BIREFRINGENCE OF PROTEIN SOLUTIONS AND BIOLOGICAL SYSTEMS

II. Studies on TMV, Tropocollagen, and Paramyosin

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ABSTRACT The intrinsic birefringences of TMV, tropocollagen, and paramyosin were calculated from flow birefringence measurements using the theory of Peterlin and Stuart. The values are −0.029, −0.029, and −0.030, respectively. The intrinsic birefringences of TMV and tropocollagen were measured as a function of the refractive index of the solvent in glycerol-water mixtures. In both cases the values were not constant and became less negative as the refractive index increased. Theoretical calculations showed that the large solvent effect could not be caused by a hydration shell of index different from that of the bulk solvent. It is concluded that either (*a*) the intrinsic birefringence calculated from the Peterlin-Stuart theory is incorrect or (*b*) the intrinsic birefringence depends markedly on the solvent. These results are of importance to the problem of quantitative polarized light microscopy since the separation of form and intrinsic birefringence contributions is based on the assumption that intrinsic birefringence is independent of solvent.

INTRODUCTION

The intrinsic birefringence of rigid molecules can be calculated from measurements of flow birefringence and refractive index increment according to the theory of Peterlin and Stuart (1). It was shown previously (2) that the theory is applicable to molecules which are not small compared to the wavelength of light. This extension was necessary because molecules which can be oriented in aqueous solution have lengths of at least 1000 Å and many molecules of interest have lengths of 3000 Å or more. Sufficient data on proteins has not so far been available to enable conclusions to be drawn regarding the relation of the sign and magnitude of birefringence to protein structure. (In this paper the word birefringence will be used to designate intrinsic birefringence only. Form and flow birefringence will be referred to explicitly.) In particular, it may be asked whether birefringence is affected by the helical content and folding of protein chains. Also, the birefringence is a parameter which is necessary for the quantitive interpretation of the total birefringence of biological structures such as muscle.

The Peterlin-Stuart theory has not been adequately verified and there is no independent method for obtaining the birefringence of a protein molecule. An indirect check on the consistency of the theory can be obtained by determining the birefringence in aqueous solution and using this value to predict the flow birefringence in solvents of different refractive index. In so doing it is necessary to assume that the intrinsic birefringence is independent of solvent. This assumption has been made in almost all quantitative studies on biological systems. For the special case of optically isotropic molecules the flow birefringence can be calculated from the refractive index increment.

The experiments were performed on three macromolecules. Tobacco mosaic virus was chosen for investigation because Lauffer (3) had shown it to be optically isotropic in a solution of glycerol-aniline-water of refractive index about 1.57. Tropocollagen was selected since it is a long rigid molecule of known dimensions (4). In addition, the birefringence can be related to total birefringence of collagenous structures such as tendon. Measurements were also made on paramyosin because it is generally regarded as a helical protein. Measurements were made in aqueous solution and glycerol-water mixtures. Glycerol was used to vary the refractive index because it does not denature proteins and data on other molecules have been obtained in this solvent system (5). The birefringences of these three molecules, of distinctly different structure proved to be very similar and in addition were markedly dependent on the composition of the solvent.

EXPERIMENTAL

A sample of TMV (sample A) was kindly provided by Dr. Norman Simmons. It was stored at 4°C in 0.01 molar phosphate buffer, pH 7. Concentration of the stock solution was determined from the optical density at 265 mμ using the extinction coefficient of 30.6 provided by Dr. Simmons. The partial specific volume was taken as 0.73 (6). Measurements of $\frac{dn}{dc}$ at 546 mμ were made on a Brice-Phoenix differential refractometer through the courtesy of Dr. Martin Mathews, yielding a value of 0.190. All solutions were made by gravimetric dilution from the stock. The sample was nearly homogeneous when first received, but over the course of a few months some aggregation was detected. Since one of the earlier measurements yielded a less negative value for the birefringence, the measurement was repeated on another TMV sample (B) provided by Dr. Robert Haselkorn. This sample gave a single peak in the ultracentrifuge and a very small variation of rotary diffusion constant with shear. It was therefore judged to be homogeneous, and it yielded a birefringence at high shear identical with that of the slightly aggregated sample.

Ichthyocol tropocollagen was a gift of Dr. Melvin Glimcher. The sample was stored at 4°C in citrate buffer, pH 3.5. It was clarified before use by centrifugation after dilution to bring the concentration below 0.2 gm/100 ml (4). The partial specific volume is somewhat uncertain. Gallop (7) gave the value 0.705 for gelatin. This value is probably low for collagen and a value of 0.75 was used in some of the calculations. The error due to uncertainty in $\bar{v}$ will be discussed below. Concentration of the stock solution was determined by the micro-Kjeldahl method (8) using a value of 17.5 per cent for the nitrogen content (7). The refractive index increment was taken as 0.187 (4). Examination

of the sample in the ultracentrifuge showed the presence of a shoulder on the leading side of the peak indicating the existence of a small amount of more rapidly sedimenting material. The rotary diffusion constant at low shear indicated the presence of some material with length greater than 3000 Å.

Clam paramyosin (water insoluble tropomyosin) was prepared by Mrs. Susan Rupp Nanney by the method of Bailey (9). The values of $\bar{v} = 0.736$ and $\frac{dn}{dc} = 0.188$ were taken from the work of Kay (10). Concentration was determined on the stock solution by Kjeldahl method using a nitrogen content of 18.2 per cent (11).

Birefringence measurements were made on a Rao apparatus, which was thermostated by circulating water from a large temperature bath set at 20°C. Although the temperature of the effluent was unchanged, it is probable that there was some temperature rise in the apparatus, particularly for the high glycerol solutions. A thermocouple placed at the bottom of the central filling hole, indicated a temperature rise of a few degrees at maximum shear even for aqueous solutions. This region is not in contact with the water cooling so that this is the maximum temperature change. The temperature of the solution in the gap between the cylinders cannot be conveniently measured. It may differ by 2 or 3 degrees from that of the water bath.

Refractive indices of glycerol-water solutions were measured on a Zeiss refractometer. The viscosity was required in order to check the length of the molecules in glycerol and was obtained from viscosity *versus* composition data of Sheely (12). This viscosity can only be regarded as approximate, owing to possible temperature variations referred to above.

RESULTS

The intrinsic birefringence was calculated from the equations $\Delta n = 2\pi n\bar{v}c(g_1 - g_2)f(\sigma p)$ and $\frac{dn}{dc} = (2\pi n\bar{v}/3)(g_1 + 2g_2)$. The symbols were defined previously (2). $f(\sigma p)$ was obtained from measurements of extinction angle using the table of Scheraga *et al.* (13). Values of p of 20, 215, and 70 were used for TMV, tropocollagen, and paramyosin, respectively. The optical parameters for rods were used in all cases (*i.e.*, $g_1 - g_2 = (1/4\pi)[m_1^2 - 1 - 2(m_2^2 - 1)/(m_2^2 + 1)]$). Measurements were made in a concentration range for which $\frac{\Delta n}{c}$ and the rotary diffusion constant θ were independent of concentration. All the samples showed a variation in θ with shear, which is a sensitive test for poldispersity. Table I summarizes the results for a shear of 6000 sec.$^{-1}$. The two values for the length of TMV (samples A and A_1) were obtained several months apart and it is evident that some aggregation has occurred. Sample B was measured shortly after preparation. The birefringences, Δn_1 of both samples were equal within experimental error. Lengths were calculated from the theory of Broersma (14) for the rotary diffusion constant of a rod. The most reliable value for the length of TMV based on the average of a number of determinations by a variety of methods was calculated to be 2980 ± 30 Å by O'Konski and Haltner (15). The length obtained for TMV sample B was 3010 Å which is in complete agreement with this value. For tropocollagen the value reported here agrees within experimental error with the value reported by

TABLE I

ROTARY DIFFUSION CONSTANT, LENGTH, AND OPTICAL PARAMETERS FOR TMV, TROPOCOLLAGEN, AND PARAMYOSIN

Molecule		θ	Length	$4\pi(g_1 - g_2)$	n_1	n_2	Δn_i
			Å				
TMV	Sample A	275	3100				
	Sample A_1	288	3370	3.3×10^{-2}	1.582	1.612	−0.029
	Sample B	215	3010	3.4×10^{-2}	1.586	1.616	−0.030
Tropocollagen		790	2830	2.8×10^{-2}	1.573	1.603	−0.030
Paramyosin		8200	1200	3.2×10^{-2}	1.591	1.621	−0.030

All values are for a shear of 6000 sec.$^{-1}$. Samples A and A_1 are the same preparation. The measurements were made several months apart and some aggregation has occurred. Sample B was measured within a few hours after preparation.

Boedtker and Doty (4). The length of 1200 Å for paramyosin can be compared with the length of 1400 Å from light scattering (10) and the crystal repeat period (16). The sample could not be studied over an appreciable shear range since the error in θ for extinction angles close to 45° becomes quite large. A plot of θ *versus* G for TMV sample A and tropocollagen is shown in Fig. 1. The presence of longer molecules lowers θ at low shear. The limiting θ at $G = 0$ is about 200 for tropocollagen which is somewhat larger than the value of about 100 expected for dimers. At high shear θ is close to the value for the monomer length, so the preparation probably contains

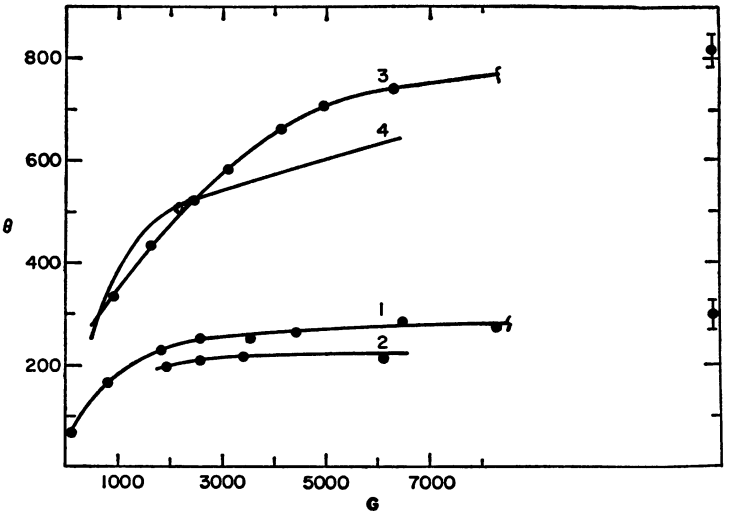

FIGURE 1 Dependence of rotary diffusion constant, Θ, on shear gradient. Curves 1 and 2 refer to TMV samples. A and A_1. Curve 3 refers to tropocollagen. Curve 4 is a theoretical plot for a polydisperse system consisting of particles with $l = 2800$ Å, $p = 200$ and $l = 5600$ Å, $p = 400$ in a ratio of 9:1. The points indicated by the filled circles with error flags are the limiting Θ values at the highest glycerol concentration corrected to the viscosity of water.

some material which is longer rather than shorter than 3000 Å. The θ *versus* shear plot is very sensitive to polydispersity and the variation in θ is not as serious as the curvature of the plot might imply. The error introduced in Δn_i by polydispersity can be estimated from the theory of Sadron (17). $\tan 2X = \sum \delta_i \sin 2X_i / \sum \delta_i \cos 2X_i$

$$(\Delta n)^2 = \sum (\delta_i \sin 2X_i)^2 + (\delta_i \cos 2X_i)^2,$$

where $\delta_i = 2\pi n \phi_i \Delta g_i f_i$ is the contribution to the birefringence by component i, X_i is the corresponding extinction angle, and Δg_i is the optical parameter of component i. A plot of the apparent rotary diffusion constant for a solution consisting of particles of length 5600 Å, axial ratio 400, and length 2800 Å, axial ratio 200 in a ratio of one to nine is shown in Fig. 1. These dimensions were chosen to approximate the tropocollagen system. Comparison of the shape of the plot with the experimental curve indicates that our sample contains molecules longer than the monomer, but not as large as the dimer, and these probably represent less than 10 per cent of the total. The effect on Δn_i can be appreciated by comparing the apparent Δn_i for systems of molecules consisting of 90 per cent of monomers with length 2800 Å and $p = 200$, and 10 per cent of particles of length (*a*) too small to orient, (*b*) 1400 Å, $p = 100$, (*c*) 5600 Å, $p = 400$. It was assumed that $4\pi\Delta g = 0.03$ for all orientable species, which corresponds to $\Delta n_i = -0.031$. This assumption is valid since p is equal to or greater than 100 for all species which contribute to the birefringence. The results are shown in Table II for a shear of 6000 sec.$^{-1}$. It is apparent that the presence of

TABLE II

EFFECT OF POLYDISPERSITY

θ	θ_2	θ_{app}	$4\pi g_{app}$	Δn_{app}	$\Delta n_{app} - \Delta n_i$
785	Very large	785	0.027	−0.0325	−0.0015
785	3080	840	0.028	−0.0320	−0.0010
785	109	630	0.029	−0.0315	−0.0005

Intrinsic birefringence at shear of 6000 sec.$^{-1}$ for systems consisting of (*a*) molecules of length l = 2800 Å, $p = 200$ and l too small to orient (*b*) $l = 2800$ Å $p = 200$ and $l = 1400$ Å, $p = 100$ (*c*) l = 2800 Å, $p = 200$ and $l = 5600$ Å, $p = 400$. The ratio of the two components in each case is 9 to 1. $\Delta n_i = -0.031$ for all components.

10 per cent of molecules with a length widely different from the main component introduces an error of at most 5 per cent in Δn_i and in each case the error increases the negative birefringence. A consideration of the other sources of error indicates that polydispersity is not the most important one for the systems under study. For particles of 3000 Å length, the uncertainty in extinction angle and retardation would introduce an error of about 2 to 3 per cent in Δn_i, while errors in $\bar{v}$ could introduce an error in Δn_i of about 2 per cent. For tropocollagen widely different values for $\bar{v}$ of 0.705 and 0.75 give Δn_i of −0.027 and −0.030. The largest error is in $\frac{dn}{dc}$, which

could amount to 5 per cent in Δn_i. It can be seen from the graph (Fig. 3 of ref. 2) that a variation in $\bar{n}$ has an appreciable effect on Δn_i. The expected error is, therefore, somewhat less than 10 per cent.

INTRINSIC BIREFRINGENCE IN GLYCEROL-WATER MIXTURES

The measurements of flow birefringence of TMV were undertaken as a means of verifying the Peterlin-Stuart theory for the special case $\Delta n_i = 0$ since it had been shown by Lauffer that the flow birefringence of TMV is zero in a glycerol-water-aniline mixture of index 1.57. A consideration of the errors discussed above clearly shows that the finding of negative birefringence cannot be attributed to experimental error. A study was therefore made of flow birefringence in glycerol-water solutions. The quantity $4\pi(g_1 - g_2)$ can be calculated as a function of solvent idex and compared with experiment. The results are shown in Figs. 2 and 3 for TMV and tropocollagen. It can be seen that there is no agreement with theory. Whereas flow birefringence should have changed sign at an index below 1.40, the optical parameter is still positive at the highest glycerol concentration. The length calculated from the rotary diffusion constant in glycerol is not significantly different from that in aqueous solution so the disagreement can not be caused by a change in the configuration of the molecule.

One possible source of this discrepancy can be considered. If the protein continues to bind water in the glycerol-water solvent, it will possess a shell of index differing

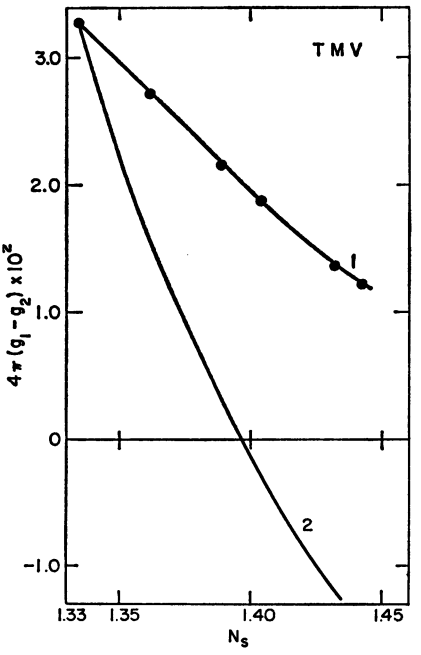

FIGURE 2 Dependence of the optical parameter for TMV sample A_1 on refractive index of solvent (N_s) for glycerol-water mixtures. Curve 2 is the theoretical dependence calculated from the intrinsic birefringence in aqueous solution.

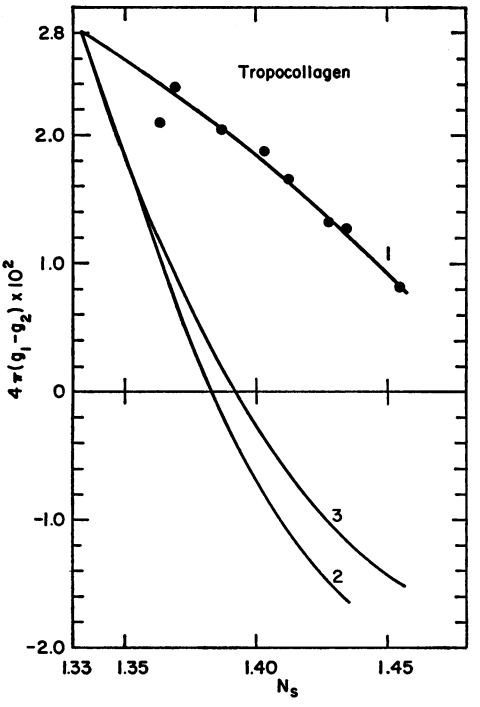

FIGURE 3 Dependence of the optical parameter for tropocollagen on the refractive index of the solvent (N_s) for glycerol-water mixtures. Curve 2 is the theoretical dependence calculated from the intrinsic birefringence in aqueous solution. Curve 3 is the theoretical dependence assuming a water of hydration shell of 0.3 gm/gm protein.

from that of protein and bulk solvent. The necessary theory for birefringence of an ellipsoidal shell was given in a previous paper (2), and an upper limit for the shell effect can be obtained as follows. Assume a value of 0.3 grams water per gram protein as a representative value for hydration and that the entire layer is retained in the glycerol-water solvent. The Δg curve for tropocollagen for such a hydration shell is shown in Fig. 2. There is a small effect, but it can do very little to bring the curves into correspondence, particularly at low glycerol concentrations. A completely unreasonable value for the hydration shell would be required to reconcile the theory with experiment.

DISCUSSION

The total birefringence of a TMV solution attains a minimum value of zero for a solvent refractive index of about 1.57 (3). The molecule is therefore believed to be optically isotropic.

The value of $4\pi(g_1 - g_2)$ for an isotropic molecule of index 1.60 in aqueous solution is 0.08. Two TMV preparations yielded values of 0.033 and 0.034. Therefore the molecule cannot be isotropic if the Peterlin-Stuart theory is correct. A similar discrepancy exist for tropocollagen, since it gave a negative intrinsic birefringence, while that of collagen is positive. The TMV measurements were made to determine

the validity of the theory for the special case of zero intrinsic birefringence. However the lack of agreement does not permit the conclusion that the theory is incorrect. Further understanding can be gained from the glycerol-water data. These data have been replotted in Fig. 4 in terms of the apparent intrinsic birefringence calculated from the Peterlin-Stuart theory as a function of solvent index. It was assumed that the average index of the particle is constant (*e.g.* $(n_1 + 2n_2)/3 = 1.60$ for TMV). The intrinsic birefringence was then derived by solving this equation with the equation for $g_1 - g_2$. It is apparent that the negative birefringence decreases smoothly as the index is raised. The highest index used, namely 1.45 is not sufficiently close to 1.60 to warrant extrapolation but at least the trend in the curve is not incompatible with zero birefringence at 1.60. The tropocollagen birefringence which is the same as that of TMV in aqueous solution, within experimental error, also follows a curve which is very similar to the TMV curve over the range of index studied.

A number of other investigators have reported on the birefringence of proteins. Boedtker and Doty (4) stated that tropocollagen has a very small negative birefringence of about −0.003. However, their value of $g_1 - g_2$ corresponds to a birefringence of −0.04 and examination of their curve of $\frac{\Delta n}{c}$ *versus* G agrees with the larger value. Hocking *et al.* (6) reported for fibrinogen indices of 1.64 and 1.69. Thus the birefringence is large and negative although the absolute values of these indices are clearly incorrect since $\bar{n}$ cannot be more than 1.60. These measurements were made in glycerol-water solutions and it seems likely that the index of water was used in their calculation instead of the index of the solvent. Edsall and Foster (18) reported $g_1 - g_2$ values for a number of proteins, including fibrinogen. The measurements were made in glycerol at various concentrations for the different proteins, and the authors are aware of the possible complications introduced by the solvent and refrain from calculating the birefringence. Their data have been recalculated and plotted in Fig. 4. The two determinations for fibrinogen at different glycerol concentrations seem to show a solvent effect, falling on a curve of slope not too different from the curves for TMV and tropocollagen. If the same phenomenon occurs in this case the birefringence of fibrinogen in aqueous solution must be at least −0.035 and is probably even more negative. (A similar variation of intrinsic

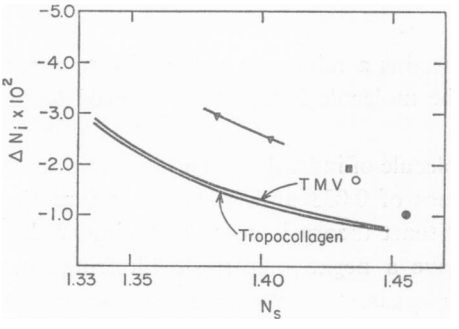

FIGURE 4 Dependence of apparent intrinsic birefringence (ΔN_i) on refractive index of solvent (N_s) for TMV and tropocollagen. It was assumed that the average index of the protein $(n_1 + 2n_2)/3$ is constant.

▼ Fibrinogen
○ Human γ-globulin
■ Zein
● Human serum albumin

Data recalculated from Edsall and Foster (19).

birefringence with solvent index has been demonstrated for myosin by Mr. J. Cassim in this laboratory. The myosin curve lies near the fibrinogen points. Thus, in three cases so far studied the same general effect is present and there is some justification in generalizing to other proteins.) The birefringences of zein, human γ-globulin, and human serum albumin also fall above the TMV-tropocollagen curve. Their birefringences in aqueous solution would presumably also be large and negative. Bovine serum albumin has been investigated in this laboratory (19). The concentration necessary to give appreciable birefringence is too high to justify using the data in quantitative calculations, both because there is interaction between the molecules which would invalidate the derivation of the distribution function and because the protein makes an appreciable contribution to the index of the solution. In fact, the concentration was sufficiently high to produce detectable optical rotation. However, it is significant that the total birefringence was found to be negative. This result is not in disagreement with the finding of Edsall and Foster for human serum albumin, since they report $4\pi(g_1 - g_2) = 0.00036$ at a concentration of 4.48 gm/100 ml. This value is one hundredth as large as TMV in aqueous solution, that is to say it is almost zero. The concentration is too high for proper application of the Peterlin-Stuart theory so the birefringence shown in Fig. 4 will be slightly in error. The small negative birefringence obtained by us at a concentration of 12 gm/100 ml could be accounted for as a concentration effect since, as can be shown by generalizing Wiener's theory, total birefringence can change sign for high concentrations of negatively birefringent molecules (20).

Since an experimental verification of the birefringence theory is still lacking, there are two alternatives to be considered in interpreting the data on the birefringence of proteins.

1. The birefringence equation, $\Delta n = 2\pi n\phi(g_1 - g_2)f(\sigma p)$, is incorrect. The equation was rederived in a previous paper (2) and the assumptions made in the derivation were discussed. The variation of intrinsic birefringence with the refractive index of the solvent could be a result of the use of an incorrect function of the refractive indices for the optical parameter $g_1 - g_2$. In previous work the orientation factor $f(\sigma p)$ was assumed to be correct. The orientation factor and the relation of extinction angle to rotary diffusion constant are both obtained from the same solution of the hydrodynamic problem. The value obtained for the length (and therefore also the rotary diffusion constant) of TMV preparation B, agreed with the accepted value obtained from a variety of other methods (15). Thus, if the birefringence equation is incorrect it is the optical parameter and not the orientation factor which must be modified.
2. The birefringence equation is correct. The intrinsic birefringence of a number of proteins both fibrous and globular is negative and depends markedly on the solvent in those cases which have been studied.

An explanation is required for the sign of the birefringence and the large solvent

effect. Differential absorption from a mixed solvent has been eliminated, but there are at least two other explanations which should be considered.

(*a*) Perturbation of electronic transitions by the solvent may be sufficient to modify the birefringence. A calculation of the birefringence of an α-helix would involve a theory similar to that applied to optical rotation and hypochromicity (21–23). The nearest strong transition occurs at about 2000 Å and gives rise to the parallel and perpendicularly polarized exciton bands. Although this transition accounts for only a small part of the polarizability of an amino acid residue, it will make an important contribution to the birefringence. The absorption of oriented polypeptide films in the 2000 Å region has recently been measured by Gratzer *et al.* (24). It is apparent from their absorption curves that the integrated absorption for the perpendicularly polarized transition is greater than for the parallel transition. This implies that the contribution to the birefringence from the peptide chromophore must be negative. The orientation was not accurately known in these experiments, but the birefringence can be calculated from the theoretical transition moments obtained by Tinoco *et al.* (25). The dipole moments are $u_1 = 1.88$ *D*, $u_2 = 2.40$ *D*. Using the residue weight of alanine and the values of λ_{max} for these transitions obtained by Gratzer *et al.* of 2060 Å and 1910 Å, the contribution to the birefringence was obtained from the equation: $\Delta(n_i^2) = 4\pi N(\alpha_1 - \alpha_2)$, where $\alpha_i = (e^2/4\pi^2 m)f_i/\nu_i^2 - \nu^2$ and $f_i = (8\pi^2 m\nu_i/he^2)\mathbf{u}_i \cdot \mathbf{u}_i$. $i = 1, 2$ refers to parallel and perpendicular bands. Δn_i was found to be -0.015. Considering the approximations made in the theory of Tinoco *et al.*, it is interesting that the result is in the range found for proteins. The birefringence is relatively independent of the wavelength splitting of the bands, but is quite sensitive to the values of the dipole moments.

The contributions of the higher frequency transitions are more difficult to assess. According to Tinoco *et al.* (25), there are a pair of strong transitions at 1650 and 1500 Å (involving the oxygen atom). Detailed calculations are not available but from the orientation of the transition moments in the isolated molecule they may be expected to contribute negative birefringence which could be as large as the contribution from the transition at 2000 Å. The remaining valence electrons on the nitrogen may be expected to decrease the birefringence somewhat. It, therefore, is probable that a more complete theoretical study would predict a negative birefringence for the α-helix. This conclusion lends some weight to the correctness of the birefringence calculated from the flow data, but if the α-helical structure determines the birefringence it is difficult to understand why the birefringence of a helical protein like paramyosin is not considerably larger than a globular protein.

Solvent effects on birefringence could be caused by a change in intensity of the transitions. However, a randomly oriented solvent would not produce intensity changes in the exciton bands by resonance interactions, to the approximation of first order perturbation theory. The effect would, therefore, have to arise from at least a second order perturbation and would be expected to be small, while the

apparent change in birefringence with solvent is large. A more extensive study of the birefringence of proteins and synthetic polypeptides in a variety of solvents is now in progress and further theoretical discussion is not fruitful at this time.

(*b*) A second explanation of the solvent effect is that the protein probably through its coulomb field, is capable of orienting a shell of water molecules. This possibility bears some formal resemblance to the "iceberg" theory (26). Ice and other lattices which water may occupy could contribute negative intrinsic birefringence if the molecules are oriented with their maximum index direction perpendicular to the protein surface. In glycerol-water mixtures the lattice may be disordered; while in organic solvents it would not be present, leaving the birefringence of the protein which is zero or positive. A calculation of the birefringence would be prohibitively difficult, since this is essentially a Kerr effect in a very large non-homogeneous field. However, a very rough consideration of the ordered domain necessary to account for a negative birefringence of -0.03 makes this explanation unlikely. Ice itself has a very low birefringence, and a shell with a volume eight times that of the molecule would be necessary in the case of tropocollagen. However, the water molecule is strongly anisotropic and it is therefore possible that a lattice of higher birefringence could be formed under the influence of the field. From the depolarization of light scattering by water vapor (27) the anisotropy can be estimated. The molecule does not have a simple shape, but if it is represented by an ellipsoid of axial ratio 2, then Δn_i is about 0.10 to 0.15. This gives an estimate of the maximum birefringence of the lattice. The average birefringence must be much less than this; even if a value of 0.05 is taken, the shell volume would still be about equal to the protein volume. Thus, a large rigidly bound hydration shell is required which should affect the rotary diffusion constant and the nuclear magnetic resonance spectrum. The rotary diffusion constant of nearly monodisperse TMV, preparation B, agrees with the value calculated for a rod of dimensions 2980×150 Å to an accuracy of 3 per cent. A shell with a volume equal to that of the virus would reduce the diffusion constant by 13 per cent. Thus, whatever hydration shell the virus may possess appears to be much too small to account for the birefringence in terms of oriented solvent. Also the type of nuclear magnetic resonance broadening expected for a large solvent lattice has not been found in protein solutions (28). A more direct answer to the question of the importance of orientation of a polar solvent may be provided by the study of uncharged polypeptides in non-polar solvents since the effect should be absent in the latter case.

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REFERENCES

1. PETERLIN, A., and STUART, H. A., Z. *Physik.*, 1939, **112**, 1.
2. TAYLOR, E. W., and CRAMER, W., *Biophysic. J.*, **3**, 000.

3. LAUFFER, M. A., *J. Physic. Chem.*, 1938, **42**, 935.
4. BOEDTKER, H., and DOTY, P., *J. Am. Chem. Soc.*, **78**, 4267, 1956.
5. HOCKING, C., LASKOWSKI, M., JR., and SCHERAGA, H. A., *J. Am. Chem. Soc.*, 1952, **74**, 755.
6. BOEDTKER, H., and SIMMONS, N. S., *J. Am. Chem. Soc.*, 1958, **80**, 2550.
7. GALLOP, P., *Arch. Biochem. and Biophysics*, 1955, **54**, 486, 501.
8. MA, T. S., and ZUAZAGA, G., *Ind. and Eng. Chem.*, 1942, **14**, 280.
9. BAILEY, K., *Pubb. stazione Zool. Napoli*, 1956, **29**, 96.
10. KAY, C., *Biochem et Biophysica, Acta*, 1958, **27**, 469.
11. BAILEY, K., *Biochem et Biophysica Acta*, 1957, **24**, 612.
12. SHEELY, M. L., *Ind. and Eng. Chem.*, 1932, **24**, 1060.
13. SCHERAGA, H. A., EDSALL, J. T., and GADD, J. O., *J. Chem. Physics*, 1951, **19**, 1101.
14. BROERSMA, S., *J. Chem. Physics*, 1960, **32**, 1626.
15. O'KONSKI, C. T., and HALTNER, A. J., *J. Am. Chem. Soc.*, 1956, **78**, 3604.
16. HODGE, A. J., *Proc. Nat. Acad. Sc.*, 1952, **38**, 850.
17. SADRON, C., *J. Physique et Radium*, 1938, **9**, series 7, 381.
18. EDSALL, J. T., and FOSTER, J. F., *J. Am. Chem. Soc.*, 1948, **70**, 1860.
19. CASSIM, J., unpublished data.
20. WIENER, O., *Abhandl. Sächs. Akad. Wissensch.*, 1912, **33**, 507.
21. MOFFITT, W., *J. Chem. Physics*, 1956, **25**, 467.
22. TINOCO, I., *J. Chem. Physics*, 1960, **33**, 1332.
23. RHODES, W., *J. Am. Chem. Soc.*, 1962, **83**, 3609.
24. GRATZER, A. B., HALZWARTH, B. M., and DOTY, P., *Proc. Nat. Acad. Sc.*, 1961, **47**, 1785.
25. TINOCO, I., HALPERN, A., and SIMPSON, W. T., *in* Polyamino Acids, Peptides, and Proteins, (M. A. Stahmann, editor), Madison, University of Wisconsin Press, 1962.
26. KLOTZ, I. M., *Science*, 1958, **128**, 815.
27. BHAGAVANTAM, S., Scattering of Light and the Raman Effect, Brooklyn, New York, Chemical Publishing Co., 1942.
28. BALAZS, E. A., BOTHNER-BY, A. A., and GERGELY, J., *J. Mol. Biol.*, 1959, **1**, 147.