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Synthesis and Potentiometric Titration of Random Copolymers of L-Leucine and L-Glutamic Acid[1a,b]

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The monomer reactivity ratios for the amine-initiated copolymerization of L-leucine and L-glutamate N-carboxyanhydrides in N,N-dimethylformamide have been determined. Six random copolymers containing up to 30% leucine were prepared and their titration curves determined. When the copolymers are in the helical conformation, the electrostatic interaction of the side-chain glutamate carboxyls was found to increase as the leucine content increases even though the carboxyls on the average become further separated. Side-chain carboxyl–carboxyl hydrogen bonding does not account for the observed effect. The most reasonable explanation is that the increase in hydrocarbon component lowers the effective dielectric constant of the medium near the remaining carboxyls.

The stability in aqueous solutions of ordered (generally helical) conformations of homopolymers of L-glutamic acid,[2-4] L-lysine,[5] L-serine,[6] L-tyrosine,[7] and L-histidine[8] has been investigated. Unless the amino acid has an ionizable side chain, the homopolymer is only sparingly soluble in water. The behavior in aqueous media of polymers of nonionic and nonpolar amino acids is of considerable interest, particularly the stability of ordered, hydrogen-bonded conformations and the importance of hydrophobic interactions. Several methods have been used to make water-soluble polymers containing nonionic residues.[9-11] A method not previously exploited in this connection is the synthesis of random copolymers in which one of the monomeric units is water-soluble and renders the copolymer soluble. By random we mean that the amino acid composition at any point along the polymer chain, when averaged over all polymer molecules, is invariant of the position in the chain. The contribution of each monomer to physical properties can hopefully be assessed using a statistical approach. Before attempting to generate random copolymers, the kinetics of copolymerization must be studied.

A copolymer of leucine and glutamic acid of unknown monomer distribution has been investigated.[11] In this paper we report on the synthesis and potentiometric titration of random leucine–glutamic acid copolymers; the stability of the helix is reported in the accompanying paper.[12] The effect of leucine residues on the ionization of the side-chain glutamic acid carboxyls is of particular interest. The helix–random coil transition can be driven by ionization of the side chains, and any alteration in their ionization may alter the helix–coil transition as well as affect the stability of the helix. Previous studies have not investigated this point.

(1) (a) This investigation was supported in part by Public Health Service Research Grant GM-08409; (b) taken in part from the Ph.D. Thesis of R. E. Nylund, University of Iowa, 1964.
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(3) M. Idelson and E. Blout, *J. Am. Chem. Soc.*, 80, 4631 (1958).
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(5) J. Applequist and P. Doty in "Polyaminoacids, Polypeptides and Proteins," M. Stahmann, Ed., University of Wisconsin Press, Madison, Wis., 1962, p. 161.
(6) (a) Z. Bohak and E. Katchalski, *Biochemistry*, 2, 228 (1963); (b) G. D. Fasman and E. Blout, *J. Am. Chem. Soc.*, 82, 2262 (1960).
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(11) (a) G. Fasman, C. Lindblow, and E. Bodenheimer, *Biochemistry*, 3, 155 (1964); (b) G. Fasman, C. Lindblow, and E. Bodenheimer, *J. Am. Chem. Soc.*, 84, 4977 (1962).
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Experimental

Copolymerization Kinetics. L-Leucine N-carboxyanhydride (NCA) and γ-benzyl-L-glutamate NCA (Pilot Chemical Co.) were copolymerized in N,N-dimethylformamide (DMF) by initiation with *n*-hexylamine. The general procedure for the polymerization and isolation was as described previously.[13] In a typical experiment the desired ratio of glutamate NCA to leucine NCA to a total of 3×10^{-3} mole of anhydride was dissolved in 25 ml. of DMF, and enough initiator was added to make the anhydride to initiator ratio equal to 700. The polymerization was run at 25° and followed by CO_2 evolution. The reaction was stopped at 5% conversion by addition of 25 ml. of 3 *N* HCl.

The copolymer samples were hydrolyzed in sealed tubes containing 3 *N* HCl at 110°. Debenzylation prior to hydrolysis was found to be unnecessary. The hydrolysate was placed on a column (Phoenix Precision Instrument Co. ion-exchange resin blend X-15, particle size 19–25 μ) pre-equilibrated with pH 3.2 citrate buffer. The L-glutamic acid was eluted with pH 3.25 citrate buffer (0.2 *M*) followed by elution of the L-leucine with pH 4.25 citrate buffer (0.2 *M*). The amounts of glutamic acid and leucine were determined by reaction with ninhydrin and analysis at 570 mμ using a Beckman DU spectrophotometer. Molar absorptivities were determined by use of standard solutions.

Synthesis of Random Copolymers. The copolymerization was carried out as in the previous section with slight modification. The total N-carboxyanhydride concentration was 10 wt. % and the anhydride to initiator ratio was 600. The polymerization was followed by CO_2 evolution and stopped at 50% monomer conversion by the addition of 3 *N* HCl.

The glutamate composition was determined from the absorption at 258 mμ of a known concentration of the copolymer in chloroform using a Beckman DU spectrophotometer. Standard curves were obtained using poly-γ-benzyl-L-glutamate. Copolymer dispersion curves (240–280 mμ) when multiplied by a constant superimposed on the poly-γ-benzyl-L-glutamate dispersion curve, indicating that the presence of leucine does not alter the absorption band of the benzyl group. The amount of leucine in the copolymer was obtained by difference. This method of analysis was much quicker and at least as accurate as that used in the copolymerization kinetics studies. The small amounts of material involved made its use impractical there. The copolymers were then debenzylated with anhydrous HBr in benzene by the procedure of Idelson and Blout.[3]

Molecular Weight Determinations. Number-average molecular weights of the debenzylated polymer were determined in DMF at 37° using a Mechrolab high speed membrane osmometer. With the low molecular weights encountered here, there was a tendency for the solute to diffuse slowly through the membrane, resulting in a decrease in osmotic pressure of about 8%/hr. Upon injecting a solution into the osmometer, the osmotic pressure was recorded as a function of time and the osmotic pressure at zero time determined by extrapolation. After each run the solution side of the membrane was flushed with solvent until the original reading with solvent on both sides of the membrane was obtained. Using this procedure osmotic pressures were reproducible although the precision was less than with higher molecular weight samples. The molecular weights were calculated from the osmotic pressure extrapolated to zero concentration. Their precision is about ±10%.

Weight-average molecular weights were estimated using the relations reported by Mitchell, Woodward, and Doty.[14]

pH Measurements. A Beckman GS pH meter was used with Beckman standard buffer (pH 6.86). In a typical titration 100 mg. of copolymer was dissolved in 100 ml. of aqueous NaCl solution by addition of enough NaOH to ionize the carboxyls completely. The carboxylate ions were then titrated with 1 *M* HCl. Duplicate titrations agreed well. Precipitation problems were encountered before the titration was complete. Nevertheless nearly all titrations used the theoretical amount of HCl (±3%).

Results

Reactivity Ratios. Analysis of the copolymerization kinetics was handled in the usual manner for addition polymerization. The copolymer composition is determined by the propagation reactions

$$\sim\!\!\sim G + M_G \xrightarrow{k_{11}} \sim\!\!\sim G$$

$$\sim\!\!\sim G + M_L \xrightarrow{k_{12}} \sim\!\!\sim L$$

$$\sim\!\!\sim L + M_G \xrightarrow{k_{21}} \sim\!\!\sim G$$

$$\sim\!\!\sim L + M_L \xrightarrow{k_{22}} \sim\!\!\sim L$$

where M_G and M_L are glutamate and leucine NCA, respectively, and the remaining species are growing peptide chains with terminal residues as indicated. Through use of the rate equation for monomer consumption and the steady-state approximation on chain concentrations, an equation may be derived which for low conversion (<10%) may be arranged[15] to

$$F(f - 1)/f = r_1F^2/f - r_2 \qquad (1)$$

where $F = (M_G)/(M_L)$, $f = m_G/m_L$ (mole ratio in copolymer), and r_1 ($=k_{11}/k_{12}$) and r_2 ($=k_{22}/k_{21}$) are the monomer reactivity ratios. The left side of eq. 1 may be plotted against F^2/f to obtain r_1 and r_2. The composition of copolymers (5% conversion) for various monomer ratios is given in Table I and shown graphically in Figure 1. Least-squares analysis gives $r_1 = 1.57 \pm 0.08$, $r_2 = 0.61 \pm 0.16$, and $r_1r_2 = 0.92 \pm 0.25$. Although analysis in terms of radical addition kinetics may not be rigorously applicable to our system,[16] the conditions under which the polymers were synthesized sensibly satisfy the requirements for using this type of analysis.

Random Copolymers. Random copolymers may be synthesized either by finding conditions under which

(13) R. E. Nylund and W. G. Miller, *Biopolymers*, 2, 131 (1964).

(14) J. C. Mitchell, A. E. Woodward, and P. Doty, *J. Am. Chem. Soc.*, 79, 3995 (1957).

(15) P. J. Flory, "Principles of Polymer Chemistry," Cornell University Press, Ithaca, N. Y., 1953, p. 178 ff.

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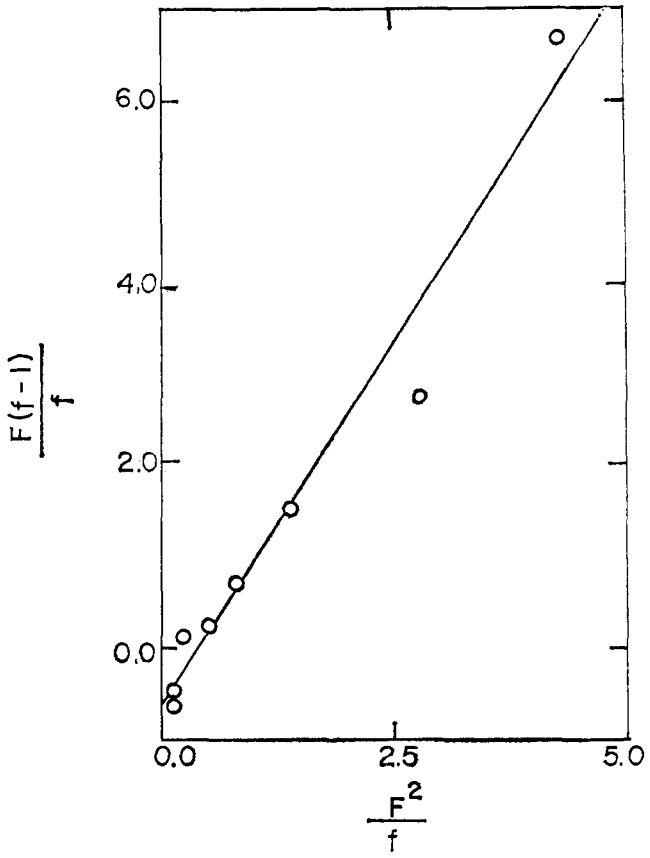

Figure 1. Determination of monomer reactivity ratios for the *n*-hexylamine-initiated copolymerization of γ-benzyl-L-glutamate NCA and L-leucine NCA.

$r_1 = r_2 = 1$, by continuously adding monomer to the polymerizing system such that the unreacted monomer composition remains constant, or by stopping the polymerization at low conversions. It is difficult to find conditions to satisfy the requirements for the first method, and the second is difficult to handle experimentally. The third is the least satisfactory though the most practical. By carrying polymerizations to 50% conversion, it was possible to prepare copolymers of sufficiently high molecular weight and yet of nearly random composition. Six copolymers of varying leucine composition were prepared in this manner and are characterized in Table II.

Table I. Copolymerization of L-Leucine NCA and γ-Benzyl-L-glutamate NCA in DMF

Mole fraction of glutamate in—	
Monomer mixture (x_G)	Copolymer— 5% conversion (X_G)
0.880	0.925
0.780	0.819
0.684	0.773
0.567	0.686
0.456	0.594
0.354	0.571
0.156	0.229
0.120	0.193

A knowledge of the monomer reactivity ratios allows the incremental glutamate composition in the copolymer

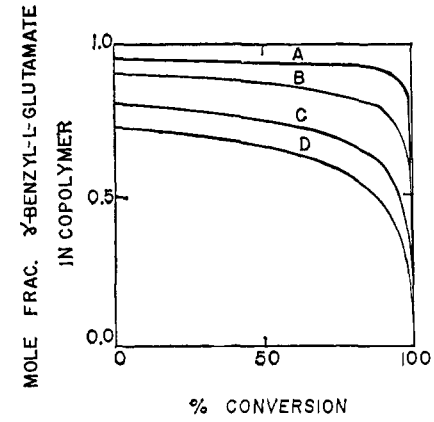

Figure 2. Incremental copolymer composition. Initial mole fraction of γ-benzyl-L-glutamate in monomer mixture: A, 0.93; B, 0.85; C, 0.72; D, 0.63.

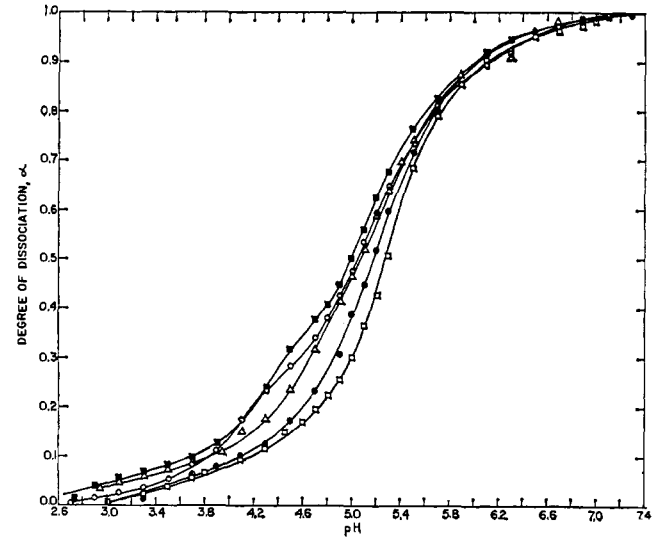

Figure 3. Titration curves in 0.200 *M* NaCl at 25°. Mole per cent leucine: ○, 0.0; ■, 5.7; △, 12.4; ●, 22.9; □, 29.1.

(X_G) to be calculated for any monomer ratio. If the glutamate NCA content, $(x_G)_0$, of the initial monomer mixture is known, the mole fraction of glutamate NCA (x_G) remaining at any degree of conversion, $1 - (M)/(M)_0$, may be determined graphically from

$$\ln (M)/(M)_0 = \int_{(x_G)_0}^{(x_G)} dx_G/(X_G - x_G) \qquad (2)$$

From eq. 1 and 2 the incremental composition of the copolymer can be calculated at various points along the chain as well as the incremental composition if the polymerization had been allowed to proceed further. Results of such calculations for four copolymers are given in Table II and Figure 2. The maximum change in glutamate composition along the chain varies from 1% in the 5.7 copolymer to 10% in the 29.1 copolymer, quite in contrast to the change in composition if the polymerizations had been run to complete conversion.

Potentiometric Titration Curves. The titration curves for several of the copolymers in 0.200 *M* NaCl at 25° are shown in Figure 3. As the leucine composition increases the titration curve is shifted toward higher pH with most of the shift occurring at low α (degree of

Table II. Random Copolymers of L-Leucine and L-Glutamic Acid

Sample designation (% leucine)	$(x_G)_0$	No. av. mol. wt. $(\overline{M}_n)$	Av. glutamate composition of copolymers Obsd.	Calcd.[a]	Incremental glutamate composition[a] Initiated end	Terminal end	90%[b]	95%[b]	98%[b]
0	1.000	12,000							
5.7	0.930	7,300	0.943 ± 0.005	0.950	0.954	0.945	0.906	0.875	0.83
12.4	0.853	7,400	0.876	0.888	0.901	0.874	0.784	0.737	0.65
16.2	0.853	10,400							
22.9	0.721		0.771 ± 0.007	0.780	0.804	0.752	0.600	0.505	0.38
24.6	0.721	12,000							
29.1	0.633	8,800	0.707 ± 0.020	0.700	0.732	0.662	0.466	0.38	0.28

[a] Calculated from eq. 1 and 2. [b] Mole fraction of glutamate at terminal end if copolymerization had been carried to per cent conversion as indicated.

ionization). The 5.7 copolymer is anomalous. Assuming all deviations from the titration curves of simple acids and bases are electrostatic in origin, the titration curve for polyelectrolytes can be described[17] by

$$\mathrm{pH} - \log \alpha/(1-\alpha) = \mathrm{p}K_0 + (1/2.3kT)(\partial G_{el}/\partial \overline{Z}) \quad (3)$$

For the leucine–glutamate copolymers, K_0 is the intrinsic dissociation constant of the γ-carboxyl and G_{el} is the electrostatic free energy of a polyion of average charge $\overline{Z}$. As charged groups remote from each other in a polymer chain do not interact, the titration

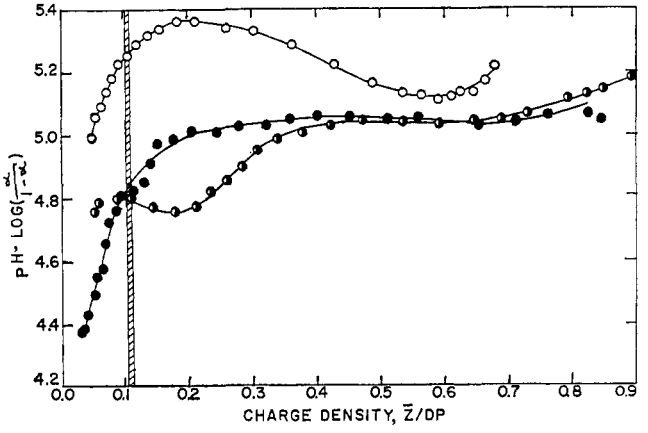

Figure 4. Titration curves in 0.200 M NaCl at 25°. Mole per cent leucine: ◑, 0.0; ●, 12.4; ○, 29.1. Hatched area indicates region in which precipitation is first observed as pH is lowered.

curves for high molecular weight linear polyelectrolytes are nearly independent of molecular weight. Rather than plotting the left side of eq. 3 against $\overline{Z}$ or α as is done frequently, it is more meaningful here to plot against the charge density. Charge density is defined as the average charge per amino acid residue, $\overline{Z}/DP$, where DP is the degree of polymerization of the copolymer. The titration data are plotted in this manner in Figures 4 and 5. The polyglutamic acid curve agrees well with those previously published.[4,18] The shape of the curves is affected by the helix–coil transition as well as by precipitation problems at low α. Although the pH at which precipitation occurs varies by 0.8–1.0 in going from pure polyglutamic acid to the 29.1 copolymer, at a fixed ionic strength and solute concentration each precipitates at approximately the same charge density (hatched area in Figures 4–6). The meaningfulness of the titrations curves at a pH below the precipitation point is unknown.

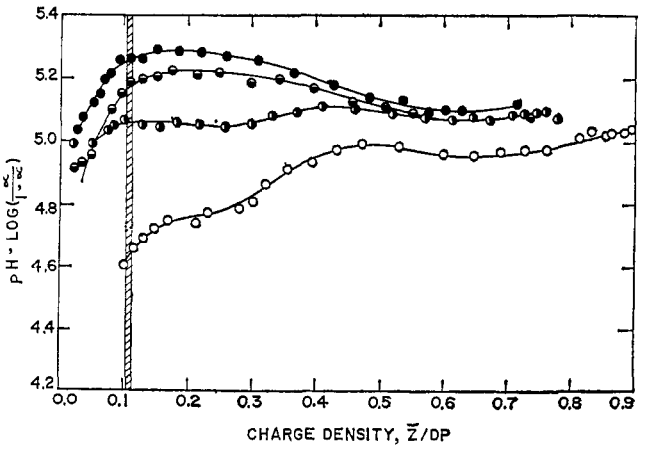

Figure 5. Titration curves in 0.200 M NaCl at 25°. Mole per cent leucine: ○, 5.7; ◑, 16.2; ◒, 22.9; ●, 24.6. Hatched area is first observed as pH is lowered.

The titration curve can be separated into several parts as indicated in Figure 7.[4,18] In the region where essentially pure random coil is being titrated (see Figures 4 and 5), the electrostatic interaction is essentially independent of leucine composition. In regions where essentially pure helix is titrated, the electrostatic interaction appears to increase considerably as the leucine content increases, even though the carboxyls on the average are farther apart. This interpretation implicitly assumes that introduction of leucine into the helical polymer does not alter $\mathrm{p}K_0$ of the remaining glutamic acid side chains. No shift in $\mathrm{p}K_0$ is expected from inductive effects transmitted through the peptide backbone. Furthermore the volume change on ionization of a carboxyl in helical polyglutamate is normal,[19] indicating the carboxyl is exposed to solvent. Only if solvent could not as freely approach the γ-carboxyl in the copolymers because of shielding by neighboring leucine side chains would one expect $\mathrm{p}K_0$ to change. Molecular models indicate solvent can approach. In addition titration

(17) (a) A. Katchalsky and J. Gillis, *Rec. trav. chim.*, **68**, 899 (1949); (b) A. Arnold and J. Overbeek, *ibid.*, **69**, 192 (1950).
(18) A. Wada, *Mol. Phys.*, **3**, 409 (1960).
(19) H. Noguchi and J. T. Yang, *Biopolymers*, **1**, 359 (1963).

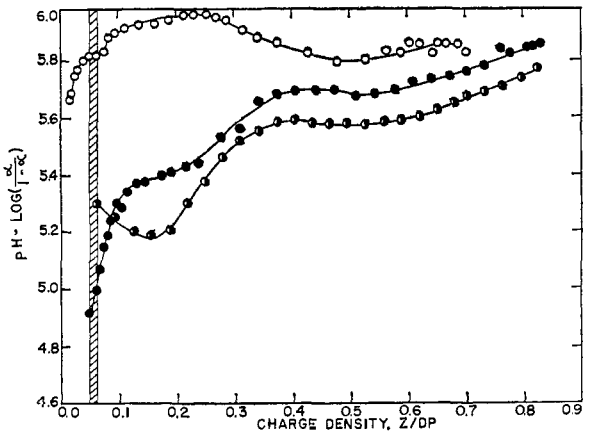

Figure 6. Titration curves in 0.020 *M* NaCl at 25°. Mole per cent leucine: ◑, 0.0[4]; ●, 12.9; ○, 29.1. Hatched area is first observed as pH is lowered.

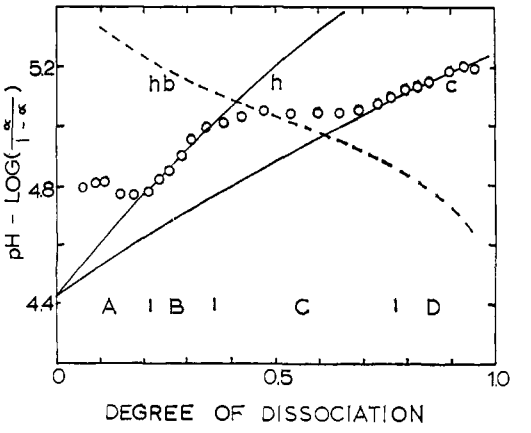

Figure 7. Titration curves: -○-, PGA; h, pure helix; c, pure random coil; hb, effect of side-chain hydrogen bonding (from eq. 4 with $\partial G_{el}/\partial \bar{Z} = 0$; $\gamma = 0$; $k = 10$). Identifiable regions of actual titration curve: A, titration of precipitated polymer; B, nearly pure helix; C, helix–coil transition; D, nearly pure random coil.

curves at varying ionic strengths (Figures 4–6) indicate the ionic strength dependence is about the same in the copolymers as in polyglutamic acid. It is therefore reasonably safe to assert that pK_0 is the same for all the polymers, both in the helical and the random coil conformations.

Discussion

The product of the reactivity ratios, 0.92 ± 0.25, is unity within experimental error and the polymerization can thereby be classified as ideal copolymerization.[15] Katchalski and Shalitin,[20] using the same solvent and an amine initiator, found that pairs of NCA's involving γ-benzylglutamate, ε-carbobenzoxylysine, and phenylalanine followed ideal copolymerization kinetics, whereas a glycine–lysine pair did not. It is not known if reactivity ratios determined in dimethylformamide will be applicable to methoxide-initiated polymerization in benzene. It is interesting to note that Fasman, *et al.*,[11] found a higher glutamate composition in all their copolymers than was in the initial monomer composition. This could happen only if glutamate NCA was incorporated in preference to leucine and the polymerization was not carried to 100% completion. Assuming the reactivity ratios are the same in benzene as in DMF, conversions of 85 to 99% would give their observed copolymer compositions.

In contrast to methoxide-initiated polymerization in benzene, it is difficult to prepare high molecular weight polypeptides in DMF. The helix–coil transition, ultraviolet absorption, and titration curves for our polyglutamic acid agreed well with those of higher molecular weight material. Since the amine-initiated polymerization is much better understood than methoxide-initiated polymerization, it seems desirable to sacrifice molecular weight for a better known polymerization mechanism. In the one sample examined (0% leucine), the ratio of weight- to number-average molecular weight was about two, suggesting a most probable molecular weight distribution. The degree of polymerization was always lowered during debenzylation. Undoubtedly peptide bond cleavage by HBr occurred, thus broadening the molecular weight distribution. It is not unlikely that all the polymers had close to a most probable molecular weight distribution.

The observed dependence of the titration curve of helical polymer upon leucine content seems surprising and anomalous. As the leucine content increases, the charges are on the average further separated. The electrostatic interaction might thereby be expected to decrease rather than increase with increasing leucine content. The latter effect is, however, observed when the electrostatic interaction is compared either at equal charge density or at equal degree of dissociation.

Evidence has been presented indicating the intrinsic dissociation constant of the side-chain carboxyl is unaltered by incorporation of leucine into the polymer. A possible effect not yet discussed is side-chain carboxyl–carboxyl hydrogen bonding. Several investigators have suggested such hydrogen bonding as a possible stabilizing effect in polyglutamic acid.[2,4,11,21] The effect of such hydrogen bonds on titration curves has been discussed[22] and a representative curve is shown in Figure 7. If one assumes that there is perfect pairing of carboxyls in pure PGA and that the effect of incorporating leucine into the side chain is to disrupt perfect pairing, an equation (4) for the titration curve of the helix in the presence of side-chain hydrogen bonding is easily derived. Here w describes the electrostatic interaction assuming $\partial G_{el}/\partial \bar{Z}$ is a linear function of $\bar{Z}$, k is the equilibrium constant for dimer formation, γ is the fraction of leucine in the copolymer, and $x = K_0 \exp(2w\bar{Z})10^{pH}$. When $\gamma = 0$ the equation reduces to that of Laskowski and Scheraga. Calculations using eq. 4 clearly indicate that as the leucine content increases, the titration curve ($pH - \log \alpha/(1-\alpha)$ *vs.* $\bar{Z}$) should fall steadily below rather than above the polyglutamic acid titration curve. It is our feeling that side-chain hydrogen bonding cannot be the dom-

$$pH - \log \alpha/(1-\alpha) = pK_0 - 0.868w\bar{Z} - \log[(1+x)^2 + k\gamma] + \log\{(1+x)^2 + k[1 + (1-\gamma)x]\} \quad (4)$$

(20) E. Katchalski and Y. Shalitin, *J. Am. Chem. Soc.*, **82**, 1630 (1960).

(21) J. T. Yang, *Tetrahedron*, **13**, 143 (1961).

(22) M. Laskowski and H. Scheraga, *J. Am. Chem. Soc.*, **76**, 6305 (1954).

inant factor in the seemingly anomalous titration curves of the helix and is unimportant in polyglutamic acid under conditions in which the polymer is soluble.

Hill,[23] using a linearized Poisson–Boltzmann equation, and Nagasawa and Holtzer,[24] using numerical integration of the nonlinearized Poisson–Boltzmann equation, have calculated the electrostatic interaction for charges smeared over the surface of a cylinder. These calculations cannot explain the increased electrostatic interaction upon increase in leucine content unless the dielectric constant of the solvent is treated arbitrarily as an adjustable parameter. However, the side-chain carboxyls carry discrete charges and discrete charge state models are more realistic. The equations for the electrostatic potential of discrete charges on a cylinder have not been solved in closed form. Tanford and Kirkwood[25] have obtained solutions for discrete charges attached to spheres of low dielectric medium immersed in a high dielectric solvent. As the discrete charges are moved from the interface and embedded further and further in the low dielectric medium, the electrostatic interaction was found to increase enormously. Embedding the charges causes more of the electric lines of force to act through the low dielectric medium and thus give rise to an increased electrostatic interaction. Molecular models of the α-helix with both glutamate and leucine side chains attached show the leucine side chain to protrude slightly further than the glutamate side chain. It is thus possible that the increased electrostatic interaction is a result of the increased hydrocarbon content in the vicinity of the carboxyls. Alternatively, the leucine could be looked upon as lowering the effective dielectric constant of the medium through which the charges interact.[26] Changes in water structure near the hydrocarbon side chains may also contribute to the apparent alteration of the effective dielectric constant. If eq. 3 is graphically integrated from a charge density of 0 to 0.2, the electrostatic free energy per unit charge density in the helix may be obtained. Results of such integration are given in Table III. The observed increase in electrostatic free energy is not unreasonable when compared to the results of Tanford and Kirkwood.

(23) T. Hill, *Arch. Biochem. Biophys.*, **57**, 229 (1955).
(24) M. Nagasawa and A. Holtzer, *J. Am. Chem. Soc.*, **86**, 531 (1964).
(25) C. Tanford and J. Kirkwood, *ibid.*, **79**, 5333 (1957).
(26) C. Tanford, *ibid.*, **79**, 5348 (1957).

Table III. Electrostatic Free Energy in the Helix

Copolymer (% leucine)	ΔG_{el},[a] cal./mole/unit charge density
0	250
5.7	200
12.4	485
16.2	570
24.6	960
29.1	1000

[a] At $\bar{Z}/DP = 0.2$, a charge density at which all the polymers are still predominantly helical.

Even though we propose that the increased electrostatic interaction in the copolymers results from a lowering of the effective dielectric constant in the region between interacting carboxylates, it does not follow that a corresponding change in pK_0 should occur. The carboxylates may be looked upon as constrained to positions on the surface of a rough cylinder of low dielectric material. Interacting carboxylates have many electric lines of force acting through the low dielectric cylinder. Changes in "roughness" of the surface of the cylinder can produce measurable changes in the electrostatic interaction. In order to shift pK_0, changes must occur in the electrostatic interaction between a carboxylate and a proton in the solvent. However, in this case the electric lines of force run predominantly through the solvent. Changes in the roughness of the low dielectric cylinder will have far less effect on the interaction.

The Stability of the Helical Conformation of Random L-Leucine–L-Glutamic Acid Copolymers in Aqueous Solution[1]

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Contribution from the Department of Chemistry, University of Iowa, Iowa City, Iowa. *Received February 8, 1965*

The stability in aqueous solution of the helical conformation of random copolymers of L-*leucine and* L-*glutamic acid containing 0 to 30% leucine has been investigated. The standard free-energy change for the transition (coil to helix) was resolved into two components independent of copolymer composition, one characteristic of glutamic acid and the other of leucine. For uncharged glutamic acid residues in 0.200 M NaCl at 25° $\Delta G^\circ = -130$ cal. mole^{-1}, $\Delta H^\circ \approx -1.0$ kcal. mole^{-1}, and $\Delta S^\circ \approx -2.8$ cal. deg.$^{-1}$ mole^{-1}. The helix–coil transition temperature for uncharged polyglutamic acid is predicted to be above 90°. For leucine $\Delta G^\circ = -840$ cal. mole^{-1}, indicating the helix with leucine side chains is more stable than the uncharged polyglutamic acid helix relative to their respective random coil conformations.*

(1) Taken in part from the Ph.D. thesis of R. E. Nylund, University of Iowa, 1964.