Fluorescent Probe Analysis of the Lipid Architecture of Natural and Experimental Cholesterol-Rich Membranes†

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ABSTRACT: Steady-state and time-resolved fluorescence anisotropy of 12-(9-anthroyl)stearic acid was examined in the presence of red blood cell membranes and phospholipid dispersions containing various amounts of cholesterol. The fluorescence emission spectrum, quantum yield, and fluorescence lifetime of AS are changed little by the presence of cholesterol. The rotational correlation time, ϕ for 12-(9-anthroyl)stearic acid fluorescence anisotropy at 37° is 7.8×10^{-9} sec in normal red blood cells and 8.5×10^{-9} sec in blood cells containing twice the normal complement of cholesterol. A similar increase in ϕ was observed in dipalmitoyllecithin vesicles containing high contents of cholesterol. The energy of activation for rotation of 12-(9-anthroyl)stearic acid is lowered by the presence of cholesterol in the membrane. Analysis of the time-resolved fluorescence indicates that the decay of anisotropy is biphasic, including a fast phase which is unresolved, followed by a slower phase which, for normal red blood cell membranes at 37°, has a rate constant of 0.135×10^{8} sec^{-1}. These data indicate that the addition of cholesterol to natural or artificial membranes (liposomes) restricts molecular motion in the hydrophobic portion of the membrane. The physical change in the membrane which results from altered membrane lipid composition may explain the increased rate of destruction of cholesterol-rich "spur" red cells *in vivo*, and their unusual cell morphology.

Although there is no universally accepted model for membrane architecture, there is much evidence to indicate that many properties of membranes can be accounted for by a dynamic, fluid lipid bilayer structure (Gitler, 1972; Singer and Nicholson, 1972). Techniques used to detect the dynamic properties of the membrane include spin-label (De Vaux and McConnell, 1972; Huang *et al.*, 1970; Traüble and Sackman, 1972) and fluorescent probe analysis (Cogan *et al.*, 1973; Vanderkooi and Chance, 1972; Ygeurabide and Stryer, 1971) and nuclear magnetic resonance (nmr) (Chan *et al.*, 1972; Metcalfe *et al.*, 1971; Lee *et al.*, 1973).

Since the cholesterol of mature cells is unesterified and confined to the plasma membrane (van Deenan and De Gier, 1964), it can be expected that it becomes interposed among the fatty acid chains within the bilayer, thereby affecting the physical characteristics of the membrane. Alteration of the hydrocarbon region of the membrane can explain the reduction in mobility of fluorescent and electron paramagnetic resonance (epr) probes which have been observed in cholesterol-containing membranes. Kroes *et al.* (1972) have observed that a spin-label derivative of stearic acid becomes immobilized in pig red cells which are altered by dietary cholesterol intake. Similar ordering of fluid hydrocarbon chains by cholesterol was found in artificial membranes using fluorescent (Cogan *et al.*, 1973) and epr probes (Hubbell and McConnell, 1971). Furthermore, nmr spectroscopy indicates that cholesterol restricts the motion of fluid phospholipid hydrocarbon chains (Darke *et al.*, 1971; Oldfield and Chapman, 1972).

In this report, we describe the use of a fluorescent probe, 12-(9-anthroyl)stearic acid, to detect the effect of cholesterol in altering the structure of phospholipid dispersions and red blood cell membranes. The fluorescent anisotropy of the stearic acid is examined in red cells in which cholesterol content had been altered *in vivo* as a result of disease (spur cell anemia) or *in vitro* by incorporating cholesterol into the membrane. Phospholipids in aqueous dispersions are known to form a molecular bilayer, and this system is used as a model for the biological membrane. 12-(9-Anthroyl)stearic acid was chosen as the fluorescent probe for two reasons. First, X-ray analysis (Lesslauer *et al.*, 1972) and spectroscopic studies (Badley *et al.*, 1973) indicate that the anthroyl moiety of 12-(9-anthroyl)stearic acid is located in the hydrocarbon region of the membrane. Since the ring region of cholesterol extends in the membrane to about 11 Å from the surface (Rothman and Engelman, 1972), it can be expected that the presence of cholesterol will affect the motion of the fluorescent probe. Second, the fluorescent lifetime of 12-(9-anthroyl)stearic acid is long (Waggoner and Stryer, 1970); consequently, its fluorescence emission properties can be affected by motion occuring during the excited-state lifetime.

Materials and Methods

Cholesterol and L-α-dipalmitoyllecithin were obtained from Sigma Chemical Co. (St. Louis, Mo.) and General Biochemicals (Chagrin Falls, Ohio), respectively. 12-(9-Anthroyl)-stearic acid was the kind gift of Drs. A. Waggoner and L. Stryer (Yale University). It appeared as one spot under ultraviolet (uv) irradiation after thin-layer chromatography using ethanol–H_2O (95:5) as solvent.

Preparation of Membranes. LIPOSOMES were prepared by sonifying 40 mg of L-α-lecithin with either 0, 23, 40, or 80 mg of cholesterol in 10 ml of 0.154 M NaCl in a fluted metal container surrounded by an ice slurry. A Branson sonifier was used at 70 W for 1 hr with a standard tip. For studies with red cells, 0.4 part of normal human serum, previously heated to 56° for 30 min, was added to the liposomes, and the liposome–serum mixture was centrifuged at 21,800*g* for 30 min to sediment undispersed lipid. Except in dispersions containing

† From the Johnson Research Foundation, Department of Biophysics and Physical Biochemistry and the Department of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19174. *Received November 16, 1973.* This work was supported by U. S. Public Health Service Grants GM12202, AM15441, and AM05228, an U. S. Public Health Service Postdoctoral Fellowship (GM55116) to J. V. and by a U. S. Public Health Service Research Career Development award (AM38345) to R. A. C.

80 mg of cholesterol, only trace amounts of lipid were sedimented. For studies of liposomes, the dispersed lipid without added serum was centrifuged at 2800*g* for 30 min and at 108,000*g* for 30 min. The supernatants were assayed for cholesterol (Zlatkis *et al.*, 1953) and phospholipid (Bartlett, 1959).

For direct study, liposomes were diluted with 0.154 M NaCl to give a solution containing 0.4 mg of phospholipid/ml. 12-(9-Anthroyl)stearic acid from a stock solution in methanol was added to a final concentration of 13 μM (Figures 1–3 and 5–6) or 40 μM (Figures 7 and 8 and Table III) and the mixture containing this higher 12-(9-anthroyl)stearic acid concentration was sonified for 3 min at 40 W. The same anisotropy values were obtained for both concentrations of dye, suggesting that energy transfer among the dye molecules did not occur.

Studies of Red Cells. Red cells were collected in heparin, washed three times with Hank's balanced salt solution to remove the buffy coat, and resuspended at a cell concentration of 10% v/v in Hank's solution to which was added penicillin (1000 U/ml). One volume of a fresh cell suspension was mixed with one volume of either serum, previously heated to 56° for 30 min, or of a serum–liposome mixture and incubated in stoppered vessels at 37° in a shaker bath for 20 hr. No change occurred in the lipid content or appearance of normal red cells incubated in normal serum under these conditions. The cells were sedimented at 1000*g*, washed once with 0.154 M NaCl, and either extracted for measurement of lipid content or lysed for preparation of ghosts (Dodge *et al.*, 1963). Cell lipids were extracted with 160 volumes of isopropyl alcohol and chloroform (Rose and Oklander, 1965) for measurement of cholesterol and lipid phosphorus. Ghost membranes were suspended in 0.154 M NaCl at a concentration of 0.2–0.4 mg of ghost protein/ml and were labeled with 12-(9-anthroyl)-stearic acid by incubating the membranes for 2 hr at room temperature, at a final concentration of 10–30 μM 12-(9-anthroyl)stearic acid. Measurements were made within 24 hr of ghost preparation. The two patients with spur cells whose cells and serum were studied had advanced cirrhosis of the alcoholic and were in all ways similar to the group of patients previously reported (Cooper *et al.*, 1972).

In order to ascertain that the 12-(9-anthroyl)stearic acid was incorporated into the membrane, labeled membranes were centrifuged at 15,000*g* for 30 min. No 12-(9-anthroyl)stearic acid was found in the supernatant, either by measurement of absorbance or fluorescence.

Fluorescent Measurement. Steady-state fluorescence was measured at 90° relative to the exciting beam, using an Hitachi MPF-2A fluorescence spectrometer. The sample compartment was maintained at constant temperature with the use of circulating water through the cell block. For anisotropy measurements, a Glan-Thompson prism or a Polacoat 4B polarizer was used to polarize the exciting beam, and the emitting beam was polarized with a Polacoat 4B polarizer. Fluorescence anisotropy, r, is defined as

$$r = (I_{||} - I_{\perp}G)/(I_{||} + 2I_{\perp}G) \quad (1)$$

where $I_{||}$ and $I_{\perp}$ are the fluorescence intensities observed with the analyzing polarizer parallel and perpendicular to the polarized excitation beam. A Correction factor, G, equal to $I_{||}'/I_{\perp}'$, the primes indicating excitation polarized in a perpendicular direction, was used to correct for the unequal transmission of differently polarized light.

For a spherical molecule in an isotropic medium, the Perrin relationship (Perrin, 1934) holds for conditions of constant illumination

$$1/r = (1/r_0)[1 + (RT\tau/\bar{\eta}V_0)] \quad (2)$$

which may rearranged to

$$\bar{\eta}V_0 = RT\tau/[(r_0/r) - 1] \quad (3)$$

where r_0 is anisotropy in the absence of probe motion (taken to be 0.275), V_0 is molecular volume, τ is the fluorescence lifetime, R is the universal gas constant, T is the absolute temperature, and $\bar{\eta}$ is the viscosity of the medium. The value, r_0 is less than the theoretical 0.4, since the angle between the absorption and emission vectors is approximately 30° (Badley *et al.*, 1973). The rotational correlation time, ϕ, is given by:

$$\phi = \bar{\eta}V_0/kT \text{ sec} = 1/6D \quad (4)$$

where k is the Boltzmann constant and D is the rotational diffusion coefficient (Ygeurabide *et al.*, 1970). For an isotropic fluid, the change of $\bar{\eta}$ with temperature follows the exponential form

$$V_0\bar{\eta} = Ae^{\Delta E/RT} \quad (5)$$

where ΔE is the energy of activation for viscosity (Shinitzky *et al.*, 1971). ΔE was calculated from the slope of a plot (log η *vs.* $1/T$) fit by the least-squares method.

Fluorescence lifetimes were measured on an Ortec photon-counting fluorescent lifetime instrument equipped with an air spark-gap type flash lamp. A Corning glass filter with 350-nm band pass was used for excitation and an interference filter with 450-nm band pass was used for emission. The decay of fluorescence was defined by 265 data points, which were transferred from the multichannel analyzer *via* cassette magnetic tape to a PDP-10 computer for analysis. Fluorescence lifetimes (τ) were calculated by fitting a function of the form

$$F(t) = \sum_{i=1}^{n} a_i e^{-t/\tau_i} \quad (6)$$

where $n = 1$ or 2, t is the time, and $F(t)$ is the fluorescence intensity. This function is related to the observed intensities as follows

$$o(t) = \int_0^{\infty} F(t)L(\lambda - t)\mathrm{d}t \quad (7)$$

where $o(t)$ are the observed intensities and $L(t)$ are intensities of the scattered light from the lamp (Ygeurabide *et al.*, 1970). The data could usually be fit by a single exponential; however, a few samples showed a short-lived component which could be attributed to a light-scattering artifact. In these cases, two exponentials were tried (one of which had the same decay as the lamp), and were found sufficient to fit the data. The data were plotted on a Tetronix or Calcomp plotter.

For measurement of the decay of fluorescence anisotropy, a Glan-Thompson prism was used to polarize the exciting light and a Polacoat 4B polarizer was used in positions parallel and perpendicular to the exciting polarized light. The data were transferred as described above to a PDP-10 computer and the anisotropy of fluorescence was calculated according to eq 1. The fluorescence anisotropy as a function of time was fitted to an equation of the form of eq 6, and the rate constant was calculated (Tao, 1969; Ygeurabide *et al.*, 1970). To obtain statistically significant results, it was necessary to collect data for periods up to 2000 sec, during which time fluctuations in lamp intensity often occurred. To correct for this error and possible inability to detect parallel and perpendicular polar-

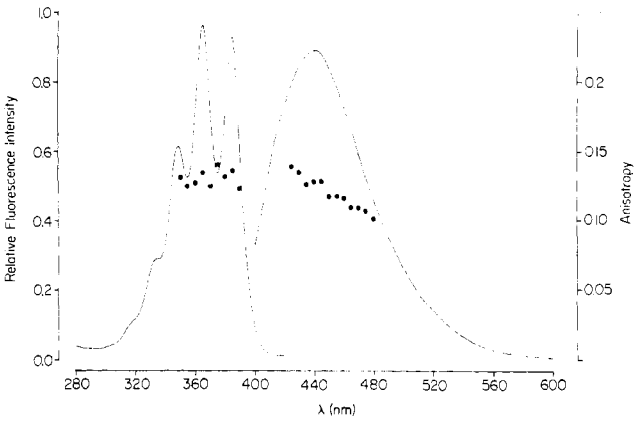

FIGURE 1: Excitation and emission spectra of 12-(9-anthroyl)stearic acid in dipalmitoyllecithin vesicles. A sonicated dispersion of dipalmitoyllecithin (0.56 mM) in 0.154 M NaCl was incubated with 13 μM 12-(9-anthroyl)stearic acid for 1 hr at 25°. On the left is the excitation spectrum (—) and corrected anisotropies (●), using 440 nm for emission, and on the right are anisotropies and emission spectra using 385 nm as excitation wavelength.

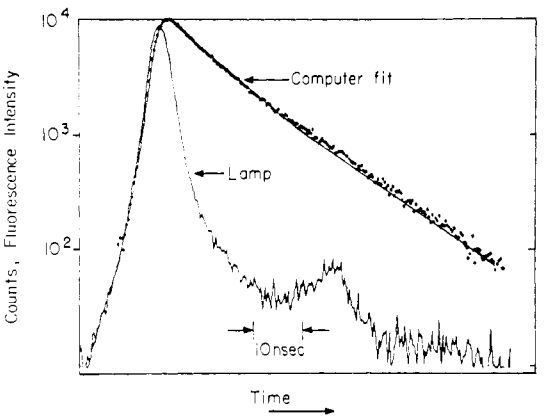

FIGURE 2: Fluorescence decay of 12-(9-anthroyl)stearic acid in red cell membrane. The membranes (0.22 mg of protein/ml) were incubated with 13 μM 12-(9-anthroyl)stearic acid in 0.154 M NaCl for 1 hr. The lamp flash is indicated in the figure and the symbols refer to experimental points. The line is computer drawn, assuming one exponential decay of 12.1 nsec and a small scatter component, and convoluted for lamp contribution. Temperature was 37°. The cholesterol to phospholipid mole ratio of red cell membranes was increased to 2.3 by incubation with cholesterol-rich liposomes.

ized light with equal facility, the light intensity measured from 60 to 64 nsec after the flash was integrated for each measurement. The ratio of this measurement in parallel and perpendicular positions was used in the computer analysis to standardize the two decay curves.

Results

Characteristics of 12-(9-Anthroyl)stearic Acid Fluorescence in Liposomes and Red Blood Cell Membranes. The excitation and emission spectra of 12-(9-anthroyl)stearic acid in L-α-dipalmitoyllecithin vesicles are shown in Figure 1. Identical spectra are obtained in mixed lecithin–cholesterol dispersions and in red blood cell ghosts to which 12-(9-anthroyl)stearic acid has been added (not shown). As noted in the figure, the fluorescence is positively polarized and nearly independent of wavelength when excited in the last absorption band.

The fluorescence of 12-(9-anthroyl)stearic acid incorporated into red blood cell membranes decays as a single exponential with respect to time (Figure 2). The fluorescent lifetime (τ) depends upon temperature and concentration of cholesterol in the membranes. As shown in Table I, with increasing temperature, a slight increase in the rate of decay is observed in both liposomes and red cell membranes of various lipid composition. Furthermore, there is a slight decrease in fluorescent lifetime in the presence of increasing amounts of cholesterol, which corresponds to a small decrease in fluorescence intensity. Since 12-(9-anthroyl)stearic acid behaves identically in the artificial membranes and in red blood cell ghosts, it can be concluded that the chromophore is located in an environment which is similar in the artificial and natural membranes. The identical excitation and emission spectra, as well as negligible difference in lifetimes and quantum yields of 12-(9-anthroyl)-stearic acid fluorescence in the membranes with or without cholesterol, effectively excludes complex formation between 12-(9-anthroyl)stearic acid and cholesterol.

Steady-State Anisotropy of 12-(9-Anthroyl)stearic Acid in Membranes of Varying Cholesterol Content. The temperature dependence of 12-(9-anthroyl)stearic acid fluorescence anisotropy in dipalmitoyllecithin liposomes containing variable amounts of cholesterol is shown in Figure 3. As predicted by eq 2, anisotropy increases with decrease in temperature. However, for pure lecithin vesicles, the anisotropy of 12(9-anthroyl)stearic acid fluorescence is a discontinuous function of temperature with a "break" at 35°, the temperature at which lecithin undergoes a phase transition. In contrast, in cholesterol-containing membranes, anisotropy increases continuously with decreasing temperature.

The fluorescence anisotropy of 12-(9-anthroyl)stearic acid was also measured in normal red blood cell membranes and in the membranes which were enriched in their original cholesterol content (either by incubation, or using so-called "spur cells;" for details, see Methods). The 12-(9-anthroyl)stearic acid anisotropy in altered membranes relative to the anisotropy found in normal cells is plotted *vs.* the cholesterol-to-phospholipid ratio (Figure 4). In spite of scatter in the data, the anisotropy of 12-(9-anthroyl)stearic acid fluorescence increases as cholesterol content is increased, indicating that the presence of cholesterol in the red cell ghosts imposes constraint on the rotational motion of 12-(9-anthroyl)stearic acid. As in the case of artificial membranes, anisotropy of 12-(9-anthroyl)stearic acid fluorescence increases at lower temperatures (Figure 5).

The viscosity, $\bar{\eta}$, of the probe environment is a function of

TABLE I: Fluorescent Lifetimes (nsec) of 12-(9-Anthroyl)stearic Acid in Membranes.[a]

Temp (°C)	Cholesterol:Phospholipid Ratio					
	Liposome				Erythrocyte	
	0	1.0	1.9	2.4	1.0	2.3
15	13.4	12.9	12.7	12.3		
25	12.6	12.2	12.7	12.2	12.7	12.6
30	12.6	12.6	12.3	12.1		
37	12.1	12.5	12.1	11.5	12.7	12.1
43	12.0	12.0	11.8	11.5		
46					10.6	12.0
48	12.1	11.6	11.7	11.5		

[a] Conditions are given in Figures 3 and 4. Fluorescent lifetimes were measured as described in Materials and Methods.

TABLE II: Energies of Activation (ΔE), Rotational Relaxation Times (ϕ), and Diffusion Constants (D) for 12-(9-Anthroyl)stearic Acid in Liposomes and Red Cell Membranes of Varying Cholesterol: Phospholipid Composition.[a]

Membrane	ΔE (cal/°K mol)	ϕ (sec $\times 10^9$) 25°	D ($sec^{-1} \times 10^7$) 25°	ϕ (sec $\times 10^9$) 37°	D ($sec^{-1} \times 10^7$) 37°	ϕ (sec $\times 10^9$) 46°	D ($sec^{-1} \times 10^7$) 46°
Liposomes							
Cholesterol–phospholipid mole ratio							
0	4.24[b] 9.79[c]	13.3	1.26	6.29	2.65	2.95	5.65
1.0	6.11	16.7	0.998	10.1	1.64	7.12	2.34
1.9	4.55	17.2	0.971	11.5	1.45	8.66	1.92
2.4	4.72	14.7	1.13	11.8	1.41	8.00	2.08
Red cells							
1.0	5.96	11.7	1.42	7.81	2.13	5.77	2.89
2.3	3.41	12.1	1.38	8.50	1.96	6.99	2.38

[a] All conditions were the same as in Figures 3 and 4. [b] Below 33°. [c] 33–45°.

temperature, and is calculated knowing probe lifetime and anisotropy (eq 3). Since V_0, the molecular volume is a constant, the energy of activation for viscosity change is related to temperature according to the classical Arrhenius equation (eq 5). Using the information presented in Table I and Figures 3 and 5, Arrhenius plots are presented (Figure 6). Straight-line relationships are observed in all membranes, except in pure dipalmitoyllecithin vesicles in which the break in the curve coincides with the temperature for the lipid phase transition (Figure 6).

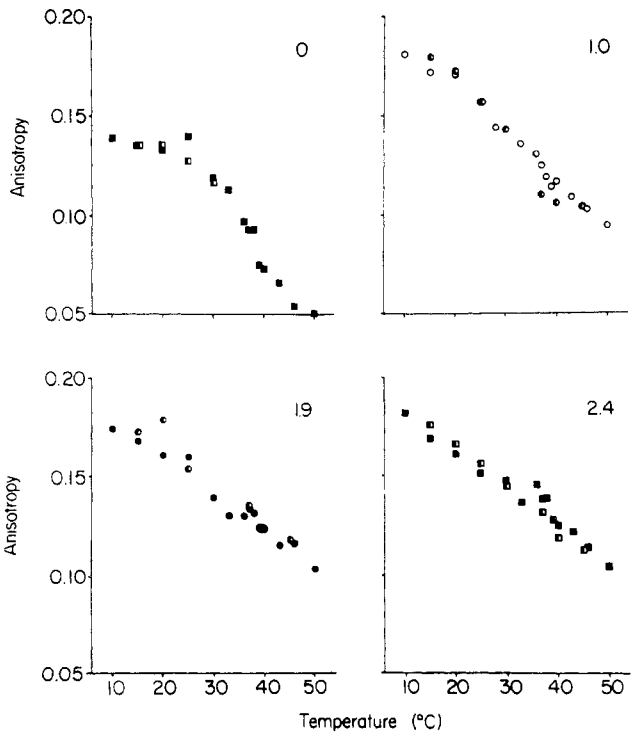

FIGURE 3: Temperature dependence of steady-state fluorescence anisotropy for 12-(9-anthroyl)stearic acid in liposomes of varying cholesterol content. Lecithin (0.56 mM) was sonicated in 0.154 M NaCl with 10 μM 12-(9-anthroyl)stearic acid and cholesterol, to give molar ratios indicated on the upper right-hand corner of each figure. Open and closed symbols refer to ascending temperature and half-solid symbols refer to descending temperature; excitation, 385 nm; emission, 440 nm.

By substituting the calculated $\bar{\eta}V_0$ to eq 4, we are able to obtain the rotational correlation time, ϕ, and the rotational diffusion constant, D, for the anthroyl moiety of the 12-(9-anthroyl)stearic acid molecule. All these calculated data are presented in Table II. A critical survey of this table allows the following conclusions. (1) In both the liposomes and the red cell membranes, the energies of activation for viscosity and of 12-(9-anthroyl)stearic acid rotational correlation time are low, ranging from 3 to 9 kcal, characteristic of diffusion-limited reactions. The presence of cholesterol further decreases the energy of activation. (2) The rotational correlation time increases with increasing cholesterol content in both liposomes and red cell membranes. (3) The high energy of activation at the lecithin phase transition (34–45°) reflects the continually changing rotational constraint on 12-(9-anthroyl)stearic acid during the phase transition and is thus related to the energy of activation for the melting process.

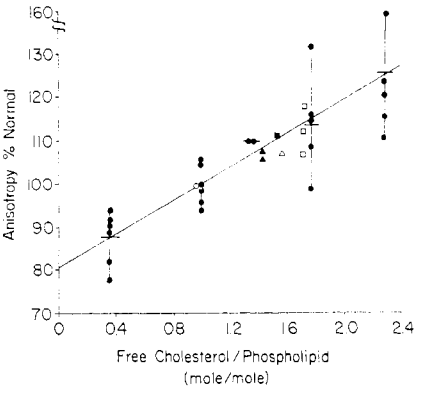

FIGURE 4: Effect of the free cholesterol:phospholipid mole ratios of red cell membranes on the fluorescence anisotropy of 12-(9-anthroyl)stearic acid. The anisotropy of 12-(9-anthroyl)stearic acid was measured in ghosts prepared from normal red cells (○), red cells from two patients with spur cells (□,△), and normal red cells incubated with liposomes of varying cholesterol:phospholipid composition (●) or serum from patients with spur cells (■,▲). Data are expressed as per cent anisotropy relative to that of normal red cells, either unincubated or incubated in normal serum. A line calculated by the least-squares method has been constructed from the mean values for anisotropy of 12-(9-anthroyl)stearic acid in red cells incubated with liposomes. Each experimental point is an average of four determinations from a single ghost preparation.

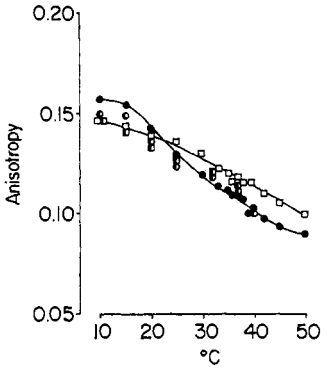

FIGURE 5: Temperature dependence of 12-(9-anthroyl)stearic acid fluorescence anisotropy in red cell membranes. Solid symbols represent ascending temperatures and half-solid figures represent descending temperatures. Normal red cells, cholesterol:phospholipid = 1.0 (○). Cholesterol-rich red cells, cholesterol:phospholipid = 2.3 (□), as in Figure 2.

Decay of Fluorescence Polarization Anisotropy. Nonrandom motion of the fluorescent chromophore can be expected on the basis of the structure of 12-(9-anthroyl)stearic acid and the likelihood that the environment in the membrane where it is located is of an anisotropic nature. Thus, the correlation time measured by the steady-state method represents a mean of several motions. Resolution of these motions is possible by resolving the decay of fluorescence anisotropy following a nanosecond light pulse (Jablonski, 1961).

The decay of polarized light in parallel and perpendicular directions relative to a polarized exciting beam studied over a 60-nsec time interval following a nanosecond light pulse, is presented in Figure 7. Approximately 15 nsec after the flash, light in these two directions becomes identical in intensity, indicating that, after this time interval, the molecule assumes

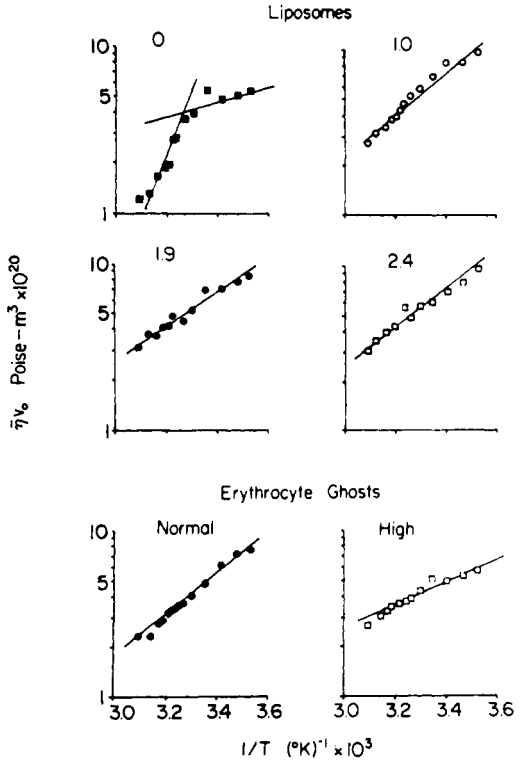

FIGURE 6: Dependence of $\bar{\eta}V_0$ with reciprocal temperature. Conditions are the same as in Figure 3 (liposomes) and Figure 2 (red cell membranes).

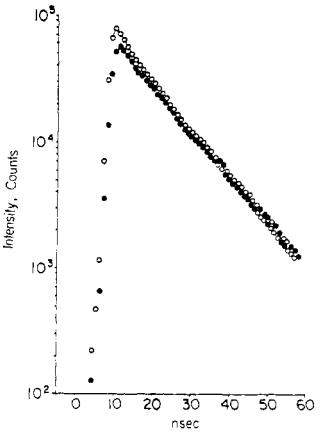

FIGURE 7: Decay of 12-(9-anthroyl)stearic acid fluorescence in liposomes parallel and perpendicular to polarized excitation beam. The decay of 12-(9-anthroyl)stearic acid fluorescence in liposomes with a cholesterol:lecithin molar ratio of 1.0 and 40 μM 12-(9-anthroyl)stearic acid in 1.54 M NaCl was measured in the parallel (○) and perpendicular (●) positions, relative to perpendicular polarized excitation.

random orientation. In most experiments, the data fit a single exponential, as shown in Figure 8. In agreement with the steady-state measurements, the anisotropy decay constants increase with increasing temperature and decrease with an increase in cholesterol content (Table III).

Although most of the anisotropy data could fit a single exponential decay, several lines of evidence suggest the existence of a fast component (or components) of the decay. First extrapolation of the decay curves to the time of the flash yields an intercept which is less than the value expected for limiting polarization. Second, in some experiments, a fast component of decay was directly observed. For example, at 37° in cholesterol–lecithin liposomes, the fast component of the decay was approximately ten times faster than the slow one. Thirdly, the steady-state measurements of 12(9-anthroyl)-stearic acid anisotropy give consistently higher values than those determined in Figure 8. This would also suggest the existence of more than one component which contributes to the anisotropy values. Since the steady-state anisotropy values and the decay of anisotropy are both qualitatively affected in

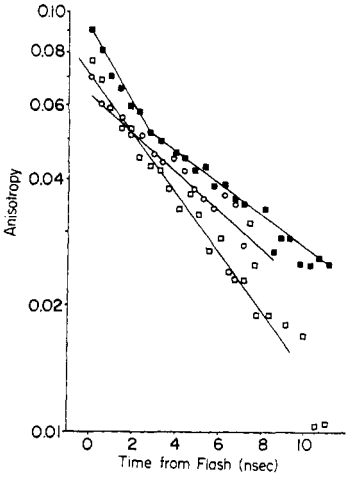

FIGURE 8: Decay of 12-(9-anthroyl)stearic acid anisotropy in liposomes as a function of time after excitation flash. The anisotropy of 12-(9-anthroyl)stearic acid fluorescence in liposomes with a cholesterol:lecithin molar ratio of 2.1 and 40 M 12-(9-anthroyl)stearic acid is shown as a function of time after vertically polarized excitation flash: (○) 25°; (■) 37°; (□) 46°.

TABLE III: Anisotropy Decay Rate Constants ($nsec^{-1}$).

Membrane	Cholesterol: Phospholipid	Temperature 25°	37°	46°
Liposome	0	0.122	0.139	0.269
	1.0	0.120	0.142	*a*
	1.9	0.128	*a*	0.148
	2.4	0.104	*a*	0.158
Erythrocytes	1.0	0.085	0.135	0.489
	2.3	0.046	0.080	0.124

[a] The data were not sufficiently accurate, or showed evidence of more than one decay. See text.

the same way by changes in temperature and cholesterol content, it is likely that both the fast and slow decay rates are increased in the presence of cholesterol.

Discussion

Measurement of fluorescence anisotropy of 12-(9-anthroyl)-stearic acid provides a simple, albeit indirect, method of detecting changes in membrane structure induced by the presence of a disproportionate excess of cholesterol. As shown by our experimental results, the fluorescence anisotropy of 12-(9-anthroyl)stearic acid in membranes decays within the time span of its excited state, a result which was predicted by Ygeurabide and Stryer (1971) on the basis of steady-state anisotropy parameters of 12-(9-anthroyl)stearic acid fluorescence in oriented cholesterol model membranes. Using time-resolved anisotropy measurements, it can be demonstrated that the rotational motion of 12-(9-anthroyl)stearic acid consists of more than one motion, including a fast phase which was not possible to resolve and a slower phase with $k = 0.135 \times 10^{-9}$ sec^{-1} in normal red blood cells at 37°.

The biphasicity of the decay of fluorescence anisotropy may be related to different rotational rates around the long axis of fatty acid chain and around the ester linkage. Although in most experiments it was impossible to accurately resolve these motions, it is unlikely that the presence of cholesterol would identically affect these motions. Indeed, Cogan *et al.* (1973) in a study of perylene in egg lecithin dispersions, find that an increase in out-of-plane rotations and a decrease of in-plane rotations occurs with increasing amounts of cholesterol in the artificial membranes.

Because of the difficulty in assessing the effective volume, V_0, of 12-(9-anthroyl)stearic acid, we have converted anisotropy values into rotational correlation times and have not calculated "viscosity of 12-(9-anthroyl)stearic acid environment." However, comparison of 12-(9-anthroyl)stearic acid anisotropy in mineral oil of known viscosity with the anisotropy found in normal red cell membrane at 37° yields an anisotropy value corresponding to a viscosity of about 1.0 P. This compares favorably with 1.3 P found under similar conditions using perylene as a fluorescent probe (Rudy and Gitler, 1972). Using the latter value for viscosity, the rotational correlation time for membrane-bound proteins can be calculated from eq 4. Assuming a sphere of 30-Å radius (reasonable size for a protein), a value of 10^{-5} sec for a rotational correlation time is obtained. In contrast, the turnover rate of the membrane-bound Na^+K^+ ATPase, the monovalent cation-transporting enzyme in erythrocyte membranes, is much slower than this—about 1 sec^{-1} (Post *et al.*, 1972). Although there are many uncertainties in this calculation, the rotational relaxation of the ATPase is at least fast enough to allow the ATPase to be a mobile carrier.

The sensitivity of 12-(9-anthroyl)stearic acid anisotropy to the fluidity of the hydrocarbon region of the membrane is illustrated by the discontinuity in its fluorescence anisotropy at the melting point of dipalmitoyllecithin. In mixed dipalmitoyllecithin–cholesterol dispersions, no discontinuity is observed in the temperature profile of 12-(9-anthroyl)stearic acid fluorescence polarization anisotropy, a result which is consistent with independent findings using differential scanning calorimetry (Phillips *et al.*, 1969). The modifying effect of cholesterol on 12-(9-anthroyl)stearic acid fluorescence anisotropy raises a question as to whether cholesterol and 12-(9-anthroyl)stearic acid are evenly distributed in the hydrocarbon matrix. Although our results do not exclude the possibility that there are patches of pure cholesterol, the additive effect of cholesterol in increasing 12-(9-anthroyl)stearic acid anisotropy tends to argue against the "lateral phase separation" such as been hypothesized to occur in some membranes (Shimshick and McConnell, 1973).

The effect of cholesterol in decreasing the anisotropy decay rates indicates rotational constraint imposed on 12-(9-anthroyl)stearic acid by cholesterol. Increased rotational constraint in the hydrocarbon region of the membrane may be related to the "condensing" effect of cholesterol observed with mixed monolayers of cholesterol and lecithin (Shah and Schulman, 1967). Cholesterol has been found to inhibit the release of K and Cl from lecithin liposomes (Papahadjopoulos and Watkins, 1967) and to reduce the rate of calcium loss from dioleyllecithin vesicles (Vanderkooi and Martonosi, 1971). The inhibitory effect of cholesterol on fusion of mammalian cells may also be related to the membrane fluidity (Papahadjapoulos *et al.*, 1973). Ladbrook *et al.* (1968) have suggested that cholesterol acts to control the fluidity of hydrocarbon chains by not only reducing their mobility in the fluid state, but also by introducing disorder among hydrocarbon chains in the gel state, thereby producing an "intermediate fluid" condition. The decreased effect of temperature on anisotropy which was observed in cholesterol-rich membranes in our studies is consistent with this view.

The importance of the modifying effect of cholesterol on the fluidity of the membrane is suggested by the observation that, in spite of great variety in composition of the polar lipid in red cell membranes among mammals, the ratio of cholesterol to total polar lipid varies little (Rouser *et al.*, 1968). Similarly, although the concentration of plasma lipid varies widely among individuals, the amount of unesterified cholesterol relative to phospholipid in plasma lipoproteins is far more constant (Cooper *et al.*, 1972). Furthermore, the mature red cell is not capable of synthesizing lipid *de novo*, although it can accomplish chain lengthening of fatty acids (Pittman and Martin, 1966). Therefore, the composition of the red cell membrane is dependent on the maintenance of a constant sterol–polar lipid ratio in the lipoproteins, since deviation from constancy is reflected by a similar deviation in plasma membrane (Cooper, 1969). In severe hepatocellular disease, common to cirrhosis of the alcoholic, the sterol–polar lipid ratio in the red cell membrane is disrupted and the cell becomes spiculated in appearance. The effect of high cholesterol ratios in altering the membrane fluidity may explain why the "spur" cell of patients have membranes which have a fold contour and are poorly deformable, resulting in a shortened life span (Cooper, 1969; Smith *et al.*, 1964).

Acknowledgments

The authors thank Drs. Martin Pring and Wolfgang Nadler for aid in computer programming and instrumentation. In addition, the advice of Dr. L. Brand (Johns Hopkins University) is gratefully acknowledged.

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