Studies of Intramolecular Transitions and Intermolecular Interactions of Polypeptides by Fluorescence Techniques†

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ABSTRACT: Polarization of fluorescence measurements reflect the hydrodynamic properties of macromolecules, and they provide a sensitive means for investigating their structure and structural transitions. The studies reported here focus on various aspects of the coil–helix transition, the structure of helices, the coil–β-conformation transition and aggregation in poly(lysine), poly(glutamic acid), poly(vinylamine), and a copolymer of glutamic acid and lysine. In the helical form, poly(lysine) and poly($Glu^{63}Lys^{37}$) contain multiple, discrete helical segments, whereas poly(glutamic acid) is entirely helical. The glutamic acid residues, which are strong helix formers, dominate the behavior of the copolymer in solution. The helices formed by poly(lysine) and by the copolymer do not aggregate until the pH is considerably past the point of maximal helix formation, due to the influence of the residual coil segments on the properties of the polypeptide chain. In contrast, the completely helical poly(glutamic acid) aggregates just after all the residues assume the helical conformation. The coil forms of all the polymers display the polyelectrolyte effect at the extremes of pH. Finally, poly(lysine) undergoes a transition from the α helix to the β conformation *via* the coil form when heated in alkali; the transition is reversible at pH 10.05, but not at pH 10.7. Molecular volumes calculated from measurements of the intrinsic viscosity and of the sedimentation coefficient are considerably larger than the theoretically calculated volumes due to hydration of the macromolecules. In contrast, the volumes calculated from polarization of fluorescence measurements are smaller than the theoretical volumes because of internal rotation in the polypeptide chain.

Polarization of fluorescence techniques employing dye macromolecule conjugates have been used to study the structure and interactions of proteins (Weber, 1953; Steiner and Edelhoch, 1962) and of synthetic polypeptides (Gill and Omenn, 1967; Gill *et al.*, 1970). The fundamental relationship describing the dependence of polarization of fluorescence upon the Brownian rotational diffusion of the macromolecule was developed by Perrin (Perrin, 1926, 1929, 1934, 1936a,b) and extended by Weber (Weber, 1952, 1953). The derivation of these relationships, which were developed for rigid spheres or ellipsoids, embodies five assumptions: (1) the equipartition of energy pertains; (2) the solvent is a continuum, *i.e.*, the microviscosity of the solution is equivalent to the measured solvent viscosity; (3) the fluorescent oscillators are randomly placed on the molecule; (4) there is no internal rotation in the molecule; and (5) changes in the orientation of the oscillators can yield all possible directions. If the fluorescent lifetime and the volume of the molecule are constant, the Perrin law of isotropic depolarization by Brownian motion predicts a linear relationship between $(1/p + {}^1/_3)$ and T/η. According to the theory, equivalent changes in T/η can be produced by a change in either temperature or viscosity and the effect on the degree of polarization would be the same. Departure of this relationship from linearity at high values of T/η has been noted for several proteins (Young and Potts, 1963; Weber and Teale, 1965) and for synthetic polypeptides (Gill and Omenn, 1967; Gill *et al.*, 1970).

Direct measurements of fluorescent lifetimes, which are essential for the accurate calculation of the rotational relaxation time, have shown that the decay times of the dyes vary under different conditions and often display complex kinetics. These lifetimes have been measured by the single-photon method (Wahl and Lami, 1967; André-Frey and Wahl, 1969; Wahl, 1972), the flash-lamp technique (Ware and Baldwin, 1964; Hundley *et al.*, 1967; Chen *et al.*, 1967; Ladoulis and Gill, 1972), and the phase-shift technique (Bailey and Rollefson, 1953; Steiner and McAlister, 1957; Muller *et al.*, 1965; Spencer and Weber, 1969).

Previous work in our laboratory has focused on four different areas. First, studies with intramolecularly cross-linked synthetic polypeptides as models for the ordered spatial structure of proteins have shown that polarization techniques are particularly sensitive in detecting irreversible structural changes (Gill, 1965, 1969). Secondly, the properties of the various fluorescent dyes and the relationship of the dye and the macromolecule to which it is conjugated indicated that DNS[1] was the dye of choice for investigating structural changes as a function of pH and fluorescein was most useful for studying transitions as a function of temperature at a given pH (Gill *et al.*, 1967, 1970). Thirdly, the transition temperature, *i.e.*, the temperature at which the polarization behavior departed from the Perrin equation due to the onset of accelerated internal rotation, was established as a valid and sensitive measure of the stability of the internal structure of the molecule (Omenn and Gill, 1966). Lastly, the stability of

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Abbreviations used are: DNS, 1-dimethylaminonaphthalene-5-sulfonyl chloride; F, fluorescein; α, α helix; β, β structure and ρ, random coil. The nomenclature of the polypeptides is described in the Revised Recommendations (1971) of the Abbreviated Nomenclature for Synthetic Polypeptides (*J. Biol. Chem. 247*, 373 (1972)).

polypeptides in solution and in the solid state under a variety of conditions was investigated (Gill and Omenn, 1967; Gill *et al.*, 1972).

In the present paper, the use of fluorescence techniques for studying the structure and structural transitions of macromolecule–dye conjugates in solution was developed in order to have an independent, sensitive hydrodynamic method which could be used for studies with small amounts of biological macromolecules. The approach was to use these techniques with synthetic polypeptides which were representative of various types of proteins—positively charged, negatively charged, and polyampholitic—to investigate intramolecular transitions, stability of the internal structure of the molecule, and aggregation. The nature of the structural alterations was established independently by measurements of the intrinsic viscosity, the sedimentation constant, and a variety of spectroscopic parameters. The lifetimes of the dye–conjugates were measured under each of the experimental conditions in order to get the proper values for use in calculating the rotational relaxation times.

Methods

Fluorescence Measurements. Fluorescent spectroscopic measurements were performed in a Farrand Model MK-1 double-grating spectrofluorimeter. Fluorescence polarization measurements were made in a Brice-Phoenix light-scattering photometer equipped with an 85-W AH3 mercury lamp source and an RCA 1P28 photomultiplier detector (Omenn and Gill, 1966). For excitation of DNS, the unpolarized excitation beam passed through the instrument's band-pass filter, which had its peak transmission at 365 nm and had less than 1% transmission at 405 nm. For excitation of fluorescein, the band-pass filter had its peak transmission at 436 nm. The samples were dissolved in the appropriate solutions and placed in standard 1-cm^2 quartz cuvets immersed in a thermostatically-controlled, water-filled quartz bath. All of the experimental solutions and the water used in the temperature bath were filtered free of particles. The temperature was varied over the range 2–80°, and the appropriate values of the solvent viscosities were measured or were obtained from the International Critical Tables (1929) in order to calculate the value of T/η. The value of p_0 was measured in an 80% glycerol solution at 2°. Radiation emitted perpendicular to the incident beam was detected after passing successively through a saturated sodium nitrite solution in a 10 × 1 mm quartz cuvet, a glass cutoff filter and a movable polaroid filter. The emission filter cut off at 450 nm (Corning 3486) for DNS and at 520 nm (Corning 3387) for fluorescein. The sodium nitrite solution, which cut off at 395 nm, effectively absorbed scattered ultraviolet radiation and eliminated substantial polarization which could be caused by excitation of the intrinsic fluorescence of the glass emission filter.

The degree of polarization was calculated according to eq 1,

$$p = \frac{I_V - I_H}{I_V + I_H} \tag{1}$$

where I_V is the intensity of the vertically polarized light and I_H, the intensity of the horizontally polarized light, and the data were analyzed by the Perrin equation

$$(1/p + 1/3) = (1/p_0 + 1/3)\left(1 + \frac{RT\tau}{V\eta}\right) \tag{2}$$

where p_0 is the degree of polarization when T/η is zero, τ is the fluorescent lifetime, V is the molecular volume, and η is the viscosity of the solution. The rotational relaxation time at 5° was calculated from the slope of the graph of $(1/p + 1/3)$ *vs.* T/η using eq 3; 5° was chosen because it was below the transition temperatures of the polymers. The transition

$$\rho_h = 3\tau\left[\frac{(1/p_0 + 1/3)}{(1/p + 1/3) - (1/p_0 + 1/3)}\right] \tag{3}$$

temperature, T_T, was determined empirically from the graph as the point at which the experimental relationship deviated from linearity to follow an exponential curve. The theoretical relaxation time was obtained from eq 4, where V_0 is the theoretical volume of the molecule (see below). The standard

$$\rho_0 = 3\left(\frac{V_0\eta}{RT}\right) \tag{4}$$

deviation in the measurement of p was ±5% and of ρ_h[5], ±10%; changes in either of these parameters greater than 20% were significant. Below 15°, T_T had a standard deviation of ±10%, and changes greater than 20% were significant. Above 15°, the standard deviation in T_T was ±5%, and changes greater than 10% were significant (Gill and Omenn, 1967).

Several considerations influence the choice of the dye used in the polypeptide conjugates: extinction coefficient, lifetime, and effect of pH (Gill *et al.*, 1967; Ladoulis and Gill, 1972). Fluorescein has a higher molar extinction coefficient (3.4×10^4) than DNS (4.3×10^3); therefore, it is useful when working with small amounts of material, for example, when studying transitions as a function of temperature. The lifetime of DNS is constant from approximately pH 3 to 14 (16–17 nsec), and the degree of polarization of DNS and its conjugates is not affected by pH over this range. On the other hand, the lifetime of fluorescein is strongly dependent upon pH (3–7 nsec) and the degree of polarization of fluorescein and its conjugates is quite sensitive to the effects of acid and base. Thus, DNS was used for studying the effects of pH on intramolecular transitions.

Fluorescent Lifetime Measurements. The system for decay time measurement utilized a pulsed flash-lamp apparatus (Unilux, Inc., Woodside, N. Y.). The flash-lamp was specifically designed and operated to provide a repetitive pulse of very short decay time (2.0 nsec) with a relatively narrow ultraviolet energy distribution, and measurements were made at a pulse frequency of 400–600 cycles/sec. The solutions of the dye–conjugates were measured at 22° in 1 × 1 cm quartz cuvettes mounted in a temperature-controlled block. For measurements with fluorescein, no excitation filter was necessary because of the narrow wavelength distribution of the lamp. An emission cutoff filter (Corning 3387) was used to provide optical conditions similar to those employed in measurements of the degree of polarization. The photodetector was an ultraviolet-sensitive, high-gain photomultiplier (Amperex XP 1023) which was mounted normal to the path of excitation. In order to minimize the intrinsic phototube time dispersion and the pulse rise and fall times, the phototube was specially shielded, had a small aperture window, and had a dynode circuit designed for fast pulse rise times. The output from the detector went to a sampling oscilloscope, and the wave form was displayed for photographic recording. The wave form was simultaneously relayed to a 100-channel voltage integrator (Waveform Eductor TDH-9, Princeton Applied

Research, Inc., Princeton, N. J.) which averaged 20–30 sampled wave forms; the stored analog data were recorded on a strip chart and then converted to digital values. Time calibration was made during each measurement by comparison to a reference sine-wave signal which had a period of 1.00 nsec. The precision of replicate measurements was 10%. The fluorescent lifetimes were calculated by regression analysis of the data with a PDP-8 digital computor. All of the analyses were made with the assumption of first-order decay of fluorescence, although the actual decay was more complex; hence, the fluorescence lifetime measurements were mean values.

Other Spectroscopic Measurements. Ultraviolet measurements were made with a Cary Model 15 spectrophotometer; ir measurements, with a Beckman Model IR-10; and optical rotatory dispersion measurements, with a Perkin-Elmer spectropolarimeter. The values of the b_0 parameter from the Moffitt–Yang treatment of the optical rotatory dispersion date (Moffitt and Yang, 1956) and the wavelength and intensity of the far-ultraviolet absorbance maximum (Rosenheck and Doty, 1961) were used to follow the transition from the coil to the helix. Far-ultraviolet spectroscopy (Rosenheck and Doty, 1961) and the relative intensity of the infrared absorption band at 1615 cm^{-1} (Lenormant *et al.*, 1958; Doty *et al.*, 1958) were used to follow the transition from the coil to the β structure. The aggregation of the β structure was detected by far-ultraviolet spectroscopy.

Viscosity. Intrinsic viscosity measurements were made at 22° using an Ubbelhode viscometer. The stability of the polymers in acid and alkali was checked by assaying the effects of storage for 2–3 hr at low or high pH on the intrinsic viscosity of the polymer solutions. In no case was there any evidence of degradation as reflected in a change in intrinsic viscosity.

The size of the molecule in solution was estimated from the viscosity measurements using the Florey–Fox equation for free-draining coils (eq 5) (Florey and Fox, 1951) and using the length of the prolate ellipsoid model (eq 6) (Yang, 1961)

$$\langle r^2 \rangle^{1/2} = \left(\frac{[\eta]M}{\phi} \right)^{1/3} \tag{5}$$

where ϕ was chosen as 2.1×10^{21}, and

$$L = 6.82 \times 10^{-8}([\eta]M)^{1/3}(p^2/\nu)^{1/3} \tag{6}$$

where $(p^2/\nu)^{1/3}$ was obtained from standard tables (Yang, 1961) as a function of the axial ratio b/a (see below).

Molecular Weight and Sedimentation Constant Measurements. The molecular weights were determined in a Spinco model E analytical ultracentrifuge using the Yphantis approach to equilibrium technique (Yphantis, 1964), and the sedimentation constants were measured in the same instrument using the schlieren technique. The sedimentation constant was calculated according to eq 7, and it was corrected

$$s_T = \frac{\ln r/r_0}{\omega^2 t} \tag{7}$$

for changes in temperature, solvent viscosity, and pressure according to eq 8 (Svedberg and Pedersen, 1940), where η is

$$s_{20,w} = s_T \left[\frac{\eta_T^0}{\eta_{20}^0} \right] \left[\frac{\eta_T}{\eta_T^0} \right] \left[\frac{1 - \bar{v}_{20}\rho_{s,20}^0}{1 - \bar{v}_T \rho_{s,T}} \right] \tag{8}$$

the viscosity of the solvent, $\bar{v}$ is the partial specific volume of the polymer, and ρ_s is the density of the solvent. The value of $s^0_{20,w}$ was obtained by extrapolating the plot of $(1/s_{20,w})$ against concentration to zero concentration. The sedimentation constant was used to calculate the frictional ratio f/f_0 according to eq 9 and 10:

$$f = \frac{M(1 - \bar{v}\rho_s)}{N s_{20,w}^0} \tag{9}$$

where N is Avogadro's number, and

$$f_0 = 6\pi\eta \left(\frac{3M}{4\pi N} \right)^{1/3} \tag{10}$$

The frictional ratios were then used to obtain the axial ratio b/a from standard tables (Westley and Cohen, 1966).

The partial specific volume measurements used in the calculation of the molecular weights, the frictional ratio f/f_0, and the theoretical volume V_0 were measured in a density gradient column using bromobenzene and *m*-xylene (Linderstrom-Lang and Lanz, 1938; Cohn and Edsall, 1943; Bauer and Lewin, 1959). These values were not determined over a wide enough pH range to detect accurately any intramolecular transitions. The values of $\bar{v}$ did not vary very much with concentration (Gill *et al.*, 1972), and the measured values often differed considerably from those calculated using the $\bar{v}$ of the individual amino acid residues (Fajans and Johnson, 1942; Cohn and Edsall, 1943).

Molecular Volumes. The theoretical volumes were calculated according to eq 11, using the values of the $\bar{v}$ and M

$$V_0 = \bar{v}M \tag{11}$$

measured experimentally. The equivalent volumes of the prolate ellipsoid model were calculated from intrinsic viscosity and sedimentation measurements using eq 12, where a is the

$$V_e(b/a) = \frac{4}{3}\pi a^2 b \tag{12}$$

length of the semiminor axis and b is the length of the semimajor axis. The latter value was obtained from eq 6, and then the length of the semiminor axis was calculated from the axial ratio. The equivalent volumes obtained from polarization of fluorescence measurements were calculated from eq 13.

$$V_e(\rho) = \left(\frac{RT}{3\eta} \right) \rho_h^5 \tag{13}$$

Other. The synthetic polypeptides poly(lysine) (no. 16B, mol wt 51,000), poly($Glu^{97}Lys^3$) (no. 37, mol wt 25,000), and poly($Glu^{63}Lys^{37}$) (no. 8A, mol wt 48,000) were purchased from the Pilot Division of New England Nuclear Co., Watertown, Mass. The poly(vinylamine) (mol wt 53,000) was a gift of Professor Paul Doty. All of the polymers were purified and conjugated with DNS or fluorescein as described previously (Gill *et al.*, 1967). The buffers were made according to standard formulae, and all chemicals and solvents were analytical grade. Furthermore, they were all checked spectroscopically for impurities and for light scattering. The pH was measured with a Radiometer Model 22 pH meter.

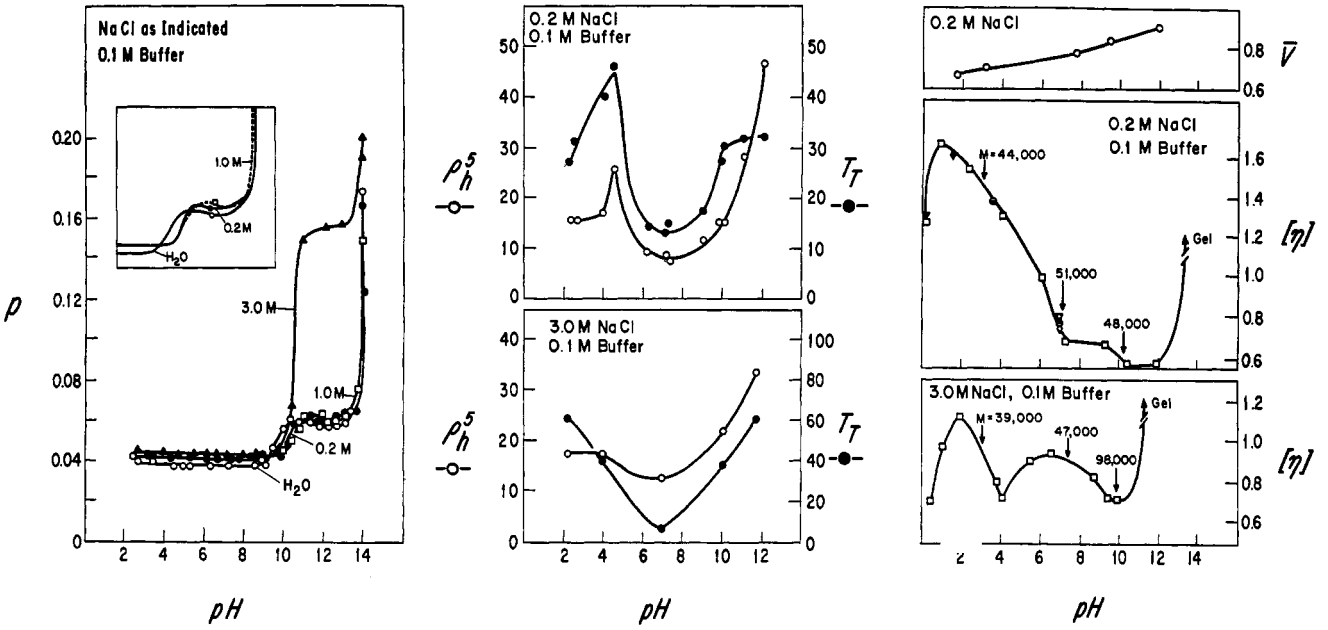

FIGURE 1: The polarization of fluorescence and the hydrodynamic properties of poly(lysine). The conditions under which each experiment was performed are indicated in the appropriate panel. In the viscosity studies, the data indicated by the triangles, diamonds, and half-colored circles represent those from samples studied in acid (solid symbols and bottom shaded circle) and after neutralization (open symbols and top shaded circle). The solutions used for polarization of fluorescence measurements contained 0.03–0.05 mg/ml, and those used for viscosity measurements initially had 15 mg/ml.

Results

The fluorescent lifetimes of the dye–macromolecule conjugates changed with pH and were different for various dye–polymer conjugates at the same pH (Table I). The lifetimes of each conjugate under the appropriate experimental conditions were obtained from direct measurements using a single exponential equation to approximate the decay process. This approximation was employed because: (1) it was found empirically that a single first-order exponential approximates the decay behavior quite well; (2) the longest component of the decay process, which is the major influence in the approximation, has a relatively high-quantum yield; and (3) the major component of the decay has the greatest effect on the measurement of the degree of polarization.

The structural transitions in poly(lysine) are shown in Figure 1, and the pH values at which the transitions were 50 and 90% completed are summarized in Table II. In water, the helix reached maximal rigidity (pH_{50} = 9.64) before all of the residues were in the helical conformation (pH_{50} = 10.0), whereas in 0.2 M NaCl + 0.1 M buffer, the development of rigidity in the molecule (pH_{50} = 10.30) paralleled the increase in helical content (pH_{50} = 10.2). The pH at which the coil-to-helix transition occurred increased as the NaCl concentration increased (Table II). In acid, both the rotational relaxation time and the transition temperature displayed a maximum, the magnitude of which decreased in 3.0 M NaCl + 0.1 M buffer (Figure 1).

The intrinsic viscosity of poly(lysine) decreased in the region where helical and coil forms coexisted (Doty *et al.*, 1957) and increased rapidly as the molecule became more helical. In acid, the intrinsic viscosity increased to a maximum around pH 1, and this effect was greatly suppressed in 3.0 M NaCl + 0.1 M buffer. This increase in intrinsic viscosity was seen only with poly(lysine) over 40,000–50,000 in molecular weight (Gill *et al.*, 1970).

When poly(lysine) was heated in solution at pH 10–11, it underwent a transition from the helical to the β conformation (Figure 2) (Gill and Omenn, 1967; Davidson and Fasman, 1967). The changes were reversible at pH 10.05, but not at pH 10.7. In water or in 0.2 M NaCl + 0.1 M carbonate, the

TABLE I: Fluorescent Lifetimes of Fluorescein–Polymer Conjugates.[a]

F–Poly(Lys)[b]		F–Poly-(vinylamine)[c]		F–Poly-($Glu^{63}Lys^{37}$)[d]	
pH	τ (nsec)	pH	τ (nsec)	pH	τ (nsec)
2.62	5.0	2.63	4.0	3.28	5.1
4.08	4.8	3.60	4.0	4.00	4.6
4.66	5.3	4.65	4.7	4.67	4.5
6.28	6.8	7.68	5.5	5.00	4.3
7.30	5.9	9.95	5.5	5.48	5.3
7.34	6.0	12.70	5.2	5.94	5.4
9.12	6.2			7.20	5.3
10.05	6.0			7.87	5.8
10.08	6.0			9.74	5.6
11.20	7.0			11.07	6.2
12.30	6.2			12.15	6.2
				12.70	6.0

[a] There were 2–4 dye molecules/polymer molecule. [b] 2 mg/ml in 0.2 M NaCl + 0.1 M buffer at 21°. [c] 2 mg/ml in 0.3 M NaCl adjusted to the appropriate pH at 22°. [d] 2 mg/ml in 0.2 M NaCl + 0.1 M buffer at 22°. The fluorescent lifetimes of the free dye were used in calculating the rotational relaxation times of poly($Glu^{97}Lys^{3}$), because the fluorescent intensity of the dye conjugate was too low for accurate measurement. The values of τ were 4.1, 4.4, 6.2, and 5.0 nsec at pH 4.00, 5.35, 9.58, and 11.00, respectively.

TABLE II: pH Values at Which the Coil–Helix Transition Was 50 and 90% Completed.

Solvent	Poly(Lys) (%)		Poly(Glu) (%)		Poly(Glu^{63}Lys^{37}) (%)	
	50	90	50[a]	90[a]	50	90
	Polarization of Fluorescence Measurements					
Water[b]	9.64	10.2	5.80	5.62	Glu: 6.12 Lys: (13.3)[c]	5.7
0.2 M NaCl + 0.1 M buffer	10.30	10.7	5.05	4.82	Glu: 5.00 Lys: (13.7)[c]	4.6
1.0 M NaCl + 0.1 M buffer	10.35	11.0				
3.0 M NaCl + 0.1 M buffer	10.72	10.9	d	d	Glu: 4.80 Lys: (13.6)[c]	4.2
	Optical Rotatory Dispersion Measurements[e]					
Water[b]	10.0	10.6	6.0	5.8	Glu: 5.2 Lys:	4.4
0.2 M NaCl	10.2	10.6	5.2	5.0	Glu: 5.2 Lys: 11.0	4.4 12.0
1.0 M NaCl	9.7	10.8				
3.0 M NaCl			4.6	4.4		

[a] Poly(Glu^{97}Lys^{3}) was used as a model for poly(glutamic acid) in the polarization of fluorescence studies. [b] The pH was adjusted with HCl or NaOH. [c] Approximate values. [d] None detected. [e] Studies of the coil–helix transition by a variety of optical methods are reviewed in Gill *et al.* (1970).

TABLE III: Assignment of the Structural Transitions in Poly(lysine) as Measured with Polarization of Fluorescence by Comparison to Data from other Spectroscopic Techniques.

Technique	Solvent	pH	Parameter	Type of Transition		
				α Helix–Coil	Coil–β Structure	β Structure–Aggregated β Structure
Polarization of fluorescence	0.2 M NaCl + 0.1 M carbonate	10.7	Temperature ± Std Dev (°C)	22 ± 1	48 ± 1	57 ± 6
			$(1/p + {}^1/_3)$ *vs.* T/η	Discontinuous transition	Discontinuous transition	Discontinuous transition
Optical rotatory dispersion	0.2 M NaCl + 0.1 M carbonate	10.7	Temperature[a] (°C)	20–25		
			b_0 value	−600 to −35		
Far-ultraviolet spectroscopy	0.3 M NaF[b]	10.7	Temperature[a] (°C)	20–25	40–46	49
			Wavelength (nm)	193–194	194–196	196–197
			Absorbance	0.70–1.05	1.05–1.80	1.80–2.00
Infrared spectroscopy	0.3 M NaCl[c]	10.7	Temperature		48°	
			Relative absorbance at 1615 cm^{-1}		0–1.0	

[a] Onset of the transition. [b] pH adjusted with NaOH. [c] pH adjusted with NaOD.

transition occurred *via* the coil form. In 3.0 M NaCl + 0.1 M carbonate, there was no clear transition prior to precipitation at pH 10.05, but there was at pH 10.7. The data upon which the assignments of the structural transitions were based are shown in the bottom part of Figure 2, and they are summarized in Table III.

The polarization of fluorescence and the hydrodynamic behavior of poly(vinylamine) were studied for comparison with poly(lysine) (Figure 3 and Table V). Poly(vinylamine) has a pK_a of 9.4, whereas poly(lysine) has a pK_a of 10.44, but because of nearest-neighbor interactions, the molecule is not completely charged until pH 4–5 in 0.1–1.0 M NaCl (Katchalsky *et al.*, 1954, 1957). It does not undergo a coil–helix transition, but, like poly(lysine), the intrinsic viscosity of the coil form displays a marked maximum in acid.

The nature of the coil–helix transition and the structure of the helix in poly(glutamic acid) differed considerably from those of poly(lysine) (Figure 4 and Table II). Just after the

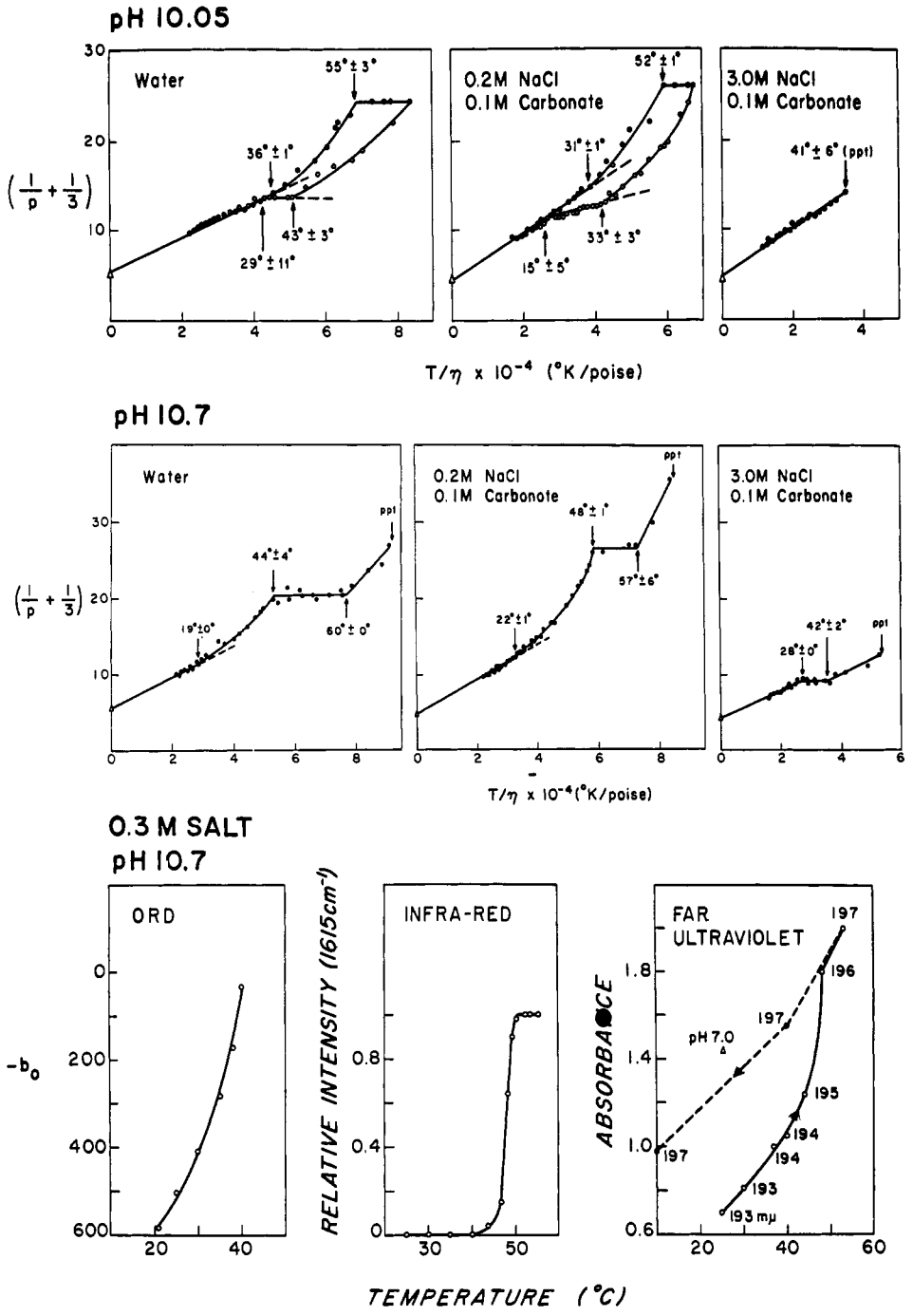

FIGURE 2: The effects of heating solutions of poly(lysine) containing 0.03–0.05 mg/ml at different pH's and at different salt concentrations. The transitions temperatures are given as the average ± std dev, and each value represents three to seven experiments. At pH 10.05 the transitions were completely reversible in water and in 0.2 M NaCl + 0.1 M carbonate; two full cycles were performed during each experiment. At pH 10.7 the experiments were reversible below *ca.* 50° in water and in 0.2 M NaCl + 0.1 M carbonate and below *ca.* 35° in 3.0 M NaCl + 0.1 M carbonate. In the bottom frame, the data establishing the nature of each transition in 0.3 M salt, pH 10.7, are shown. The interpretation of the coil-to-helix transition was based upon optical rotatory dispersion and far-ultraviolet spectroscopy; the assignment of the coil to β-structure transition was based upon far-ultraviolet spectroscopy and infrared spectroscopy; and the aggregation of the β structure was based upon far-ultraviolet spectroscopy, and, in more concentrated solutions, upon visible evidence of precipitation. The optical rotatory dispersion studies were performed in 0.2 M NaCl + 0.1 M carbonate at a concentration of 1.1 mg/ml. The infrared studies were performed in 0.3 M NaCl in D_2O, and the pH was adjusted with NaOD; the solution contained 15 mg/ml. The far-ultraviolet spectroscopy was performed in 0.3 M NaF, and the pH was adjusted with NaOH; the solution contained 0.032 mg/ml.

transition was complete, the poly(glutamic acid) helices aggregated. The aggregation occurred over a short pH interval (0.2–0.6) in water and in 0.2 M NaCl + 0.1 M buffer, but it occurred immediately in 3.0 M NaCl + 0.1 M buffer. The entire molecule became helical, and, at this point, its rotational relaxation time increased to the same value as that predicted for a completely rigid particle of the same size. The maximal rigidity of the helix occurred at approximately the same pH at which all the residues assumed the helical conformation, and the pH at which the transition occurred decreased as the NaCl concentration increased.

The intrinsic viscosity of poly(glutamic acid) decreased

TABLE IV: Structural Parameters of the Helix and the Coil.[a]

Polymer	Helix				Coil				ρ_h^5 (Helix)/ ρ_h^5 (Coil)
	pH	ρ_h^5 (nsec)	ρ_h^5/ρ_0	T_T (°C)	pH	ρ_h^5 (nsec)	ρ_h^5/ρ_0	T_T (°C)	
Poly(Lys)	11.3	29	0.33	32	7.3	8	0.10	14	3.6
Poly($Glu^{97}Lys^3$)[b]	4.7	30	1.00	16	7.0	2	0.06	Ca.0	15.0
Poly($Glu^{63}Lys^{37}$)	4.2[c]	65	1.04	47	7.2	12	0.18	10	5.4

[a] Solvent is 0.2 M NaCl + 0.1 M buffer (citrate, phosphate, carbonate). [b] Used as a model for poly(glutamic acid). [c] Glutamic acid helix.

markedly in the region of mixed helices and coils and then increased when the molecules became completely helical and aggregated. The behavior in 3.0 M NaCl + 0.1 M buffer was more complex. In alkali, the intrinsic viscosity displayed a maximum which was abolished by 3.0 M NaCl + 0.1 M buffer.

The behavior of the copolymer of glutamic acid and lysine was dominated by the properties of the glutamic acid residues (Figure 5 and Table II). There was a clearly defined coil–helix transition for the glutamic acid residues, but only a vestigial one for the lysine residues. In water, the maximal rigidity occurred before all the residues went into the helical conformation, but in salt solution, the changes in rigidity and in conformation closely paralleled each other. The pH of the coil–helix transition decreased with increasing concentrations of NaCl (Table II). When all of the glutamic acid residues were in the helical conformation, the rotational relaxation time was the same as that predicted for a rigid particle of the same size.

Some characteristics of the helical and the coil forms of the polymers are summarized in Table IV. The pH value for the helix is that at which the coil–helix transition was complete, and neutral pH was selected for the coil form. In all cases, the rotational relaxation time was longer for the helix than for the coil, indicating that the helix was the more rigid structure. Since the transition temperature was higher for the helical form, a larger amount of thermal energy was needed to disrupt the highly organized helical structure than to induce rotation in the coil. The relatively low-transition temperature for both the helical and coil forms of poly($Glu^{97}Lys^3$) may be due, at least in part, to the small size of the molecule.

The hydrodynamic properties of the polymers are summarized in Table V. The root-mean-square end-to-end distance calculated by the Florey–Fox equation (eq 5) was smaller than the length calculated for the prolate ellipsoid model (eq 6). The molecular volume calculated from hydrodynamic measurements, $V_e(b/a)$, was considerably larger than the theoretical volume, V_0. In contrast, the volume calculated from polarization of fluorescence measurements, $V_e(\rho_h)$, was smaller, and the ratio of the measured to calculated rotational relaxation times, ρ_h^5/ρ_0, was less than 1, except when the polymers aggregated.

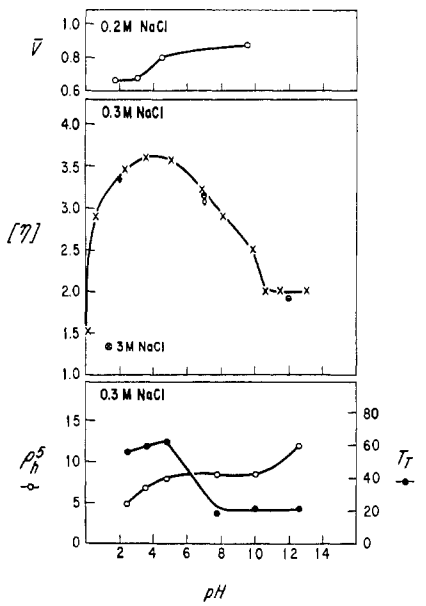

FIGURE 3: The polarization of fluorescence and hydrodynamic properties of poly(vinylamine). The concentrations and the symbols are the same as in Figure 1. The studies were done in 0.3 M NaCl, and the pH was adjusted with HCl or with NaOH. The viscosity was greatly decreased in 3 M NaCl.

Discussion

The pH at which the coil–helix transition occurred increased with increasing concentration of NaCl for the lysine helix and decreased for the glutamic acid helix (Table II). This shift was probably due to salt stabilization of the coil form: the pH had to be increased or decreased in order to titrate the amino groups of lysine or the carboxylate groups of glutamic acid, respectively, so that the uncharged polypeptide chain could then assume the helical conformation. The aggregation that followed the completion of helix formation was shown dramatically by polarization of fluorescence measurements, which were more sensitive and convenient for such studies than optical rotatory dispersion or light scattering (Schuster *et al.*, 1966; Spach and Constantin, 1968; Jennings *et al.*, 1968).

There are similarities in the behavior of the helical forms of poly(lysine) (Figure 1) and of poly($Glu^{63}Lys^{37}$) (Figure 5) which may be ascribed to the presence of interrrupted helices, *i.e.*, helical segments interspersed with short regions of coil. First, both molecules attained maximal rigidity when the majority of the residues that were capable of forming the helix had done so: the addition of a few more residues did not significantly alter the hydrodynamic behavior of the molecule, but it did change the optical rotatory behavior (Table II). Secondly, the addition of salt delayed the onset of maximal rigidity so that it coincided with the entrance of the residues into the helical conformation. The explanation for this finding probably lies in the influence of the salt on the charged, nonhelical portions of the molecule—a localized polyelectrolyte

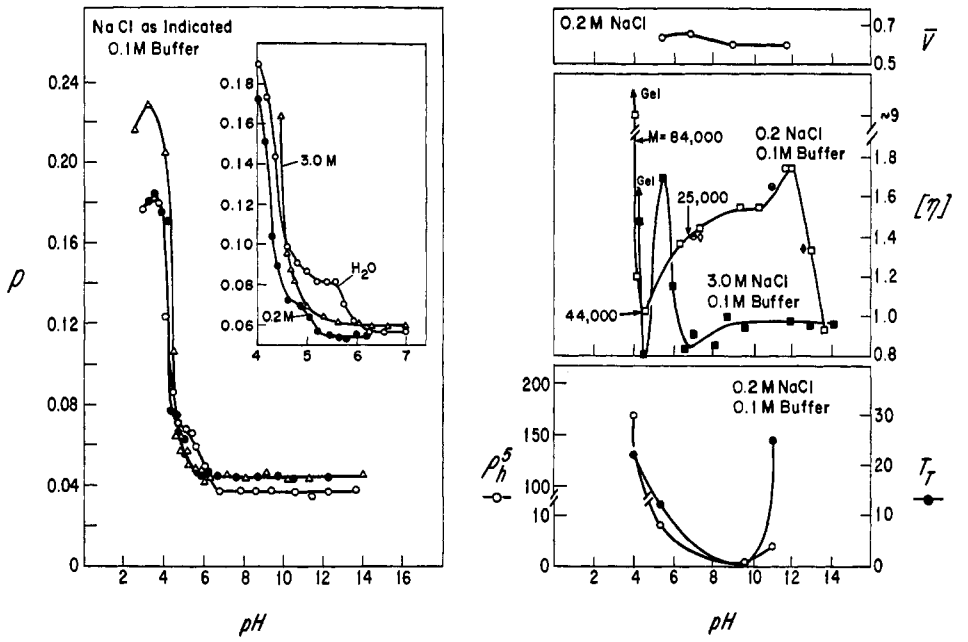

FIGURE 4: The polarization of fluorescence and hydrodynamic properties of poly(Glu[97]Lys[3]), which was used as a model for poly(glutamic acid). The concentrations of the solutions and the symbols are the same as in Figure 1. The conditions under which each experiment was performed are indicated in the appropriate panel.

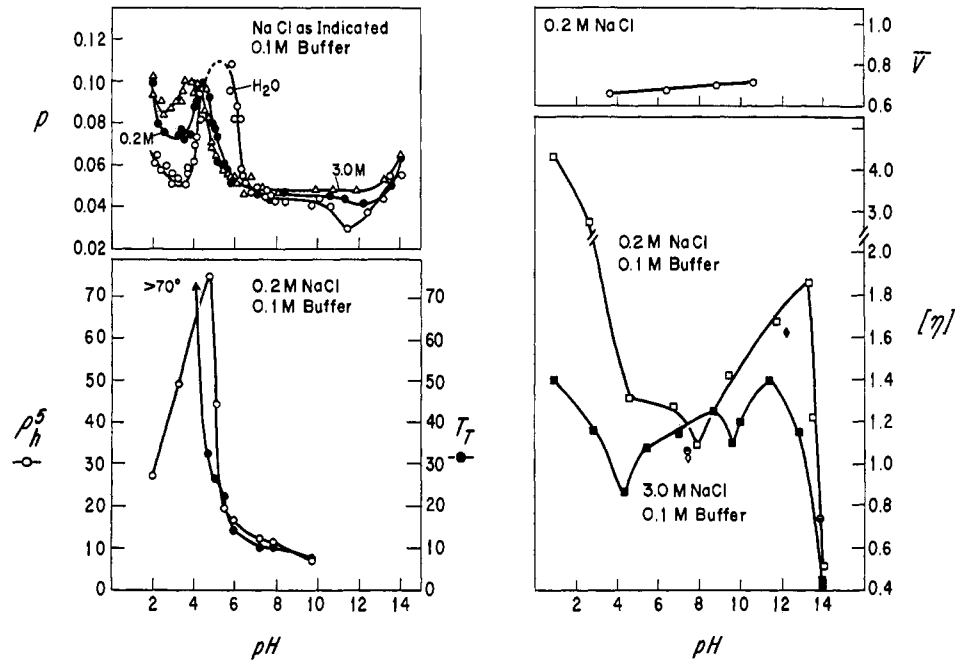

FIGURE 5: The polarization of fluorescence and hydrodynamic properties of poly(Glu[68]Lys[37]). The concentrations of the solutions and the symbols are the same as in Figure 1 except that the studies on the reversibility of the viscosity were carried out in alkali. The conditions under which each experiment was performed are indicated in the appropriate panel.

effect. In water, the rigidity of the molecule is due both to the helical segments and to repulsion between like charges in the coil portions of the molecule. In salt solutions, the electrostatic repulsions in the coil regions would be partially neutralized, thereby causing a decrease in rigidity. This mechanism would leave the helical segments as the source of rigidity; hence, the development of rigidity would parallel the amount of helix in the molecule. Thirdly, there was a decrease in polarization between the completion of helix formation and aggregation, and the onset of aggregation occurred long after the completion of the coil–helix transition: approximately pH 13.5 for poly(lysine) and approximately pH 2 for poly(Glu[68]-Lys[37]). These effects were also due to the persistence of short segments of coil after the maximal amount of helix had formed.

Applequist and Doty (1962) provided evidence for the presence of interrupted helices in poly(lysine) during the transition from the coil to the helix from several other types of measure-

TABLE V: Structural Parameters of Synthetic Polymers Calculated from Hydrodynamic and from Polarization of Fluorescence Measurements in 0.2 M NaCl + 0.1 M Buffer Solutions.

pH	$\langle r^2 \rangle^{1/2}$ (Å)	$s^0_{20,w}$	f/f_0[b]	b/a[b]	$L/2 = b$[a] (Å)	a (Å)	Vol × 10^{-4} Å^3 $V_0(\bar{v}M)$	$V_e(b/a)^{a,b}$	$V_e(\rho)$[c]	ρ_h^5/ρ_0
					Poly(Lys)					
0.50	339	1.75	2.990	52.0	359	7	3.4	7		
3.05	328	2.64	1.634	11.8	287	24	3.5	69	1.5	0.25
7.25	257	2.91	1.175	3.8	166	44	3.9	135	1.1	0.16
10.28	244	2.28	1.046	2.0	119	60	4.3	179	1.9	0.27
12.61		1.16	1.304	5.8			4.7		(3.4)[d]	(0.43)[d]
					Poly(vinylamine)[e]					
1.05	428	5.01	1.105	2.9	291	100	3.5	1220		
2.40	443	4.43	1.093	2.7	247	91	3.5	857	0.4	0.07
4.55	450	2.89	1.112	3.0	264	88	4.2	856	0.7	0.10
9.21	406	2.30	1.036	1.8	177	98	4.6	712	0.7	0.09
12.67	370	1.50	1.478	8.4	302	36	4.9	164	1.0	0.13
					Poly(Glu^{97}Lys^{3})					
4.55	230	4.20	0.799	5.5[f]			1.5		7.8	3.1
6.90	256	1.24	2.667	40.0	264	7	1.6	5	0.4	0.15
12.10	274	3.25	1.173	3.8	177	46	1.8	157	0.7	0.23
12.67	260	3.18	1.276	5.4	189	35	1.8	97	(0.9)[d]	(0.30)[d]
					Poly(Glu^{63}Lys^{37})					
3.05	354	3.68	1.286	5.6	260	46	3.1	230	3.8	0.73
5.95	308	3.17	1.515	9.5	258	27	3.2	79	1.3	0.25
11.11	333	2.22	1.842	16.3	312	19	3.4	47	0.5	0.10

[a] Function of the intrinsic viscosity. [b] Function of the sedimentation constant. [c] At 5°. [d] Calculated from extrapolated data. [e] The sedimentation constants were measured in 0.2 M NaCl, and the intrinsic viscosities were measured in 0.3 M NaCl. The pH was adjusted with HCl or NaOH in all cases. [f] Oblate ellipsoid.

ments. Their arguments were based upon: (1) thermodynamic studies of the equilibrium between the helical and coil forms, including the breadth of the transition; (2) the loss of rigidity as measured by flow birefringence when the molecule was 90% helical; and (3) the presence of a minimum in the intrinsic viscosity and a maximum in the sedimentation constant in the transition region. They calculated that the average length of the helical segments at pH 9.98–10.10 was 13–14 residues.

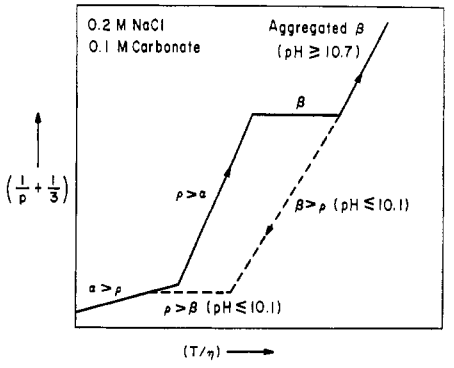

FIGURE 6: Schematic representation of the transitions that occur in poly(lysine) upon heating in alkaline solution. The transitions are reversible at pH ≤10.1, and they are irreversible at pH ≥10.7. The symbols represent the various conformations: α, α helix; β, β structure; and ρ, random coil. The predominant conformation in each region is indicated.

When poly(lysine) was heated in solution at pH 10–11, it underwent transitions from the coil to the helical to the β conformations. These transitions were reversible at pH ≤10.1 and were irreversible above pH 10.7. Figure 6 shows an interpretive summary of the various transitions, which is based on the experimental data in Figure 2 and in Table III. Between pH 10 and 11, the coil–helix transition is only partially completed, so that the molecule consists of mixed helices and coils. Upon heating, the helical segments melt out, and then the coil form undergoes transition to the β conformation. At pH 10.7, further heating causes the β structure to aggregate and to precipitate. At pH 10.05, however, the β structure can still revert to the coil form and then to the original mixture of helices and coils. The fact that the transition between the helix and the β conformation occurs through the coil form may explain the different effects seen at pH 10.05 and 10.7 in 3.0 M NaCl + 0.1 M carbonate (Figure 2). At the lower pH, there is probably enough coil present for the β conformation to form directly and then to aggregate immediately. In contrast, at the higher pH, there is less coil form, so some helical structure must melt out before the transition to the β structure can occur, and distinct transitions can be identified.

The charged coil forms of the polymers all displayed a polyelectrolyte effect at the extremes of pH: in acid with poly(lysine) and poly(vinylamine) and in alkali with poly(glutamic acid) and poly(Glu^{63}Lys^{37}). This effect was characterized by a

maximum in the intrinsic viscosity which was decreased by high salt (Figures 1, 3, and 4). Similar behavior was observed with progressive dilution of polyelectrolytes in water or in very dilute salt solutions (Fuoss and Strauss, 1948, 1949; Fuoss, 1951; Eisenberg and Pouyet, 1954) and with serum albumin in acid (Yang and Foster, 1954; Tanford *et al.*, 1955).

Finally, each of the various methods was uniquely useful for studying a particular molecular property. The change in the degree of polarization was the most sensitive indicator of the intramolecular coil to helix and coil to β structure transitions and of intermolecular aggregation. The rotational relaxational time (ρ_h) reflected the overall rigidity of the molecule, and, by comparison to the theoretical value (ρ_0), it showed whether the molecule contained many or few rotational units ($\rho_h/\rho_0 < 1$) or whether it had aggregated ($\rho_h/\rho_0 > 1$). The transition temperature, T_T, was a sensitive measure of the stability of the internal structure of the molecule. The volume of the molecule calculated from the intrinsic viscosity and the sedimentation coefficient, $V_e(b/a)$, was generally much greater than the theoretical volume, V_0, and this finding indicates that the molecule was hydrated. In contrast, the volume calculated from the rotational relaxation time, $V_e(\rho_h)$, was generally less than the theoretical volume, since the polymers have a considerable amount of internal rotation. Only when the molecule was completely helical or when the helices aggregated did $V_e(\rho_h)$ equal or surpass the theoretical volume.

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