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Helix-Coil Transition of Poly-L-glutamic Acid and Poly-L-lysine in D_2O*

Pearl Appel and Jen Tsi Yang

ABSTRACT: The specific rotations at 233 mμ of poly-L-glutamic acid and poly-L-lysine in D_2O were measured as a function of *p*D and compared with those in H_2O. In both cases the helix-coil transition shifted toward the alkaline side; i.e., $(pD - pH)_{tr} = \sim 0.6$. Thus, deuteration appeared to favor the formation of the helix for poly-L-glutamic acid but the coil for poly-L-lysine. This apparent contradiction was resolved by studying the titration behavior of the two polypeptides. Just as in simple acids, both the glutamic acid and lysine side groups in the polypeptide chains became weaker Brønsted acids upon deuteration. The difference in the intrinsic dissociation constant, $pK_i(D_2O) - pK_i(H_2O)$, was about 0.5 for poly-L-glutamic acid and 0.7 for poly-L-lysine. When the rotations were replotted against the degree of dissociation instead of against *p*D or *p*H, the experimental data in D_2O and H_2O coincided. Thus the deuterated helices of the two polypeptides in D_2O seem to have the same stability as the protonated ones in H_2O.

The deuterium isotope effect on protein structure and function has been extensively investigated. Several protein molecules have shown a change in the stability of their helical conformation in a D_2O medium; these results have been variously interpreted in terms of hydrogen bonds (Hermans and Scheraga, 1959; Maybury and Katz, 1956), the structure of water (Von Hippel and Harrington, 1960), hydrophobic forces (Berns *et al.*, 1963), or internal rotation (Berns, 1963) being responsible for maintaining the native conformation of the protein molecules. One important factor not considered is the weakened acidity of ionizable groups in deuterium systems. Deuterated weak acids (Brønsted) have smaller dissociation constants than the corresponding protonated ones (LaMer and Chittum, 1936; Lumry *et al.*, 1951; Li *et al.*, 1961). We anticipated that polyelectrolytes, both acidic and basic, would show similar changes in their dissociation behavior when ionizable hydrogen atoms were replaced by deuterium atoms. We chose poly-L-glutamic acid and poly-L-lysine as model polymers in this study because their conformations have been well characterized.

* From the Cardiovascular Research Institute and Department of Biochemistry, University of California, San Francisco. *Received February 18, 1965.* Presented at the 48th Annual Meeting of the Federation of American Societies for Experimental Biology (*Federation Proc. 23*, 216, 1964). This work was aided by grants from the U.S. Public Health Service (HE-06285, GM-10880, and GM K3-3441).

We followed their helix-coil transitions in H_2O and D_2O by both optical rotation and potentiometric titration. Our data show that in D_2O the Bronsted dissociation constants of these deuterated polyions do decrease, but the isotope effect on the stability of their helical conformations is negligible when the change in acidity of the ionizable side groups is considered.

Experimental Procedures

Materials. Poly-L-glutamic acid was prepared using the *N*-carboxyanhydride of γ-benzyl-L-glutamate (Blout and Idelson, 1956; Blout and Karlson, 1956). The anhydride was purchased from Pilot Chemicals, Inc. Our preparation had a molecular weight of 50,000, as estimated from the intrinsic viscosity of its sodium salt in 0.2 M NaCl (Wada, 1960). Poly-L-lysine hydrobromide was obtained from Pilot Chemicals, Inc. (lot L-22). It had a molecular weight of about 110,000. The polymer was exhaustively dialyzed against double-distilled water prior to its use. The concentrations of both poly-L-glutamic acid and poly-L-lysine were determined by the micro-Kjeldahl method.

Poly-L-glutamic acid and poly-L-lysine were deuterated by dissolving their salts (in coiled form) in 99.5% D_2O (purchased from Abbott Laboratories) and lyophilizing them to dryness; this process was repeated twice. Deuterium chloride was prepared by causing benzoyl chloride to react with D_2O at high temperature (Brown and Groot, 1942) or by diluting a concentrated HCl solution (12 N) with a large volume of 99.5% D_2O. Sodium deuterium oxide was prepared by dissolving NaOH pellets directly in D_2O. The final D_2O concentrations in both cases were kept above 99%.

Polarimetry. Optical rotations were measured with a Cary Model 60 recording spectropolarimeter at 27°. The 233-mμ trough of the Cotton effects of a helical conformation was chosen as a qualitative measure of the helix-coil transition. (The quantitative aspects of the trough method for determining the helicity of proteins are still uncertain at present [Yang, 1965; Yang and McCabe, 1965; Iizuka and Yang, 1965].) The high levorotation of the helical form at this wavelength made it possible to use very dilute samples, thus minimizing the problem of aggregation at low (for poly-L-glutamic acid) and high (for poly-L-lysine) *p*H values. The concentrations used ranged from 0.005 to 0.1% for poly-L-glutamic and from 0.02 to 0.07% for poly-L-lysine. A 1-cm cell was used in all measurements. The data were expressed in terms of $[m']$,[1] which is equal to $(10\alpha/d \cdot m)[3/(n^2 + 2)]$, α being the rotations in degrees, m the molar (residue) concentration, d the cell length in decimeters, and n the refractive index of the solvent. In water, $3/(n^2 + 2) = 0.764$ at 233 mμ.

Titration. The *p*H values of the solutions were measured with a Radiometer (Type *p*H 25) *p*H meter equipped with a scale expander (Type *p*H A630P), using glass (Type B222) and calomel (Type K100) electrodes. The *p*D values for samples in 99.5% D_2O were calculated by adding 0.4 to the apparent *p*H, i.e., *p*D = *p*H(apparent) + 0.4 (Mikkelsen and Nielsen, 1960). To check this correction factor, we measured the apparent *p*H of a 0.01 M DCl in 0.02 M NaCl solution and found it to be 0.4 unit less than the *p*H of a 0.01 M HCl in 0.02 M NaCl solution (Glasoe and Long, 1960). A further calibration (Lumry *et al.*, 1951) was done by comparing *p*H-meter readings of acetate buffers in H_2O and in D_2O to the *pK* values known from conductivity measurements (LaMer and Chittum, 1936). This again gave a correction of 0.4 unit.

All titrations were performed in a jacketed glass vessel attached to a constant-temperature bath (purchased from Bonus Laboratories, Reading, Mass.) at 25° under nitrogen flush which had been saturated with the solvent vapor. Standard acid or base was delivered from a Gilmont ultramicroburet of 1-ml capacity. Usually 0.01-ml increments were added to the solution to be titrated and the reaction mixture was stirred with a Teflon-coated magnetic bar before recording of the *p*H readings.

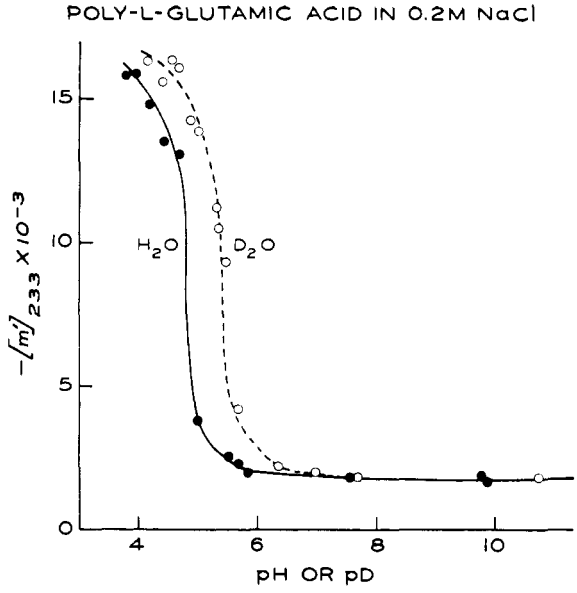


FIGURE 1: Reduced mean residue rotation of poly-L-glutamic acid in acid solution (0.2 M NaCl). ●, protonated in H_2O; ○, deuterated in D_2O.

Results

Figure 1 shows the change in $[m']$ at 233 mμ as a function of acidity for both protonated and deuterated poly-L-glutamic acid. The profiles of the two curves are very similar, and both curves exhibit the well-known helix-coil transition for this polymer (Doty *et al.*, 1957; Wada, 1960). The levorotation at low *p*H or *p*D approached a plateau when the poly-L-glutamic acid was very dilute, say, 0.005%. One sample (0.08%), however, did show a downward curvative (decrease in

[1] Abbreviation used in this work: $[m']$, reduced mean residue rotation.

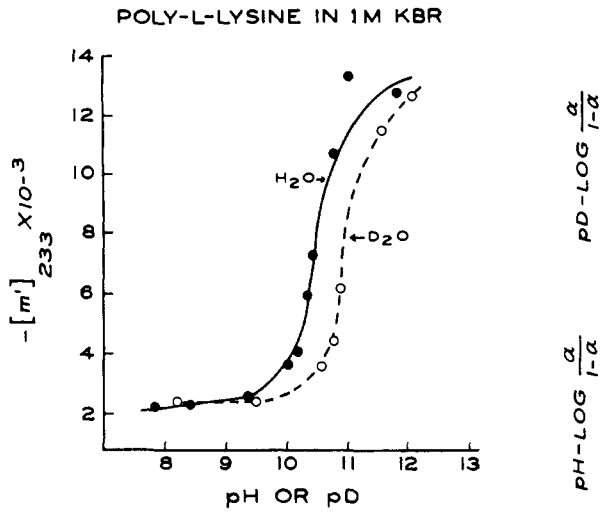


FIGURE 2: Reduced mean residue rotation of poly-L-lysine in alkaline solution (1 M KBr). ●, protonated in H_2O; ○, deuterated in D_2O.

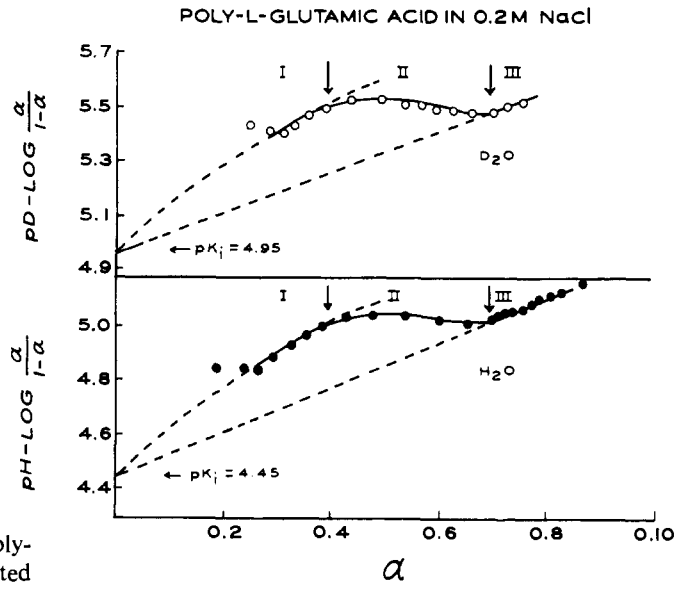


FIGURE 3: Apparent dissociation constant of poly-L-glutamic acid in 0.2 M NaCl as a function of degree of dissociation. ●, protonated in H_2O; ○, deuterated in D_2O.

levorotation) below *p*H 4.4, similar to that reported earlier (Doty *et al.*, 1957; Wada, 1960; Idelson and Blout, 1958). This probably was the result of aggregation of poly-L-glutamic acid molecules in moderately concentrated solutions. On the other hand, Applequist and Breslow (1963) managed to obtain a constant levorotation at low *p*H values even with a 0.5% solution. He attributed the downward curvature to some unknown factors in the preparations, in the techniques, or in the metastable nature of the poly-L-glutamic acid solutions below *p*H 4.

Similar rotation studies were carried out for protonated and deuterated poly-L-lysine solutions. The helix-coil transitions of poly-L-lysine in both H_2O D_2O are shown in Figure 2 (see also Applequist and Doty, 1962). The salient feature in Figures 1 and 2 is that the helix-coil transition of the two polypeptides shifted toward the alkaline side upon deuteration. The transition point increased from *p*H 4.8 to *p*D 5.4 for poly-L-glutamic acid and from *p*H 10.4 to *p*D 11.0 for poly-L-lysine. This seemed to indicate that deuteration increased the stability of the helical form for poly-L-glutamic acid but decreased it for poly-L-lysine. To resolve this apparent contradiction, we therefore studied the titration behavior of the two polymers. Since many organic acids are known to have a larger *pK* in D_2O than in H_2O, similar change in acidity is expected to operate for polyions upon deuteration. This behavior provides a simple explanation for the apparent shift in the helix-coil transition of poly-L-glutamic acid and poly-L-lysine (Figures 1 and 2).

For polyions the apparent dissociation constant, K_a, can be expressed as

$$pK_a = p\mathrm{H} - \log(\alpha/(1-\alpha)) = pK_i + (0.434/RT)(\partial F_{el}/\partial Z) \quad (1)$$

where α is the degree of dissociation, K_i the intrinsic dissociation constant at $\alpha = 0$, and F_{el} an electrostatic free energy term that vanishes when the total number of charges, Z, on the polyion is zero. Following Wada's suggestion (1960), we plotted (Figures 3 and 4) the apparent pK_a values of poly-L-glutamic acid and poly-L-lysine against α in both H_2O and D_2O. Region I represents the helical form for poly-L-glutamic acid and the coiled form for poly-L-lysine; region III is the coiled form of poly-L-glutamic acid and the helical form of poly-L-lysine. In both cases region II is the helix-coil transition zone. The titration curves in H_2O and D_2O for each polypeptide are similar and, in fact, can be superimposed by shifting the ordinate scale. The pK_a values of poly-L-glutamic acid and poly-L-lysine at various degrees of dissociation were about 0.5 and 0.7 unit higher in D_2O than in H_2O, respectively. This difference agrees well with the reported ΔpK_a (D_2O versus H_2O) for simple carboxylic acids and amines (Högfeldt and Bigeleisen, 1960).

By extrapolating the data in Figure 3 to $\alpha = 0$, where the electrostatic free energy term in equation (1) drops out, we found an intrinsic *pK* of 4.45 and 4.95 for poly-L-glutamic acid in H_2O and D_2O (0.2 M NaCl), the former value agreeable with that obtained by Nagasawa and Holtzer (1964b). Following these authors' suggestion (Nagasawa and Holtzer, 1964a), the extrapolated lines were made slightly curved, since Nagasawa and Holtzer have justified such treatment through their calculation of the electrostatic potential with the aid of a computer. For poly-L-lysine in Figure 4 we extrapolated the experimental data to $\alpha = 1$, where the polymer is uncharged. The intrinsic *pK* for poly-L-lysine in 1 M salt is 11.0 in H_2O and 11.7 in

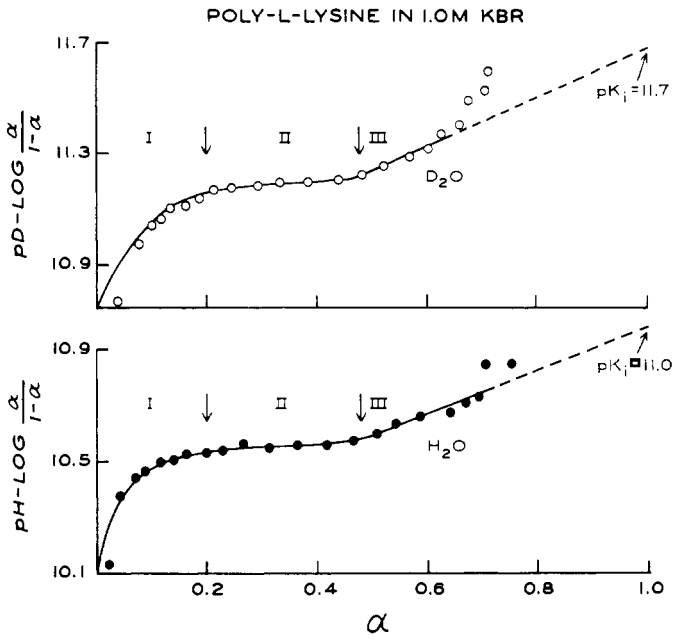


FIGURE 4: Apparent dissociation constant of poly-L-lysine in 1 M KBr as a function of degree of dissociation. ●, protonated in H_2O; ○, deuterated in D_2O.

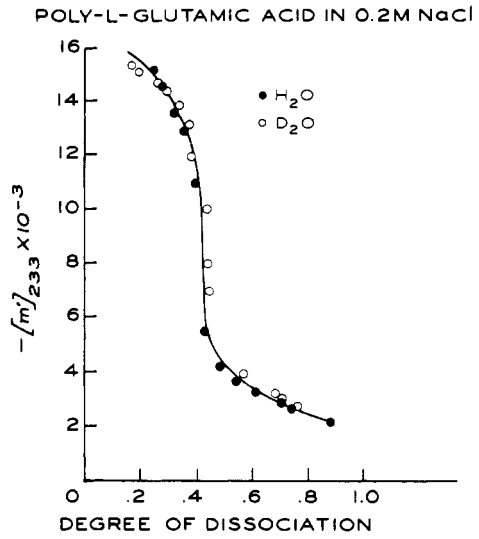


FIGURE 5: Optical rotation of poly-L-glutamic acid in 0.2 M NaCl as a function of degree of dissociation.

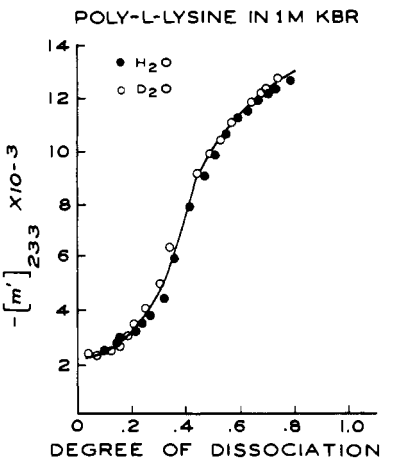


FIGURE 6: Optical rotation of poly-L-lysine in 1 M KBr as a function of degree of dissociation.

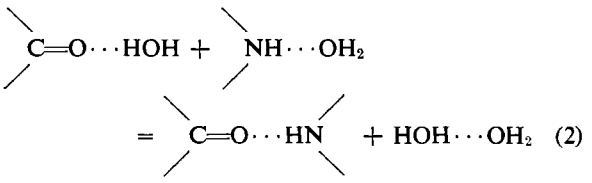
D_2O. Titrations of poly-L-lysine in 0.2 and 0.5 M KBr showed that the pK_t extrapolated to zero salt concentration was 10.6 in H_2O and 11.2 in D_2O. The former value is in good agreement with the value of 10.63 reported by Greenstein (1933) for the pK of the α-NH_3^+ group of lysyllysine in 0.02 M salt concentration.

The fact that ΔpK_t equals ΔpK_a at any α value for the two polymers indicates that the electrostatic free energy term in equation (1) remains essentially the same in H_2O and D_2O. From Figures 3 and 4 we see that the helix-coil transition (region II) occurs between $\alpha \cong 0.3$–0.7 for poly-L-glutamic acid and between $\alpha \cong 0.2$–0.5 for poly-L-lysine, irrespective of whether the polypeptides are deuterated and dissolved in D_2O or protonated and dissolved in H_2O. (The demarcation between region II and the helical region is rather uncertain because of the appearance of the aggregates in the latter.) The correct way to compare such transitions is then in terms of degree of dissociation rather than of pH and pD. Replotting optical rotation data in Figures 1 and 2 as a function of α (Figures 5 and 6) removes the difference between the transitions of the protonated poly-L-glutamic acid and poly-L-lysine in H_2O and the deuterated molecules in D_2O. The seemingly increased

stability of the helical poly-L-glutamic acid after deuteration is purely due to the fact that fewer —COOD groups are ionized at any chosen *p*D than the —COOH groups at the corresponding *p*H. The reverse is true for deuterated poly-L-lysine, where more undissociated $—ND_3^+$ groups are present at a chosen *p*D than the $—NH_3^+$ groups at the corresponding *p*H.

Discussion

We have demonstrated in this study that the deuterium isotope effect on the polypeptide conformation is negligible and that the apparent shift in the helix-coil transition as a result of deuteration can be explained simply in terms of the weakening acidity (Brønsted) of the side groups of polyions in D_2O. Once this change in degree of dissociation is taken into consideration, the deuterated polypeptides in D_2O appear to have the same conformational stability as the protonated ones in H_2O. This, of course, does not imply that the N—H and N—D bonds of the amide linkages have the same bond strengths. The helix-coil transition process involves not only the formation or disruption of the hydrogen bonds among the amide linkages but also a change in water of hydration associated with the transition; that is (Noguchi and Yang, 1963):



(2)

Thus the deuterium isotope effect will manifest itself not only in the intrachain hydrogen or deuterium bonds, but also in the solvent-solvent (water) and solvent-polymer interactions. A change in the bond strength of the peptide hydrogen bonds upon deuteration could very well be canceled by similar changes in the hydrogen bonds between water molecules and between polypeptide chain and water molecules. The net result may be deceptively small; at least we are unable to detect such changes in the conformational stability of the polypeptides by their rotatory properties. In this respect two recent reports concerning the deuterium effect on DNA are in accord with our findings on the polypeptides. Bunville (1964) used an approach similar to ours and found that the helix-coil transition of DNA in D_2O and H_2O occurred at the same degree of dissociation (on the basis of potentiometric titration). Maslova *et al.* (1964) reported that DNA in D_2O was relatively more stable to acid and less to base denaturation than in H_2O. Here again they found that the dissociation constants for the purine and pyrimidine bases differed in D_2O and H_2O; this could account for the apparent change in the conformational stability upon deuteration.

Although we have postulated that deuterated helices of simple polypeptides in D_2O have the same conformational stability as the protonated ones in H_2O and the apparent shift in helix-coil transition upon deuteration is purely the result of a change in the acidity of the ionizable side groups, these findings are of course not directly applicable to the complicated protein molecules. When the helical segments are buried inside the molecule, where no water of hydration as postulated in equation (2) exists, the substitution of deuterium for hydrogen would certainly alter the equilibrium between the helical and disordered forms of a polypeptide chain, primarily because of changes in the zero-point energies of the various vibrational frequencies (Scheraga, 1960). Likewise, the tertiary structure and hydrophobic interactions in the protein molecules can be affected when the exchangeable hydrogen is replaced by deuterium. Nevertheless, we cannot overlook the contributions of the deuterium isotope effect on the conformational stability that arises from the increase in *pK* for the ionizable side groups, especially when a change in *p*H is involved in such conformational studies. (Of course, unlike proteins, poly-L-glutamic acid and poly-L-lysine have one ionizable group per amino acid residue, and the electrostatic interactions in these polymers are more predominant than those in the protein molecules.) In this respect we note the findings of Berns (1963) that both protio- and deuteriophycocyanin were less stable thermally in H_2O than in D_2O when the thermal transitions were conducted in acetate buffers, but the reverse was true in phosphate buffers. Perhaps part of this alteration of conformational stability with respect to *p*H is related to the changes in dissociation constants of the ionizable groups upon deuteration, just as we found that poly-L-glutamic acid and poly-L-lysine underwent apparently opposite shifts in the helix-coil transition.

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Effect of Salts and Dioxane on the Coiled Conformation of Poly-L-glutamic Acid in Aqueous Solution*

Eisaku Iizuka† and Jen Tsi Yang

ABSTRACT: The conformation of poly-L-glutamic acid at *p*H 7.3 was studied in LiBr, NaCl, KF, and CsCl solutions by viscometry and optical rotatory dispersion (ORD). The intrinsic viscosity, $[\eta]$, of the polyion decreased with increasing salt concentrations, indicating contraction of the polypeptide chains. Above 6 M salt, $[\eta]$ began to drop more steeply, suggesting further change in conformation, possibly owing to aggregation. Visible rotatory dispersion in all cases obeyed a one-term Drude equation; the dispersion constant, λ_c, varied between 204 mμ (in low salt) and 223 mμ (up to 8 M LiBr), whereas the levorotations dropped markedly with increasing salt concentrations. Correspondingly, the b_0 values of the Moffitt equation (with λ_0 = 212 mμ) changed from +50 in water to −50 in high salt solutions.

Similar studies of poly-L-glutamic acid in dioxane-water solutions at *p*H 7.3 (apparent) showed that both the viscosities and levorotations in the visible region also reduced with increasing dioxane concentrations. But at 40% dioxane (v/v) the ORD no longer obeyed a one-term Drude equation; at 50% dioxane the conformation was essentially helical (b_0 became −670). Similar drastic changes were observed in the ultraviolet region, where the Cotton effects revealed a coil-to-helix transition. These conclusions were further corroborated by measurements of rotations as a function of *p*H. In all cases a sharp transition was observed, but the transition *p*H depended on the salt and dioxane concentrations used.

The helix-coil transition of poly-L-glutamic acid in aqueous solutions has been demonstrated by several physical techniques such as viscometry, optical rotation, and infrared spectroscopy (Doty *et al.*, 1957), and more recently also by dilatometry and refractometry (Noguchi and Yang, 1963). In 0.2 M NaCl-dioxane (2:1, v/v) the helical conformation did not break up unless more than 40% of the carboxyl groups were ionized through change in *p*H and temperature, thus implying the stability of these helices against a substantial degree of electrostatic repulsion among the side groups (Doty *et al.*, 1957). Wada (1960) has since shown that the transition point can be shifted by the addition of NaCl. This is attributed to the increase in the apparent dissociation constant of the —COOH groups with ionic strength in spite of the reduction in electrostatic repulsion in the presence of counter ions. Earlier, the levorotation of poly-L-glutamic acid in its coiled conformation was found to undergo a sharp drop with increasing salt concentration (Yang, 1962). In this paper we attempt to present a more detailed study of the effect

* From the Cardiovascular Research Institute and the Department of Biochemistry, University of California, San Francisco. *Received February 18, 1965.* This work was aided by grants from the U.S. Public Health Service (GM-K3-3441, GM-10880, and HE-06285). Part of this work was presented at the 46th Annual Meeting of the Federation of American Societies for Experimental Biology, Atlantic City, 1962 (Yang, 1962).

† On leave from the Ministry of Agriculture and Forestry, under the auspices of the Office of the Prime Minister, Japan.