BIREFRINGENCE OF PROTEIN SOLUTIONS AND BIOLOGICAL SYSTEMS. I

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ABSTRACT The quantitative interpretation of birefringence of biological structures such as muscle requires a knowledge of intrinsic birefringence of the components. The intrinsic birefringence of fibrous structures as determined by variation of solvent index is positive while the intrinsic birefringence of proteins in solution is negative as calculated by the Peterlin-Stuart theory. As a first step in clarifying this discrepancy the basis of the Peterlin-Stuart theory has been reexamined. The theory has been recalculated from the standpoint of light scattering and extended to particles whose length is not small compared to the wavelength. The birefringence of a system of particles possessing a shell with index different from the bulk solvent has been obtained in order to interpret measurements in mixed solvents.

INTRODUCTION

Polarized light microscopy has made some important contributions to the study of certain biological structures, such as muscle, nerve myelin, and mitotic spindle (1–3). Its special advantages are that systems may be studied in the living state, and rapid structural changes, such as occur in muscle contraction, may be detected. Quantitative microscopic measurements of birefringence are potentially capable of providing independent evidence with which to test the validity of detailed structural proposals. For example, a relatively complete structure for striated muscle has been derived from electron microscopic and x-ray diffraction studies (4), yet the exact interpretation of birefringence measurements in terms of this structure is far from satisfactory. The problem to be considered is whether or not existing theories of birefringence are adequate for quantitative studies. An approximate equation derived by Wiener (5) for the birefringence of parallel rods has often been used in this connection,[1] but it is not clear at what point the assumptions used in the derivation

[1] The Wiener equation is $n_{\parallel}^2 - n_{\perp}^2 = \phi_1\phi_2(n_1^2 - n_2^2)^2/[(1 + \phi_1)n_2^2 + \phi_2 n_1^2]$ where $n_{\parallel}$, $n_{\perp}$, are the refractive indices of the system for light polarized parallel and perpendicular to the rods; n_1 and n_2 are the indices of the particles and solvent and ϕ_1 and ϕ_2 are the volume fractions of the particles and solvent. It should be noted that this equation becomes identical with the flow birefringence equation (1) for the case of isotropic particles if ϕ_1 is very small.

will break down and an adequate experimental verification of this theory is lacking. The theoretical problem for concentrated systems will be discussed elsewhere. In this paper attention will first be directed to the experimental problem of verifying the Wiener theory (or any other theory) of birefringence.

In most biological systems the structural elements are themselves birefringent. Thus, the measured birefringence is made up of two terms, the form birefringence of oriented structural elements and the intrinsic birefringence of the elements. The Wiener equation is concerned only with the first term although it can be generalized in a straightforward way to include intrinsic birefringence. Interpretation of birefringence measurements in terms of structure therefore requires that the intrinsic birefringence be known or determined experimentally. It has usually been obtained by measuring the birefringence in solvents of different refractive index. Wiener's equation predicts a minimum of zero when the index of the solvent matches that of the rods. Any residual birefringence is attributed to the intrinsic birefringence of the rods. This approach is unsatisfactory since it is necessary to assume that the "intrinsic birefringence" is unchanged by the presence of organic solvents. Even though the relation of birefringence to protein structure is not known in detail, it is to be expected that the birefringence will depend to some extent on the secondary and tertiary structure, and can not be completely independent of solvent.

It is necessary to consider other methods of testing the theory. The intrinsic birefringence of macromolecules can be determined from flow birefringence measurements using the theory of Peterlin and Stuart (6). A test of Wiener's equation could be made by comparing the measured and calculated birefringence of a system of known structure composed of subunits of known intrinsic birefringence. This approach, however, immediately leads to a contradiction. The intinsic birefringence of the A band of vertebrate striated muscle is positive (7, 8). The structure determined by electron microscopy and x-ray diffraction would require myosin molecules to be packed more or less parallel to the axis of the sarcomere (4). Thus the intrinsic birefringence of myosin should be positive, while flow birefringence measurements yield a large negative value (9). A positive intrinsic birefringence, as measured by variation of solvent index, is quite general for protein fibers. Schmitt has stated that "the intrinsic birefringence is positive in sign in all protein fibers except those conjugated with nucleic acid" (10). The list of such fibers includes muscle, mitotic spindle and aster, collagen, fibrin, myonemes of protozoa, flagella and sperm tails. However, the intrinsic birefringence calculated from flow birefringence data is negative for a number of proteins, namely fibrinogen, serum albumin, γ-globulin, myosin, paramyosin, tropocollagen, and tobacco mosaic virus (9, 11, 12). Since the two groups overlap, there is some fundamental discrepancy between the two methods of obtaining intrinsic birefringence.

The disagreement cannot be attributed to a failure of the Wiener theory, because it is the sign and not simply the magnitude which differs. The sign is determined

directly from the minimum value of the birefringence *versus* refractive index curve. Although the use of organic solvents may have modified the protein structure, it would be surprising that the solvent effect could be so large that a negative intrinsic birefringence had been converted into a positive one.

Before proceeding further with the problem of birefringence in a concentrated system it is first necessary to investigate the validity of the Peterlin-Stuart theory. This theory is usually regarded as resting on firmer theoretical ground than Wiener's, because it is a dilute solution approximation. The main restriction on its use is that the particles should be small compared to the wavelength of light (13), a restriction which is almost never fulfilled. Both theories are concerned with predicting the refractive indices of a molecule from measurements of the refractive index of oriented structures or solutions; *i.e.*, they are theories of the index of a mixture. A great deal of experimental and theoretical work has been devoted to this subject (14, 15) and some information on the validity of birefringence theories can be gained from a consideration of the validity of the underlying refractive index theory. This aspect of the problem will be discussed in a subsequent communication. In this report it is shown that the theory of flow birefringence can be extended to particles which are not small compared to the wavelength of light. The equations are generalized to cover the effect of a shell of different refractive index from the bulk solvent, a situation which may well occur when the index of the solvent is varied. The problem is treated in terms of the theory of light scattering as developed by Van de Hulst (16).

DEFINITIONS OF THE PRINCIPAL SYMBOLS

Δn	= total birefringence of the solution
n_1, n_2	= refractive indices of particle for light polarized along the principal axes
n	= refractive index of the solvent
m_1, m_2	= refractive indices of particle relative to solvent
a, b	= major and minor axes of ellipsoidal particle
p	= axial ratio of particle $p = a/b$
σ	= G/θ where G is shear gradient (sec.$^{-1}$) and θ is rotary diffusion constant
$f(\sigma p)$	= orientation factor
V	= volume of particle
α_1, α_2	= principal excess optical polarizabilities
g_1, g_2	= principal excess optical polarizabilities per unit volume $\alpha_i = Vg_i$
ϕ	= volume fraction of particles
L_1, L_2	= depolarization factors
λ	= wavelength
k	= wave number, $k = 2\pi n/\lambda$
$\mathbf{a}_1, \mathbf{a}_2, \mathbf{a}_3$	= unit vectors defining fixed coordinate system (xyz)
$\mathbf{b}_1, \mathbf{b}_2, \mathbf{b}_3$	= unit vectors defining principal axes of particle
θ, ψ	= angles between $\mathbf{a}_3$, $\mathbf{b}_1$ and $\mathbf{a}_2$, $\mathbf{b}_1$ which define orientation of particle with respect to coordinate axes.
$F(\theta\psi)$	= orientation distribution function of particles
$\mathbf{E}_0$	= applied electric field vector

$E_{P_0}, E_{r_0}, E_P, E_r$	= compounds of the applied and scattered electric field
$\mathbf{p}$	= excess dipole moment vector of particle
p_x, p_y	= components of excess dipole moment of particle in directions $\mathbf{a}_1$ and $\mathbf{a}_2$
$\mathbf{S}$	= scattering amplitude matrix
$\mathbf{S}_{(0)}$	= scattering amplitude matrix for scattering at zero angle
$\bar{\mathbf{S}}_{(0)}$	= average scattering amplitude matrix at zero angle
$S_i (i = 1 \text{ to } 4)$	= components of scattering amplitude matrix
$\mathbf{P}$	= scattering matrix expressed in terms of the excess polarizabilities $\mathbf{S}_{(0)} = ik^3\mathbf{P}$
P_{ik}	= components of matrix $\mathbf{P}$
D	= dielectric constant
Im	— denotes the imaginary part of the quantity in brackets following the symbol.

THEORY OF FLOW BIREFRINGENCE

Peterlin and Stuart (6) have shown that the birefringence of a dilute solution of rigid macromolecules of ellipsoidal shape oriented in a shear gradient is given by $\Delta n = (2\pi\phi/n)(g_1' - g_2')f(\sigma, p)$. $g_1' - g_2'$ is an optical parameter, $g_i' = (1/4\pi)(n_i^2 - n^2)/(1 + [(n_i^2 - n^2)/n^2]L_i)$ and

$$L_i = \frac{1 - e^2}{e^2}\left(-1 + \frac{1}{2e}\log_n\left(\frac{1+e}{1-e}\right)\right), \qquad e^2 = 1 - \frac{1}{p^2}, \qquad L_1 + 2L_2 = 1.$$

It is convenient to introduce a change of notation.

$$g_i = (1/4\pi)(m_i^2 - 1)/[1 + (m_i^2 - 1)L_i] = g_i'/n^2$$

The birefringence equation becomes $\Delta n = 2\pi n\phi(g_1 - g_2)f(\sigma p)$ (equation 1). In most applications the axial ratio is greater than 20, so that $L_1 = 0$, $L_2 = 1/2$ and the optical parameters reduce to a simple form.

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

The g factors arise as follows: the excess dipole moment of a particle in a constant field E' applied along its a or b axis is $p_1 = \alpha_1 E'$, $p_2 = \alpha_2 E'$. The polarizability of an ellipsoid is given by $\alpha_i = (V/4\pi)(m_i^2 - 1)/[1 + (m_i^2 - 1)L_i]$ where V is the volume of the ellipsoid. The change in notation was introduced to make $\alpha_i = Vg_i$, so that g_i is the excess polarizability per unit volume. If E' is the field of a light wave, the same considerations apply with the restriction that since the field is to be constant in space, the largest dimension of the particle must be small compared to the wavelength of light in the medium, *i.e.*, $\lambda/2\pi n \gg 2a$. This condition restricts the theory to particles about 200 Å long. The birefringence equation follows directly from electrostatic theory. For simplicity consider a solution of ellipsoidal particles oriented with their long axes parallel to a given direction. The solvent molecules are in random orientation. The dielectric constants are required for fields applied parallel and perpendicular to the orientation direction. Let E be the field applied parallel to this

direction and let D_p be the corresponding dielectric constant. Then $(D_p - 1)E/4\pi = P$ and $P = \sum_{k=1}^{2} N_k \alpha_1^k E_k$. N_k = number of particles per cc of type k, α_1^k is the polarizability of a particle of type k in the specified direction and E_k is the field at particle k. In general, E_k is very difficult to calculate since it depends on the applied field and the field of the induced dipoles of all the other particles. However, it will be proportional to E. The problem can be simplified by introducing excess polarizabilities relative to the solvent. The solvent is considered to be a continuous medium of dielectric constant D. The field in the solvent in the absence of the particles is E_a which is proportional to E. Therefore, the equation becomes $(D_r - 1)E_a/4\pi = N\alpha_1 E'$ where $D_r = D_p/D$; N, α_1 and E' are the number of particles per cc, the excess polarizability of a particle for a field parallel to the long axis, and the field at a particle, respectively. If the solution is sufficiently dilute, the field at a given particle produced by the induced moments of all the other particles will be negligible and $E' = E_a$. Therefore $(D_r - 1)/4\pi = N\alpha_1$. A similar equation holds for the perpendicular direction. Therefore $\Delta D_r = 4\pi N(\alpha_1 - \alpha_2)$ and $\Delta n = 2\pi n\phi(g_1 - g_2)$. This result is identical with equation 1 for $f = 1$. It is evident that evaluation of the local field causes the applied field to drop out of the equation so that replacing E by E_a will not affect the result.

The important contribution of Peterlin and Stuart was in evaluating the function $f(\sigma, p)$. The validity of f will be accepted in this work; it is the correctness of the approximations relating to the optical parameters which concerns us.

The method outlined above, which is essentially that employed by Peterlin and Stuart, does not take advantage of the fact that m is close to 1 for molecules of biological interest. Therefore, the problem can be treated in terms of light scattering, using the Rayleigh-Gans approximation. A relation between zero angle scattering and refractive index was derived by Rayleigh (17) and further developed by Van de Hulst (16). This approach applies to particles of arbitrary size and is therefore of general interest in treating birefringence problems.

It will be applied to a number of important cases: (*a*) the refractive index of a dilute solution of randomly oriented anisotropic particles, small compared to the wavelength; (*b*) the birefringence of a dilute solution of preferentially oriented anisotropic particles, small compared to the wavelength; (*c*) the birefringence of a dilute solution of preferentially oriented anisotropic particles which satisfy the conditions for Rayleigh-Gans scattering.

Case (a), Random Orientation. Consider a plane wave from the negative z direction incident on a slab of medium of thickness l containing N particles per cc (Fig. 1). The turbidity is assumed to be very small. The scattering of a single particle will give rise to a spherical wave which is obtained by a linear transformation of the incident wave. The general form of the scattered wave is given by Van de Hulst (16).

$$\begin{pmatrix} E_P \\ E_r \end{pmatrix} = \begin{pmatrix} S_2 & S_3 \\ S_4 & S_1 \end{pmatrix} \frac{e^{-ikr+ikz}}{ikr} \begin{pmatrix} E_{P_0} \\ E_{r_0} \end{pmatrix} \qquad \text{(equation 2)}$$

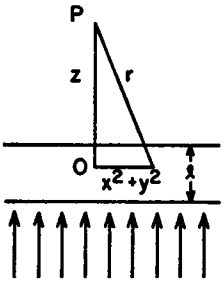

FIGURE 1

The functions S_i, $i = 1$ to 4 are components of the scattering amplitude matrix S and each is a function of the two angles defining the scattering and the orientation of the particle. E_{P_0}, E_{r_0}, E_P, E_r are the components of the incident and scattered wave parallel and perpendicular to planes of reference containing the directions of propagation of the incident and scattered beams. (The symbols P and r are used to imply parallel and perpendicular.) r is the distance from the particle to the point of observation. The field at P is equal to the incident field plus the scattered field of all the particles. However, since the turbidity is very small, only those particles in a cylinder corresponding to the first few Fresnel zones as seen from P will contribute to the field. The scattering is therefore obtained by summing the contributions from all particles in the active volume, using for S the value $S_{(0)}$ which denotes the zero angle scattering. Because there is no phase shift the scattering of N particles in random orientation is proportional to N times the scattering of a single particle averaged over all orientations. $S_{(0)}$ can be replaced by $\bar{S}_{(0)}$. To evaluate the components of $\bar{S}_{(0)}$ the optical properties of the particles must be introduced. The particle is represented by an ellipsoid of revolution with principal axes' directions defined by the unit vectors $\mathbf{b}_1$, $\mathbf{b}_2$, $\mathbf{b}_3$ (Fig. 2). α_1 and α_2 are the principal excess polarizabilities.

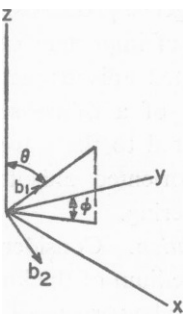

FIGURE 2

$\mathbf{a}_1$, $\mathbf{a}_2$, $\mathbf{a}_3$ are unit vectors along x, y, and z. Since properties of the particle are identical in the $\mathbf{b}_2$, $\mathbf{b}_3$ directions, $\mathbf{b}_2$ may be taken in the $x\,O\,y$ plane. The orientation of a particular particle is specified by the angles θ and ψ between the $\mathbf{a}_3$, $\mathbf{b}_1$ and $\mathbf{a}_2$, $\mathbf{b}_1$ axes.

The sets of axes are related by the equation $\mathbf{a}_i = \sum_{k=1}^{3} C_{ik}\mathbf{b}_k$. The incident wave is chosen such that $\mathbf{E}_0 = E_{P_0}\mathbf{a}_2 + E_{r_0}\mathbf{a}_1$. Since the particles are assumed to be small compared to the wavelength, the scattered field is produced by dipoles induced in the particles. $\mathbf{p} = \sum_{k=1}^{3} \alpha_i E_{0i}\mathbf{b}_i$, where p is the dipole moment vector and E_{0i} is the component of the incident field in the $\mathbf{b}_i$ direction. The x and y components of $\mathbf{p}$ are $p_x = E_{r_0}P_{11} + E_{P_0}P_{12}$ and $p_y = E_{r_0}P_{21} + E_{P_0}P_{22}$, where $P_{ik} = P_{ki} = C_{i1}C_{k1}\alpha_1 + C_{i2}C_{k2}\alpha_2 + C_{i3}C_{k3}\alpha_2$. The components of the scattered field at a distance r from the origin for zero angle scattering are $E_P = p_y k^2 e^{-ikr}/r$, and $E_r = p_x k^2 e^{-ikr}/r$. After substitution for p_x and p_y the equations can be combined to give

$$\begin{bmatrix} E_P \\ E_r \end{bmatrix} = \begin{bmatrix} P_{22} & P_{12} \\ P_{12} & P_{11} \end{bmatrix} \frac{k^2 e^{-ikr}}{r} \begin{bmatrix} E_{P_0} \\ E_{r_0} \end{bmatrix} \qquad \text{(equation 3).}$$

By reference to equation (2), with $z = 0$, it follows that $\mathbf{S}_{(0)} = ik^3\mathbf{P}$ where $\mathbf{P}$ is the matrix formed by the P_{ij} components.

The average scattering amplitude is obtained by averaging over all orientations of the particle. $\overline{P}_{12} = 0$, $\overline{P}_{11} = \overline{P}_{22} = 1/3(\alpha_1 + 2\alpha_2) = \bar{\alpha}$. Therefore $\overline{S}_{1(0)} = \overline{S}_{2(0)} = \overline{S}_{(0)}$ and $\overline{S}_{3(0)} = \overline{S}_{4(0)} = 0$. Equation (2) becomes

$$\begin{bmatrix} E_P \\ E_r \end{bmatrix} = \begin{bmatrix} \overline{S}_{(0)} & 0 \\ 0 & \overline{S}_{(0)} \end{bmatrix} \frac{e^{-ikr+iks}}{ikr} \begin{bmatrix} E_{P_0} \\ E_{r_0} \end{bmatrix} \qquad \text{(equation 4).}$$

From the form of equation (4) it is evident that the problem has been reduced to a scalar theory and (4) may be replaced by a single equation $E = (\overline{S}_{(0)}e^{-ikr+iks}/ikr)E_0$ (equation 5), where E_0 is the amplitude of the scalar wave. The contribution to the field at P can now be obtained by summing the field obtained from (5) for all the particles in the active volume. The result is $E = (-2\pi Ni/k^2)\overline{S}_{(0)}$. The total field at P is $E' = E_0(1 - 2\pi Nl\,\overline{S}_{(0)})/k^2$.

If the scattering medium is replaced by a continuous medium of index m, the field at P is $E' = E_0 e^{-ikl(m-1)} = E_0(1 - ikl(m - 1))$. Equating these expressions for E' gives $m = 1 - (i\,2\pi N/k^3)\overline{S}_{(0)}$. Since there is no absorption m is real, while $\overline{S}_{(0)}$ is in general complex. Therefore $m = 1 - (2\pi N/k^3)\,\mathrm{Im}\,(\overline{S}_{(0)})$. The equation holds for particles of arbitrary size and shape. In the special case of small ellipsoidal particles $\overline{S}_{(0)} = ik^3\bar{\alpha} = ik^3V(g_1 + 2g_2)/3$. Since the volume concentration is small, $(m - 1)/NV = \frac{1}{n}\frac{dn}{d\phi}$. m is the index of the solution relative to the solvent of index n. Therefore $\frac{dn}{dc} = 2\pi n\bar{v}(g_1 + 2g_2)/3$, where $\phi = \bar{v}c$, $\bar{v}$ is the partial specific volume and c is the concentration in gm/cc. This equation has been attributed to Peterlin and Stuart (13) although it was not derived in their original paper. It provides one relation involving the two unknowns g_1 and g_2 and experimental quantities. The derivation presented here is essentially that of Van de Hulst (16, chapters IV, V, VI). It has been given in

detail to clarify the next section, and to exhibit the similarity between a theory of refractive index of a mixture and birefringence.

Case (b), Preferred Orientation. The orientation of the particles is specified by a distribution function $F(\theta, \psi)$ which is the probability that the b_1 axis lies within the element of solid angle $d\omega$ in the direction θ, ψ. The derivation is similar to case (*a*). The average amplitude matrix $\bar{S}_{(0)}$ is evaluated using the distribution function F. $\cos a_1b_1 = \sin\theta \sin\psi = C_{11}$, $\cos a_2b_1 = \sin\theta\cos\psi = C_{21}$ etc. Therefore $P_{11} = \alpha_2 + (\alpha_1 - \alpha_2)\cos^2 a_1b_1$, $P_{22} = \alpha_2 + (\alpha_1 - \alpha_2)\cos^2 a_2b_1$, $P_{12} = (\alpha_1 - \alpha_2)\cos a_1b_1 \cos a_2b_1$. The average value of P_{11} is given by $\bar{P}_{11} = \alpha_2 + (\alpha_1 - \alpha_2)\overline{\cos^2 a_1b_1}$ where $\overline{\cos^2 a_1b_1} = \int F \cos^2 a_1b_1\, d\omega$. Similar expressions hold for P_{22} and P_{12}.

The system is now birefringent and the proof could be completed as before except that the refractive index would be represented by a matrix. It is more convenient to proceed to the case of flow birefringence. For an incident plane polarized beam E_{r_0}, the scattered beam at zero angle will have components E_P and E_r, *i.e.*, it will be eliptically polarized. In the flow birefringence cell, the z axis is parallel to the axis of rotation of a pair of concentric cylinders. Viewed through an analyzer set in the direction a_2, a dark cross is visible; the smallest angle between the cross and $\mathbf{a}_1$ is the extinction angle. In this location the beam is plane-polarized. We require the refractive index at this location for incident beams polarized along $\mathbf{a}_1$ and $\mathbf{a}_2$, respectively. Elliptical polarization of the scattered beam arises from the presence of non-zero P_{12} terms. To find this location, the coordinate system is rotated to make the P matrix diagonal. If the diagonal form is denoted by $\begin{pmatrix} q_2 & 0 \\ 0 & q_1 \end{pmatrix}$ where

$$q_1 = \frac{\bar{P}_{11} + \bar{P}_{22}}{2} + \left[\left(\frac{\bar{P}_{11} - \bar{P}_{22}}{2}\right)^2 + \bar{P}_{12}^{\,2}\right]^{1/2} = \bar{S}_{1(0)}/ik^3$$

$$q_2 = \frac{\bar{P}_{11} + \bar{P}_{22}}{2} - \left[\left(\frac{\bar{P}_{11} - \bar{P}_{22}}{2}\right)^2 + \bar{P}_{12}^{\,2}\right]^{1/2} = \bar{S}_{2(0)}/ik^3$$

the fields become

$$E_r = \left[1 - \frac{2\pi}{k^2} Nl\bar{S}_{1(0)}\right]E_{r_0}, \qquad E_P = \left[1 - \frac{2\pi}{k^2} Nl\bar{S}_{2(0)}\right]E_{P_0}.$$

The scattering medium can be replaced by a continuous medium with refractive indices m_1 and m_2 for the fields E_{r_0} and E_{P_0} in two perpendicular directions. The same argument applied in case (*a*) now gives two expressions for the refractive index,

$$m_i = 1 + (2\pi N/k^3)\ \mathrm{Im}\,[\bar{S}_{i(0)}], \qquad i = 1, 2.$$

The birefringence is

$$\Delta m = (2\pi N/k^3)\ \mathrm{Im}\,[\bar{S}_{1(0)} - \bar{S}_{2(0)}] \qquad \text{(equation 6)}.$$

Equation (6) applies to particles of arbitrary size and shape. In the special case of small ellipsoidal particles,

$$\Delta n = 2\pi Nn[(\overline{P}_{11} - \overline{P}_{22})^2 + 4\overline{P}_{12}{}^2]^{1/2}$$

$$= 2\pi n\phi(g_1 - g_2)[(\overline{\cos^2 a_1b_1} - \overline{\cos^2 a_2b_1})^2 + 4(\cos a_1b_1 \cos a_2b_1)^2]^{1/2}.$$

The quantity appearing as the square root is the Peterlin-Stuart function $f(\sigma p)$. Therefore $\Delta n = 2\pi n\phi(g_1 - g_2)f(\sigma p)$, which is equation (1). This equation taken together with the equation derived in the previous section, can be solved for g_1 and g_2 and therefore n_1 and n_2 can be obtained.

Case (*c*). The conditions for Rayleigh-Gans scattering are (1) $m - 1 \ll 1$ and (2) the phase shift for light passing "through" the particle is small (16). In the birefringence case the particles are partially oriented so that they tend to be perpendicular to the incident light. Therfore the distance travelled in a cylindrical particle by radiation scattered at zero angle will satisfy these conditions as long as the particle radius is small (*i.e.*, $2b \leqq \lambda/10$). For a system of very long particles a fraction will be so oriented that the condition is not fulfilled. Since the particles are long the distribution of orientations will be quite sharp. Thus the fractional number of such particles is very small and in addition they are nearly parallel to the incident beam and therefore make a negligible contribution to the birefringence.

The general equation was given in a previous section. (equation (6) of section *b*). It is necessary to obtain $S_{1(0)}$ and $S_{2(0)}$ for Rayleigh-Gans scattering. The particle can be divided into volume elements, each of which gives Rayleigh scattering. For an arbitrary angle, the scattering from different elements has to be referred to a common origin. The scattering is now proportional to αR, where $R = \frac{1}{V}\int e^{i\delta}\, dV$. δ is the phase difference for scattering from different volume elements. However, for zero angle, $R = 1$; the scattering amplitude is identical with the previous case. This can be seen intuitively, since for this direction there is no geometric path difference for radiation scattered by different volume elements, and the optical path through the particle was assumed not to differ significantly from the medium. The birefringence equation (1) can, therefore, be taken over without change, and the restriction of particle length in the original derivation of Peterlin and Stuart is removed.

A comment should be made regarding the relation of these results to the general problem of light scattering. There is an obvious parallel between the Zimm method (18) of extrapolating the scattering to zero angle to avoid calculating intraparticle interference and the results of the last paragraph. However, the zero angle scattering differs in certain respects from the general case. The scattering amplitude was obtained by adding the scattering from individual particles. The intensity is proportional to $(NA)^2$, where A is the scattering by a single particle or its value suitably averaged. For non-zero angles the intensity of scattering by each particle has to be added; thus, the intensity is proportional to NA^2 times a factor to account for inter- and

intra-particle interference. The zero angle scattering is larger by a factor of order N. Thus the refractive index is controlled by the true zero angle scattering and not just by the scattering at very small angles.

It should also be noted that whereas the true zero angle scattering of anisotropic particles is not depolarized, the depolarization obtained from intensity measurements does not extrapolate to zero at zero angle. However this effect does not contribute to the birefringence because the component of the beam received by the detector from depolarization of scattering at non-zero angles is negligible compared to the zero angle component. This problem is treated in detail by Van de Hulst (16, chapter V).

LIMITATIONS ON THE SIZE OF THE PARTICLES

Some further insight into the maximum particle dimensions which are acceptable in the Rayleigh-Gans approximations for birefringence can be obtained from a consideration of the scattering by rods of arbitrary size. The case of parallel rods oriented with their long axes perpendicular to the direction of the incident beam, and of length $l \gg \lambda$ was treated by Van de Hulst (16). The birefringence equation $\Delta m = (2\pi N/k^3)\ \mathrm{Im}\ [S_{1(0)} - S_{2(0)}]$ holds for particles of arbitrary size. It is only necessary to consider the values of $S_{1(0)}$ and $S_{2(0)}$, for beams polarized parallel and perpendicular to the rod axis. $S_{1(0)} - S_{2(0)} = (kl/\pi) \sum (b_n - a_n)$ where a_n, b_n, are coefficients in a Hankel function expansion of the scattered fields. In terms of the phase angle expansion of Van de Hulst

$$\mathrm{Im}\ (b_n) = \sin \beta_n \cos \beta_n, \qquad \mathrm{Im}\ (a_n) = \sin \gamma_n \cos \gamma_n.$$

$$\mathrm{Im}\ [S_{1(0)} - S_{2(0)}] = (kl/\pi)[\sin \beta_0 \cos \beta_0 + 2 \sin \beta_1 \cos \beta_1 - \sin \gamma_0 \cos \gamma_0 - 2 \sin \gamma_1 \cos \gamma_1],$$

retaining terms up to γ_1, β_1:

$$\gamma_0 = \beta_1 = \frac{\pi x^4}{32}(m^2 - 1), \qquad \beta_0 = \frac{\pi x^2}{4}(m^2 - 1), \qquad \gamma_1 = \frac{\pi x^2}{4}\frac{(m^2 - 1)}{(m^2 + 1)},$$

where $x = kb$. This is a valid approximation if both x and mx are less than 0.4. For a typical protein in aqueous solution $m = 1.2$. For these values of x and m, γ_0, γ_1, β_0, β_1 are sufficiently small that $\sin \gamma = \gamma$, $\cos \gamma = 1$, etc. and

$$\mathrm{Im}\ [S_{1(0)} - S_{2(0)}] = \frac{kl}{\pi}(\beta_0 - 2\gamma_1) = \frac{k^3 V}{4\pi}\left[(m^2 - 1) - \frac{2(m^2 - 1)}{m^2 + 1}\right] = k^3 V(\alpha_1 - \alpha_2).$$

This is the result obtained previously for Rayleigh scattering. The maximum value of $2b$ satisfying the condition on x is 400 Å, for green light.

The calculation was made assuming optical isotropy. If $\Delta mi \neq 0$, where Δmi denotes relative intrinsic birefringence, there is an error of about 2 per cent for

$\Delta mi = 0.03$, which is a representative value for proteins. Thus for this particular case of parallel rods oriented perpendicular to the incident beam the birefringence equation (1) is valid for very long rods of diameter up to 400 Å. The result is relevant to some biological systems consisting of parallel oriented rod elements. In the flow birefringence case, the rods are not perpendicular to the incident beam, but for very long rods the extinction angle can be less than 5 or 10 degrees. Thus these dimensions can probably be taken as an estimate of the upper size range to which the birefringence equation may be applied.

EXPERIMENTAL VERIFICATION

The two indices can be obtained from measurements of Δn and $\frac{dc}{dn}$. A plot of the experimental quantity $4\pi(g_1 - g_2)$ for rods *versus* intrinsic birefringence for a number of values of $\bar{n} = (n_1 + 2n_2)/3$ is given in Fig. 3. The indices of most proteins and

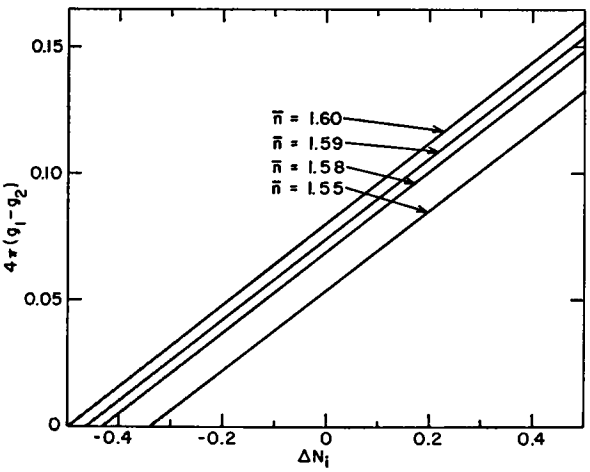

FIGURE 3 Dependence of the optical parameter $4\pi(g_1 - g_2)$ on intrinsic birefringence Δn_i *for* $\bar{n} = (n_1 + 2n_2)/3 =$ constant.

viruses fall in the range 1.57 to 1.60. The relation is linear within the error of the scale of the graph. In general there is no independent method of obtaining the intrinsic birefringence. For the special case of optically isotropic rods, $g_1 - g_2$ could be calculated from the refractive index increment and compared with experiment. Lauffer (19) found the birefringence of TMV to be zero in a solvent of index 1.57 (glycerol-water-aniline) indicating that the particles are isotropic. If the intrinsic birefringence is independent of solvent it is not necessary to risk the possible complications introduced by organic solvents to obtain an indirect check of the theory. $g_1 - g_2$ can be measured in aqueous solution to obtain n_1 and n_2. $g_1 - g_2$ can then be calculated for various solvent indices and compared with experiment. In Lauffer's experiment

and in almost all the work on birefringence in biological systems, it is assumed that the intrinsic birefringence does not depend on the solvent. As noted in the introduction there is a complete disagreement regarding the sign of the intrinsic birefringence, obtained from flow birefringence and from variation of the solvent index. Measurements in a mixed solvent which might be expected not to affect the configuration of proteins, such as glycerol-water mixtures would provide a bridge between the two methods of obtaining intrinsic birefringence. Experiments on a number of fibrous proteins in mixed solvents will be described in succeeding communications.

To interpret such experiments the effects of a mixed solvent on the birefringence must first be considered. If the particle preferentially binds one component of the solvent it may become encased in a shell of refractive index different from that of the bulk solvent. Proteins are usually considered to bind water; therefore, such an effect is apt to occur in glycerol-water mixtures. The shell would be expected to contribute positive form birefringence. In view of the interest in ordered structures in the solvent surrounding the molecule it may be necessary to consider that the shell is also birefringent. If so the intrinsic birefringence, calculated in aqueous solution, would not properly apply to the protein molecule and could depend markedly on the solvent.

The solution of the problem of the polarizability of a shell has been given by Labrum (20). The particle and the shell are represented by a pair of confocal ellipsoids. The only change in the birefringence equation occurs in the optical parameter α_i

$$\alpha_i = \frac{2/3\left[(D-D_s)(D_i-D_s)(A_{pi}-A_{0i}) + D_s\left(\frac{D_s-D_i}{R_0} + \frac{D-D_s}{R_p}\right)\right]}{(D-D_s)(D_i-D_s)\left(A_{0i}A_{pi}-A_{0i}^2+\frac{2A_{0i}}{R_0}\right)+\frac{2D_s(D-D_s)A_{0i}}{R_p}+\frac{2D(D_s-D_i)A_{pi}}{R_0}-\frac{4DD_s}{R_0R_p}}$$

D, D_s, D_i are the dielectric constants of the solvent, shell, and particle, respectively;

$$R_0 = ab^2 \qquad R_p = (a^2 - p^2)^{1/2}(b^2 - p^2)$$

$$A_{01} = \int_0^\infty \frac{dS}{(S + a^2)R_0}, \qquad A_{p1} = \int_{-p^2}^\infty \frac{dS}{(S + a^2)R_p},$$

$$A_{02} = \int_0^\infty \frac{dS}{(S + b^2)R_0}, \qquad A_{p2} = \int_{p^2}^\infty \frac{dS}{(S + b^2)R_p}$$

The surfaces S = constant are confocal ellipsoids. $S = p^2$ and $S = 0$ are the inner and outer boundary surfaces of the shell. The major and minor axes of the particle and the shell are $(a^2 - p^2)^{1/2}$, $(b^2 - p^2)^{1/2}$ and a, b. (There is a misprint in the equation as given by Labrum. The corrected equation is stated above.) The equation is greatly simplified for the case of a long rod.

$$A_{01} = A_{p1} = 0 \qquad A_{02} = \frac{1}{R_0} \qquad A_{p2} = \frac{1}{R_p}$$

$$\text{Let } K = \frac{R_0}{R_p} = \frac{\text{Volume of particle + shell}}{\text{Volume of particle}} = \frac{V'}{V}$$

$$\alpha_1 = \frac{V'}{4\pi} \frac{D_1 - D_s + (D_s - D)K}{KD}$$

$$\alpha_2 = \frac{V'}{4\pi} \frac{2[K(D_2 + D_s)(D_s - D) + (D_s + D)(D_2 - D_s)]}{K(D_s + D)(D_2 + D_s) - (D - D_s)(D_2 - D_s)}$$

The birefringence equation can be written in the same form as before; $\Delta n = 2\pi n\phi'(g_1 - g_2)f(\sigma p)$, where $\phi' = NV'$ and $\alpha_i = V'g_i$.

This equation will be applied to birefringence measurements in mixed solvents in a subsequent paper. As an example of its use the effect of the concentric 40 Å diameter hole in the TMV. molecule (21) on its apparent birefringence will be calculated. To apply the equation, the hole is taken as the particle and the shell becomes the virus.

$$g_1 = \frac{1}{4\pi} \frac{K - 1}{K} (m_1^2 - 1), \qquad g_2 = \frac{1}{4\pi} \frac{K - 1}{K} \frac{2(m_2^2 - 1)}{m_2^2 + 1 - (m_2^2 - 1)/K(m_2^2 + 1)}$$

K is approximately 15; the hole occupying about 7 per cent of the volume. The second term in the denominator of g_2 can be neglected to an error of 0.2 per cent. Since $[(K - 1)/K]\phi' = \phi$, the previous birefringence equation (1) is obtained. If TMV were optically isotropic, with $m = 1.2$, ignoring the hole would introduce an apparent negative birefringence of 5×10^{-4} which is less than the experimental error.

A similar result for a spherical shell has been given by Van de Hulst based on the solution of Mie scattering by spherical shells obtained by Guttler (22). For a small sphere such that the first term of the series is sufficient,

$$g' = \frac{1}{4\pi} \frac{(m^2 - 1)(2m^2 + 1)}{(m^2 + 2)(2m^2 + 1) + 9m^2/(K - 1)}.$$

The same result is obtained from Labrum's equation if the particle is allowed to become a spherical shell. A moderately small hole, $K = 15$ gives $\phi'g' = \phi g$ to an error of 0.1 per cent. These two cases of concentric ellipsoidal and spherical shells are probably the only ones that can be solved in a simple fashion. It is unlikely that displacing the hole from a central or axial position would affect the physical problem. Thus, it can be concluded that departure of the particle shape from simple geometry in the form of cavities (which may or may not be filled with solvent) will not affect the birefringence for cavities up to about 10 per cent of the volume of the particle. This result is of some interest, since denaturation of proteins usually leads to a small volume change (order of 3 per cent) which is attributed to an internal free volume. (23)

DISCUSSION

The flow birefringence equation of Peterlin and Stuart has been rederived by means of light scattering theory. The restriction to ellipsoidal particles with major axis small compared to the wavelength has been removed. The equation (1) is unchanged, but may now be applied to the class of particles with minor axis small compared to the wavelength, and refractive index close to that of the medium. This class includes most molecules of biological interest. As was discussed in the introduction, the birefringence of fibrous proteins calculated from flow birefringence measurements does not agree, even with regard to sign, with the values obtained by variation of solvent index. Since this discrepancy may be the result of a faulty birefringence theory it is important to state the assumptions made in the derivation. If the distribution function is accepted, and there is a good reason to suppose that it is correct, (24) then the derivation essentially depends on the Rayleigh-Gans approximation which has been successfully applied to the scattering of a variety of macromolecules. However, two assumptions have been made either of which may be questioned:

1. The problem was formulated in terms of excess polarizabilities thereby avoiding a calculation of the local field. It can be easily shown that this assumption, when used to calculate the refractive index of a mixture of spherical particles does not yield the Lorentz-Lorenz equation even at infinite dilution. This treatment should therefore be regarded as an approximate way of dealing with the local field.
2. Scattering theory is concerned with polarizabilities. To obtain the principle refractive indices it must be assumed that the particle can be represented by a continuous distribution of matter of ellipsoidal shape. The relation of refractive indices to polarizability strictly applies to macroscopic particles, and the minimum size of particle which can still be regarded as a continuous dielectric must be determined by experiment. The polarizability difference ($g_1 - g_2$) may be more reliable than the refractive index difference and for certain applications a knowledge of this quantity is sufficient.

Further theoretical and experimental work is required to determine the errors introduced by these assumptions.

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