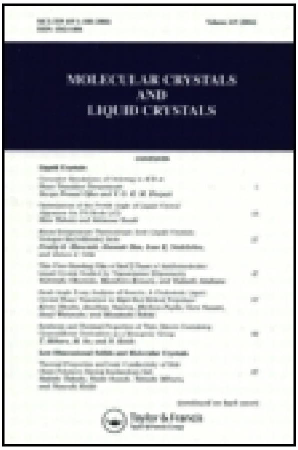

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Optical Properties of Oxidized Cholesterol Bilayer Lipid Membranes

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Bilayer Lipid Membranes (BLM) formed from oxidized cholesterol in the aqueous phase were prepared to investigate their optical characteristics. As an optical model of BLM, we adopted a uniaxial layer model where the optical axis is perpendicular to the membrane surface. The refractive indices were measured precisely by means of the ellipsometry, and in consequence, in the temperature range from 10°C to 40°C, the ordinary and extraordinary refractive indices for 633 nm decreased from 1.471 to 1.458 and 1.490 to 1.471, respectively. Its positive birefringence also decreased according to temperature. The thickness was also measured, and the value of 7.1 $\pm$ 0.9 nm at 30°C was obtained.

1 INTRODUCTION

Biomembranes are basic organizational structures of living bodies. The importance of those as fields for active transport, biosynthesis, energy metabolism and information transmission, has recently been recognized increasingly.[1]

Biomembranes are composed of hydrophobic bilayer lipid core. Hence, experimental bilayer lipid membranes (BLM) with suitable modification are useful model systems for the biomembranes.[2] The refractive index and the thickness are the most fundamental material values to study the BLM, and several optical models have already been proposed from early days of the BLM study. The three typical models are as follows:

a) The single layer model; this was the first and the simplest model proposed by Huang and Thomspon.[3] They supposed that BLM are homogeneous and isotropic.

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b) The triple layer model; this was propsed by Tien.[4] In this case the central hydrocarbon layer has a different refractive index from the bifacial layers of the polar groups.

c) The uniaxial layer model; this was proposed by Cherry and Chapman.[5] They assumed that BLM are optically uniaxial where optical axis is perpendicular to the membrane surface.

Based on the single layer model, the Brewster angle θ_{iB} is given by

$$\tan \theta_{iB} = \frac{n}{n_1} \tag{1}$$

provided that n_1 is the refractive index of aqueous phase and n is that of BLM. However, we have measured very small value of 34.6° for oxidized cholesterol BLM in LiBr aqueous solution with the refractive index 1.445 as shown in Figure 8. So, the refractive index of oxidized cholesterol BLM should become below unity if this model was adopted. The drawbacks of the triple layer model and other complicated models will be described in Section 4.

Ohki has calculated the dielectric constant of BLM according to Kirkwood's theory, and concluded the anisotropy of BLM.[6] This result seems to support the uniaxial layer model. Hence, we have adopted the uniaxial layer model, and we have examined the assumption that the optical axis is perpendicular to the membrane surface.

In this paper, the refraction indices of oxidized cholesterol BLM and their temperature dependences were measured precisely by means of the ellipsometry based on this model. The thickness of the BLM was also measured.

2 PRINCIPLES OF MEASUREMENTS

2.1 Reflection matrix

The polarization of the reflected light from isotropic media is the same as that of the incident light when they are parallel or perpendicular. Even for the case of uniaxial media, this is true when the optical axis is in the plane of incidence.

When the optical axis is not in the plane of incidence, the optical properties of uniaxial media are complicated. We define the reflection matrix $\mathbf{R}$ by

$$\mathbf{E}_r = \mathbf{R}\mathbf{E}_i \tag{2}$$

$$\mathbf{R} = \begin{pmatrix} r_{11} & r_{12} \\ r_{21} & r_{22} \end{pmatrix} \tag{3}$$

where $\mathbf{E}_1$ and $\mathbf{E}_r$ are two-dimensional vectors of the incident electric field and the reflected electric field, respectively. Their coordinates are illustrated in Figure 1. We define **R**'s eigenvalue c_i and eigenvector $\mathbf{e}_i$ by

$$\mathbf{R}\mathbf{e}_i = c_i \mathbf{e}_i \qquad (i = 1, 2) \tag{4}$$

Generally speaking, c_i and $\mathbf{e}_i$ are complex, but we can regard c_i as pure imaginary and $\boldsymbol{\theta}_i$ is a real vector, because the thickness of the BLM is very small in comparison with the wavelength, so, the phase difference between two lights which are reflected by the boundary I–II and the boundary II–III,

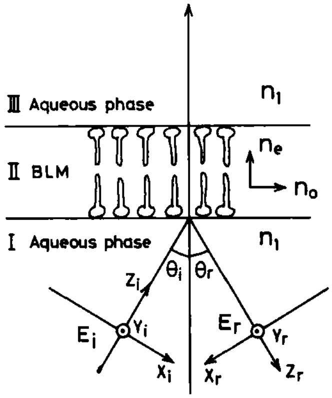

FIGURE 1 The uniaxial layer model of BLM. $\mathbf{E}_i$ and $\mathbf{E}_r$ are two-dimensional vectors whose directions are in the planes normal to Z_i and Z_r, respectively.

respectively, are almost π. So, when the azimuth of the incident linearly polarized light equals to the direction of $\mathbf{e}_i$, the azimuth of reflected light becomes the same as that of incident light.

We have calculated the direction of eigenvector $\mathbf{e}_i$, as the function of the directional angles of the optical axis of σ and ρ defined in Figure 2. The ways to obtain the eigenvectors are as follows. The light transmitted through anisotropic media splits into two directions, and those states can be cal-

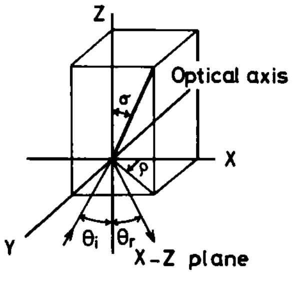

FIGURE 2 Definition of directional angles σ and ρ. The incident and reflected lights are in X-Z plane.

culated by Maxwell equations. In our case, those two lights are refracted at II–III boundary, so, we must consider each propagation and azimuth of four lights in the uniaxial layer. In this case, the attention should be paid on the difference of phase velocities $v_{e\pm}$ of the extraordinary lights which are propagated to the positive and negative directions of z axis, respectively.

$$v_{e\pm} = \frac{c}{n_\pm} \tag{5}$$

where

$$\frac{1}{n_\pm^2} = \frac{1}{n_0^2} + [\sin^2\sigma + (\cos^2\sigma - \sin^2\sigma\cos^2\rho)\sin^2\theta_{t\pm} \mp \sin\sigma\cos\sigma\cos\rho\sin 2\theta_{t\pm}]\left(\frac{1}{n_e^2} - \frac{1}{n_0^2}\right) \tag{6}$$

$$n_1 \sin\theta_i = n_\pm \sin\theta_{t\pm} \tag{7}$$

provided that n_0 and n_e are the ordinary and the extraordinary refractive indices of the BLM, respectively, and θ_i is the incident angle. (Phase velocities $v_{0\pm}$ of the ordinary lights are represented simply by $v_{0\pm} = c/n_0$.) These things as mentioned above make the reflection matrix too complicated to write down explicitly. Therefore, we considered each boundary condition at I–II and II–III boundaries, solved numerically 8 simultaneous linear equations, and obtained eigenvectors e_i. The results of the calculations are illustrated in Figure 3.

When the optical axis is perpendicular to the BLM surface, the reflection matrix of the BLM is represented simply. When the thickness d of the uniaxial layer is sufficiently smaller than wavelength λ, we obtain

$$r_{11} = -2ir_p\delta_p \exp(i\delta_p) \tag{8}$$

$$r_{22} = -2ir_s\delta_s \exp(i\delta_s) \tag{9}$$

$$r_{12} = r_{21} = 0 \tag{10}$$

where

$$r_p = \frac{n_0\cos\theta_i - n_1\sqrt{1 - n_1^2/n_e^2\sin^2\theta_i}}{n_0\cos\theta_i + n_1\sqrt{1 - n_1^2/n_e^2\sin^2\theta_i}} \tag{11}$$

$$r_s = \frac{n_1\cos\theta_i - n_0\sqrt{1 - n_1^2/n_0^2\sin^2\theta_i}}{n_1\cos\theta_i + n_0\sqrt{1 - n_1^2/n_0^2\sin^2\theta_i}} \tag{12}$$

$$\delta_p = \frac{2\pi}{\lambda} n_0 \sqrt{1 - n_1^2/n_e^2\sin^2\theta_i}\; d \tag{13}$$

$$\delta_s = \frac{2\pi}{\lambda} n_0 \sqrt{1 - n_1^2/n_0^2\sin^2\theta_i}\; d. \tag{14}$$

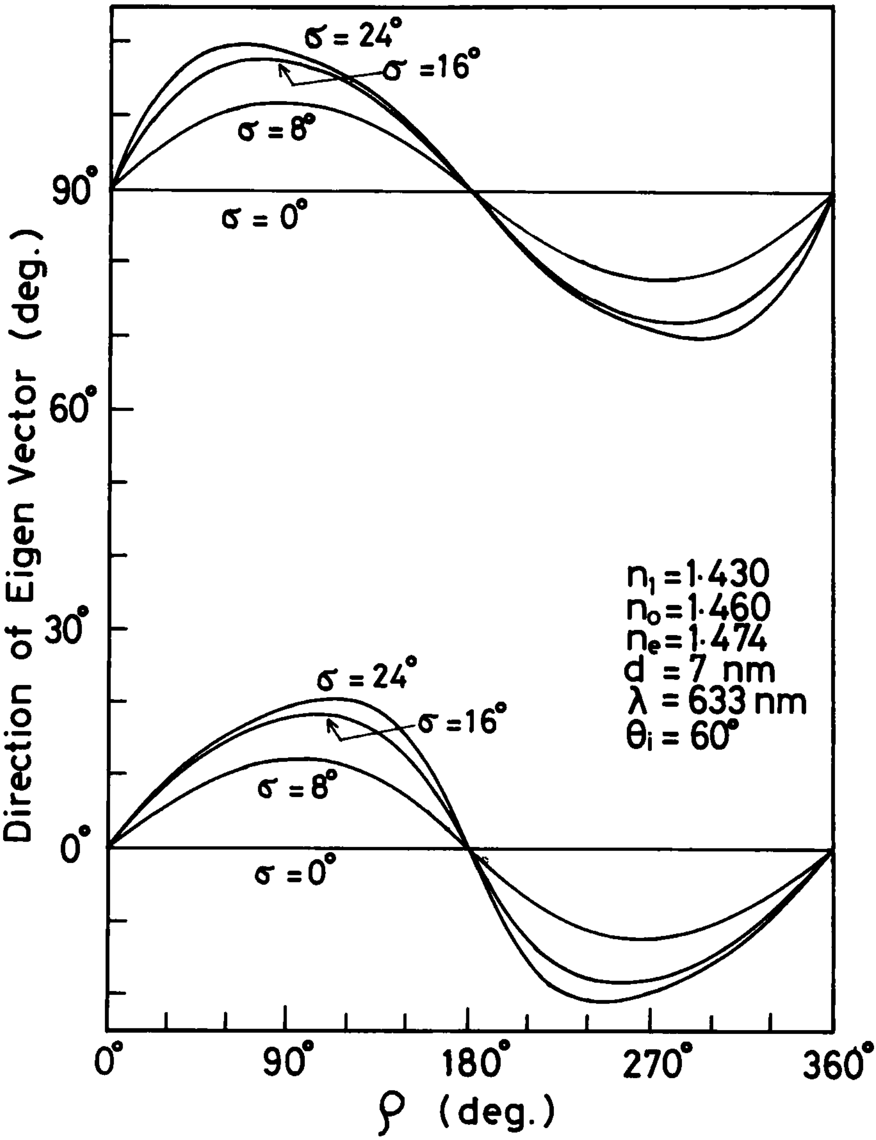

FIGURE 3 Calculated eigenvectors with the function of the direction of the optical axis.

was lyphilized. After, purified cholesterol was added to distilled pure grade *n*-octane, and the mixture was refluxed at boiling temperature ($\sim$125°C) in oxygen gas for 6 hours. Its supernatant was used for the BLM formation.

Teflon cell: For the purpose of optical observation, we designed a Teflon cell illustrated in Figure 4a. On this cell, an aperture of 3 mm is diameter was punched in the centre of the cylindrical structure, and a pyrex glass tube was fixed to cover the cell (see Figure 4c).

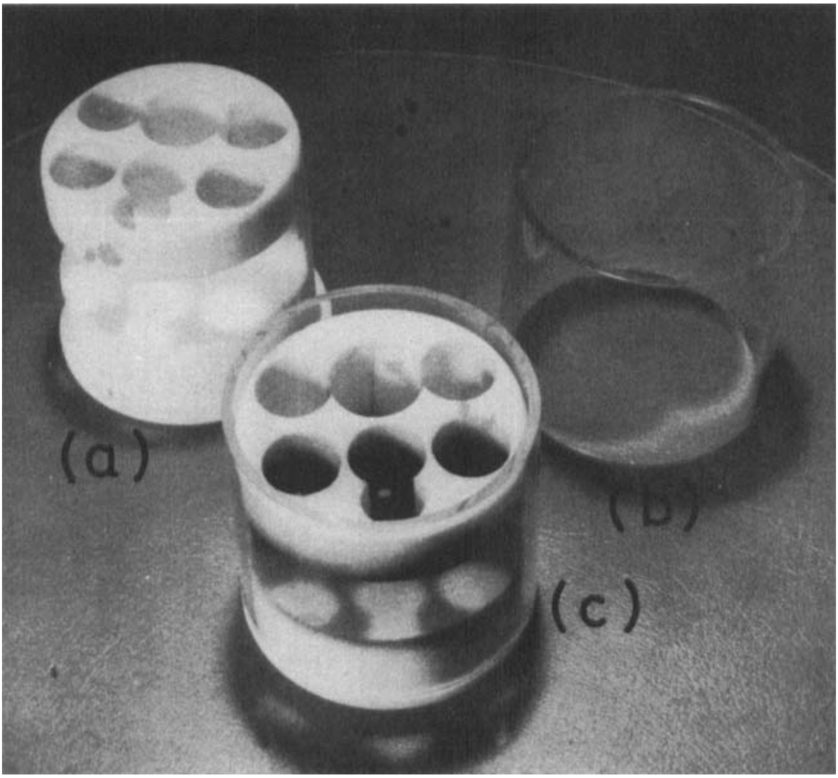

FIGURE 4 (a) Teflon cell with 60 mm in diameter and 57 mm in height. (b) Pyrex glass tube. (c) Pyrex glass tube fixed to cover Teflon cell.

Aqueous phase: The alkali halide aqueous solutions were prepared with doubly-distilled and deionized water which was kept in glass containers throughout, and alkali halide such as NaCl, LiCl, LiBr, etc., and they were poured into the Teflon cell.

Formation of BLM: A blush made in China for calligraphists was used to form the BLM onto the aperture of the Teflon cell, and we observed the membrane thinning, exhibiting interference fringes and finally forming the BLM. This procedure took about 10 to 40 minutes, and after, the BLM were usually stable for several hours.

Thermostat: The Teflon cell was fixed in a thermostat with a copper pipe into which water at stabilized temperature was circulated. The temperature range of this thermostat was from 10°C to 40°C with a stability better than ±0.5°C.

3.2 Ellipsometer

We improved the performance of the ellipsometer (Shimadzu EP–10) by using the 633 nm He–Ne laser (5 mW) as a light source, and observed the reflected light by the BLM (see Figure 5). Using a polarizer and a Fresnel rhomb, the laser light is changed into the circularly polarized light, which was then led to the polarizer of the ellipsometer. This part also fulfills the role of an isolator.

The polarizer and analyzer are Glan-Thompson prisms, and the compensator is a quarter-wave plate. Each of these parts is fixed on holder which

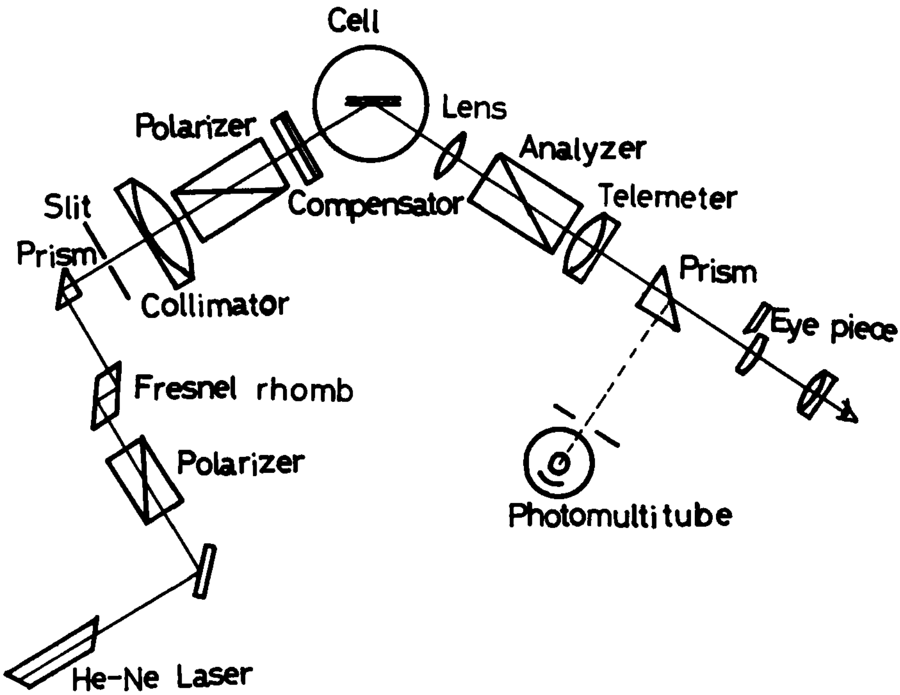

FIGURE 5 Diagram of the ellipsometer.

enables us to read the azimuthal angle up to 1 minute accuracy. The spotsize of the incident light on the BLM is about 1 mm.

We could measure the azimuth of reflected light with accuracy up to ± 0.5 degree at each experiment, and by the statistical treatment, we are able to determine the Brewster angle more accurately.

4 RESULTS AND DISCUSSIONS

4.1 Refractive index

In Section 2.1 (Figure 3), it is shown that if the optical axis of the BLM is tilted from the normal axis of the BLM surface (tilt angle is σ), the direction of the eigenvector deviates from 0° or 90° except for the special values of ρ, i.e., 0° and 180°. The value of σ can be considered as constant when temperature and the state of aqueous phase are specified. On the other hand, ρ depends on the geometrical alignment of the cell and may be different at each experiment by accidental initial conditions.

Figure 6 shows the experimental results of the direction of eigenvectors as a function of temperature. The experimental values are averages of several measurements. The deviations from 0° or 90° are less than 2°. Therefore, for the case of oxidized cholesterol BLM in the temperature range from 10°C to 40°C, it seems to be reasonable to assume that the optical axis is approximately perpendicular to the BLM surface. In order to justify this assumption, ρ dependence of the direction of the eigenvector must be investigated. For this purpose we should use, for example, a cell which can rotate about the normal axis of the BLM surface.

In Figure 7, we show an example of measured variation of the azimuthal angles with the incident angles. (The azimuthal angle of the incident light is 45°). The solid line in Figure 7 was drawn by the least square method according to Eq. (18) where it is assumed that the optical axis is perpendicular to the BLM surface. The Brewster angle can be obtained by finding the incident angle which corresponds to the azimuthal angle of 90°.

Next, we measured the Brewster angle by changing the refractive indices of aqueous phase n_1. The results are shown in Figure 8. These aqueous phases were prepared by the several kinds of alkali halide solutions shown in Figure 8, and each index of aqueous phase was measured by the Abbé refractometer. There is no observable change in the refractive index of the BLM with the kind of solutes. The solid line in Figure 8 was drawn by the least square method using the Eq. (14), with n_0 and n_e as fitting parameters and in consequence, n_0 and n_e at 30°C were determined as 1.4600 ± 0.0024 and 1.4741 ± 0.0039, respectively.

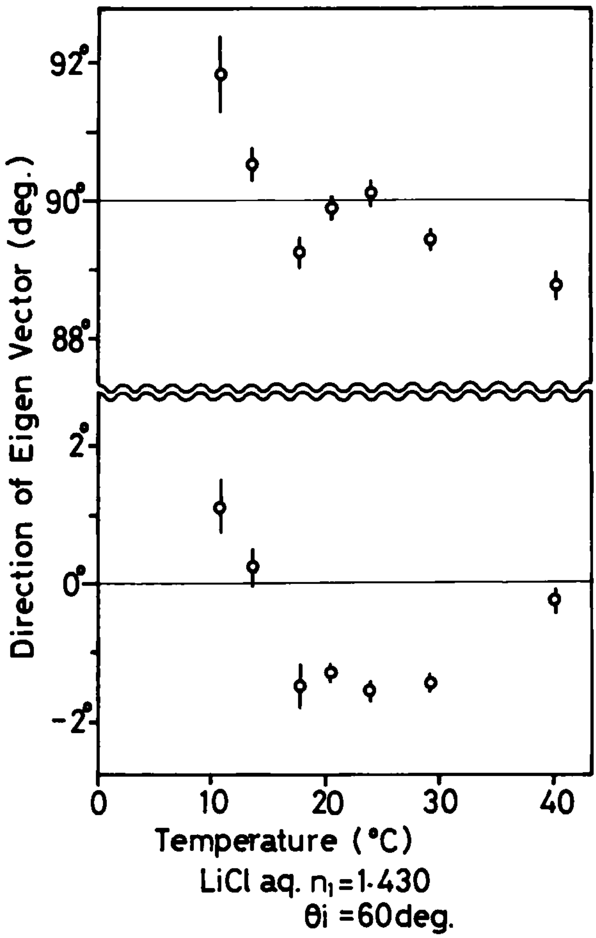

FIGURE 6 The direction of eigenvector with the function of temperature.

The temperature dependences of the ordinary and extraordinary refractive indices of the BLM obtained in these ways are plotted in Figure 9. In the temperature range from 10°C to 40°C, n_0 decreases from 1.4706 $\pm$ 0.0025 to 1.4579 $\pm$ 0.0024 and n_e from 1.4897 $\pm$ 0.0042 to 1.4712 $\pm$ 0.0038, and its positive birefringence also decreases according to the temperature. The decreases of n_0 and n_e with temperature are due to the decrease of the density of the BLM, and the decrease of its birefringence is considered to be due to the increase of the disorder of molecules.

Now, the other optical models which do not take anisotropy into account such as the triple layer model should be reexamined. Extending the triple

2.2 Refractive index

From Eq. (11), the Brewster angle θ_{iB}, the incident angle at which r_p is equal to zero, is given by the following equation,

$$\tan^2 \theta_{ib} = \frac{n_0^2 - n_1^2}{n_e^2 - n_1^2} \frac{n_e^2}{n_1^2}. \tag{15}$$

Equation (15) shows that a small difference between n_0 and n_e has a large effect on the Brewster angle. We can determine n_0 and n_e by measurements of the Brewster angles as function of each refractive index n_1 of several aqueous phases.

Generally, the Brewster angle can be measured directly. However, in this experiment we are dealing with BLM which have an extremely low reflectance. It is about 10^{-4} even at normal incidence and less than 10^{-6} in the wide range near the Brewster angle. Therefore, we propose to use an ellipsometer for precise determination of the Brewster angle for the BLM.

In the ellipsometry both the azimuth ψ and phase difference Δ of reflected light, which are defined by

$$E_{ry}/E_{rx} = \tan \psi \exp(-i\Delta) \tag{16}$$

are measured. In the case that the optical axis is perpendicular to the BLM surface and the azimuth of incident light is $\pi/4$, from Eqs. (8) and (9), Eq. (16) becomes

$$E_{ry}/E_{rx} = r_{22}/r_{11} = \frac{r_s \delta_s}{r_p \delta_p} \exp(i\delta_s - i\delta_p) \tag{17}$$

whence,

$$\tan \psi = \frac{r_s \delta_s}{r_p \delta_p}, \tag{18}$$

$$\Delta = \delta_p - \delta_s. \tag{19}$$

So, when the thickness is much smaller than wavelength, ψ's value is independent of the thickness. We can obtain ψ's value and Δ's value from the azimuth of the analyzer and the polarizer of the ellipsometer, respectively. Therefore, we will be able to determine the Brewster angle precisely by measuring ψ at various values of the incident angles, and finding the value of the incident angle where ψ becomes $\pi/2$.

2.3 Thickness

Several optical methods for the measurement of the thickness of the BLM were proposed. Many investigators adopted the method to measure the absolute reflectance, but this method is very difficult to measure the thickness precisely because reflectance is extremely low as mentioned above.

We cannot determine the thickness by means of the ellipsometry either, because the value of Δ is smaller than experimental errors. So, we adopted an interference method.[7]

When membranes still exhibit interference fringes, they can be regarded as isotropic media, and the intensity of the reflected ordinary light is expressed as

$$I = \frac{4r_a^2 \sin^2 \delta_a}{(1-r_a^2)^2 + 4r_a^2 \sin^2 \delta_a} I_0 \qquad (20)$$

where I_0 is the intensity of the incident light, and r_a is given by Fresnel's formula

$$r_a = \frac{n_1 \cos \theta_i - n_a \cos \theta_{ta}}{n_1 \cos \theta_i + n_a \cos \theta_{ta}} \qquad (21)$$

provided that n_a is the refractive index of membranes still exhibiting interference fringes, and θ_{ta} is the angle of refraction.

In the process of thinning, the intensity of reflected light changes oscilatorilly, and when r_a is sufficiently smaller than unity the maximum intensity of the reflected ordinary light I_{max} which is given at the phase difference $\delta_a = (n + \frac{1}{2})\pi$, is approximately represented by

$$I_{max} = 4r_a^2 I_0. \qquad (22)$$

On the other hand, from Eq. (9) for the ordinary light, intensity of the reflected light from the BLM is given by

$$I_{BLM} = 4r_s^2\delta_s^2 I_0 = 4r_s^2\left(\frac{2\pi}{\lambda} n_0 \cos \theta_{ta} \cdot d\right)^2 I_0. \qquad (23)$$

Therefore, by measuring the ratio of I_{max}/I_{BLM} for several aqueous phases with various refractive indices, it is possible to estimate the value of n_a and the thickness of the BLM.

3 EXPERIMENTAL METHODS

3.1 Preparation of BLM

Solutions: We used oxidized cholesterol dissolved in *n*-octane which is known to yield highly stable BLM. We followed the procedure for preparing oxidized cholesterol BLM forming solution as described by Tien.[8] Cholesterol was purified further by recrystallization in nitrogenated ethanol, and

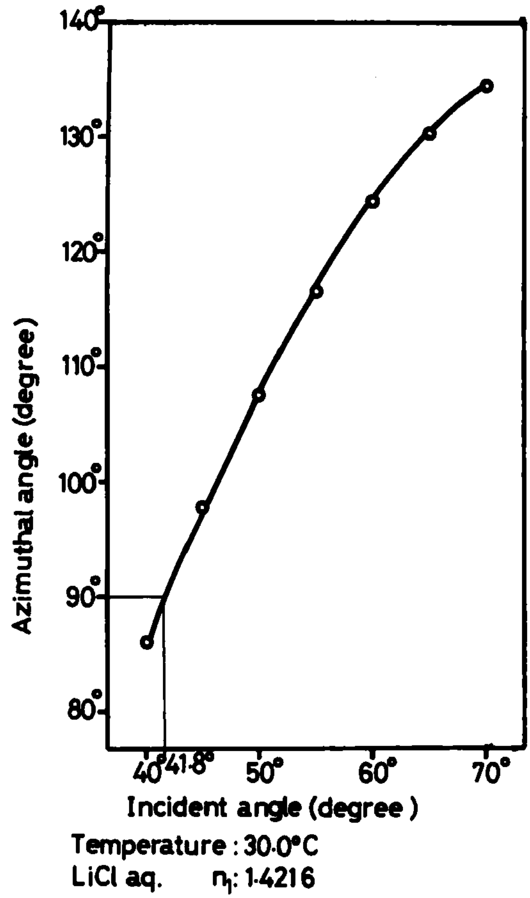

FIGURE 7 Determination of the Brewster angle and the azimuth of the incident light is 45°. The azimuthal angle of reflected light was measured by the ellipsometry, and phase difference Δ is nearly 180°. When n_1 draws near n_0 and in the incident angle is large ($\sim 70°$), the azimuth over 135° of the reflected light was observed (whence $r_p > r_s$). This is characteristic of anisotropic media.

layer model, Simon and Cidder derived the Brewster angle relation of optically isotropic film where the profile for the dielectric constant is symmetrically disposed about the centre of the membrane and varies continuously from a value of ε_1 in the outside media to a value of ε_2 in the film interior;[9]

$$\tan^2 \theta_{iB} = \frac{\bar{\varepsilon}}{\varepsilon_1} \frac{\varepsilon_1 - \varepsilon_1^2/\bar{\varepsilon}}{\varepsilon_1 - \varepsilon_1^2 \overline{\overline{\varepsilon^{-1}}}} \tag{24}$$

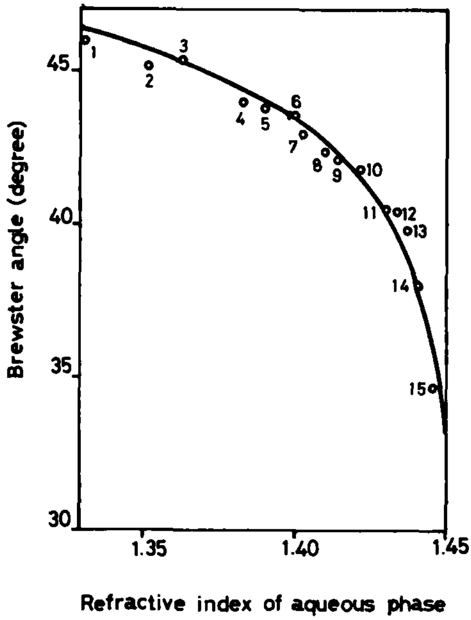

FIGURE 8 Variation of the Brewster angle with the refractive index of aqueous phase. Temperature: 30.0° ± 0.5°C. The solutes of aqueous phase are 1. only water, 2. NaCl, 3. KI, 4. LiBr, 5. LiBr, 6. NaBr, 7. LiCl, 8. LiBr, 9. NaI, 10. LiCl, 11. LiCl, 12. LiBr, 13. NaI, 14. LiBr, 15. LiBr.

where

$$\bar{\varepsilon} = \int \varepsilon \, dz, \tag{25}$$

$$\overline{\varepsilon^{-1}} = \int \frac{1}{\varepsilon} \, dz. \tag{26}$$

Taking note that ε_1 equals n_1^2, Eq. (24) becomes the same formula as Eq. (15) if $\bar{\varepsilon}$ and $\overline{\varepsilon^{-1}}$ are replaced by n_0^2 and $1/n_e^2$, respectively. In order to explain the results shown in Figure 8 by Eq. (24), the condition $\bar{\varepsilon} < (\overline{\varepsilon^{-1}})^{-1}$ must be satisfied. But $(\overline{\varepsilon^{-1}})^{-1}$ cannot become greater than $\bar{\varepsilon}$ mathematically. Hence, an optical model without anisotropy cannot account for the optical properties of the BLM.

Cherry and Chapman proposed the uniaxial layer model and analyzed their experimental results on egg lecithin.[5] The results were: $n_0 = 1.363 \pm 0.004$ and the birefringence $n_e - n_0 = 0.022 \pm 0.003$. Our study on oxidized cholesterol also confirm this model.

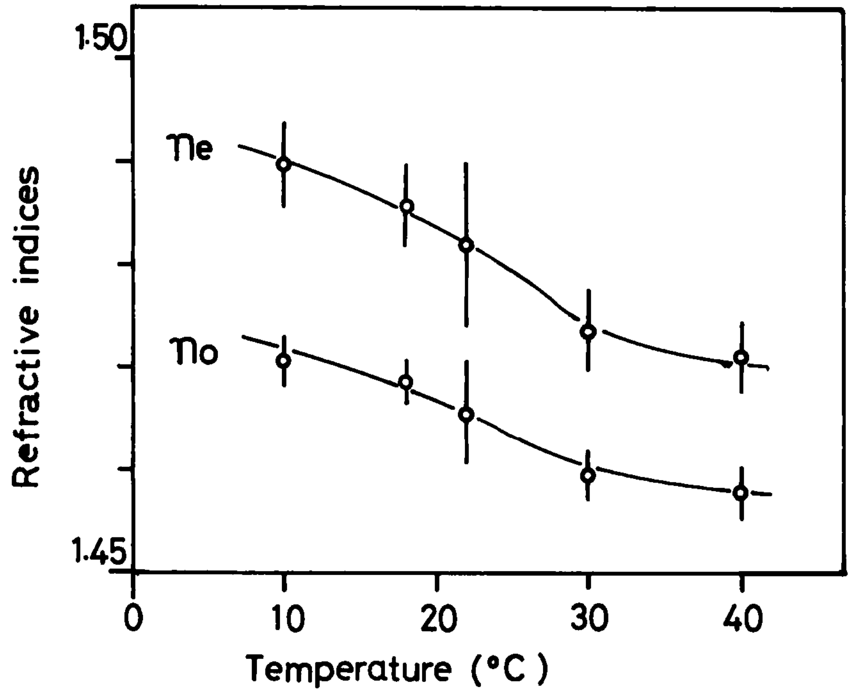

FIGURE 9 Temperature dependences of the ordinary and extraordinary refractive indices of oxidized cholesterol BLM for 633 nm.

We consider it meaningful that the direction of optical axis should be examined for the other BLM. Other models such as the anisotropic triple layer model need so many parameters that the material values of the BLM cannot be determined experimentally. Therefore, the tilted uniaxial layer model analyzed in this paper is the most reasonable model corresponding to the experiments.

4.2 Thickness

In Figure 10, we show an example of the strip chart recorder tracing illustrating the formation of thin lipid membrane under ordinary light and thinning to the BLM. From this figure we can obtain the ratio of I_{max}/I_{BLM}. We obtained the thickness of oxidized cholesterol BLM and the value of n_a from 108 experimental data which was measured with various refractive indices of aqueous phases at 30°C, and the results are as follows; the thickness is 7.12 ± 0.93 nm and the value of n_a is 1.396 at 30°C. The value of n_a is close to the refractive index of *n*-octane.

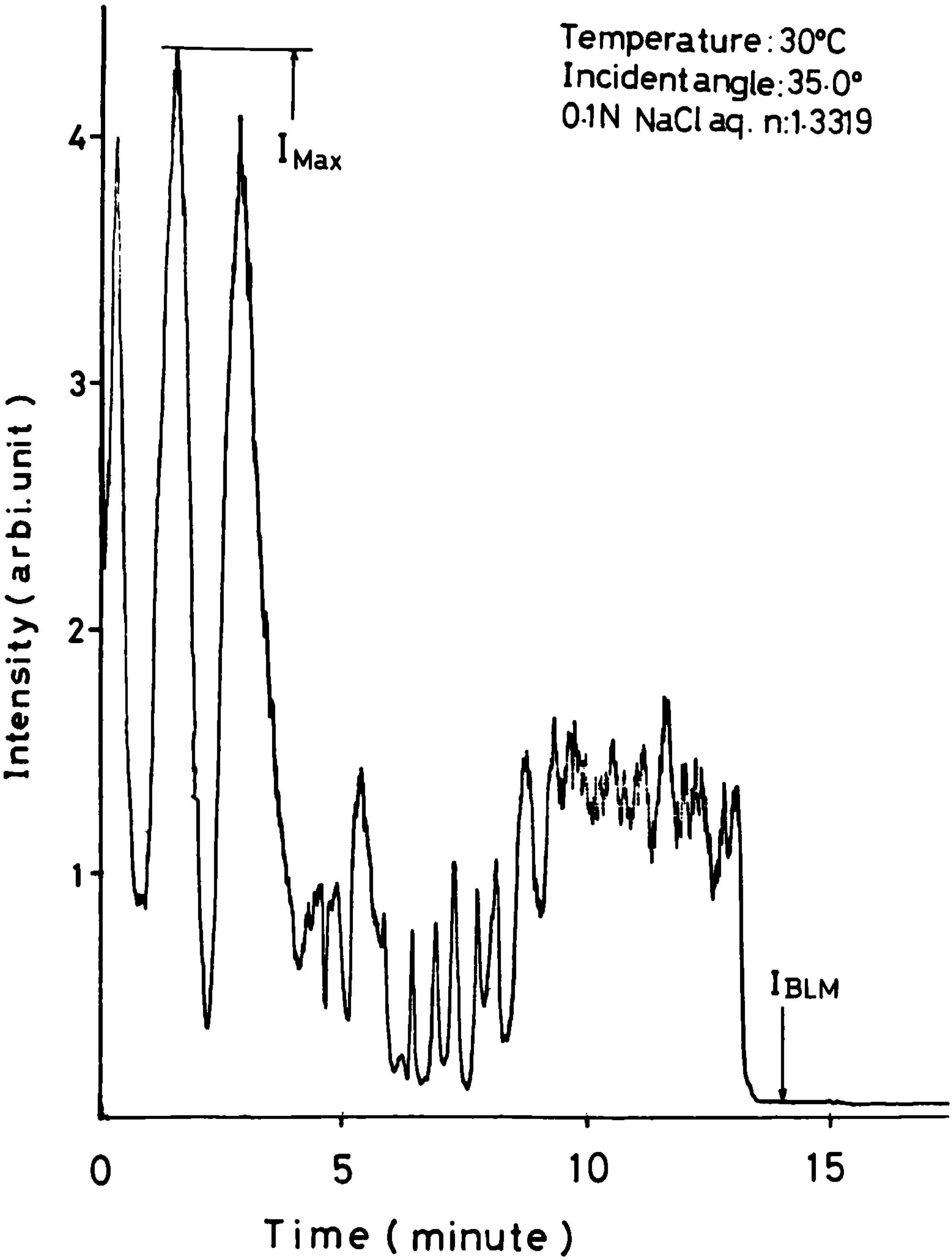

FIGURE 10 Strip chart recorder tracing illustrating the formation of thin lipid membrane under perpendicular polarized light (633 nm) and its thinning to the BLM. This was recorded through a low pass filter (cut off 0.1 Hz–6 dB/oct).

On the other hand Tien reported that the thickness of oxidized cholesterol BLM is 4 ± 1 nm[7] and this value is different from our results. The determination of thickness is influenced by the value of refractive indices of BLM, therefore, we consider that this disagreement mainly depends on the value of the refractive indices.

5 CONCLUSION

In this study, we made the BLM by oxidized cholesterol on the Teflon cell in the aqueous phase and investigated their optical characteristics. As an optical model of the BLM, we studied and analyzed the uniaxial layer model where the optical axis is in the arbitrary direction, and from experimental results, we adopted the uniaxial layer model where the optical axis is perpendicular to the BLM surface.

The refractive indices could be obtained from the ellipsometry, and we obtained the refractive indices in the temperature range from 10°C to 40°C, no decreases from 1.4706 ± 0.0025 to 1.4579 ± 0.0024 (for 633 nm), and N_e from 1.4897 ± 0.0042 to 1.4712 ± 0.0038 (for 633 nm). Its birefringence also decreases according to the temperature. The accuracy of the measurements was obtained up to the effective number of four figures.

We also measured the thickness of oxidized cholesterol BLM, and the value of 7.12 ± 0.93 nm at 30°C was obtained.

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