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Hydrogen Ion Titration of Poly-S-carboxymethyl-L-cysteine

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Potentiometric titration curves have been determined for aqueous solutions of poly-S-carboxymethyl-L-cysteine. A pH-induced transition of the polymer conformation is manifest from the occurrence of a transition region around pH 5, where the solution has an essentially infinite buffer action. The titration curve at higher ionization corresponds to that of a random coil. At lower ionization the polymer is associated to form a β-structure and its titration behavior is qualitatively accounted for by the Gouy model for a uniformly charged sheet. Deviation from the model would partly be ascribed to the aggregation of sheets in the β-form. The standard free energy of transition from random coil to β-form has been estimated for the unchanged polymer and is found to depend on the molecular weight of polymer. It is suggested that fine structure of the β-form and its mode of aggregation vary with polymer chain length.

It has been accepted that some synthetic polypeptides, which exist as the β-conformation in solid, can retain the β-structure even in solution, if some specific solvent systems are chosen,[1-3] However, most of these polypeptides are insoluble in aqueous media and thus have been examined in mixtures of organic solvents. A conformational transition takes place from random coil to β-form at a certain solvent composition, as the solvent mixture becomes less polar. Evidence for the coil-β transition and the formation of the β-form was mainly derived from measurements of optical rotation and infrared spectrum. However, thermally induced formation of β-form of poyl-L-lysine in water was elaborated by two groups of workers.[4-6]

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Recently, pH-induced coil-β transition was found for a water-soluble polypeptide, poly-S-carboxymethyl-L-cysteine.[7,8] The transition was observed as large changes in optical rotation, intrinsic viscosity and infrared spectrum around pH 5. The polymer is associated into an intermolecular β-conformation upon lowering pH, while it is in a random coil conformation at higher pH. Its ultraviolet rotatory properties were also investigated as a function of pH and correlated with the conformational transition.[9] .Is can be expected that the transition is also observed on potentiometric titration curves of the polymer, provided that ionized carboxyl groups in the β-form have different affinity or electrostatic interaction with hydrogen ions from those in the random coil. The pH titration method would provide another useful approach to elucidate the detailed structure of the β-form and its transition to random coil. Preliminary titration curves have already published.[7,8]

The helix-coil transition of poly-L-glutamic acid in aqueous meida was successfully investigated by means of the titration method.[10-13] The results clearly showed the presence of three regions on a titration curve, each corresponding to the pure helix, the pure coil and the intervened transition region, and made it possible to estimate the standard free energy of transition.

Experimental

Materials. Poly-S-carboxymethyl-L-cysteine used was the same samples as described previously.[8] The intrinsic viscosities (in d*l* g^{-1}) of the three samples were 0.230 for E602, 0.089 for E527 and 0.041 for E515, in 0.2M NaCl at pH 7, 25°C. Most experiments were performed on the highest molecular weight sample, E602.

Carbonate-free 1N NaOH was standardized against potassium acid phthalate, and 1N HCl was standardized against Na_2CO_3 and checked against the NaOH. Glass-redistilled CO_2-free water was used to make up all the solutions.

pH Measurements. A Horiba Model P pH-meter of a potentiometric type, accurate to ±0.003 pH unit, was used with a Horiba standard buffer (pH 6.86 at 25°C).

Hydrogen Ion Titration. Titrants were delivered from calibrated micrometer syringes with 0.5 m*l* capacities. All the measurements were carried out under nitrogen flushing. Temperature was 25°C, unless otherwise stated. A specially designed titration vessel with three necks, two of which were installed with a glass electrode and a reference electrode, respectively, and the other was fitted with a syringe, thermometer and nitrogen inlet, was placed on a magnetic stirrer which was grounded to the earth.

To a weighed amount of sample was added 10 m*l* of solvent with appropriate ionic strength of NaCl. The suspension was first titrated with 1N NaOH up to pH 11.5, when the sample had been in solution at about pH 6. Then the alkaline solution was back-titrated with 1N HCl. Each addition of HCl yielded white gel upon the tip of the syringe needle but the gel redissolved within a few minutes. However, below about pH 4, the solution was so viscous and turbid that observed pH values were unreliable.

The degree of ionization, α, was calculated from the difference between total and free concentrations of hydrogen ions. Free hydrogen ion concentration was computed by assuming that liquid junction potentials and mean activity coefficients of the solution were the same as those of solution containing no polymer.

Results

The titration curves in a forward direction, *i. e.*, from acid to alkaline, and those in a backward direction were not necessarily conicident with each other. The forward curve generally shifted to slightly more alkaline side than the backward curve. This would be caused mostly by the different rates of reactions in suspension and in homogeneous solution. The suspension became clear when its pH was raised to 5 or higher. The homogeneous solution could be kept clear down to pH 4 or even lower during the beckward titration, which was more reproducible. The backward titration may be regarded as closer to the equilibrium titration

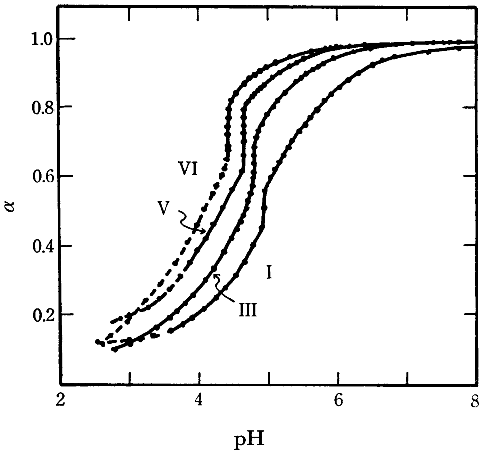

Fig. 1. Potentiometric titration curves at different ionic strengths.

Initial NaCl concentration: I, 0.00M NaCl; III, 0.05M; V, 0.20M; VI, 1.00M. Dashed parts indicate the region where turbidity was manifest.

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behavior of the system. All the results presented below are concerned with those of the backward titration process, which moves from the alkaline to the acid side, using acid titer.

A characteristic feature common to all the titration curves should be noted. As the titration proceeds from alkaline to acid side, a region always appears where the solution exhibits an essentially infinite buffer action or where the pH of solution remains unaltered upon addition of some amount of acid. This is illustrated in Fig. 1. The location of this particular region is slightly dependent on polymer concentration and temperature and is markedly dependent on ionic strength. The characteristic region is located around pH 5 or slightly lower. Previous results[7-9] have shown that various properties suddenly change around pH 5 and conformational change from coil to β-from takes place here. Thus the characteristic pH titration curve may be reasonably identified with the pH where the polymer undergoes a conformational change. In this sense, the specific pH region will be referred to as the transition pH or the transition region. At pH higher than this, the polymer is in a randomly coiled form, but at lower pH it is in an intermolceular β-conformation. Occurrence of such a transition region during the titration would imply that the affinity of hydrogen ions with ionized carboxyl groups is higher in the coil form than in the β-form. The observed sharpness of the transition would be related to the cooperative formation of hydrogen bonds upon lowering pH.

The titration curve slightly shifted to higher pH with increasing polymer concentration. The transition pH in 0.2M NaCl was 4.64 for 0.154 g dl^{-1}, 4.68 for 0.295 g dl^{-1} and 4.72 for 0.608 g dl^{-1}. The last pH value is in complete agreement with that found previously[8] for the titration. As the concentration was as high as 0.5 g dl^{-1}, gelation occurred at the transition pH and made it difficult to obtain reliable values at lower pH. The concentration dependence would be ascribed to stronger electrostatic interaction between ionized groups at higher concentrations or in larger aggregates. Most experiments were, therefore, performed at polymer concentrations around 0.150 g·dl^{-1} or 0.0093N.

Typical titration curves at different ionic strengths are shown in Fig. 1. As the ionic strength is higher, more carboxyl groups are ionized at a given pH, and the transition pH is more acid but the degree of ionization is higher. The latter indicates that the β-form is stabilized by the ionic atmosphere formed around charged groups, which is in accord with the previous results.[8,9]

The effect of temperature on the titration in 0.2M NaCl is shown in Fig. 2. The observed shift of the titration curve indicates that at higher temperature the β-form is less stable owing to the increased ionization.

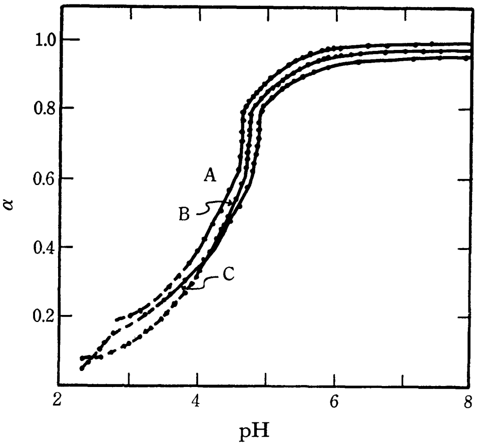

Fig. 2. Titration curves in 0.2M NaCl at different temperatures.
A, 25°C; B, 16°C; C, 3°C. Dashed parts indicate the region where turbidity was manifest.

Discussion

Electrostatic Free Energy and Polymer Conformation. Potentiometric titration of polyelectrolytes having a uniform distribution of ionizable groups can be described by the expression[14-16)]

$$\mathrm{pH}-\log\frac{\alpha}{1-\alpha}=\mathrm{p}K_{\mathrm{int}}+\frac{0.434}{ZkT}\frac{\partial G_{\mathrm{el}}}{\partial\alpha} \qquad (1)$$

where $\mathrm{p}K_{\mathrm{int}}$ is the negative logarithm of intrinsic dissociation constant, Z the total number of ionizable groups in a molecule (or its associated from), and G_{el} is the electrostatic free energy of the molecule (or its associated form) with $Z\alpha$ ionized groups. Since the last term, $\partial G_{\mathrm{el}}/\partial(Z\alpha)$, represents the work necessary to remove a hydrogen ion from the molecule (or its associated form) with $Z\alpha$ charges, Eq. 1 may be written[17,18]

$$\mathrm{pH}-\log\frac{\alpha}{1-\alpha}=\mathrm{p}K_{\mathrm{int}}-0.434\frac{\varepsilon\psi_0}{kT} \qquad (2)$$

where ε is the elementary charge and ψ_0 is the electrostatic potential at the molecular surface.

Figure 3 shows the modified plots, $\mathrm{pH}-\log\alpha/(1-\alpha)$ *vs.* α, at various ionic strengths. As was also noted in Fig. 1, each of the curves in Fig. 3 can be separated into at least three regions, corresponding to the random coil at higher α, the β-

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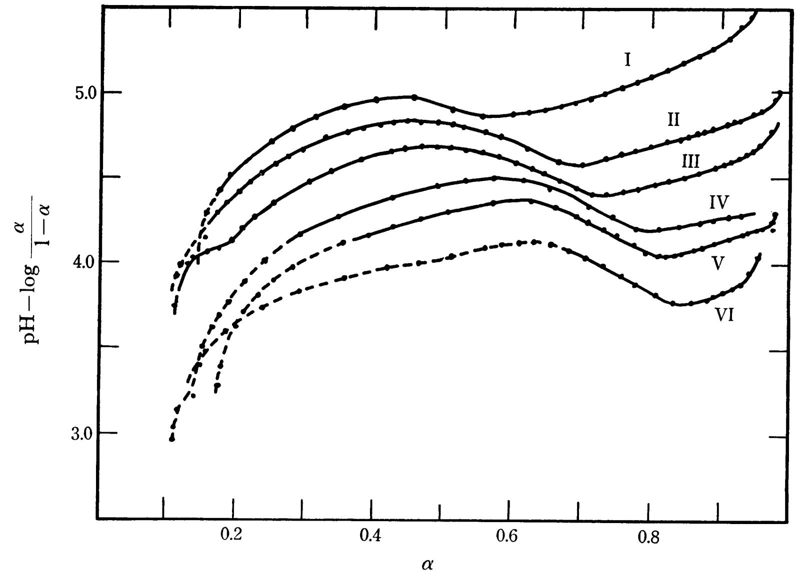

Fig. 3. Modified titration curves at different ionic strengths.
Initial NaCl concentration: I, 0.00M; II, 0.01M; III, 0.05M; IV, 0.10M; V, 0.20M; VI, 1.00M. Dashed parts indicate the region where turbidity was manifest.

form at lower, α, and the intermediate transition region. The portion of curves at very low α could not be traced down to $\alpha=0$, owing to its large curvature, to determine the pK_{int} value. However, the pK_{int} value seems to be considerably lower than that ($pK_{\text{int}}=4.5$) for poly-L-glutamic acid.[12,13] In fact, the δ-carboxyl group of the monomer, S-carboxymethyl-L-cysteine, has a pK value, 3.12, in 0.2M NaCl, as will be seen in Fig. 6 below. As there is no way for the extrapolation to $\alpha=0$, the pK_{int} value is assumed to be 3.00 for both random coil and β-form, irrespective of the ionic strength. Then the specific titration behavior of the polymer in each region may be accounted for by different electrostatic interactions, each characteristic of the conformation in that region. The electrostatic interaction or the electrostatic free energy has been evaluated for various systems, either analytically or numerically, using idealized model appropriate to the system.

Although a detailed structure of the β-form in solution is not known yet, its fundamental structure may be sheet-like, as in the solid, in which polypeptide chains are aligned and held together by inter- or intra-molecular hydrogen bonds. The two-dimensional hydrogen bonded structure can stack together through side chain interactions to form intersheet aggregates. To the first approximation or at some stage of its formation, the β-structure would have the sheet-like structure in solution. Then the β-form of ionized polypeptides in solution would be most adequately represented by a uniformyl charged plane, *i. e.*, by the Gouy model. The electrostatic potential at a charged plane dipped in an electrolyte solution is given by[19,20]

$$\psi_0 = \frac{2kT}{\varepsilon}\sinh^{-1}\frac{\kappa\sigma}{4n\varepsilon} \tag{3}$$

where σ is the surface charge density, n is the number concentration of salt, and κ is the Debye-Hückel parameter.

Charged groups in the present polymer are side chain carboxyls and they are directed to both sides of the molecular sheet alternately along with polypeptide chains or hydrogen bonds in the β-structure. Thus an electric double layer is formed at each side of the molecular sheet, and the area assigned to a charge should be twice as large as the area occupied by an amino acid residue, A_0. The surface charge density may be given by

$$\sigma = -(\varepsilon/2A_0)\,\alpha \tag{4}$$

Substitution of Eq. (4) into Eq. (3) leads to an expression for the surface potential of a charged β-form

$$\psi_0 = -\frac{2kT}{\varepsilon}\sinh^{-1}\frac{125\pi}{DN_{\text{A}}kT}\,\frac{\varepsilon\alpha}{A_0\sqrt{C_s}} \tag{5}$$

where C_s is the molar concentration of salt, D the dielectric constant of solvent, and N_{A} the Avog-

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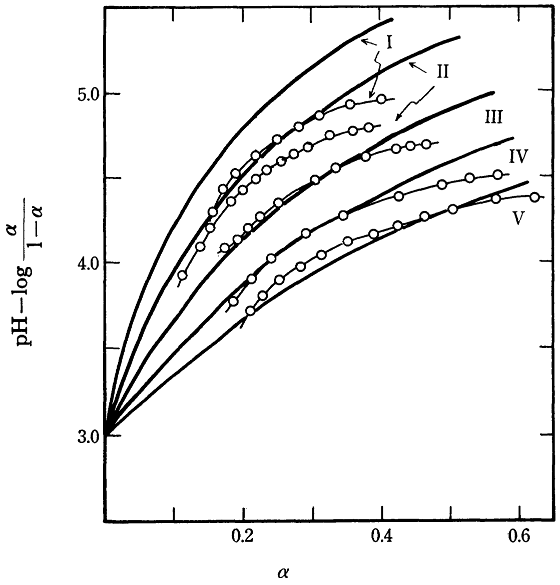

Fig. 4. Calculated and experimental titration curves of the β-form.

Thick curves are calculated by Eq. (6) with $\mathrm{p}K_{\mathrm{int}}=3.00$ and $A_0=16.8\,\mathrm{A}^2$. Circles show experimental values. NaCl concentrations (in M) are:

	I	II	III	IV	V	
C_s	0.01	0.02	0.05	0.10	0.20	(Calcd)
Initial	0.00	0.01	0.05	0.10	0.20	(Exp.)

adro number.

Thus the potentiometric equation for the charged β-form is obtained as

$$\mathrm{pH}-\log\frac{\alpha}{1-\alpha}=\mathrm{p}K_{\mathrm{int}}$$
$$+0.868\sinh^{-1}\frac{125\pi}{DN_{\mathrm{A}}kT}\frac{\varepsilon\alpha}{A_0\sqrt{C_s}} \qquad (6)$$

This can be brought to direct comparison with experimental results. Assuming that $\mathrm{p}K_{\mathrm{int}}$ is 3.00 and the area per residue, A_0, is 16.8 A^2, theoretical curves can be obtained for different C_s values. Figure 4 shows the calculated curves together with the experimental plots in the region of the β-form.

Agreement is rather good, except for the lowest ionic strength, if the use of the idealized model and the assumed values for the parameters are taken into account. At low ionic strengths, the effect of increase in ionic strength during the titration is considerably high. While the β-structure has not been well defined, the sheet-like structure postulated above would stack into a larger aggregate owing to the side-chain interaction. Only at the initial stage of its formation, *i. e.*, at pH far from the transition pH, the β-structure would be more like a sheet. Upon lowering pH, the intersheet aggregation would become predominant and cause the β-form to deviate from the sheet-like structure. In this sense, the agreement mentioned above appears to be fortuitous, and in fact, deviation is noticed in different curvatures between experimental and calculated curves.

Randomly coiled polyelectrolytes have been treated on the basis of various models, and the Poisson-Boltzmann equations have been solved by means of the Debye-Hückel approximation or electronic computors. Among them, Kotin and Nasasawa[21] obtained computor solutions, postulating a rod-like model for a charged random coil, and found that the titration data of polyacrylic acid and polymethacrylic acid could be described by these solutions.[21,22] Although no precise comparison has been made, the present titration data at higher ionization appear to conform to the Kotin-Nagasawa solution, if the $\mathrm{p}K_{\mathrm{int}}$ value is assumed to be 3.00 and the molecular dimension is properly chosen.

Standard Free Energy for the Coil-β Transition. The pH-induced transition of the polymer is of electrostatic one, and the process bringing polymer molecules from an uncharged β-form to fully charged random coils can be divided into two steps: first, transforming the uncharged molecules from β-form to random coils and secondly, charging up the random coil molecules. More significant in the discussion of conformational change is the free energy charge accompanying the first step, *i. e.*, the charge-independent part, which will be denoted by $-\Delta G^{\circ}_{\mathrm{c}\rightarrow\beta}$ per residue.

The second step, during which random coils are being charged, yields a free energy change per residue

$$\mathrm{d}G^{(\mathrm{c})}=2.30\,kT\left(\mathrm{p}K^{(\mathrm{c})}-\mathrm{pH}^{(\mathrm{c})}+\log\frac{\alpha}{1-\alpha}\right)\mathrm{d}\alpha \qquad (7)$$

where $\mathrm{p}K^{(\mathrm{C})}$ is the pK in the random coil form and $\mathrm{pH}^{(\mathrm{C})}$ is the pH of the hypothetical random coil solution. The free energy change per residue accompanying the ionization process through the actual titration course can be expressed by

$$\mathrm{d}G=2.30kT\left(\mathrm{p}K-\mathrm{pH}+\log\frac{\alpha}{1-\alpha}\right)\mathrm{d}\alpha \qquad (8)$$

where pK is the apparent pK in the actual polymer. In equilibria, Eqs. (7) and (8) lead to the Henderson–Hasselbach equations, respectively, which permit the calculation of the pK values from titration data.

It follows from an earlier statement that a thermodynamic relation

$$\Delta G=-\Delta G^{0}_{c\rightarrow\beta}+\Delta G^{(\mathrm{c})} \qquad (9)$$

holds for integrated values of the above quantities. Equation (9) can be written as

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$$\Delta G^{0}_{c\to\beta} = 2.30\,kT\int_0^1 (\mathrm{p}K^{(c)} - \mathrm{p}K)\,\mathrm{d}\alpha \qquad (10)$$

$$= 2.30\,kT\int_0^1 (\mathrm{pH}^{(c)} - \mathrm{pH})\,\mathrm{d}\alpha \qquad (11)$$

Equations (10) and (11) provide methods of evaluating charge-independent part of the free energy of transition.

The methods of evaluating the transition free energy have been presented by Zimm and Rice,[11] Nagasawa and Holtzer,[14] and Leyte and Mandel.[23] The helix-coil transitions of poly-L-glutaimc acid,[14,14–26] poly-L-lysine,[25] and copolymers of L-leucine and L-glutamic acid[24] were treated by these methods, and an undefined conformational transition of polymethacrylic acid[23] was also examined in the same way. From the method of derivation it is clear that Eqs. (10) and (11) have a general validity for any conformational change, even if it accompanies an association-dissociation equilibrium.

Equation (10) was applied to the present coil-β transition to estimate the charge-independent part of transition free energy. Graphical integration was performed on the modified titration curve such as shown in Fig. 3, after both coil and β-form regions, drawn in solid curves, were extrapolated to lower ionization in such a way to give an identical pK_{int} value (3.00) at $\alpha=0$. The values of $\Delta G^{0}_{c\to\beta}$ obtained are plotted against the square root of ionic strength in Fig. 5. The charge-independent part of transition free energy is still dependent on the ionic strength, as was also observed in the helix-coil of transition, of poly-L-glutamic acid,[14] and varies from −700 cal mol^{-1} in water to −480 cal mol^{-1} at higher ionic strength. These values suggest that the β-form of unionized poly-S-carboxymethyl-L-cysteine is generally much more stable than the

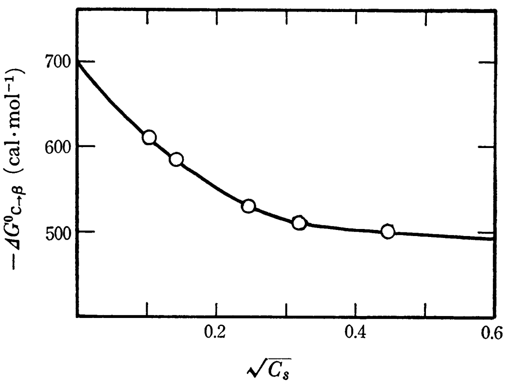

Fig. 5. Dependence of $-\Delta G^{0}{}_{c\to\beta}$ on the square root of ionic strength.

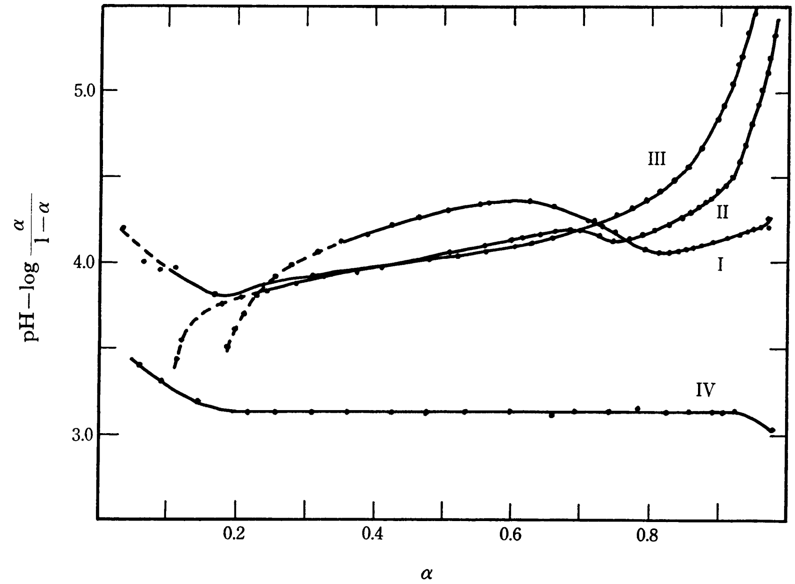

Fig. 6. Modified titration curves for different samples in 0.2M NaCl. I, E602, $[\eta]=0.230$; II, E527, $[\eta]=0.089$; III, E515, $[\eta]=0.041$, IV, δ-carboxyl group of S-carboxymethyl-L-cysteine. ($[\eta]$: dl g^{-1} in 0.2M NaCl at pH 7, 25°C). Dashed parts indicate the region where turbidity was manifest.

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α-helix of unionized poly-L-glutamic acid,[14,24–26] relative to their random coil forms. However, this conclusion can not be extended to the other poly-S-carboxymethyl-L-cysteine samples, because of marked molecular weight dependence of $\Delta G^{\circ}_{c\to\beta}$, as will be shown below.

The coil-β transition of the polymer in 0.2M NaCl was also examined at different temperatures, as illustrated in Fig. 2, and the charge-independent transition free energy was estimated at each temperature. Rough estimates lead to a corresponding transition enthalpy of about -2.4 kcal mol^{-1} and a transtion entropy of -6.5 e.u., giving a transition temperature around 100°C. Both negative values obtained would reflect the nature of hydrogen bonding and hydrophobic interaction.

The Effect of Molecular Weight upon the Titration Behavior. Figure 6 shows modified plots of titration curves for three samples of poly-S-carboxymethyl-L-cysteine in 0.2M NaCl, together with that for δ-carboxyl group of its monomer, S-carboxymethyl-L-cysteine, in 0.2M NaCl. The three polymer samples differ from one another in their molecular weight, as revealed in their intrinsic viscosity of random coil form.

It can be seen that the δ-carboxyl group of the monomer has a constant pK value, 3.12, reasonably close to the pK_{int} value assumed for the polymers. The lowest molecular weight sample, E515, does not show any sharp transition region on its titration curve. Its rotatory properties have shown only trivial changes with pH,[9,10] in accordance with the titration result. Berger, Noguchi and Katchalski[27] have reported that titration curves of poly-S-carboxymethylcysteine prepared by other synthetic methods can be described by the Linderstrøm-Lang equation, suggesting the absence of clear transition region.

With increasing molecular weight, the transition region becomes more distinct. The molecular weight dependence of titration behavior is considerably stronger at higher ionization, where random coil forms are predominant. At lower ionization, however, no such regular dependence is observed. If the charged β-form could be represented by the Gouy model, in which hydrogen-bonded polypeptide chains would form an infinitely extended sheet, its titration behavior would not depend on the molecular weight of constituent polymer. Thus the observed molecular weight dependence indicates deviation of the β-structure from an infintely extended sheet model. The sheet structure of the β-form would have a finite size with appreciable edge effect, which would depend on the chain length of polymer. More possibly, the sheet-like structures would form a globular aggregate by side chain interaction.

The charge-independent transition free energy of these three polymer samples was estimated by Eq. (10) and found to be -80 ± 80 cal mol^{-1} for E515, -180 cal mol^{-1} for E527, and -500 cal mol^{-1} for E602, in 0.2M NaCl at 25°C. The uncharged β-form is less stable as the molecular weight becomes smaller. It is not yet known whether such dependence of stability of β-form is extended to a higher molecular weight range. However, it is conceivable that the dependence reflects variations of a detailed structure of the β-form and its mode of aggregation with polymer chain length and is closely related with the intermolecularly associated structure of the β-form.

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