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

I. STUDIES ON POLY-γ-BENZYL-L-GLUTAMATE

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ABSTRACT The constancy of the Moffitt optical rotatory dispersion parameters for polypeptides in different solvents was tested by dispersion measurements on poly-γ-benzyl-L-glutamate in fifty-five solvents and solvent mixtures. b_0 was not constant but varied linearly with the refractive index of the solvent according to the equation $-b_0 = 1701 - 730.3\ n_s$. This variation could not be explained by changes in configuration of the polypeptide. a_0 also showed a trend with solvent index but the values were widely scattered. λ_0 did not show a statistically significant dependence on solvent index. The variation in b_0 can be interpreted as an effect of solvent polarizability on the frequencies of optically active transitions.

INTRODUCTION

The analysis of optical rotatory dispersion measurements is the most important procedure available for the estimation of the helical content of proteins and polypeptides (Urnes and Doty, 1961). The method is based on an evaluation of the parameters characterizing the dispersion according to Moffitt's equation (Moffitt, 1956; Moffitt and Yang, 1956)

$$[m']_\lambda = \frac{a_0\lambda_0^2}{\lambda^2 - \lambda_0^2} + \frac{b_0\lambda_0^4}{(\lambda^2 - \lambda_0^2)^2}$$

where

$$[m']_\lambda = \frac{3}{n_s^2 + 2}\frac{M_0}{100}[\alpha]_\lambda$$

$[\alpha]_\lambda$ is the specific rotation, n_s the solvent refractive index, M_0 the mean residue molecular weight, a_0 and b_0 are empirical constants, and λ_0 the characteristic wavelength which is selected for best fit of the data to a two term equation (Sogami, Leonard, and Foster, 1963). Although this equation was originally derived from a

particular theory, further theoretical (Moffitt, Fitts, and Kirkwood, 1957) and experimental studies have shown that it should be regarded only as an empirical relation.

The primary postulate of the analysis of dispersion data states that the magnitude of the parameter b_0 is determined only by the helical content of the protein or polypeptide (Urnes and Doty, 1961). The effects of side chains, of neighboring non-helical regions, and of the solvent environment are assumed to be negligible. Although a large body of experimental evidence on the optical rotation of small molecules has shown that the rotation is quite sensitive to solvent it is presumed that the dispersion of macromolecules can be expressed in such a way that a "helix parameter" is obtained.

The purpose of this study was to test the constancy of b_0 for a single polypeptide in a large variety of solvents. Poly-γ-benzyl-L-glutamate was chosen for study since its properties have been characterized in more detail than any other polypeptide. It is generally believed to be completely helical and it is soluble in a large number of solvents.

It was found that b_0 was not independent of solvent but varied linearly with solvent refractive index. The cause of this solvent effect will be discussed in terms of the effect of solvent polarizability on the frequencies of the optical active transitions.

MATERIALS AND METHODS

(*a*) *Polypeptide.* Poly-γ-benzyl-L-glutamate of molecular weight 255,000 was obtained from Pilot Chemicals, Inc. (Watertown, Massachusetts). It was further purified by repeated precipitation from dioxane solution with 95 per cent ethanol, and vacuum dried at 50° for 24 hours.

(*b*) *Solvents.* All solvents were reagent grade. Many of the solvents were purified by distillation just prior to use. The solvents are listed below with their abbreviated nomenclature which will be used hereafter: amylbenzene (ABZ), aniline (AL), anisole (AS), benzene (BZ), bromobenzene (BBZ), bromoform (B), 1-bromopropane (BP), chlorobenzene (CBZ), chloroform (C), 2-chloro-2-methylpropane (CMP), *m*-cresol (CS), *m*-dibromobenzene (DBBZ), dibromochloromethane (DBCM), 1, 2-dibromoethane (EDB), dibromomethane (DBM), dichloroacetic acid (DCA), 1, 2-dichloroethane (EDC), cis-1, 2-dichloroethane (CIS-DCEY), dichloromethane (MC), dimethylformamide (DMF), *p*-dioxane (DIOX), iodobenzene (IBZ), methyl salicylate (MS), pyridine (PYR), 1, 1, 2, 2-tetrabromoethane (TBE), 1, 1, 2, 2-tetrachloroethane (TCE), tetramethylurea (TMU), toluene (T), trichloroethylene (TCEY), 2, 2, 2-trifluoroethanol (TFE), *m*-xylene (MX), *o*-xylene (OX), *p*-xylene (PX).

(*c*) *Solutions.* The solutions were prepared by weighing the polypeptide into red glass containers with low actinic transmission, and adding a measured volume of solvent. Solutions were clarified, if necessary, by filtering through medium sintered glass crucibles, or by ultracentrifugation. Concentrations were redetermined either by the evaporation of a known volume of solution, or by the micro-Kjeldahl method (Ma and Zuazaga, 1942).

(*d*) *Optical Rotation.* Rotation measurements were made with a Rudolph photoelectric polarimeter (model 80S), equipped with a thermostated cell compartment,

and an oscillating polarizer. The instrument was converted into a spectropolarimeter by coupling it to a Bausch and Lomb (Bausch and Lomb Optical Company, New York) grating monochromator (model 33-86-40), and an Osram (Osram GMBH, Berlin, Germany) high pressure mercury lamp (model HBO 200w). A slit width of 0.5 mm on the monochromator, and a symmetrical angle of 1° on the oscillating polarizer were maintained during all measurements. The wavelength range covered in dispersion measurements was from about 350 to 595 mμ. Measurements were made in the concentration range, 0.5 to 1.0 g/dl, in a 2dm tube at a temperature of 20°C unless otherwise stated. Repeated measurements indicated an uncertainty of about ±0.5° in the specific rotation.

(*e*) *Flow Birefringence and Intrinsic Viscosity.* Measurements of molecular lengths from flow birefringence and intrinsic viscosity are discribed in detail in the accompanying report (Cassim and Taylor, 1965).

(*f*) *Infrared Spectra.* Infrared spectra were recorded on a Beckman spectrophotometer (Beckman Instruments, Inc., Palo Alto, California) (model IR7) using a 1 mm cell fitted with rock salt windows. The solutions were at a concentration of 0.5 g/dl.

(*g*) *Refractive Index.* The refractive indices of the solvents were measured on a thermostrated Zeiss refractometer (Carl Zeiss, Inc., New York) using a sodium lamp source. All measurements were made at 20°C unless otherwise stated.

(*h*) *Computations.* Statistical analyses of dispersion data to obtain best fit values for λ_0 were performed on an IBM 7094 computer.

RESULTS

The optical rotatory dispersion of poly-γ-benzyl-L-glutamate (hereafter PBLG) was measured in fifty-five organic solvents and solvent mixtures. The dispersion data were fitted to the Moffitt equation, using a λ_0 of 212 mμ. The parameters, a_0 and b_0 were determined graphically from the intercept and slope, respectively, of the Moffitt plot in the manner suggested by Moffitt and Yang (1956). The dispersion in the refractive index of the solvent was neglected in making the calculations. Trial calculations using dispersion formulas for a number of solvents showed that in the wavelength range utilized, this neglect can result in a maximum variation of only 10° in the values of a_0 and b_0. However, repeated measurements indicated that errors due to other sources result in an uncertainty of about ±10° in the calculated values of a_0 and b_0.

The linearity of the Moffitt plots in a few solvents is shown in Fig. 1. The linearity was equally good in all the solvents studied. The b_0 values, as well as the other optical parameters, in various solvents are listed in Table I. The values are in accord with those of other investigators using the same solvents (Blout, de Lozé, Bloom, and Fasman, 1960; Mitchell, Woodward, and Doty, 1957; Karlson, Norland, Fasman, and Blout, 1960; Simmons, Cohen, Szent-Györgyi, Wetlaufer, and Blout, 1961).

The b_0 values listed in Table I show a variation of 201°. However, this variation is not random as can be seen by plotting b_0 against the index of refraction of the solvent (Fig. 2). Least-squares analysis of the data, excluding the *m*-cresol point, yielded the equation $-b_0 = 1701 - 730.3\ n_s$. Forty-nine points out of fifty-four points, fell on the line within the experimental uncertainty of ±10° in b_0.

This regularity is not shown by a similar plot of the a_0 parameter (Fig. 3). Even though there is a trend indicating a direct variation of a_0 with the index of refraction, the points show considerably greater scatter. For example, at an index of about 1.495 there is a spread of 180° among the a_0 values in different solvents. There is also a correlation with

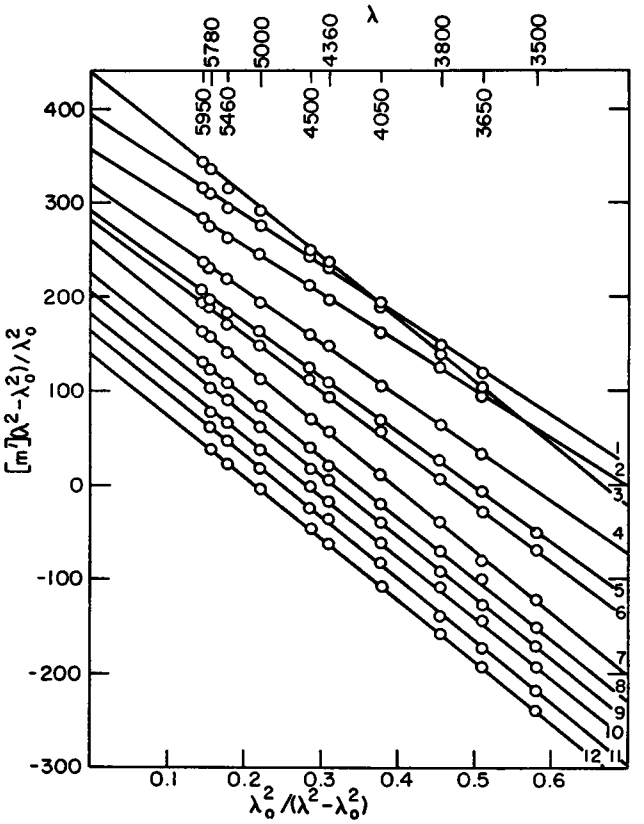

FIGURE 1 Moffitt plots of dispersion data for PBLG in some organic solvents: 1. B, 2. TBE, 3. CS, 4. DBM, 5. EDB, 6. TCEY, 7. C, 8. MC, 9. EDC, 10. TMU, 11. CMP-MC, 12. DMF. Solvent symbols are defined in Materials Section.

the nature of the solvent, the a_0 values for aromatic solvents being higher than those for alkyl halides of the same refractive index.

Since these results contradict the generally accepted conclusion that b_0 is independent of solvent and has a value of $-630°$ for a complete helix (Urnes and Doty, 1961; Moffitt and Yang, 1956; Yang, 1961; Doty, 1959), a number of experiments were carried out to show that the b_0 variation was not an artifact. If b_0 depends on solvent refractive index we expect the values obtained for solvent mixtures to agree with values for pure solvents of the same refractive index. The dispersion of PBLG was measured in various mixtures of MC and TBE. The refractive index of mixtures of these two solvents is a linear function of the volume fraction within experimental error. A plot of b_0 as a function of solvent composition (Fig. 4) shows the same linearity as Fig. 2, with an identical slope within experimental uncertainty. It should also be noted that the a_0 parameter varied linearly with the solvent composition.

A possible explanation for the apparent variations of the b_0 values in Fig. 2 is that the helical content is not constant in the different solvents that were used. In view of the

TABLE I

OPTICAL PARAMETERS OF PBLG IN ORGANIC SOLVENTS

	Solvent	Volume *Ratio*	Refractive index of solvent n_s	Moffitt parameters for λ_o = 212 mμ a_o	$-b_o$	$[\alpha]_{578}^{20}$
1.	TFE–MC	2–3	1.364	114	706	0.63
2.	TFE–MC	1–2	1.375	138	702	2.46
3.	TFE–MC	1–3	1.388	166	688	5.06
4.	CMP–MC	1–1	1.403	165	667	5.68
5.	DIOX	—	1.422	190	660	8.41
6.	MC	—	1.424	223	656	11.52
7.	DMF	—	1.430	140	654	3.65
8.	BP–C	1–1	1.440	214	650	11.44
9.	EDC	—	1.445	211	660	10.64
10.	C	—	1.446	256	651	15.48
11.	CIS–DCEY	—	1.447	260	642	16.10
12.	TMU	—	1.452	176	632	7.59
13.	DMF–BZ	1–1	1.466	263	620	16.36
14.	EDC–TCEY	1–2	1.467	234	634	13.20
15.	DMF–AS	1–1	1.474	227	624	13.40
16.	TCEY	—	1.478	238	616	14.30
17.	ABZ–CS	91–9	1.479	322	626	23.17
18.	DIOX–CS	1–1	1.482	233	606	13.90
19.	MC–CS	1–1	1.483	331	620	23.06
20.	EDC–EDB	1–1	1.492	251	630	15.68
21.	EDC–CS	1–1	1.493	346	620	25.23
22.	C–CS	1–1	1.494	340	610	23.92
23.	TCE	—	1.494	282	606	19.12
24.	DMF–BBZ	1–1	1.495	245	612	14.84
25.	BZ–DMF	91–9	1.495	357	614	26.45
26.	BZ–DMF	95–5	1.498	360	610	26.80
27.	PX–CS	91–9	1.500	320	610	22.90
28.	MX–CS	91–9	1.501	331	608	23.90
29.	T–CS	91–9	1.501	382	600	29.41
30.	BZ–CS	97.5–2.5	1.502	403	604	31.70
31.	BZ–CS	91–9	1.505	424	602	33.18
32.	OX–CS	91–9	1.509	364	606	27.47
33.	PYR	—	1.510	358	586	27.06
34.	PX–CS	1–1	1.518	377	600	29.52
35.	T–CS	1–1	1.519	398	596	31.61
36.	AS–CS	91–9	1.520	355	587	27.29
37.	BZ–CS	1–1	1.521	421	592	33.64
38.	CBZ–CS	91–9	1.526	414	586	33.22
39.	MC–TBE	1–1	1.530	302	576	21.73
40.	CS–DMF	91–9	1.531	396	588	31.67
41.	EDB	—	1.538	280	583	19.56
42.	DBM	—	1.540	320	564	24.32
43.	EDC–TBE	1–1	1.540	290	568	20.92
44.	MS–CS	91–9	1.540	281	584	19.92
45.	CS	—	1.541	437	656	34.82
46.	DBCM	—	1.547	364	564	28.47
47.	BBZ–CS	91–9	1.559	434	572	36.55
48.	EDB–B	1–1	1.568	327	554	25.35
49.	AL–CS	91–9	1.582	390	548	32.88
50.	EDB–TBE	1–1	1.587	316	542	24.80
51.	DBBZ–CS	91–9	1.596	384	532	32.25
52.	B	—	1.598	392	538	32.72
53.	IBZ–CS	91–9	1.615	440	520	39.53
54.	B–TBE	1–1	1.617	362	522	30.64
55.	TBE	—	1.635	349	505	32.72

Solvent index values refer to sodium D line.

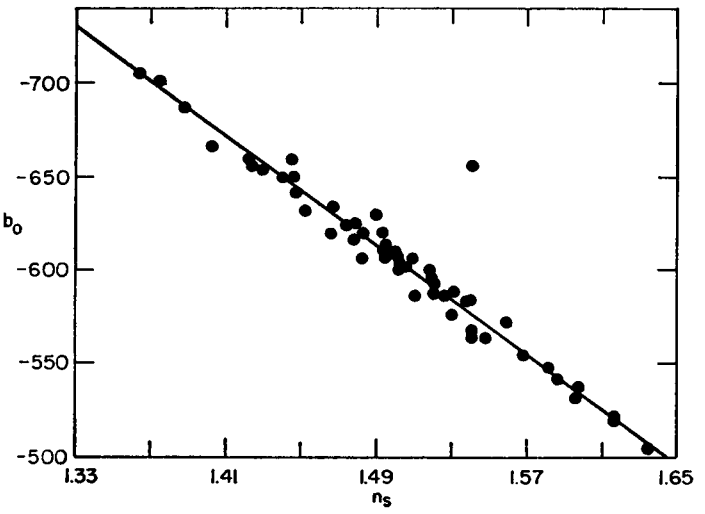

FIGURE 2 b_o as a function of solvent refractive index. n_s. (sodium D line) The equation of the line is $-b_o = 1701 - 730.3\ n_s$. The one point deviating from the curve is the value obtained in *m*-cresol.

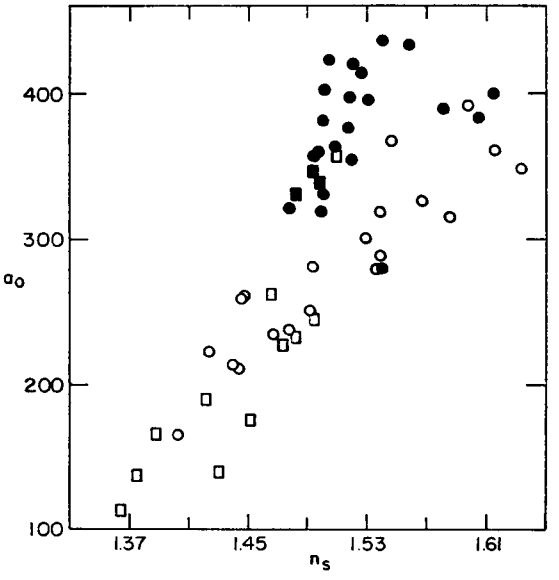

FIGURE 3 a_o as a function of solvent refractive index, n_s. (sodium D line)
○ alkyl halides
● aromatic compounds
■ mixture of alkyl halides and aromatic compounds
□ others.

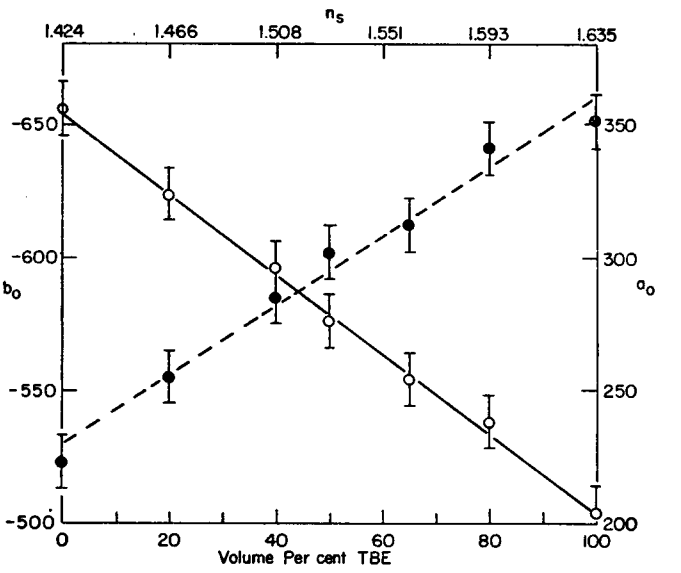

FIGURE 4 Variation of a_0(●) and b_0(○) with solvent composition in mixtures of methylene chloride and tetrabromoethane (TBE).

regular dependence of b_0 on refractive index rather than on the chemical properties of the solvent this explanation would require a remarkable coincidence; and in any case, would not account for b_0 values significantly more negative than 630°, the value generally assigned to a complete helix. Yet this point is sufficiently important, that it was felt necessary to provide auxiliary evidence that a configuration change had not occurred.

The b_0 value in tetrabromoethane (TBE) from Table I is −505°. If a value of −630° indicates a 100 per cent helix, then the helical content in TBE should be about 80 per cent (assuming a linear interpolation between b_0 values and helical content). Thus the molecule would be in a transition region between the helical and coil configurations.

It has been shown (Doty and Yang, 1956; Doty, 1957; Blout, Doty, and Yang, 1957; Karlson *et al.*, 1960) that increasing the DCA volume fraction in a solution, consisting of PBLG dissolved in a solvent in which the helical form is the stable configuration, results in a helix-coil transition when the DCA concentration reaches a critical value. This critical value then determines the relative stability of the helical configuration in that solvent (Fasman, 1962). If the molecule is not completely helical in TBE, then a small amount of DCA should have been sufficient to bring about a large change in the b_0 value. However, a transition was not obtained until the volume fraction of DCA was about 58 per cent (Fig. 5), and the transition was still as sharp as that obtained in EDC-DCA mixtures. This value should be compared with the values of 76 per cent DCA for the transition in EDC (Doty and Yang, 1956) and 68 per cent in chloroform (Karlson *et al.*, 1960).

Doty and Yang (1956), Zimm and Bragg (1959) and Calvin, Hermans, and Scheraga (1959) have shown that the transition behavior of PBLG in EDC-DCA solutions is extremely temperature-sensitive. For PBLG of high molecular weight in EDC-DCA solutions, the temperature range for a complete helix-coil transition is about 20°C with the

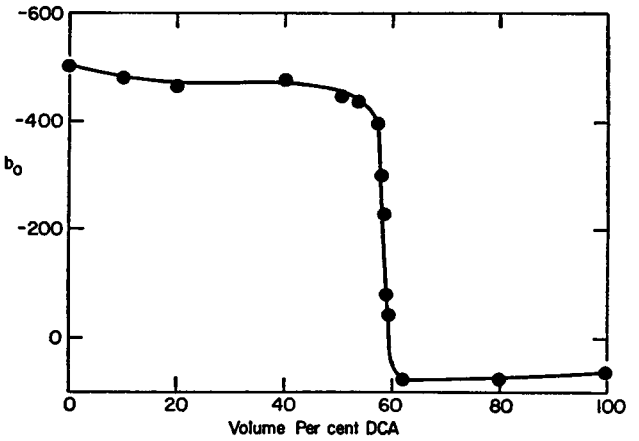

FIGURE 5 b_0 as a function of solvent composition in mixtures of tetrabromoethane and dichloroacetic acid (DCA).

helical configuration being favored at higher temperatures. The b_0 values in TBE were determined in the experimentally accessible temperature range of 2—70°C (Fig. 6). Within experimental uncertainty, the b_0 values remained independent of temperature. On the other hand, PBLG in TBE, which was in a DCA-induced transition region at 20°C, showed the characteristic thermal sensitivity noted in EDC-DCA solutions. The results of the experiments with solvent mixtures in conjunction with the thermal experiments show that PBLG in TBE can not be in a helix-coil transition region.

A second method of testing for a modification of the helical structure of PBLG is to

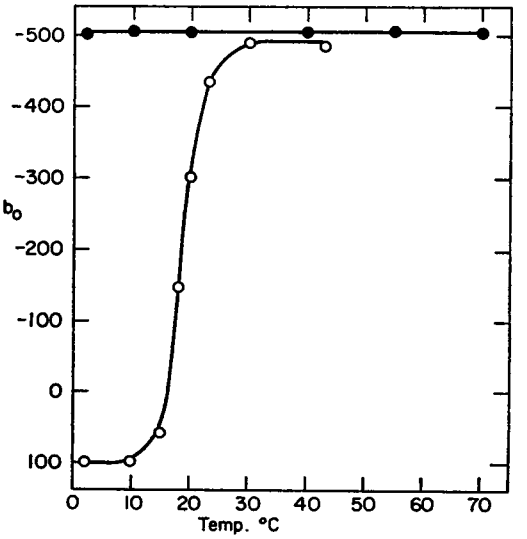

FIGURE 6 b_0 as a function of temperature in tetrabromoethane (●) and tetrabromoethane-dichloroacetic acid (57.5 per cent DCA by volume) (○).

measure the length of the molecule in the various solvents. The length was determined by flow birefringence and intrinsic viscosity measurements. The gradient dependence of molecular lengths was not excessive, and the length became constant at moderate shear. Since molecular association occurred in some of the solvents, it was necessary to use 0.5 per cent formamide or 1.0 per cent TMU as deaggregants. These additions did not cause any significant change in the optical rotatory dispersion. Values are given in Table II of the previous paper (Cassim and Taylor, 1965). In a variety of solvents covering the refractive index range, the lengths were constant with an average value of 1800 ± 100 A. Thus the hydrodynamic data support the conclusion that the structure remains the same in all solvents.

It has been shown (Yang and Doty, 1957; Fasman and Blout, 1961; Wada, 1961) that the appearance of the β-form in synthetic polypeptide solutions is accompanied by a positive contribution to the rotatory power. Analyses of the dispersion data of β-form in solution (Wada, 1961; Imahori, 1960) yielded positive b_0 values. Thus the presence of the β-form would lead to a less negative b_0. Doty, Holtzer, Bradbury, and Blout (1954) and Blout and Asadourian (1956) have shown by infrared studies that low molecular weight PBLG exists in the β-form in solutions. However, Mitchell, Woodward, and Doty (1957) have given ten as the critical degree of polymerization for this effect.

The infrared spectra in ethylene dibromide, bromoform, and tetrabromoethane were recorded in the wave number range 1500 to 2000 cm^{-1}. Amide I absorption maxima appeared at about 1650 cm^{-1} and amide II at about 1552 cm^{-1}, with no indications of a shoulder in the region 1625 to 1635 cm^{-1} or in the region 1520 to 1530 cm^{-1}. Therefore the results are characteristic of the α-helix (or random coil form) with no evidence for the presence of the β-form (Ambrose and Elliott, 1951; Elliott, 1953). If the change of 201° in b_0 in TBE were due to the presence of β-form it should have been detectable in the infrared spectrum.

The relative amount of β-form in solution is often concentration dependent (Yang and Doty, 1957; Wada, 1961; Blout and Asadourian, 1956). The dispersion parameters of PBLG in TBE, measured over a solute concentration range of 3.0 to 0.25 g/dl were independent of concentration. Bradbury, Elliott, and Hanby (1962) working with poly-O-benzyl-L-serine have shown that the dispersion of the polypeptide in the β-form is also time dependent. No time dependence was noted for the dispersion parameters in TBE, even though measurements were made over a period of months.

As can be seen from Fig. 2, the result in *m*-cresol is the one point which deviates appreciably from the least-squares straight line. Since the helical configuration in *m*-cresol has been well established by various physical methods (Yang and Doty, 1957; Fasman and Blout, 1961; Doty, Bradbury, and Holtzer, 1956; Downie, Elliott, Hanby, and Malcolm, 1957; Yang, 1959; Philippoff and Gaskins, 1959), the anomalous b_0 value cannot be a reflection of a configuration change of the molecular backbone. In order to obtain some understanding of the phenomenon responsible for this b_0 variation of 80°, the dispersion of PBLG was measured as a function of solvent composition in a mixture of *m*-cresol and ethylene dibromide. The refractive index change with solvent composition is negligible for this solvent pair. A plot of a_0 and b_0 as a function of solvent composition is shown in Fig. 7. b_0 remained approximately constant from 0 to 86 per cent *m*-cresol. Between 86 per cent and 87.5 per cent, the b_0 value changed very sharply and then remained constant to 100 per cent. a_0 increased very rapidly at first, and then tended to level off, but increased again in the region of the b_0 transition.

The b_0 parameter is extremely sensitive to the choice of λ_0 used in the solution of the

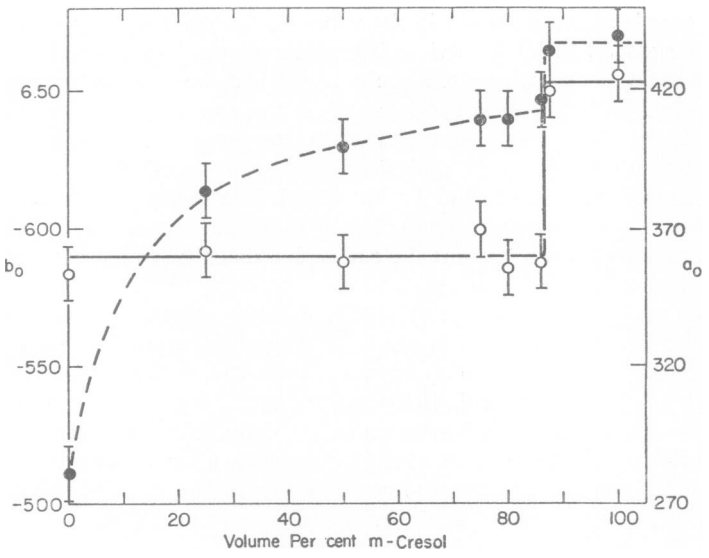

FIGURE 7 a_0(●) and b_0(○) as a function of solvent composition in ethylene dibromide-*m*-cresol mixtures.

Moffitt equation (Urnes and Doty, 1961; Ridgeway, 1963). The error in the graphical estimation of λ_0, if the dispersion measurements are not extended below 300 mμ, is about ± 5 mμ (Moffitt and Yang, 1956). For comparative purposes, we chose to use a λ_0 value of 212 mμ. This is the value which has been commonly used for PBLG in the usual frequency range. However, to determine if there was any influence of solvent on the most probable value for this parameter, the dispersion data were evaluated by the statistical method described by Sogami *et al.* (1963). In stable and transparent solvents, for example, EDC, DMF, C, the best values of λ_0 obtained were 212 mμ with a confidence limit of ± 2 mμ in accord with the results reported by Leonard and Foster (1963). However, in high refractive index solvents and in aromatic solvents, due to some reduction of solvent transparency at the shorter wavelengths, the confidence limit was increased to ± 4 mμ. Therefore, the most probable value of λ_0 over the entire refractive index range of 1.364 to 1.635 should be taken as 212 ± 4 mμ. The best-fit values of λ_0 showed no statistically significant trend with solvent refractive index.

DISCUSSION

The experiments described in the previous section (effects of DCA addition, thermal sensitivity, molecular length, intrinsic viscosity, infrared spectra, concentration dependence, and time dependence) gave no evidence of any modification of the helical content of the molecule in the solvents used in this study. Therefore, the variation of the b_0 parameter seems to be a result of the sensitivity of this parameter to solvent effects even when these do not affect the helical configuration. Fortunately, b_0 varied linearly with a bulk property of the solvent, the refractive index, and its behavior is

therefore predictable. The values in all solvents with the exception of *m*-cresol are given by the equation $-b_0 = 1701 - 730.3\, n_s$.

The solvent effect on the a_0 parameter is much more complex. It is noteworthy, that for a given type of solvent there appears to be a linear dependence of the a_0 parameter on the refractive index of the solvent, but the relationship is obscured by scattering of the data. There was also an indication of a relationship between the a_0 parameter and the structure of the solvent molecule. The a_0 values in aromatic solvents in most cases tended to be significantly higher than those in non-aromatic solvents of comparable refractive index.

Two possible sources of the variation in b_0 are changes in λ_0 or in refractive index through dispersion. Fitting of λ_0 for several solvents by the statistical procedure of Sogami *et al.* (1963) showed no trend with solvent index. Inclusion of index dispersion in the Lorentz factor $(n_s^2 + 2)/3$ for a number of high and low index solvents yielded variations in b_0 of only about 10° over the wavelength range.

In view of the solvent refractive index dependence of the b_0 parameter, for comparative purposes, it is useful to transform all b_0 values to some reference index. From a practical standpoint, the index of water is perhaps the most suitable value.

From the equation $-b_0 = 1701 - 730.3\, n_s$ it follows that $b_0^{1.333} = b_0 + 730.3 \cdot (1.333 - n_s)$ where $b_0^{1.333}$ is the b_0 value at the refractive index of water. By employing the above equation, the previously published b_0 values for presumed helical polypeptides have been transformed to the reference index. The data are listed in Table II. It is apparent that b_0 determinations do not cluster about a single value. Polypeptides in organic solvents give values close to −720°. Our PBLG results in fifty-four organic solvents and solvent mixtures yield a value of about −730°. However, polypeptides in aqueous solutions seem to cluster about a b_0 value −615°.

The significance of the markedly different values in aqueous solution will be discussed in a subsequent communication. The difference can not be simply related to the high dipole moment of water, since there was no correlation with dipole moment for the organic solvents used in this study.

The uniqueness of the dispersion of PBLG in *m*-cresol has been cited by Yang and Doty (1957) and Downie *et al.* (1957) on the basis of a high a_0 value. Our results show that a_0 is not anomalous when compared with values in other aromatic solvents, but that b_0 is 80° more negative than expected from the correlation of b_0 with solvent index. (By coincidence the value corresponds to b_0 values found in solvents commonly employed so this effect was not noticed previously). Yang (1958) and Yang and Doty (1957) noted a high intrinsic birefringence in *m*-cresol, and we have also reported similar results (Cassim and Taylor, 1965). Also in ethylene dibromide-cresol mixtures, b_0 did not change continuously with solvent composition but showed a sharp increase at 86 per cent *m*-cresol. This type of behavior suggests a specific solvent effect. Yang and Doty (1957) have suggested that the behavior in *m*-cresol may reflect a helical packing of the benzyl side chains. Orientation of solvent molecules may also play a role. Since it is actually the b_0 value which is anomalous, if this explanation

TABLE II

COMPARISON OF b_0 VALUES FOR VARIOUS POLYPEPTIDES AT THE SAME REFERENCE INDEX

Polypeptide	Solvent	$-b_0$	n_e	$-b_0^{1.333}$	Reference
poly-β-benzyl-D-aspartate	C	631	1.446	714	1
poly-γ-benzyl-L-glutamate	DIOX	630	1.422	710	2
		670		750	3
		682		762	4
		630		710	5
	C	625	1.446	708	6
		670		753	7
		610		693	8
	DMF	666	1.430	737	9
		620		691	10
		609		680	11
	EDC	635	1.445	717	12
		635		717	13
		595		677	14
poly-ε-N-carbobenzoxy-L-lysine	DMF	625	1.430	698	15
copoly-L-lysine-L-glutamic acid (equimolar)	2-chloroethanol	636	1.442	716	16
poly-L-methionine	C	630	1.445	712	17
	MC	630	1.424	697	18
poly-γ-methyl-L-glutamate	DMF	600	1.430	671	19
	DIOX	672	1.422	737	20
	trifluoroethanol	816	1.279	777	21
poly-L-leucine	DCA	650	1.466	747	22
poly-L-glutamic acid	H_2O pH 4.4	610	1.333	610	23
	H_2O 0.18M NaCl acetate buffer pH 4.75	600	1.333	600	24
	H_2O pH 4.88	625	1.333	625	25
	H_2O pH 4.0	580	1.333	580	26
poly-L-lysine	H_2O, 0.2M NaBr pH 11.9	650	1.333	650	27
copoly-L-tyrosyl-L-glutamic acid, 5 per cent tyrosine	H_2O 0.1M phosphate buffer pH 4.0	615	1.333	615	28

References for Table II: 1. Blout and Karlson (1958), 2. Moffitt and Yang (1956), 3. Simmons *et al.* (1961), 4. Mitchell *et al.* (1957), 5. Leonard and Foster (1963), 6. Moffitt and Yang (1956), 7. Karlson *et al.* (1960), 8. Leonard and Foster (1963), 9. Moffitt and Yang (1956), 10. Mitchell *et al.* (1957), 11. Leonard and Foster (1963), 12. Moffitt and Yang (1956), 14. Blout (1960), 14. Leonard and Foster (1963), 15. Applequist and Doty (1962), 16. Doty, Imahori, and Klemperer (1958), 17. Fasman (1962), 18. Simmons *et al.* (1961), 19. Goodman, Listowsky, Masuda, and Boardman (1963), 20. Goodman, Schmitt, and Yphantis (1962), 21. Goodman *et al.* (1963), 22. Blout (1960), 23. Blout (1960), 24. Simmons *et al.* (1961), 25. Fasman, Lindblow, and Bodenheimer (1964), 26. Miyazawa and Blout (1962), 27. Applequist and Doty (1962), 28. Urnes, Imahori, and Doty (1961).

is correct b_0 may not be entirely unaffected by side chain configuration. In the next section we will discuss variation of the dispersion parameters in terms of the effects of solvent interactions on the frequencies of the active transitions. A change in side chain configuration could affect the dispersion parameters without the requirement that the side chains be arranged on a helix, if the change permitted side chain or solvent dipoles to interact with the peptide group.

Theoretical Discussion of Solvent Effects. The application of rotatory dispersion measurements to the structure of polypeptides and proteins has been dominated by the optimistic belief that the dispersion behavior of macromolecules is in some way simpler than that of the small molecules from which they are assembled. This has led some investigators to regard all deviations of $-b_0$ from the value 630° as experimental error or evidence for differences in helical content. The present investigation has shown that the dispersion parameters depend on the solvent. The main advantage of the Moffitt plot is that the b_0 parameter depends only on the solvent refractive index and therefore can still be used as a helical parameter after correction for solvent effects. Since this situation is not very different from the problem of solvent effects on the rotation of small molecules, it is pertinent to reconsider the earlier literature on solvent effects and to attempt to explain at least qualitatively the variation in the dispersion parameters.

The Lorentz Field Problem. If the Lorentz factor $(n_s^2 + 2)/3$ is incorrect, an apparent variation of the dispersion parameters will be introduced when measurements are compared over a wide refractive index range. Since the factor is an approximate expression for spherical molecules, its use for rod-like polymers requires justification.

In the Lorentz theory the solvent in a sphere surrounding the molecule is treated as a cubic lattice of point dipoles in which case the dipole field sums to zero and the effective field arises from polarization at the boundary of the sphere. A more rigorous treatment of the problem involves a calculation of the field of induced solvent dipoles using a pair correlation function and would in general lead to a correction dependent on factors other than solvent index. However in the case of a rod-like polymer, a large correction would also arise from the asymmetry of the solute.

We will first assume that the Lorentz expression is correct for a spherical molecule and evaluate the effect of asymmetry. The effective field on a rod-like molecule was given previously (Cassim and Taylor, 1965) in connection with the theory of birefringence.

$$E'' = (n_s^2 + 2)/3(1 - \delta)E$$

$$E^{\perp} = (n_s^2 + 2)/3(1 + \delta/2)E$$

where E'' and $E^{\perp}$ are the effective fields parallel and perpendicular to the long axis of the molecule and δ is a factor dependent on molecular structure and solvent polarizability.

The optical rotation can be expressed in terms of a residue rotation tensor G_{ij}. For a solution of molecules in random orientation the measured rotation is determined by the product of the rotation tensor and the effective field averaged over all orientations. Although the average effective field is equal to the Lorentz field, the average rotation is not equal to the Lorentz field times the average value of the rotation tensor. This occurs because in the present case both the rotation tensor and the effective field depend on the orientation of the molecule so that the mean value of the product is not equal to the product of the mean values. (The occurrence of this type of effect for anisotropic molecules has been treated by Kirkwood [1936] for the related problem of dielectric polarization.)

The derivation of the expression for the average rotation is straight forward but tedious and since it leads to a very small correction it will not be reproduced in detail. Following the method used by Tinoco and Hammerle (1956) the rotation in radians per cm $[\varphi]$, is given by

$$[\varphi] = \frac{16\pi^3}{\lambda^2}\left(\frac{n_s^2 + 2}{3}\right)N\bar{G}$$

$$\bar{G} = c/(3\pi ih) \sum_A (\nu_{A0}^2 - \nu^2)^{-1}[\bar{G} + \delta/6(G_{11} - G_{22})]$$

$$\bar{G} = 1/3(2G_{22} + G_{11})$$

G_{11} and G_{22} are the molecular rotation parameters for light incident parallel and perpendicular to the helix axes. For the Lorentz field case $\delta = 0$ and the equation reduces to the result given by Tinoco and Hammerle (1956).

Evaluation of the contribution from the δ term requires a knowledge of the optical rotation of oriented molecules. The dispersion of oriented PBLG in ethylene dichloride was shown to be normal by Tinoco (1959) with a λ_0 of 270 mμ. To compare the results with the average rotation, the dispersion equation given by Tinoco must be rewritten in Moffitt form with $\lambda_0 = 212$ mμ. The values of the dispersion parameters are $a_0 = 3400°$, $b_0 = -160°$. If we take for δ the value which yields good agreement for the refractive index dependence of the birefringence of PBLG, the extra term yields a change in a_0 and b_0 of 20° and 2°, respectively, over the index range 1.364 to 1.635. Therefore we can conclude that deviations from the Lorentz field arising from asymmetry of the molecule will have a negligible effect on the dispersion parameters.

It should be emphasized that these results follow from a generalization of the Lorentz treatment. We have only shown that if the Lorentz field factor is correct for small molecules, it is adequate for asymmetric polymers. Whether the $(n_s^2 + 2)/3$ factor should appear in the theory at all is a question for which we can provide no satisfactory answer. Although this factor is a good approximation when applied to polarizabilities, in the optical rotation case, it is not clear that a valid distinction can be made between the contributions to the effective field from solvent dipoles *versus*

the dipoles induced in the groups of the solute molecule itself. A similar difficulty occurs when the Lorentz factor is applied outside the domain of static polarizabilities. The theory predicts that the integrated intensities of all transitions should be enhanced in going from the vapor to the solution phase (Chako, 1934), while no such effect has been found experimentally. Jacobs and Platt (1948) have suggested that the discrepancy may arise because short range interactions within the molecule may outweight the effect of the surrounding solvent. Thus experimental and theoretical considerations (Schuyer, 1953) suggest that the Lorentz factor is too large.

Experimentally the justification for the Lorentz factor is that in a few cases the rotation corrected for the effective field is a better constant than if the factor is omitted (Beckmann and Cohen, 1936). If the Lorentz factor is omitted in the case of PBLG the dispersion parameters still depend on index, but the variation is about half as large. It was also noted by Kneten *et al.* (1962) that inclusion of the Lorentz factor increased the variation in the rotation of hexahelicene in solvents of varying index. Therefore we conclude that the change in b_0 is real, although the variation may be somewhat exaggerated if the Lorentz factor overestimates the effective field.

Effect of Solvent Spectral Shifts on Dispersion Parameters. Attempts to correlate changes in optical rotation with solvent dipole moment have been moderately successful in some cases (Rule and McLean, 1931; Lowry, 1935). In a general theoretical treatment Beckmann and Cohen (1936) attributed changes in rotation to deformation, *e.g.*, small changes in position of atoms in the active group arising from permanent dipole interactions with the solvent. This theory predicts a correlation of rotation with the molar polarization of the solvent, $(\epsilon - 1)/(\epsilon + 2)$ where ϵ is the dielectric constant. In our experiments the rotation parameters could not be correlated with dielectric constant even though the data covered a range from $\epsilon = 2.2$ (dioxane) to $\epsilon = 37$ (dimethylformamide).

A more reasonable mechanism is the effect of solvent on the frequencies of the optically active transitions. This mechanism was discussed briefly by Lowry (1935), but as yet no attempts appear to have been made to test this hypothesis. Since experimental data on spectral shifts in polypeptides are not available, we are restricted to showing that the mechanism is plausible and that it could account for the b_0 variation as well as much of the change in a_0.

To analyze solvent effects, it is more convenient to begin with the dispersion equation proposed by Shechter and Blout (1964) (hereafter the S-B equation). These authors have shown that the dispersion arises largely from Cotton effects at 193 and 225 mμ. The transitions responsible are the strong $\pi - \pi^*$ of the amide group and the weak $n - \pi^*$ of the lone pair electrons of the peptide oxygen. The dispersion can be represented by a two term approximation to the theoretical series

$$[m']_\lambda = A_1\lambda_1^2/(\lambda^2 - \lambda_1^2) + A_2\lambda_2^2/(\lambda^2 - \lambda_2^2)$$

$\lambda_1 = 193$ mμ, $\lambda_2 = 255$ mμ, and $A_1 = 2710°$, $A_2 = -1810°$ for PBLG in dioxane. However the Cotton effect around 225 appears to be made up of contributions from

both the $n - \pi^*$ and the $\pi - \pi^*$ transition which is split by exciton degeneracy (Holzwarth and Doty, 1965). Thus the interpretation of the parameters in the S-B equation is complicated by the fact that A_2 and λ_2 do not refer to a single transition.

A general survey of solvent frequency shifts by McConnell (1952) led to the conclusion that all $n - \pi^*$ transitions in planar molecules are blue shifted in solvents of increasing polarity while strong $\pi - \pi^*$ transitions are red shifted. Frequency shifts of 1000 cm^{-1} are quite common.

Thus with increasing refractive index λ_1 should be shifted to longer wavelength while λ_2 should be unaffected or shifted to shorter wavelength. Since A_1 and A_2 are large numbers of opposite sign and the solvent effect would bring the wavelengths λ_1 and λ_2 closer together, we have a favorable case for frequency shifts to affect optical rotation.

To show that the mechanism is plausible, we compute the shifts necessary to account for the change in b_0. To compare the S-B and Moffitt equations each term of the S-B equation is expanded in powers of $(\lambda^2 - \lambda_0^2)^{-1}$, and coefficients are equated retaining only terms up to $(\lambda^2 - \lambda_0^2)^{-2}$ in the final result.

$$a_0 = (A_1\lambda_1^2 + A_2\lambda_2^2)/\lambda_0^2 \quad (1)$$

$$b_0 = [A_1\lambda_1^2(\lambda_1^2 - \lambda_0^2) + A_2\lambda_2^2(\lambda_2^2 - \lambda_0^2)]/\lambda_0^4 \quad (2)$$

These equations yield values of $a_0 = 207°$ and $b_0 = -643°$ for PBLG in dioxane in fairly good agreement with our values of 190° and −660° in this solvent. A red shift in λ_1 and a blue shift in λ_2 of 2.5 mμ ($\sim$600 cm^{-1}) leads to the values $a_0 = 331$ and $b_0 = -528°$ which should be compared with values of 349° and −505° obtained in tetrabromoethane the highest index solvent used in this study. Thus small spectral shifts are sufficient to produce the variation in b_0 and much of the change in a_0 as well.

In order to account for the dependence of the dispersion parameters on refractive index rather than dielectric constant, it is necessary to consider the mechanism of frequency shifts in more detail. The discussion is based on the general theoretical treatment of McRae (1957) who distinguishes four classes of interactions. All transitions undergo a "general red shift" through dispersive interactions, *e.g.*, the reaction field produced by dipoles induced in the solvent by the transition dipole moment of the solute. The shift is proportional to the transition probability, the factor $(n_s^2 - 1)/(2n_s^2 + 1)$ and a parameter depending on the solvent absorption spectrum. Thus this effect is important only for strong transitions. If either the solute or solvent possesses permanent dipole moments further shifts arise from polarization of solvent by solute dipoles, orientation of solvent permanent dipoles and the affect of solvent dipoles on solute dipole moment (the quadratic Stark effect). The electrostatic interactions can lead to a blue shift when the solvation energy of the excited state is less than the ground state. Hydrogen bonding to the solvent is a particular case of this type of interaction and usually leads to a blue shift (Jaffé and Orchin, 1962). Inclusion of these effects will lead to frequency shifts approximately propor-

tional to $(\epsilon - 1)/(\epsilon + 2) - (n_0^2 - 1)/(n_0^2 + 2)$ where n_0 is the solvent index at zero frequency.

In helical polypeptides the peptide oxygen is internally hydrogen bonded which serves to insulate the transitions from the influence of solvent dipoles. Also the side chains may tend to prevent orientation of solvent dipoles in the vicinity of the peptide group. The polarities of the solvents used in this study are probably not sufficient to lead to an important Stark effect, so the dominant interaction is the general red shift which is appreciable only for the strong $\pi - \pi^*$ transition. An approximate calculation using the simple red shift equation of Bayliss (1950) $\Delta\nu = k(n_s^2 - 1)/(2n_s^2 + 1)$ where $k = 1.07 \times 10^{-14}\, f/a^3\nu$, and f and a are the transition probability and the cavity radius, respectively, showed that the red shift is sufficient to explain the b_0 variation.

By combining Bayliss's equation with equation (2) for b_0 it can be shown after some manipulation that a plot of b_0 *versus* $(n_s^2 - 1)/(2n_s^2 + 1)$ should be linear, and this prediction was verified [b_0 is essentially linear when plotted against n_s, $(n_s^2 - 1)/(2n_s^2 + 1)$ or $(n_s^2 - 1)/(n_s^2 + 2)$]. A similar result should hold for the a_0 data, but the scatter is too large to draw a conclusion. Since a_0 and b_0 are related by equations (1) and (2) the general trend in a_0 can be predicted from the b_0 data. The larger scatter probably arises since a_0 is a small difference of two large numbers while b_0 is a sum of two numbers of comparable magnitude. Any deviation from the simple frequency shift due to specific solvent properties such as molecular size, absorption spectrum, *etc.*, could have a much larger effect on a_0 than on b_0.

In view of the number of Cotton effects involved and the absence of spectral data, these considerations of spectral shifts do not constitute a proof that this mechanism does account for the b_0 variation. Since there is intensity borrowing among the active transitions and hypochromic effects can depend on solvent (Vala and Rice, 1963) it is also possible that the magnitudes of the Cotton effects are solvent dependent.

It might be argued that the difference in b_0 between helix and coil is preserved and the decrease in $-b_0$ is compensated by a change in b_0 coil. This implies that the characteristic wavelength for a coil varies regularly with solvent index. Even if this were the case the mechanism responsible could also be spectral shifts of the transitions. Evidence on this point is lacking since the coil form of PBLG can not be obtained in a wide variety of solvents. However as will be shown in a subsequent communication, there is an index dependence of b_0 for polyglutamic acid in organic solvents while the b_0 of the coil form is essentially constant.

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