does not permit us to exclude oxidation of *p*-terphenyl Grignard as a possible chemiluminescent mechanism.

Formation of hydrocarbon anions in Grignard solutions is not well documented, although there is some evidence that it does occur. Brown and Jones observed that butyllithium reacts with hydrocarbons to give anions in good yield.[31] Winkler and Winkler[32] also have studied electron transfer between phenyllithium and aromatic hydrocarbons. Russell, *et al.*,[33] have studied electron-transfer reactions between a variety of donors and acceptors including *n*-butylmagnesium bromide and butyllithium as donors. Acceptors were aromatic systems such as benzofuran, phenazine, benzo[*c*]cinnoline, and acridine. The extent of transfer varied from *ca.* 1% or less to 60% ion formation, as did the time to reach equilibrium, although in all cases it appeared the reaction was slow, *ca.* 5 min or longer.

(31) H. W. Brown and R. C. Jones, *J. Chem. Phys.*, **36**, 2809 (1962).
(32) H. J. S. Winkler and H. Winkler, *J. Org. Chem.*, **32**, 1695 (1967).
(33) G. A. Russell, E. G. Jantzen, and E. T. Strom, *J. Amer. Chem. Soc.*, **86**, 1807 (1964).

Anion formation in solutions of I could occur as follows.

$$C_6H_5MgBr + II \longrightarrow C_6H_5\cdot + [C_6H_5{-}C_6H_4{-}C_6H_5]^-MgBr^+$$

The *p*-terphenyl anion thus formed could react with oxygen or peroxide to give chemiluminescence. Chemiluminescent reactions between peroxides and radical anions of aromatic hydrocarbons are well known.[34]

The hydrocarbon anion oxidation mechanism is consistent with all observations of the present study. Only small amounts of anions would need to be formed since the chemiluminescence efficiency is low. The long exchange lifetimes reported for Grignards and aromatic compounds[33] are consistent with the observed effect of II on the chemiluminescent intensity.

Acknowledgment. This work was supported in part through funds provided by the U. S. Atomic Energy Commission under Contract AT(30-1)-905.

(34) E. A. Chandross and F. I. Sonntag, *ibid.*, **88**, 1089 (1966).

The Stability of the Polyglutamic Acid α Helix[1,2]

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Abstract: The intrinsic mixed acidity constant (pK_0) of glutamic acid polymers has been determined from potentiometric titrations of poly-DL-glutamic acid in aqueous solutions of various NaCl concentrations and at two temperatures (25 and 48°), thus making the difficult extrapolations of poly-L-glutamic acid titration curves more certain. These experiments indicate that pK_0 is nearly independent of temperature but varies from 4.58 in 0.01 *M* NaCl to 4.32 in 0.40 *M* NaCl. Similar studies of the titration of poly-L-glutamic acid as a function of temperature and NaCl concentration give the standard Gibbs free-energy change per amino acid residue for the transition from un-ionized α helix to un-ionized random coil ($\Delta G°/N$) for each of the various conditions; $\Delta G°/N$ depends on temperature, but only slightly, if at all, on salt concentration. From the temperature dependence of the free energy, we find that $\Delta H°/N$ is 975 ± 50 cal/(residue mole), and that $\Delta S°/N$ is 2.67 ± 0.1 cal/(residue mole deg). We find that there is no measurable effect of polymer concentration on these thermodynamic parameters and conclude that nonequilibrium aggregation is not present. It is demonstrated that, for poly-L-glutamic acid, three independent measures of helix content agree, namely ultraviolet absorption, titration, and optical rotatory dispersion. The helix content of un-ionized poly-DL-glutamic acid is estimated, from its extinction coefficient at 200 mμ, to be 62%. The implications of these experiments for molecular theories of the conformation of polyamino acids in aqueous solutions are examined. It is found that assignment of the contribution of individual molecular forces (*e.g.*, hydrogen bonding, hydrophobic bonding) to the over-all free energy of transition, whether that assignment is semiemprical or *a priori*, will have to be made with considerably more precision than has thus far been possible, if such theories are to be meaningful.

In view of the pervasiveness of the α-helical conformation in proteins and polypeptides, and of the several theoretical attempts to explain its stability, careful measurements of this stability, and of its entropic and enthalpic composition for a particular case, seem desirable. We present such measurements in this paper. In a sense, the helix–coil transition itself has been rather successfully treated theoretically. The statistical mechanical theories formulated by Peller[3] and by Zimm and Bragg[4] have added rigor to Schellman's suggestion[5] that the helix–coil transition of proteins and polypeptides must be somewhat cooperative. Among the many factors which must contribute to helix stability, however, only the cooperativeness has been elucidated. The fundamental interactions which lead to helix stability are simply lumped and put into the theories as parameters; despite numerous experimental and theo-

(1) This investigation was supported by PHS Research Grant RG-05488 from the division of General Medical Sciences, Public Health Service.
(2) A preliminary report of this work was presented at the 151st National Meeting of the American Chemical Society, Division of Colloid and Surface Chemistry, Pittsburgh, Pa., March 1966.

(3) L. Peller, *J. Phys. Chem.*, **63**, 1194 (1959).
(4) B. H. Zimm and J. K. Bragg, *J. Chem. Phys.*, **31**, 526 (1959).
(5) J. A. Schellman, *Compt. Rend. Trav. Lab. Carlsberg, Ser. Chim.*, **29**, 230 (1955).

retical studies, the detailed molecular interactions which determine these parameters remain unclarified. Any complete molecular theory of the helix–coil transition would have to predict the attendant standard free energy, enthalpy, and entropy changes as well as describe the course of the transition as a function of those quantities.

Zimm and Rice[6] have formulated a theory which extends the earlier statistical approach to admit charge-induced transitions and includes a suggestion of how to determine, from experiment, the standard free-energy change accompanying the charge-free helix–coil transition. Their theory retains the use of a parametric expression of the stabilizing interactions; they make no attempt to derive these forces from first principles.

A more detailed theoretical attempt to predict the stability of the α helix has failed, in that it is only capable of "predicting" which conformation will be the most stable one, when that information is already available.[7] For example, it is known that poly-β-methyl-L-aspartate forms a left-handed helix in helicogenic solvents, whereas essentially all other L-amino acid polymers form right-handed helices or other conformational structures. Ooi, *et al.*, succeed in explaining this only by use of a plethora of adjustable parameters, the net numerical result being in some cases extremely sensitive to the particular choice of values. For example, the statement on p 4421, paragraph 2 of ref 7 makes it clear that choice of the radius of the hydrogen atom to 0.075 Å is crucial to the argument. Note further that, in any case, these calculations ignore solvation, which could, of course, change everything.

The lack of reliable theoretical values for the free energy, enthalpy, and entropy of the helix–coil transition makes the experimental determination of these quantities all the more desirable. The experiments can provide the numerical values required for one of the parameters of the statistical theories and serve as a guide for future, more detailed theories. It is to this *experimental* problem that we have addressed ourselves, in the hope that the results will be used by someone to develop a rigorous theory of the molecular origins of the forces which give rise to the measured thermodynamic stability parameters.

The method originally suggested by Zimm and Rice[6] for the experimental determination of the standard free-energy change accompanying the transition of uncharged helix to uncharged coil is attractive for two reasons. First, the experiments (potentiometric titrations with acid or base) are relatively simple and can be performed readily using rather widely varying conditions of electrolyte concentration and temperature. Second, it has been shown that the connection, as established by Zimm and Rice, between the experimental data and the desired free energy is independent of the detailed statistical mechanical arguments used in its original derivation; in fact, it follows directly from a straightforward thermodynamic analysis.[8]

Nagasawa and Holtzer[8] used this approach to determine the helix–coil free-energy change in poly-L-glutamic acid, and pointed out, at the same time, several causes for uncertainty in its practical application. Normally, when the free energy of a reaction has been measured, the next step is determination of the temperature dependence of this free energy in order to obtain the corresponding entropy and enthalpy changes. Miller and Nylund[9] and Hermans[10] carried out such measurements without, however, first eliminating or reconsidering the difficulties enumerated by Nagasawa and Holtzer.[8] While this course was no doubt attractive, the fact remains that the limits of validity of the method had not been carefully established. Before extending the earlier measurements, therefore, we chose to reexamine the problems and ambiguities that Nagasawa and Holtzer originally discussed. While this work, therefore, does not represent the first determination of these quantities (namely, the standard free energy, entropy, and enthalpy), it has, we believe, placed the method upon a firmer basis and resulted in a significant diminution of the uncertainties which are common to the earlier values.[8-10] In addition, the range of temperature and of salt concentration covered has been increased.

The experimental problems which had remained unresolved are three in number and deserve to be explicitly stated, along with our method of attack on each.

(1) To obtain the coil titration curve, the experimental data from about $\alpha > 0.75$ must be extrapolated to $\alpha = 0$, α being the fraction of carboxyls ionized. A much shorter extrapolation of the helix data is required; it is necessitated by the interposition of an aggregation region between $\alpha = 0$ and the region of molecularly dispersed helices (see Figure 2). Nagasawa and Holtzer[8] made the shorter helix extrapolation and from it ascertained the intrinsic mixed acidity constant (K_0),[11] the $\alpha = 0$ intercept being pK_0. They then made the more difficult coil extrapolation by assuming that both helix and coil have the same pK_0. From these sort of data, it appeared that pK_0 was not a function of ionic strength, but this was a point of uncertainty. They suggested that potentiometric titration curves of the racemic polymer (poly-DL-glutamic acid) be determined, on the grounds that these were likely to show much longer coil regions and could therefore be used to improve the accuracy of the extrapolations to pK_0. We have followed that suggestion and found that the DL titration curves are even more helpful than had been expected. Not only is there a long, smooth region, but the region of precipitation is confined to much lower fractional ionization than in the L-glutamic acid polymer. The temperature dependence of pK_0's obtained from such titration curves, it will be seen, provides a check on the assumed coincidence of the helix and coil pK_0's and confirms Nagasawa and Holtzer's assumption to that effect. Their supposition that pK_0 is independent of ionic strength was, however, incorrect; use of the proper pK_0's markedly alters the measured free energy. We have thus ascertained the correct pK_0's by performing the titrations of poly-DL-glutamic acid at the various ionic strengths.

(6) B. H. Zimm and S. A. Rice, *Mol. Phys.*, 3, 391 (1960).
(7) T. Ooi, R. A. Scott, G. Vanderkooi, and H. A. Scheraga, *J. Chem. Phys.*, 46, 4410 (1967).
(8) M. Nagasawa and A. Holtzer, *J. Am. Chem. Soc.*, 86, 538 (1964).
(9) W. G. Miller and R. E. Nylund, *ibid.*, 87, 3542 (1965).
(10) J. Hermans, Jr., *J. Phys. Chem.*, 70, 510 (1966).
(11) The intrinsic mixed acidity constant, K_0, the negative of whose logarithm is the $\alpha = 0$ intercept of the titration curve, is not a rigorously thermodynamic quantity

$$K_0 = a_H(P^-)/(P^0)$$

where parentheses indicate polymer concentrations rather than activities; see E. J. King, "Acid-Base Equilibria," Pergamon Press, The Macmillan Co., New York, N. Y., 1965, p 18.

(2) We have also reexamined the contribution of polymer–polymer interaction to the activity of polymer. Nagasawa and Holtzer[8] concluded that such interaction is negligible from a comparison of the titration curves of two samples of poly-L-glutamic acid, one having twice the polymer concentration of the second. Unfortunately, these samples were 0.02 *M* in NaCl, whereas the titration was done with 0.1000 *M* NaOH; thus the salt concentration not only changed during the course of both titrations, but changed differently in the two cases. We made a more stringent check by comparing titrations done with NaOH solution of concentration equal to the salt concentration of the polymer solution, and by using a lower salt concentration (0.01 *M*) where polymer–polymer interactions would be stronger. These experiments confirm the conclusion reached by Nagasawa and Holtzer; there is no measurable polymer–polymer contribution to nonideality. However, as our analysis will show, the nonideality requirement is less demanding than was previously supposed.

(3) It has been previously taken for granted that aggregation of poly-L-glutamic acid does not persist beyond the so-called "aggregation region."[8–10,12] Were this assumption incorrect, an aggregation–disaggregation reaction would contribute to the measured free-energy change. In order to determine whether the helical region is really one of incipient precipitation, we have looked for time dependence of the pH of a solution whose extent of ionization places it in the helical region; we have also titrated isoionic samples prepared in several different ways. The system which we have assumed to be a solution of partially charged, unaggregated helices is, in these ways, shown to be, without doubt, an equilibrium system. However, such experiments cannot rule out the possibility that the system contains an equilibrium mixture of aggregates of various sizes. Evidence from physical studies in this laboratory, however, justifies the assumption that the "helix region" is a region of *unaggregated* helices;[13] this work will be reported in a future paper.

Having resolved these experimental problems, we are able to present values for the standard Gibbs free energy, entropy, and enthalpy changes of the charge-free helix-to-coil transition that are, we believe, more firmed based than earlier ones. Knowledge of the numerical values of these thermodynamic parameters makes it possible to assess, rather closely, the burden any theory must bear if its goal is to predict the stability of the poly-L-glutamic acid α helix from first principles. This, in turn, provides lower limits on the difficulties to be expected in prediction of protein conformations. This assessment, it will be made apparent below, leads to a rather poor prognosis for such efforts.

The process of determining pK_0's by titration of poly-DL-glutamic acid has also revealed something about the helix-to-coil transition in this polymer. These titration curves seem to show aspects of a conformational transition. The need for a method to test for such a transition in this racemic polymer led us to reinvestigate the usefulness of changes in peptide bond ultraviolet absorption as a means of determining helix content. This method we show to be valid, in that the helix content of poly-L-glutamic acid thus measured agrees with values from optical rotatory dispersion and from titration.[8,9] With some confidence then, we use measurements of extinction coefficient changes in the case of poly-DL-glutamic acid and, indeed, find a partial helix–coil transition; the uncharged racemic polymer is 26% helical at 25° in 0.01 *M* NaCl.

(12) A. Wada, *Mol. Phys.*, **3**, *409* (1960).

(13) R. B. Hawkins, Ph.D. Dissertation, Washington University, St. Louis, Mo., 1967.

Experimental Details

Materials. Sodium poly-L-glutamate was obtained from Pilot Chemicals, Inc., Watertown, Mass. Four different lots (G-40, G-61, G-72, and G-48; the nominal[14] degrees of polymerization were 620, 740, 680, and 170, respectively) were used in this work. The first three samples gave experimentally indistinguishable titration curves in 0.10 *M* NaCl at room temperature (G-48 was not used in titrations at all); it was assumed, therefore, that they would be indistinguishable under all conditions. All samples were prepared by methoxide-initiated polymerization of the N-carboxy anhydride of benzyl-L-glutamate followed by anhydrous HBr ester hydrolysis.[15] Amberlite MB-1 mixed-bed ion-exchange resin was used to prepare poly-L-glutamic acid from the sodium salt. Sodium poly-DL-glutamate (Lot 764; nominal degree of polymerization, 185) was from the same source and was prepared in an analogous manner.

Dialysis tubing (Union Carbide Corp., Food Products Division, Chicago, Ill.) was cleaned by boiling in sodium bicarbonate solution, rinsing with distilled, deionized water, and finally soaking in several rinses of water until the wash water in equilibrium with the dialysis tubing had a specific resistance of at least 800,000 ohm cm.

Sodium chloride, sodium acetate, acetic acid, phosphate salts, and *p*-dioxane were all reagent grade; methanol was Fisher Spectranalyzed. Standard aqueous buffers (phosphate and potassium acid phthalate) were prepared using the recipe of Bower and Bates[16] from dry salts purchased from the National Bureau of Standards. Standard buffers in 16.3% (w/w) methanol–water solution were prepared and used following the method of Bates, Paabo, and Robinson.[17]

Equipment. Measurements of pH were made with a Radiometer pHM4c meter and Radiometer electrodes (Copenhagen). Specifically, G200B or G202B glass electrodes were used in conjunction with K100 saturated calomel electrode at or below ambient temperature, or a K401 saturated calomel electrode above ambient. The K100 has an open liquid junction and is preferable, but its junction potential was not stable above ambient temperature; the K401, which has a porous fiber junction, did not present this problem. Either a 2.5-ml Ultra Precision micrometer buret (Kontes) or, for larger titrant volumes only, a 10-ml Manostat micrometer buret was used. Optical densities at 200 mμ were measured using a Cary 14 spectrophotometer and matched 1-mm quartz cells.

Methods. Poly-L-glutamic acid solutions were prepared for potentiometric titration by deionization of the sodium salt. The most convenient method is to stir the sodium poly-L-glutamate solution with a several-fold excess of ion-exchange resin for about 10 min, then to remove resin by filtration through a medium porosity frit. Polyacid samples prepared by this "batch" method showed the same titration curve as those deionized on a column of this resin. A third deionization method was used in one case (0.10 *M* NaCl, 25° determination no. 70) to provide a check on possible contamination of solution with resin; 0.1 g of sodium poly-L-glutamate was mixed with an excess of 1 *M* HCl, then dialyzed against five 1-l. changes of water. This sample gave the same titration curve as batch and column deionized solutions, indicating no resin contamination.

Samples in mixed aqueous-organic solvents were deionized by the batch method, using resin which had been washed several times

(14) The nominal degrees of polymerization were those supplied by Pilot Chemicals, as they determined them from intrinsic viscosity at pH 7 in 0.2 *M* NaCl solution. The intrinsic viscosity in this medium at some unspecified temperature (probably room temperature) was related to the degree of polymerization by Wada.[12]

(15) E. R. Blout and M. Idelson, *J. Am. Chem. Soc.*, **78**, *497* (1956).

(16) V. E. Bower and R. G. Bates, *J. Res. Natl. Bur. Std.*, **59**, *261* (1957).

(17) R. G. Bates, M. Paabo, and R. A. Robinson, *J. Phys. Chem.*, **67**, 1833 (1963).

with the solvent. Pure solvent, batch deionized, required only negligible base for a blank titration to the polymer end point.

The deionized solution was mixed with NaCl solution of the concentration required to bring the final solution to the desired molarity of NaCl without reducing the polymer concentration by more than 20%. The polymer concentrations of these final solutions were in the range 0.012–0.025 residue molar. A marked increase in turbidity always accompanied addition of salt to the deionized solution and, at higher NaCl concentration, some precipitate formed. The turbidity–precipitation invariably cleared on titration with NaOH before the region of molecularly dispersed helices was reached.

Poly-L-glutamic acid in 0.01 *M* NaCl and usually in 0.05 *M* NaCl solutions were titrated with 0.01 and 0.05 *M* NaOH, respectively, thus assuring constant counterion concentration during the experiment. For higher salt concentration, 0.10 or 0.25 *M* NaOH was used, since the change in counterion concentration over the entire course of the titration was negligible. In some cases, sufficient NaOH solution was added to the deionized polymer solution to cause 10–15% ionization of the carboxyls before mixing with NaCl; this avoids precipitation, but does not change the titration curve. The titrations at 25° were performed on 10 ml of solution in a beaker. For titration at temperatures other than ambient, the sample was contained in a double-walled beaker, with thermostated water being circulated through the jacket; sufficient polymer solution (10–30 ml) was used to immerse the electrodes deeply enough to avoid fluctuations in the indicated pH. A flux of N_2, saturated with water (or mixed solvent) at the temperature of the titration, was provided above the solution during titration. The end point of the titration was always determined to within 0.25% of the total volume of added base. Blank titrations required essentially no base. The electrodes were standardized with phthalate buffer[16] and checked with phosphate buffer[16] before each day's titrations. After each titration, the pH of standard phthalate buffer was remeasured; it was required to be within 0.03 unit of the correct value after the titration, or the experiment was rejected.

Samples for measurement of the extinction coefficient were prepared by dialysis, so as to obtain solvent whose chloride content was very nearly identical with that of the solution. This precaution seemed worthwhile, in view of the rather large chloride extinction at 200 mμ ($\epsilon \cong 24\ M^{-1}\ cm^{-1}$). For samples more than 25% ionized, a solution of the sodium salt of the polymer dissolved in water was first dialyzed against a phosphate or acetate buffer of the required pH and then dialyzed exhaustively against sodium chloride solutions of the appropriate concentration. Samples prepared in this way were thus buffered only by the polymer itself; the polymer provides adequate buffer capacity to make the experiment as done here convenient only when more than 25% ionized. Samples at less than 25% ionization were dialyzed against a buffer 0.01 *M* in sodium acetate and containing acetic acid of the concentration needed to give the desired pH. In both cases, after the final dialysis, a stock solution was obtained whose polymer concentration was determined by the micro-Kjeldahl method. Two samples were prepared by dilution of the stock solution with dialyzate; the extinction coefficients of the two, measured against dialyzate, were found to agree within 2% in all cases.

Treatment of Data. The fraction of carboxyls ionized, α, is given by

$$\alpha = [(H^+) + (NaOH) - (OH^-)]/(\text{polymer})$$

where (H^+) refers to the molarity of free H^+; (NaOH), the molarity of added titrant; (OH^-), the molarity of free OH^-; and (polymer), the total molarity of glutamic and glutamate residues. In practice, the value of (OH^-) is negligible. The value of (H^+) was obtained from titration of solvent, using the assumption that the activity coefficient of hydrogen ion is not significantly affected by polymer in the concentrations used.

The pH of a polyelectrolyte solution is given by[8,18,19]

$$\text{pH} = \text{p}K_0 - \log((1 - \alpha)/\alpha) + (1/RT \ln 10)(\partial G_{ion}/\partial\alpha) \quad (1)$$

where αG_{ion} is the incremental change in electrostatic and conformational free energy which accompanies an increment, $\partial\alpha$, in the degree of ionization. The integral

$$\oint [\text{pH} + \log((1 - \alpha)/\alpha)]\, d\alpha = (1/RT \ln 10)\oint dG_{ion} = (1/RT \ln 10)\Delta G°/N \quad (2)$$

is taken over the charge cycle, helix at $\alpha = 0$ to coil at $\alpha = 1$ to coil at $\alpha = 0$. To illustrate, for a poly-L-glutamic acid titration, the extrapolated portions have been drawn as well as the experimentally determined portions for the 0.6° curve in Figure 2; hence the cycle over which this integral must be taken is shown. We thus obtain $\Delta G°/N$, which is the standard Gibbs free-energy change, per residue, of the transition un-ionized α helix to un-ionized coil.[8]

The integration was done as follows. The helix portion of the curve was extrapolated by eye using the predetermined $\alpha = 0$ intercept as a guide, as was the coil portion. The relevant experimental points plus two others ($\alpha = 0.2$ and 0.4 for coil, $\alpha = 0.5$ and 0.7 for helix) read from the drawn extrapolations were fit by computer to quadratic least-squares curves, whose intercepts were fixed as pK_0; the computer's quadratics were invariably nearly coincident with the drawn curves. The computer then calculated the area, to obtain the above-mentioned integral, whose upper bound is a curve leading from $\alpha = 0$ to 1 along, successively, the extrapolated portion of the helix curve and the remainder of the experimental curve, and whose lower bound is the coil curve from $\alpha = 0$ to 1.

The free energies were obtained in this manner from data taken at a series of temperatures between 0.6 and 59°. These values of $\Delta G°/N$ *vs.* temperature were fitted with the best (least-squares) straight line; the slope of the line is $-\Delta S°/N$, the standard entropy change of the transition. The corresponding enthalpy change is obtained immediately as $\Delta H°/N = \Delta G°/N + T\Delta S°/N$.

In addition, the fraction of residues in helical regions (f_H) can be obtained from the titration curves;[8] this was done by computer for all titrations. The value of f_H can also be estimated from the extinction coefficient (ϵ). We chose as the coil and helix limits (see Figure 6) $\epsilon_{200,coil} = 5500\ M^{-1}\ cm^{-1}$, $\epsilon_{200,helix} = 3240\ M^{-1}\ cm^{-1}$. Assuming that residues in coiled regions or in helical regions contribute independently, one immediately obtains f_H from ϵ as

$$f_H = (\epsilon_{coil} - \epsilon)/(\epsilon_{coil} - \epsilon_{helix}) \quad (3)$$

This method has been questioned by McDiarmid and Doty;[20] their objections are dealt with below (Discussion section).

Experimental Results

Titrations of Poly-DL-glutamic Acid. The titration curves are shown in Figure 1. These are extrapolated to $\alpha = 0$, at which point $\text{pH} + \log((1 - \alpha)/\alpha) = \text{p}K_0$ (see eq 1), where K_0 is the intrinsic mixed acidity constant[11] of a glutamic acid residue in an aqueous solution containing added sodium chloride of the indicated concentration. There is, as can be seen, a variation of the measured pK_0 with molarity of NaCl. However, since the 0.40 *M* NaCl curve shown in Figure 1 essentially coincides with one obtained at 48°, pK_0 does not depend on temperature to within experimental error (±0.02 unit). The pK_0's shown in Figure 1 are $M_{NaCl} = 0.01$, $pK_0 = 4.58$; $M_{NaCl} = 0.05$, $pK_0 = 4.46$; $M_{NaCl} = 0.10$, $pK_0 = 4.40$; and $M_{NaCl} = 0.40$, $pK_0 = 4.32$. These were then accepted as the appropriate pK_0's for poly-L-glutamic acid and are used as such for the calculation of the thermodynamic parameters. A complete discussion of our use of the pK_0's of the racemic polymer appears below.

Titrations of Poly-L-glutamic Acid. Poly-L-glutamic acid solutions of the same four concentrations of added sodium chloride were titrated potentiometrically at various temperatures between 0.6 and 48°. The 0.1 *M* NaCl data were extended to 59°. All of the titrations are listed in Table I. Several titration curves, plotted according to eq 1, are shown in Figure 2; as an example, the helix and coil extrapolation to the pK_0 given above are indicated for the 0.6° curve. The helix–coil

(18) A. Katchalsky and J. Gillis, *Rec. Trav. Chim.*, **68**, 879 (1949).
(19) A. Arnold and J. Overbeek, *ibid.*, **69**, 192 (1950).
(20) R. McDiarmid and P. Doty, *J. Phys. Chem.*, 70, 2620 (1966).

Table I. Titrations of Poly-L-glutamic Acid

Temp, °C	M_{NaCl}	$\Delta G°/N$[a]	Detn[h]	$M_{polymer}$[b]	M_{NaOH}
0.6	0.0100	272	57	0.01608	0.0100
	0.0500	261	43	0.01700	0.250
		265	58	0.01725	0.0500
	0.100	227	42	0.01980	0.250
		280	44	0.01613	0.250
	0.400	271	45	0.01595	0.250
11.0	0.0100	247	62	0.01760	0.0100
	0.0500	242	21	0.01920	0.100
		201	23	0.01744	0.100
		202	59	0.01790	0.0500
	0.100	243	16	0.01680	0.100
		195	22	0.01762	0.100
		215	25	0.01724	0.100
	0.400	180	24	0.01744	0.100
		187	27	0.01664	0.100
25	0.0100	192	54	0.02532	0.0100
		192	55	0.02188	0.0100
		192	56	0.01241	0.0100
	0.0500	180	C[c]	0.01918	0.100
		175	61	0.01524	0.0500
		179	77	0.01748	0.0500
		189	78	0.01755	0.0500
		192	79[d]	0.01742	0.0500
	0.100	197	B[e]	0.01920	0.100
		154	3	0.01712	0.100
		174	15	0.01780	0.100
		200	40	0.01708	0.250
		182	60	0.01579	0.100
		190	70[e]	0.01824	0.100
		171	71[f]	0.01520	0.100
		158	49	0.01620	0.250
	0.200	175	A[c,g]	0.01895	0.100
		159	1A[g]	0.01914	0.100
	0.300	142	1	0.01480	0.100
	0.400	119	4	0.01310	0.100
		131	10	0.01450	0.100
37.5	0.0500	144	33	0.01787	0.250
	0.100	133	30	0.01598	0.250
	0.400	130	34	0.01691	0.250
48.0	0.0100	146	81	0.01494	0.0100
	0.0500	148	37	0.01422	0.250
	0.100	118	35	0.01643	0.250
	0.400	102	38	0.01707	0.250
54.9	0.100	119	52	0.01547	0.250
59.0	0.100	113	51	0.01646	0.250

[a] Calories/(residue mole) (infinitely dilute reference state, molality basis). [b] Residue molarity. [c] Titration data from Nagasawa and Holtzer (ref 8). [d] Titration was halted in the helix region overnight, then completed. The curve is smooth throughout the titration. [e] Sample was deionized by addition of HCl, which was subsequently dialyzed out. [f] Sample was dissolved in 0.1 *M* NaOH for 90 min before using. [g] pK_0 was obtained by interpolation of the data given above. At 0.200 and 0.300 *M* NaCl, pK_0 = 4.35 and 4.33, respectively, were used. [h] This refers to the determination number.

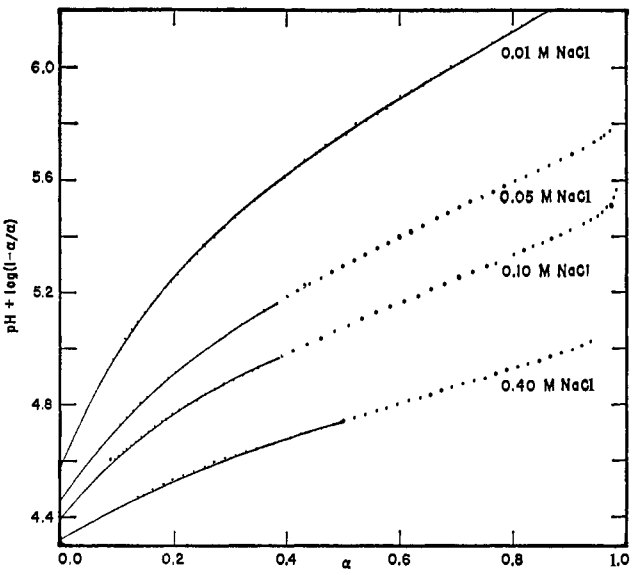

Figure 1. Potentiometric titration curves of poly-DL-glutamic acid. Zero charge intercepts of extrapolated curves indicate pK_0's. Titrations are at room temperature.

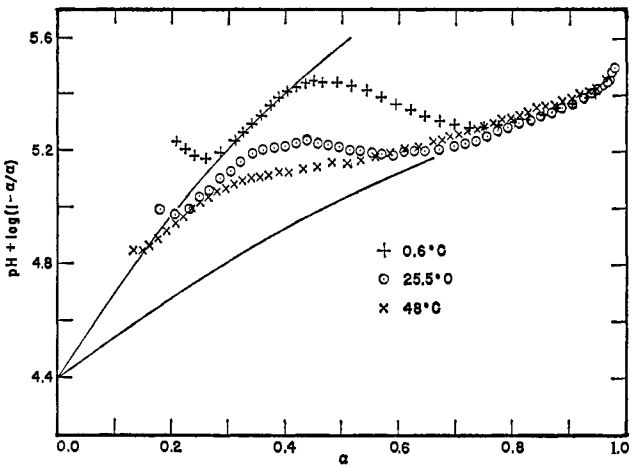

Figure 2. Titration curves of poly-L-glutamic acid in 0.10 *M* NaCl at various temperatures. Extrapolations are shown for the 0.6° titration.

free-energy changes listed in Table I are plotted in Figure 3. Two titrations in 0.01 *M* NaCl at 25° which differ only in their polymer concentrations are shown in Figure 4.

Poly-L-glutamic acid was also titrated in two mixed aqueous–organic solvents; these were 16.3% (w/w) methanol–water, 0.1 *M* in NaCl, and 10% (v/v) *p*-dioxane–water, 0.1 *M* in NaCl. These data are shown in Figure 5. For the 16.3% methanol titration, buffers for standardization were dissolved in this solvent;[17] these data are thus correctly referred to a 1 *m* standard state in 16.3% methanol. Such buffers are not available for the dioxane solvent; hence, for these experiments, the electrodes were standardized with aqueous buffers.[16] The dioxane data, then, are referred to a 1 *m* standard state in pure water; correction of these data to a more appropriate standard state would not change the shape of the curve, but would only shift it vertically.

Thermodynamic Parameters of the α Helix to Random-Coil Transition. The standard Gibbs free-energy changes per residue for the un-ionized α helix to un-ionized random-coil transition ($\Delta G°/N$) are given in Figure 3. Some of these data points represent the mean of several determinations, some only one, as is evident from Table I. For example, the free-energy change for the transition in 0.10 *M* NaCl at 25°, the best established datum, was found from eight separate experiments to be 178 cal/(residue mole); the average deviation from the mean was 8%, the range, 25%. The single line drawn (Figure 3) is the best (least squares) through these data, each experiment being given equal weight. It indicates that the standard enthalpy change per residue mole for the same transition ($\Delta H°/N$) is

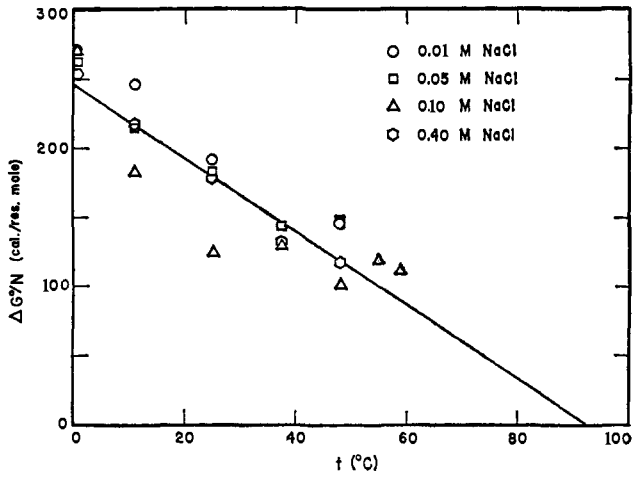

Figure 3. Free-energy change per residue of the uncharged helix–uncharged coil transition as a function of temperature at various salt concentrations. Solid line is the linear least-squares fit of all data.

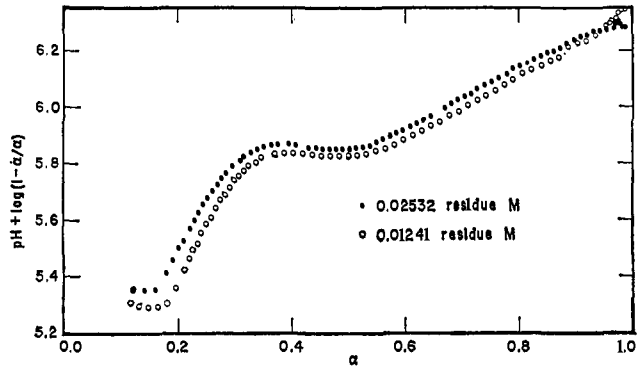

Figure 4. Potentiometric titration curves of poly-L-glutamic acid at various polymer concentrations; polymer in 0.01 *M* NaCl at 25°.

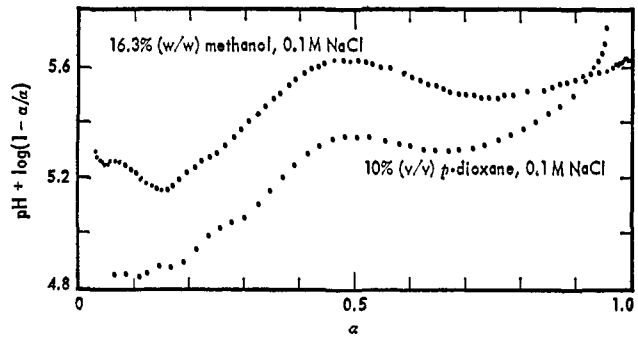

Figure 5. Potentiometric titration curves of poly-L-glutamic acid in methanol–water–salt and in *p*-dioxane–water–salt mixtures. The pH scale used for the *p*-dioxane titration is referred to a standard state of 1 *m* in pure water, whereas the methanol titration is referred to a 1 *m* standard state in 16.3% (w/w) methanol. These data are not corrected for free hydrogen ion; thus α = (NaOH)/(polymer).

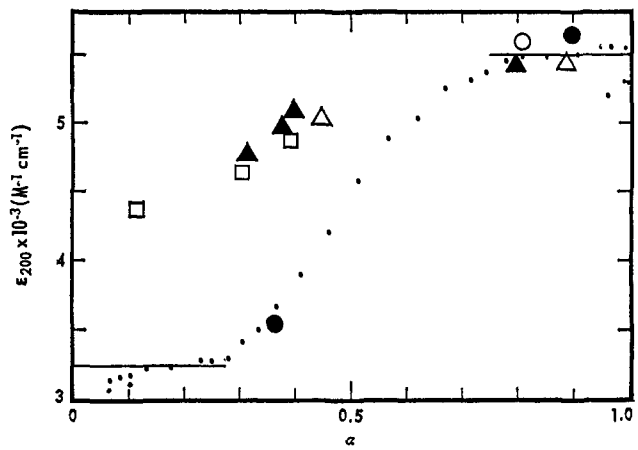

Figure 6. Extinction coefficient at 200 mμ of poly-L-glutamic acid and poly-DL-glutamic acid in aqueous solution at room temperature, as a function of fraction of carboxyls ionized: ●, poly-L-glutamic acid, DP 680, 0.10 *M* NaCl; ○, poly-L-glutamic acid, DP 170, 0.01 *M* NaCl; • poly-L-glutamic acid, 0.20 *M* NaCl (extinction coefficients from ref 9); △, poly-DL-glutamic acid, 0.10 *M* NaCl; ▲, poly-DL-glutamic acid, 0.01 *M* NaCl; □; poly-DL-glutamic acid, 0.01 *M* $NaC_2H_3O_2$ + $HC_2H_3O_2$.

975 ± 50 cal/(residue mole); the standard entropy change ($\Delta S°/N$) is 2.67 ± 0.1 cal/(residue mole deg).[21]

The Extinction Coefficient and Helix Content of Poly-DL-glutamic Acid. The extinction coefficients at 200 mμ of poly-L-glutamic and poly-DL-glutamic acids as a function of α in various media are shown in Figure 6. The small dots were taken from Miller and Nylund's work on the L polymer.[9] The extinction coefficient is apparently the same for all these polymers whether in 0.01 or 0.10 *M* NaCl. Likewise, the extinction coefficient is not affected by using 0.01 *M* $NaC_2H_3O_2$ + $HC_2H_3O_2$ buffer instead of 0.01 *M* NaCl at the same pH. Analysis of the poly-DL-glutamic acid extinction coefficient, according to eq 3, leads us to conclude that the uncharged, racemic polymer is 62% helix in 0.01 *M* NaCl.

Evaluation of Results

The Values of pK_0. The assumptions made by Nagasawa and Holtzer[8] and tacitly accepted by later investigators[9,10] can now be subjected to more careful scrutiny. The question to be answered about pK_0 are as follows. (1) Do α-helical and randomly coiled poly-L-glutamic acid have the same pK_0? (2) Does pK_0 depend on temperature? (3) Does pK_0 depend on ionic strength?

The first of these questions is an important one; if $pK_{0,coil} - pK_{0,helix}$ were to be as large as 0.1 unit, $\Delta G°/N$ would, in general, be decreased by about 50%. The answer, as best as we can determine, is that they are the same; two items of evidence support this conclusion. One of these items is obtained directly from the appearance of the titration curves. The helix and coil titration curves are extrapolated smoothly; those titrations which are amenable to the most certain extrapolations[22] indicate that the intercept of the helix curve (pK_{0h}) is the same as that of the coil curve (pK_{0c}). We could not absolutely preclude from this, however, a

(21) The error estimates given for the entropy and enthalpy should be taken to mean, "We would be surprised if this value is in error by more than this amount." Statistical analysis of these data indicates that the probable error of the measured enthalpy is 16 cal and the probable error of the entropy is 10^{-4} eu; the method of analysis of such data is described by A. G. Worthing and J. Gefner, "Treatment of Experimental Data," John Wiley and Sons, Inc., New York, N. Y., 1943, pp 249–250. The statistical uncertainties are obviously much too small.

(22) Those amenable to most certain extrapolation are titrations done at low temperatures and high ionic strength. The helix portions are relatively long and have small slopes and little curvature. The coil portions are either linear or very nearly so.

value of $pK_{0c} - pK_{0h}$ as large as 0.1. The second piece of relevant evidence stems from the data for poly-DL-glutamic acid; in analyzing these data, the answers to questions 1 and 2 become interwoven. Samples of the DL polymer in 0.400 *M* NaCl were titrated at two different temperatures, 25 and 48°. Both curves give the same pK_0 within an experimental error of ±0.02 unit. We take this to mean, in answer to the second question, that pK_0 is temperature independent. But, since poly-L-glutamic acid's helix content is known to be temperature dependent,[23] the pK_0's so measured for poly-DL-glutamic acid are almost certainly the pK_0's of systems of different helix content. Thus, unless there are compensatory changes in pK_0 and helix content with temperature, we can conclude that pK_0 is dependent neither on temperature nor on helix content. The hypothesis that pK_0 is temperature independent is supported by the temperature independence of the ionization constants of simple carboxylic acids; the p*K* of acetic acid, for example, changes only 0.01 unit over the temperature range included in our studies.

The third question has already been answered; the pK_0's do depend on ionic strength in the manner stated above. This dependence on ionic strength, like the lack of it on temperature, is qualitatively confirmed by the ionization behavior of acetic acid. In poly-DL-glutamic acid, pK_0 varies from 4.32 in 0.40 *M* NaCl to 4.58 in 0.01 *M* NaCl; the same change in sodium chloride concentration produces a change in the p*K* (mixed acidity constant) of acetic acid from 4.48 to 4.67.[24] This agreement lends credence to our answers to all three questions.

The titration curves of poly-DL-glutamic acid above $\alpha = 0.75$ always lie above the coil portion of the corresponding poly-L-glutamic acid curve. This is neither surprising nor worrisome in view of the demonstration by Nagasawa, Murase, and Kondo[25] that stereoregular polymethacrylic acids titrate differently from atactic polymer. On the other hand, pK_0 must be independent of the polymer's stereochemistry.

The Extrapolation to pK_0. This difficult and previously dubious extrapolation has been rendered considerably more certain by our knowledge of the values of pK_0 to which this extrapolation should be made. The only remaining uncertainty, then, is in the shape of the extrapolated curves. We have chosen to extrapolate quadratically; the data clearly indicate a curved extrapolation and are well fit by a quadratic. Nagasawa and Holtzer have demonstrated that the Poisson–Boltzmann equation does indeed predict nonlinear titration curves for the range of electrostatic potentials encountered in the media of the ionic strengths used here.[26] The Poisson–Boltzmann equation is not, however, rigorously correct.[27] While it is true that Manning and Zimm, using the more satisfactory Mayer theory, have predicted that the linear region of titration curves of rod-like polyelectrolytes will persist to higher electrostatic potentials than one would expect from the Poisson–Boltzmann equation,[28] they have made no estimate of the potential at which linearity might finally be expected to fail. Since our titration curves do not seem to be linear, we have extrapolated along curved lines. In any case, the variations in the values of $\Delta G°/N$ calculated from linear extrapolations, so long as they are brought to the above-determined pK_0's instead of curved ones, are within experimental error.

Using the method described by Nagasawa and Holtzer,[8] Hermans obtained thermodynamic parameters for the poly-L-glutamic acid helix–coil transition which are in fair agreement with those reported herein.[10] However, this agreement must be, to some extent, fortuitous, because his coil titration curves were extrapolated by drawing straight lines based on the coil (*i.e.*, above $\alpha = 0.75$) segment of the curve. Using this procedure, Hermans obtains values of pK_{0c} that are sometimes equal to, but sometimes considerably higher than, pK_{0h}. These pK_0's vary in an apparently random way over about 0.22 p*K* unit as the temperature goes from 4 to 55°. Considering the difficulties inherent in such a long extrapolation, it would seem hazardous to extrapolate to an inconsistently varying pK_0, particularly when such variation is not found in simple carboxylic acids. The justification presented by Hermans for this procedure[10] is that, "Nagasawa and Holtzer have indeed shown that the extrapolations are linear at ionic strengths of 0.1 *M* and higher." This is manifestly incorrect; Nagasawa and Holtzer, in fact, include an explicit statement to the contrary, and the coil extrapolation for 0.20 *M* NaCl shown in their paper[8] (Figure 1) is clearly curved.

The Titration End Point. The end point is determined experimentally as the volume of added base at which the change in pH per unit volume of added base is greatest. For determination of $\Delta G°/N$, this end point, whose inaccuracy is never more than ±0.25% of the volume of base added, is more than adequate; $\Delta G°/N$ calculated from a titration in 0.05 *M* NaCl at 11° changed by only 0.1% when recalculated using an end point differing by 0.5% from the experimentally determined one.

Though it does not materially affect the free-energy change, erratic behavior is shown by all of the titration curves near the basic end point (*i.e.*, as α approaches 1). The curve ordinarily sweeps up, then down sharply, as α rises; this usually appears in our curves above $\alpha = 0.95$. Hermans claims to have straightened such curves at high (and low) α by choosing slightly different end points, then recalculating the curves until an appropriate end point was found.[10] We have tried to repeat this in two different ways: (1) iteration until an end point was found which reduces curvature near $\alpha = 1$, as suggested by Hermans, and (2) least-squares fitting the experimental curve (pH as a function of volume of added base) in the region immediately surrounding the end point to a polynomial of the third degree; the exact end point should be the analytically determined inflection point of this polynomial. The first of these methods corresponds to the possibility that the experimental end point is not necessarily the one which gives the straightened titration curves. The second is predicated on the physically more reasonably hypothesis that, if the precisely correct experimental end point were used, there would not be the divergence at high α;

(23) J. Y. Cassim and E. W. Taylor, *Biophys. J.*, **5**, 573 (1965).
(24) H. S. Harned and B. Owen, "The Physical Chemistry of Electrolytic Solutions," 3rd ed, Reinhold Publishing Corp., New York, N. Y., 1963, pp 676, 677.
(25) M. Nagasawa, T. Murase, and K. Kondo, *J. Phys. Chem.*, **69**, 4005 (1965).
(26) M. Nagasawa and A. Holtzer, *J. Am. Chem. Soc.*, **86**, 531 (1964).
(27) L. Onsager, *Chem. Rev.*, **13**, 73 (1933).
(28) G. S. Manning and B. H. Zimm, *J. Chem. Phys.*, **43**, 4250 (1965).

i.e., that only more accurate interpolation between experimental points is needed. We found, however, that neither of these methods determines an end point which prevents sharp deviation near $\alpha = 1$. We have no explanation for the apparent success of the method in Hermans' hands but not in our own.

Aggregation. It is possible that aggregation could persist beyond what has previously been considered to be the "aggregation region" of the titration curve (*i.e.*, that portion at lower ionization than the helical segment). While we cannot yet eliminate this possibility entirely, we can say that, whatever the state of aggregation, the titration curves above $\alpha = 0.25$ are representative of polymer at equilibrium with respect to aggregation. The evidence for this, obtained from a series of titrations in 0.1 *M* NaCl at 25°, follows. (1) Two samples (determinations B and 3) were deionized on a resin column: $\Delta G°/N = 197$ and 154 were obtained. (2) Four samples (40, 60, 49, 15) were deionized by stirring with resin; $\Delta G°/N = 200$, 182, 158, 174. (3) A sample (70) was deionized by mixing with an excess of HCl, which was then dialyzed out; the sample formed a flocculent white precipitate which redissolved completely before the helix region was reached; $\Delta G°/N = 151$. (4) No salt was added to two solutions (40, 49) until they had been titrated to $\alpha = 0.1$. There was no precipitation or marked turbidity; $\Delta G°/N = 200$, 158. (5) Salt was added to our resin deionized solutions (B, 3, 15, 60), causing opacity and partial precipitation, which disappeared before the helix region was reached; $\Delta G°/N = 200$, 154, 174, 182. (6) A solution was titrated to beyond the basic end point with 0.100 *M* NaOH, then back to $\alpha = 0$ with 0.100 *M* HCl; the two titration curves thus obtained were superposable. (7) A sample was deionized, brought to 0.05 *M* in NaCl, and then divided. One portion (78) was titrated immediately; the other (79) was titrated to $\alpha = 0.345$ and then allowed to stand overnight, after which the titration was completed. These two titration curves also superpose. Clearly the system under study is an equilibrium one, since it can be prepared in so many ways, all differing in the extent of aggregation en route, with the same result.

Applequist has suggested that incomplete debenzylation of the poly-γ-benzyl-L-glutamate could tend to cause aggregation, but that such residual benzyl groups can be removed by keeping the polymer in 0.1 *M* NaOH for 90 min.[29] This was done, and the solution (71) was then nearly neutralized with 0.1 *M* HCl and then dialyzed exhaustively against water. The solution of sodium poly-L-glutamate thus obtained was deionized and titrated in 0.1 *M* NaCl at 25°. Its titration curve was in agreement with those obtained from untreated samples.

It there are aggregates in equilibrium which gradually disperse with titration, their disaggregation would make a positive contribution to the measured value of $\Delta G°/N$, since the free-energy change we measure is for all changes which accompany ionization. However, evidence has been obtained by Hawkins and Holtzer[13] that the helical region represents a molecularly dispersed system. Their results will be presented in the near future.

(29) J. Applequist, personal communication, 1966.

The Validity of the Thermodynamic Parameters. Our particular application of eq 2 presumes that nonideality resulting from polymer–polymer interactions ($\ln \gamma_p'$) is negligible. Since, to obtain $\Delta G°/N$, the integral of the coil titration curve is subtracted from the integral of the experimental titration curve, this nonideality requirement is not very stringent. In order that $\Delta G°/N$ be correct, it is only necessary that interpolymer interaction in the experimental solution (at charge α) be about the same as the intercoil interactions. That is, if at every α, $\gamma'_{exptl} \cong \gamma'_{coil}$, it is not necessary that $\gamma'_{exptl} = \gamma'_{coil} = 1$.

We have, however, experimental evidence that γ'_{exptl} is indeed near unity. Since these interpolymer interactions are primarily electrostatic, they would be greatest at the lowest ionic strength, 0.01 *M* NaCl. Figure 4 shows titration curves of poly-L-glutamic acid in 0.01 *M* NaCl at 25°, obtained from solutions of different polymer concentration. For the upper curve (determination 54) at $\alpha = 0$, the concentration of L-glutamic acid residues was 0.02532 *M*, and at $\alpha = 1$, 0.00717 *M*. For the lower curve (determination 56) at $\alpha = 0$, the concentration was 0.01241 *M*, and at $\alpha = 1$, 0.00554 *M*. The change in concentration through the titration results from the addition of 0.01 *M* NaOH, and the concentration thus changes linearly with α. The upper of these two curves gives 192 cal/(residue mole) for $\Delta G°/N$; the lower, also 192. The difference between the curves themselves cannot be ascribed to concentration dependence (*i.e.*, polymer–polymer nonideality) since that would cause no discrepancy at low α (where polymer is less charged) and the greatest discrepancy at high α, just the opposite of what is seen. If, however, the initial solution of higher polymer concentration were not quite isoionic, but 2% ionized, whereas the other solution were essentially un-ionized, this is just the discrepancy which would result.

Data which are relevant to the results we report have been obtained in several other laboratories. Miller and Nylund,[9] using an experimental method very similar to the one described earlier,[8] obtain, for poly-L-glutamic acid in 0.20 *M* NaCl at 25°, $\Delta G°/N = 130 \pm 9$ cal/(residue mole); this is to be compared with our value of 167 ± 20 cal, obtained from two titrations. Our values, $\Delta H°/N = 975 \pm 50$ cal/(residue mole) and $\Delta S°/N = 2.67 \pm 0.1$ cal/(residue mole deg), obtained from 45 titrations at 7 temperatures, are the same as results based on titrations at three temperature (10, 25, and 40°) reported by Miller and Nylund. They give no details about the studies of the temperature dependence. Unless several titrations were done at each temperature, the close agreement of Miller and Nylund's data with ours must be fortuitous, as is apparent from the scatter of the data shown in Figure 3.

In the work of Hermans, examined in the discussion of the extrapolation to pK_0, he reports $\Delta G°/N = 105$ cal/(residue mole) at 25°, $\Delta H°/N = 1120$ cal/(residue mole), and $\Delta S°/N = 3.43$ eu/residue in 0.10 *M* KCl.[10] The earlier discussion makes apparent that these values are probably less reliable than the ones reported here.

Rialdi and Hermans have reported a calorimetric measurement of the un-ionized helix to un-ionized coil transition.[30] They find $\Delta H°/N = 1100 \pm 200$ cal/

(residue mole), in good agreement with the values obtained from analysis of titration curves. Although the general calorimetric method they employ has been described earlier,[31] because of the dearth of experimental data, we have been unable to derive from their report any very precise notion of their use of this method in this particular case. Thus, while we wish to give Rialdi and Hermans' measurement full credit, we do not wish to suggest that it confirms our value of $\Delta H°/N$.

Ionic Strength Dependence of the Measured Thermodynamic Parameters. The measured $\Delta G°/N$ appears as if it may be somewhat dependent on ionic strength, as can be seen in Figure 3. That $\Delta G°/N$ is dependent on the choice of pK_0 is readily seen from Nagasawa and Holtzer's results;[8] they find, using $pK_0 = 4.45$ for all ionic strengths, that $\Delta G°/N$ (25°) varies monotonically from 260 cal/(residue mole) in 0.01 M NaCl to 130 cal/(residue mole) in 0.20 M NaCl. We find, using the more appropriate choice of pK_0 for each ionic strength, 192 and 125 cal, respectively. It is apparent then that the modest variation of $\Delta G°/N$ with ionic strength observed here could well be a manifestation of the residual experimental inaccuracy of our choices of pK_0; that is, $\Delta G°/N$ is not dependent on ionic strength within the limits of our experimental accuracy.

Some dependence within these limits, however, would not be surprising. Since the highly charged coil which determines the extrapolated coil curve clearly does not have the same mean conformation at different salt concentrations, the un-ionized coil states attained by our extrapolations may not be quite the same. There is also the possibility that there is a small effect of salt concentration on hydrogen bond strength, or, for that matter, on whatever other stabilizing effects are present.

Application of the Theory of the Helix–Coil Transition.[3,4,32] It is of interest to interpret our results in terms of the previously mentioned statistical theories of the un-ionized helix to un-ionized coil transition. From these, we can estimate the helix content of un-ionized poly-L-glutamic acid. The helix content (f_H) is given in terms of two free-energy changes, one of which we can and will calculate directly from our data. These two are $-RT \ln s$, the free-energy change for the transfer of one residue at a helix–coil interface from a coiled state to a helical state; and $-RT \ln \sigma$, the free-energy change for the transfer of a coiled residue amid coiled residues into a helical state, while one residue at a helix–coil interface is transferred from helix to coil (*i.e.*, the free energy of starting a helical sequence at constant f_H). Since the transition from coil to helix involves one initiation and N (N being the degree of polymerization) transfers, the standard free-energy change for the helix-to-coil transition is

$$\Delta G° = RT \ln (s^N \sigma)$$

so that the quantity we have measured, $\Delta G°/N$, is related to the parameters of the helix–coil theory by

$$\Delta G°/N = RT \ln s + (RT/N) \ln \sigma$$

Since N was at least 600 in our experiments, we ignore the term in σ and are thus able to obtain $RT \ln s$ almost exactly as $\Delta G°/N$. At 25°, $\Delta G°/N = 178$ cal/(residue mole), giving $s = 1.60$.

From Applequist's equation 24,[32] the fraction helix can be calculated for various values of σ.

$$2f_H = 1 + (s - 1)/[(1 - s)^2 + 4\sigma s]^{1/2}$$

Use of this formula assumes long chains and that a single-coil residue can exist between helical segments.

Applying this to poly-L-glutamic acid at 25°, then, we find that, if $\sigma = 10^{-2}$, $f_H = 0.961$; if $\sigma = 10^{-3}$, $f_H = 0.996$; and if $\sigma = 10^{-4}$, $f_H = 0.999$. Snipp, Miller, and Nylund report that $\sigma = 3 \pm 2 \times 10^{-3}$ for poly-L-glutamic acid in aqueous solution.[33] Using $\sigma = 3 \times 10^{-3}$, $s = 1.60$, the fraction helix is 0.987. Thus, while there is not enough coil to affect the measured value of $\Delta G°/N$ significantly, un-ionized poly-L-glutamic acid may not be fully helical.

The Helix Content of Poly-L- and Poly-DL-glutamic Acids. One could question the validity of comparison of the extinction coefficient of a poly-DL-glutamic acid sample whose degree of polymerization is 185 with a much more highly polymerized sample of poly-L-glutamic acid. For example, light scattering might contribute significantly to the extinction coefficient, thus invalidating comparison between polymers of very different size. To examine this point, we measured the extinction coefficients of two poly-L-glutamic acid samples (one of DP = 680, the other of DP = 148) at high degrees of ionization; these extinction coefficients are shown in Figure 6 and are in quantitative agreement. Thus, the contribution of light scattering to the measured extinction coefficient is inappreciable.

A study of the variation of extinction coefficient with polymeric charge by McDiarmid and Doty casts doubt on the correctness of interpreting changes in extinction as changes in helix content.[20] They show that the contribution of the carboxyl group itself to the extinction coefficient depends on its charge state. Nevertheless, extinction coefficient as a measure of helix content can be checked against other methods; Zimm and Rice presented a method for determination of f_H from titration.[6] Nagasawa and Holtzer demonstrated that this titration method yields values of f_H which agree with those obtained from optical rotatory dispersion for poly-L-glutamic acid.[8] Figure 7 shows a comparison of f_H determined from our titration curves and from extinction coefficients measured by Miller and Nylund.[9] These are in acceptable agreement, demonstrating that, at least for poly-L-glutamic acid, extinction coefficient is as good an indicator of helix content as are optical rotatory dispersion and titration. It appears, then, that extinction coefficient *is* a reliable way to measure helix content of poly-L-glutamic acid.

Since polymers of L-amino acids form right-handed helices and D-amino acids form left-handed helices, we expected, *a priori*, that the racemic polymer would

(30) G. Rialdi and J. Hermans, Jr., *J. Am. Chem. Soc.*, **88**, 5719 (1966).
(31) J. Hermans and G. Rialdi, *Biochemistry*, **4**, 1277 (1965).
(32) J. Applequist, *J. Chem. Phys.*, **38**, 934 (1963).
(33) R. L. Snipp, W. G. Miller, and R. E. Nylund, *J. Am. Chem. Soc.*, **87**, 3547 (1965). It seems to us that what these authors refer to as σ is a quantity which Zimm and Rice[6] identify as σ', which replaces σ when the polymer is charged. If that is the case, perhaps we err in using the value given by Snipp, *et al.*, to deal with the charge free transition.

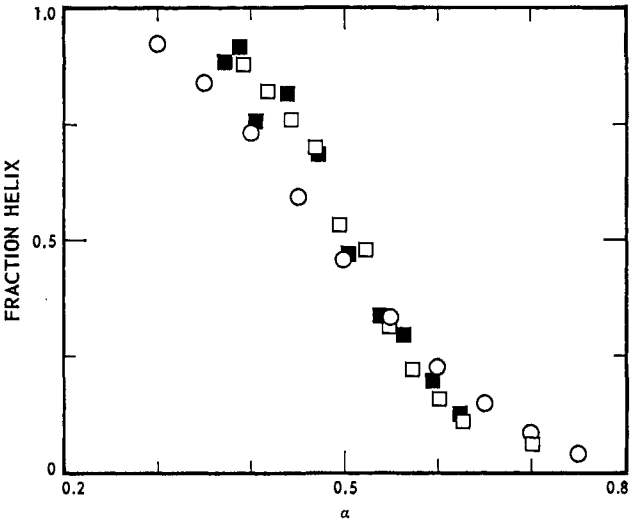

Figure 7. Fraction of residues in helical regions as a function of fraction of carboxyls ionized, for poly-L-glutamic acid. All data taken at room temperature. Helix content determined by: ○, ϵ_{200}, 0.20 *M* NaCl (extinction coefficients from ref 9); □, titration in 0.20 *M* NaCl (data from ref 8); ■, titration in 0.20 *M* NaCl.

not be helical. As noted above, this is not borne out, uncharged racemic polymer being 62% helix. However, even if the D and L residues are distributed randomly along the polymer chain, there will inevitably be short regions of pure D and of pure L residues which will form helices rather readily, and regions of nearly pure L or D which could force an isolated enantiomeric residue, if reluctantly, into a helix.

Titration of Poly-L-glutamic Acid in Aqueous–Organic Mixtures. The titrations of poly-L-glutamic acid dissolved in 16.3% (w/w) methanol–water, 0.1 *M* NaCl, and in 10% (*v*/*v*) *p*-dioxane–water, 0.1 *M* NaCl, were done in the hope that we would find aggregation less severe.[34] As can be seen in Figure 5, this is not the case. There is, however, a curious, reproducible hump in both titration curves, interposed between the aggregation region and the helix region, indicating some sort of transition. We guess it to be associated with a transition from incipient precipitation to a state in which the polymer exists as soluble aggregates of various sizes. Only further experimentation can clarify this point.

Some Comments on Molecular Theories of Polypeptide Conformation

Let us consider what is required of a molecular theory, even a semiempirical one, of the α helix to random-coil transition in aqueous solution. We have found that, at 25°, the standard free-energy change of this transition is 178 cal/(residue mole). The estimate of σ as 3×10^{-3} implies,[6,33] then, that poly-L-glutamic acid would be about 99% helical in aqueous solution, were it soluble. Thus, because of the cooperativeness of the transition, only a very small free energy of stabilization per residue leads to a molecular population that is overwhelmingly helical. (A similar situation prevails for globular proteins; the denaturation free energy of globular proteins, in the approximation of additivity, could similarly be very large, being the sum of the small amounts of stabilization provided by each residue.) Such a theory, to be meaningful, must give the free energy of transfer of a residue from the helical to the randomly coiled state to within, say, 50 cal/mole, in order merely to predict correctly which conformation is the stable one. As the reader shall see, this demand cannot possibly be met be any extant theory, semiempirical or fundamental, or by extension of any extant theory.

Many effects have been implicated as contributors to the α-helical stability of poly-L-glutamic acid, *i.e.*, to the over-all free energy of the transition. Among these are (1) backbone peptide hydrogen bonding,[35] (2) hydrophobic bonding among the side-chain methylenes, (3) side-chain carboxyl hydrogen bonding, and (4) electric dipolar interactions.[36] Let us examine critically attempts which have been made to determine the quantitative contributions of the first two of these effects and compare the results to the requirements enunciated above. The third is discussed below (under "Molecular Models"). The fourth has been less thoroughly studied than the first two and will not be discussed.

Peptide Hydrogen Bonding. There have been two careful attempts to estimate the free energy and enthalpy of peptide hydrogen bond formation in water from examination of model compounds. Kauzmann[37] estimated these quantities from thermodynamic data presented (and originally analyzed in a slightly different manner) by Schellman;[38] Klotz and Franzen[39] estimated them from direct spectroscopic measurement of hydrogen-bond formation. Throughout the discussion of these attempts, which follows, the reader should keep in mind that the measured total free-energy change of the helix-to-coil transition at 25° is only 178 cal/(residue mole), and therefore that an estimate with an uncertainty of more than 50 cal/(residue mole) is an entirely uninformative one.

Schellman calculated the extent of dimerization of urea in water on the assumption that the nonideality of urea–water mixtures is the consequence of aggregation of urea molecules.[38] He points out that a urea dimer can have either one hydrogen bond (linear dimer) or two (cyclic dimer) and analyzes the data so as to correct for the presence of cyclic dimers. To obtain the standard free energy of the reaction which most closely approximates peptide hydrogen bond formation, Kauzmann[37] subtracts the contribution of translational entropy (cratic entropy)[40] from the standard (infinitely dilute reference state, molality basis) free-energy change to obtain the unitary[40] free energy (ΔG_u) of disruption of a urea hydrogen bond. This free energy then includes only those entropy changes associated with changes in solvent contact and rotational motion that accompany dimerization. He thus assumes tacitly that the reduction of rotational freedom accompanying urea dimerization is similar to the loss of chain entropy of peptide hydrogen

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bond formation. The value of ΔG_u thus obtained is 400 ± 100 cal/mole of hydrogen bonds. Having presented this value, Kauzmann[37] then quotes Schellman's[38] conclusion: "Hydrogen bonds, taken by themselves, give a marginal stability to ordered structures, which may be enhanced or disrupted by interactions of side chains." In view of our experimental result, the first part of this statement can be misleading; if hydrogen bonds taken by themselves do provide 400 cal of stabilization, it is not a marginal stability but is, in fact, twice as large as the actual stability. Kauzmann also, correcting for the cyclization (following Schellman), finds $\Delta H°$ of the same reaction to be 1400 ± 100 cal; this is favorable to helix formation and is roughly similar to the 975 cal measured for the helix-to-coil transition in poly-L-glutamic acid.

Klotz and Franzen used infrared spectroscopy to measure the extent of hydrogen bonding of the model compound, N-methylacetamide, in water at 25 and 60°. From these data, they find that the standard (infinitely dilute reference state, molality basis) Gibbs free energy of the dimer-to-monomer reaction is −3100 cal/mole, the standard enthalpy 0.0 kcal, and the standard entropy 10 eu/mole. The enthalpy that they were able to measure is not that of the dimerization in infinitely dilute solution, but under conditions such that 50% of the amides are hydrogen bonded, corresponding to about 10 *M* solute concentration. They note that the measured value of the standard enthalpy change of the dimer-to-monomer reaction varies "a few tenths of a kilocalorie" with extent of aggregation, a large number compared with the measured helix stability.

From these data, Klotz and Franzen[39] conclude, "It seems clear from these experiments that the intrinsic stability of interpeptide hydrogen bonds in aqueous solution is small," and later, "The results with this model system indicate that for protein molecules in aqueous solution, interpeptide hydrogen bonds cannot contribute significantly to the stabilization of macromolecular organization, except perhaps in a few regions with a very low local dielectric constant due to a specific high concentration of hydrocarbon-like residues." These conclusions were, of course, stated before the stability of an α helix had been measured. In light of the measured values, though, these hardly seem the conclusions to draw from the above data; what, in fact, the data of Klotz and Franzen suggest is that interpeptide hydrogen bonds are so much weaker than water–peptide hydrogen bonds that they represent an *overwhelming destabilizing* influence, as we will demonstrate.

Let us consider these data further. The standard free energy of hydrogen-bond disruption given includes the cratic term; when this is subtracted, one finds $\Delta G_u = -700$ cal. The measured 178 cal that stabilizes the α helix of poly-L-glutamic acid indicates that this still would have to be considered a tremendously destabilizing interaction. Thus if one accepts the ΔG_u calculated from Klotz' data, we must *supply* about 875 cal of stabilizing free energy from effects other than peptide hydrogen bonds. In addition, the enthalpy which Klotz and Franzen[39] give, $\Delta H° = 0.0$ kcal/mole, provokes another serious question: what provides the 975 cal of enthalpy that we have measured for the poly-L-glutamic acid α helix? As we will show below, it is unlikely that this is supplied by either hydrophobic interactions or by side-chain hydrogen bonding.

The difficulty to be encountered in assessment of individual contributions to the helix stability by studies of model compounds is apparent from the conflicting conclusions of the Schellman–Kauzmann and Klotz and Franzen methods. The Schellman–Kauzmann result is that interpeptide hydrogen bonding would be an enormously stabilizing influence ($\Delta G_u = 400 \pm 100$ cal); Klotz and Franzen find that this would overwhelmingly destabilize the helix ($\Delta G_u = -700$ cal).

Hydrophobic Bonding among Side Chains. The standard Gibbs free energy of transferring one methylene group from water of a hydrocarbon environment (ΔG_{HC}) has been calculated in several different ways.[41,42] Comparison of the solubility of a given amino acid in water and in ethanol leads Tanford[41] to assign ΔG_{HC} the value, −730 cal/mole of methylene. The amino acids whose solubilities are consistent with this result are alanine, valine, and leucine. If analysis of this sort has more general validity, the difference in the free energy of transfer between glutamic and aspartic acids should also be −730 cal/mole, since these two molecules differ precisely by one methylene; in fact, the data Tanford presents give instead +10 cal/mole. Further, if the standard free energy of transfer of a side chain from ethanol to water can be obtained by simple addition of the free energies of transfer of its parts (*i.e.*, the methylenes, carboxyls, hydroxyls, etc.), glutamine and asparagine would differ by +10 cal/mole, as do glutamic and aspartic acids; Tanford's data, however, give a difference of −90 cal/mole. He alludes to these facts. Whether this inconsistency is a failure of the additivity assumption or reflects experimental inaccuracy is not relevant to this discussion; the simple fact is that this analysis results in a 100-cal "peculiarity." Application of such a directly measured hydrophobic bonding free energy to the poly-L-glutamic acid helix would not be straightforward even if the data were otherwise perfectly consistent, since only a small part of the surface of each methylene is removed from contact with water when the coil becomes helical. Since the entire stability of the poly-L-glutamic acid α helix is 178 cal/(residue mole), Tanford's analysis is, again, of no help in predicting either the sign or the magnitude of the helix–coil free-energy change. It could not, for example, tell us that, at low charge density, poly-L-glutamic acid in aqueous solution is a stable helix, but poly-L-glutamic acid is never more than 15% helical.[43] Nevertheless, these are the facts.

Emerson and Holtzer[42] have obtained the free energy of transfer of the linear hydrocarbon portion of ionic detergents from aqueous medium to the interior of a micelle; their result is ΔG_{HC} = 620–860 cal/mole of methylene, depending on the ionic strength of the aqueous medium and on whether a cationic or anionic detergent is used. Again, the magnitude of the total helix stabilization is less than the range of these values.

An Estimate of the Effectiveness of Urea as a Denaturant. Using maleic acid as a model, Levy and Magoulas[44] have tried to assess the effect of 7 *M* urea

(41) C. Tanford, *J. Am. Chem. Soc.*, **84**, 4240 (1962).
(42) M. F. Emerson and A. Holtzer, *J. Phys. Chem.*, **69**, 3718 (1965).
(43) M. E. Noelken, Ph.D. Thesis, Washington University, St. Louis, Mo., 1962.

on the free energy of hydrogen-bond formation. They conclude that urea does not weaken hydrogen bonds "significantly;" by significantly, they mean within an uncertainty of 100–200 cal. This, too, is not a useful result; the entire stability of the poly-L-glutamic acid α helix lies within their limits of uncertainty, allowing us to conclude that urea is capable of completely destroying the α helix of poly-L-glutamic acid, of having no effect at all, or of doubling its stability.

Molecular Models. Models of poly-L-glutamic acid were examined in the hope of gaining some insight into the helix-stabilizing influences. Corey–Pauling–Koltun models were used (Ealing Corp., Cambridge, Mass.); if, and only if, great care is taken in setting all peptide bonds to the correct angles, these models can be formed into a right-handed α helix fully hydrogen-bonded along its backbone. The glutamic side chains then project away from the α-helical core; when viewed from above the helical axis, the side chains are seen to form four lines which spiral slowly around the core in a right-handed sense. Between the lines of side chains is a groove wide enough to allow water molecules to penetrate to the helical core. Two side chains that are adjacent along the helical axis are spaced closely enough to prevent penetration of water directly between their hydrocarbon portions. The water molecules thus have access to most, but not all, of the surface area of the hydrocarbon part of the side chains.

The possibility of side-chain carboxyl–carboxyl hydrogen bonding in the models can also be examined, though the molecule cannot necessarily be expected to behave in the same way. Many adjacent pairs of carboxyls can be singly hydrogen-bonded with difficulty, but steric effects prevent further hydrogen bonding; *i.e.*, no carboxyl can be hydrogen bonded to two others.[45]

In view of the preceding comments regarding the difficulties of even deciding whether or not an effect stabilizes the α helix, it would be most unwise to try to draw firm conclusions from examination of a model. We thus make no attempts to assess the importance of hydrophobic bonding. We will, however, meekly suggest that stabilization from side-chain hydrogen bonding would probably be meager[46] and, further, that advocates of hydrophobic stabilization (through interaction of side-chain methylenes), if they wish to be convincing, will have to reckon with the annoying fact that a relatively small fraction of the hydrophobic surface is effectively removed from contact with the solvent when the coil-to-helix transition occurs.

(44) M. Levy and J. P. Magoulas, *J. Am. Chem. Soc.*, **84**, 1345 (1962).

(45) Using Dreiding Stereomodels (Büchi, Switzerland), one can link the side-chain carboxyls in long hydrogen-bonded chains; this is mentioned simply to indicate the caution required when using models which are not space filling.

(46) It initially seemed to us that a difference in pK_{0h} and pK_{0c} could be interpreted as a measure of side-chain hydrogen bonding. This view is superficial; the first ionization of a hydrogen-bonded carboxyl could result in formation of a single carboxylate–carboxyl hydrogen bond. Such bonds are stronger than carboxyl–carboxyl bonds.

Conclusion

We see, then, that even to formulate a semiempirical theory of the helix–coil transition, much more experimental precision would be required than has hitherto been possible. The entire observed stabilization of the poly-L-glutamic acid α helix could be provided by any of the above discussed effects, and none of the experimental methods so far available are nearly precise enough to sort them out. We see, also, the rigor which would be required to calculate s and σ from the known molecular properties of similar compounds, and, by implication, the far greater rigor which would be required of a theory whose goal is the calculation, from first principles, of the stable conformation of a globular protein. A single effect whose magnitude is of the order of a few kilocalories, for instance, would have to be assessed with an absolute accuracy of about 1%, since the conformation is determined by the sum of many such effects.

An example of these difficulties is provided by a recent study[47] which resulted in a prediction of the conformation of the enzyme, ribonuclease, which a recent X-ray determination showed to be almost totally incorrect.[48] Since the method employed requires a large number of physically indeterminate parameters, the calculation of a known structure would represent no accomplishment whatsoever; the failure to predict the correct structure of ribonuclease should not be interpreted as a call for further parametric adjustment. Instead, we must recognize, with deep disappointment, that, assuming additivity, the structure of a protein is determined by the algebraic sum of many large (compared to the over-all stability) effects, some stabilizing, some destabilizing; and that we cannot assess *any* of them with sufficient accuracy to assure that even the sign of the sum is correct. This total failure is most starkly illustrated by a frustratingly simple example: poly-L-glutamic acid forms a very stable α helix, whereas poly-L-aspartic acid forms almost no helix,[20,43] and no one has the vaguest notion why.

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