Molecular Crystals and Liquid Crystals
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/gmcl16

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Eisaku Iizuka [a]
[a] Institue of High Polymer Research, Faculty of Textile Science and Technology, Shinshu University, Ueda, 386, Japan
Version of record first published: 28 Mar 2007.

To cite this article: Eisaku Iizuka (1977): The Liquid Crystals of Deoxygenated Sickel-Cell Hemoglobin, Molecular Crystals and Liquid Crystals, 42:1, 67-80

To link to this article: http://dx.doi.org/10.1080/15421407708084497

Mol. Cryst. Liq. Cryst., 1977, Vol. 42, pp. 67–80

Printed in Holland

The Liquid Crystals of Deoxygenated Sickle-Cell Hemoglobin

EISAKU IIZUKA

Institute of High Polymer Research, Faculty of Textile Science and Technology, Shinshu University, Ueda 386, Japan

(*Received October 12, 1976*)

Gels of deoxygenated sickle-cell hemoglobin appear to be a collection of rodlike particles which are equivalent with those fibers widely accepted to be present in these gels. These particles start to align at some peculiar points such as the edges of a quartz spacer in an optical cell, are oriented perpendicular to the edges, and form structures that satisfy the descriptions of liquid crystals of the nematic type. Ordering of the liquid crystals is more uniform when the solution thickness is smaller. Rubbing of the surfaces and edges of spacer prior to its insertion into the optical cell also enhances the ordering of these liquid crystals.

Deoxygenated sickle-cell hemoglobin (deoxyHb S) is concentrated solutions aggregates to form gels that are temperature dependent. This is believed to be the result of close ordering of deoxyHb S into linear aggregates. Finch *et al.*[1] interpreted each aggregate as a tubular fiber made up of six thin strings of hemoglobin molecules linked end to end at intervals of 64 Å in wet fibers and each string was wound around the tubular surface with a helical pitch of about 3000 Å.

Monomeric deoxyHb S in supersaturated solutions aggregates to form a compact nucleus for a single fiber (nucleation), the fiber then grows by the relatively rapid sequential addition of both monomer and small aggregated species (polymerization), and the fibers align one another and crystallize into the parallel, ordered arrangements (crystallization).[2] These rodlike structures are formed both in sickled cells[3] and cell-free solutions.[4] They extended most of the length of the sickled cell, forming either square or hexagonally packed bundles with lattice constant of 170–180 Å.[1] Subsequently, Hofrichter *et al.*[2] proposed three models for the fibers all involving and compatible with the optical results of Finch *et al.*

The appearance of these fibers—as clusters of parallel rods—resembles descriptions of liquid crystals of the nematic type;[4] similarities of sickling to liquid crystal formation were recognized by earlier workers[5,6] who noted the birefringence of deoxyHb S in red cells and in cell-free solutions. However, it remains unknown whether the formation of well-ordered structure needs some peculiar points to start in the cells or solutions. Lyotropic liquid crystals of polypeptides are characterized by a sharp decrease in the viscosity upon the formation of liquid crystals,[7,8] so are the thermotropic liquid crystals.[9] But the liquid crystals of deoxyHb S are formed at temperatures close to the sol–gel transition temperature. In this work, I wish to report some new findings on the gels of deoxyHb S.

EXPERIMENTAL

Concentrated solutions of hemoglobin S (15–53%(wt/v)) in phosphate buffer (ionic strength 0.1) containing some methemoglobin S (7.8–10.5%) was supplied by Professor J. T. Yang of The Cardiovascular Research Institute, University of California, San Francisco. Deoxygenation of these cell-free solutions was carried out in an ice-cooled flask by exposing them at a low atmospheric pressure (about 20 mm Hg) for 20–30 min and later under nitrogen flash for another 20–30 min followed by the addition of a trace of sodium dithionite (about 0.02M). The deoxygenated solution was then sealed in a 1-mm quartz cell at 0°C with a quartz spacer to shorten the path length to desired length between 30 and 210 microns and surrounded by a water jacket to regulate the temperature of the solution to 37°C. The protein concentrations were only very approximate.

Polarizing micrographs of the gels of deoxyHb S were taken with a Nikon microscope, and small-angle light scattering patterns of the gels were photographed using a He–Ne gas laser of 2-mW as a light source. Details of these measurements were described elsewhere.[10] Visible and infrared linear dichroisms of the gels were measured, respectively with a Jasco ORD/UV-5 spectropolarimeter (630–450 nm^{-1}) and with a Jasco DS-301 infrared spectrophotometer (1800–1000 cm^{-1}). For the infrared absorption measurements, the original solution was repeatedly vacuum-concentrated at about 20 mm Hg and diluted with heavy water. It was then introduced into a CaF_2 cell with a CaF_2 spacer inside.[11] Possible increase in the methemoglobin content during these procedures cannot be ruled out. The optical cell was tilted 45° with respect to the entrance slit of the apparatus to eliminate the error in the observed dichroic ratio due to the polarization of the monochrometer.[12]

The birefringence of the oriented gels was measured with a double refractometer manufactured by Shibayama Scientific Instrument Co., Ltd. Circular

dichroism (CD) measurements were also carried out with the Jasco spectropolarimeter. The optical cell was mounted on a cell holder that can be rotated in a plane perpendicular to the incident beam.[13] The small-angle laser light scattering was also measured in magnetic fields up to 25 kilogauss applied parallel to the cell surface (and perpendicular to the incident laser beam) with an electromagnet, a modified model of JM-151 manufactured by Japan Electron Optics Laboratory Co., Ltd.[14]

RESULTS AND DISCUSSION

1 Observation Under Crossed Nicols

A markedly birefringent phase appears over the full range of observation within a few minutes at 37°C when the quartz spacer is rubbed both its edges and surface (with a filter paper) prior to introduction into the optical cell (Figure 1). The feature of the pattern of the micrograph differs from solution to solution; however, for solutions 30 μ thick, uniform, parallel lines are always seen perpendicular to the edges of the spacer whether the direction of rubbing is parallel or perpendicular to the edges (Figure 1, c and d). When the spacer is not rubbed, the gel is only slightly birefringent (the left-hand micrograph of Figure 1, b) and the markedly birefringent phase is seen only in limited regions adjacent to parts of the edges (Figure 1, f) suggesting that rubbing of the spacer surface stimulates the growth of the well-ordered structure and that the edge effect is also enhanced when the edge is rubbed. Although the edge effect is more important than the rubbing effect with respect to the direction of the parallel lines under crossed nicols, these lines have a tendency to be parallel to the direction of rubbing (Figure 1, f). For gels whose thickness are larger, say 210 μ, uniform, parallel lines are no longer observed even though the spacer surface is rubbed (Figure 1, g). In this connection, the spacer introduced into the optical cell was always in contact with one inner surface of the cell on one side of its surface. It was possible to photograph the extreme line of the birefringent phase that had started to grow at the edge of the spacer (Figure 1, e). Sometimes spherulitic structures were observed in the gel (Figure 1, h). They could have been formed in the presence of some impurities in solution, as was the case of concentrated solutions of poly(γ-benzyl-L-glutamate).[15]

These experimental results suggest that the birefringent structure is caused by some nucleation process starting at some peculiar points such as the edges of the quartz spacer in an optical cell, as a result of irregularities on the surface of the spacer after rubbing (such as roughness and surface charges), and in the presence of impurities in solution. The well-ordered structure is

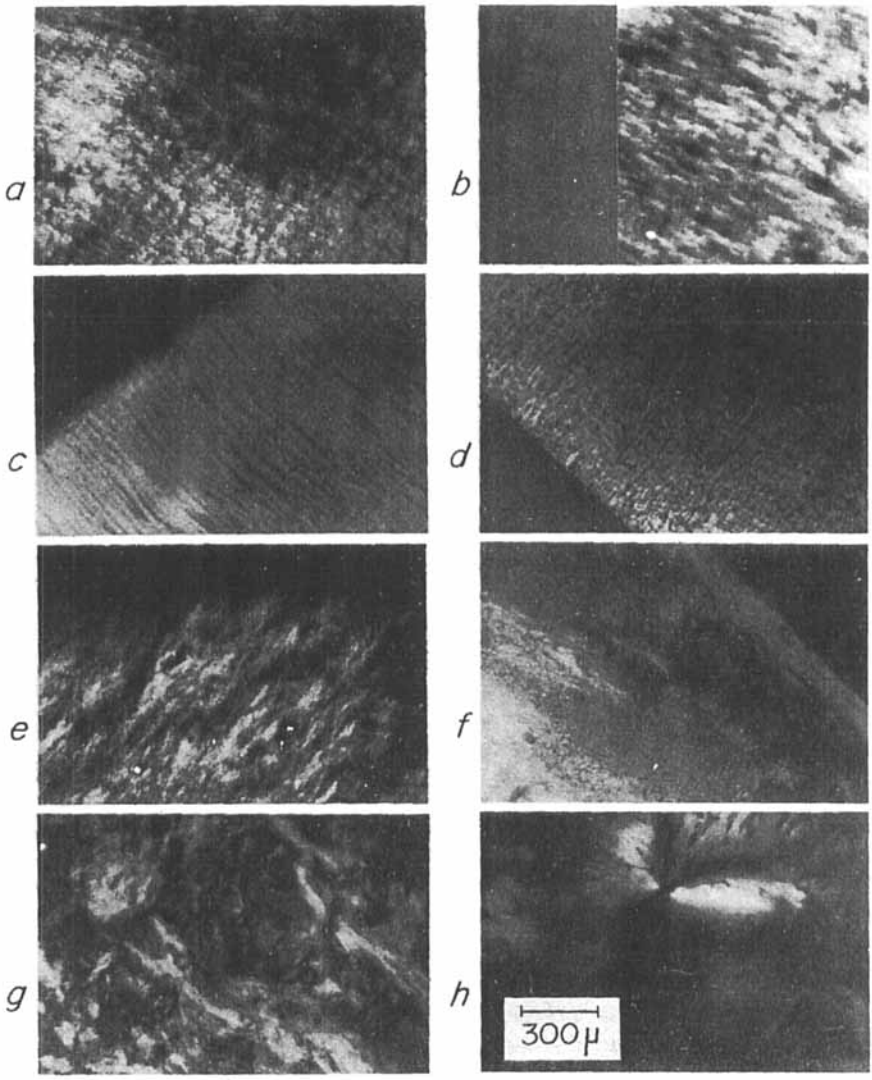

FIGURE 1 Polarizing micrographs of the liquid crystals of deoxyhemoglobin S. Measured at 37°C in a quartz optical cell with a quartz spacer which was rubbed both its edges and surface with a filter paper unless stated otherwise prior to introduce it into the optical cell. Polymer concentration, 24%(wt/v). (a) a typical pattern, (b) the left-hand pattern was taken without rubbing the spacer, (c) taken with a spacer rubbed parallel to its (upper left) edge, (d) taken with a spacer rubbed perpendicular to its (lower right) edge, (e) a pattern showing that the process of nucleation advances, (f) taken with a spacer whose surface was rubbed parallel to its (upper right) edge only in its middle part taking care not to rub the edge, (g) taken with a specimen whose thickness was 166 μ (The other specimens were 30 μ thick except for the specimen in (f) (69 μ), (h) a spherulitic structure that appeared rarely.

obtained when the solution is thin and the edges and surface of the spacer with which the solution is in contact have been rubbed.

2 Small-Angle Laser Light Scattering

The V_h pattern of the birefringent phase (taken with the polarizer placed horizontally and the analyzer, vertically) is either of the circular or of the $\pm 45°$ type and the latter is more common (Figure 2). This indicates the presence of birefringent assemblies of the protein molecules of the order of length comparable to the wavelength of light used (6328 Å). Furthermore, these assemblies are rodlike having maximum polarizability directions parallel ($\omega = 0°$) or perpendicular ($\omega = 90°$) to their long rod axes.[10] For the gels that show horizontal, parallel lines when observed under crossed nicols, the upper (and the lower) included angle of the cross (V_h) pattern is smaller than 90° and the H_h pattern (taken with the polarizer and the analizer both placed horizontally) is long lengthwise (Figure 2, c and d); when these lines are vertical, both the V_h and the H_h patterns are tilted by 90° (Figure 2, e). These indicate that the gel is made up of rodlike particles that are ordered parallel to these lines (and in the direction that the nucleation advances starting at the edges of the spacer in the optical cell) according to the previous result.[10]† The appearance of these ordered structure satisfies the descriptions of liquid crystals of the nematic type.

A marked increase in the viscosity upon the liquid crystal formation can be attributed to the increase in the degree of aggregation of deoxyHb S molecules with resultant increase in length and stiffness. That the liquid crystals form at high rather than low temperature can be explained thermodynamically. The presence of some peculiar points for a well-ordered structure is the case for low-molecular weight thermotropic liquid crystals in which the surface of the electrodes is rubbed for the display use.

3 Linear Dichroism

The hemoglobin molecule is a slightly elongated spheroid of dimensions $65 \times 55 \times 50$ Å.[17] Despite the lack of a significant shape anisotropy, the ellipsoid that describes the absorption of polarized light by the hemoglobin molecule is highly anisotropic because the heme chromophore behaves like a nearly perfect planar absorber (in single crystals of various heme

† In this paper, only the case for $\omega = 0°$ was assumed to calculate the scattering intensity. The same result should be obtained for the case $\omega = 90°$ because the expressions for the intensity given by Rhodes and Stein for oriented rods that were used in the calculation are same whether $\omega = 0°$ or 90°.[16]

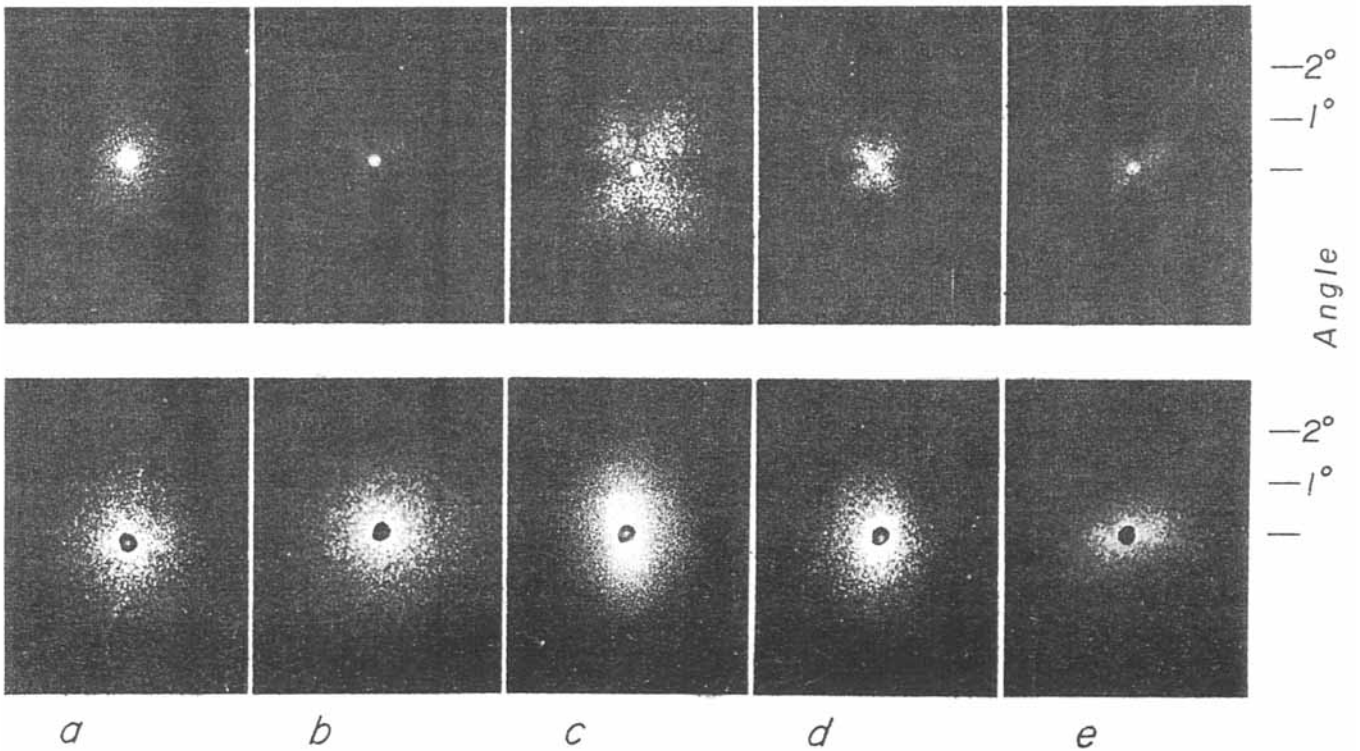

FIGURE 2 Small-angle laser light scattering of the liquid crystals of deoxyhemoglobin S. Upper, the V_h patterns and the lower, the H_h patterns. Distinct parallel lines were observed under crossed nicols for the cases (c)–(e). When the light scattering patterns were taken, these lines were horizontal (c, d), or vertical (e). Experimental conditions, same as in Figure 1.

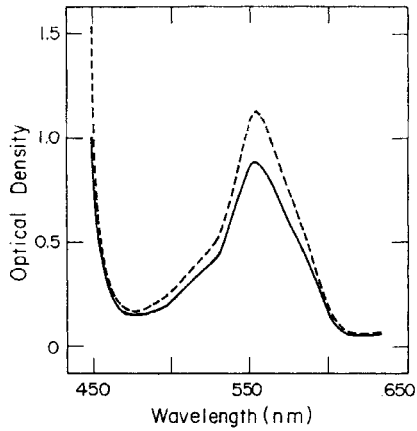

FIGURE 3 Linear dichroism of the oriented liquid crystals of deoxyhemoglobin S. The electric vector of the incident beam is parallel (solid line), or perpendicular (broken line) to the rodlike particles of this protein. Polymer concentration, 23%(wt/v); measured at 37 C.

proteins) for the electronic transition at the Soret band.[18] Oriented gels (liquid crystals) of deoxyHb S are dichroic, showing a stronger absorption when the electric vector of the incident light is normal to the axis of the rodlike particle, and a weaker absorption when the vector is parallel to the particle axis; the dichroism of the Soret band appears to be similar to that of the visible band at 550 nm (Figure 3). These are consistent with the optical results of Finch *et al.*[1] and the maximum polarizability direction of the rodlike particle is perpendicular to the rod axis.

The polarization ratio, $P(=D_{\perp}/D_{\parallel})$, at 550 nm is 1.30–1.31 for two oriented specimens of thickness 30 μ and 69 μ. It is known that the long axis of a sickle cell is the preferred direction for the orientation of the long axis of the hemoglobin fibers,[19,20] and that the highest polarization ratio at the Soret band (430 nm) is 3.0 ± 0.1 for sickled cells from 10 different patients.[19] In single crystals of ferrimyoglobin complexes, the polarization ratios at the peaks of the Soret band (426 nm) and main visible band (542 nm) are 1.25 and 1.26, respectively.[18] These findings, however, cannot tell how high the degree of orientation in these cell-free liquid crystals of deoxyHb S is at this moment.

Since the direction of orientation of the molecular segments is not uniform in the hemoglobin molecule, infrared dichroism has not been detected for oriented liquid crystals of deoxyHb S in the wavelength range measured. Only the absorption spectra of this protein are presented both in the isotropic and the liquid crystalline states (Figure 4). The bands of the absorption spectra appear to be broader in the latter state. The amide II band is still

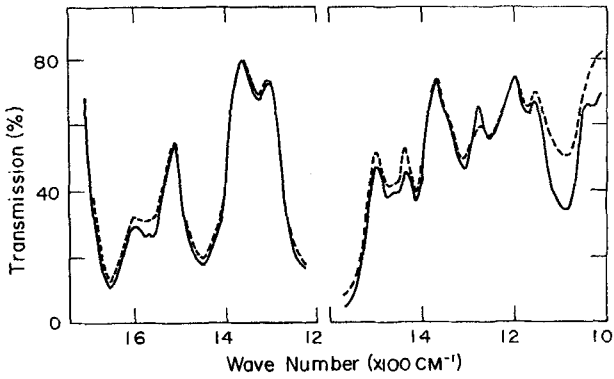

FIGURE 4 Infrared absorption spectra of the concentrated solutions of deoxyhemoglobin S in H_2O (right-hand curves) and in D_2O (left-hand curves). Solid lines, measured at 27°C (isotropic); broken lines, measured at 37°C (liquid crystalline). Polymer concentration, 24%(wt/v).

at about 1550 cm^{-1} in D_2O, indicating that in the α-helical segments of deoxyHb S molecule the hydrogen atoms of the peptide groups are not exchanged by deuterium atoms in this measurement. The absorption centered at 1450 cm^{-1} may be the amide II band for the deuterated peptide groups in the coiled segments.

4 Linear Birefringence

Oriented liquid crystals of deoxyHb S exhibit negative birefringence as well as negative polarization dichroism as was expected (Table I). The magnitude of the former is only 0.0011 for the specimen having the polarization ratio of 1.30–1.31 at 550 nm. This value is about 1/20 of that of electrically or magnetically oriented liquid crystals of polypeptides such as poly(γ-benzyl-L-glutamate) and poly(γ-ethyl-L-glutamate).[14]

5 Circular Dichroism

The CD of hemoglobin has been measured and the bands have been assigned by several workers.[21–24] The bands between 600 and 240 nm are related

TABLE I

Birefringence and polarization ratio of the oriented liquid crystals of deoxyhemoglobin S

	$-(\Delta n/c_v)_{547\text{-nm}}$	$(D_\perp/D_\parallel)_{550\text{-nm}}$
Specimen A	0.00117	1.31
Specimen B	0.00094	1.30

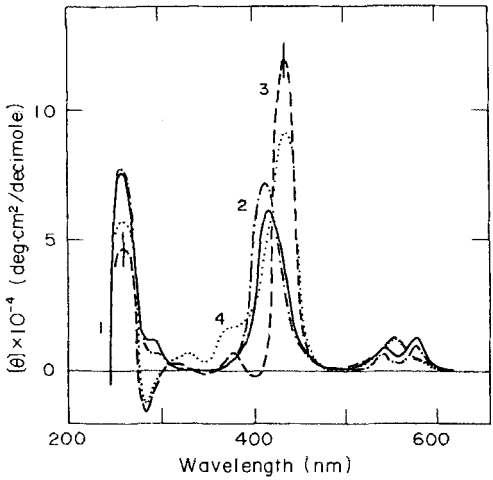

FIGURE 5 Circular dichroism spectra of the isotropic solutions of hemoglobin S. 1, oxyhb 0.047%(wt/v); 2, oxyhb 15.0%(wt/v); 3, deoxyhb 0.057%(wt/v); 4, deoxyhb 16.0%(wt/v).

to the heme groups of this protein molecule except the 285-nm band that appears only in deoxygenated state and is considered to be due to the tyrosine residues present at the osculating planes of the α- and the β-chains of this protein molecule.[22] In the isotropic state, there appears to be no difference between the CD of normal hemoglobin (Hb A) and that of this abnormal hemoglobin (Hb S) (Figure 5). Oxyhemoglobin S (Hb S) shows similar CD in concentrated solutions as in dilute solutions. This is also the case for deoxyHb S; however, the intensity of the Soret band (centered at 434 nm) is lower in concentrated solutions probably because of scattering effect.

The measured CD of the oriented liquid crystals of deoxyHb S shows a dependence of the microscopic angle, α, that the fast optical axis of the oriented particle of hemoglobin molecules makes with respect to the plane of polarization of the polarizer of the CD instrument. Experimentally, a macroscopic rotation angle, θ, is defined as the tilt angle of the optical cell (around the incident beam and around the normal of the surface of the optical cell) with respect to the vertical position. The rotation angle is measured clockwise when facing the light source. This angle is related to α by[25]

$$\theta - \theta' = \alpha$$

where θ′ is the angle of θ at which the fast optical axis of the specimen has an angle $\alpha = 0°$ with respect to the fast optical axis of the Pockel's cell of the CD instrument. θ′ is called the orientation angle and is a function of the wavelength since different chromophores of the specimen can have different directions. Turnis-Schneider and Maestre[25] have developed a general

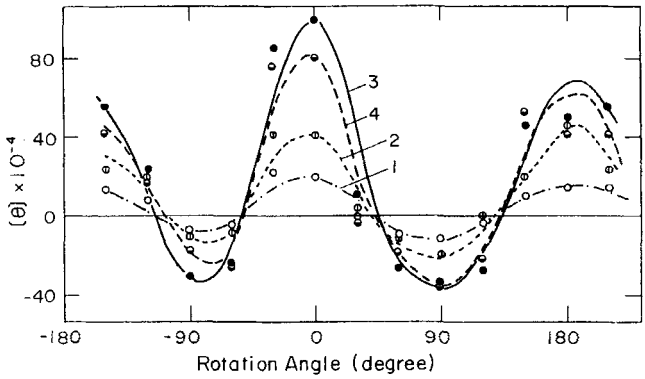

FIGURE 6 Periodic variation of the circular dichroism of the oriented liquid crystal of deoxyhemoglobin S as a function of the rotation angle at specific wavelengths. (1) 550 nm, (2) 445 nm, (3) 360 nm, (4) 270 nm.

formula for the CD of oriented films of DNA, which can be reduced to a simple form for reasonably thin (0.01–0.1 cm) or/and partially oriented (less than 5% orientation) films:

$$\delta_{app} = \delta_{real} - 0.298p \cos 2\alpha$$

where p is the linear dichroism. The apparent CD of oriented liquid crystals of deoxyHb S shows a periodic variation (sinusoidal with a period of π) with respect to the rotation angle, θ, or the orientation angle, α; however, it does not follow exactly this equation (Figure 6). This would be due to possible irregularity of the gap (where the solution is filled up) thickness as thin as 30 μ together with the fact that the entrance slit of the instrument is not circular but rectangular. The scattering effect, if any, is another possible cause which can be strong in some cases.

According to the above equation, the average of two δ_{app} values at any chosen wavelength, whose rotation angle are 90° apart gives the real CD, noting that cos 2α in the two experiments will be equal in magnitude but opposite in sign.[25] In this study, however, the twelve curves which had been obtained at every 30° were averaged and corrected for the change of the CD intensity when the optical cell was turned over to introduce the incident beam from the opposite side of the optical cell.† Those CD results that did not show good sinusoidal variation were excluded because of possible artifacts. The averaged CD of the oriented liquid crystal of deoxyHb S is

† The CD measurements were made only from two directions (concerning the three-dimensional structure of the liquid crystal) perpendicular to the long rod axis and the measurements from the direction parallel to the long rod axis could not be made.

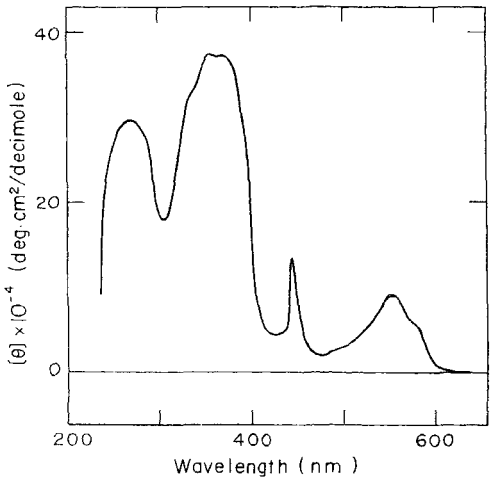

FIGURE 7 (Intrinsic) circular dichroism spectrum of the oriented liquid crystal of deoxyhemoglobin S. Measured at 37 C and averaged. Even though the measurement was done at photomultiplier voltages below 900 V to avoid artifacts, the spectrum differs from specimen to specimen. See the text.

characterized by a marked increase in intensity at the main visible band (550 nm) and at around 260 nm, the narrowing and the red shift of the Soret band from 430 nm to 445 nm, the red shift of the 285-nm band to some 303 nm, and the appearance of an intense band centered at 360 nm (Figure 7). This CD curve is the average of two curves obtained with two different specimens (of polymer concentrations 23.0 and 24.3 %(wt/v)); the two curves are very similar and the averaged curve should be real.

Even though the CD measurements were carried out at photomultiplier voltages below 900 V to secure the reliability of the result, it was not always fixed and seemed to depend on the degree of the liquid crystal orientation: the magnitude of the CD varied and sign of the 445-nm band became negative in some cases; however, the 360-nm band was always positive and very intense. These occured for specimens whose apparent CD was the same at two different specimen-photomultiplier tube distances (14 and 26 cm). The 360-nm band is not a linear birefringence effect and may be a manifestation of the reflection bands similar to those of cholesteric liquid crystals as postulated by Holzwarth and Holzwarth.[26] Recent finding by Sophianopoulos and his coworkers[27] that the CD of deoxyHb S measured at a fixed rotation angle varied with the growth of nucleation will be interpreted in line with this point. At any rate, these CD results appear to point to well-ordered heme groups that are restricted to rotate and a highly regular long range organization in the liquid crystalline state. To clarify further the CD

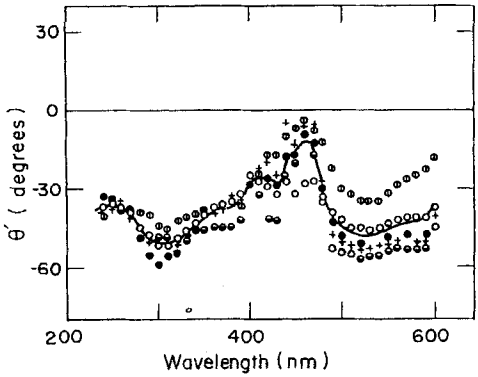

FIGURE 8 θ'-values as a function of wavelength for the oriented liquid crystal of deoxyhemoglobin S. The solid curve is the average of all the computed values. Different symbols in figure are for different values of θ_1. See the text.

of any ordered structure, experimental differential light-scattering correction as made by Maestre and his coworkers[28,29] must be introduced.

The orientation angle, θ, can be solved at any wavelength using the equation:[25]

$$\frac{\delta(\theta_1) - \delta(\theta_1 + \pi/2)}{\delta(\theta_2) - \delta(\theta_2 + \pi/2)} = \frac{\cos 2(\theta_1 - \theta')}{\cos 2(\theta_2 - \theta')}$$

where θ_1 and θ_2 are the readings of the cell holder and should be different enough in value so as to keep the uncertainty in the value θ' small. The angle, θ', for the specimens adopted in Figure 7 is very roughly constant ($-45°$) at the whole wavelength range measured (600–240 nm) (Figure 8). Since the fast optical axis of the Pockel's cell is at 45° with the vertical position (and with the long axis of the deoxyHb S particle), this agrees with the fact that the maximum polarization is perpendicular to the particle axis and agrees also with all the experimental evidence so far mentioned. Some deviation of the θ'-value from $-45°$ especially around 460 nm may indicate the presence of a chromophore or chromophores that is (are) not in a common plane. However, these conclusions are true only when the scattering effect was negligibly small.

6 Effects of a Magnetic Field on the Liquid Crystals of DeoxyHb S

DeoxyHb S is paramagnetic and the rodlike assemblies of this protein in which the heme groups are roughly perpendicular to the rod axis is expected to have enough resultant dipole moment to orient in a magnetic field. Sickled cells whose long axes are prefered directions for the deoxyHb S

fibers actually align with their long axes perpendicular to the magnetic lines of force (3.5 kilogauss).[30] No change has been detected, however, in the patterns of both the polarizing micrograph and the small-angle laser light scattering when cell-free liquid crystals of deoxyHb S is placed in magnetic fields up to 25 kilogauss, suggesting that no structural change occurs in these liquid crystals. This may be due to the lack of fluidity of oriented liquid crystals. Any sign of the magnetic-field effect has not been detected as well during the process of liquid crystal formation when temperature was changed from 0°C to 37°C, pointing to the priority of the direction prescribed by the nucleation starting at peculiar points (the edges of a spacer in this case) over the direction determined by magnetic torques exerting on the protein molecule owing to its anisotropy of the magnetic susceptibility.

CONCLUSIONS

The experimental evidence obtained so far appears to support the following hypotheses concerning the gels of deoxyhemoglobin S:

1) Nucleation of deoxyhemoglobin S starts at some peculiar points such as the edges and surface irregularities of a quartz spacer introduced in an optical cell and impurities present in solution.

2) Rodlike particles of deoxyhemoglobin S molecules align in the direction the process of nucleation advances (that is, perpendicular to the edges of a spacer) and form structures that satisfy descriptions of liquid crystals of the nematic type.

3) The growth of oriented liquid crystals is better attained when the solution thickness is smaller, say, 70 microns or less (30 microns), and the spacer is rubbed in the direction the liquid crystals orient prior to its insertion into an optical cell.

4) In the oriented liquid crystals, the heme groups of this protein molecules are ordered and restricted to rotate and there is a highly regular long range organization.

5) Edge effects are more important than magnetic torques exerting on the hemoglobin molecule concerning the ordering of the liquid crystals of this protein.

Acknowledgments

I wish to thank Professor J. T. Yang for the supply of hemoglobin S used. Part of this work was completed at his laboratory and supported by USPHS grant HL-18818 awarded to him. Thanks are also due to Professor M. F. Maestre of Space Science Laboratory, University of California, Berkeley for his valuable comments, especially on the circular dichroism of deoxyHb S liquid crystals.

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