The microscopic protein structure of the lens with a theory for cataract formation as determined by Raman spectroscopy of intact bovine lenses*

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Intact bovine lenses have been studied using the polarized Raman spectroscopic technique. A brief theoretical and experimental review of Raman spectroscopy is presented. From the dependence of the Raman depolarization ratio on the propagation direction of the incident radiation we have determined that the uniaxial qualities of the lens result from microscopic anisotropy and have established the quantitative positional correlation of specific chemical bonds with respect to the lens optic axis. In particular, the hydrogen bonded linear CONH groups of the antiparallel β-pleated sheet are preferentially oriented in directions orthogonal to the lens optic axis. The Raman spectra of intact lenses do not exhibit bands at positions characteristic of either the α-helix or the random coil protein structure. The antiparallel β-pleated sheet protein microstructure and the lens fiber cross-sectional macrostructure exhibit a remarkable similarity. This similarity may be causal and is consistent with the protein concentration of the lens, the birefringent properties observed by both Lenhard and Brewster, the CONH bond angle distribution with respect to the optic axis, and the lens anatomy. It is suggested that cortical cataracts are caused by fluctuations in protein orientational order.

Key words: birefringence, lens protein, anti-parallel β-pleated sheet, Raman spectroscopy, protein orientational order, cataract.
Lenhard performed a careful study of the positional dependence of the optical anisotropy of the lens. He determined the index ellipsoid associated with each area of the lens and attributed its uniaxial qualities to “micells” of typical length 10 to 1,000 A. Apparently, Lenhard’s comprehensive results quenched research in lens birefringence; since 1933 the optical anisotropy of the intact lens has not been re-examined.

The lack of interest in lens birefringence exhibited during the past several decades is particularly surprising in view of the paucity of information on the details of the chemical microstructure of the intact lens and the intimate relationship between structural order and transparency of solid substances. Accordingly, the purpose of this paper is to: (1) determine if the uniaxial qualities of the lens result from form birefringence, or are a manifestation of microscopic properties, (2) specify the lens protein structure and identify specific chemical bonds and groups, (3) describe the positional correlation of specific chemical bonds in the proteins with the optic axis of the lens, (4) establish whether or not there is any protein ordering in the lens, and (5) elucidate the possible relationship between protein structural ordering and lens transparency.

To accomplish the tasks specified above, it is desirable that one examine the intact lens. Unfortunately, the familiar investigative techniques commonly employed in most research laboratories are not applicable to an examination of the intact lens. For instance, water, the major chemical constituent of the lens exhibits strong infrared absorption in the spectral region most rich in protein absorption bands. Therefore, infrared spectroscopy, a simple yet powerful investigative tool, cannot be usefully employed to study the intact lens.

With the advent of laser sources, it is now possible to excite the photoluminescence spectrum of the intact lens and to examine the polarization properties of the emitted radiation. We have performed an extensive study of the propagation and polarization dependence of the photoluminescence of intact bovine and human lenses. The results of this study will be published elsewhere. Suffice it to say here that the lens photoluminescence spectra do not provide the detailed structural and chemical information we seek.

There is one noninvasive technique, Raman spectroscopy, which when judiciously applied to a study of the intact lens yields significant information on both structural and chemical properties. Because the subject of this paper is of primary interest to ophthalmologists who, we assume, are quite unfamiliar with the principles of Raman spectroscopy, it is useful to give a concise simple mathematical description of the Raman effect.

Theory

Consider a molecule which is exposed to an applied electric field \( \vec{E} \). The applied field will generate in the molecule an induced dipole moment \( \vec{p} \) according to the equation:

\[
\vec{p} = \vec{\alpha} \cdot \vec{E}
\]

where \( \vec{\alpha} \) is the polarizability tensor of the molecule. Presently, for simplicity, we treat the polarizability as a scalar quantity. The polarizability
of the molecule can be expanded in a Taylor series about the molecular normal coordinate $Q_m$ of the $m^{th}$ normal vibrational mode. Then:

$$\alpha = \alpha_0 + \sum_{m,n} \left( \frac{\partial^2 \alpha}{\partial Q_m \partial Q_n} \right) Q_m Q_n + \ldots$$ (2)

Here the "0" indicates that the subscripted quantity is evaluated at the equilibrium position of the normal coordinate. Both the applied electric field and the normal coordinates vary periodically in time according to:

$$E_i = E_{i0} \cos \omega_i t$$
$$Q_m = Q_{m0} \cos \omega_m t$$ (4)

where $\omega_i$ is the frequency of the applied electric field and $\omega_m$ is the molecular vibrational frequency associated with the $m^{th}$ normal mode. If we treat the applied electric field and induced dipole moment as scalars, we can substitute Equations 2, 3, and 4 into Equation 1 to obtain

$$P = \alpha_0 E_{i0} \cos \omega_i t + \frac{1}{2} \sum_{m,n} \left( \frac{\partial^2 \alpha}{\partial Q_m \partial Q_n} \right) Q_m Q_n + \ldots$$ (5)

Here we retain only the first two terms in the Taylor series expansion of the polarizability. Upon re-expressing the cosine product in the second term of Equation 5, that equation becomes:

$$P = \alpha_0 E_{i0} \cos \omega_i t + \frac{1}{2} \sum_{m} \left( \frac{\partial \alpha}{\partial Q_m} \right) Q_m E_{i0} \cos \omega_i t + \ldots$$ (6)

Equation 6 has considerable physical significance. It indicates that under the action of an applied electric field oscillating at a frequency $\omega_i$, a molecule will experience an induced dipole moment which not only oscillates at the frequency $\omega_i$, but also at the frequencies $(\omega_i + \omega_m)$ and $(\omega_i - \omega_m)$ where $m = 1, 2, \ldots, N$; $N$ being the number of normal modes of the molecule. Now it is a fact of classical electromagnetic theory that oscillating dipoles radiate energy in the form of electromagnetic waves which have a frequency equal to the frequency of oscillation. Thus, the molecule reradiates, i.e., scatters, incident monochromatic radiation. The first term in Equation 6 corresponds to Rayleigh scattering for which the scattered radiation has the same frequency as the incident radiation. The second term of Equation 6 gives rise to first-order Raman scattering for which the scattered radiation contains Stokes emission at $(\omega_i - \omega_m)$ down-shifted in frequency from the exciting radiation and anti-Stokes emission at $(\omega_i + \omega_m)$ up-shifted in frequency. The Raman scattering process is illustrated schematically in Fig. 2.

Notice that in order for the $m^{th}$ vibrational mode to be "Raman active"

$$\left( \frac{\partial \alpha}{\partial Q_m} \right) \neq 0.$$ (7)

For each mode satisfying Equation 7, there will appear in the spectrum of the scattered radiation...
a pair of Raman "lines" equally displaced in frequency from \( \omega_i \), the frequency of the exciting radiation.

Traditionally, the Raman spectrum of a given substance, like the infrared spectrum, has been used as a chemical fingerprint. Specific chemical bonds give rise to characteristic frequencies \( \omega_i \), which depend only slightly upon the substance containing that bond. However, the Raman effect is characterized by a change in optical activity with a given normal vibration whereas infrared absorption is a manifestation of a change in dipole moment with a normal vibration. While we have treated the optical activity as a scalar in Equations 2 through 7, it is in reality a second-rank symmetric tensor. Such a tensor yields significantly more information on the structure of a given system and on the symmetry character of the normal coordinate than does the dipole moment vector associated with infrared absorption.

Specifically, the Raman spectral line corresponding to the \( m \) th mode has associated with it one or more Raman tensors the number of such tensors being equal to the degeneracy (at most three) and characteristic of the symmetry of the \( m \) th normal coordinate. Let the \( m \) th normal mode be non-degenerate. Then the Raman tensor for that mode can be written as:

\[
\langle a'_{ij} \rangle_m = \left( \frac{\delta a'_{ij}}{3Q_m} \right) \text{ with } i,j = x,y,z \tag{8}
\]

and \( x,y,z \) refer to the symmetry axes of the molecule.

Consider a collection of identical molecules each with a polarizability tensor \( \sigma \). If those molecules were arranged in an ordered system such as a molecular single crystal, the polarizability of the crystal would be proportional to \( \sigma \). Then the Raman component of the induced dipole moment associated with the \( m \) th normal mode would be:

\[
(P_m)_i \approx \frac{3}{4} \langle a'_{ij} \rangle_m E_i \text{ with } i,j = x,y,z \tag{9}
\]

The intensity of the scattered radiation polarized in the \( i \) direction, \( I_i \), is proportional to \( |(P_m)_i|^2 \). Thus, by properly selecting the polarization directions of the incident and scattered radiation one can determine which of the tensor components \( \langle a'_{ij} \rangle_m \) are nonzero and thereby establish the symmetry character of the \( m \) th normal mode.

In contrast to the case of an ordered single crystal, a disordered material such as a polycrystal, liquid, gas, or biological structure is composed of molecules which do not exhibit perfect orientational and positional correlation. Thus it is not possible to determine the specific nonzero tensor components \( \langle a'_{ij} \rangle_m \). Nevertheless, by applying the proper orientational average to the Raman tensor \( \langle a'_{ij} \rangle_m \) it is still possible to extract mode symmetry information. We define the mean value \( \overline{\sigma}_m \) and anisotropy \( \gamma_m \) of the Raman tensor associated with the \( m \) th mode as follows:

\[
\overline{\sigma}_m = \frac{1}{3} \left( \langle a'_{xx} \rangle_m + \langle a'_{yy} \rangle_m + \langle a'_{zz} \rangle_m \right) \tag{10}
\]

and

\[
\gamma_m = \frac{1}{6} \left\{ \left( \langle a'_{xx} \rangle_m - \langle a'_{yy} \rangle_m \right)^2 + \left( \langle a'_{yy} \rangle_m - \langle a'_{zz} \rangle_m \right)^2 + \left( \langle a'_{zz} \rangle_m - \langle a'_{xx} \rangle_m \right)^2 + 6 \left( \langle a'_{xx} \rangle_m + \langle a'_{yy} \rangle_m + \langle a'_{zz} \rangle_m \right)^2 \right\} \tag{11}
\]

It is possible to relate \( \gamma_m \) and \( \overline{\sigma}_m \) to an observable quantity, the depolarization ratio, \( \rho \), by applying the proper orientational average to \( \langle a'_{ij} \rangle_m \). Thus, theoretically it can be shown that for measurements made with linearly polarized incident radiation:

\[
\rho_m = \frac{3\gamma_m}{25(\overline{\sigma}_m)^2 + 4\gamma_m}. \tag{12}
\]

Experimentally, one determines \( \rho_m \) from the scattering configurations shown in Fig. 3 for which:

\[
\rho_m = \frac{I_{1v} - I_{1v}'}{I_{1v}}. \tag{13}
\]

Here, \( I_{1v} \) is the intensity of the Raman scattered radiation collected through an analyzer set with its transmission axis perpendicular to (vertical with respect to) the scattering plane when the exciting radiation is polarized in (horizontal with respect to) the scattering plane. Similarly, to measure \( I_{1v}' \) the analyzer is set with its transmission axis perpendicular to the scattering plane when the exciting radiation is linearly polarized perpendicular to the scattering plane. [Note: The scattering plane is defined as the plane containing the propagation directions of the incident and scattered radiation.]

According to Equation 12 the depolarization ratio of a given Raman line of a disordered substance can take on values in the range

\[
0 \leq \rho_m \leq \frac{3}{4} \tag{14}
\]

However, the only Raman active vibrations for which \( \overline{\sigma}_m \neq 0 \) are the totally symmetric non-degenerate vibrations which ordinarily are designated by the group theoretical symbol \( A_1 \). For these \( A_1 \) vibrations Equation 14 applies; for all others \( \rho_m = \frac{3}{4} \) since \( \overline{\sigma}_m = 0 \). In addition,
$$\rho = \frac{I_{HV}}{I_{VV}}$$

Fig. 3. The experimental polarization configurations for measuring the Raman depolarization ratio, ρ. The electric field vector and propagation directions of the incident laser radiation and Raman scattered radiation are denoted by \( \vec{E}_i, \vec{k}_i \) and \( \vec{E}_s, \vec{k}_s \), respectively.

The above classical analysis of the Raman effect has been necessarily simplified in order to facilitate an exposition of the underlying physical principles. Those readers interested in the modern quantum mechanical description of the Raman effect are referred to appropriate journal articles.

**Apparatus and procedure**

Bovine eyes were obtained from a local slaughterhouse. The intact lens was carefully removed from the eye and immersed in a beaker containing sterile saline. A thin metal wire was used to support the lens in a position in which its optic axis was either parallel or perpendicular to the direction of propagation of the exciting laser radiation. A block diagram of the experimental setup for measuring Raman spectra is shown in Fig. 4. Monochromatic, linearly polarized radiation from an argon ion laser passes through diaphragms \( D_1 \) and \( D_2 \) and through a half-wave (\( \lambda/2 \)) plate, the purpose of which is to rotate the plane of polarization to any desired position. The laser light is then focused by a lens to a minimum spot size of \( \sim 100 \, \mu\text{m} \) in the bovine lens and the transmitted component is collected by a power meter. Radiation Raman and/or Rayleigh scattered at an angle of \( 90 \pm 15^\circ \) is focused by an F1 collection lens through an analyzer and onto the entrance slit of a Jarrel-Ash double monochromator. The
Fig. 5. The VV polarized Raman and photoluminescence spectra of an intact bovine lens. The wavelength and power of the incident laser radiation are designated \( \lambda_0 \) and \( P_\text{in} \), respectively. The abscissa is linear in wavelength.

Dispersed radiation passed by the monochromator is detected by a cooled phototube coupled to a digital photon-counting system. Ultimately, a display of scattered intensity versus wavelength is produced on a strip chart recorder.

The typical time required to record the Raman spectrum of a bovine lens ranged from 20 minutes to 1 hour. During this time the transmitted laser power was continuously monitored in order to detect radiation-induced changes in the turbidity of the lens. No such changes were observed.

If accurate depolarization measurements are to be made, it is imperative that the state of polarization of the incident laser radiation remains unaltered as it propagates through the bovine lens. Therefore, before recording a Raman spectrum, the degree of polarization of the transmitted radiation was checked by placing an appropriately oriented analyzer between the bovine lens and the power meter. With the optic axis of the lens parallel to or orthogonal to the propagation direction of the incident radiation, the measured degree of polarization was greater than 0.98.

In addition to intact lenses, the polarized Raman spectra of lens homogenate and of a lysozyme solution were examined. Five hundred milligrams of lysozyme grade I obtained from Sigma Chemical Co. was dissolved in 3 c.c. of deionized water. The lens homogenate was prepared by homogenizing 10 fresh lenses in 20 ml. of 0.2 M phosphate buffer, pH 7.4, under nitrogen and then centrifuging at 10,000 g for 20 minutes. The lens supernatant or lysozyme solution was placed in a sample cell for examination in the Raman spectrometer.

Experimental results

The VV polarized Stokes Raman spectrum of an intact bovine lens excited at three different laser radiation wavelengths is shown in Fig. 5. For 4,579 Å excitation one detects a sharp line spectrum superimposed upon a broad continuous photoluminescence background that extends from \( \lambda \approx 4,700 \) to 6,000 Å. As can be seen from Fig. 5, radiation of longer wavelength at 4,765 Å and in particular 4,880 Å is much less efficient at exciting
Fig. 6. The polarized Raman spectra of an intact bovine lens excited with the incident laser radiation propagating along the lens optic axis. The abscissa is linear in wavelength rather than wavenumber. The spectra were recorded with a spectral slit width of 3 cm.$^{-1}$.

The polarized Stokes Raman spectra of an intact bovine lens excited by incident light propagating along and orthogonal to the lens optic axis are shown in Figs. 6 and 7, respectively. In those figures $k_\parallel$ represents a vector in the direction of propagation of the incident [scattered] radiation and the $z$-axis is parallel to the optic axis of the lens. While the corresponding spectra of Figs. 6 and 7 appear to be the same at first glance, one finds upon more careful examination that the depolarization ratios, $\rho_\parallel$ of several of the observed Raman lines when measured with $k_\parallel$ are significantly lower than the ratios $\rho_\perp$ measured with $k_\perp$. The shift in wave-numbers, $\delta \nu_m$ (where

$$\delta \nu_m = \frac{1}{\lambda_1} - \frac{1}{\lambda_m},$$

$\lambda_1$ being the laser excitation wavelength and $\lambda_m$ being the wavelength at which the $m^{th}$ Raman line occurs) of each of the Raman lines observed in the spectrum of the intact bovine lens is tabulated in Table I. Since the lens fiber membranes constitute less than 1 per cent of its bulk mass, we attribute the spectra of Figs. 6 through 8 to proteins in the lens. As can be seen from Table I, most of the observed lines have been assigned to the vibrations of specific chemical bonds or groups. These assignments are based upon a comparison of the Raman shifts of the bovine lens with those observed in the Raman spectra of amino acids and specific proteins.$^{15,16}$ Also shown in Table I is the depolarization ratio $\rho_\parallel$ of each Raman line measured with incident light propagating parallel [perpendicular] to the lens optic axis.

Discussion

While most of the assignments of Table I are self-explanatory, the modes of the peptide CONH groups are of particular interest. The peptide groups exhibit two characteristic group frequencies in the Raman spectrum, a singlet at 1,660 cm.$^{-1}$ designated amide I and a triplet at 1,240 cm.$^{-1}$, 1,262 cm.$^{-1}$, and 1,274 cm.$^{-1}$ designated amide II.$^{15}$ The amide I singlet results from the C=O stretching vibration whereas the amide III triplet is associated with C—N stretching and N—H in plane bending vibrations.
In agreement with Yu and co-workers, we assign the strong line at 1,675 cm$^{-1}$ to the amide I singlet. This specific assignment constitutes definitive proof that the proteins of the lens have an antiparallel $\beta$-pleated sheet structure. It is of particular interest to note that no Raman lines are observed between 1,630 cm$^{-1}$ and 1,654 cm$^{-1}$, the expected frequency shifts for the amide I band of the parallel $\beta$-pleated sheet and $\alpha$-helix structures, respectively.

Based on their optical rotary dispersion and circular dichroism studies of $\alpha$-crystallin, Li and Spector concluded that that substance exists in the $\beta$-structure with either a pure parallel or mixed parallel and antiparallel arrangement. Thus, the assignment of the lens protein structure as antiparallel $\beta$-pleated sheet is consistent with their work. On the other hand, while Li and Spector find that up to 65 per cent of $\alpha$-crystallin has the random coil structure neither we, nor Yu and co-workers, find any evidence for the random coil form in the intact lens. Quite possibly this discrepancy results because Li and Spector studied lens homogenate, not the intact lens. The homogenization process certainly could lead to a disruption of long-range order in a pleated sheet structure accompanied by coiling of the severed protein strands.

The antiparallel $\beta$-pleated sheet structure...
as given by Marsh, Corey, and Pauling\(^{22}\) is shown in Fig. 8, in which is also indicated the rectangular unit cell associated with the two-dimensional hexagonal lattice. Note that the hexagonal structure exhibits two distinct bond lengths in the ratio 3:1 and that all bond angles are \(\sim 120^\circ\). For comparison with the \(\beta\)-pleated sheet structure, we show in Fig. 9, a microscope photograph of the cross-section of cortical bovine lens fibers. In Fig. 10 is shown the rectangular unit cell of two-dimensional \(c2\text{mm}\). symmetry\(^{23}\) for an idealized (i.e., distortionless) fiber cross-section. The similarity between Figs. 8 through 10 is remarkable. Not only are the sides of the basic hexagon of the fiber macrostructure in the ratio of approximately 3:1, but also the bond angles of the macrostructure are \(\sim 120^\circ\). It is unreasonable to expect a biological structure such as the lens to be perfectly ordered. Nevertheless, the lens is a gel consisting of \(\sim 35\) per cent protein\(^{24}\). Therefore, the proteins must be closely packed inside the fiber membranes. One

### Table I. Raman shifts in cm\(^{-1}\) and depolarization ratios for intact bovine lenses. The depolarization ratios \(\rho_0\) and \(\rho_1\) were measured with 4,880 Å incident radiation propagating parallel and perpendicular, respectively, to the lens optic axis. The Raman shifts are accurate to \(\pm 3\) cm\(^{-1}\) and the depolarization ratios are accurate to within \(\pm 15\) per cent

<table>
<thead>
<tr>
<th>Raman shift (\Delta\nu) (cm(^{-1}))</th>
<th>Depolarization ratios (\rho_0)</th>
<th>(\rho_1)</th>
<th>Assignments*</th>
</tr>
</thead>
<tbody>
<tr>
<td>765</td>
<td>0</td>
<td>0</td>
<td>Trp</td>
</tr>
<tr>
<td>837</td>
<td>0</td>
<td>0</td>
<td>Tyr</td>
</tr>
<tr>
<td>861</td>
<td>0</td>
<td>0</td>
<td>Tyr</td>
</tr>
<tr>
<td>883</td>
<td>0</td>
<td>0</td>
<td>Tyr</td>
</tr>
<tr>
<td>940</td>
<td>0</td>
<td>0</td>
<td>Tyr</td>
</tr>
<tr>
<td>1,009</td>
<td>0.14</td>
<td>0.06</td>
<td>Phe</td>
</tr>
<tr>
<td>1,036</td>
<td>0.07</td>
<td>0.06</td>
<td>Phe</td>
</tr>
<tr>
<td>1,132</td>
<td>0.28</td>
<td>0</td>
<td>Skeletal modes</td>
</tr>
<tr>
<td>1,179</td>
<td>0.23</td>
<td>0</td>
<td>Tyr</td>
</tr>
<tr>
<td>1,213</td>
<td>0.17</td>
<td>0.06</td>
<td>Tyr</td>
</tr>
<tr>
<td>1,243</td>
<td>0.23</td>
<td>0.19</td>
<td>Amide III</td>
</tr>
<tr>
<td>1,259</td>
<td>0.24</td>
<td>0.12</td>
<td>Amide III</td>
</tr>
<tr>
<td>1,273</td>
<td>0.24</td>
<td>0.12</td>
<td>Amide III</td>
</tr>
<tr>
<td>1,340</td>
<td>0.36</td>
<td>0.31</td>
<td>Trp</td>
</tr>
<tr>
<td>1,450</td>
<td>0.50</td>
<td>0.64</td>
<td>CH bending</td>
</tr>
<tr>
<td>1,552</td>
<td>0.25</td>
<td>0.15</td>
<td>Trp</td>
</tr>
<tr>
<td>1,619</td>
<td>0.50</td>
<td>0.40</td>
<td>Trp, Tyr, and Phe</td>
</tr>
<tr>
<td>1,675</td>
<td>0.26</td>
<td>0.16</td>
<td>Amide I</td>
</tr>
<tr>
<td>2,881</td>
<td>0.09</td>
<td>0.08</td>
<td>Aliphatic CH stretching</td>
</tr>
<tr>
<td>2,941</td>
<td>0.15</td>
<td>0.14</td>
<td>Aliphatic CH stretching</td>
</tr>
<tr>
<td>3,068</td>
<td>0.13</td>
<td>0.14</td>
<td>Aromatic CH stretching</td>
</tr>
<tr>
<td>3,308</td>
<td>0.15</td>
<td>0.14</td>
<td>Superposition of H(_2)O and H-bonded N-H stretching frequencies</td>
</tr>
</tbody>
</table>

*Trp, tryptophan; Tyr, tyrosine; Phe, phenylalanine.
can easily envision the unit cell of the idealized fiber macrostructure as being built up from an integral number of geometrically similar but much smaller microcells, i.e., those of the \( \beta \)-pleated sheet. Since the position of each fiber is determined by its neighbors, the fiber membranes do not prevent long-range interfiber protein correlation in the macrostructure.

If the lens proteins have a globular conformation, the relationship between the \( \beta \)-pleated sheet microstructure and lens fiber macrostructure would most likely be fortuitous. However, the tertiary structure of native lens proteins has not been established. Therefore, the relationship between the fiber macrostructure and protein microstructure may indeed be causal.

If the protein microstructure and fiber macrostructure of the lens are intimately related, one would not expect the protein molecules to be randomly oriented in the lens, i.e., the lens would best be described as microscopically anisotropic. The fact that several of the Raman lines of Table I exhibit propagation dependent depolarization ratios with \( \rho_\parallel \neq \rho_\perp \) is direct evidence of microscopic anisotropy. It is this anisotropy and not form birefringence which is responsible for the uniaxial quality of the lens observed by Brewster and others. Though the proteins themselves may exhibit some degree of ordering in the lens, specific chemical bonds, for instance the C—H bond, which are located at many orientationally inequivalent sites within each protein strand would appear to have a random or nearly random orientation in the macrostructure. Thus the aliphatic C—H stretching vibrations produce a propagation independent depolarization ratio as can be seen from Table I.

In order to quantitatively characterize the protein order in the lens we focus on the polarization properties of the amide I vibration of the CONH peptide group. This group is of specific interest for several reasons: (1) parallel stacking of the CONH groups is a signature of the antiparallel \( \beta \)-pleated sheet structure, (2) the CONH group constitutes a linear bond the orientational distribution of which can be used to determine the corresponding protein chain distribution, (3) there is a clear dependence of the depolarization ratio of the amide I vibration on the direction of propagation of incident radiation [see Table I].

In Fig. 11, we show the amide I region of the polarized Raman spectra of a lysozyme-H\(_2\)O solution and of a bovine lens homogenate. Each of the above described liquids was relatively nonviscous. We therefore assume that the peptide groups are randomly oriented in such solutions. The depolarization ratios of the amide I vibrations in the lens homogenate and lysozyme solution are both 0.27 as determined from Fig. 11. On the basis of Equation 14 and these depolarization ratios we ascribe the amide I vibration to the \( A_\text{II} \) symmetry species of the \( C_{\infty v} \) point group. The Raman tensor corresponding to the amide I vibration is of the form:

\[
\begin{pmatrix}
a & a & 0 \\
a & 0 & a \\
0 & a & b
\end{pmatrix}
\]

Now consider the diagram shown in Fig. 12. In that figure, \( x, y, \) and \( z \) correspond to the symmetry axes of the peptide group with the \( z \) axis along the CONH bond while \( x'', y'', \) and \( z'' \) represent an orthogonal set of axes fixed in the lens, \( z'' \) being parallel to the lens optic axis. Note that the orientational position of the peptide group with respect to the optic axis is determined solely by the angles \( \vartheta \) and \( \phi \). The Raman tensor of Equation 15 which is written in terms of the \( x, y, z \) axes of Fig. 12 can be transformed to the \( x'', y'', z'' \) system by the following similarity transformation:

\[
(\mathbf{g}'')_{\text{amide I}} = \mathbf{S} \left(\begin{array}{ccc}
a & a & 0 \\
a & 0 & a \\
0 & a & b
\end{array}\right) \mathbf{S}^{-1}
\]

where the rotation matrix, \( \mathbf{S} \), transforms the \( x, y, z \) axes into the \( x'', y'', z'' \) axes, i.e.,

\[
\begin{bmatrix}
x'' \\
y'' \\
z''
\end{bmatrix} = \mathbf{S} \begin{bmatrix}
x \\
y \\
z
\end{bmatrix}.
\]


When transformed as described above, the Raman tensor becomes:

\[
\begin{pmatrix}
  a \sin^2 \phi + G(\phi) \cos^2 \phi & \frac{b-a}{2} \sin \phi \sin 2\phi & \frac{b-a}{2} \sin 2\phi \cos \phi \\
  \frac{b-a}{2} \sin \phi \sin 2\phi & a \cos^2 \phi + G(\phi) \sin^2 \phi & \frac{b-a}{2} \sin 2\phi \sin \phi \\
  \frac{b-a}{2} \sin 2\phi \cos \phi & \frac{b-a}{2} \sin \phi \sin \phi & a \sin^2 \phi + b \cos^2 \phi
\end{pmatrix}
\]

where

\[
G(\phi) = a \cos^2 \phi + b \sin^2 \phi.
\]

If all of the CONH bonds in a given system pointed along the z axis of Fig. 12, the depolarization ratios \(\rho_\parallel\) and \(\rho_\perp\) of the amide I vibration would be given by:

\[
\rho_\parallel = \frac{I_{\|}}{I_{\perp}} = \frac{[(a'\cdot\cdot'\cdot')_{\text{amide I}}]^2}{[(a''\cdot\cdot''\cdot'')_{\text{amide I}}]^2} = \frac{\frac{b-a}{2} \sin^2 \phi \sin 2\phi}{[a \cos^2 \phi + (a \cos^2 \phi + b \sin^2 \phi) \sin^2 \phi]^2}
\]

\[
\rho_\perp = \frac{I_{\perp}}{I_{\parallel}} = \frac{[(a''\cdot\cdot''\cdot'')_{\text{amide I}}]^2}{[(a''\cdot\cdot''\cdot'')_{\text{amide I}}]^2} = \frac{\frac{b-a}{2} \sin 2\phi \sin \phi}{[a \sin^2 \phi + b \cos^2 \phi]^2}
\]

Note that there are two values of \(\rho_\perp\) because incident radiation can propagate in a direction orthogonal to the optic axis along either \(x''\) or \(y''\). Suppose that instead of all being aligned along the z axis, the CONH bonds of a multibond system are oriented randomly. In this case the probability \(P(\phi, \phi')\) of finding a CONH bond making an angle between \(\phi\) and \(\phi + d\phi\) and between \(\phi'\) and \(\phi' + d\phi'\) with respect to the z' axis is given by

\[
P(\phi, \phi') = \frac{1}{4\pi} \sin \phi \sin \phi' d\phi \ d\phi'.
\]

The probability of finding a CONH band at least somewhere in space is unity. Thus:

\[
\int_0^\pi \int_0^{2\pi} P(\phi, \phi) = 1.
\]

To calculate the depolarization ratio of the amide I group for a random distribution of bond orientations, we must average over all possible orientations using the probability function of Equation 22. Let \(F(\phi, \phi')\) be some general function of \(\phi\) and \(\phi'\). The average of that function is written \(\langle F(\phi, \phi') \rangle\) and is given by:

\[
\langle F(\phi, \phi') \rangle = \int_0^\pi \int_0^{2\pi} F(\phi, \phi') P(\phi, \phi')
\]

Now if we make the substitution

\[
x = \frac{b}{a}
\]

in Equations 19 through 21 and calculate the depolarization ratios of the random system described above we find:

\[
\rho_\parallel = \frac{\langle I_{\|} \rangle}{\langle I_{\perp} \rangle} = \frac{(x - 1)^2}{3x^2 + 4x + 8}
\]

\[
= \frac{3y_{\text{amide I}}}{45(y_{\text{amide I}})^2 + 4y_{\text{amide I}}}
\]

where \(y_{\text{amide I}}\) and \((\bar{\alpha})_{\text{amide I}}\) are respectively, the mean value and anisotropy of...
Fig. 11. Polarized Raman spectra of (a) bovine lens homogenate and (b) a lysozyme H₂O solution. The dashed lines indicate the background levels assumed for measuring the amide I band depolarization ratios which were determined to be 0.27 in both cases. The spectra were recorded using spectral slit widths of 5 cm⁻¹. The abscissa is linear in wavelength rather than wavenumber.

the Raman tensor of Equation 15 calculated using Equations 10, 11, and 25. Upon calculating the average value of \( \rho_{I(1)} \) and \( \rho_{I(2)} \) using the probability function of Equation 22, we find that:

\[
\rho_{I(1)} = \rho_{I(2)} = \rho_H \quad (27)
\]

where \( \rho_H \) is given by Equation 26. Thus, as expected, the depolarization ratio for the vibrational mode of a randomly oriented chemical bond in a macroscopic system is independent of the direction of propagation of the incident radiation.

Instead of assuming a random distribution of amide bonds let us assume a distribution compatible with the uniaxial anisotropy of the bovine lens. First suppose that the peptide groups are preferentially aligned in directions orthogonal to the optic axis of the lens but are randomly oriented about that axis. Such a situation can be represented mathematically by the probability function

\[
P_\alpha(\phi,\theta) = (A_\alpha) \left( \sin^4 \phi \right) \left( \sin \phi d\phi \, d\theta \right) \quad (27)
\]

where

\[
A_\alpha = \int_0^\pi \int_0^{2\pi} \sin^{n+1} \phi \, d\phi \, d\theta \quad (28)
\]
The second term on the right of Equation 27 has the effect of skewing the random distribution represented by the third term toward \( \phi = 90^\circ \). Using the probability function of Equation 27, and treating \( n \) and \( x \) as variables, we can calculate the average depolarization ratios.

\[
\rho_{\text{H}}(n,x) = \frac{(x-1)^2}{4} \frac{\langle \sin^4 \phi \sin^2 2\phi \rangle}{\langle [1 + (x-1) \sin^2 \phi \sin^2 \phi] \rangle} = \frac{(x-1)^2}{3(x-1)^2 + 2(n+5)(x-1) + (n+3)(n+5)}
\]

\[
\rho_{\text{L}(1)}(n,x) = \frac{(x-1)^2}{4} \frac{\langle \sin^2 2\phi \sin^4 \phi \rangle}{\langle [1 + (x-1) \sin^2 \phi \sin^2 \phi] \rangle}
\]

\[
\rho_{\text{L}(2)}(n,x) = \frac{(x-1)^2}{4} \frac{\langle \sin^2 2\phi \cos^4 \phi \rangle}{\langle [1 + (x-1) \cos^2 \phi \cos^2 \phi] \rangle}
\]

\[
\rho_{\text{C}}(n,x) = \frac{3(x-1)^2}{2 \left( \frac{3}{n+2} \right) x^2 + 2x + (n+4)}
\]

In Equations 29 and 30 the subscript “s” stands for sine function weighting of the probability function. As expected, the integration over \( \phi \) removes the distinction between \( \rho_{\text{L}(1)} \) and \( \rho_{\text{L}(2)} \).

Following a similar procedure as used to obtain Equations 29 and 30, we can calculate the average depolarization ratios assuming the CONH groups are preferentially aligned along the lens optic axis. The appropriate probability function is:

\[
P_{\text{s}}(\phi,\theta) = B_s \cos^3 \phi \sin \theta \sin \phi \, d\phi \, d\theta
\]

where \( n = 2w; \ w \gg 0 \)

and

\[
B_s = \left[ \int_0^\pi \int_0^{2\pi} \cos^n \phi \sin \theta \sin \phi \, d\phi \, d\theta \right]^{-1}
\]

Then

\[
\rho_{\text{H}}(n,x)_s = \frac{(x-1)^2}{3(x-1)^2 + 2(n+5)(x-1) + (n+3)(n+5)}
\]

\[
\rho_{\text{L}(1)}(n,x)_s = \rho_{\text{L}(2)}(n,x)_s = \frac{(x-1)^2}{(n+3)x^2 + 4x + \frac{8}{n+1}}
\]

The subscript “c” in Equations 33 and 34 denotes cosine function weighting of the probability function.

To test the validity of the probability function given in Equation 27, we set \( \rho_{\text{H}}(n,x)_s \) and \( \rho_{\text{L}}(n,x)_s \) equal to the measured values of the depolarization ratios 0.16 and 0.26, respectively, for the amide I Raman line. Equations 29 and 30 can each then be solved for \( x \) as a function of \( n \). Each equation, being quadratic, will yield two values of \( x \) for each value of \( n \). The solutions \( x^{(1)}_s(n), x^{(2)}_s(n) \) of Equation 29 and \( x^{(1)}_c(n), x^{(2)}_c(n) \) from Equation 30 are plotted in Fig. 13, (a). Barring other constraints, the probability function of Equation 27 is viable if there exists at least one set of values of \( n \) and \( x \) that simultaneously satisfies Equations 29 and 30 with \( \rho_{\text{H}}(n,x)_s = 0.16 \) and \( \rho_{\text{C}}(n,x)_s = 0.26 \). Such a set of values for \( n \) and \( x \) occurs at points in Fig. 13, (a) where \( x^{(1)}_s(n) \) intersects either \( x^{(1)}_H(n) \) or \( x^{(2)}_H(n) \) and where \( x^{(2)}_c(n) \) intersects either \( x^{(1)}_H(n) \) or \( x^{(2)}_H(n) \).

Given the error in the measured values of the depolarization ratio, Fig. 13, (a) admits two \( n-x \) sets

1) \( n = 8 \ x = b/a = 4.8 \)

2) \( n = 0 \ x = b/a = -0.3 \).

Kondilenko and Korotkov26 have shown that the value of \( x \) for the m\textsuperscript{th} vibration of
a linear bond can be determined from the depolarization ratio \((\rho_n)_m\) measured using unpolarized light incident upon a medium in which that bond is randomly oriented. Here:

\[
(\rho_n)_m = \frac{2\rho_m}{1 + \rho_m} \tag{36}
\]

where \(\rho_m\) is given by Equations 12 and 13. Furthermore,\(^{20}\)

if

\[
(\rho_n)_m < 0.5 \quad \text{then} \quad x = b/a > 1 \tag{37}
\]

From the measurements shown in Fig. 11, we found using Equation 13, that \(\rho_{amide I} = 0.27\); therefore \((\rho_n)_{amide I} = 0.43 < 0.5\) and the \(n-x\) values labeled II in Equation 35 are not admissible. In the preceding discussion we have justifiably assumed that the tensor components \(a\) and \(b\) of Equation 15 are relatively insensitive to the local environment of the peptide group.

The probability function given in Equation 31 can be tested in the same manner as described above. Accordingly, we show in Fig. 13, (b) the solutions \(x^{(1)}(n)\), \(x^{(2)}(n)\), \(x^{(3)}(n)\), and \(x^{(4)}(n)\) obtained from Equations 33 and 34 by setting the left-hand sides equal to 0.16 and 0.26, respectively. Clearly, no intersection of these curves occurs in the region \(x > 1\); the cosine weighted probability function is not applicable to the intact bovine lens.

On the basis of the above analysis we conclude that the CONH bonds of the antiparallel \(\beta\)-pleated sheet are arranged in an angular distribution that is not random but is skewed toward directions orthogonal to the lens optic axis. While other probability functions may yield acceptable values of \(\rho_\perp\) and \(\rho_\parallel\) for the amide I band, the function \(A_s \sin^a \theta \, d\theta \, d\phi\) (where \(A_s = 0.196\)) is attractively simple. Moreover, the bond distribution it represents is perfectly consistent with Lenhard's\(^4\) results if we assume...
The relationship between lens microstructure and opacity. Benedek has shown that the cornea and the lens are transparent to visible radiation because the Fourier components of the density fluctuations in those biologic materials have wavelengths less than half of the wavelength of the transmitted light. He further suggested that opacities develop in cataractous lenses as a result of light scattering due to long wavelength density fluctuations caused by an increased concentration of high molecular weight protein aggregates. To test Benedek’s hypothesis several biochemical investigations have been undertaken to search for high molecular weight aggregates in cataracts and in the lenses of older animals. High molecular weight aggregates have indeed been found in the nuclei of old cow lenses and in the nuclei of human cataracts though the evidence for the latter is conflicting. However, no high molecular weight aggregates have been found in the cortex of cataractous lenses. It is evident from the work of Trokel, Phillipson, and Miller, Zuckerman, and Reynolds that cortical cataracts like nuclear cataracts are a manifestation of increased turbidity. What then is the mechanism responsible for cortical cataracts?

We suggest that the mechanism is long wavelength fluctuations in protein order. Because the proteins are densely packed in the lens and locally ordered in the antiparallel $\beta$-pleated sheet their angular orientations with respect to the lens optic axis are probably correlated over large distances of the order of several thousand Angstroms or more. In Fig. 14, (a) we represent the wavelength, $\lambda$, of visible light. In the upper diagram of Fig. 14, (b) we show the lens proteins as long rods regularly spaced and perfectly ordered. The pseudolattice representation of Fig. 14, (b) is adopted for illustrative purposes only and does not reflect the true ordering which would be characterized by small frozen fluctuations in the positions and orientations of the proteins about the lattice equilibrium configuration shown. In the lower diagram of Fig. 14, (b) is plotted the density fluctuation $\Delta d(x)$ corresponding to the ordering exhibited in the upper diagram. The meaning of $\Delta d(x)$ is as follows: we measure the average density and call it $<d>$. If $d(x)$ is the density measured at point $x$ then

$$\Delta d(x) = d(x) - <d> \quad (38)$$

The density fluctuation $\Delta d(x)$ has only one Fourier component. Its wavelength, $\lambda_b$, is smaller than half the wavelength of visible light, $\lambda$, and the proteins arranged as in Fig. 14, (b) do not scatter light, i.e., the medium is transparent.
The protein chains are highly polar. Therefore, a slight tilting of one of them, induced for example by the breaking of hydrogen bonds, will cause its neighbor to tilt and so on down the line until distant neighbors do not sense the initial distortion. This situation and the density fluctuation $\Delta \rho_\ell(x)$ appropriate to it are represented in Fig. 14, (c). Notice that the Fourier transform of $\Delta \rho_\ell(x)$ contains a component with a wavelength $\lambda_{\ell2}$ that is considerably greater than $\lambda/2$. A lens characterized by fluctuations in protein order of the type shown in Fig. 14, (c) would scatter light effectively and appear cataractous. Disorder-induced opacity of the type described above is biologically easy to envision. Hydrogen bonds in proteins can be easily broken when the pH of the solution in which they are dissolved is altered. Note that disorder-induced opacity is not predicated upon the presence of high molecular weight aggregates.

Barber has observed that the application of pressure to a normal lens can produce instant yet reversible opacification. He attributed the reversible opacification to the migration of water between the lens fiber membranes. Since the lens is gel-like and highly viscous, it seems unlikely that water could diffuse between the membranes "instantaneously." It is quite possible, however, that the long wavelength protein distortions engendered in the region of applied pressure are responsible for the observed opacity.

Summary and conclusions

Using the Raman technique to extract structural as well as "fingerprint" information we have shown that the lens proteins are organized in an antiparallel $\beta$-pleated sheet with the CONH bonds preferentially aligned in directions orthogonal to the lens optic axis. We have suggested that cortical cataracts are a manifestation of fluctuations in long-range orientational protein order and have thus obviated the need to invoke the presence of high molecular weight aggregates to explain lens opacity. To test this hypothesis of disorder-induced opacity we have begun a study of the polarized Raman spectra of rat galactose cataracts with varying degrees of quantitatively determined turbidity.

Polarized Raman spectroscopy is clearly superior to other techniques for studying the intact lens. In principle, this technique could safely be used to study the lens in vivo.

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