PHASE TRANSITIONS IN PHOSPHATIDYLCHOLINE DISPERSION OBSERVED WITH AN INTERFERENCE REFRACTOMETER

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ABSTRACT An interferometer is used to measure the refractive index change accompanying the crystal-to-liquid-crystal phase transition in the dispersion of phosphatidylcholines. Two separate methods of obtaining the refractive index change are employed: the first method analyzes the intensity transmitted through a spatial filter and the second method utilizes a piezoeletric crystal-based electronic compensator. The results of the two methods agree well. The accuracy of the apparatus (6×10^{-6}) permitted us to use a very dilute sample to detect the phase change. Only a fraction of a milligram of dry lecithin is needed to observe the change. The result confirms conclusively that the major reason for the turbidity change at the transition temperature is the alteration in the refractive index of the lipid membranes. The fractional change in the refractive index does not agree well with the fractional change in the density of lipid molecules in vesicles.

INTRODUCTION

Refractive index measurements have been widely used to determine solute concentration of a solution containing a known solute. The applications of refractometry to living systems (1) are based on such functional relationships between refractive index values and solid contents of organisms. However, the possibility of employing refractometry for the detection of conformational changes of nucleic acids, proteins, and cell membranes has not been explored.

If solute molecules are macromolecules, the polarizability per volume of solution depends on the partial molar volume of the macromolecule, because the volume of a solution is the sum of the volumes occupied by the solvent and the solute. Hence, the refractive index of the solution must change if a conformational change induces a volume change on the macromolecule. This change is, in general, very small and cannot be easily detected with commercially available refractometers. Besides, biological cell components are often available only in small amounts. Therefore, the sensitivity of an interferometer is needed for the detection of such changes.

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It has been known for some time that the turbidity of an aqueous dispersion of a pure phospholipid undergoes two sharp changes (2,3) at temperatures corresponding to the phase transition temperatures observed first with differential scanning calorimetry and X-ray diffraction (4–6). The turbidity change observed at the higher temperature with samples containing sonicated dipalmitoyl phosphatidylcholine (DPPC) exhibited little or no thermal hysteresis and was accounted for at least partly by the change in the refractive index of lipid vesicles (2). The low-temperature transition showed a large thermal hysteresis, and produced no detectable change in the refractive index of lipid with the Abbe refractometer (model 3L, Bausch and Lomb Inc., Rochester, N.Y.).

The relative change of the density of lipid molecules occurring at the main transition of the DPPC membrane is measured to be 3.5% (7,8). This change causes a relative volume change of a few parts in 10,000 of the solution's volume containing 1% lipids. This is barely detectable with the Abbe refractometer. A more accurate instrument is desirable for the correct understanding of the refractive index and turbidity changes. Furthermore, the sensitivity of an interferometer may permit observations of possible alterations in biological membranes caused by external agents such as ions, hormones, and drugs, as well as the environmental temperature.

In a medium of refractive index n, both the wavelength and the speed of light are reduced by the factor of n. If two coherent beams are allowed to interfere after they pass through equal lengths of two different media, the interference pattern will shift if the refractive index of one medium changes differently from the refractive index of the other. We used this property of the interferometer to measure the temperature dependence of molecular structure in lipid membranes. The accuracy of measuring refractive indices with an interference refractometer increases with the beam path length in the sample. However, the turbidity of the sample limits this length. With a sample holder of 1 cm beam path and with samples containing 1% lipid concentration, we have achieved an accuracy of nearly three orders of magnitude better than that of the Abbe refractometer. This means that a quantitative comparison between the light scattering and the refractive index changes and between the lattice expansion and discontinuities of this optical parameter can be made for the phase transitions in lipid vesicles.

METHODS

The Apparatus

An interferometer has been in use as a refractometer to measure refractive index changes of clear liquids (9,10). The present work is, as far as the authors have found, the first to use an interferometer to measure refractive index changes of turbid solutions.

The basic optics employed was from a Michelson interferometer. As shown in Fig. 1, the beam splitter S reflects a portion of the light at a 90° angle with respect to the incident beam toward mirror M_1. The remainder of the light is transmitted through S toward mirror M_2. A water-jacketed spectrophotometer cell is placed in the beam before each of two mirrors. The light beams reflected by the mirrors travel back through the sample cells and are reunited after

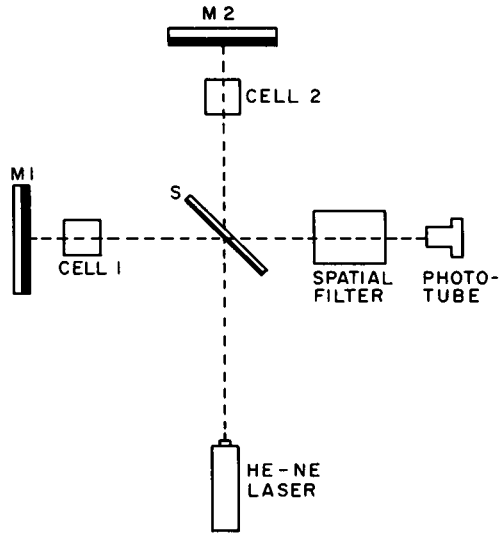

FIGURE 1 Schematics of the interference refractometer. S: beam splitter; M_1 and M_2: mirrors.

passing through the beam splitter again. When the observer focuses an eye toward M_1 through S, he will see the superposition of the two beams.

If cell 1 in Fig. 1 contains the sample, cell 2 will have the reference solution. As the temperature of the sample and the reference is varied by pumping water through the cell jackets, their refractive indices will change and their changes will, in general, be different. Such differential changes of the refractive index of the sample against the reference will generally cause the shift of interference fringes. The refractive index change of the suspended particles can be obtained by adding the change of the reference alone to the differential change. Therefore, it is important to use a matched pair of spectrophotometer cells. If the walls of the sample cuvette and the reference cuvette have the same homogeneous refractive index and an equal thickness, the differential refractive index change of the walls would not enter the measurement. Likewise, the light paths in the two cells must be equal within the precision that the differential measurement requires. With matched cells obtained from Hellma Cells, Inc. (Jamaica, N.Y.), a white light fringe can be easily restored after the cells are placed, and again after the cells are filled with distilled water. The lengths of the light beams inside the cells are estimated to be within 3 μm of each other.

Two methods of obtaining the refractive index change were used. Both methods employ basically the same optics described in the above paragraph. In method I the output beam was passed through a spatial filter (R250 F-20, Gaertner Scientific Co., Chicago, Ill.) to single out a narrow beam and to expand it before the intensity is monitored with a photomultiplier. Method II does not employ a spatial filter or a slit. It uses an automatic compensator that nullifies any differential index change occurring in the two cells. Both methods are detailed below. The preliminary result of this work has been reported in the American Physical Society Meeting (11).

Method I: The Intensity Analysis

The light intensity transmitted through a pinhole oscillates sinusoidally as the temperature (or other variable) of the sample is continuously varied through the range T to $T + \Delta T$. The number of fringes or cycles in intensity, m, passing through the pinhole in front of the detec-

tor can be given in terms of changes Δn_1 and Δn_2 of the refractive indices of the two media over this range:

$$m = 2(d_1\Delta n_1 - d_2\Delta n_2)/\lambda, \quad (1)$$

where d_1 and d_2 are the lengths of the light beams determined by the inner walls of cell 1 and 2, respectively, and λ is the wavelength of the light in vacuum. The factor 2 in Eq. 1 comes from the fact that each light beam makes a round trip through the cell, toward and then away from the mirror. If the two cells are exactly matched, $d_2 = d_1 = d$. Assuming a sinusoidal line shape for the interference fringe, the light intensity I received through a pinhole is:

$$I = I_0 \cos(4\pi d\Delta(n_1 - n_2)/\lambda) + C. \quad (2)$$

I_0 is the amplitude of the light intensity variation and $C - I_0$ is the light intensity at its minima. C and I_0 are simple functions of the light transmission through the cells. Since the turbidity depends on the temperature, both C and I_0 are functions of the temperature. Hence, it becomes somewhat complicated to extract from the experimental intensity profile the temperature dependence of the refractive index with Eq. 2.

Method II: The Method of an Electro-Optic Compensator

The above method of calculating refractive index changes is tedious partly because the intensity maxima and minima may vary. One can obtain the change directly with a calibrated optical compensator. An electro-optic compensator described below gives the change in terms of an analogue signal.

One mirror of a Michelson interferometer is attached to one end of a piezoelectric translator (model ED-25 Jodon Engineering Associates, Inc., Ann Arbor, Mich.), so that the translator can electrically control the optical path length in this arm. The fringe pattern is viewed by two fiber optics ribbons, each coupled to the face of an RCA 6199 photomultiplier tube (RCA Solid State, Somerville, N.J.). The thickness of the ribbons is 0.25 mm and they are 6 mm high. The separation between the ribbons is 3 mm, which corresponds to about half of the fringe width at the viewing plane. The anode current from each phototube is monitored with an electrometer. The electrometer outputs are fed into a difference amplifier. The error signal (corresponding to a fringe shift) generated by the difference amplifier is integrated at an adjustable rate. The output of this integrator is a measure of the optical path change or, assuming constant geometrical path, the refractive index change. This output is supplied to the y axis of an x-y recorder and sent to the translator after further amplication. The translator moves in such a way as to reduce the error to zero, thus holding the fringe in place. Notice that in this method the light intensity variation caused by a turbidity change during a temperature sweep has no effect on the measurement of a refractive index change. The schematics of the method are shown in Fig. 2.

Sample Preparation

When the particle size and the particle density are large, the light beam cannot be formed through the sample because of the scattering. At low concentrations of lipid molecules, the particle size dependence of the light scattering outweighs its particle density dependence. Hence, it is desirable to disperse the sample into small vesicles. Weighed mixtures of phosphatidylcholine and doubly distilled water were irradiated with low-intensity sound waves until the sample of 0.5–1 ml volume showed a hint of bluish color. Caution was taken not to overheat the sample during sonication. A well-defined light beam was formed with a well-sonicated 1% DPPC/water mixture in the temperature range, 20°C ~ 50°C. L-α-dipalmitoyl-lecithin (DPPC)

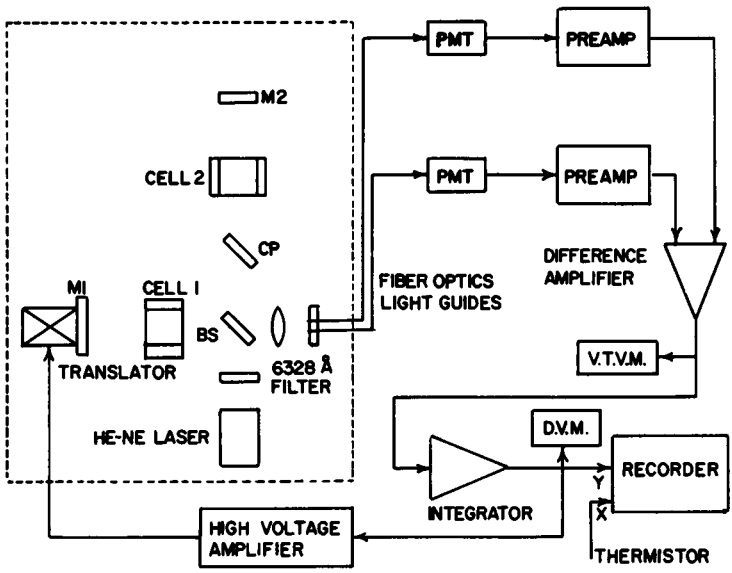

FIGURE 2 Schematics of the automatic interference refractometer. BS, beam splitter; CP, compensating plate.

was purchased from Calbiochem (San Diego, Calif.), and was purified by silica gel column chromatography by eluting with mixtures of varying proportions of chloroform and methanol. This purification procedure was found essential to obtain a pure sample, which only gave a sharp change in the light scattering at approximately 41°C. Lipids obtained from all suppliers gave a broad and shallow change in the light scattering if used without purification. It should be noted that presence of D-α-DPPC lowers the transition temperature (R. C. MacDonald and P. N. Yi. Unpublished work.).

RESULTS

The Intensity Analysis

Fig. 3 is the tracing of the light intensity vs. temperature with an x-y recorder when one cell of the interferometer contains the 1% lipid dispersion and the other cell has deionized doubly distilled water. The temperature of the sample was measured with a Yellow Springs thermolinear thermistor (Yellow Springs Instrument Co., Yellow Springs, Ohio) by amplifying its output with an operational amplifier before it was fed into the x-axis of the recorder. The temperature was swept from 25°C to 46°C at the rate of 1°C/min. Each cycle of the light intensity tracing in Fig. 3 means one full fringe translation across the pinhole located in front of the detector. The fringe shift near 41°C occurs much faster than at other temperatures. This represents a more rapidly changing refractive index at the phase transition temperature of L-α-DPPC. One should recall that the fringe shift is proportional to the relative refractive index change of the lipid with respect to the refractive index change of water. When both cells contained water, no more than half of a fringe cycle was recorded between 25°C

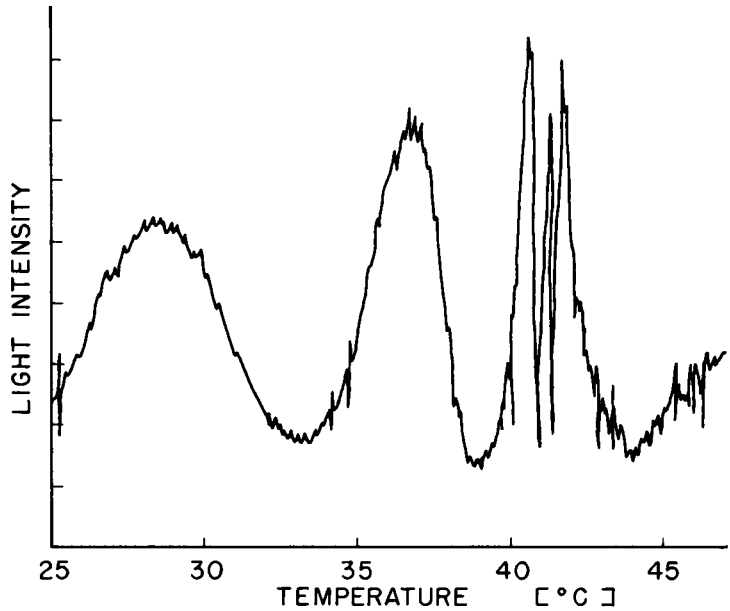

FIGURE 3 Light intensity through a slit vs. temperature. The sample is aqueous dispersion of DPPC (light intensity not calibrated).

and 45°C. Hence, the difference in the beam paths of the two cells are very small, and for the purpose of measuring the change between 40°C and 42°C, the difference is negligible.

The relative change of the refractive index of the sample was calculated by dividing each cycle into increments of one-tenth of a fringe and reading off the temperature at which these increments occurred. The increment of the relative refractive index

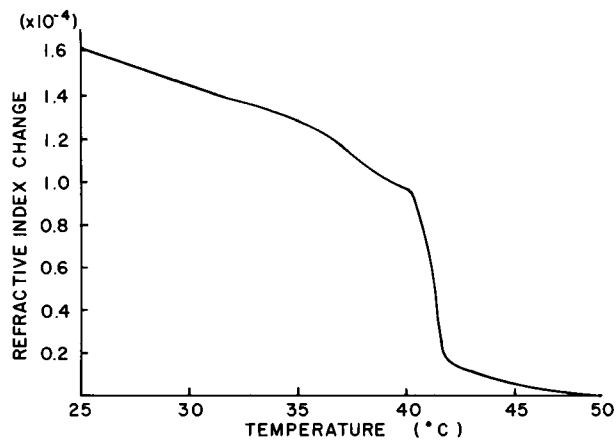

FIGURE 4 Refractive index variation vs. temperature for aqueous dispersion of DPPC (arbitrary origin).

$\Delta(n - n_w)$ is then calculated by using Eq. 2 with $\lambda = 6328$ Å, and $d = 1$ cm. Thus, the temperature dependence of the relative refractive index change is plotted for the 1% dispersion of DPPC (Fig. 4). The noise level is such that the uncertainty in dividing one fringe cycle into 10 segments is estimated to be, at most, 5% of a single fringe cycle.

This change in the refractive index of the dispersion is, of course, caused by the alteration in the refractive index of the lipid portion, and the latter change is in turn the result of the modification in the electric polarizability per molecule, and in the packing density of lipid molecules within the lipid bilayer.

One may define the index n_l of the lipid vesicle in terms of the empirical relation (2)

$$n = f_w n_w + f_l n_l, \tag{3}$$

in which n and n_w are the indices of the dispersion and the water phase, respectively; likewise, f_w and f_l are the volume fractions of water and lipid, respectively. n_l, as defined above, is uniquely determined by measurable quantities. This n_l is related to the isotropic component (α_l) of the polarizability of lipid per unit volume of vesicles by the Clausius-Mossotti equation:

$$3(n_l^2 - 1)/(n_l^2 + 2) = \alpha_l. \tag{4}$$

The decrease of n_l over the temperature interval 40° ~ 42°C is $0.8(\pm 0.05) \times 10^{-2}$. With Eq. 4 and the previously measured value of n_l, the percentage decrease of α_l over the same interval is $1.5 \pm 0.2\%$. The percentage decrease of dn/dc over the temperature interval 40–42°C is $4(\pm 1)\%$. The major source of the error comes from the averaging of different runs.

The Compensator Method

The temperature dependence of the refractive index difference $n_l - n_w$ is plotted directly with an x-y recorder by monitoring the input voltage of the high-voltage amplifier as a function of the sample temperature. The result is given in Fig. 5. The refractive index change is obtained by calibrating the input voltage change. This voltage was calibrated by injecting a linearly increasing external signal into the mirror translator and recording the intensity variation detected as a function of the translation voltage using one fiber optics ribbon. This procedure was carried out without the compensation. The intensity vs. voltage and the number of fringes vs. voltage are given in Fig. 6. The scanning speed of the calibration curve was made approximately equal to the voltage sweep per second of an actual experiment. The sample temperature was cycled bi-directionally several times to examine possible nonreproducible effects. All graphs exhibited a cooling curve displaced from the heating curve. The possible source of this discrepancy resides either in the crystal hysteresis or in the temperature difference in the two cells, reversed when the temperature sweep was turned around. Since the oppositely directed voltage sweeps gave the same calibration curves for the fringe vs. voltage, the net effect of the crystal hysteresis on the curves for the refractive index change vs. temperature is limited to the ends of the temperature scan.

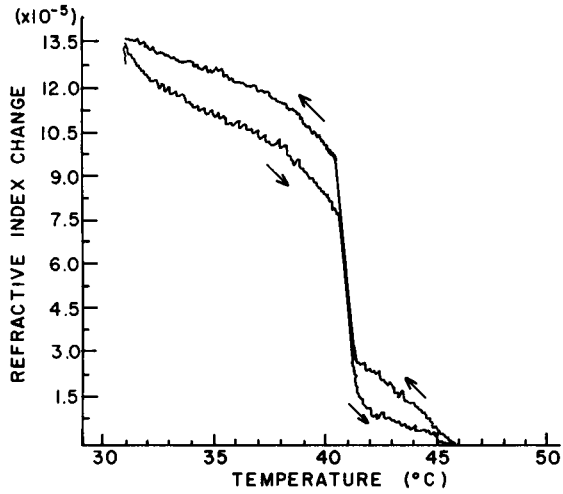

FIGURE 5 Automated printout of the refractive index change vs. temperature obtained by method II.

The result obtained by the compensator method agrees very well with the result calculated from the intensity analysis. The agreement between the two results demonstrates the correctness of both methods.

DISCUSSION

The present work has demonstrated that an interferometer can be used to measure refractive index changes accompanying a conformational alteration occurring in a macromolecule suspended in water. This technique can be extended to study structural changes occurring in other biological systems. Volume and optical changes observed in an axon during nerve activity (12–14), changes during enzyme denaturation, and membrane permeability increase above the physiological temperature are a few possible areas where the interferometer, particularly the automatic refractometer described in method II of the Methods section, can be a useful tool. The automatic path compensator employed in method II uses a mirror translator that is essentially a piezoelectric crystal. Hence a question may be raised as to the reproducibility and linearity of the crystal and of the refractometer output. Indeed, both Jodon ED-25 and El Don PMD-20 (Ann Arbor, Mich.) exhibit a considerable hysteresis for certain conditions. As it is easy to see from Fig. 5, the effect of the irreversibility on the refractive index change at the phase transition point is small.

The automatic interference refractometer described by Kinder and Plesse also utilizes a compensator (9). In this method a glass plate is rotated to offset the optical path change. The optical path change is determined by the time interval taken to "recapture" the zero-order white light fringe. Kinder and Plesse's photoelectric pulse amplitude modulation technique is, however, more complicated without being more

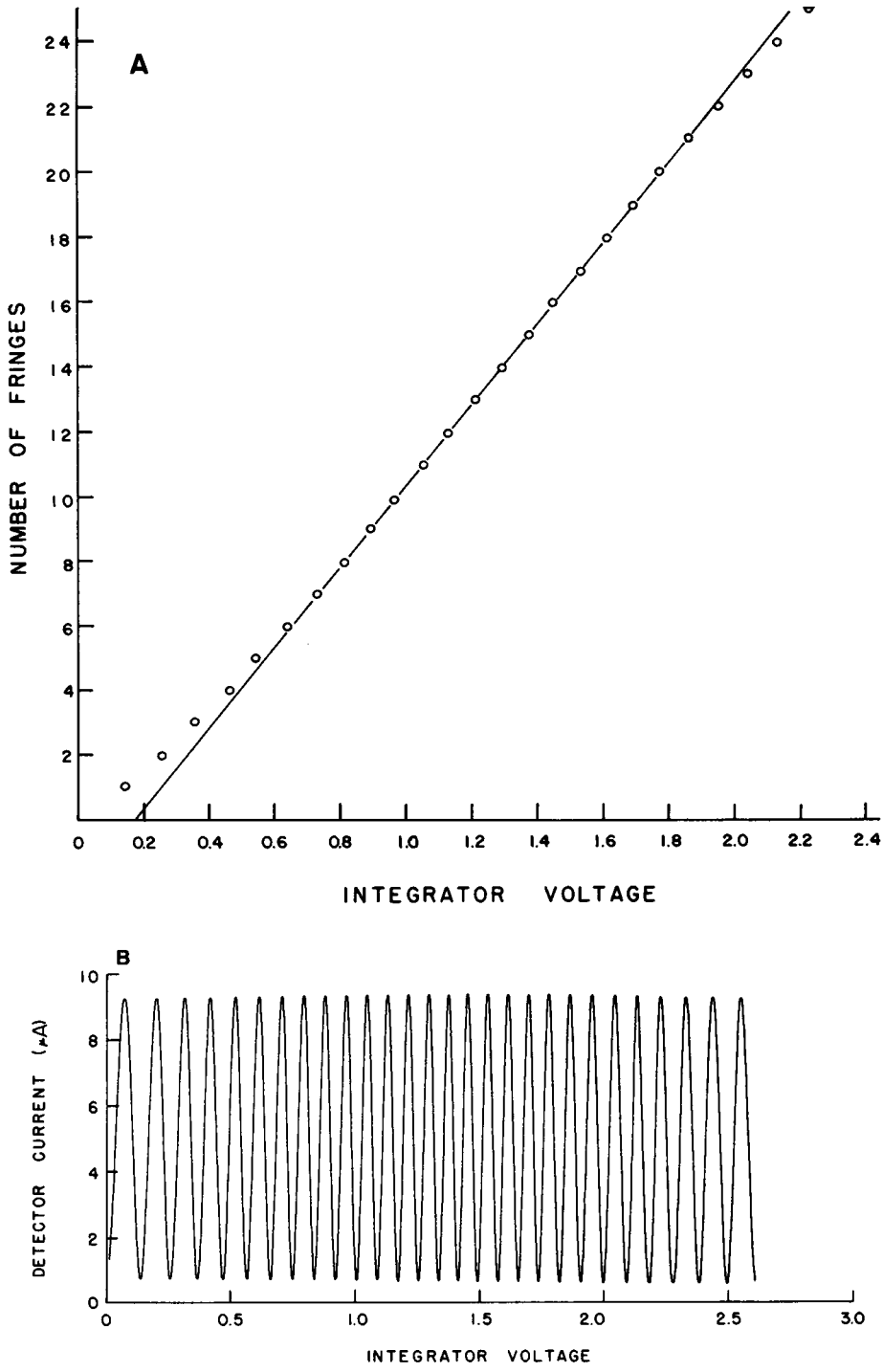

FIGURE 6 Number of fringes vs. integrator voltage (A) and intensity vs. integrator voltage (B).

accurate. On the other hand, the white light "recapture" technique can give absolute as well as relative refractive index measurements.

This work has also demonstrated that the refractive index change indeed occurs at the crystal-to-liquid-crystal transition temperature of the DPPC membranes. This result conclusively confirms the earlier work of Yi and MacDonald (2), obtained with an Abbe refractometer. The magnitude of the change in the refractive index is not large enough to account for the entire change of the light scattering occurring at the same temperature. However, since the accuracy of the present work is comparable to the light scattering experiment, one may be able to use both sets of data to study vesicle size changes and membrane thickness changes.

The low-angle light scattering (15) and the optical density measurement (2) exhibit a large decrease in turbidity somewhere between 30°C and 35°C. The decreased turbidity of the DPPC dispersion gives rise to an increased light transmission. The increased signal produces the enhanced peaks observed above 30°C in Fig. 3. Fig. 4 shows that there is no abrupt change in the index of refraction in the temperature range 25°–34°C. The temperature coefficient of the refractive index also appears to remain constant in this temperature interval. Therefore, the turbidity change that occurs in the above temperature range is not a direct reflection of the change in the molecular structure of the membranes. Rather, it appears to involve a change in the shape of the membranes or the aggregations of vesicles. Other evidences for these possibilities have been discussed in ref. 2.

From Fig. 4 one can easily notice a slope change in the temperature dependence of the refractive index at slightly below 35°C. The position of this change agrees very well with the temperature of "pre-transition" observed with calorimetry (4,5) and dilatometry (7,8). This temperature behavior near the "pre-transition" appears to contrast with the two-step change observed in the density measurement. The temperature of the sample in the water-jacketed cell changed continuously at the rate of 1°C/min. It is quite possible, particularly at temperature much higher than room temperature, that the temperature could fluctuate by a small fraction of a degree along the beam path. Such thermal inhomogeneity would tend to round off any sharp changes. The density change at the "pre-transition" was measured to be approximately 1/10 of the density change at the upper transition (7). If the ratio of the refractive index changes at the "pre-transition" and at the main transition is same as the ratio of the density changes, the order of magnitude change in the refractive index expected near 35°C in Fig. 3 is about 8×10^{-6}. This change is certainly big enough to be observable with the instrument, yet it could be smoothed out when the temperature sweep rate is 1°C/min. It must be pointed out that the slope change near 35°C is also observable in the 90° light scattering (2).

It can be concluded that the turbidity change is only an indirect consequence of the "pre-transition." If this turbidity decrease is the result of a disaggregation phenomenon, as the evidence suggests (2,15), the disaggregation should, in turn, involve a change in the surface property of vesicles without an alteration in the temperature de-

pendence of the molecular packing. The ionic head groups may reorient themselves so as to give a radial displacement of charged layers which produce a short-range repulsion between membrane vesicles. If one assumes that the pre-transition is an onset of the rotation of lipid molecules about its fatty chain axis (4,5), then the reorientation of the ionic head group reduces the moment of inertia to prepare for an increased rotational speed.

It is interesting to compare the fractional change in the density and the fractional change in the refractive index measured across the crystal-to-liquid crystal transition. Nagle has measured the density change to be 3.5 ± 0.3% for DPPC (7). Employing a sensitive scanning dilatometer, Blazyk et al. reported (8) the same value as Nagle. The present work has measured the change of the refractive index of DPPC in bilayer and has found that the decrease of the electric polarizability (at optical frequencies) per lipid volume occurring in the temperature interval 40°C ~ 42°C is only 1.5 ± 0.2%. Hence, the decrease of the index, caused by the volume expansion, is partially offset by an increase of the polarizability per molecule. Nevertheless, a refractometer, such as described in the present work, can be a useful tool in detecting conformation changes occurring in biological macromolecules.

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