INTRINSIC BIREFRINGENCE OF POLY-γ-BENZYL-L-GLUTAMATE, A HELICAL POLYPEPTIDE, AND THE THEORY OF BIREFRINGENCE

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ABSTRACT The intrinsic birefringence of macromolecules can be obtained directly from flow birefringence measurements in a solvent whose refractive index matches that of the solute. A small and positive value (approximately 0.01) was found for the helical polypeptide, poly-γ-benzyl-L-glutamate. The birefringence in solvents of varying index calculated from the Peterlin-Stuart theory using this value of the intrinsic birefringence did not agree with experimental values. Considerations of polydispersity and shear deformation indicated that the discrepancy could not be attributed to these effects. Also it could not be explained in terms of specific solvent effects. It is concluded that optical properties cannot be derived from the continuum model employed by Peterlin and Stuart. Much better agreement was obtained with a helical dipole necklace model.

INTRODUCTION

The birefringence of solutions of oriented macromolecules provides information on the optical and geometrical anisotropy of the molecules which is of importance in the study of molecular structure and in the interpretation of the birefringence of biological systems such as muscle. The theory of Peterlin and Stuart (1939) has usually been employed to calculate the "intrinsic birefringence" of the molecule, *i.e.,* the difference in the indices of refraction of the molecule for light polarized along the principal axes. We have previously reported birefringence measurements on a number of fibrous proteins and tobacco mosaic virus (TMV) (Taylor and Cramer, 1963*a*) which suggested serious discrepancies in the Peterlin-Stuart theory (henceforth P-S theory). The calculated intrinsic birefringence depended on solvent refractive index and in addition the birefringence was negative in sign while evidence from biological systems, such as muscle and tendon, indicated that the sign should be positive (Taylor and Cramer, 1963*b*).

In this report we wish to consider two main questions. What is the sign and magnitude of the intrinsic birefringence of an α-helix? What are the sources of error in the P-S theory and can a theoretical or semiempirical equation be obtained which would yield better agreement with experiment?

We chose poly-γ-benzyl-L-glutamate for this study since it is generally believed to be completely helical and its properties have been studied more extensively than any other polypeptide. It will dissolve in a variety of solvents of sufficiently high refractive index that the form birefringence can be obtained without recourse to theory. In addition, it is uncharged and soluble in non-polar solvents so that any effects arising from interactions of charged molecules with polar solvents will not be important.

The intrinsic birefringence was found to be small and positive while the values calculated from the P-S theory exhibited the same type of index dependence as the proteins of our earlier study. A theoretical analysis suggests that optical properties cannot be derived from the type of model used by Peterlin and Stuart in which a molecule is treated as a continuous dielectric imbedded in a continuous medium of different dielectric constant. A dipole necklace model led to much better agreement with experiment but the model is too simple to be regarded as more than semiquantitative.

MATERIALS AND METHODS

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

(*b*) *Solvents.* The solvents were all of reagent grade. Some were further purified by distillation just prior to use. Solvents utilized in this investigation were: bromoform (B), 1-bromopropane (BP), Chloroform (C), 2-chloro-2-methylpropane (CMP), *m*-cresol (CS), 1, 2-dibromoethane (EDB), 1, 2-dichloroethane (EDC), dichloromethane (MC), dimethylformamide (DMF), *p*-dioxane (DIOX), formamide (F), pyridine (PYR), 1, 1, 2, 2-tetrabromoethane (TBE), tetramethylurea (TMU), and trichloroethylene (TCEY).

(*c*) *Solutions.* The solutions were prepared by dissolving a known weight of dried polypeptide in a known 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*) *Flow Birefringence.* Birefringence measurements were made on a Rao apparatus (model B-10), which was thermostated by circulating water from a large temperature bath set at 20°C. The gum rubber packing rings at the top and bottom windows of the cell were replaced by teflon rings to avoid contamination of organic solvents. The extinction angle and the retardation were measured both in the clockwise and counterclockwise directions at any given velocity gradient to reduce any error due to zeroing of the polarizer. The four extinction angles were measured in each direction and averaged.

The retardation was measured by means of a quarter-wave plate at a wavelength of 546 mμ utilizing a narrow band interference filter (Baird Atomic, Cambridge, Massachusetts).

(*e*) *Refractive Index.* The refractive indices of the solvents were measured at 20°C on a thermostated Zeiss refractometer (Carl Zeiss, Inc., New York) using a sodium lamp source.

(*f*) *Viscosity.* The viscosities of solvents were obtained from the literature whenever possible. If unobtainable, they were measured in Ubbelohde viscometers with solvent flow times of at least 90 seconds at 20 ± 0.01°C. Viscometers were calibrated against water.

Intrinsic viscosities of PBLG in various solvents were obtained by extrapolation of specific viscosity measurements in the concentration range from 0.025 to 0.3 g/dl. Gradient dependence of the viscosity was negligible.

RESULTS

The birefringence of PBLG was determined in a variety of organic solvents and solvent mixtures (Table I). From the Peterlin-Stuart equation the optical parameter $g_1 - g_2$ can be obtained. $\Delta n = 2\pi n_s \bar{v} c\ (g_1 - g_2)\ f(\alpha, p)$ where Δn is the

TABLE I

OPTICAL PARAMETERS OF PBLG IN ORGANIC SOLVENTS

Solvent	Solvent refractive index n_s	Optical factor $4\pi(g_1 - g_2) \times 10^2$	Intrinsic birefringence $\Delta n_i \times 10^2$
CMP-MC*	1.403	2.08	−1.08
DIOX*	1.422	1.98	−0.65
MC*	1.424	1.98	−0.76
DMF	1.430	2.27	−0.25
BP-C*	1.440	1.80	−0.34
EDC‡	1.445	2.04	−0.07
C*	1.446	1.80	−0.20
TMU	1.452	2.20	+0.17
TCEY‡	1.478	1.80	+0.40
EDC-EDB‡	1.492	1.90	+0.62
PYR	1.510	1.63	+0.74
EDB‡	1.538	1.80	+1.15
EDC-TBE‡	1.540	1.44	+0.89
CS	1.541	2.47	+1.71
EDB-B‡	1.568	1.54	+1.15
EDB-TBE‡	1.587	1.39	+1.08
B‡	1.598	1.20	+0.96
TBE‡	1.635	1.32	+0.98

All values are for $G\eta_0$ of 100 poise second^{-1}.
Solvent indices in column 2 refer to the sodium D line.
deaggregants used:
* 0.5 per cent F
‡ 1.0 per cent TMU

birefringence of the solution, and n_s, $\bar{v}$, c, $f(\alpha, p)$ are the refractive index of the solution, the partial specific volume of the solute, the concentration of solute and the orientation factor, respectively. The molecules were sufficiently asymmetric to justify the use of orientation factors and optical parameters for infinite axial ratio.

$$g_1 = (1/4\pi)(m_1^2 - 1);\ g_2 = (1/4\pi)2(m_2^2 - 1)/(m_2^2 + 1)$$

where $m_1 = n_1/n_s$ and $m_2 = n_2/n_s$. n_1 and n_2 are the refractive indices of the molecule for light polarized parallel and perpendicular to the long axis. By combining the P-S equation with the refractive index increment equation $dn/dc = 2\pi n_s \bar{v}$ $(g_1 + 2g_2)/3$, n_1 and n_2 can be calculated. To avoid measurements of refractive index increment in every solvent the average index $\bar{n}$ was calculated from $dn/dc =$ 0.115 for PBLG in chloroform (Doty, Bradbury, and Holtzer, 1956). The values of n_1 and n_2 were obtained by graphical solution of the equation for $g_1 - g_2$ with the equation $(n_1 + 2n_2)/3 = \bar{n} = 1.594$. $\bar{v}$ was taken as 0.787 (Mitchell, Woodward, and Doty, 1957; Luzzati, Cesari, Spach, Masson, and Vincent, 1961). $f(\alpha, p)$ was obtained from the tables of Scheraga, Edsall, and Gadd (1951).

A plot of the intrinsic birefringence $\Delta n_i = n_1 - n_2$ *versus* solvent index is shown in Fig. 1. (Solvent index values refer to sodium D line.) Since Δn_i is a property of the molecule it should be constant in different solvents, but the values ranged from $-$ 0.01 for $n_s = 1.403$ to +0.01 for $n_s = 1.635$. When the solvent index matches the average solute index, form birefringence is negligible so that the sign

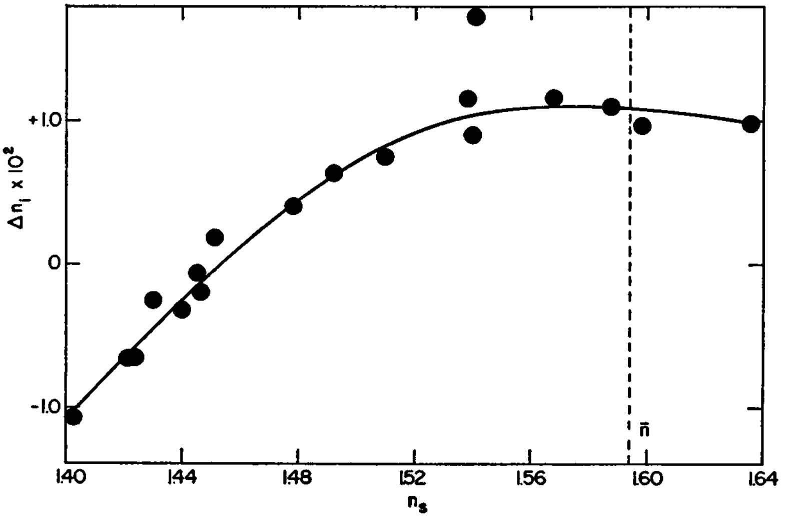

FIGURE 1 Dependence of apparent intrinsic birefringence Δn_i on refractive index of solvent n_s. The point showing the greatest deviation from the curve is the value in cresol. All values are for shear corrected for solvent viscosity, $G\eta_0$, of 100 poise seconds $^{-1}$. Solvent index values refer to the sodium D line.

of the intrinsic birefringence is independent of the validity of the expression for the optical parameter or orientation factor. Thus the sign of the intrinsic birefringence is positive but the magnitude (0.01) while it does not depend on optical theory could be in error if the orientation factor is not correct.

The P-S theory fails to predict a constant intrinsic birefringence but it must be determined whether this failure arises from an incorrect theory or because the experimental system does not satisfy the assumptions made in the derivation. The theory applies to monodisperse rigid rods at sufficiently high dilution that solute interactions are negligible. In addition the structure of the molecule must remain unchanged in all of the solvents under study. Since the PBLG system does not meet all of these restrictions the various sources of error must be evaluated.

Concentration Effects. Both the extinction angle χ and birefringence increment $\Delta n/c$ were concentration dependent in the experimental range of 0.05 to 0.4 g/dl. To obtain values at infinite dilution cot 2χ and $\Delta n/c$ were plotted against concentration and extrapolated to $c = 0$ (Yang, 1959). In all the solvents studied the plots were linear. Typical results in bromoform for various shear gradients are shown in Fig. 2 and Fig. 3. The concentration dependence of χ and $\Delta n/c$ tends to

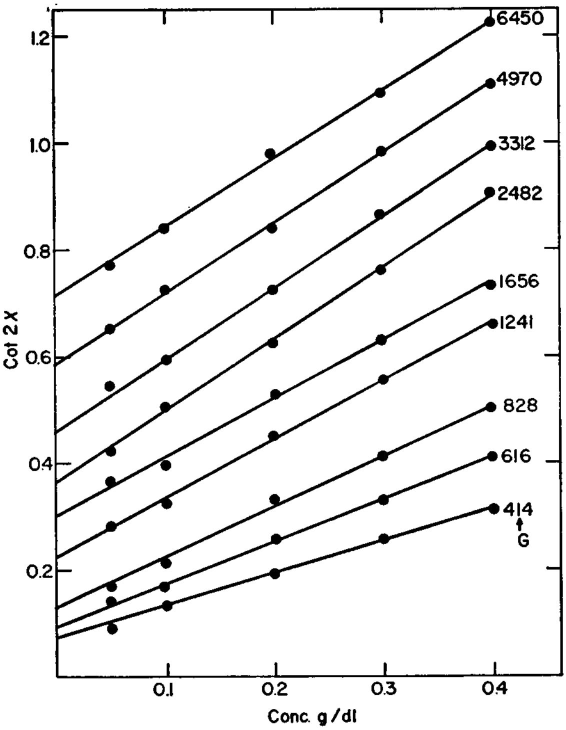

Figure 2 Extinction angles as a function of concentration at various rates of shear, *G*, in bromoform.

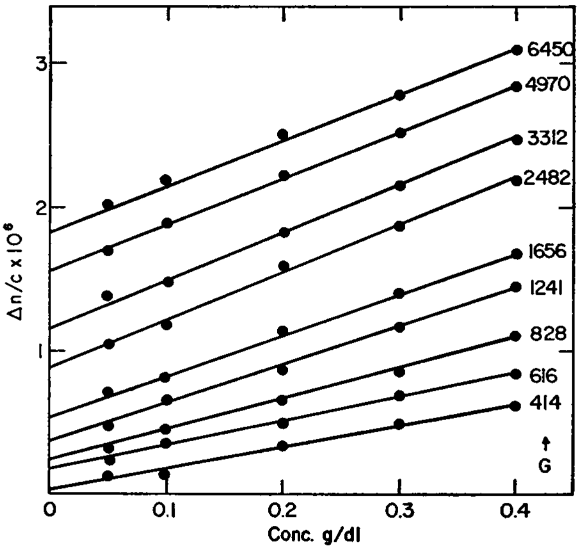

FIGURE 3 Birefringence increments, $\Delta n/c$, as a function of concentration at various rates of shear, G, in bromoform.

cancel in the calculation of $g_1 - g_2$ and the values at finite concentrations did not differ appreciably from the extrapolated values.

Effect of Solvent on Helical Content. To test for a loss in helical structure in the various solvents, the length of the molecule was calculated from measurements of extinction angle and intrinsic viscosity. Optical rotatory dispersion was measured in all of the solvents. In some solvents the infrared spectrum was determined to check for the presence of β-form. The determination of helical content from rotatory dispersion is discussed in detail in the accompanying report (Cassim and Taylor, 1965). The dispersion measurements gave no evidence of a loss of helical content or decrease in stability of the helix in the high index solvents. The presence of β-form could not be detected in the infrared spectra.

Molecular lengths were calculated from the rotatory diffusion constant θ, using the Perrin equation (Perrin, 1934) corrected by the theory of Broersma (1960) for rigid rods. Results in various solvents are shown in Fig. 4. Aggregation occurred in some solvents but this could be largely eliminated by the addition of 0.5 per cent formamide or 1.0 per cent tetramethylurea (see Table I). In eight solvents the length became independent of shear at a value of 1750 ± 50 A. In five other cases, the solvent viscosity was too low to allow the shear independent range to be reached but since the curves are nearly parallel over the shear range, the corresponding length values must be less than 2100 A with the exception of trichloroethylene. Since lengths calculated from birefringence measurements are relatively sensitive to aggregation the amount of aggregation is fairly small. The dependence of $g_1 - g_2$

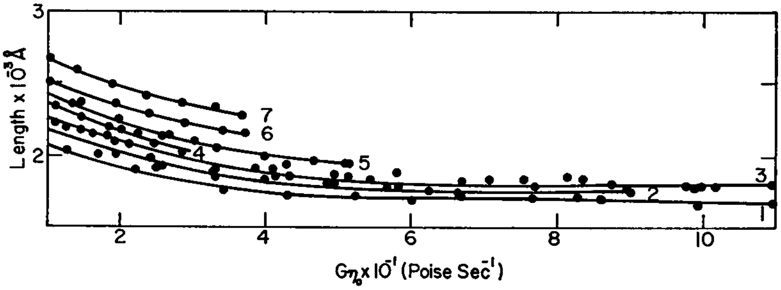

FIGURE 4 Calculated molecular length as a function of the rate of shear corrected for solvent viscosity, $G\eta_0$, in various solvents: 1. CS, 2. PYR and DIOX, 3. DMF, EDB, B, TBE, and TMU, 4. MC, 5. EDC, 6. C and 7. TCEY. Since in some cases the results in different solvents are nearly equal, they are represented by a single curve.

or Δn_t on refractive index could have been determined from the solvents in which the lengths were essentially the same and in any case variation of the optical parameters did not correlate with the degree of aggregation.

Lengths were also obtained from the intrinsic viscosity at zero gradient (Yang, 1961*a*) from the equation

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

The axial ratio p was obtained from Simha's equation (Simha, 1940) and the molecular weight from the viscosity-molecular weight data of Doty *et al.* (1956). p^2/ν has been tabulated as a function of p by Yang (1961*b*). Results are shown in Table II and can be seen to agree with the birefringence lengths at high shear. The agreement may be fortuitous since the methods are based on different hydrodynamic

TABLE II

HYDRODYNAMIC PROPERTIES OF PBLG IN ORGANIC SOLVENTS

Solvent	Solvent refractive index n_s	Intrinsic viscosity $[\eta]$	Molecular length: From flow birefringence at high shear	Molecular length: From intrinsic viscosity
			A	*A*
DIOX	1.422	—	1740	—
DMF	1.430	4.26	1800	1830
TMU	1.452	4.40	1800	1850
PYR	1.510	—	1740	—
EDB	1.538	4.20	1800	1820
CS	1.541	—	1700	—
B	1.598	4.28	1800	1840
TBE	1.635	4.32	1800	1840

models and measure different averages if the system is polydisperse. However the absolute values are not as important as the fact that the viscosity lengths are essentially the same in five solvents.

The hydrodynamic evidence in conjunction with rotatory dispersion measurements justifies the conclusion that the differences in intrinsic birefringence cannot be explained by aggregation, a loss of helical content or a collapse of the rod-like structure.

Effects of Flexibility and Polydispersity. Recent evidence has suggested that PBLG is not completely rigid (Luzzati *et al.*, 1961; Daniel and Katchalski, 1962; Spach, Freund, Daune, and Benoît, 1963). Since the optical properties are represented by a polarizability ellipsoid, small departures from a straight rod-like shape are structural features belonging to a higher level of approximation than the continuous medium treatment of Peterlin and Stuart. However, the difference between a rigid and a slightly flexible rod may appear as a difference in intrinsic birefringence. If the particles are deformable by the shear gradient, this effect could lead to an apparent shear dependence of $g_1 - g_2$.

Evidence for deformation is given by the behavior of the extinction angle at low shear gradients. For a rigid particle $-(d\chi/dG)_{G=0,\, c=0} = (\tan\alpha)_o = 1/12\theta = A\eta_o$ where A is a constant determined by the size and shape of the molecule and η_o is solvent viscosity (Peterlin and Stuart, 1939). Therefore a plot of $(\tan\alpha)_o$ *versus* η_o should be a straight line passing through the origin. If the particle is deformable the plot will have an intercept determined by the internal viscosity (Cerf, 1951). $(\tan\alpha)_o$ values were obtained from the intercepts of $(\cot 2\chi)/G$ *versus* G plots. As shown in Fig. 5 the $(\tan\alpha)_o$ plot exhibits a positive intercept (a similar plot for myosin, a rigid molecule, passed through the origin [unpublished results]). The value is quite small compared to the results for a deformable particle such as polystyrene (Cerf, 1950). Thus the degree of flexibility must be small but it could introduce some uncertainty in the absolute values of $g_1 - g_2$.

Plots of $4\pi(g_1 - g_2)$ *versus* $G\eta_o$ are shown in Fig. 6. It is evident that this parameter is shear dependent but tends to reach a plateau at high shear. This type of behavior is exhibited by all proteins and polypeptides that have been studied, and is usually attributed to polydispersity. Although part of the variation at low shear may be accounted for in this way, a second factor can be recognized. It is important to note that the curves in different solvents tend to be displaced parallel to each other. Since aggregation is quite small in many of the solvents and all particles contributing to the birefringence are long enough to have the same optical factors the effect of polydispersity will appear as an incorrect orientation factor for a particular value of $G\eta_o$. Thus the ratio of $g_1 - g_2$ values for two values of $G\eta_o$ should be the same in different solvents and therefore the slopes of the $g_1 - g_2$ curves should increase with the magnitude of $g_1 - g_2$. Instead, all values increased by a constant amount for the same change in $G\eta_o$. The curve in bromoform ($n_s =$

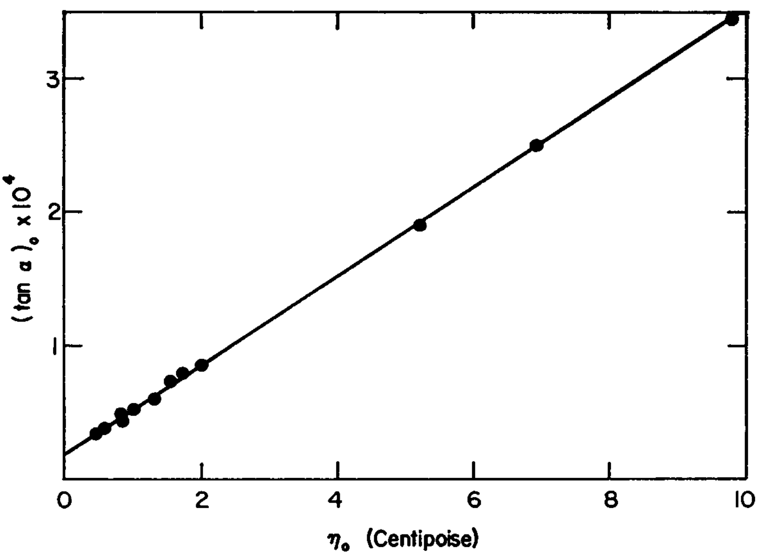

FIGURE 5 Behavior of the initial slope of the extinction angle curve, $(\tan \alpha)_0$ as a function of solvent viscosity η_0.

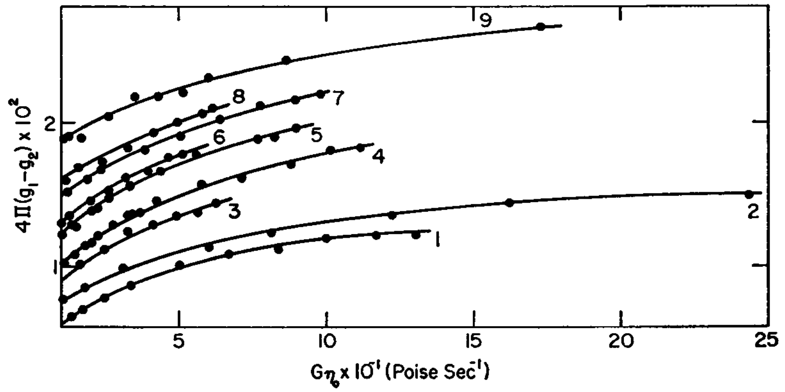

FIGURE 6 Dependence of the optical factor $4\pi\,(g_1 - g_2)$ on the rate of shear corrected for solvent viscosity, $G\eta_0$, in various solvents: 1. B, 2. TBE, 3. PYR, 4. C, 5. DIOX, and MC, 6. EDC, 7. TMU, 8. DMF and 9. CS. Since in some cases the results in different solvents are nearly equal, they are represented by a single curve.

1.598) has essentially the same shape as in other solvents even though the form birefringence is negligible in this case. Thus part of the increase in $g_1 - g_2$ with shear appears to be due to a change in the intrinsic optical properties and could arise from shear deformation. At high $G\eta_0$ values the lengths become independent of shear and although this may be partly due to a cancellation of small errors, shear dependent effects must be fairly small in this region.

To avoid long extrapolations $g_1 - g_2$ values were compared at $G\eta_o = 100$ poise second^{-1} even though the plateau has not quite been reached at this shear. Thus the calculated Δn_t values (Fig. 1) could be 20 per cent too small, but even a 50 per cent error in the magnitude of $g_1 - g_2$ would not affect the conclusion that the P-S equation is incorrect.

This point is illustrated in Fig. 7 in which $4\pi(g_1 - g_2)$ is plotted as a function

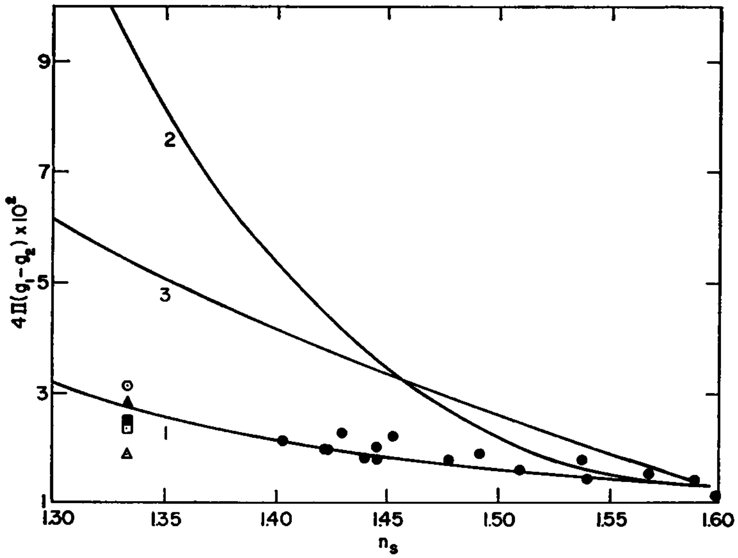

Figure 7 Dependence of optical factor, 4π $(g_1 - g_2)$, on refractive index of solvent. Curves 1, 2, and 3 are calculated from a semiemperical equation, Peterlin-Stuart equation and helical dipole model, respectively. All cases are calculated for $\Delta n_t = 0.01$. (For further details see text). Experimental points for PBLG are shown by filled circles ●. For comparison, values for proteins and poly-L-glutamic acid (PLGA) are also included: O TMV and paramyosin, ▲ tropocollagen, ■ F-actin, △ myosin and □ PLGA.

of n_s for $G\eta_o = 100$ poise seconds^{-1}. (Curve 1 was obtained from the theory discussed in a following section but the best line fitting the data is not sufficiently different to warrant a separate plot). The P-S curve for $\Delta n_t = 0.01$ (curve 2) has a much steeper slope than the experimental one. Since the $g_1 - g_2$ *versus* shear curves are approximately parallel, extension of the curves to the shear independent region would raise the values by approximately the same amount. Thus the curve representing the experimental data would be displaced upward without change of slope. Since Δn_t would also be increased, the P-S curve would be raised by an equal amount. Therefore, although there is some uncertainty in the magnitude of $g_1 - g_2$, the variation in this parameter with refractive index cannot be reconciled with the the P-S theory.

DISCUSSION

The specific birefringence, $\Delta n/f(\alpha,\ p)\bar{v}c$ for PBLG was measured in solutions ranging in refractive index from 1.403 to 1.635. When the index of the solvent is equal to the average index of the solute, the form contribution is negligible and the intrinsic birefringence is obtained without recourse to optical theory. A value of approximately +0.01 was obtained by this method, but in view of the errors discussed in the previous section, the value could be 50 per cent low. Thus the intrinsic optical anisotropy of an α-helix is relatively small. For example it is the order of one-tenth the birefringence of glutamic acid or glycine crystals.

From a knowledge of the intrinsic birefringence we can determine directly the validity of the P-S theory. The optical parameter calculated from this theory is plotted in Fig. 7 and can be seen to deviate markedly from the experimental values as the difference in solute and solvent indices increases.

Since the theoretical value is much too large, we can understand why the intrinsic birefringences of fibrous proteins are consistently negative when calculated from this theory. The form contribution is overestimated and this error must be compensated in the calculation by assigning a large negative intrinsic birefringence to the molecule.

In Table III the specific birefringences of a number of proteins and polypeptides are given for the index 1.333 (water). In view of difficulties of absolute birefringence measurements the spread in the values is comparable to the experimental error.

The molecules have in common a high axial ratio and similar average refractive indices. Thus the contribution from form birefringence should be very similar although the magnitude is difficult to calculate exactly. Therefore the intrinsic bire-

TABLE III

SPECIFIC BIREFRINGENCE OF PROTEINS AND POLYPEPTIDES

Molecule	Specific birefringence $\Delta n/f(\alpha, p)\bar{v}c \times 10^2$	Reference
Poly-L-glutamic acid	1.6*	Cassim (unpublished observations)
PBLG	1.9*	
TMV	2.1	Taylor and Cramer (1963*a*)
Tropocollagen	1.9	" " " "
Myosin	1.35	Cassim (unpublished observations)
Paramyosin	2.1	Taylor and Cramer (1963*a*)
F-actin	1.7	Tobias and Taylor (unpublished observations)

All data refer to refractive index 1.333.
* Extrapolated to index of water.

fringences must also be roughly equal even though the detailed molecular structures are quite different. PBLG is an α-helix; myosin is partly α-helical; tropocollagen is a triple helix with different parameters than the α-helix; *F*-actin is built from globular protein subunits. Thus within the limits set by a rather large experimental error, the birefringence technique is not capable of distinguishing differences in molecular structure among highly asymmetric proteins. Since birefringence is essentially a scattering phenomenon (Taylor and Cramer, 1963*b*), we might not expect to "resolve" differences between helical and globular molecules when both are rod-like in shape.

The specific birefringences given in Table III, plus data in the literature on other proteins (reviewed in Taylor and Cramer, 1963*a*) provides additional support for the conclusion that the P-S equation overestimates the birefringence.

There is no evidence for a difference in birefringence in organic and aqueous solvents in addition to the continuous variation with refractive index. Thus if an ordered water shell surrounds a protein molecule in aqueous solution, it does not contribute appreciably to the birefrigence. However, a large solvent effect did occur for PBLG in *m*-cresol. The meaning of the increased birefringence is not clear but an anomaly also occurs in the optical rotatory dispersion in this solvent (Cassim and Taylor, 1965) which may be indicative of solvent or side chain orientation.

With the exception of cresol the results for a variety of proteins and polypeptides tend to fall on a smooth curve when plotted against index which argues against the presence of large specific solvent effects. Therefore the results are not very sensitive to finer details such as the size of the solvent molecule or the distribution function for solute-solvent correlation. Since the same variation of the calculated intrinsic birefringence with index occurs for proteins and polyglutamic acid as for PBLG it is also unlikely that side chain orientation can be invoked to explain the effect.

Theory of Birefringence A rigorous birefringence theory requires the calculation of the polarizability tensor of a macromolecule from the properties of the atoms of groups and also a calculation of the effective field arising from the mutual interaction of the molecule with the solvent and applied field. Since this is a formidable theoretical problem we will first consider an idealized system, namely, a dilute gas of oriented macromolecules. This system serves to illustrate certain features of a theory of birefringence without the added complication of the calculation of the effective field. The results of the calculations can still be compared roughly with the experiments by extrapolation of birefringence data to $n_s = 1$.

We will first consider a system of macromolecules whose general shape can be approximated by an ellipsoid of revolution. We choose a set of axes **i**, **j**, **k** and suppose that the molecules are oriented with the axes of the ellipsoid parallel to **i** and **j**. A plane polarized electromagnetic wave will induce a dipole moment in the molecule $\mathbf{p} = \alpha \cdot \mathbf{E}$ where α is the polarizability tensor. For simplicity we will assume that the incident wave is propagated in the **k** direction with its plane of polarization parallel to either **i** or **j**. In this case the polarizability tensor is diagonal in the **i j k** system and we have

$$n_i^2 - 1 = 4\pi P/E = 4\pi N\alpha_{ii}$$

where N is the number of molecules per unit volume.

The birefringence is simply

$$\Delta n = 2\pi N(\alpha_{ii} - \alpha_{jj}) \tag{1}$$

If the particle orientation is specified by a distribution function then it can be shown (Taylor and Cramer, 1963*b*) that the right-hand side of equation (1) is multiplied by the factor f appearing in the P-S equation.

The quantity α_{ii} is given by

$$\alpha_{ii} = \frac{e^2}{4\pi^2 m}\sum_q f_{(q)ii}/(\nu_q^2 - \nu^2) \tag{2}$$

where $f_{(q)ii}$ is the element of the oscillator strength tensor for the transition of frequency ν_q. Since the evaluation of Δn from equation (1) requires a knowledge of the direction and magnitude of the dipole moments of all electronic transitions, it is necessary to make a number of approximations in order to evaluate α_{ii}. Equation (1) is actually a dispersion equation and the dispersion of many substances can be represented by replacing the summation in equation (2) by a single term. We replace equation (2) by $\alpha_{ii} = \alpha_i\, \lambda^2/(\lambda^2 - \lambda_o^2)$ where λ_o is some characteristic wavelength and α_i is the static polarizability.

λ_o is usually less than 150 mμ, thus $\lambda^2/(\lambda^2 - \lambda_o^2)$ will usually be close to unity in the visibile region. In addition a partial cancellation of the wavelength dependent part of α will occur in taking the difference. Therefore the use of static polarizabilities should lead to only a small error in Δn and could not account for the large discrepancy noted in the present experiments.

The P-S treatment makes the further assumption that a molecule can be regarded as a continuous dielectric and therefore

$$\alpha_i = \frac{v}{4\pi}\frac{n_i^2 - 1}{1 + L_i(n_i^2 - 1)}$$

where v is the molecular volume, L_i is a shape factor and n_i is the refractive index of the "molecule" for light polarized along the i axis.

This assumption can hardly be justified. A medium can be treated as continuous if it can be divided into a large number of volume elements whose dimensions are small compared with the dimension of the sample but still large enough to contain a large number of atoms. These conditions are not satisfied by proteins and polypeptides.

The assumption may be based on a misunderstanding of some common results.

The average polarizability of molecules in solution can be calculated from the Lorentz theory (Lorentz, 1952) by treating molecules as dielectric spheres. Calculated values are in good agreement with measurements of polarizability in the gas state. Applications of the theory are essentially concerned with average properties of macroscopic samples and thus provide no justification for applying the theory to calculate small differences in the principal polarizabilities of a single molecule. Since $(\alpha_1 - \alpha_2)/\bar{\alpha} \cong 0.05$, a very accurate calculation of the α's is necessary to obtain even an approximate value for the birefringence.

Dipole Necklace Model of Birefringence A better approximation to the static polarizability could be obtained by treating all the atoms as polarizable dipoles and calculating their mutual interactions (Silberstein, 1927). However this approach requires a detailed knowledge of atomic positions and in any case would be completely intractable when applied to solutions since induced solvent dipoles markedly affect the birefringence. We therefore adopt a very simple model which can be applied in solution and which will at least provide the basis for a semiempirical equation.

The molecule is represented as a chain of spherical residues of radius a. Let α_1 and α_2 be the polarizabilities of the isolated residues. The polarizabilities of the molecule α_i, α_j are to be calculated from interactions between point dipoles induced at the center of each residue. The theory will contain a parameter determined by the positions of the residues which will be referred to as the structure factor. We derive this parameter for straight, zig-zag and helical models.

(*a*) *Straight chain model.* The residues are arranged on a line with coordinates $0, \pm 2ka$, $k = 1, 2,$ *etc*. The dipole moment of residue m is $\mathbf{p}_m = \alpha \cdot \mathbf{E}$ where $\mathbf{E} = \mathbf{E}_0 + 2\sum_{m=1}^{\infty} \mathbf{E}_{mn}$ and $\mathbf{E}_{mn}$ is the field at m due to moments induced in all other residues

$$\mathbf{E}_{mn} = (\nabla\nabla + K^2 U)e^{-iKr_{mn}}\mathbf{p}_n/r_{mn} \tag{3}$$

K is the wave number, $K = 2\pi/\lambda$, ∇ is the gradient operator and U is the unit tensor. For the sake of clarity we again treat the case of a wave incident perpendicular to the long axis, with polarization either parallel or perpendicular to the axis. Since we are interested in the static polarizability and the interaction is dominated by the region for which $r_{mn} \ll \lambda$ we set $K = 0$ in equation (3), which reduces to

$$\mathbf{E}_{mn} = \frac{3\mathbf{p}_n \cdot \mathbf{r}}{r_{mn}^5}\mathbf{r} + \frac{p_n}{r_{mn}^3}$$

The solution is obtained by a series of successive approximations. For the field parallel to the chain we have for the 0^{th} approximation

$$\mathbf{p}_i^{(0)} = \alpha_1 \mathbf{E}_0$$

The extra field of all remaining residues is $\mathbf{E}_1 = \mathbf{p}_i^{(0)}/(2a^3)\sum_1^{\infty}(1/k^3)$. The extra moment $\mathbf{p}_i^{(1)} = \alpha_1 \mathbf{E}_1$ *etc*. The successive terms form a geometric series and the final result is

$$\mathbf{p}_i = \alpha_i \mathbf{E}_0 \text{ where } \alpha_i = \alpha_1/(1 - F\alpha_1/a^3) \text{ and } F = (1/2) \sum_1^{\infty} 1/k^3$$

Similarly, for the field perpendicular to the axis we have

$$\alpha_j = \alpha_2/[1 + F\alpha_2/(2a^3)]$$

(It is necessary to assign polarizabilities α_1 and α_2 to the residues to account for intrinsic birefringence in solution.) The birefringence equation is simply $\Delta n = 2\pi N(\alpha_i - \alpha_j)$.

(*b*) *Ziz-zag model.* The residue centers are in a plane with center to center distance $2a$, and an angle between lines joining centers of 120°. The result differs from the first model only in the value of F. There are three values for the three field directions but it can be shown that $F_1 = (F_2 + F_3)/2$

$$F_1 = (10.392)^{-1} \sum_1^{\infty} 1/k^3 + \sum_0^{\infty} \frac{(3/2)[(k + 1/2)^2 - 1/16]}{[3(k + 1/2)^2 + 1/4]^{5/2}}$$

(*c*) *Helical model.* We choose four residues per turn and a translation b per residue. For simplicity the residues are in contact, $b^2 = 2a^2$. The coordinates of successive residues are $(a, 0, 0)$, $(0, a, b)$, $(-a, 0, 2b)$, $(0, -a, 3b)$ etc. Calculations of F_1 and F_2 are straightforward but tedious and will not be reproduced here.

All series summations were carried out for ± 30 residues on an IBM 7094 computer. Series convergence errors were estimated from the corresponding integrals.

Values of α_1 and α_2 were chosen to correspond with the intrinsic birefringence of PBLG. The form birefringence is essentially zero when the solvent matches the average index of the molecule. For a molecular model this means that induced dipole interactions between the residues are compensated by dipole interactions with the solvent. The intrinsic birefringence is therefore given by the polarizabilities of the isolated residues. It should be noted that the residues are isolated in the sense that induced dipole interactions are zero, but any changes in polarizability arising from permanent dipoles or covalent bonding in the polymer are absorbed in the choice of experimental α values. The polarizabilities of spherical particles can be obtained from the Lorentz theory so we take $\alpha_1 = (v/4\pi)3(n_1^2 - 1)/(n_1^2 + 2)$. Since the intrinsic birefringence of PBLG is approximately + 0.01, the indices were assigned the values $n_1 = 1.60$, $n_2 = 1.59$. Let $\alpha_i = (v/4\pi)g_i$, and $Nv = \varphi$ where φ is the volume fraction. The birefringence equation becomes

$$2(\Delta n)/\varphi = g_i - g_j = [g_1/(1 - F_1 g_1/3) - g_2/(1 + F_2 g_2/6)]$$

Values of $g_i - g_j$ calculated from the three models and from the P-S theory are shown in Table IV. Birefringence data on PBLG as a function of solvent index was extrapolated to $n_s = 1$. The extrapolation was made from a plot of Δn *versus* $(n_s - 1.6)^2$ which was approximately linear. In view of the long extrapolation and

TABLE IV

BIREFRINGENCE OF NECKLACE MODELS

MODEL	F_1	F_2	g_i	g_j	$g_i - g_j$
Peterlin-Stuart theory	—	—	1.560	0.867	0.693
Straight necklace	0.600	—	1.291	0.920	0.371
Zig-zag necklace	0.465	—	1.220	0.940	0.280
Helical necklace	0.265	0.314	1.128	0.962	0.166
Experimental (PBLG)*	—	—	—	—	0.08 ± 0.04

* Data from the index range 1.403 to 1.635, extrapolated to $n_s = 1$.

the errors discussed in the Results section, the value could be in error by at least 50 per cent, and is probably too low.

The birefringences of the necklace models are all less than the value for a continuous dielectric. The lowest value is given by the helical model and it is moderately close to the experimental result. A calculation of $g_i - g_j$ to an accuracy of 20 per cent requires an accuracy of 2 per cent in the individual values, and in view of the simplicity of the model and the sensitivity of the result to the structure factor F the agreement may be fortuitous. Care must therefore be taken in drawing conclusions from the fact that the helical model gave the best agreement. The largest value was obtained with the straight chain model. This is to be expected since the fields of the residues introduce terms of the form $(3 \cos^2 \theta - 1)/r^3$. The difference in the interactions between adjacent residues for the two orientations of the applied field will be largest for $\theta = 0$ and $\pi/2$ which are the values for the straight necklace model. The difference is reduced as the θ values approach each other and the helical model has the smallest difference in the angles. As the first two models are planer, only the helical model gives a realistic three dimensional distribution of the residues. Although the value is of the correct magnitude a number of non-helical distributions would probably give similar results. Experimentally, it was found that a number of proteins and polypeptides with quite different arrangements of the residues had very similar birefringence values.

Birefringence in Solution In solution the birefringence is dependent on the polarizability of the solvent. Two effects have to be considered. (*a*) The effective field acting to polarize the molecule is determined by the field of solvent dipoles induced both by the applied field and by the dipole moment of the solute. (*b*) The part of the solvent polarization arising from solute dipoles depends on the orientation of the solute and will therefore contribute to the birefringence.

In the P-S theory these effects are concealed in the notation by using excess polarizabilities relative to the solvent. Although the derivation is thereby simplified the polarizabilities are no longer molecular properties since they depend on the refractive index of the solvent as well as the solute. The same equation can be obtained by starting with the polarizability of an isolated ellipsoid but the derivation will not be given here since the results are essentially contained in a comprehensive review of dielectric theory by Fuller-Brown (1956). We note only that the P-S equation is equivalent to Onsager's theory (Onsager, 1936) generalized to ellipsoidal particles and that the solvent polarization difference contributes up to 10 per cent of the birefringence.

To apply the dipole model to solutions, the surrounding solvent molecules must be treated as a collection of point dipoles. Any birefringence theory must satisfy a consistence condition namely that the birefringence must be zero for an optically isotropic molecule if the solvent index matches the index of the molecule. This condition can be satisfied if the solvent and solute dipoles are treated equivalently. Thus we can not use a dipole model for the solute and a continuum model for the solvent.

In order to evaluate the contribution of solute and solvent dipoles to the effective field we will generalize the lattice method of Lorentz (1952) to non-spherical particles. In the Lorentz treatment a large sphere is drawn around the particle. Matter outside the sphere can be treated as continuous, yielding a field $(n_s^2 - 1)/3\mathbf{E}_0$. Solvent inside the sphere is treated as a cubic lattice of point dipoles, in which case the field sums to zero. The total effective field is then

$$\mathbf{E}_0 + (n_s{}^2 - 1)/3\ \mathbf{E}_0 = (n_s{}^2 + 2)/3\ \mathbf{E}_0.$$

This result is generally regarded as only approximately correct but a more rigorous treatment would involve averaging the field using a pair distribution function. This function will depend on the particular solute-solvent system and is generally unknown. Since the experimental data did not show large specific solvent effects the birefringence is apparently not sensitive to solvent packing. Thus the error introduced by treating the solvent according to a lattice model should be small compared with the deviation from the Lorentz result introduced by asymmetry of the solute molecule.

We consider first the effective field on a residue of the straight chain model. It is assumed that solute residues and solvent molecules may occupy the points of the same lattice. The addition of a solute molecule requires the removal of a line of solvent dipoles of polarizability α_s. The lattice dipole field will no longer be zero and we calculate the field by a series of successive approximations as before. Dipole interactions of the type solute-solvent and solute-solvent-solute will be taken into account but solvent-solvent interactions will be considered in first approximation only. This restriction will not be serious for the calculation of the effective field on the solute but will be important for the average solvent field. Since we are primarily concerned with the difference in solvent polarization induced by the solute and this effect contributes about 10 per cent of the total birefringence, an approximate calculation of this term will be sufficient. We will therefore restrict the calculation of solvent polarization to the adjacent solvent layer. In this region the solute-solvent terms will outweight the solvent-solvent interactions.

The field $\mathbf{E}$ is applied parallel to the long axis of the straight chain model. The 0^{th}

approximation is $\mathbf{p}_i^{(0)} = \alpha_1\mathbf{E}$ and $\mathbf{p}_s^{(0)} = \alpha_s\mathbf{E}$ where subscript s refers to solvent. The extra field of all solute residues is $\mathbf{p}_1^{(0)}S_1$ as before, where $S_1 = 1/(2a^3)\sum_1^\infty 1/k^3$. The field of solvent dipoles is made up of two terms, the field of all solvent residue in a sphere minus the field of the line of dipoles removed to accommodate the particle. The first term is zero and the second is $-\mathbf{p}_s^{(0)}S_1$. The extra solute moment is

$$\mathbf{p}_i^{(1)} = \alpha_1(\alpha_1 - \alpha_s)S_1\mathbf{E}$$

The extra field on a solvent residue at distance $2at$ arising from the difference in solute-solvent polarizability yields an extra solvent moment

$$\mathbf{p}_s^{(1)} = \alpha_s(\alpha_1 - \alpha_s)S_2\mathbf{E}$$

where

$$S_2 = 1/(2a)^3 \sum_{-k}^{k} (2k^2 - t^2)/(k^2 + t^2)^{5/2}$$

Because of the cylindrical symmetry of the problem we approximate the solvent lattice by placing $2\pi t$ dipoles on a ring of radius $2at$. The second approximation is

$$\mathbf{p}_i^{(2)} = \alpha_1\mathbf{p}_i^{(1)}S_1 + \alpha_1 \sum_1^t 2\pi t\mathbf{p}_s^{(1)}S_2$$

Since solvent-solvent terms are not included

$$\mathbf{p}_s^{(2)} = \alpha_s\mathbf{p}_i^{(2)}S_2$$

Higher approximations can be written down by inspection. By grouping terms into geometric series we obtain finally

$$\mathbf{p}_i = \alpha_i'(1 - q_1\alpha_s^2)/(1 - q_1\alpha_s\alpha_i')\mathbf{E}$$

where $\alpha_i' = \alpha_i(1 - F\alpha_s/a^3)$ and $q_1 = 1/(2a)^6\sum_1^t 2\pi t(S_2)^2$. The summation is to be taken over solvent dipoles in a large sphere, so the condition $k + t$ equal to a constant must be imposed in evaluating q_1.

A similar calculation for the field perpendicular to the chain axis yields $\mathbf{p}_t = \alpha_t'(1 + q_2\alpha_s^2)/(1 + q_2\alpha_s\alpha_t')\mathbf{E}$. In this case the field on a solvent dipole depends on the angle φ between the applied field and the radius vector to the dipole. The summation over a ring of dipoles was approximated by $2\pi t \cos^2\varphi$ which yields $q_2 = q_1/2$. Although the positioning of solvent molecules is somewhat artificial and contradicts the original assumption of a cubic lattice the values of q_1 and q_2 should be at least approximately correct and some important conclusions can be drawn.

The effective polarizability is made up of three factors. The polarizability of the particle in vacuum α_i, a factor $(1 - F\alpha_s/a^3)$ which is a cavity field arising from the exclusion of solvent dipoles by the particle, and a factor $(1 - q_1\alpha_s^2)/(1 - q_1\alpha_s\alpha_i')$ which is a reaction field produced by polarization of the surroundings by the solute.

To evaluate q_1 and q_2, values of $\sum_2 = (2a)^3S_2$ were computed up to $t = 6$ beginning with $k = 30$ (Table V). Convergence is slow and in fact the sums begin to increase

TABLE V

VALUE OF Σ_2 FOR $k + t = 31$

t	Σ_2
1	−0.14766
2	−0.00348
3	−0.00239
4	−0.00252
5	−0.00262
6	−0.00264

slightly at $t = 4$. This occurs because the terms for $k \neq 0$ progressively change sign as t increases and for $t \gg k$ they approach k/t^3. By evaluation of the corresponding integrals it can be shown that the magnitudes of the terms are less than $2/t^2$. Therefore $q_i < 8\pi/(2a)^6 \sum_1^t 1/t^3$. Substitution of values of the parameters appropriate to aqueous solution yields $p_i - p_j \cong 1.005(\alpha_i - \alpha_j)E$. Thus the reaction field can be neglected and the remaining factors are not sensitive to the distribution function for solvent molecules.

Finally we require an expression for the extra polarization of the solvent. The extra polarization of a solvent molecule at distance $2at$ is $\alpha_s(\alpha_i' - \alpha_s)S_2$ for the parallel field, and we will retain only the term for the first solvent layer, $t = 1$. Substitution of these results into the equation $(n_s^2 - 1)\mathrm{E} = 4\pi\mathrm{P}$ we have

$$3\left(\frac{n_s^2 - 1}{n_s^2 + 2}\right) = 4\pi(N\alpha_i' + N_s[\alpha_s(\alpha_i' - \alpha_s)s_1 + \alpha_s])$$

where N and N_s are the number of solute and solvent molecules per cc. The Lorentz factors $(n_s^2 + 2)/3$ is introduced by the presence of the continuous distribution of matter outside the sphere. After combining this result with the equation for the perpendicular field and substituting for the α's in terms of the g's we have finally

$$\Delta n = \frac{1}{2n_s}\left(\frac{n_s^2 + 2}{3}\right)^2 \varphi[g_i' - g_j' + s_1 g_s(g_i' - g_s) - s_2 g_s(g_j' - g_s)]f(\alpha,p) \quad (4)$$

where s_1 and s_2 are the values computed for the summation over the first solvent layer. $f(\alpha, p)$ is the P-S distribution function which can be taken over without change since it does not depend on the expressions for the optical parameters.

The helical model can be treated in the same manner. Since the structure factor is smaller the reaction field would not be larger than for the straight chain case and this effect can therefore be ignored. To calculate the solvent polarization we consider only the adjacent solvent layer. The solvent packing is approximated by placing eight dipoles on a ring of radius $3a$ which slightly underestimates closest packing. The series summations are tedious since there are six different values for the fields at the solvent positions. $s_1 = 0.0629$, $s_2 = 0.1280$.

The birefringence calculated from the helix model is shown in Fig. 7, curve 3. As the calculated polarizability difference of the isolated molecule was 50 per cent too large, the theoretical curve will overestimate the experimental values for PBLG. At an index of 1.33 the result is about one half the value given by the P-S theory (curve 2), but it is still 50 per cent larger than the experimental value.

A value of the structure factor F of 0.15 leads to approximate agreement for the isolated molecule. If this value is used in calculating g'_i and g'_j, curve 1 is obtained which fits the experimental data reasonably well. Thus the theory predicts the correct shape of the birefringence *versus* solvent index curve, although the obsolute value is in error through a failure to give the correct results for the polarizabilities of the isolated molecule.

Although the theory is not entirely satisfactory, the computational difficulties encountered by even a very simple model suggest that further progress in this direction will be difficult. The birefringences of a variety of proteins and polypeptides over a wide index range can be represented by a semiempirical equation with the structure factor equal to 0.15. To apply the equation to other molecules the structure factor may be regarded as a parameter to be determined experimentally.

Equation (4) can be rewritten in the form:

$$\frac{2n_s\Delta n}{f(\alpha,\,p)\bar{v}c}\left(\frac{3}{n_s^2+2}\right)^2 - g_s^2(s_1 - s_2) = g_i'(1 + g_s s_1) - g_j'(1 + g_s s_2) \qquad (5)$$

The quantities on the left-hand side are known if we take for s_1 and s_2 the values calculated from the helix model. A second relation is obtained from the average index of the solution. The extra solvent polarization terms do not cancel but they contributed only a few per cent of the total so that approximately:

$$\frac{1}{\bar{v}}\frac{dn}{dc}\left(\frac{6n_s}{n_s^2+2}\right) = \bar{g}' - g_s \qquad (6)$$

g_s is the solvent polarizability per unit volume and $\bar{g}' = (g'_i + 2g'_j)/3$. Equations (5) and (6) can be solved for g'_i and g'_j. To obtain the molecular parameters g_i and g_j, measurements must be made over a range of refractive indices and the value of F which satisfies the condition $g_i - g_j =$ constant must be determined by trial and error. (For example a plot of $g'_i/(1 - Fg_s/3) - g'_j/(1 + Fg_s/6)$ *versus* g_s should yield a straight line of zero slope for the correct F value.) For reference purposes definitions of the symbols are given below

$$g_i' = g_i(1 - Fg_s/3) \qquad g_j' = g_j(1 + Fg_s/6)$$

$$g_i = g_1/(1 - Fg_1/3) \qquad g_j = g_2/(1 + Fg_2/6)$$

$$g_1 = 3(n_1^2 - 1)/(n_1^2 + 2)$$

and similar equations for g_2 and g_s. n_1 and n_2 are the refractive indices of the protein and n_s is the index of solvent. $s_1 = 0.0629$ and $s_2 = 0.1280$.

Relation of the Dipole Model to the Dielectric Approximation. The continuous dielectric treatment applied to a molecule predicts a specific birefringence which is two or three times too large. If the diameter of the particles is increased to 1000 A the dielectric treatment would probably be a reasonable approximation. However for diameters larger than 500 A the phase difference for light passing through the particles *versus* the solvent can no longer be neglected (Taylor and Cramer, 1963*b*). Thus in the intermediate range from about 50 to 500 A, specific birefringence may be a function of particle diameter. At present there is no experimental evidence available to support this conjecture. Previous measurements on TMV (150 × 3000 A) gave about the same specific birefringence as fibrous proteins (Taylor and Cramer, 1963*a*). The intrinsic birefringence of TMV may be zero (Lauffer, 1938) in which case the total birefringence would be almost twice as large as expected from the dipole model.

Since fibers occurring in biological systems are often around 200 A in diameter, further studies on birefringence *versus* particle diameter are necessary.

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