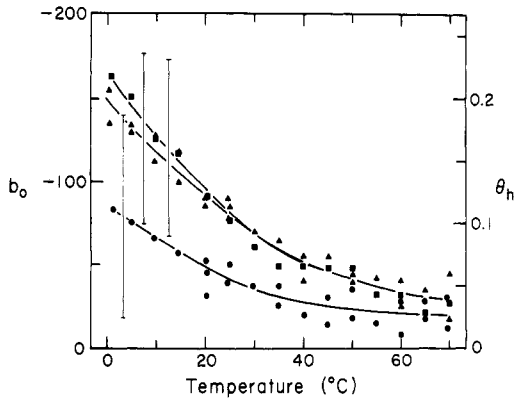

Figure 3. Effect of salt on the thermally induced helix–coil transition for poly(HBG,His) copolymer IV-H (17.9% His, $\overline{DP}_w$ = 243) at pH 3.0: (●) in water; (▲) in 0.1 N KCl; (■) in 0.5 N KCl.

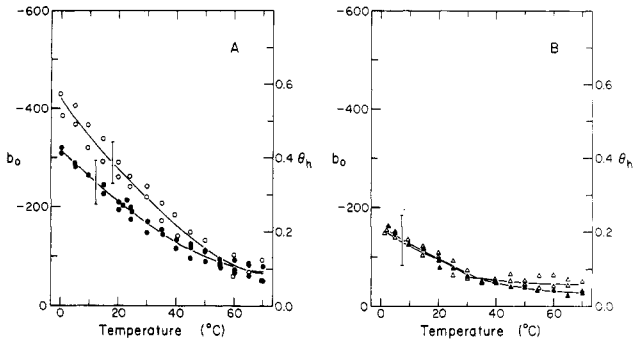

Figure 4. Temperature dependence of b_0 for poly(HBG,His) copolymers at pH 3.0: (A) (○) 8.7% His, $\overline{DP}_w$ = 444 (fraction III-B) in water; (●) 9.7% His, $\overline{DP}_w$ = 165 (fraction III-G) in water; (B) (▲) 18.4% His, $\overline{DP}_w$ = 308 (fraction IV-B) in 0.5 N KCl; (▲) 17.9% His, $\overline{DP}_w$ = 243 (fraction IV-H) in 0.5 N KCl.

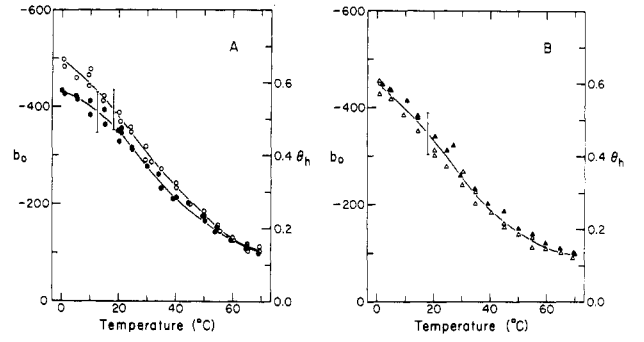

Figure 5. Temperature dependence of b_0 for poly(HBG,His) copolymers in water at pH 9.0: (A) (○) 8.7% His, $\overline{DP}_w$ = 444 (fraction III-B); (●) 9.7% His, $\overline{DP}_w$ = 165 (fraction III-G); (B) (▲) 18.4% His, $\overline{DP}_w$ = 308 (fraction IV-B); (▲) 17.9% His, $\overline{DP}_w$ = 243 (fraction IV-H).

added salt at pH 3, as shown in Figure 3 for copolymers IV-H with high content of charged guest residues. Figure 3 shows that the helix content increases with the concentration of added salt from 0 to 0.5 N KCl at pH 3. It is apparent from this figure that the conformation of this copolymer is affected by long-range electrostatic interactions between the charged histidine side chains at low ionic strength. However, the small difference between the melting curves in 0.1 N KCl and 0.5 N KCl indicates that the long-range electrostatic repulsions are completely shielded in a solution of 0.5 N KCl and, therefore, that the fractions of higher histidine content (IV-B and IV-H) in 0.5 M KCl may be used in the analysis to obtain σ and s.

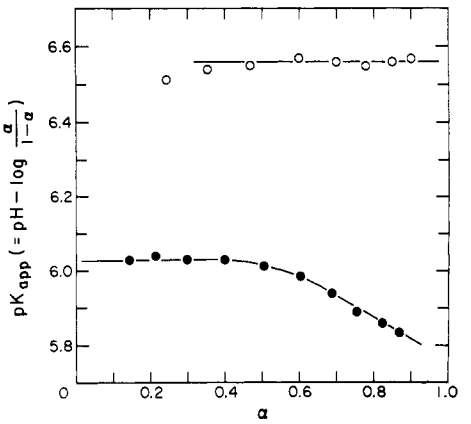

Figure 6. Plot of the apparent pK [=pH − log (α/(1 − α))] vs. the degree of ionization, α (α = 1 is the fully charged state), for (○) N-acetyl-N'-methylhistidineamide and (●) poly(HBG,His) copolymer III-D (9.3% His) in 0.1 N KCl at 25 °C.

For the fractions with low histidine content (<10% His), no differences could be detected between the melting curves obtained in water and in 0.1 N KCl solution at pH 3.0. This indicates that, for the copolymers of low histidine content, the charged histidine side chains are sufficiently separated from one another so that the helix–coil transition is not influenced by electrostatic repulsions. Figures 4 and 5 indicate the following general aspects of the melting curves for poly(HBG,His). First, the presence of histidine residues decreases the helix content of all fractions relative to that of the homopolymer poly(HBG) of comparable $\overline{DP}_w$. Second, for a given histidine content, the helix content increases with increasing $\overline{DP}_w$ between fraction III-B ($\overline{DP}_w$ = 444) and fraction III-G ($\overline{DP}_w$ = 165). However, this trend could not be observed for the copolymer fractions of higher histidine content (between IV-B and IV-H) because their chain lengths are too similar. Third, for a given chain length, the helix content decreases with increasing histidine content. Fourth, for the same copolymer fraction, the helix content decreases as the histidine residues become charged. These facts reveal that the histidine residue behaves as a helix breaker and that the charged histidine residue is a stronger helix breaker than the uncharged species.

C. b_0 for the Complete Helix and Coil. For the homopolymer poly(HBG) studied in paper 2,[4] the value of b_0 for the complete helix was taken to be −750 and that for the complete coil as zero. Because these values vary with the nature of the side chain,[42] fraction IV-B with a histidine content of 18.4 mol % was examined in trifluoroethanol (TFE) at 1.5 °C, with b_0 corrected for the dispersion of the refractive index of the solvent.[43]

The experiments gave a value of −710 for the copolymer in trifluoroethanol. The smallest observed value of b_0 in water is about −10. These results demonstrate that the values used for the homopolymer and also other copolymers in this series are reasonable for the histidine copolymers.

D. Titration Data. The results of the titration experiments with the copolymer and N-acetyl-N'-methylhistidinamide are given in Figure 6, plotted as pK_{app} vs. the degree of ionization, α (α = 1 when all histidine side chains are charged). The apparent pK_a (vs. α) of poly(L-histidine), which was reported by Terbojevich et al.,[34] exhibited a curved region at intermediate values of α that is characteristic of a pH-induced conformational transition. However, the copolymers examined in this study have a much simpler titration behavior than the histidine hom-

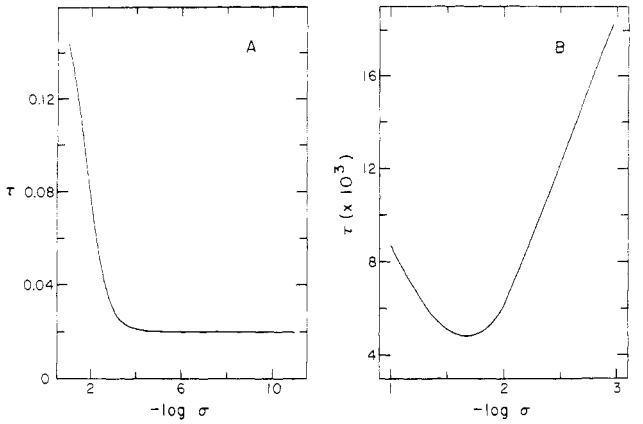

Figure 7. Determination of the best temperature-independent value of σ as the one that corresponds to the lowest value of τ for the histidine copolymers using the Allegra theory at (A) pH 3.0 and at (B) pH 9.0.

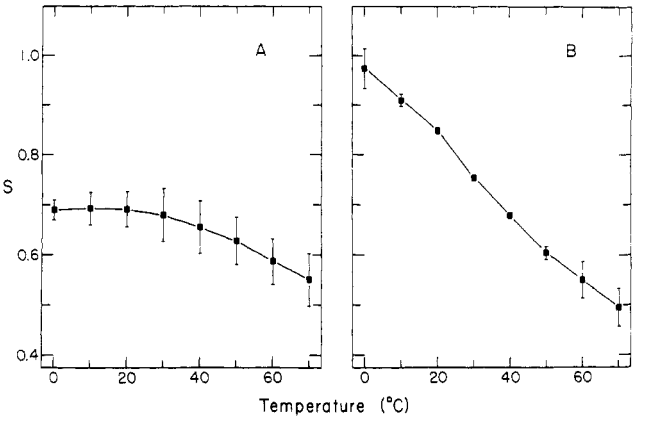

Figure 8. Temperature dependence of s for poly(L-histidine) in aqueous solution (A) at pH 3.0 for $\sigma = 1.0 \times 10^{-5}$ and (B) at pH 9.0 for $\sigma = 2.1 \times 10^{-2}$. The error symbols are described in the text. The solid line is drawn to pass through all the points.

opolymer. The apparent pK_a is constant (=6.02) at low degree of ionization ($\alpha < 0.4$) and decreases slightly with increasing degree of ionization ($\alpha > 0.4$).

The pK_{app} of *N*-acetyl-*N'*-methylhistidinamide was found to be 6.56 ± 0.03 and is considerably higher than the pK_0 of the histidine residues of the copolymers in this study. Figure 6 demonstrates that at pH 3 or 9, which we used in this study, the histidine residues of the copolymers are completely charged or uncharged, respectively.

Discussion

A. Helix–Coil Parameters for Poly(L-His). The melting curves described in the Results section were analyzed according to the LAPS (Lifson–Allegra–Poland–Scheraga) hierarchy of approximations to obtain the Zimm–Bragg parameters σ and s for poly(L-His).[44] This procedure has been discussed extensively in earlier papers of this series.[3,4] The approximations corresponding to the theories of Lifson[45] and Allegra[46] were both used to fit the data; the exact theory of Lehman and McTague[47] was used only in a few cases because of the large amount of computing time required and because the Lifson and Allegra methods have been shown to be adequate for the range of σ and s values found in these experiments.[3,8] The data in Figures 4 and 5 were used for these calculations. The results of these calculations for a few representative samples are shown in Table III.

Both the first-order (Lifson) and second-order (Allegra) approximations give results that agree well with each other. The higher order (Allegra) approximation will be used in all subsequent discussion of the L-histidine parameters.

In all cases, the melting data were analyzed with σ taken independent of temperature. The best value of σ was obtained by application of the "goodness of fit" criterion expressed in terms of the parameter τ defined in paper 2.[4] The best fit for all copolymer data was obtained by minimizing τ. Figure 7 shows a graph of τ vs. σ. At pH 3 there is no minimum in τ, and values of σ below 1×10^{-5} fit equally well. For $\sigma \leq 1 \times 10^{-5}$ the computed values of s are found to be independent of σ; for values of $\sigma > 1 \times 10^{-5}$, however, the computed values of s depend on σ. Since these large values of σ correspond to a larger τ value, they are thus not applicable. This problem of indeterminancy of σ has been encountered in earlier papers, viz., for glycine,[5] serine,[7,15] valine,[10] glutamic acid,[11] asparagine,[14] lysine,[13] arginine,[17] and threonine.[19] This behavior arises when it becomes very improbable for the guest residue to nucleate a helical segment in a pure-guest region of the random copolymer. Such nucleation becomes more improbable as the value of $\alpha\sigma_A s_A$ of the guest amino acid becomes small compared to the value of $\sigma_B s_B$ of the host amino acid, where α is the (generally small) relative composition of the guest residue (see Appendix B of paper 1 of this series[3]). In copolymers with histidine as the guest residue, the independence of τ on σ (at values of $\sigma \leq 1 \times 10^{-5}$) occurs for charged histidine residues at pH 3.0, because the host residue (HBG) has a large value of s and σ, the guest residue has a small value of s and σ ($\sigma < 1 \times 10^{-5}$), and the composition of the guest residue is low. At pH 9, $\alpha\sigma_A s_A$ is not so small compared to $\sigma_B s_B$, and a minimum in the τ vs. log σ curve appears.

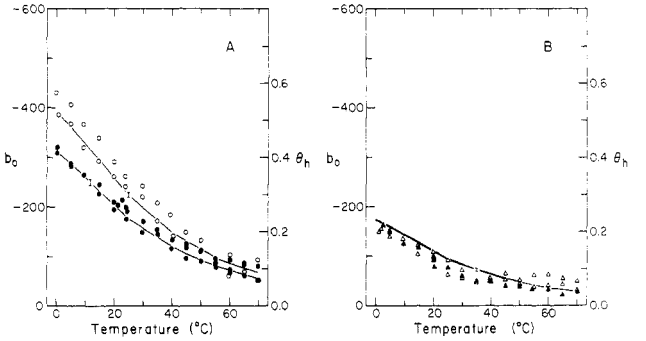

Figure 9. Comparison of the calculated melting curves, obtained with the parameters of the Allegra theory (with $\sigma = 1.0 \times 10^{-5}$) for L-histidine, with the experimental points at pH 3.0: (A) (○) 8.7% His, $\overline{DP}_w$ = 444 (fraction III-B) in water; (●) 9.7% His, $\overline{DP}_w$ = 165 (fraction III-G) in water; (B) (△) 18.4% His, $\overline{DP}_w$ = 308 (fraction IV-B) in 0.5 N KCl; (▲) 17.9% His, $\overline{DP}_w$ = 243 (fraction IV-H) in 0.5 N KCl. The error symbols indicate errors in the calculated values of θ_h arising from errors in composition and chain length.

The values of s obtained for the uncharged polymers as well as for the charged polymers in the presence and absence of salt are shown in Table IV. Figure 8 shows the temperature dependence of s with the estimated error in s. The error symbols were calculated by fitting each melting curve at the best-fit value of σ and determining the standard deviations of these values of s.

Figures 9 and 10 show the computed melting curves along with the experimental points. The errors shown here are due to the uncertainties in molecular weight and composition. The calculated values of θ_h are in good agreement with experimental data.

The thermodynamic quantities $\Delta G°$ (the free energy), $\Delta H°$ (the enthalpy), and $\Delta S°$ (the entropy) for the conversion of a coil residue of L-histidine to a helical one at the end of a long helical sequence can be obtained from

Table III
Comparison of the Values of θ_h Calculated with Approximate and Exact Theories for Finite Chains[a]

L-His content, mol %	$\overline{DP}_w$	temp, °C	pH	$(\theta_h)_{exptl}$	$(\theta_h)_{theor.}$ Lifson[b,d]	Allegra[c,d]	Lehman-McTague[c,d]
8.7	444	0	3.0	0.556	0.544	0.522	0.520
		30		0.300	0.271	0.266	0.264
		60		0.117	0.116	0.115	0.114
17.9	243	0	3.0	0.220	0.217	0.233	0.227
		30		0.082	0.108	0.113	0.111
		60		0.038	0.048	0.049	0.049
9.7	165	0	9.0	0.580	0.607	0.609	0.603
		30		0.371	0.366	0.367	0.365
		60		0.164	0.173	0.173	0.172
18.4	308	0	9.0	0.603	0.599	0.597	0.595
		30		0.328	0.333	0.332	0.330
		60		0.151	0.140	0.140	0.139

[a] The parameters used for PHBG are those of Table II in paper 2.[4] [b] The parameters used for L-histidine were obtained by fitting the data with the Lifson theory.[45] [c] The parameters used for L-histidine were obtained by fitting the data with the Allegra theory.[46] [d] The values of σ used in these calculations for L-histidine at pH 3 and 9 were 1.0×10^{-5} and 2.1×10^{-2}, respectively.

Table IV
Values of the Zimm-Bragg Parameters s for Poly(L-histidine)

temp, °C	s[a] pH 3.0[b]	pH 9.0[c]
0	0.69	0.98
10	0.70	0.91
20	0.69	0.85
30	0.68	0.76
40	0.66	0.68
50	0.63	0.60
60	0.59	0.55
70	0.55	0.50

[a] Calculated with the Allegra theory.[46] [b] Calculated with $\sigma = 1.0 \times 10^{-5}$. The data for fractions III-B and III-G were obtained in water, and those for fractions IV-B and IV-H in 0.5 N KCl. [c] Calculated with $\sigma = 2.1 \times 10^{-2}$. All measurements at pH 9.0 were made in water.

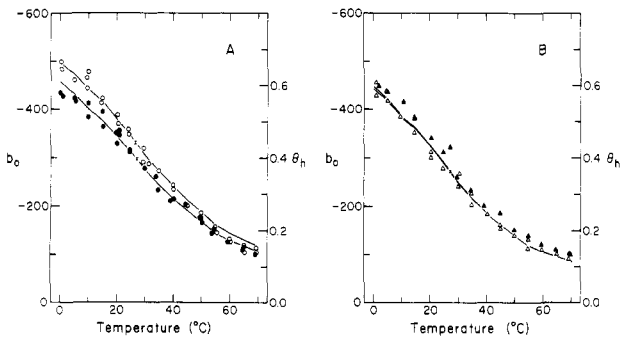

Figure 10. Comparison of the calculated melting curves, obtained with the parameters of the Allegra theory (with $\sigma = 2.1 \times 10^{-2}$) for L-histidine, with the experimental points at pH 9.0: (A) (○) 8.7% His, $\overline{DP}_w$ = 444 (fraction III-B) in water; (●) 9.7% His, $\overline{DP}_w$ = 165 (fraction III-G) in water; (B) (△) 18.4% His, $\overline{DP}_w$ = 308 (fraction IV-B) in water; (▲) 17.9% His, $\overline{DP}_w$ = 243 (fraction IV-H) in water. The error symbols indicate errors in the calculated values of θ_h arising from errors in composition and chain length.

the value of s and its temperature dependence. Figure 11 shows plots of $\Delta G°$ ($=-RT \ln s$) vs. temperature with error symbols calculated from the standard deviations in s. $\Delta H°$ was calculated from the slope of the ln s vs. $1/T$ curve using the van't Hoff equation [$-dR \ln s/d(1/T) = \Delta H°$], and $\Delta S°$ was obtained from the relation $\Delta G° = \Delta H° - T\Delta S°$. These thermodynamic parameters are listed in Table V and should be regarded only as estimates in view of the large errors involved in their computation.

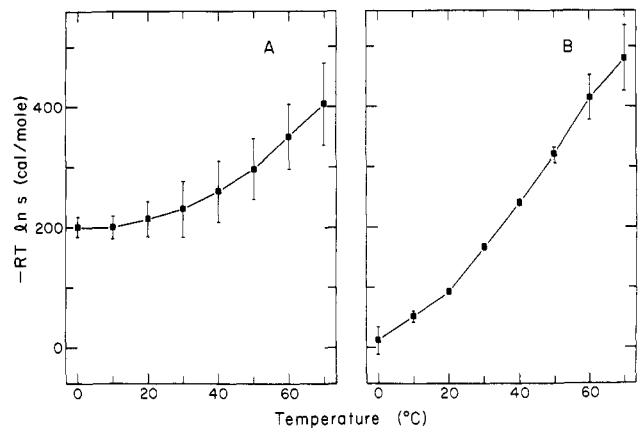

Figure 11. Plot of $-RT \ln s$ (i.e., $\Delta G°$) vs. T for poly(L-histidine) in water and/or salt solution (A) at pH 3.0 and (B) at pH 9.0. The solid line has been drawn through the points obtained from the Allegra analysis with (A) $\sigma = 1.0 \times 10^{-5}$ and (B) $\sigma = 2.1 \times 10^{-2}$. The error symbols were calculated from the standard deviation in s.

Table V
Thermodynamic Parameters for L-Histidine[a]

	pH 3.0	pH 9.0
$\Delta G°_{20}$, cal/mol	214 ± 29	94 ± 4
$\Delta H°_{20}$, cal/mol	−253 ± 230	−1660 ± 60
$\Delta S°_{20}$, eu	−1.6 ± 0.9	−6.0 ± 0.2
σ	1.0×10^{-5}	2.1×10^{-2}

[a] Calculated as described in the text.

B. Titrations. The titration behavior of poly(HBG,-His) (in Figure 6) is similar to that of poly(HBG,L-Glu)[11] and poly(HBG,L-Asp).[18] The titration curves of these three sets of copolymers do not exhibit the features that are characteristic of the pH-induced conformational transition in the homopolymers poly(L-Glu)[48] and poly(L-His).[34] The values of pK_{app} for poly(HBG,L-Glu) and poly(HBG,L-Asp) increase with increasing α; however, that of poly(HBG,L-His) decreases with increasing α. Only the directions of the curves differ. This behavior arises from the difference in the state of ionization of the guest residues in the copolymers; i.e., glutamic acid and aspartic acid residues are ionized at high pH, whereas the histidine residue is ionized at low pH.

In the case of the homopolymers poly(L-histidine) and poly(L-glutamic acid), the slope of the pK_{app} vs. α curve at low degrees of ionization (where the polymers are in the

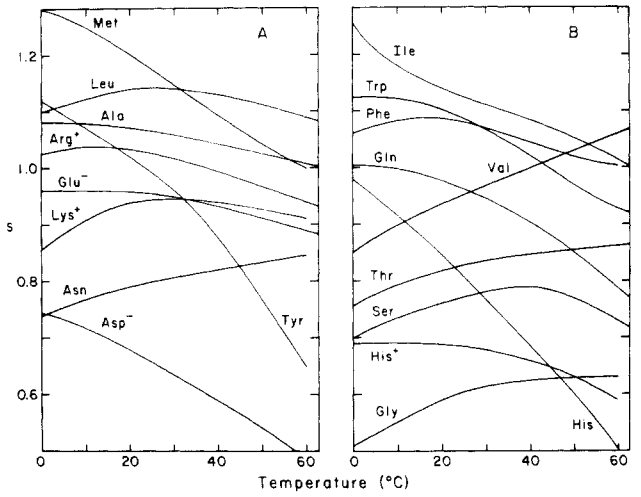

Figure 12. Plots of s vs. T for 18 of the naturally occurring amino acid residues investigated thus far by the host–guest technique.

helical conformation) is greater than that at high degrees of ionization (where the polymers are in the coil form); i.e., the electrostatic free energy of the helix is higher than that of the coil form. Although Figure 6 shows a dependence of pK_{app} on α at high degrees of ionization, we attribute this to the large experimental error in these titrations and conclude that there is no large difference between the electrostatic free energies of the helical and coil states of the copolymers in 0.1 N or 0.5 N KCl, as was observed for poly(HBG,L-Glu)[11] and poly(HBG,L-Asp).[18] Since the electrostatic free energy of the charged helix and the charged coil are similar in the copolymers used in this study, the only way that a pH-induced helix–coil transition can occur is for σ and s to differ for charged and uncharged side chains, as is the case for poly(HBG,L-Glu);[11] such differences arise from short-range interactions (between the side chain and its own backbone). As the pH decreases, poly(HBG,His) undergoes a helix–coil transition, because of the lower values of s for charged histidine. As was discussed[11] for poly(HBG,L-Glu), we attribute the decrease in pK_0 of the histidine side chain of the copolymers over that of the corresponding *N*-acetyl-*N'*-methylamide derivative (viz., 6.03 compared to 6.56) to environmental effects on the copolymers (primarily the nonpolar character of the HBG side chains).

C. Comparison with Other Results. Previous CD studies of poly(L-His)[49,50] indicated that the histidine homopolymer adopts an ordered β-structure rather than an α-helix conformation at high pH (low degree of ionization). Thus, no information about the helix–coil transition in poly(L-His) is available from these studies. Application of the "host–guest" technique here has revealed that L-His is a pronounced helix breaker in water and in salt solution over the temperature range 0–70 °C and that the charged L-His residue is a somewhat stronger breaker than the uncharged one at low temperature.

The "host–guest" technique provides information about the intrinsic character of charged and uncharged L-histidine residues separately by ignoring the interaction between the host and guest residues (a valid assumption[51]) and classifies L-histidine as a helix breaker. On the contrary, information obtained from the frequencies of occurrence of histidine in various conformational states in proteins of known crystal structure pertains to a mixture of charged and uncharged histidine residues and is influenced by residues that interact with histidine residues. (It is well-known that the histidine residue can chelate to metal ions in metalloenzymes and can also serve as a constituent of a charge relay system in proteases). Such analyses of proteins classified the L-histidine residue as a helix maker[52,53] or as a neutral residue.[54–56] Thus, L-histidine is intrinsically a helix breaker but is frequently observed in a helix region in proteins, probably because of favorable interactions with other residues in the helix state.

Since histidine residues in proteins have a pK_a of around 6.6,[1] each histidine residue is a mixture of protonated and deprotonated forms and their ratio is very sensitive to changes in the pH of its environment. Since the conformations of proteins are rather insensitive to small changes in pH near neutrality, the variable ratio of charged/uncharged histidine residues indicates that protonated and deprotonated histidine residues probably have similar conformational preferences. This idea is supported by the result that the value of s (0.66) for charged His is similar to that (0.70) for uncharged His at physiological temperature (37 °C).

Finally, in Figure 12, we summarize the s vs. T behavior of the 18 amino residues investigated thus far in this series. Of the 20 naturally occurring amino acid residues, the data for only proline and cystine (cysteine) have yet to be determined. However, Kidera et al.[57] have made a preliminary estimate of the values of s for proline and cystine at 20 °C by extrapolation, using the values of s of the 18 other amino acid residues and various other properties of these residues (Table VI).

Table VI
Zimm–Bragg Parameters for the 20 Naturally Occurring Amino Acids

amino acid	s (20 °C)[a]	σ[a] × 10^4	amino acid	s (20 °C)[a]	σ[a] × 10^4
Ala	1.07	8.0	Ile	1.14	55
Arg^+	1.03	0.1	Leu	1.14	33
Asn	0.78	0.1	Lys^+	0.94	1.0
Asp^-	0.68	70	Met	1.20	54
Asp	0.78	210	Phe	1.09	18
Cys	0.92[b]	NA[c]	Pro	0.66[b]	80[d]
Gln	0.98	33	Ser	0.76	0.1
Glu^-	0.97	6.0	Thr	0.82	0.1
Glu	1.35	100	Trp	1.11	77
Gly	0.59	0.1	Tyr	1.02	66
His^+	0.69	0.1	Val	0.95	1.0
His	0.85	210			

[a] Obtained by the host–guest technique in this series, using the Allegra theory,[46] except for Cys and Pro. [b] Extrapolated from various properties of the amino acids and the values of s of the remaining 18 amino acids at neutral pH.[57] [c] NA = not available. [d] Calculated by Matheson and Scheraga.[58]

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Registry No. Ala, 56-41-7; Arg^+, 17806-42-7; Asn, 70-47-3; Asp^-, 50808-62-3; Asp, 56-84-8; Cys, 52-90-4; Gln, 56-85-9; Glu^-, 11070-68-1; Glu, 56-86-0; Gly, 56-40-6; His^+, 70805-60-6; Ile, 73-32-5; Leu, 61-90-5; Lys^+, 17806-41-6; Met, 63-68-3; Phe, 63-91-2; Pro, 147-85-3; Ser, 56-45-1; Thr, 72-19-5; Trp, 73-22-3; Tyr, 60-18-4; Val, 72-18-4; N^{α}-Z-His(N^{im}-Ioc), 77205-69-7; His(N^{im}-Ioc)-NCA·HCl, 88376-81-2; γ-benzyl L-glutamate *N*-carboxyanhydride,

3190-71-4; γ-benzyl L-glutamate, 1676-73-9; N^{α}-(benzyloxycarbonyl)-L-histidine, 14997-58-1; isobutyloxycarbonyl chloride, 543-27-1; poly(γ-benzyl L-glutamate-*co*-N^{im}-(isobutyloxycarbonyl)-L-histidine), 88376-80-1; poly(hydroxybutylglutamine-*co*-L-histidine), 88391-93-9; 4-amino-1-butanol, 13325-10-5; L-histidine, 71-00-1; *N*-acetyl-*N'*-methylhistidinamide, 6367-11-9; poly(L-histidine) (homopolymer), 26062-48-6.

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