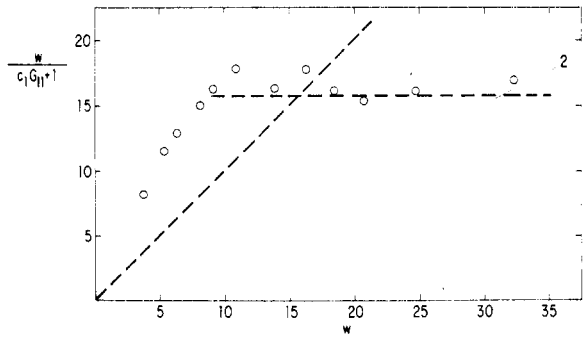

Figure 7. Clustering of water in serum albumin.

are those in which the hydrophilic–hydrophobic tension is most marked. It is in these cases that the water–polymer interaction is forced to assume its most highly structured character.

References and Notes

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Helix–Coil Stability Constants for the Naturally Occurring Amino Acids in Water. IX. Glutamic Acid Parameters from Random Poly(hydroxybutylglutamine-*co*-L-glutamic acid)[1]

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ABSTRACT: The synthesis and characterization of water-soluble random copolymers containing L-glutamic acid with N^5-(4-hydroxybutyl)-L-glutamine and the thermally induced helix–coil transitions of these copolymers in water and in 0.1 *N* KCl are described. The incorporation of L-glutamic acid was found to increase the helix content of the polymer at low pH and to decrease it at high pH even though the presence of 0.1 *N* KCl effectively eliminated the difference between the electrostatic free energies of the helix and the coil. The Zimm–Bragg parameters σ and s for the helix–coil transition in poly(L-glutamic acid) in water and in 0.1 *N* KCl were deduced from an analysis of the melting curves of the copolymers in the manner described in earlier papers. The synthesis of *N*-acetyl-*N'*-methylglutamic acid amide and its titration, as well as that of the copolymers and poly(L-glutamic acid), in 0.1 *N* KCl are described.

There have been several previous investigations of the helix–coil transitions of poly(L-glutamic acid) in water and in salt solutions.[3-9] Both the homopolymer and copolymers in which glutamic acid is the principal component have been studied, and the effects of temperature and pH on the helix–coil transition have been observed. Using the Zimm–Rice theory,[10] the helix–coil stability constants for this amino acid have been evaluated.[3-10] These previous studies have, however, been hampered by the precipitation of poly(L-glutamic acid) at low pH and the difficulty of treating the electrostatic interactions at high pH. In the present paper, the use of random copolymers, in which glutamic acid is the minor component, eliminates these problems so that the Zimm–Bragg[11] helix–coil transition parameters for uncharged and charged glutamic acid can be calculated. For random copolymers in which the glutamic acid side chains are charged, 0.1 *N* KCl provides sufficient shielding of the charges to allow calculation of the Zimm–Bragg parameters using a near-neighbor theory, as in previous papers in this series.[12-19] The importance of short-range[20] and medium-range[21] interactions has been discussed elsewhere, and their effects on the conformation of glutamic acid residues in proteins and polypeptides will be examined in this paper and in the following paper.[22]

The synthesis of water-soluble random copolymers of L-glutamic acid with N^5-(4-hydroxybutyl)-L-glutamine (HBG) is described in section I. The experimental characterization of these copolymers and their melting and titration behavior are presented in section II along with the titration of poly(L-glutamic acid) and *N*-acetyl-*N'*-methylglutamic acid amide. Finally, in section III, the data are analyzed to determine the helix–coil stability parameters of L-glutamic acid in the neutral and charged forms in water and in 0.1 *N* KCl. These results are then compared with previous results for polypeptides and proteins.

I. Experimental Section

Preparation and Characterization of the Copolymers. The copolymers were prepared by first copolymerizing the *N*-carboxyanhydrides (NCA) of γ-*tert*-butyl L-glutamate and γ-benzyl L-glutamate in dioxane with triethylamine or sodium methoxide as an initiator. The γ-*tert*-butyl blocking group was removed using trifluoroacetic acid, and the benzyl blocking group was replaced by reaction with 4-amino-1-butanol.

A. Materials. Dioxane was purified shortly before use by refluxing and distilling over sodium. Hexane was dried over calcium sulfate and decanted just before use. Triethylamine (TEA) was refluxed and distilled with acetic anhydride, and then dried and distilled over KOH. Sodium methoxide was prepared by placing freshly cut sodium in anhydrous methanol and diluting with benzene. Ethyl acetate was dried over Linde Molecular Sieves (4A) and decanted just before use. Purified grade dichloroacetic acid

(DCA) from Fisher Scientific Co., absolute ethanol from U.S. Industrial Chemicals Co., and anhydrous methanol from Mallinckrodt Chemical Works were used without further purification. Benzene and ether from Mallinckrodt Chemical Works were of analytical reagent grade. 2,2,2-Trifluoroethanol (TFE) obtained from Aldrich Chemical Co. Inc. was stirred over sodium bicarbonate and distilled. Dimethyl sulfoxide (DMSO) was dried over Linde Molecular Sieves (4A) and then distilled at reduced pressure. Acetyl chloride was distilled from potassium carbonate. Dimethylformamide was dried over Linde Molecular Sieves (4A) and vacuum distilled immediately before using. Chloroform was used without further purification. Trifluoroacetic acid was distilled from a small amount of phosphorus pentoxide. Potassium phthalimide was purchased from Eastman Kodak Co. and used without further purification. L-Glutamic acid was purchased from Aldrich Chemical Co. Inc. Fisher Certified tetrahydrofuran (THF) that gave a negative test for peroxides[23] was used without further purification, and distilled from calcium hydride in the preparation of 4-chlorobutyl acetate. Standard solutions of KOH (0.1 *N*) were made up from ampoules of a concentrated KOH solution purchased from Anachemia Chemicals Ltd. by diluting with distilled deionized degassed water. Standard solutions of HCl (0.5 *N*) were purchased from BDH Chemicals Ltd. and prepared in the same manner. Potassium acid phthalate (Primary Standard), potassium dihydrogen phosphate, and sodium hydrogen phosphate were of analytical reagent grade and purchased from Mallinckrodt Chemical Works.

Using the L-leucyl dipeptide method of Manning and Moore[24] the starting L-glutamic acid was found to contain no detectable amounts (within 0.1%) of D residues.

PHBG of $\overline{DP}_w$ = 720 was fraction V of paper II.[13]

B. Synthesis. The synthesis of 4-amino-1-butanol and *N*-acetyl-*N'*-methylglutamic acid amide is described in the Appendix.

***N*-Carboxyanhydrides.** γ-*tert*-Butyl L-glutamate was prepared according to the procedure of Roeske[25] in an overall yield of 57%: mp 193–194° (lit. mp 190–191°); $[\alpha]^{24}D$ +10.6° (*c* 1, H_2O) [lit. $[\alpha]^{25}D$ +18.1° (*C*1, H_2O)]. All attempts to repeat the preparation of the NCA of γ-*tert*-butyl L-glutamate reported by Roeske resulted in loss of the *tert*-butyl protecting group. However, the NCA was synthesized successfully using the method that Hirschmann et al.[26] reported for N^{ϵ}-*tert*-butyloxycarbonyl-L-lysine NCA. Two recrystallizations from ethyl acetate/hexane gave a 52% yield of short needles: mp 99–100° (lit. mp 95–96°); $[\alpha]^{24}D$ −20.6° (*c* 2, EtOAc) [lit. $[\alpha]^{25}D$ −19.0 (*c* 2, EtOAc)].

γ-Benzyl L-glutamate NCA was prepared as in paper VII[18] of this series.

Poly(γ-Bzl-L-Glu$^{m'}$:γ-*tert*-Bu-L-Glu$^{n'}$) [P(BzG:BuG)]. Copolymers I–IX. Random copolymers of γ-*tert*-butyl L-glutamate and γ-benzyl L-glutamate containing from 0 to 20% γ-*tert*-butyl-L-glutamic acid were synthesized by polymerization of the NCA's in dioxane with triethylamine or sodium methoxide as an initiator. The two NCA's were dissolved in dioxane (at a concentration of about 10 mmol of total NCA per 80 ml of solvent) in the molar ratio desired for the copolymer product. For polymers I–V and IX, triethylamine initiator was added to give an A/I ratio of 25, and the reaction flask was sealed with a "Drierite" drying tube and allowed to stand at room temperature for a week. For polymers VI–VIII, sodium methoxide initiator was added to give an A/I ratio of 15, and the polymerization was stopped after 8 hr. In both cases, the viscous mixture was then introduced into 400 ml of vigorously stirred absolute ethanol. The white precipitate was collected on a filter funnel, washed thoroughly with ethanol, and dried to constant weight over P_2O_5 in vacuo. The yields ranged from 81 to 93%.

Poly[N^5-(4-OHBu)-L-Gln^m:L-Glu^n] [P(HBG:Glu)]. Copolymers X–XVIII. One of the γ-benzyl L-glutamate homopolymers (V) was treated with hydrogen bromide in TFA to convert it to polyglutamic acid, according to the reported procedure of Idelson and Blout.[27] The trifluoroacetic acid solution was then freeze dried, the white polymer was dissolved in water and a small amount of 0.1 *N* NaOH added (pH 8), and the solution was dialyzed exhaustively against water. Freeze drying of the remaining solution gave a yield of the sodium salt of polyglutamic acid of 94%. Homopolymer IX was treated with 4-amino-1-butanol to convert it to poly(hydroxybutylglutamine), as in paper II.[13]

In order to remove the *tert*-butyl protecting group, the γ-benzyl L-glutamate:γ-*tert*-butyl L-glutamate copolymers (I–III) were dissolved in TFA, allowed to stand for 15 min, and then lyophilized. In order to replace the benzyl group, copolymer I was treated with 4-amino-1-butanol by dissolving in dioxane as previously described.[15] However, copolymers II and III became insoluble in dioxane upon addition of 4-amino-1-butanol. Therefore, the solvent system dimethyl sulfoxide:dioxane (1:1) was used to keep the copolymers in solution during the replacement reaction. The yields were 91 and 98%. Copolymers VI–VIII were dissolved in TFA, allowed to stand for 30 min, and then precipitated with ether. The precipitate was collected on a filter funnel and washed with ether. It was then dissolved in 20 ml of dioxane, reprecipitated with ether, and collected on a filter funnel. After washing thoroughly with ether, the white precipitate was dried to constant weight over P_2O_5 in vacuo. The copolymers were then dissolved in dimethyl sulfoxide:dioxane (1:1) and treated with 4-amino-1-butanol. Yields ranged from 76 to 83%. Polymers I–III and VI–VIII were dialyzed extensively against 0.1 *N* HCl (to protonate all side-chain carboxyls) and then exhaustively against water before being lyophilized. Copolymer IV was reacted with 4-amino-1-butanol in dioxane without removing the *tert*-butyl protecting group. A yellow color developed after a few days as the reaction proceeded. The reaction mixture was poured into 2 *N* HCl (which removed the *tert*-butyl protecting group) and a yellow precipitate appeared. After filtration and dialysis against 0.01 *N* HCl and then exhaustively against water, the solution was lyophilized and gave a yield of white polymer of 88%.

C. Spectral Analysis. The spectra of aqueous solutions of polymers X–XVIII showed no absorption at 257 nm, indicating that the benzyl groups were completely removed (within 1.0%) in the final stage of synthesis.

D. Presence of Sulfur. Analysis for sulfur was performed on fraction C of polymer XVI by Galbraith Laboratories, Inc., Knoxville, Tenn. The analysis showed 0.13 ± 0.03% sulfur, which presumably is due to the presence of dimethyl sulfoxide during the treatment with 4-amino-1-butanol.

E. Removal of *tert*-Butyl Protecting Group. The Fourier transform ^1H NMR of sample XVI-C was measured on a Brüker HX-90 spectrometer with an NMR3 Digilab data system using 2500 pulses. The absence of a peak corresponding to the *tert*-butyl protecting group indicates that less than 1% of the side chains contain a *tert*-butyl ester.

F. Fractionation. The water-soluble copolymers were fractionated with methanol and ether by the procedure described in paper II,[13] after which they were dissolved in water, lyophilized, and dried in vacuo.

G. Titrations. The polymers were titrated to determine both their composition (for fractions of copolymers X–XVI, see below) and their titration curves. These potentiometric titrations were carried out on an instrument previously described,[28] using Marksson glass and reference electrodes, No. 880 and 881, respectively. The meter was calibrated just before and after each use with phthalate and phosphate buffers made up according to Bates.[29] The titration vessel was thermostated at 25 ± 0.1°, and all titrations were performed under a nitrogen atmosphere.

Ten milliliters of a solution containing 15 to 50 mg of polymer dissolved in 0.1 *N* KCl was placed in the titration vessel and allowed to reach thermal equilibrium. The titrant, which was 0.1 *N* KOH for the copolymers and 0.5 *N* HCl for the sodium salt of poly(L-glutamic acid), was delivered with an Agla Micrometer syringe with a Teflon needle. Each determination was carried out at least twice, and the titrations were demonstrated to be reversible. The individual activity coefficient for the hydrogen ion needed for the calculation of the p*K*'s of the polymers was obtained by measuring the pH of several dilute HCl solutions at an ionic strength of 0.1. The value for the activity coefficient was 0.78. The apparent p*K* at any degree of ionization was calculated using the well-known equation[30]

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

where α is the fraction ionized and is determined in the usual manner.[9] The curves of pK_{app} vs. α often curved up or down strongly at high and low degrees of ionization. This problem has been observed before,[3] and is attributed to the sensitivity of pK_{app} to small errors in the determination of the end points. To correct for these errors, the end-point volumes of titrant were adjusted by a small amount to give the best smooth curves. This adjustment averaged about 5% of the total amount of titrant. The error in the determination of pK_{app} is estimated to be ±0.05 p*K* units. The titration of the *N*-acetyl-*N'*-methylglutamic acid amide was carried out as above except that a carefully weighed sample was dissolved in 10 ml of 0.1 *N* KCl in the titration vessel. No end-point adjustments were made, and the error in pK_a is estimated to be ±0.02 p*K* units.

H. Determination of Concentration. The concentrations of all polymer solutions were determined using a micro Kjeldahl nitro-

Table I
Compositions and Chain Lengths of the Unfractionated Poly(γ-Bzl-L-Glu$^{m'}$:γ-*tert*-Bu-L-Glu$^{n'}$) Copolymers

Polymer no.	*tert*-Bu-Glu content of reaction mixture, mol %	Av mol wt[a] × 10^{-3}	$\overline{DP}$
I	5	140	650
II	10	260	1200
III	13	260	1200
IV	20	220	1100
V	0	160	730
VI	7	180	810
VII	10	170	770
VIII	15	140	650
IX	0		

[a] By viscometry, using the relation of Fujita et al.[31] for polymers in dichloroacetic acid.

gen analysis as described previously.[13] Each concentration was determined at least three times. The error in the determination was estimated to be ±3%.

I. Determination of Composition. Since the composition of these copolymers could not be determined by amino acid analysis (i.e., the hydrolysis products are glutamic acid and the decomposition products of 4-amino-1-butanol), these data were obtained using the information from titration experiments, nitrogen analyses, and molecular weight determinations. The mole fraction of *free* glutamic acid in the copolymer, X_{Glu}, was obtained from the following equation

$$X_{Glu} = 2[n_{OH-} - (n_N + n_{OH-})/\overline{DP}_w]/[n_N + n_{OH-} - (n_N + n_{OH-})/\overline{DP}_w] \quad (2)$$

where n_{OH-} is the number of equivalents of base required to titrate a sample containing n_N moles of nitrogen, determined as in section H. The term $(n_N + n_{OH-})/\overline{DP}_w$ is a correction for the titration of two end groups per polymer chain. $\overline{DP}_w$ is the degree of polymerization determined as in section L but using the approximate composition, $X_{Glu} \simeq 2n_{OH-}/(n_N + n_{OH-})$. This approximation introduced no error greater than 1% in X_{Glu}. The value of n_{OH-} used was the value which gave the best pK_{app} vs. α curve as discussed in section G.

J. Optical Purity. The starting materials as well as the final copolymers were checked for racemization by the L-leucyl dipeptide method of Manning and Moore.[24] The L-Leu-L-Glu and L-Leu-D-Glu were separated on a Technicon amino acid analyzer with sodium citrate elution buffer of pH 3.1. Dipeptide standards were prepared from D,L-glutamic acid.

K. Viscosity. The viscosities of samples I–VIII were determined in dichloroacetic acid at 25 ± 0.1° in a Cannon-Ubbelohde semimicro dilution viscometer as described earlier,[13] and the relation of Fujita et al.[31] was used to obtain only a rough molecular weight [using poly(γ-benzyl L-glutamate) as a model]. The viscosity of sample XVII was determined in 0.2 *M* NaCl at pH 7.3 in the manner described above and compared with the data of Idelson and Blout[27] to obtain a molecular weight for this polymer.

L. Determination of Molecular Weights. Molecular weights for the fractions from samples X–XVI and XVIII were determined by conventional sedimentation equilibrium using a Spinco Model E ultracentrifuge as reported earlier[13] with one major exception. The polymers were dissolved in a dilute HCl solution of pH 2.3 to suppress any ionization of the side-chain carboxyl groups. At this pH less than 0.5% of the side chains will be charged. This same solvent was used for determining c_0 (the concentration of polymer before centrifugation) needed for calculation of the molecular weights. The concentration dependence of the weight-average molecular weight $\bar{M}_w$ was determined for each sample, and $\bar{M}_z$ was computed at the lowest concentration run made on each fraction. The molecular weights are accurate to within ±5%.

The partial specific volumes ($\bar{v}$) of the P(HBG:Glu) copolymers, required for the calculation of molecular weight, were determined from the amino acid content as described by Cohn and Edsall.[32] A value of $\bar{v}$ = 0.816 for PHBG was used in the calculation of $\bar{v}$ for the copolymers.[13]

Table II
Characterization of the Fractionated Copolymers

Fraction[a]	L-Glutamic acid content, mol %	$\overline{M}_w \times 10^{-3}$ [b]	$\overline{M}_z/\overline{M}_w$	$\overline{DP}_w$
X-B	5.1	123	1.04	630
X-G	6.5	52	1.00	265
XI-F	11.0	116	1.10	610
XII-C	15.8	130	1.30	690
XIII-E	16.7	88	1.25	465
XIII-G	21.3	49	1.11	265
XIV-D	7.1	90	1.17	460
XIV-G	7.0	53	1.06	270
XV-C	10.4	85	1.13	440
XV-F	12.0	58	1.20	305
XVI-B	16.1	81	1.11	430
XVI-D	17.3	57	1.14	300
XVI-F	16.2	37	1.07	195
XVII	100	90	[c]	700
XVIII-B	0	170	1.5	830
XVIII-D	0	39	1.20	195

[a] Samples X–XVI were obtained from I–IV and VI–VIII, respectively. Samples XVII and XVIII were obtained from V and IX, respectively. The host was HBG in all cases, and the letter corresponds to the fraction obtained in the fractionation procedure. Sample XVII was not fractionated. [b] This value was determined for samples X–XVI and XVIII by conventional sedimentation equilibrium (with an extrapolation to zero concentration). The value for sample XVII was obtained by viscosity in 0.2 *M* NaCl at pH 7.3 using the data of Idelson and Blout.[27] [c] This value could not be obtained for sample XVII because viscosity measurements were used.

M. Optical Rotatory Dispersion and Circular Dichroism Measurements. The optical rotatory dispersion (ORD) and circular dichroism (CD) measurements were made with a Cary Model 60 spectropolarimeter equipped with a Model 6001 CD attachment, as described earlier.[13] Solutions for measurement in water at pH 2.3 were made up as described in section L, while those at pH 8 were made by dissolving some polymer in water and then titrating it with 0.1 *N* KOH to the proper pH. For measurements in 0.1 *N* KCl, the polymer was dissolved in a 0.1 *N* KCl solution and titrated to the desired pH with 0.1 *N* KOH or 0.1 *N* HCl. The experimental error in helix content, θ_h (which is taken as $-b_0/750$, where b_0 is the slope of the Moffitt–Yang plot obtained as described previously[13]), results from: (a) the error in concentration (±3%), (b) the error in values of b_0 for the complete helix and the complete coil (±3%), and (c) the error in the slope of the Moffitt–Yang plot (±2%).

II. Results

A. Characterization of the Copolymers. Table I summarizes the composition and the average degree of polymerization $\overline{DP}$ of the unfractionated P(BzG:*tert*-Bu-Glu) copolymers, and Table II shows the data for the fractionated P(HBG:Glu) copolymers that were investigated. The usual decrease in $\overline{DP}_w$ attributed to transaminolysis,[13,33] upon conversion of these polymers to their hydroxybutylglutamine 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. While the triethylamine initiated copolymers (X–XIII in Table II) show an increase in free glutamic acid content with decreasing molecular weight, there is no general change in the amount of glutamic acid from the composition intended. Compare, for example, copolymer I with the average of the fractions of X and the same with copolymers VIII and XVI. These analytical results suggest that there are no large departures from

randomness in these copolymers. Further evidence for randomness is presented in section IIIB. In any case, paper I[12] 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. Although samples XII-C, XIII-E, and XVIII-B have a higher $\bar{M}_z/\bar{M}_w$ ratio than the others, it is not significant since the helix content changes slowly for these large DP's.[13] The molecular weights obtained at various concentrations (0.1 to 0.3% w/v, data not shown) showed little if any concentration dependence within the experimental error, and thus the final $\bar{M}_w$'s were for the most part averages of the values at different concentrations.

Using the Manning–Moore[24] dipeptide procedure, hydrolyzates of copolymers XIII, XIV–XVI, and XVIII (6 *N* HCl for 1 day) were found to contain less than 2 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-glutamic acid.

B. ORD and CD Data for the Copolymers. The ORD and CD data for representative samples of fractions of P(HBG:Glu) at high and low temperature and high and low pH in water and 0.1 *N* KCl are shown in Figure 1. Both the ORD and the CD data clearly indicate the presence of a right-handed α-helical structure in all of the spectra.[34,35] At pH 2.3 the helix content is seen to increase with increasing glutamic acid content, as expected for a helix-forming residue. With increasing temperature and increasing pH, the ORD and CD spectra become characteristic of larger amounts of random coil mixed with smaller amounts of helix, indicating that these copolymers undergo a thermally and pH induced transition from the α helix to the random coil in water.

Measurements of b_0, shown in Figures 2 and 3, for the 13 copolymer fractions used to obtain σ and s at pH 2.3 and 8 studied over the range of λ 280–420 nm as a function of temperature confirm the above conclusion. The procedures used to obtain these curves were the same as those used previously.[17] The curves exhibited no concentration dependence, and they were demonstrated to be reversible in all cases. The size of the error symbols in Figure 2 reflects the experimental errors in θ_h arising from errors in concentration and in the slope of the Moffitt–Yang plot (see section IM for the magnitudes of these errors). All the curves were reproducible.

An examination of Figures 2 and 3 reveals some interesting things about the thermally induced melting curves of glutamic acid copolymers. First, at low pH, the melting curves of the copolymers are consistently above the curves for the host homopolymer (PHBG) of comparable DP. This difference between the homopolymer and the copolymers increases as the glutamic acid content is raised. Also, even though the DP increases from 265 to 465 for fractions XIIIG and XIIIE the compensatory effect of lowering the glutamic acid content (from 21.3 to 16.7%) causes the melting curves to become almost identical. Figure 3D shows that, at pH 2.3, a copolymer with 16.2% glutamic acid is much more helical than a host homopolymer of the same DP (195) and slightly more helical than a homopolymer with a much larger DP (830). Melting curves for copolymer fractions XIVG, XVF, and XVIB and homopolymer fractions XVIIIB and XVIIID were the same in water and 0.1 *N* KCl at pH 2.3, although the data for these samples in 0.1 *N* KCl only are shown in Figure 3. Since these samples cover a wide range of composition and chain lengths, it

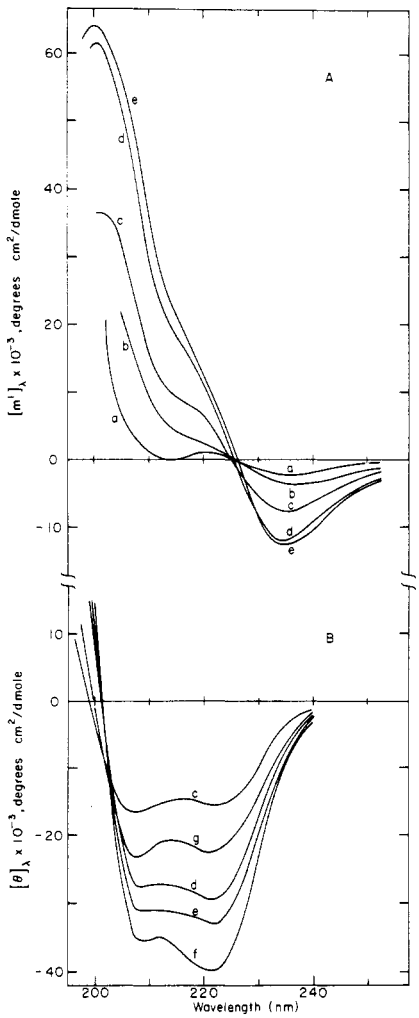

Figure 1. (A) ORD and (B) CD data in water (a–e) and 0.1 *N* KCl (f and g) for representative samples of glutamic acid copolymers: (a) 21.3% Glu at pH 8, $\overline{DP}_w$ = 265 (fraction XIII-G) at 75°; (b) 6.5% Glu at pH 8, $\overline{DP}_w$ = 265 (fraction X-G) at 70°; (c) 21.3% Glu at pH 8, $\overline{DP}_w$ = 265 (fraction XIII-G) at 26°; (d) 21.3% Glu at pH 2.3, $\overline{DP}_w$ = 265 (fraction XIII-G) at 26°; (e) 15.8% Glu at pH 2.3, $\overline{DP}_w$ = 690 (fraction XII-C) at 26°; (f) 7.0% Glu at pH 2.3, $\overline{DP}_w$ = 270 (fraction XIV-G) at 2°; (g) 7.0% Glu at pH 2.3, $\overline{DP}_w$ = 270 (fraction XIV-G) at 50°.

seems that 0.1 *N* KCl has no measurable effect on these copolymers at low pH.

The same copolymers at pH 8 have very different melting behavior than they have at low pH. In water at *low* temperature, the three fractions of low glutamic acid content have nearly the same helix content as they have at pH 2.3. However, as the temperature increases, the helix content steadily decreases below that of the polymers at low pH, and below that of the homopolymer. The high-composition copolymers exhibit melting curves which are continuously below both the homopolymer and the same copolymers at low pH. Thus, there appears to be a difference not only between the copolymers at low and high pH, but also between copolymers at the same pH, but with different composition. At pH 8 in 0.1 *N* KCl, the helix content is consistently lower than for the same copolymers at low pH, the difference becoming greater as the glutamic acid content is increased.

The last thing to note about these melting curves is that two of them (fractions XIIC and XVIB shown in Figures 2C and 3C, respectively) do not extend above ~45° because the polymer precipitates, and thus no melting data were

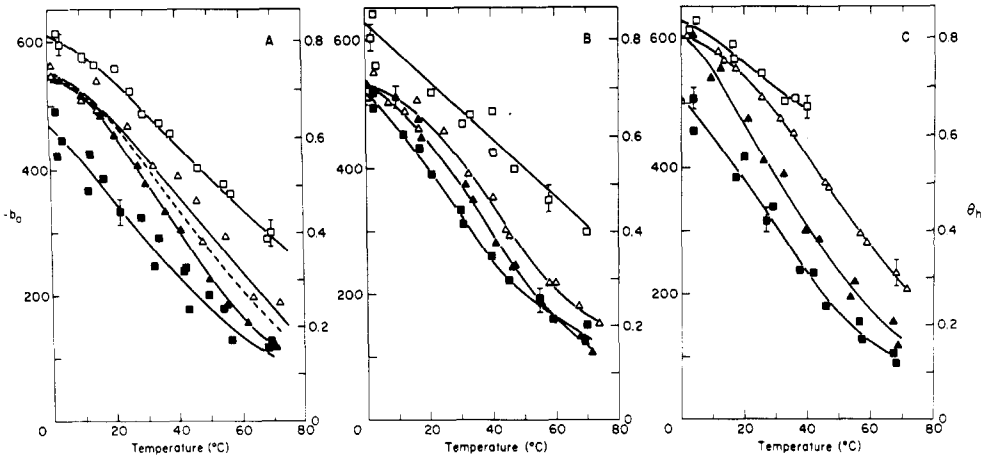

Figure 2. Temperature dependence of b_0 for Glu copolymers in water at pH 2.3 and 8. PHBG of $\overline{DP}_w$ = 720 (fraction V of paper II[13]) is included for comparison and is indicated by the dashed lines in A: (A) 21.3% Glu, $\overline{DP}_w$ = 265 (fraction XIII-G) at pH 2.3 (□) and pH 8 (■); 5.1% Glu, $\overline{DP}_w$ = 630 (fraction X-B) at pH 2.3 (△) and pH 8 (▲); (B) 16.7% Glu, $\overline{DP}_w$ = 465 (fraction XIII-E) at pH 2.3 (□) and pH 8 (■); 6.5% Glu, $\overline{DP}_w$ = 265 (fraction X-G) at pH 2.3 (△) and pH 8 (▲); (C) 15.8% Glu, $\overline{DP}_w$ = 690 (fraction XII-C) at pH 2.3 (□) and pH 8 (■); 11.0% Glu, $\overline{DP}_w$ = 610 (fraction XI-F) at pH 2.3 (△) and pH 8 (▲). The points are the experimental ones, and the lines represent the smoothed experimental curves. The size of the error symbols reflects the experimental errors in θ_h arising from errors in the determination of concentration and in the slope of the Moffitt–Yang plot.

obtained above this temperature. When the solutions were cooled, the polymers redissolved and showed the same melting behavior as before the precipitation. This behavior has been observed before in copolymers of HBG with valine[17] and leucine.[19]

C. b_0 for the Complete Helix and Coil. For the homopolymer PHBG studied in paper II,[13] the value of b_0 for the complete helix was taken to be −750 and for the complete coil as zero. Because these values vary with the nature of the side chain,[36] fraction XIIIG with a glutamic acid content of 21.3 mol % was examined in trifluoroethanol and dichloroacetic acid, with b_0 corrected for the dispersion of the refractive index of the solvent.[37] The experiments gave a value of −732 for the polymer in trifluoroethanol and +42 for it in dichloroacetic acid. These values demonstrate that the values used for the homopolymer and also other copolymers in this series are not unreasonable ones for these copolymers.

D. Titration Data. The results of the titration experiments with the copolymers, the homopolymer of glutamic acid, and *N*-acetyl-*N'*-methylglutamic acid amide are given in Figure 4, plotted as pK_{app} vs. the degree of ionization, α (α = 1 when all glutamic acid side chains are charged). In Figure 5 the titration results are replotted as pK_{app} vs. the average charge per residue, $\bar{Z}$/DP. This method of plotting the data allows comparisons between polymers of different composition at equal charge densities. The straight line extrapolations of the helical and coil regions of the poly(L-glutamic acid) titration curve are included for purposes of qualitative comparison only and are not intended to represent a rigorous extrapolation to $\bar{Z}$/DP = 0. The titration data for polymers of similar composition were usually well within experimental error of each other, and in such cases only representative titration curves are shown.

The titration curve of poly(L-glutamic acid), curve 5 in Figure 4, shows the well known three regions in its titration curve.[3] The region from α = 1 to 0.7 is the titration of the random coil (the pK_{app} dropping almost linearly with α as α decreases). Then after enough charges have been neutralized, the polymer starts to undergo a coil-to-helix transition from about α = 0.7 to 0.2, the lower nearly linear portion being the titration of the full helix. The last portion of the curve from α = 0.2 to 0 is the titration of the precipitated polymer with another nearly linear part near the end.

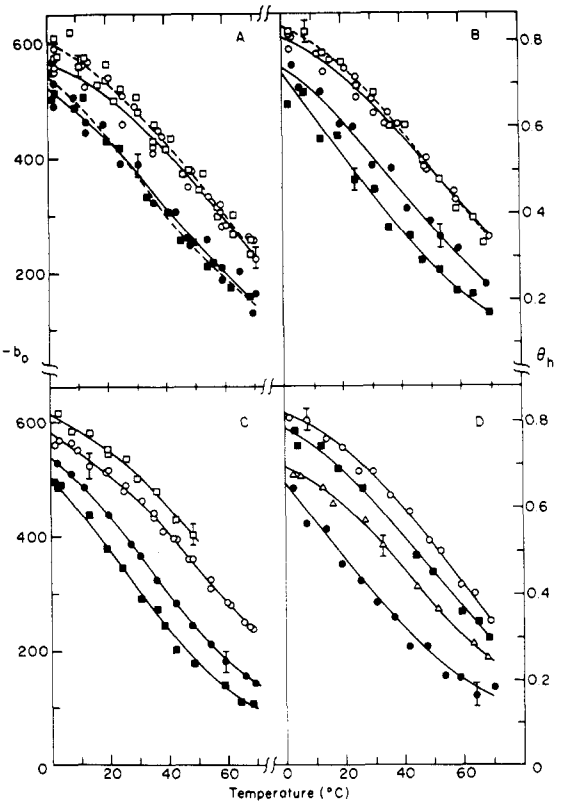

Figure 3. Temperature dependence of b_0 for Glu copolymers and PHBG homopolymers in 0.1 *N* KCl: (A) (—) 7.0% Glu, $\overline{DP}_w$ = 270 (fraction XIV-G) at pH 2.3 (○) and pH 8 (●); (- - -) 12.0% Glu, $\overline{DP}_w$ = 305 (fraction XV-F) at pH 2.3 (□) and pH 8 (■); (B) 7.1% Glu, $\overline{DP}_w$ = 460 (fraction XIV-D) at pH 2.3 (○) and pH 8 (●); 17.3% Glu, $\overline{DP}_w$ = 300 (fraction XVI-D) at pH 2.3 (--□--) and pH 8 (—■—); (C) 10.4% Glu, $\overline{DP}_w$ = 440 (fraction XV-C) at pH 2.3 (○) and pH 8 (●); 16.1% Glu, $\overline{DP}_w$ = 430 (fraction XVI-B) at pH 2.3 (□) and pH 8 (■); (D) 16.2% Glu, $\overline{DP}_w$ = 195 (fraction XVI-F) at pH 2.3 (○) and pH 8 (●); PHBG, $\overline{DP}_w$ = 830 (fraction XVIII-B) (■); PHBG, $\overline{DP}_w$ = 195 (fraction XVIII-D) (△). The points are the experimental ones, and the lines represent the smoothed experimental curves. The error symbols are the same as in Figure 2.

The pK_0 of poly(L-glutamic acid) may be obtained by extrapolating any one of these nearly linear portions to α = 0.

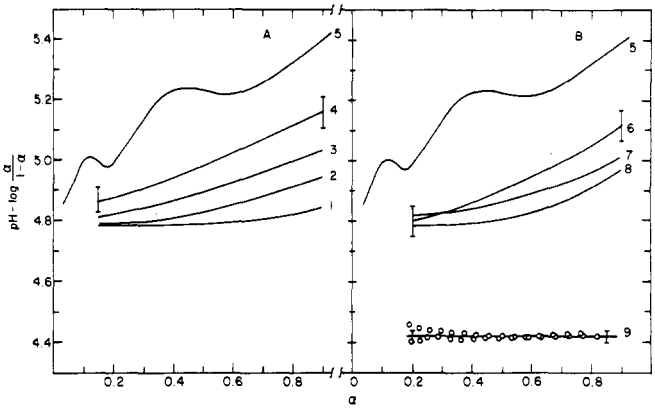

Figure 4. A plot of the apparent pK [pH − log $\alpha/(1-\alpha)$] vs. the degree of ionization, α (α = 1 is the fully ionized state), for the copolymers and poly(L-glutamic acid) in 0.1 N KCl at 25°: (1) 5.1% Glu (fraction X-B); (2) 11.0% Glu (fraction XI-F); (3) 15.8% Glu (fraction XII-C); (4) 21.3% Glu (fraction XIII-G); (5) poly(L-glutamic acid) (polymer XVII); (6) 16.1% Glu (fraction XVI-B); (7) 10.4% Glu (fraction XV-C); (8) 7.1% Glu (fraction XIV-D); (9) N-acetyl-N'-methylglutamic acid amide.

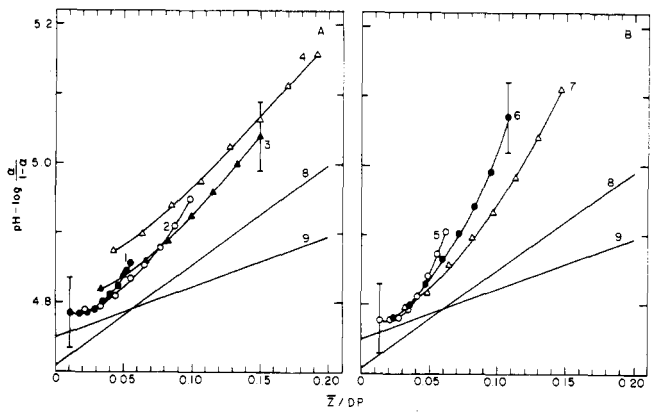

Figure 5. A plot of the apparent pK [pH − log $\alpha/(1-\alpha)$] vs. the average charge per residue $\bar{Z}$/DP (the maximum value of $\bar{Z}$/DP is the mole fraction of Glu in the copolymer): (1) 6.5% Glu (fraction X-G); (2) 11.0% Glu (fraction XI-F); (3) 16.7% Glu (fraction XIII-E); (4) 21.3% Glu (fraction XIII-G); (5) 7.0% Glu (fraction XIV-G); (6) 12.0% Glu (fraction XV-F); (7) 16.2% Glu (fraction XVI-F). Lines 8 and 9 represent linear extrapolations of the helix and coil portions of the poly(L-glutamic acid) titration curve, respectively. The extrapolations are included for comparison and are not intended to represent rigorous extrapolations to $\bar{Z}$/DP = 0.

However, this extrapolation is not straightforward because of the length of the extrapolation compared with the range over which experimental data are obtained. A linear extrapolation of the helix and coil regions gives a pK_0 of 4.75 ± 0.05 which agrees well with previous work in which a linear extrapolation was employed[3] (pK_0 = 4.72). Other authors[4,5,9] have used a curvilinear extrapolation of the titration curves, and Olander and Holtzer[9] have indicated that this curvature leads to a pK_0 of poly(L-glutamic acid) of 4.40 at an ionic strength of 0.1.

The copolymers examined in this study have a much simpler titration behavior than the homopolymer. The pK_{app} at α = 0 for all the copolymers is the same (within experimental error) at about 4.8. In Figure 4 it can be seen that, as the degree of ionization rises, the pK_{app} also increases. Each of the titration curves also shows a slight upward curvature; there is, however, no obvious helix–coil transition as there was for the homopolymer. As expected, the change in pK_{app} between α = 0 and α = 1 increases as the glutamic acid content increases.

The pK_a of the N-acetyl-N'-methylglutamic acid amide is 4.42 ± 0.02 and is considerably below the pK_0 of the copolymers in this study. It is, however, very close to the pK_0 of poly(D,L-glutamic acid) (pK_0 = 4.40)[9] titrated at the same ionic strength (μ = 0.1). Rinaudo and Domard[38] have titrated N-acetyl-N'-ethylglutamic acid amide without added electrolyte and obtained a pK_a of 4.45. The equivalent weight determined from our titrations was 202 ± 3 in excellent agreement with the calculated molecular weight (202.2).

From the titration data in Figure 5, it can be seen that at a given charge density, the value of pK_{app} − pK_0 is much larger for the copolymers than for helical or coil linear extrapolations of the poly(L-glutamic) acid titration curve. This would also be true (although the magnitude of the difference would be smaller) if curvilinear extrapolations were used. In general, the slope of the curves in Figure 5 is greater for copolymers with lower glutamic acid content.

III. Discussion

A. Helix–Coil Parameters for Poly(L-glutamic acid). The melting curves described in section II 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-glutamic acid).[39] This procedure has been discussed extensively in earlier papers of this series.[12,13] The approximations corresponding to the theories of Lifson[40] and Allegra[41] were both used to fit the data; the exact theory of Lehman and McTague[42] 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.[12,17] Since the melting behavior of the copolymers at low pH was the same in water and in 0.1 N KCl, both sets of data were combined for these calculations. The two sets of data at high pH in water and in 0.1 N KCl were analyzed separately. The results of these calculations for a few representative samples are shown in Table III. Both the second-order (Allegra) and first-order (Lifson) approximations give results which agree well with each other. The higher order (Allegra) approximation will be used in all subsequent discussion of the L-glutamic acid parameters.

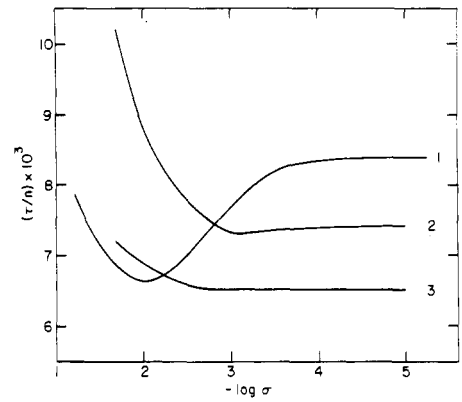

Figure 6. Determination of the best temperature-independent value of σ as the one which corresponds to the lowest value of τ for the glutamic acid copolymers at pH 2.3 (1), at pH 8 in water (2), and at pH 8 in 0.1 N KCl (3).

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 II.[13] The best fit for all copolymer data was obtained by minimizing τ. Figure 6 shows a graph of τ divided by n, the

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

L-Glutamic acid content, mol %	$\overline{DP}_w$	Solvent conditions	Temp, °C	$\theta_{h,expt}$	$\theta_{h,theor}$ Lifson[b,d]	$\theta_{h,theor}$ Allegra[c,d]	$\theta_{h,theor}$ Lehman–McTague[c,d]
7.1	460	pH 2.3	0	0.809	0.794	0.795	
			30	0.660	0.615	0.613	
			60	0.427	0.375	0.373	
7.1	460	pH 8	0	0.737	0.749	0.749	
		0.1 *N* KCl	30	0.541	0.503	0.503	
			60	0.301	0.261	0.261	
11.0	610	pH 2.3	0	0.807	0.814	0.815	
			30	0.641	0.652	0.652	
			60	0.371	0.410	0.410	
11.0	610	pH 8	0	0.827	0.737	0.738	
		Water	30	0.520	0.480	0.480	
			60	0.233	0.226	0.226	
17.3	300	pH 2.3	0	0.836	0.813	0.813	0.812
			30	0.677	0.667	0.669	0.668
			60	0.425	0.431	0.434	0.433
17.3	300	pH 8	0	0.723	0.685	0.686	
		0.1 *N* KCl	30	0.451	0.441	0.442	
			60	0.225	0.217	0.218	
21.3	265	pH 2.3	0	0.811	0.819	0.820	0.819
			30	0.657	0.682	0.685	0.684
			60	0.452	0.445	0.451	0.449
21.3	265	pH 8	0	0.627	0.631	0.633	
		Water	30	0.387	0.385	0.386	
			60	0.187	0.165	0.165	

[a] The parameters used for PHBG are those of Table II in paper II.[13] [b] The parameters used for L-glutamic acid were obtained by fitting the data with the Lifson[40] theory. [c] The parameters used for L-glutamic acid were obtained by fitting the data with the Allegra[41] theory. [d] The values of σ used in these calculations for L-glutamic acid at pH 2.3 and 8 were 1.0×10^{-2} and 6×10^{-4}, respectively.

number of samples in each set of data, vs. σ. At low pH the best fit is obtained for $\sigma = 1.0 \times 10^{-2}$. At pH 8 in water, the best value is $\sigma = 6 \times 10^{-4}$. There is also a very shallow minimum near $\sigma = 6 \times 10^{-4}$ for the data at pH 8 in 0.1 *N* KCl, and this value was used for both sets of data at pH 8. The absence of a deep minimum in the τ vs. σ curve has been observed before.[16,19] In order to estimate the uncertainty in σ, the best value of σ for each fraction at each temperature was determined using the values of s listed in Table IV, and the standard deviation in these values of σ was determined. For the data at pH 2.3 the standard deviation in σ was 9×10^{-3}, and for the data at pH 8 in 0.1 *N* KCl it was 4×10^{-3}. The minimum values of τ/n for the data at pH 2.3 and at pH 8 in 0.1 *N* KCl are nearly the same, but the minimum value for pH 8 in water is larger than the other two. This is so even though the same types of errors should be present in all three sets of data. The reason for the poorer fit at pH 8 in *water* is probably the importance of long range electrostatic interactions between the charged side chains under these conditions. These types of interactions are not provided for in the theory.[12] The effect of electrostatic interactions would explain the qualitative difference between the melting curves of the high and low composition copolymers in water at pH 8 as discussed in section IIB. Apparently, the presence of 0.1 *N* KCl reduces the difference between the electrostatic free energy of the helix and the random coil in these copolymers enough (see section IIIB) so that it can be neglected in our calculations.

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 7 shows these data plotted against temperature. The error symbols were

Table IV
Values of the Zimm-Bragg Parameter s for Poly(L-glutamic acid)

Temp, °C	s[a] pH 2.3[b]	s[a] pH 8 in 0.1 *N* KCl[c]	s[a] pH 8 in water[c]
0	1.47	0.98	0.96
10	1.42	0.97	0.96
20	1.35	0.97	0.96
30	1.28	0.97	0.95
40	1.21	0.95	0.93
50	1.13	0.94	0.91
60	1.07	0.93	0.89
70	1.01	0.90	0.86

[a] Calculated using the Allegra[41] theory. [b] Calculated with $\sigma = 1.0 \times 10^{-2}$. [c] Calculated with $\sigma = 6 \times 10^{-4}$.

calculated by fitting each melting curve at the best fit value of σ and determining the standard deviations of these s values. No estimates were made for the standard deviations for the charged data in water because of the likelihood that nonrandom errors due to electrostatic interactions were present. The large standard deviations in the low pH values of s are due to the large value of σ used in the calculations. At such large values of σ, the change in $\theta_{h,calcd}$ with a change in s is relatively small. Thus, a large range of s values will give almost the same calculated melting curves and fit the data nearly as well as the best fit value. Figures 8 and 9 show the computed melting curves along with the

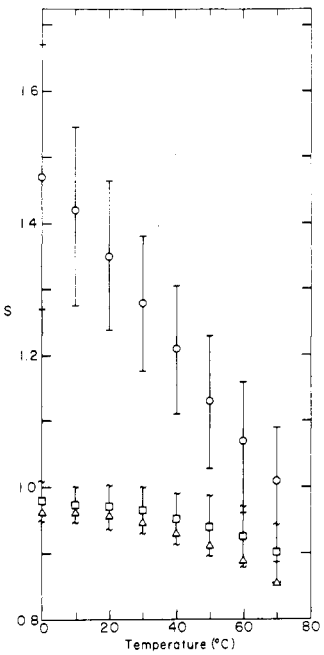

Figure 7. A plot of s vs. T for poly(L-glutamic acid) at pH 2.3 (○), at pH 8 in 0.1 N KCl (□), and at pH 8 in water (△). The error symbols for the values at pH 2.3 (⌶), and at pH 8 in 0.1 N KCl (⌶), are described in section IIIA.

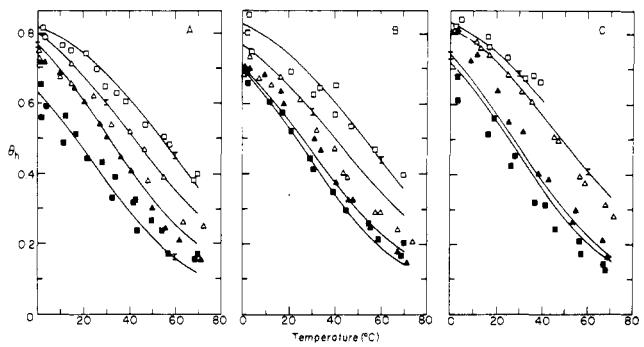

Figure 8. Comparison of the calculated melting curves, obtained from the parameters of the Allegra theory for L-glutamic acid given in Table IV and those of PHBG of Table II in paper II,[13] with the experimental points for copolymers in water: (A) 21.3% Glu, $\overline{DP}_w$ = 265 (fraction XIII-G) at pH 2.3 (□) and pH 8 (■); 5.1% Glu, $\overline{DP}_w$ = 630 (fraction X-B) at pH 2.3 (△) and pH 8 (▲); (B) 16.7% Glu, $\overline{DP}_w$ = 465 (fraction XIII-E) at pH 2.3 (□) and pH 8 (■); 6.5% Glu, $\overline{DP}_w$ = 265 (fraction X-G) at pH 2.3 (△) and pH 8 (▲); (C) 15.8% Glu, $\overline{DP}_w$ = 690 (fraction XII-C) at pH 2.3 (□) and pH 8 (■); 11.0% Glu, $\overline{DP}_w$ = 610 (fraction XI-F) at pH 2.3 (△) and pH 8 (▲). The error symbols indicate errors in the calculated values of θ_h arising from errors in composition and chain length (see Figure 2 for additional errors in experimental points).

experimental points. The errors shown here are due to the uncertainties in $\bar{M}_w$ and composition. It should be noted that no account was taken of possible errors in the Zimm–Bragg parameters for PHBG. The values of s calculated from the melting curves of PHBG in 0.1 N KCl agreed with the values obtained in water[13] within 1%. For the charged polymers, where no deep minimum in the plot of τ/n vs. σ was observed, values of σ below the value used result in calculated melting curves which are nearly indistinguishable from those shown.

The thermodynamic quantities ΔG^0 (the free energy), ΔH^0 (the enthalpy), and ΔS^0 (the entropy) for the conversion of a coil residue of L-glutamic acid to a helical one at the end of a long helical sequence can be obtained from the value of s and its temperature dependence. Figure 10 shows a plot of $\Delta G^0(-RT \ln s)$ vs. temperature with error symbols calculated as in Figure 7. The data for all the uncharged polymers and the charged polymers in 0.1 N KCl were fit to a straight line by a weighted least-squares method using the deviations in Figure 10 as weighting factors as described in paper IV.[15] The resulting least-squares lines are shown in Figure 10. The parameters calculated by this method are given in Table V with the errors calculated by the least-squares method. These thermodynamic values should be regarded only as estimates in view of the large errors involved in their computation.

B. Titrations. For a polymeric acid in which all deviations from the titration curve of a simple acid are assumed to be electrostatic in nature, the titration curve is given by the expression[43,44]

$$pK_{app} = pH - \log\left(\frac{\alpha}{1-\alpha}\right) = pK_0 + (0.434/RT)\frac{\partial G_{el}}{\partial \alpha} \quad (3)$$

where G_{el} is the electrostatic free energy of the polymer. The titration curves of poly(L-glutamic acid) have been interpreted using this equation by several workers, and values of s for the helix–coil transition of L-gluamic acid without electrostatic interactions have been calculated using the theory of Zimm and Rice.[10] Curve 5 in Figure 4 shows a titration curve for poly(L-glutamic acid) in 0.1 N KCl, the almost straight portions from α = 0.2 to 0.30 and from α = 0.7 to 0.9 representing the titrations of pure helix and pure coil, respectively. If these curves are appropriately extrapolated to α = 0, one finds the intrinsic pK of the carboxyl groups in the polymer. Integration of eq 3 between α = 0 (uncharged) and α = 1 (fully charged) along the extrapolated titration curves of the helix and the coil gives the electrostatic free energy of both of these conformations, and indicates that the electrostatic free energy of the coil state is lower than that of the helix, thus causing the helix–coil transition. The titration curves of the copolymers are much simpler (i.e., they are nearly linear from low to high values of α), indicating that there is no large difference between the electrostatic free energies of the helical and coil states of the copolymers in 0.1 N KCl. Although there are electrostatic interactions present in both the helix and the coil, as shown by the rise in pK_{app} as the degree of ionization increases, and by the increase in G_{elec} of *both* helix and coil with increasing glutamic acid composition, this does not affect the melting behavior because the electrostatic free energy is nearly the same in both. The good fit of the melting data at pH 8 in 0.1 N KCl using the Allegra theory,[41] which does not take long range electrostatic interactions into account, also indicates that electrostatic interactions do not affect the melting behavior of the copolymers under these conditions. Furthermore, the value of s at pH 8 in 0.1 N KCl calculated for each fraction individually with $\sigma = 6 \times 10^{-4}$ is independent of the composition within our experimental error. Thus, the use of 0.1 N KCl and random copolymers with low glutamic acid content allows the calculation of σ and s for charged L-glutamic acid without electrostatic interactions between side chains. It should also be noted that the absence of an electrostatic effect on the helix–coil transition indicates that there are no large blocks of glutamic acid in the copolymers since the electrostatic effects in such blocks would be similar to those obtained in poly(L-glutamic acid). A similar reduction in the electrostatic effects on the helix–coil transition has been noted for poly(Lys-Ala-Ala) which was found to be 40% helical even when fully charged in 0.1 N KCl at 30°.[45]

It should be noted that, even though $\Delta G_{elec} = 0$ for the helix–coil transition in these copolymers, nevertheless, the

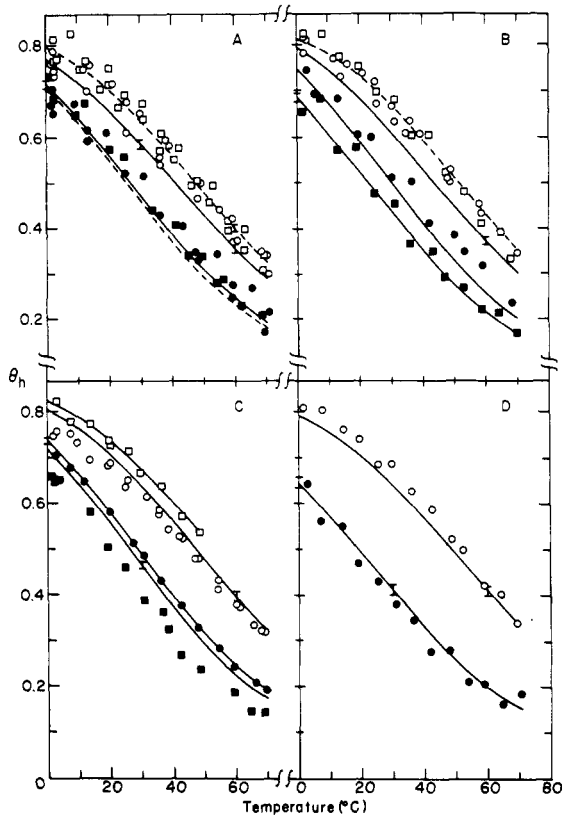

Figure 9. Comparison of the calculated melting curves, obtained from the parameters of the Allegra theory for L-glutamic acid given in Table IV and those of PHBG of Table II in paper II,[13] with the experimental points for copolymers in 0.1 *N* KCl: (A) (—) 7.0% Glu, $\overline{DP}_w$ = 270 (fraction XIV-G) at pH 2.3 (○) and pH 8 (●); (- - -) 12.0% Glu, $\overline{DP}_w$ = 305 (fraction XV-F) at pH 2.3 (□) and pH 8 (■); (B) 7.1% Glu, $\overline{DP}_w$ = 460 (fraction XIV-D) at pH 2.3 (○) and pH 8 (●); 17.3% Glu, $\overline{DP}_w$ = 300 (fraction XVI-D) at pH 2.3 (--□--) and pH 8 (—■—); (C) 10.4% Glu, $\overline{DP}_w$ = 440 (fraction XV-C) at pH 2.3 (○) and pH 8 (●); 16.1% Glu, $\overline{DP}_w$ = 430 (fraction XVI-B) at pH 2.3 (□) and pH 8 (■); (D) 16.2% Glu, $\overline{DP}_w$ = 195 (fraction XVI-F) at pH 2.3 (○) and pH 8 (●). The error symbols are the same as in Figure 8 (see Figure 3 for additional errors in the experimental points).

helix content at $\alpha = 0$ and 1 differs (at a given temperature), i.e., there *is* an isothermal pH-induced helix–coil transition (cf. curves with open and closed symbols in Figure 3). This arises from the fact that σ and s differ for charged and uncharged glutamic acid residues, because of relatively short-range (e.g., side chain backbone) interactions and *not* from long-range electrostatic interactions.

When the titration curves are plotted as pH − log $\alpha/(1-\alpha)$ vs. $\bar{Z}$/DP as in Figure 5, it can be seen that the slope increases as the HBG content increases, while the value of pK_0 is nearly constant. This indicates that the electrostatic free energy of *both* the helix and coil increases, at a given charge density, as the HBG content increases. A similar effect has been observed by Nylund and Miller[6] as the L-leucine content was increased (up to 30%) in random copolymers of L-leucine and L-glutamic acid, and it was attributed to a lowering of the effective dielectric constant between the charges due to the increased hydrocarbon content in the vicinity of the carboxyls and, possibly, to changes in the water structure near the hydrocarbon chains. This interpretation is also consistent with the titration curves for our copolymers since the HBG side chains would increase the hydrocarbon nature of the medium surrounding the carboxyl groups.

Table V
Thermodynamic Parameters for L-Glutamic Acid[a]

	pH 2.3	pH 8 in 0.1 *N* KCl
ΔG°_{20}, cal/mol	−174 ± 49	17 ± 19
ΔH°, cal/mol	−1070 ± 330	−190 ± 110
ΔS°, eu	−3.1 ± 1.1	−0.72 ± 0.37

[a] Calculated as described in the text.

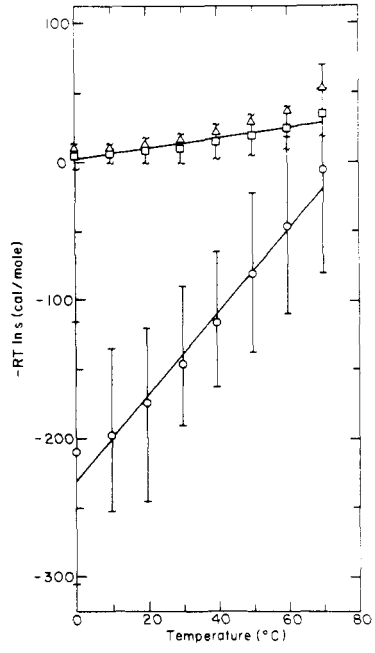

Figure 10. A plot of $-RT \ln s$ (i.e., ΔG^0) vs. T for poly(L-glutamic acid) at pH 2.3 (○), at pH 8 in 0.1 *N* KCl (□), and at pH 8 in water (△). The error symbols for the values at pH 2.3 (I) and at pH 8 in 0.1 *N* KCl (I) are described in section IIIA.

The relatively nonpolar environment of the side chain carboxyls may also explain the difference between the pK_a of *N*-acetyl-*N'*-methylglutamic acid amide (pK_a = 4.42) and the pK_0 of the copolymers (p$K_0 \simeq$ 4.8) measured in 0.1 *N* KCl. The effects of the solvent environment on the ionization process can be explained in terms of a medium effect, which reflects differences in chemical and electrostatic interactions of the isolated ions with the solvent, and a salt effect which is characterized by electrostatic interactions among the ions.[46] In order to estimate the solvent conditions which would lead to the observed values of pK_0, the data of Spivly and Shedlovsky[47] for acetic acid in water–ethanol mixtures were used to approximate the medium effect, and the Debye–Hückel equation was used to calculate the salt effects. The data of Spivly and Shedlovsky indicate that a mixture of about 20% (w/w) ethanol in water with a dielectric constant of 67.0[48] would be expected to give a pK_a for the *N*-acetyl-*N'*-methylglutamic acid amide of 4.8 when the ionic strength is 0.1. This is only a very rough approximation, but it indicates that it is reasonable to assume that the influence of the rest of the polymer molecule (effectively serving as a medium similar to 20% ethanol) could be responsible for the difference between pK_0 in the polymers and the pK_a of the model compound. It is interesting to note that the pK_0 of poly(D,L-glutamic acid)[9] is nearly the same as the pK_a for our model compound when both are titrated in 0.1 *N* KCl. If our in-

terpretation is correct, this would indicate that the carboxyl groups in poly(D,L-glutamic acid) are exposed to the solvent to a greater degree than the carboxyls in the copolymers.

C. Comparison with Other Results. Values for the helix–coil transition parameters of L-glutamic acid obtained from titration curves are based on the assumption, pointed out by Zimm and Rice,[10] that the helix forming ability is independent of the charge state except for the side chain–side chain repulsions between charged groups. In contrast, our results show that, even under conditions where electrostatic repulsions do not contribute to the helix–coil transition, there are significant differences between the Zimm–Bragg parameters for charged and uncharged L-glutamic acid. Furthermore, it seems likely that the random coil in the copolymers will be different from the random coil of charged poly(L-glutamic acid) which has a higher charge density. The latter would be expected to be restricted to a relatively extended structure in order to minimize the electrostatic free energy. Thus, the values of σ and s obtained from our experiments with copolymers cannot be compared directly with values obtained by titration. Figure 11 illustrates the difference between the two methods, where the subscripts u and c indicate that the glutamic acid side chains are uncharged or charged, respectively. The Helix_c and Coil_c states correspond to those observed in the copolymers in which the electrostatic interactions are small. The "Extended Coil_c" state corresponds to the random coil of charged poly(L-glutamic acid). Coil_c and Extended Coil_c differ in that the ensemble of states in the latter is expected to be more extended than that in the former. The free energy of electrostatic interaction, which contributes to this difference in conformation, is *not* included in the free energy of the three charged states in Figure 11, viz. Helix_c, Coil_c, and Extended Coil_c. The reason for this is that, experimentally, ΔG^0_3 for the copolymers was shown not to contain an electrostatic contribution; also, the Zimm–Rice method,[10] which evaluates ΔG^0_5 from titration data by extrapolating the titration curve of the extended coil to the uncharged state, eliminates the electrostatic contribution to ΔG^0_5, i.e., they compute the free energy difference between uncharged helix and uncharged (but extended) coil. Thus, ΔG^0_6, in our usage here, arises only from the different geometrical forms of the coil, with the electrostatic contribution omitted.

Figure 11 can also be used to show that there is a difference between the intrinsic pK's of glutamic acid in the helix and coil state, as follows. ΔG^0_1 and ΔG^0_3 can be obtained from the parameters in Table V at pH 2.3 and 8, respectively. Neglecting conformational differences between Coil_u and Coil_c, ΔG^0_2 and ΔG^0_4 are related to the intrinsic pK's of the helix and the coil by the following equations.

$$\Delta G^0_2 = 2.303RT\text{p}K_{0,\text{helix}} \quad (4a)$$

$$\Delta G^0_4 = -2.303RT\text{p}K_{0,\text{coil}} \quad (4b)$$

Using the values of ΔG^0_{20} and ΔS^0 in Table V, we calculate $\Delta G^0_1 = -158$ cal/mol and $\Delta G^0_3 = -21$ cal/mol at 25°; with these, and with the fact that the free energy change around a cycle must be zero, we obtain the following

$$\Delta G^0_1 + \Delta G^0_3 = -(\Delta G^0_2 + \Delta G^0_4) = -179 \pm 75 \text{ cal} \quad (5)$$

Using eq 4a and 4b, this corresponds to a value of 0.13 ± 0.06 for the quantity ($\text{p}K_{0,\text{helix}} - \text{p}K_{0,\text{coil}}$) at 25° in 0.1 N KCl. This difference in the intrinsic pK's for the helix and coil may be due to the differences in the interactions between the side chains and the backbone in the helical and coil conformations. It may also reflect differences in the interactions with the neighboring side chains (e.g., hydrogen bonds or hydrophobic interactions) between the helix and coil states. The reasons for the differences in the intrinsic pK's are, by eq 5, the same as the reasons for the difference in the helix–coil transition behavior for charged and uncharged glutamic acid residues. To the extent that the differences are due to side chain–backbone interactions, they should be the same in the copolymers as in poly(L-glutamic acid). The effects of neighboring side chains would be expected to be somewhat different in the copolymers and in poly(L-glutamic acid).

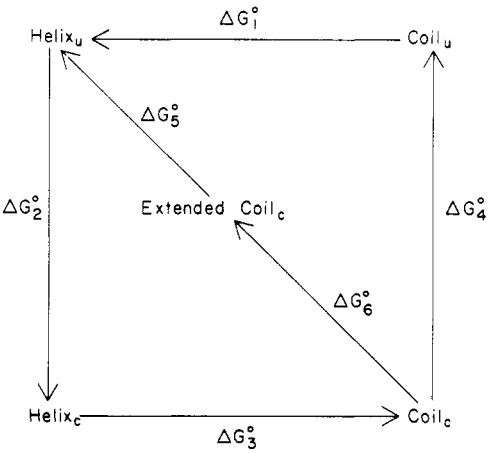

Figure 11. Elementary processes in an isothermal pH-induced helix–coil transition. The subscripts u and c indicate that the glutamic acid side chains are uncharged or charged, respectively.

A difference in the intrinsic pK's of the coil and the helix in poly(L-glutamic acid) as large as 0.1 could change the values obtained by the titration method for the free energy change of the coil-to-helix transition by as much as 50%.[9] If the intrinsic pK of the helix is higher than that of the coil, as expected from our results (see eq 5), the coil-to-helix free energy change would be more negative than the value computed by using the same $\text{p}K_0$ for the helix and the coil. Due to the long extrapolations involved in obtaining the $\text{p}K_0$'s from poly(L-glutamic acid) titrations, it has not been possible to rule out differences in the intrinsic pK's of the helix and the coil as large as 0.1.[9] Using the value of 4.40 for the $\text{p}K_0$ of the helix and the coil, Olander and Holtzer[9] obtained a free energy change per residue of −178 cal for the coil-to-helix transition of poly(L-glutamic acid) in 0.1 N NaCl at 25°. The enthalpy change for the same transition was found to be −975 ± 50 cal/(residue mol), and the entropy change was −2.67 ± 0.1 cal/(residue mol deg). While these thermodynamic parameters are similar to those for uncharged glutamic acid in our study (see Table V), it must be emphasized that the "coil" states in the two experiments are different and that the titration method relies on the assumption that the $\text{p}K_0$'s of the helix and coil are the same.

It would be useful to be able to evaluate ΔG^0_6 since this would give the free energy difference between the "coil" states in our experiments and in the titration experiments. Unfortunately, the experimental errors and the uncertainties in the $\text{p}K_0$'s of the various states would seem to preclude any meaningful evaluation of the free energy change from presently available experimental data. Intuitively, it seems reasonable to expect ΔG^0_6 to be positive due to the loss of entropy in the "Extended Coil_c" state.

The values of the initiation parameter σ reported in the literature[4,7,49] vary between 2.5×10^{-3} and 5×10^{-3} for poly(L-glutamic acid) titrated at an ionic strength of 0.1 or

0.2. These values are intermediate between our values of σ for charged and uncharged glutamic acid.

D. Conclusion. Since the ultimate goal of this work is to aid in understanding the factors responsible for determining protein conformation, it is interesting to compare the conformational preferences of glutamic acid residues in proteins whose crystal structures have been determined with what might be expected from our results. This matter is discussed more fully in the accompanying paper[22] in which it is shown that specific medium-range interactions can modify the conformational preference of glutamic acid residues *in proteins*. Specifically, the presence of a positively charged side chain four residues from a glutamic acid increases the likelihood for the negatively charged glutamic acid residue to be helical in proteins. Thus, in proteins, both short-range and medium-range interactions have a strong influence on the conformation of glutamic acid residues. On the other hand, in the copolymers described in this work, short-range interactions are expected to predominate. As a result it should be a relatively poor approximation to use the experimentally determined Zimm–Bragg σ and s parameters for charged glutamic acid (without accounting for medium-range interactions) to predict the fraction of glutamic acid residues in proteins which are helical or, conversely, to estimate the Zimm–Bragg parameters from protein data. This is demonstrated by the fact that our results indicate that charged glutamic acid, which is the predominant form at neutral pH, is nearly indifferent to forming a helix (i.e., $s \simeq 1$) while recent surveys of several proteins[50,51] indicate that glutamic acid has the highest probability to be helical of all the amino acids.

While charged glutamic acid is indifferent as a helix maker, uncharged glutamic acid is the strongest helix former studied by this method to date. The reason for this stability is not clear from this work or from the results of titration experiments. It has been suggested[52,53] that hydrogen bonding between the side chains might be responsible for the high stability of the poly(L-glutamic acid) helix, but other authors[6,9] have disagreed with this interpretation. Since these hydrogen bonds could, of course, occur in both the helix and the coil, it would be necessary for them to stabilize the helix preferentially in some way. In the copolymers, both carboxyl–carboxyl and carboxyl–amide side chain hydrogen bonds are possible. Since there is no noticeable change in the values of s as the composition is changed, it would be necessary for both types of hydrogen bonds to be of equivalent strength in their stabilization of the helix, if such stabilization were present. The value of σ for uncharged glutamic acid is the largest reported for any of the amino acids studied by this method. As with the large value of s, the reason for this value of σ is not clear from our work.

The differences in helical stability between charged and uncharged glutamic acid are due to changes in side chain–backbone interactions and changes in the interactions with the surrounding medium (possibly including other side chains) due to the negative charge on the side chain. This difference in the helical stability of charged and uncharged glutamic acid residues requires a difference in the intrinsic pK's of the helix and coil states. These results call into question one of the fundamental assumptions of the Zimm–Rice treatment[10] as discussed in section IIIC.

The results described here demonstrate the importance of short- and medium-range interactions on the conformations of polypeptides and proteins containing glutamic acid. Short-range effects such as side chain–backbone interactions are responsible for the differences between charged and uncharged glutamic acid observed in the copolymers. In order to account for the titration curve of poly(L-glutamic acid) it is necessary to include the medium- and long-range effects due to electrostatic repulsions between neighboring side chains as shown by Zimm and Rice.[10] The effect of the nature of the fourth residue on either side of a glutamic acid residue in proteins, as discussed in the accompanying paper,[22] indicates that short-range effects may be dominant in determining the helical probability of glutamic acid residues unless there is the possibility for interaction with a positive charge on the $i + 4$ or $i - 4$ side chain.

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Appendix

A. Synthesis of 4-Amino-1-butanol. All materials are described in the Experimental Section.

4-Chlorobutyl Acetate.[54] Into a 3-l. three-necked round-bottom flask was placed acetyl chloride (1.07 l., 15 mol) and THF (1.22 l., 15 mol). The mixture was stirred and refluxed for 2 days. The low-boiling fraction was distilled into a 2-l. round-bottom flask and refluxed for two additional days. The low-boiling components were removed by distillation and the combined residues were distilled in vacuo. The fraction boiling at 73–77° (8 mm) afforded 85 g (38%) of a colorless liquid: n^{20}D 1.4358 (lit. n^{20}D 1.4325); ^{1}H NMR (neat) τ 8.16 (m, $-CH_2CH_2-$) and 7.98 (s, CH_3C) (total 7.0 H), 6.36 (m, 2.0 H, CH_2Cl), and 5.87 ppm (m, 2.0 H, CH_2O); ir (neat) 5.77 (s), 6.92 (m), 7.19 (m), 7.30 (m), 8.1 (br s) and 9.6 μ (br s).

***N*-(4-Acetoxybutyl)phthalimide.**[55] A solution of 4-chlorobutyl acetate (365 g, 2.42 mol) in DMF (400 ml) was added dropwise to a stirred mixture of potassium phthalimide (436 g, 2.36 mol) in DMF (800 ml) at 100° over several hours. Upon completion of the addition, the mixture was heated (at 100°) and stirred for 3 hr and allowed to cool overnight. The solution was poured into 6 l. of crushed ice and stored at 4°. The white solid was collected, washed with water, and dried in vacuo to give 555 g (90%): mp 58–60°. A second crop was recovered from the filtrate after the addition of water. The white solid was collected, washed with water, and recrystallized from alcohol–water to give 24 g (total yield 579 g (94%)): mp 60–61°; ^{1}H NMR (DMSO-d_6) τ 8.36 (m, 4.0 H, $-CH_2CH_2-$), 7.98 (s, 3.0 H, $CH_3C(O)$), 6.39 (m, 2.0 H, NCH_2), 5.96 (m, 2.1 H, CH_2O), and 2.12 ppm (s, 4.0 H, phenyl); ir (KBr) 3.34 (w), 5.74 (m), 5.83 (s), 6.83 (w), 6.99 (w), 7.17 (m), 7.32 (m), 8.00 (m), 9.55 (m), 13.90 (m), and 14.04 μ (m).

4-Amino-1-butanol. A mixture of *N*-(4-acetoxybutyl)phthalimide (318 g, 1.21 mol) and 20% potassium hydroxide (2-l.) was refluxed for 1 hr. The bulk (≈1500 ml) of the water was distilled through a Vigreux column (30 cm) and discarded. The column was replaced with a distilling head and the residue was distilled with a continuous dropwise addition of water until the head temperature returned to ~100°. The distillate was concentrated by distilling through the Vigreux column to less than 1 l. and then the concentrate was continuously extracted with hot chloroform. The chloroform was distilled off at atmospheric pressure, and then the residue was distilled in vacuo to yield 71.0 g (66%) of a colorless liquid: bp 96–101° (8 mm); n^{29}D 1.4631 (lit.[56] n^{20}D 1.4625); ^{1}H NMR (DMSO-d_6) τ 8.61 (m, 3.9 H, $-CH_2CH_2-$), 7.44 (m, 2.1 H, $NHCH_2$), 7.14 (s, 2.9 H, NH_2 and OH), and 6.60 ppm (m, 2.1 H, CH_2O), the addition of D_2O resulted in the loss of the peak at 7.14 ppm, τ, and a new peak appearing at 6.34 ppm (s, 3.1 H, HDO); ir

(neat) 2.98 (s), 3.06 (s), 3.43 (s), 3.53 (s), 6.22 (m), 6.98 (m), 7.29 (m), and 9.5 μ (br s).

B. Synthesis of *N*-Acetyl-*N'*-methylglutamic Acid Amide. *tert*-Butyloxycarbonyl-γ-benzyl Glutamate.[57] γ-Benzyl L-glutamate (4.74 g, 20 mmol) was reacted in a 1-l. round-bottmed flask with *tert*-boc azide (3.1 ml, 22 mmol) and TEA (5.4 ml, 39 mmol) in DMSO (100 ml) at room temperature. The solid dissolved overnight. Water (300 ml) was added to the DMSO solution and extracted three times with ether. Sulfate buffer (0.1 *N* $KHSO_4$, 0.2 *N* K_2SO_4) (210 ml) was added and the aqueous phase was extracted three times with ethyl acetate. The combined ethyl acetate extracts were washed three times with a few milliliters of water and dried over Na_2SO_4 (anhydrous). Evaporation of the solvent gave an oil that was converted to the dicyclohexylamine salt by dissolving in ether (300 ml) and adding dicyclohexylamine (4.4 ml, 22 mmol). After stirring for several hours, the solid was collected and dried affording 6.77 g (65%): mp 141–142° (lit.[58] mp 138–139°); $[\alpha]^{22.5}D$ +12.9° (*c* 1, methanol) (lit.[58] $[\alpha]^{20}D$ +11.9° (*c* 1, methanol).

N^{α}-*tert*-Butyloxycarbonyl-γ-benzyl Glutamate α-Methylamide. *tert*-Butyloxycarbonyl-γ-benzyl glutamate dicyclohexylamine salt (1.04 g, 2 mmol) was weighed into a separatory funnel, 0.5 *N* H_2SO_4 (4.0 ml) was added, and the aqueous solution was twice extracted with ethyl acetate. The combined ethyl acetate extracts were washed with water and dried over $CaSO_4$ (anhydrous).

The oil was dissolved in THF (10 ml), *N*-methylmorpholine (0.22 ml, 2 mmol) was added, and the solution was cooled to −20°. Isobutyl chloroformate (0.26 ml, 2 mmol) was added, and after 5 min 0.8 ml of 2.5 *M* CH_3NH_2–dioxane was added. The cooling bath was removed and the reaction mixture was warmed to room temperature overnight. The salts were filtered off and the filtrate was evaporated to dryness in vacuo. The solid was recrystallized from ethyl acetate–hexane affording 505 mg (72%): mp 125–126°; $[\alpha]^{22}D$ 0 ± 0.5 (*c* 1, methanol); ^1H NMR ($CDCl_3$) τ 8.57 (s, 9.3 H), 7.97 and 7.53 (m and m, 4.2 H), 7.23 (d, J = 5 Hz, 3.0 H), 5.83 (m, 1.0 H), 4.87 (s, 2.0 H), 4.57 (d, J = 7 Hz, 0.8 H), 3.56 (s, 0.8 H), and 2.66 ppm (s, 4.9 H). TLC: (i) single spot, R_f 0.36 in chloroform:acetic acid (19:1); (ii) single spot, R_f 0.64 in *n*-butyl alcohol:acetic acid:water (3:1:1); (iii) single spot, R_f 0.86 in methanol:chloroform (1:1).

Anal. Calcd for $C_{18}H_{26}N_2O_5$: C, 61.69; H, 7.48; N, 8.00. Found: C, 61.56; H, 7.38; N, 7.98.

N^{α}-Acetyl-γ-benzyl Glutamate α-Methylamide. N^{α}-*tert*-Butyloxycarbonyl-γ-benzyl glutamate α-methylamide (285 mg, 0.81 mmol) was dissolved in 3.4 *N* HCl–dioxane (5 ml) for 15 min. The solvent was removed and the residue was dried in vacuo over KOH.

The crude hydrochloride was treated with acetic anhydride (77 μl, 0.81 mmol) and TEA (220 μl, 1.62 mmol) in THF (10 ml). After stirring at room temperature for 1 hr, the solid was filtered off and washed with THF. The combined filtrates were evaporated, and the residual solid was recrystallized from ethyl acetate–hexane affording 142 mg (59%) of colorless needles: mp 175–176°; $[\alpha]^{22}D$ −7.8 ± 0.5 (*c* 1, methanol); ^1H NMR ($CDCl_3$) τ 8.06, 7.86, and 7.59 (s, m, and m, 6.9 H), 7.24 (d, J = 5 Hz, 2.9 H), 5.53 (m, 1.0 H), 4.89 (s, 2.1 H), 3.14 (m, 2.0 H), and 2.67 ppm (s, 5.0 H).

Anal. Calcd for $C_{15}H_{20}N_2O_4$: C, 61.63; H, 6.90; N, 9.59. Found: C, 61.87; H, 7.05; N, 9.71.

N-Acetyl-N'-methylglutamic Acid Amide. To the stirred solution of N^{α}-acetyl-γ-benzyl glutamate α-methylamide (70 mg) in absolute methanol (5 ml) was added Pd (10% on charcoal) (6 mg), and a stream of hydrogen gas, presaturated with methanol, was bubbled through the suspension for 2 hr at room temperature. The reaction mixture was filtered through a bed of Celite in methanol and washed twice with methanol. The filtrate was taken to dryness in vacuo and dried over P_2O_5. The crude product was dissolved in warm ethyl acetate (3 ml) to which a few drops of methanol had been added. The crystallization was completed by addition of hexanes affording 28 mg (58%) of the title compound in colorless prisms: mp 130°; $[\alpha]^{22}D$ −20.5 ± 0.5 (*c* 0.44, water).

Anal. Calcd for $C_8H_{14}N_2O_4$: C, 47.52; H, 6.98; N, 13.86; neutralization equivalent, 202. Found: C, 47.68; H, 6.95; N, 13.96; neutralization equivalent 202. TLC: 40 γ single spot R_f 0.57 in solvent system chloroform:methanol:NH_4OH (17%) 20:20:9. Soluble in water, methanol, and acetone; insoluble in ether. (Compare *N*-acetyl-*N'*-ethylglutamic acid amide: mp 130–131°; $[\alpha]^{22}D$ −23 (*c* 1 water).[59])

References and Notes

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The Effect of Neighboring Charges on the Helix Forming Ability of Charged Amino Acids in Proteins[1]

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ABSTRACT: It has been found that the fraction of glutamic acid residues which are helical in proteins is larger than might be expected from the experimentally determined value of the helical stability constants of glutamic acid. In order to understand this difference, the effect of neighboring charged side chains on the glutamic acid residues in proteins of known structure is examined. It is found that a positively charged side chain four residues away from a glutamic acid greatly enhances its probability to be helical. Similar results are obtained for aspartic acid, lysine, arginine, and histidine.

The helix–coil stability constants for charged poly(L-glutamic acid) in 0.1 N KCl have been determined[3] experimentally using the host–guest technique[4-11] under conditions where the long-range electrostatic interactions do not contribute significantly to the helix–coil transition. These results indicated that an isolated charged glutamic acid residue at 20° is essentially indifferent as far as its helix forming or breaking tendency is concerned. On the other hand, recent surveys of several proteins whose crystal structures have been determined indicate that glutamic acid has the highest probability to be helical of all the amino acids.[12,13] In comparing the helical stability in random copolymers with the probability that a residue will be helical in a protein, it is important to remember that the former seems to be determined by short-range interactions,[4,5,14] while the latter will also include the effects of specific medium- and long-range interactions.[12,15] In this paper, it is shown by a statistical analysis of the conformations of amino acid residues in proteins of known structure that a specific medium-range interaction (viz., the presence of a positively charged side chain four residues away from a glutamic acid residue in a protein) greatly enhances the likelihood that glutamic acid will be helical. It is also shown that this type of interaction (i.e., with oppositely charged side chains) influences the conformational preference of aspartic acid, lysine, arginine, and histidine.

I. Methods

The proteins used in this investigation are the 14 listed in Table V of ref 12 plus bovine pancreatic trypsin inhibitor[16] and trypsin.[17] The definition of an α helix is that used by Burgess et al.,[12] and various frequencies of occurrence (as indicated below) were recorded.

The statistical significance of any difference in the likelihood for a residue to be helical in the presence or absence of a specific interaction was determined using Fisher's "goodness of fit" criterion, based on an exact treatment of 2×2 contingency tables.[18] The four entries in each contingency table were the number of times, $n_{\alpha\beta}$, that a given amino acid residue was found to occur in the data set with conditions specified by the subscripts. When $\alpha = 1$, say, the residue being studied is helical, and when $\alpha = 2$, it is not helical. When $\beta = 1$, the type of medium-range interaction being studied is present, and it is absent when $\beta = 2$. For example, if the ith residue is glutamic acid, $\beta = 1$ may mean that the $(i + 4)$th residue is positively charged, whereas $\beta = 2$ indicates that it is not positively charged. If the interaction being studied does not affect the likelihood for the amino acid under consideration to be helical, one would expect n_{11}/n_{21} to be roughly the same as n_{12}/n_{22}. Fisher's method measures the probability, P, that deviations of n_{11}/n_{21} from n_{12}/n_{22} as large or larger than those actually observed in the data could occur by chance; e.g., if P is 0.01, then the probability is 1 in 100 that such a difference could occur by chance.

In order to evaluate P, it is necessary to consider the contingency table further. With the margins of the table fixed [i.e., with $(n_{11} + n_{12})$, $(n_{11} + n_{21})$, $(n_{12} + n_{22})$, and $(n_{21} + n_{22})$ fixed], one can calculate the values expected for each entry in the table if the conditions specified for the rows (i.e., values of β) do not affect the distribution between the columns (i.e., values of α). We define an expected frequency $e_{\alpha\beta}$ as

$$e_{\alpha\beta} = (n_{\alpha 1} + n_{\alpha 2})(n_{1\beta} + n_{2\beta})/(n_{11} + n_{12} + n_{21} + n_{22}) \quad (1)$$

Further, it is convenient to define a quantity, Q, by the equations

$$Q = \sum_{h=0}^{m} |(n_{11} - h)!(n_{12} + h)!(n_{21} + h)!(n_{22} - h)!|^{-1} \quad (2a)$$

for $n_{11} \leq e_{11}$