THE EFFECTS OF SOLVENT ENVIRONMENT ON THE OPTICAL ROTATORY DISPERSION PARAMETERS OF POLYPEPTIDES

II. Studies on Poly-L-Glutamic Acid

JOSEPH Y. CASSIM *and* EDWIN W. TAYLOR
From the Department of Biophysics, University of Chicago, Chicago

ABSTRACT The Moffitt b_0 parameter of poly-L-glutamic acid in the presumed helical state varied with solvent composition, ranging in magnitude from less than 600° in aqueous solution to 800° in methanol. b_0 was also dependent on temperature throughout the excessable temperature range. The value in aqueous solution is at least 100° smaller than the values for a number of polypeptides in organic solvents, when compared at the same refractive index. Therefore the optical rotatory dispersion data do not provide evidence that the molecule is completely helical in aqueous solution. Since other types of evidence for helical content are not sufficient to establish that PLGA is a complete helix, the helical content of proteins and polypeptides determined by rotatory dispersion measurements should be regarded as uncertain by about 20 per cent.

INTRODUCTION

In a previous communication (Cassim and Taylor, 1965*a*) we have shown that the "helical content" parameter b_0 of the Moffitt equation[1] (Moffitt, 1956) for poly-γ-benzyl-L-glutamate (PBLG) is not independent of solvent but varies linearly with the refractive index of the solvent according to the equation $-b_0 = 1701 - 730.3\ n_s$. When the previously published $-b_0$ values for helical polypeptides were compared at the same solvent index (1.333), it was noted that the values in organic solvents were about 100° higher than those reported for aqueous solutions. The question to be considered is whether this difference is due to the inability of polypeptides to form complete helices in aqueous solution, or to the dependence of b_0 on factors other than helical content and solvent index.

In order to clarify this problem we have investigated the dependence of the b_0

---

[1] $[m']_\lambda = [3/(n_s^2 + 2)](M_0/100)[\alpha]_\lambda = a_0\lambda_0^2/(\lambda^2 - \lambda_0^2) + b_0\lambda_0^4/(\lambda^2 - \lambda_0^2)^2$ where $[\alpha]_\lambda$ is the specific rotation at wavelength λ, n_s, the refractive index of the solvent, M_0, the mean residue molecular weight, a_0 and b_0 are empirical constants having the same dimensions as $[\alpha]_\lambda$, and λ_0 is the characteristic wavelength which is selected for best fit of the data to a two term equation.

parameter for poly-L-glutamic acid (PLGA) on solvent, pH and temperature, and attempted to correlate the optical rotatory dispersion behavior with other methods of estimating helical content (far UV hypochromicity, intrinsic viscosity, and hydrogen exchange). The dependence of b_0 on factors other than helical content requires caution in the use of the b_0 value but the simplest interpretation of the available evidence is consistent with a helical content of 80 per cent for PLGA in aqueous solution of low pH at room temperature. However, whether the variation in b_0 is attributed to an incomplete helix or to the effect of other parameters, the helical contents previously assigned to polypeptides and proteins in aqueous solution must be regarded as uncertain by about 10 to 20 per cent.

MATERIALS AND METHODS

(*a*) *Polypeptides.* The polypeptides used in this investigation were obtained from Pilot Chemicals, Inc. (Watertown, Massachusetts). Poly-L-glutamic acid (PLGA) with DP's of 700 and 830 was dialyzed exhaustively against water at pH 7 and lyophilized. Poly-L-lysine hydrobrobromide (PLL) with DP of 550 was utilized without further purification.

(*b*) *Solvents.* The solvents were all of reagent grade purity. Some were further purified by distillation just prior to use. Double distilled water was used throughout. The solvents utilized in this investigation were: dimethylformamide, dimethylsulfoxide, *p*-dioxane, ethanol, formamide, glycerol, isopropanol, methanol, propanol, and tetramethylurea.

(*c*) *Solutions.* Stock solutions were prepared in double distilled water and clarified by ultracentrifugation. Concentrations were determined by the micro-Kjeldahl method (Ma and Zuazaga, 1942). Solutions were prepared by volumetric dilutions of the stock solution. In the case of pure organic solvents, solutions were prepared by dissolving a known weight of dried polypeptide in a known volume of solvent. A correction was made for the bound water content of the dried polypeptide which was determined by the micro-Kjeldahl method. pH was adjusted in all cases by the addition of either HCl or NaOH.

(*d*) *Optical Rotatory Dispersion.* The equipment utilized in this study has been described in detail elsewhere (Cassim and Taylor, 1965*a*). The wavelength range investigated was 350 to 578 mμ. The rotatory dispersion data were analyzed by means of the Moffitt equation, with the value of λ_0 taken as 212 mμ. The solvent refractive index at the sodium D line was used throughout. No correction was made for dispersion of the refractive index, since trial calculations indicated that this source of error was negligible in the wavelength range utilized. Repeated independent measurements indicated an uncertainty of about ±10° in the calculated b_0 values.

(*e*) *pH.* The pH of the solutions was measured with a Radiometer pH meter (type pHm 4h) (Radiometer, Copenhagen, Denmark). The meter was calibrated with standard buffer solutions. pH measurements in mixed solvents and in organic solvents are uncorrected values. All pH measurements were made at room temperature.

(*f*) *Viscosity, Flow Birefringence, and Index of Refraction.* The methods used have previously been described in detail (Cassim and Taylor, 1965*b*).

(*g*) *Far Ultraviolet Absorption Spectra.* The absorption spectra of PLGA, spanning the wavelength range from 2600 to 1850 A, were obtained with the Cary recording spectrophotometer model 14 (Applied Physics Corp., Monrovia, California). Dry nitrogen was passed through the cell chamber to improve the high frequency sensitivity. Measurements were made in jacketed quartz cells with 5 mm pathlengths. For low temperature measurements ice water was circulated through the cell jacket.

The pH of the polypeptide solutions was adjusted with HCl or NaOH. Since at these low wavelengths the absorption of the hydroxyl ion is significant (Imahori and Tanaka, 1959; Rosenheck and Doty, 1961), for high pH measurements the pH of both the polypeptide solution and the reference solution was adjusted to the same value. For low pH measurements the same amount of HCl was added to both solutions.

(*h*) *Hydrogen-Exchange Determinations.* The hydrogen-exchange kinetics of PLGA solutions were studied by utilizing a tritium-Sephadex column method developed by Englander (1963). Coarse grade G-25 Sephadex and tritiated water (activity 25 mc/gm) were obtained from A. B. Pharmacia (Pharmacia Fine Chemicals, New Market, New Jersey) and New England Nuclear Corporation, Boston, Massachusetts, respectively. A Packard tri-carb automatic liquid scintillation spectrometer (Series 314 EX) Packard Instrument Co., LaGrange, Illinois, was used for counting. Aliquots of the column effluent and initial tritiated solutions were counted in diatol scintillation mixture (Herberg, 1960). Polypeptide concentrations were determined by measurements of optical rotation at 365 mμ which had previously been calibrated by the micro-Kjedahl method.

To achieve a good separation of the tritiated PLGA from the tritiated solvent, it was necessary to remove the low molecular weight fraction from the PLGA samples. PLGA solutions, 3 per cent, in 2.0 M NaCl, pH 7.5 were passed over Sephadex columns, and effluent from the ascending portion of the PLGA region was pooled, dialyzed exhaustively against water and lyophilized. For the exchange experiments, 1 per cent solutions of the lyophilized PLGA were prepared in the appropriate solvents at pH 7.5. Tritiated water (1 to 100 parts by volume) was added and solutions were allowed to equilibrate overnight. Sufficient HCl was then added to adjust the pH to 4.4. The sample was passed over a Sephadex column, and effluent fractions were collected and measured for polypeptide concentration and tritium activity. The kinetics of the loss of tritium by the polypeptide were studied by allowing the tritiated polypeptide to incubate within the column with tritium-free solvent for given periods of time. Exchangeable hydrogen atoms per residue were determined from the ratio A/A_0 where A_0 is the specific activity of the initial tritiated solution (CPS per mole of exchangeable hydrogen in the solvent) and A is the specific activity of the column effluent (CPS per mole residue of polypeptide).

RESULTS

(*a*) *Optical Rotatory Dispersion in Aqueous-Organic Solutions.* The optical rotatory dispersion of poly-L-glutamic acid (PLGA) was studied in methanol-aqueous solutions. In this solvent system the added complication of refractive index variation with solvent composition was avoided, because of the near identity of the refractive indices of water and methanol. The rotatory dispersion was measured in a number of methanol-aqueous solutions at pH 4.4, varying in methanol concentration from 0 to 100 per cent. The use of pH 4.4 is based on the current concept that PLGA assumes a helical configuration in aqueous solution below pH 5. (Urnes and Doty, 1961). The b_0 values were obtained by a graphical solution of the Moffitt equation in the manner suggested by Moffitt and Yang (1956). $-b_0$ increased monotonically with methanol concentration as shown in Fig. 1. The $-b_0$ value rose rapidly with methanol concentration up to about 50 per cent, while from 50 to 100 per cent the b_0 change was only about 14°. Since the uncertainty in b_0 is about ±10°, this change is hardly significant. The b_0 values leveled off at about $-760°$.

The reversibility of this b_0 change with methanol addition was investigated. Concentrated solutions of PLGA were prepared in 75 per cent methanol and allowed to remain overnight. The solutions were then systematically diluted with water and the rotatory dispersion was measured. The results (also shown in Fig. 1) indicated that this effect is completely reversible.

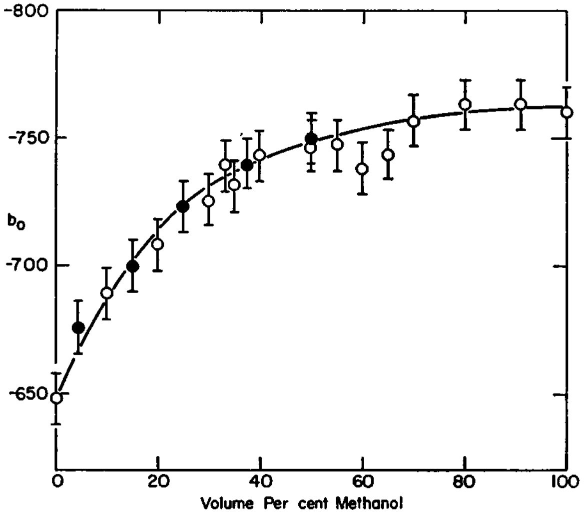

FIGURE 1 Dependence of b_0 for poly-L-glutamic acid on solvent composition for aqueous-methanol mixtures, pH 4.4, 20°C. (○) dilution of aqueous solution of PLGA with methanol (●) dilution of 75 percent methanol solution of PLGA with water.

To determine if similar results could be obtained with other polypeptides, the rotatory dispersion of poly-L-lysine (PLL) was investigated under similar conditions. In this case the investigation was carried out at pH 11.6, since Applequist and Doty (1962) have shown that PLL in aqueous solution appears to be helical only at pH values above 11. The dependence of the b_0 parameter on solvent composition is shown in Fig. 2. The general shape of the curve was similar to that for PLGA, although the plateau value of $-755°$ was reached at a higher methanol concentration (80 per cent). It should be noted that the $-b_0$ values of PLGA and PLL in methanol correspond to the range of values of PBLG extrapolated to the same index.

The rotatory dispersion experiments were extended to other aqueous-organic mixtures. The results are shown in Tables I and II for PLGA at pH 4.4 and PLL at

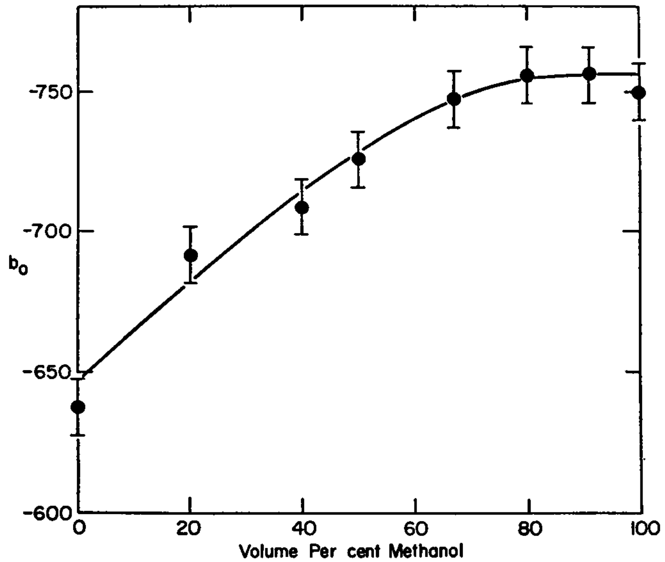

FIGURE 2 Dependence of b_o for poly-L-lysine on solvent composition, aqueous-methanol mixtures, pH 11.6, 20°C.

pH 11.6, respectively. In most solvent mixtures the $-b_0$ values are significantly greater than in pure aqueous solution without correcting for solvent index differences. However if it is assumed that these polypeptides are completely helical and show the same dependence of b_0 on refractive index as PBLG, the results can be compared at the same index ($b_0^{1.333}$). The corrected b_0 values show an even larger increase and the slight decrease in value at higher concentrations in dimethylformamide, glycerol, and ethanol disappears.

(*b*) *Hydrodynamic Behavior in Aqueous-Organic Solutions.* The intrinsic viscosity of PLGA was studied in aqueous-methanol mixtures at pH 4.4. Measurements were made in the absence of salt to avoid precipitation of salt in organic solvents. η_{sp}/c plots were linear in the concentration range studied (0.03 to 0.3 gm/dl). A plot of the intrinsic viscosity and Huggins' k' (the concentration dependence of the reduced specific viscosity) as a function of solvent composition is shown in Fig. 3. The parameter k' is defined by the equation $\eta_{sp}/c = [\eta] + k'[\eta]^2c$. Although k' is basically an empirical parameter, it is believed to be a function of the intermolecular interaction and can possibly serve as a measure of molecular aggregation. It is noteworthy that the intrinsic viscosity curve is very similar to the b_0 curve, rising very rapidly with methanol concentration up to 50 per cent and then leveling off at higher concentrations. On the other hand, the k' curve remains almost independent of methanol concentration up to 25 per cent. The maximum value of k' observed was 0.35. At 25 per cent methanol where the k' change is still negligible, the extent of the change in intrinsic viscosity and b_0 is about two thirds of the total change.

TABLE I

THE EFFECTS OF ORGANIC SOLVENTS ON $-b_0$ VALUE FOR PLGA, pH 4.4, 20°C

Solvent added	Volume per cent	Solvent refractive index n_s	$-b_0$	$-b_0^{1.333*}$
None	—	1.333	648	648
Dimethylformamide	50	1.393	676	720
	75	1.416	668	729
Tetramethylurea	26.3	1.362	710	731
	41.7	1.392	722	765
Glycerol	22	1.364	658	681
	42	1.391	672	715
	81.5	1.447	664	748
Dioxane	33.3	1.366	650	674
	83.3	1.416	652	713
Ethanol	50	1.359	752	771
	83.3	1.366	746	770
Isopropanol	50	1.367	730	755
Dimethylsulfoxide	70	1.432	635	708
Methanol	100	1.329	760	757
Formamide	100	1.445	578	660

Solvent index values refer to sodium D line.

Reference solvent, water.

* $-b_0^{1.333}$ was calculated on the assumption that the polypeptide is completely helical and the index dependence is the same as for PBLG, $-b_0 = 1701 - 730.3\ n_s$ (Cassim and Taylor, 1965*a*).

TABLE II

THE EFFECTS OF ORGANIC SOLVENTS ON $-b_0$ VALUE FOR PLL, pH 11.6, 20°C

Solvent added	Volume per cent	Solvent refractive index n_s	$-b_0$	$-b_0^{1.333*}$
None	—	1.333	638	638
Ethanol	50	1.359	700	719
	80	1.366	720	744
Propanol	50	1.366	683	707
Tetramethylurea	50	1.402	648	699
Methanol	100	1.329	750	747

Reference solvent, water.

* $-b_0^{1.333}$ was calculated on the assumption that the polypeptide is completely helical and the index dependence is the same as for PBLG, $-b_0 = 1701 - 730.3\ n_s$ (Cassim and Taylor, 1965*a*). Solvent index values refer to sodium D line.

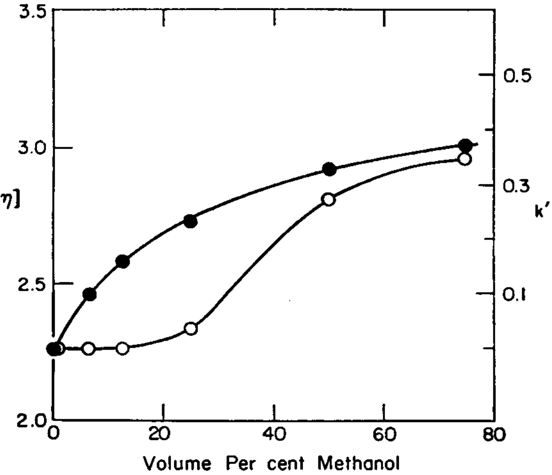

FIGURE 3 Dependence of the intrinsic viscosity (●) and Huggins' k′ (○) for poly-L-glutamic acid on solvent composition, aqueous-methanol mixtures, pH 4.4, 20°C.

The intrinsic viscosity of PLGA was also studied in several other aqueous-organic mixtures (Table III). In every case there was a significant increase in the intrinsic viscosity upon the addition of organic solvents. No correlation was found between the changes in intrinsic viscosity and k', but an increase in b_0 was always associated with an increase in viscosity.

The molecular length of PLGA was calculated from the rotatory diffusion constant measured by flow birefringence in various methanol-aqueous solutions in a manner previously described in detail (Cassim and Taylor, 1965*b*). The results indicated an increase of the apparent molecular length with methanol concentration which paralleled the increase in intrinsic viscosity.

TABLE III

EFFECTS OF ORGANIC SOLVENTS ON THE HYDRODYNAMIC PROPERTIES OF PLGA, pH 4.4, 20°C

Solvent added	Volume per cent	Intrinsic viscosity [η]	Huggins' constant k'
None	—	2.26	0.0
Dimethylformamide	50	2.80	0.233
	75	2.73	0.012
Dioxane	33.3	2.46	0.451
Isopropanol	50	2.89	0.042
Ethanol	50	2.94	0.435

Reference solvent, water.

The degree of polymerization of the sample was stated to be 830 by the manufacturer (Pilot Chemicals, Inc.)

(*c*) *Stability of PLGA Solutions.* In the course of these rotatory dispersion and hydrodynamic experiments, it was noted that there was a marked variation in the stability of PLGA solutions below pH 5 for the various solvents used. Significant precipitation occurred in aqueous solutions which were allowed to remain at low pH for a few days. The tendency to precipitate was greatly reduced by the addition of methanol up to 90 per cent by volume. The addition of other organic solvents in the quantities listed in Table I, also resulted in stable solutions which remained optically transparent over long periods of time.

(*d*) *Effects of Temperature on Rotatory Dispersion.* The Gradual temperature change of the specific rotation and rotatory dispersion of PLGA and PLL in solution have previously been observed and interpreted in terms of a helix-coil transition with the coil configuration being favored at higher temperatures (Doty, Wada, Yang, and Blout, 1957; Doty, 1959; Applequist and Doty, 1962; Fasman, Lindblow, and Bodenheimer, 1964). In view of this interpretation, if a polypeptide is considered to be completely helical at a given temperature and pH, the b_0 values should be constant in the vicinity of this temperature. The rotatory dispersion of PLGA was measured in aqueous solution at pH 4.9 as a function of temperature over the range 2–50° C. The b_0 parameter decreased monotonically with tempreature over this whole range with no indications of a plateau in the 20–25°C region (Fig. 4). Our results are in good agreement with the data of Fasman *et al.* (1964) over the temperature range common to both sets of measurements (20–50°C). Even in the vicinity of 2°C there was no indication that $-b_0$ had reached a maximum value. For comparison, the rotatory dispersion in 70 per cent methanol was also examined as a function of temperature. In contrast to the results in aqueous solutions, the b_0 values were essentially independent of temperature (Fig. 4, upper curve). The curve drawn was

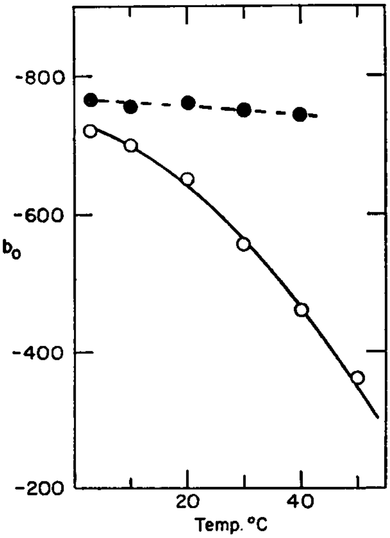

FIGURE 4 Temperature dependence of b_0 for poly-L-glutamic acid in aqueous solutions (○) and in 70 per cent methanol solutions (●) at pH 4.9. pH values refer to room temperature (22°C).

obtained by fitting the data to a straight line by least-squares analysis. Since there is an experimental uncertainty of ±10° in the b_0 values, the small slope shown in Fig. 4 may not be statistically significant.

(*e*) *Effects of Temperature on the pH-induced Helix-Coil Transition.* pH-induced transitions of PLGA and PLL in solutions have been investigated previously at room temperature (Doty *et al.*, 1957; Idelson and Blout, 1958; Wada, 1960; Miyazawa and Blout, 1962; Goldstein and Katchalski, 1960; Applequist and Doty, 1962). In view of the temperature effects noted in the preceding section, it was of interest to determine the effects of temperature variation on the pH-induced transition profile. The transition of PLGA in aqueous solution was measured at 2°, 20°, and 40°C (Fig. 5). It can be seen that (*a*) transitions occurred at all three temperatures in a narrow pH range, 4.7–6.0 (however, the transition was slightly broader at the higher temperature) (*b*) the maximum $-b_0$ value reached in each transition was temperature-dependent, 720° at 2°C, 648° at 20°C, and 548° at 40°C, (*c*) the transitions were displaced towards more acidic pH with rising temperatures (this effect is somewhat more pronounced than suggested by Fig. 5 since the pH measurements were made at room temperature and the carboxyl groups have a small negative heat of ionization), (*d*) at all three temperatures $-b_0$ went through a maximum value and decreased with further decrease in pH.

(*f*) *Anomalous Behavior of b_0 at Low pH.* If b_0 is proportional to helical content, then helical content must be a single valued function of b_0. Since $-b_0$ goes through a maximum we have designated this behavior as anomalous. Further studies on the low pH behavior of b_0 were not possible in aqueous solution since in the region

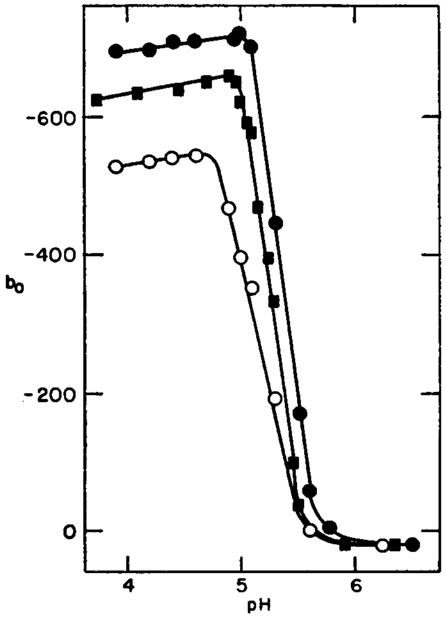

FIGURE 5 pH dependence of b_0 for poly-L-glutamic acid in aqueous solutions at various temperatures, (●) 2°C, (■) 20°C, and (○) 40°C. pH values refer to room temperature (22°C).

below pH 4 solutions became turbid and precipitated rapidly. Therefore the pH-induced transitions were determined in 70 per cent methanol, 70 per cent dimethylsulfoxide, and formamide (Fig. 6). For comparison the data in aqueous solutions are also included. The transition profiles in these three solvents were very similar. $-b_0$ rose very sharply within a narrow pH region to a peak value. With further reduction of pH the value first decreased and then became independent of pH. The peak and plateau values are shown in Table IV. Since the refractive indices of these solvents are different, for comparison, the $b_0^{1.333}$ values are also included. It was noted: (*a*) the peak $b_0^{1.333}$ values in 70 per cent methanol and 70 per cent dimethylsulfoxide are similar, while the peak in formamide lies approximately midway between these values and the value in aqueous solutions, (*b*) the pH change from the peak to the beginning of the plateau region was approximately 2.5 units in all three non-aqueous solutions, and the difference between the peak and plateau b_0 values was about 90° (*c*) in the accessible portion of the transition in aqueous solutions the decrease in the $-b_0$ values with decreasing pH was similar to those in non-aqueous solutions (35° decrease in $-b_0$ per pH unit). If it is assumed that $-b_0$ will continue to change in the same manner as observed for the non-aqueous solutions, $-b_0$ should reach a plateau at about pH 2.5 with a value of 565°. It is noteworthy that PLGA is completely uncharged at this pH (Wada, 1960).

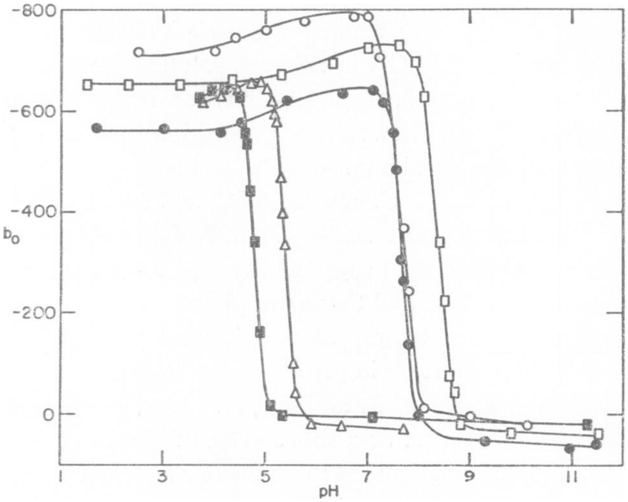

FIGURE 6 pH dependence of b_0 for poly-L-glutamic acid in various solutions, (△) water (no salt), (■) 0.2 M NaCl, (○) 70 percent methanol, (□) 70 percent dimethylsulfoxide and (●) formamide at 20°C.

It should be noted that b_0 values for the coil form in these solvents are very similar, ranging from 25° in 70 per cent methanol or water to 60° in formamide. Thus changes in nonhelical rotation can not account for the large b_0 differences of the helical form.

(*g*) *Effects of Temperature on the Far UV Hypochromism.* Helical contents of proteins and polypeptides have been estimated from the hypochromism of peptide bond absorption at 190 mμ (Imahori and Tanaka, 1959; Rosenheck and Doty, 1961; Doty and Gratzer, 1962). The molar extinction coefficient at 190 mμ for PLGA in aqueous solution was determined at 2° and 22°C (Table V). The results have been corrected for absorption of the carboxyl ion (ϵ_{190} = 1000). An uncertainty of at least ±50 should be assessed to the values listed in Table V. The results at 22°C are in good agreement with previously published values (Imahori and Tanaka, 1959; Rosenheck and Doty, 1961; Tinoco, Halpern, and Simpson, 1962). Lowering the temperature to 2°C caused a decrease in ϵ_{190} of 250 at low pH, but did not affect the high pH value. Since decreasing the temperature will affect the state of ionization of the carboxyl group its effect on ϵ_{190} should be considered. Due to a slight negative heat of ionization of the carboxyl groups of PLGA, cooling will increase the degree of ionization. Since the carboxyl groups are nearly completely ionized at pH 8.8 (Wada, 1960) cooling should have no effect on the state of ionization at this pH. At low pH, increased ionization would tend to increase rather than decrease ϵ_{190} so this effect cannot explain the experimental results.

TABLE IV

MAXIMUM AND MINIMUM $-b_0$ VALUES FOR "HELICAL" PLGA IN VARIOUS SOLVENTS AT 20°C

Solvent	Solvent refractive index n_s	Peak $-b_0$	Peak $-b_0^{1.333*}$	Plateau $-b_0$	Plateau $-b_0^{1.333*}$
Water	1.333	655	655	—	—
0.2M NaCl	1.334	645	646	—	—
Formamide	1.445	645	727	560	642
70 per cent methanol	1.342	795	802	710	717
70 per cent dimethylsulfoxide	1.432	735	808	650	723

* $-b_0^{1.333}$ was computed from the assumptions that the molecule is helical and shows the same index behavior as PBLG, $-b_0 = 1701 - 730.3\ n_s$ (Cassim and Taylor, 1965*a*).
Solvent index values refer to sodium D line.

(*h*) *Tritium-Hydrogen Exchange Experiments.* Hydrogen exchange studies have been used by a number of authors to determine helical content (Linderstrøm-Lang, 1958; Leach, 1959; Scheraga, 1961; Blout, de Lozé, and Asadourian, 1961; Englander, 1963). The tritium-hydrogen exchange kinetics of PLGA was studied in aqueous and 50 per cent methanol solutions at pH 4.4 by the tritium exchange-out method described by Englander (1963). Semilog plots of exchangeable H per residue as a function of exchange-out time were linear and extrapolated to values of 0.95 and 0.75 for PLGA in 50 per cent MOH and in aqueous solution, respectively. The halftime of exchange was 56 minutes in 50 per cent methanol and 11 minutes in aqueous solution. The uncertainty in the extrapolated value of exchangeable H per residue at zero time in 50 per cent methanol was estimated to be about ± 0.05. However, in the case of aqueous solutions, due to experimental difficulties resulting from a very fast rate of exchange, the uncertainty was ±0.25.

Thus the tritium exchange method confirmed the conclusion that PLGA is helical in methanol but it did not allow us to distinguish unequivocally between the helical content in aqueous solution *versus* methanol.

TABLE V

MOLAR EXTINCTION COEFFICIENTS AT 190 mμ FOR PLGA IN AQUEOUS SOLUTIONS

	ϵ_{190}		
pH*	22°C	2°C	$\Delta\epsilon_{190}$
3.6	4150	3900	−250
4.8	4325	4100	−225
8.8	6890	6850	− 40

* pH values refer to room temperature ~22°C.

DISCUSSION

PLGA has been used as a helical model for the estimation of helical content of proteins in solution by optical rotatory dispersion (Urnes, Imahori, and Doty, 1961; Beychok and Blout, 1961; Yang and Samejima, 1963; Shechter and Blout, 1964*a*; Samejima and Yang, 1964), by hydrogen-deuterium exchange (Blout *et al.*, 1961; Beychok, de Lozé, and Blout, 1962) and by far UV hypochromism (Imahori and Tanaka, 1959; Rosenheck and Doty, 1961; Doty and Gratzer, 1962). However the results of the present study showed that the "helix" parameter b_0 could take values ranging from −565° (extrapolated value in aqueous solution, pH 2.5) to −800° (70 per cent methanol-water, pH 7). Since b_0 exhibits large variations under experimental conditions which should preserve the molecule in a completely helical state, it is necessary to consider two questions:

(*a*) Is PLGA completely helical in aqueous solution at low pH?

(*b*) Is b_0 dependent only on helical content?

Discussion of the problem is complicated by the fact that an affirmative answer to one question has generally provided the evidence with which to answer the other.

It is appropriate to reconsider the evidence which has been used to support a completely helical state for PLGA at low pH.

(1) $-b_0$ at low pH is about 630° in agreement with the values for other helical polypeptides (PBLG, polylysine, polycarbobenzoxylysine, polymethionine, *etc.*). If

b_0 depends only on helical content, the near equality of the values for different polypeptides indicates that b_0 is a "helical parameter" and therefore PLGA is a complete helix.

(2) Hydrodynamic measurements are consistent with a long, relatively rigid rod.

(3) The titration curve exhibits an anomaly characteristic of helix-coil transition. The theory of Zimm and Rice (1960), which assumes that the low pH state is a pure helix, is in good agreement with the titration data.

The strongest evidence is provided by the rotatory dispersion data and it can be seen that the argument depends critically on the existence of a unique value of b_0 for a completely helical polypeptide. In a previous study of PBLG in a large variety of organic solvents (Cassim and Taylor, 1965*a*) it was found that $-b_0$ varied linearly with solvent refractive index and the value, extrapolated to the index of water, was 730°. Thus there is a discrepancy of about 100° between the values of a number of polypeptides in organic solvents and the values for PLGA and polylysine in aqueous solution. This discrepancy may be even larger since the value of $-b_0$ usually reported is the maximum value. As can be seen in Fig. 6, $-b_0$ began to decrease as the pH was reduced below 4.4 even though the transition to a helix was presumably complete at this pH. This effect is evident in the curves published by a number of authors (Doty, *et al.*, 1957; Idelson and Blout, 1958; Wada, 1960). Although a plateau was not reached in aqueous solution because of precipitation of the molecule at low pH, a constant value was obtained in formamide, 70 per cent dimethylsulfoxide and 70 per cent methanol.

Thus, b_0 depends on factors other than helical content and in comparing PLGA with PBLG it would seem reasonable to refer both molecules to the uncharged state. Since there is a difference of about 160° in the b_0 values, the magnitude of this parameter does not provide evidence for a completely helical state of PLGA and the proof must depend on hydrodynamic and titration data.

Measurements of intrinsic viscosity and radius of gyration over a range of molecular weights are not available and consequently the evidence from hydrodynamic and light scattering measurements is not sufficient to settle the question of helical content. The evidence from the titration curve is more difficult to evaluate since estimation of helical content is dependent on calculations based on an approximate theory. Helical content is determined by the parameters, s, the equilibrium constant for the addition of a residue to a helical segment and σ which is a measure of the difficulty of initiating a new helical segment. The value of σ calculated by Zimm and Rice (1.4×10^{-3}) is larger than for PBLG and consequently a larger value of s is necessary to maintain a complete helix (Zimm and Bragg, 1959). The value of s estimated from the titration curve (Nagasawa and Holtzer, 1964) is sufficiently small that the helical content could be uncertain by 20 per cent.

Therefore, on the basis of the available evidence it can only be concluded that

PLGA is largely helical at low pH but the exact value could be between 80 and 100 per cent.

The lower $-b_0$ value of PLGA in aqueous solution *versus* methanol does not provide unequivocal evidence for a lower helical content since this difference might be attributed to the high dipole moment of water. In a previous study (Cassim and Taylor, 1965*a*) it was pointed out that the decrease in $-b_0$ for PBLG with increasing solvent polarity could be explained as a red shift of the π-π^* transition. Although no correlation with dielectric constant up to D = 37 (dimethylformamide) was found in this study of organic solvent effects, it is still possible that water must be regarded as an anomalous solvent. The change in b_0 is in the direction expected for a dipole moment effect.

In a number of solvents (methanol, ethanol, glycerol, dimethylsulfoxide, *etc.*) of various dielectric constants the b_0 values of PLGA after correction for refractive index differences are in the range expected for a helical polypeptide. Addition of water to any of these solvents leads to a decrease in $-b_0$ which is not simply related to the dielectric constant of the mixture. In formamide the dielectric constant is higher than in water and the $-b_0$ value is increased. Thus contrary to the suggestion of Shechter and Blout (1964*b*) there is no simple relation between b_0 and dielectric constant.

Two lines of evidence suggest that PLGA is not completely helical in aqueous solution; $-b_0$ is temperature-dependent in aqueous solution and independent of temperature in methanol; the intrinsic viscosity increases with the addition of organic solvents. Changes in b_0 with temperature have generally been taken as evidence for a helix coil transition. If b_0 is affected by interaction with solvent dipoles, b_0 could be a function of temperature but since raising the temperature should be equivalent to lowering the dielectric constant (*i.e.*, reducing solvent dipole orientation) we would expect $-b_0$ to increase rather than decrease with temperature.

The change in b_0 with temperature can be correlated with the change in hypochromicity. Since Applequist and Breslow (1963) have shown that the pH induced transition profile of PLGA is similar when measured by hypochromicity and b_0, the parameters should be comparable measures of partial helical content.

Rosenheck and Doty (1961) have interpreted the decrease in hypochromicity with increasing temperature as a loss in helical content. If we take the helical content at 22° as 80 per cent, then the increased $-b_0$ at 2° would indicate 88 per cent helix at this temperature. Since $\Delta\varepsilon_{190}$ is 2950 at 2°, the value for a complete helix would be 3300. We would then predict the value at 22° to be further reduced by about 300 while 250 was found experimentally. Thus the changes in b_0 and ε_{190} with temperature are consistent with each other and with a change in helical content.

The intrinsic viscosity and the length calculated from the rotatory diffusion constant increased in methanol-water mixtures in parallel with the increase in $-b_0$. The Huggins' k' parameter remained small which suggested that the viscosity increase was not due to aggregation. In addition while intrinsic viscosity correlated with b_0, k' did not. Further evidence against aggregation is provided by the stability of polypeptide solutions in the organic solvents *versus* the gradual precipitation of aqueous solutions below pH 5.

Although the viscosity data support the interpretation of the increase in b_0 as an increase in helical content the results cannot be accepted without reservations. In order to compare intrinsic viscosity and b_0 in a variety of organic solvents, the viscosity measurements were made in the absence of salt. The polypeptide was about 20 per cent ionized in aqueous solution but η_{sp}/c *versus* concentration plots were linear. Thus the increase in η_{sp}/c at low concentrations characteristic of flexible polyelectrolytes, was not present and extrapolations to $c = 0$ were unequivocal. The electroviscous effect is small for compact protein molecules at low pH (Buzzell and Tanford, 1956) and should not be serious in these experiments. In any case the absence of salt should tend to reduce any difference between viscosities in aqueous *versus* organic solvents since the molecule is essentially uncharged in the latter. However the value of k' of approximately zero in aqueous solution is unexpectedly small. Thus the viscosity data should be regarded as preliminary to a more extensive study as a function of molecular weight, ionic strength, and solvent composition.

In summary, $-b_0$ for a number of polypeptides in organic solvents and aqueous-organic mixtures is about 730° at refractive index 1.333. In aqueous solution, at low pH $-b_0$ for PLGA is less than 600° and it is temperature-dependent. The simplest interpretation of the dispersion data requires a helical content of about 80 per cent at room temperature. In the absence of precise determinations of helical content by independent methods the question of the helical content of PLGA and the stability of an α-helix in aqueous solution must be regarded as not yet settled.

In determining the helical content of proteins in aqueous solution it must be emphasized that the correct value of b_0 for a complete helix may be uncertain by about 20 per cent and the appropriate value for a given protein may also depend on change state, and the local effective field. Also changes in b_0 with the addition of organic solvents to aqueous protein solutions (Doty, 1957; Tanford, De, and Taggart, 1960; Tanford and De, 1961; Doty, 1959) may be in part due to factors other than changes in helical content.

The relation of the present results to the helical content scale proposed by Shechter and Blout (1964*a* and *b*) and Shechter, Carver, and Blout (1964) requires a brief comment. The values of the parameters of the Shechter-Blout (S-B) equation refer to a particular polypeptide in a particular solvent. The use of the ratio of the magnitudes of the Cotton effects to define helical content involves the same kind of assumptions as were originally made for the Moffitt b_0 parameter. Thus the S-B scale is an empirical relation which must be justified by experiment, and the dependence of b_0 on factors other than helical content requires a similar variation in the Cotton effects since b_0 is functionally related to the S-B parameters. The S-B equation has the important advantage that the parameters are related to quantities which are measurable at least in principle and we have used the equation to analyze the b_0 variation with refractive index (Cassim and Taylor 1965*a*). However as a means of determining helical content the S-B scale does not appear to have any advantages over the b_0 method.

This research was supported by United States Public Health Service General Medical Research Grant GM-10992.

Received for publication, January 11, 1965.

REFERENCES

Applequist, J., and Breslow, J. L., 1963, *J. Am. Chem. Soc.*, **85**, 2869.

Applequist, J., and Doty, P., 1962, *in* Polyamino Acids, Polypeptides and Proteins, M. A. Stahmann, editor, 161, Madison, The University of Wisconsin Press.

Beychok, S., and Blout, E. R., 1961, *J. Mol. Biol.*, **3**, 769.

Beychok, S., de Lozé, C., and Blout, E. R., 1962, *J. Mol. Biol.*, **4**, 421.

Blout, E. R., de Lozé, C., and Asadourian, A., 1961, *J. Am. Chem. Soc.*, **83**, 1895.

Buzzell, J. G., and Tanford, C., 1956, *J. Phys. Chem.*, **60**, 1204.

Cassim, J. Y., and Taylor, E. W., 1965*a*, (references to accompanying paper I).

Cassim, J. Y., and Taylor, E. W., 1965*b*, (references to accompanying paper).

Doty, P., 1957, *Collection Czech. Chem. Comm.*, 22, 5.

Doty, P., 1959, *Rev. Mod. Physics*, **31**, 107.

Doty, P., and Gratzer, W. B., 1962, *in* Polyamino Acids, Polypeptides and Proteins, M. A. Stahmann, editor, 111, Madison, The University of Wisconsin Press.

Doty, P., Wanda, A., Yang, J. T., and Blout, E. R., 1957, *J. Polymer Sc.*, **23**, 851.

Englander, S. W., 1963, *Biochemistry*, **2**, 798.

Fasman, G. D., Lindblow, C., and Bodenheimer, E., 1964, *Biochemistry*, **3**, 155.

Goldstein, L., and Katchalski, E., 1960, Bull. Research Council Israel, Sect. A, **9**, 138.

Herberg, R. J., 1960, *Anal. Chem.*, **32**, 42.

Idelson, M., and Blout, E. R., 1958, *J. Am. Chem. Soc.*, **80**, 4631.

Imahori, K., and Tanaka, J., 1959, *J. Mol. Biol.*, **1**, 359.

Leach, S. J., 1959, *Rev. Pure and Appl. Chem.*, **9**, 33.

Linderstrøm-Lang, K. V., 1958, *in* Symposium on Protein Structure, A. Neuberger, editor, 23, London, Methuen and Co., Ltd.

Ma, T. S., and Zuazaga, G., 1942, *Ind. Eng. Chem., Anal. Ed.*, **14**, 280.

Miyazawa, T., and Blout, E. R., 1962, *in* Polyamino Acids, Polypeptides and Proteins, M. A. Stahmann, editor, 159. Madison, The University of Wisconsin Press.

Moffitt, W., 1956, *Proc. Nat. Acad. Sc.*, **42**, 736.

Moffitt, W., and Yang, J. T., 1956, *Proc. Nat. Acad. Sc.*, **42**, 596.

Nagasawa, M., and Holtzer, A., 1964, *J. Am. Chem. Soc.*, **86**, 538.

Rosenheck, K., and Doty, P., 1961, *Proc. Nat. Acad. Sc.*, **47**, 1775.

Samejima, T., and Yang, J. T., 1964, *J. Mol. Biol.*, **8**, 863.

Scheraga, H. A., 1961, Protein Structure, New York, Academic Press.

Shechter, E., and Blout, E. R., 1964*a*, *Proc. Nat. Acad. Sc.*, **51**, 695.

Shechter, E., and Blout, E. R., 1964*b*, *Proc. Nat. Acad. Sc.*, **51**, 795.

Shechter, E., Carver, J. P., and Blout, E. R., 1964, *Proc. Nat. Acad. Sc.*, **51**, 1029.

Tanford, C., and De, P. K., 1961, *J. Biol. Chem.*, **236**, 1711.

Tanford, C., De, P. K., and Taggart, V. G., 1960, *J. Am. Chem. Soc.*, **82**, 6028.

Tinoco, I., Jr., Halpern, A., and Simpson, W. T., 1962, *in* Polyamino Acids, Polypeptides and Proteins, M. A. Stahmann, editor, 147, Madison, The University of Wisconsin Press.

URNES, P., and DOTY, P., 1961, *Advances Protein Chem.*, **16**, 401.
URNES, P., IMAHORI, K., and DOTY, P., 1961, *Proc. Nat. Acad. Sc.*, **47**, 1635.
WADA, A., 1960, *J. Mol. Phys.*, **3**, 409.
YANG, J. T., and SAMEJIMA, T., 1963, *J. Biol. Chem.*, **238**, 3262.
ZIMM, B. H., and BRAGG, J. K., 1959, *J. Chem. Phys.*, **31**, 526.
ZIMM, B. H., and RICE, S. A., 1960, *J. Mol. Phys.*, **3**, 391.