Evaluation of the thermal coefficient of the resistance to fluorophore rotation in model membranes

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ABSTRACT The thermal coefficient of the frictional resistance to fluorophore rotation (b), a parameter related to the change in the local viscosity with temperature, was determined for anthroyloxy-fatty acid probes in micelles and dimyristoyl lecithin (DMPC) and dioleoyl lecithin (DOPC) unilamellar and multilamellar vesicles. The value of b and the percent change in anisotropy with temperature (%dA/dT) remained constant with membrane depth and only depended on composition. These parameters were also the same when either in-plane, or in-plane and out-of-plane fluorophore motions were observed. This result indicates that the membranes expand isotropically. The magnitude of b was found to be primarily dependent on the packing of the hydrocarbon chains with higher b values relating to more closely-packed chains. b was responsive to the gel to liquid crystal phase transition of DMPC and the bilayer to hexagonal phase transition of egg-phosphatidylethanolamine. When the enthalpy values for the fluorophore transfer from one phase to another are calculated, the values are larger than those measured by calorimetry and reflect a discrepancy between the microscopic enthalpy experienced by the fluorophore due to a change in environment versus the macroscopic enthalpy of the system as a whole.

INTRODUCTION

The study of membrane fluidity has received a great deal of attention because fluidity or inverse viscosity is thought to control the activity and function of membrane proteins. A prominent technique in the study of membrane viscosity is fluorescence anisotropy (A). This parameter is related to the viscosity surrounding a spherical fluorophore (η), or to the resistance to fluorophore rotation by its environment, through the Perrin Eq. 1:

$$\langle Ao\rangle/\langle A\rangle - 1 = RT * \langle\tau\rangle/V\eta, \quad (1)$$

where Ao is the anisotropy in the absence of rotational motion, R is the gas constant, T the absolute temperature, τ the fluorescence lifetime, and V the volume of the rotating sphere. Comparison of the anisotropy to its limiting value (Ao), yields the average cosine square of the rotational angle, σ, the fluorophore undergoes during its lifetime:

$$\langle\cos^2\sigma\rangle = [1 + 2\langle A\rangle/\langle Ao\rangle]/3.$$

Membrane fluidity is very anisotropic with different viscosities in each plane or axis of symmetry. By measuring the anisotropy decay of fluorescent fatty acid probes in membranes, Tilley et al. (1979) found that the membrane viscosity decreases systematically towards the center of the bilayer. If fluorophore rotations are intrinsically anisotropic or in an anisotropic environment (i.e., a membrane), its motion can no longer be described by a single rotational rate and the viscosity derived from the Perrin equation is not equal to the true macroscopic viscosity. Membrane fluidity can then be characterized using a probe that occupies a single membrane position with time-resolved anisotropy (e.g., Vincent et al., 1982; Kutchai et al., 1983) that can resolve the rotational motion in the x-y and y-z plane of the membrane.

To distinguish between the viscosity derived from spherical fluorophore rotations in an isotropic environment to those in an anisotropic lipid environment, the term "microviscosity" or simply the observed anisotropy assuming an isotropic system is commonly used. This parameter has been commonly employed to empirically characterize membrane fluidity (Shinitzky and Barenholz, 1978) and the values obtained are often related to the viscosities of bulk solvents. In general, the microviscosity is a useful operational term to characterize a membrane. However, this technique is highly dependent on the probe and the location of its emission dipole with respect to its rotating axis, the membrane, and the position it occupies in the particular membrane therefore making quantitative comparisons between different systems difficult. Furthermore, the most commonly used microviscosity probe, diphenylhexatriene or DPH can occupy different positions in the membrane thus complicating detailed analysis (e.g., Lentz et al., 1976; Straume and Litman, 1987).

Here, we will attempt to characterize membrane fluid-

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ity in terms that are independent of probe rotation, position, or orientation in the membrane. The parameter to be used is the thermal coefficient of the frictional resistance to fluorophore rotation (b) or the thermal coefficient of the viscosity derived from Eq. 1 previously determined for isotropic solvents and proteins (Weber et al., 1984; Scarlata et al., 1984; and Rholam et al., 1984). The thermal coefficient of the viscosity is based on an empirical expansion that relates the viscosity at any temperature to the viscosity at an arbitrary temperature (ηo, To):

$$\eta = \eta o * \exp [b(To - T)]. \quad (2)$$

For convenience, we let $To = 273$ K so that the expression $(T - To)$ becomes the temperature in degrees centigrade (t). Eq. 2 can be incorporated into the Perrin equation and the natural logarithm taken to yield (3):

$$Y = \ln (\langle Ao \rangle / \langle A \rangle - 1) - \ln (RT * \langle \tau \rangle / V)$$
$$= N + b * t, \quad (3)$$

where N is equal to $-\ln \eta o$. Y is then the negative logarithm of the viscosity as seen by the fluorophore. By measuring Y as a function of temperature, the thermal coefficient of the viscosity (b) is obtained. Weber et al. (1984) initially determined b for different fluorophores in isotropic solvents and found that it depends only on the solvent surrounding the fluorophore and is independent of the type of rotational motion the fluorophore undergoes. This point was further exemplified by the observation that b as measured by fluorescence spectroscopy is identical to the macroscopic value from flow measurements.

When this analysis is applied to native globular proteins under viscous conditions (80% glycerol-buffer, -40 to 20°C) such that all large backbone motions of the protein are dampened, at least two values of b are obtained (Scarlata et al., 1984 and Rholam et al., 1984). The low temperature b is always equal to that of the external solvent. The high temperature value(s) of b and the temperature at which the transition from the first occurs (Tc) is distinctive of the individual protein. Tc is found to be related to the rotational amplitude where the tyrosine and/or tryptophans begin to couple with their neighboring amino acid environment. The magnitude of the protein-determined b value(s) appears to be related to the flexibility of the fluorophore subdomain with more flexible environments giving lower values. Because the transition from one b to another is very sharp, each regimen may represent a distinct state of the fluorophore's environment and the enthalpy change in going from one state to the next can be calculated.

Recently, Chong and Thompson (1987) used this analysis to investigate the properties of the fluorescent cholesterol analogue, dehydroergosterol in phospholipid bilayers. By comparing its behavior to diphenylhexatriene, the authors found that the probe may undergo lateral phase separation similar to cholesterol.

Here, we will characterize the thermal coefficient of the viscosity of model membranes using several probes at different membrane depths to determine if highly anisotropic, closely-packed environments can be adequately described by this method and to determine the physical significance of the enthalpy changes that occur in going from one b value to the next. The use of model membranes may lead to a better understanding of the structural and chemical factors that contribute to the magnitude of b because their composition can be easily controlled, they are quasi-two dimensional fluids and their phase transition behavior has been well studied. By studying the nature of b, we can better relate protein b values to their structural and dynamic properties.

MATERIALS AND METHODS

Sample preparation

9-*cis*-dioleoyl lecithin (DOPC), dimyristoyl lecithin (DMPC), and egg phosphatidylethanolamine made from transphosphatidylation of egg lecithin (eggPE) were purchased from Avanti Polar Lipids, Inc., Birmingham, AL and used without further purification. *n*-octylglucoside was from Sigma Chemical, Co., St. Louis, MO. Fluorescent probes were purchased from Molecular Probes, Inc., Eugene, OR. Probe purity was assessed by thin-layer chromatography in chloroform and in chloroform-ethanol (80:20). Spectral-grade glycerol was from Aldrich Chemical Co., Inc., Milwaukee, WI.

Multilamellar vesicles (MLVs) were prepared by mixing probe and lipids in chloroform at a 0.5 mol% ratio and drying the sample under nitrogen and then under vacuum. Afterwards, the powder was suspended in 0.05M Hepes- 0.16M KCl, pH6, by extensive vortexing and left overnight to hydrate. Small unilamellar vesicles (ULVs) were prepared from multilamellar by sonicating with a W225 sonicator (Heat Systems-Ultrasonics, Inc., Farmingdale, NY) under nitrogen for 20 min and then spinning at 39,000 g for 30 min to remove multilamellar vesicles and titanium particles. Gramicidin was incorporated into vesicles as previously described (Scarlata, 1988). DOPC and egg-PE vesicles were stored at 5°C and DMPC vesicles at 37°C.

Measurements

Fluorescence emission spectra and anisotropies were measured on a Greg-PC (I.S.S., Champaign, IL). Background intensities never exceeded 1% of the sample signal. The anisotropy was measured at an exciting wavelength of 381 nm and an emitting wavelength of 460 nm except where indicated. Each anisotropy value recorded is the average of at least four measurements with a maximum error of 0.001. Phase-modulation lifetimes were measured on an SLM-4800 (SLM, Inc., Champaign, IL) using a modulation frequenty of 30 MHz. Average modulation values were used and heterogeneity analysis was not attempted. Temperatures were varied using a LT-50 (Neslab Instruments, Inc., Portsmouth, NH) circulating bath. At low temperatures the optical module was purged with nitrogen to prevent frosting. The limiting anisotropy of the probes were taken from the Perrin plots obtained by dissolving the probes in 80% glycerol-ethanol and varying

the temperature from −40 to 0°C. The probes 2-, 6-, and 12- (9)-anthroyloxystearic acid whose anthroyloxy labels are partway down the hydrocarbon chain all gave similar limiting anisotropies of 0.312 ± .001 while the probes whose labels are on the tail of the hydrocarbon chain, 11-(9)-anthroyloxy-undecanoic acid and 16-(9)-anthroyloxypalmitic acid, gave a value of 0.322 ± 0.001.

Calculations

Viscosity values for glycerol-water mixtures were from Miner and Dalton (1953). Y values were calculated from the anisotropies and lifetimes using Eq. 3 with a constant hydrodynamic volume of 150 ml/mol. This latter value is taken from the average of the limiting slope of the Perrin plots of the probes. We note that in this treatment the hydrodynamic volume of the probe is assumed to remain constant with temperature. Also, because we are primarily interested in the magnitude of the change in Y with temperature (b), knowledge of the precise hydrodynamic volume is not necessary. The value of b was determined by a least-square fit of the points comprising the slope of the Y versus temperature plot.

The calculation of the enthalpy change is a slight variation of Scarlata et al., (1984) and evaluates the enthalpy change that occurs during the transition from lipid phase 1 to 2 as revealed by the abrupt change in the temperature dependence of fluorophore rotation (b). We assume that the enthalpy and entropy difference between the two phases are temperature independent. The observed b value is a sum of the thermal coefficient of the pure lipid states ($b1$ and $b2$) times the relative fraction of fluorophores comprising these states ($f1$ and $f2$):

$$b(\text{observed}) = b1 * f1 + b2 * f2. \quad (4)$$

Where these fractions are determined by the equilibrium constant, K:

$$f1 = K/(K + 1) \text{ and } f2 = 1/(K + 1). \quad (5),$$

At the midpoint of the transition, Tm, $\Delta G = 0$. Therefore,

$$\Delta G = \Delta H(1 - T/Tm) \quad \text{since} \quad \Delta S = \Delta H/Tm \quad (6)$$

$$K = \exp[(-\Delta H(1 - T/Tm)/RT]. \quad (7).$$

Tm, $b1$, and $b2$ are measured experimentally. Therefore, we can input these values into the above equations and for a given enthalpy determine b at any temperature. By inserting b into Eqs. 1–3 the anisotropy and Y values at various temperatures can be calculated. The resulting Y-plot for a given enthalpy can then be compared with the experimental Y-plot, and enthalpy that gives the lowest variance can be determined.

RESULTS

Isotropic solvent studies

The behavior of the anisotropy and lifetime from −30° to 20°C of 9-methyl-anthracene (MA) and two fatty acid probes, 12-(9)-anthroyloxy-stearic acid (12 AS) and 11-(9)-anthroyloxy-undecanoic acid (11AU), were first characterized in 80% glycerol-20% ethanol. 12 AS has an anthroyloxy label partway down the hydrocarbon chain of fatty acid while 11 AS has an anthroyloxy label on the last carbon of the undecanoic hydrocarbon chain. The results for the two fatty acid probes are shown in Fig. 1. At low temperatures all three probes display the same

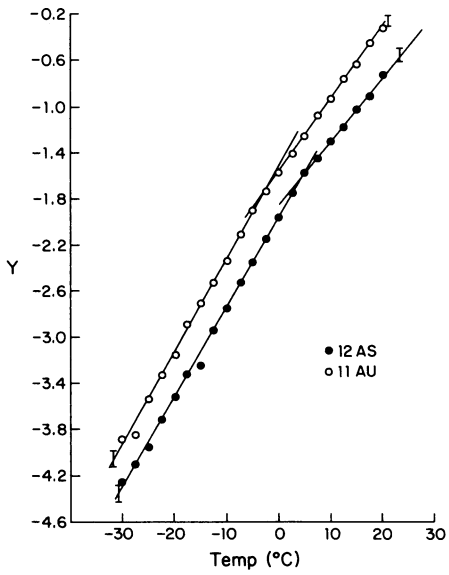

FIGURE 1 The Y versus temperature plot of 11 AU and 12 AS in 80% glycerol-20% ethanol.

slope, $b(1)$ which is close to the 8.0%°C^{-1} value obtained macroscopically by flow viscometry (Miner and Dalton, 1953). At higher temperatures the two fatty acid probes show a second reduced slope $b(2)$ (Table 1) while the slope of the free probe (MA) remains constant.

The b value of 12 AS in n-octyl-glucoside micelles was determined to be 3.4%°C^{-1} in the temperature range of 10° to 40°C.

DOPC vesicles

To determine whether b changes with membrane depth, the anisotropies and lifetimes of DOPC multilayers (MLVs) and small unilamellar vesicles (ULVs) labeled with anthroyloxy fatty acids (0.5 mol %) were measured from 5° to 40°C. The room temperature anisotropy and lifetime values closely matched those of Kutchai et al., (1983). The Y versus temperature plots are presented in Fig. 2. Because we are only interested in the changes of Y with temperature, no effort was made to accurately

TABLE 1 Y-Plot parameters of probes in 80% glycerol-20% ethanol

Probe	$b(1)$	$b(2)$	Tc
MA	8.2%	–	–
12 AS	7.8%	5.7%	5.0
11 AU	8.2%	6.3%	−2.5

b values are in °C^{-1} and have an error of +0.013°C^{-1}. Tc values are in °C and have an error of ±0.08°C.

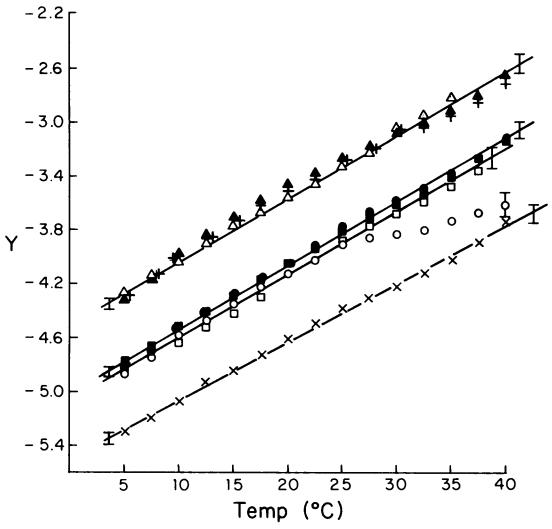

FIGURE 2 Y versus temperature plots of DOPC small unilamellar vesicles (ULVs) or multilamellar vesicles (MLVs) labeled with 0.5% probe where ● is for 2 AS-ULVs, ○ 2 AS-MLVs, × 6 AS-ULVs, ■ 12 AS-ULVs, □ 12 AS-MLVs, ▲ 11 AU-ULVs, △ 11 AU-MLVs and + 16 AP-ULVs.

determine its exact magnitude. We note that to accomplish this one must precisely determine the rotational volume as well as the contribution of in-plane and out-of-plane rotations to the anisotropy (Vincent et al. [1982]) due to the viscosity gradients that exist in the planes of the bilayer as well as with membrane depth. From Fig. 2 we find that almost all samples display a linear Y versus temperature plot in this temperature range. The exception was 2AS-DOPC MLVs which displayed significant curvature above 27°C. We interpret this behavior as being due to either probe aggregation or changes in its relative position in the membrane with temperature which would change the probe environment and thus the value of b.

From the data tabulated in Table 2 we find that although the anisotropy decreased steadily with membrane depth, the change in viscosity surrounding the fluorophore, as expressed by b, remained constant. Also, the relative change in anisotropy with temperature (dA/dT) = (A[initial]-A[final]/A[final]/dT) for all the probes remained constant with probe position. The data listed for multilayers display the same trend as the bilayers; however, these results are less reliable due to larger errors in the anisotropy as a result of sample turbidity. In all cases, the anisotropy returned its original value after cooling the sample from 40° to 5°C.

The data listed in Tables 1 and 2 were collected using an excitation wavelength of 381 nm. This wavelength allows one to observe in-plane and out-of-plane motions in equal proportions (Vincent et al., 1982). Exciting at 316 nm, on the other hand, produces emission that has been depolarized only by out-of-plane motion. Therefore, by comparing the b values at 381 and 316 nm, we should be able to determine whether b is the same in both planes of the membrane. The results are listed in Table 3. Because the anisotropies are so low exciting at this wavelength (Ao = 0.1), colder temperatures (−15° to 20°C) were used to obtain more significant anisotropy data. Glycerol was added to the buffer to prevent freezing to a final concentration of 55% wt/wt. The presence of 5–55% glycerol did not affect the values of the anisotropy or b of 6AS or 12AS labeled ULVs indicating that changes in the solvent viscosity are not transmitted into the membrane at these depths and that we are observing local motions such that vesicle tumbling does not contribute to the anisotropy. From the data in Table 3 we can conclude that the change in viscosity around the fluorophore as well as the relative change in anisotropy with temperature is constant in all planes of the membrane.

TABLE 2 b and %dA/dT values for anthroloxy probes in DOPC

Probe	ULVs		MLVs	
	b	$\%dA/dT$	b	$\%dA/dT$
2 AS	4.75	1.6	4.8	1.6
6 AS	4.81	1.4	–	–
12 AS	4.85	1.6	4.6	1.5
11 AU	4.61	1.7	4.8	1.3
16 AP	4.60	1.7	–	–

b values are in $\%°C^{-1}$ and have a 0.14% error for ULVs and a 0.23% error for MLVs. %dA/dT values are in the same units and have a 0.15 and 0.3 error in ULVs and MLVs, respectively.

DMPC Vesicles

The behavior of the anisotropy and lifetime with temperature of 11AU-labeled DMPC (0.5 mol %) was measured. The gel to liquid crystal transition behavior is clearly observed (Fig. 3). The same results, within error, were obtained in labeled MLVs. Because the emission spectra

TABLE 3 b and %dA/dT values of AS-DOPC ULVs at two excitation wavelengths

Probe		b	$\%dA/dT$
6 AS	(381)	4.8	1.3
	(316)	4.8	1.4
12 AS	(381)	4.9	1.7
	(316)	4.6	1.5

Values in parenthesis are the exciting wavelength. See the legend of Table 2.

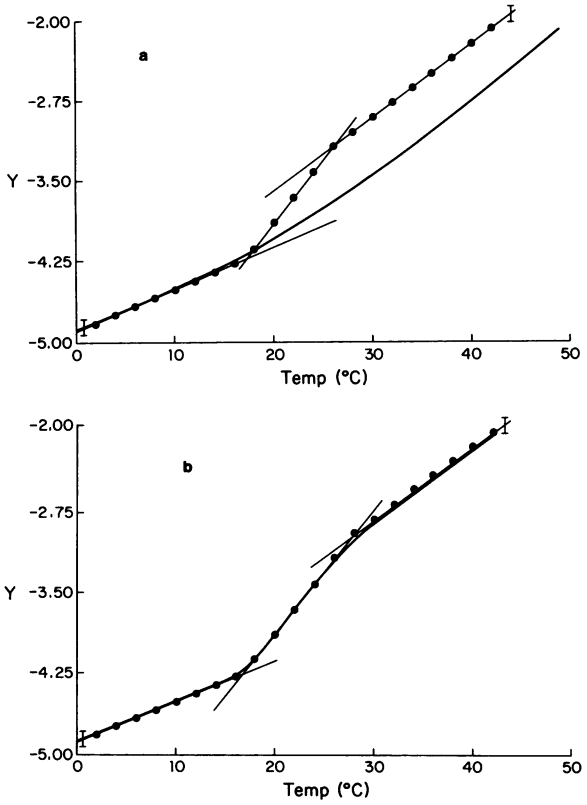

FIGURE 3 *Y* versus temperature plots of 11 AU-DMPC. The faint line was derived by a least-square analysis of the experimental data points. The bold line was computer generated from the enthalpy calculations assuming *a* one transition and *b* two transitions.

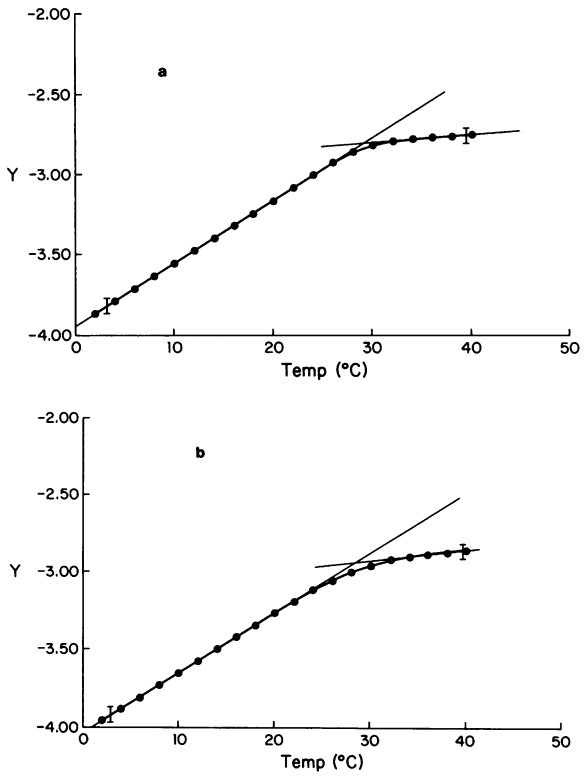

FIGURE 4 *Y* versus temperature plot of 11 AU-eggPE at a molar ratio of *a* 0.1% and *b* 1%. The faint line was derived from a least square analysis of the experimental data while the bold line was synthesized from the enthalpy-generated *Y*-plot.

remained constant with temperature in all cases, the probe probably did not form domains during the transitions. When gramicidin, an integral membrane peptide was incorporated into the vesicles at a lipid-to-protein ratio of 23:1, the gel to liquid crystal phase transition was no longer seen and a straight line was obtained in the 10° to 40°C temperature range having a *b* value of 7.8%°C^{-1}. The disappearance of the phase transition due to the presence of proteins has been routinely observed (Lee, 1983).

EggPE

The *Y* values of eggPE ULVs doped with 0.1 mol% 11AU were determined from 5° to 40°C. The resulting plot (Fig. 4 *a*) shows a clear transition at 29°C. This transition was accompanied by an increase in solution turbidity. The values of the anisotropy were reversible upon returning slowly to 5°C. The temperature of the bilayer to hexagonal phase transition is reported to be 25°–39°C (Cullis and de Kruijff, 1978). Because the hexagonal phase is composed of highly-scattering particles, the transition at 29°C probably reflects the bilayer to hexagonal phase transition. To determine if the presence of fatty acid probe affects the transition, the experiment was repeated at a probe to lipid ratio of 1% (Fig. 4 *b*) and the data were identical except for a slight broadening of the transition.

Enthalpy calculations

The enthalpy of the apparent phase transitions as determined by change in the *b* were determined for DMPC and eggPE as described in Materials and Methods. Errors were calculated from the errors of the experimental *Y* values. In several cases the phase transition was very sharp and, due to the resolution of the experimental data, an exact enthalpy could not be obtained. The values in these cases are reported as "min" values indicating that these enthalpies were determined using the highest possible errors in *Y* that gave the broadest transition region. Sharper transitions gave enthalpies over 100 kcals.

The results of eggPE are listed in Table 4 and the synthesized *Y*-plot generated from Eqs. 1–7 are shown in Figs. 4, *a* and *b*. From the data in Table 4 we see that the sample doped with 1% probe to lipid gave a low, resolv-

TABLE 4 *Y*-plot derived ΔHs for EggPE and DMPC phase transitions

Lipid	ΔH(*Y*-plot)	Variance	ΔH
eggPE(0.1% 11AU)	61 (min)	7*E-5	~0 (DOPE)*
eggPE(1.0% 11AU)	31 ± 6	1*E-4	~0 (DOPE)*
11 AU-DMPC (1-*trans*)	15 ± 6	1*E-1	6.8-9.6*
11 AU-DMPC (2-*trans*)	81,46	1*E-4	6.8-9.6*

ΔH is in kcal/mol. (min) refers to the enthalpy determined using the maximum error in *Y* values and if this error is reduced, the enthalpy becomes very large and in most cases, no longer resolvable. 1-*trans* refers to a single transition fit and 2-*trans* refers to a two transition fit with the first values corresponding to the higher temperature transition.
*DOPE value of van Dijck et al., 1976.
‡DMPC value of A. Lee, 1983.

able enthalpy while the sample with 0.1% probe-to lipid ratio was very high and not readily resolvable even though plots are very similar. EggPE consists primarily of *cis*-unsaturated lipids (Avanti Polar Lipids, Inc., personal communication). The literature value cited for this transition (van Dijck et al., 1976) is for DOPE. These authors did not detect an enthalpy change in *cis*-unsaturated phsophatidylethanolamines but did detect a small enthalpy difference (4–8 kcal/mol) for their saturated and *trans*-unsaturated counterparts.

When this analysis was applied to DMPC, the *Y* plots generated from the enthalpy values could not mimic the sudden increase in *Y* or decrease in viscosity that occurs in the transition region (Fig. 3 *a*). Therefore, the data were modeled assuming that the transition region can be treated as an intermediate state with a unique value of *b*. As the temperature rises the fluorophore passes from the gel to the transition region and then to the lipid crystal state with a certain enthalpy associated with these two transitions. The assumption of two transitions allows a good fit of the data (Fig. 3 *b*). However, the assumption of two transitions causes the enthalpy to increase so extensively that it is no longer resolvable.

DISCUSSION

Because the viscosity of a solvent reflects the strength of its intermolecular forces, the temperature dependence of the viscosity (*b*) would reflect the stability of these forces. When *b* is determined by fluorescence spectroscopy it corresponds to the temperature behavior of the local microviscosity around the probe due to the restriction to fluorophore rotation caused by its environment. The purpose here is to characterize the structural factors that contribute to the magnitude of *b*. In isotropic solvents, *b* appears to rely on a combination of intramolecular forces.

TABLE 5 *b* Values of isotropic solvents

Solvent	Temperature range (°C)	$b\ \%C^{-1}$
Diethylether	−40–20	1.14
Acetone	−30–30	1.12
Methanol	−40–25	1.85
Ethanol	−33–30	2.17
n-Propanol	0–50	2.45
Hexane	0–50	1.00
Benzene	0–50	1.14
Water	0–20	3.10
Butanol	−40–20	4.10
Isooctane	−20–10	0.08
Glycerol	0–30	9.23
80% Glycerol-water	−40–20	8.00

b values were extracted from the viscosity data reported in the CRC Handbook of Chemistry and Physics, 64th edition, CRC Press, Boca Raton, FL.

Comparing the values of methanol, ethanol, *n*-propanol, and butanol (Table 5) we find that *b* increases with chain length in primary alcohols. Hydrogen bonding between the hydroxyl groups of butanol can serve to position the hydrocarbon chains so as to increase dispersion interactions. Heavily hydrogen-bonded solvents like glycerol and glycerol-water mixtures show very high *b* values. Water displays an unexpectedly low *b* value probably because of the large free volume associated with its molecular packing due to repulsive forces.

We first compared the anisotropy and lifetime behavior of a side-chain (12 AS) and an end-on (11 AU) anthroyloxy fatty acid probe to 9-methyl anthracene. While all three probes show the same *b* value at low temperatures, the two fatty acid probes show different *b* values at higher temperatures. This behavior is similar to the case of small peptides and free fluorophores (Scarlata et al., 1984). Based on that study we interpret the behavior seen in Fig. 1 for the fatty acid probes as follows. At low temperatures the motions of the anthroyloxy group are small and any increase in the rotational amplitude with temperature is due to the relief of restrictional forces from the solvent. However, at a certain temperature, *Tc*, the fluorophore amplitude is large enough that its motion becomes limited by the surrounding fatty acid chain and thereafter any increase in amplitude with temperature is modified by these groups. Because this effect is similar in both the side-chain probe and the tail probe, the second *b* value is indicative of folding of the hydrocarbon chain around the probe.

Here the values of *b* were determined from the temperature dependence of the steady state anisotropy and lifetime and no attempts to heterogeneity analysis were made. The data are analyzed in terms of the average rotational amplitude seen by the fluorophore during its

lifetime assuming that fluorophore rotation is fast enough such that all the space allowed to it by its environment is filled. Computer simulations support this assumption (Scarlata, 1988). Time resolved anisotropy studies have shown that in highly anisotropic systems (i.e., gel-phase DPPC), probes do not completely depolarize but reach a limiting value (r_∞) at longer times due to the high viscosity of the environment (Kawato et al., 1977). The magnitude of this quantity is a function of the probe, the membrane, the probe position in the membrane, and the temperature. r_∞ is interpreted to represent a rotational boundary that cannot be overcome by the probe during its lifetime and modeled as the maximum angle than can be achieved by a probe undergoing isotropic rotations (Kawato et al., 1977 and Weber, 1978). Using a modified Perrin equation that takes into account this boundary condition, the anisotropy a probe would have in an isotropic solvent can be obtained.

The viscosity around a fluorophore depends on total amount of factors that restrict fluorophore rotation regardless of whether these are overcome during the lifetime of the probe. In this study we are interested in all of the physical limitations to probe rotation and not how the probe would rotate if it were in an isotropic solvent. Correcting the anisotropy for r_∞ would eliminate the substantial restrictive forces that contribute to the apparent viscosity, which is precisely what we are interested in observing and thus lead to inaccuracies because these forces would be neglected. Recently, Weber has shown that limited rotations of fluorophores in anisotropic media can be analyzed without geometric restrictions or the use of r_∞ by a thermodynamic equilibrium approach (Weber, G., manuscript submitted for publication), which completely supports this treatment of the data. We also note that in this analysis, Y is the negative log of the microviscosity around the probe which is highly dependent on the probe position and orientation, but the change in Y with temperature is independent of the location of the probe and its type of rotation in DOPC membranes.

If lipids did not take on a bilayer form, we would expect the viscosity change with temperature to depend on both the external solvent and the hydrocarbon chain, i.e., 12 AS octyl-glucoside micelles in water displays a b value higher than water but lower than DMPC or DOPC vesicles. When lipids form bilayers, extensive dispersion forces occur along the hydrocarbon chains that result in much higher viscosities that change more rapidly with temperature. The magnitude of b obtained in the fluid states of 11 AU labeled DOPC and DMPC bilayers (4.8 and 7.2%°C^{-1}) show that chain packing around the fluorophore can give b values similar to butanol or to 80% glycerol-water solutions, respectively. DOPC has a *cis* double bond in the ninth position (18:1) whereas DMPC is saturated (14:0). Therefore, the presence of one double bond is enough to disrupt lipid packing and dispersion interactions to lower b by almost 30%. This effect is also observed in the large difference in the gel to liquid crystal phase transition temperatures (−18°C for DOPC and 22°C for DMPC). The bilayer (La) phase of eggPE has the same value of b at DOPC which is reasonable because eggPE primarily consists of unsaturated lipids. The thermal coefficient of the hexagonal phase is very low and similar to isooctane. Because the chains of the hexagonal phase are not closely packed and less ordered than the lamellar phase, a low b value is expected.

The results of this study show that the relative changes in anisotropy of anthroyloxy fatty acid probes with temperature and b values are surprisingly constant throughout the DOPC bilayers even though the anisotropy decreases steadily with membrane depth (Tilley et al., 1979). Because the anisotropy is related to the free volume around the fluorophore, the uniformity of dA/dT with depth indicates that the bilayer is expanding isotropically with temperature. We have also shown that a very anisotropic system like DOPC can be analyzed in terms of b even though it is calculated by an equation that pertains to isotropically rotating fluorophores (Eq. 1). Therefore, by characterizing this membrane system in terms of b or dA/dT, we are not subject to variations in probe position or orientation in the membrane.

The data obtained here can be compared with the fluorescence depolarization studies of Lentz et al. (1976) on DPH labeled vesicles. The values of b extracted from their data for DMPC and DOPC multilayers and bilayers in the fluid state are close to those reported here. This may indicate that in the fluid phase b is independent of probe which is the case in isotropic solvents. However, in the phase transition region DPH-DMPC displayed a much larger change in viscosity with temperature than the 11 AU-DMPC. In the gel phase, the behavior of $1n\,\eta$ as seen by DPH motions in DMPC multilayers and bilayers was nonlinear with the microviscosity remaining constant below 8°C. The authors theorize that the transition and gel phase DPH behavior may reflect changes in the amount of probe in different membrane sites. Changes in membrane site occupancy would change the overall viscosity felt by DPH and hence affect the value of b. This would explain the difference between the nonfluid phase anisotropy behavior of DPH-DMPC as opposed to the 11AU-DMPC data because 11 AU is expected to occupy a single membrane position (Tilley et al., 1979). Also, because the steepness of the transition region depends on the size of the vesicles and the concentration of fatty acid probe (Lee, 1983), discrepancies between the two studies are expected.

The values of b determined for the gel, transition, and

liquid crystal phases of AU-DMPC are all higher than those reported for dehydroergosterol labeled DMPC bilayers (Chong and Thompson, 1986). The authors of this study postulated that the dehydroergosterol probe is not evenly distributed in the membrane at the concentration used (4 mol %). Formation of probe domains would indeed change the effective solvent around the fluorophore and modify *b*. Here it is also possible that an uneven distribution of probe in the bilayer may occur and thus yield erroneous *b* values. However, we feel that probe aggregation in the membrane is not a serious problem for several reasons. First, very low concentrations of probe were used. Second, in eggPE the same *b* values and the same bilayer to hexagonal phase transition temperatures were obtained at two different 11 AU concentrations (0.1 and 1 mol %). If aggregation occurred we would expect the higher concentration to form a larger aggregate and thus perturb the spectral parameters. Third, we would expect the amount of aggregation to be dependent on probe position with side chain probes (e.g., 9 AS), because they cause a greater disruption of chain packing than tail end probes that sit between the layers (e.g., 16 AP), to aggregate more extensively. The observation that the same *b* value was obtained for all the probes supports the idea that the probe molecules are distributed evenly in the bilayers.

The fact that our probes displayed the same phase transition temperatures as other techniques that do not use added probes, e.g., calorimetry, (Lee, 1983) indicates that preferential partitioning of the fluorophores into the gel or fluid phase does not occur to any great extent. We note that preferential partitioning would only change the transition temperature and not the values of *b* unless aggregation occurred. However, ordering of lipids chains by the probes would increase *b* due to increased dispersion forces as previously discussed. Again, we would expect ordering to be the greatest for side chain probes as opposed to tail probes and because the same *b* values were obtained in all cases, we presume that ordering of the lipid chains by probe, if it does occur is not extensive enough to influence the value of *b*.

It is interesting to compare the phase transition anisotropy behavior of 11 AU in DMPC and eggPE. The gel to liquid crystal phase transition of DMPC spans ~6° (16°–22°C) probably due to the fluorescent probe impurity (Lee, 1983) and is accompanied by a large drop in the anisotropy (0.095 to 0.040). The eggPE bilayer to hexagonal phase transition is sharper and does not have a large anisotropy drop at this temperature. These results imply that the fluidities of both phases as felt by the fluorophore in eggPE are similar.

Here we calculated the enthalpy change that occurs when a fluorophore is transferred from one lipid phase characterized by one thermal coefficient to another which we shall call "*b* enthalpy". In this treatment we assumed that the value of *b* derived from the fluorophore probe rotations reflects the *b* of the pure lipid phase. Because *b* can be determined at temperatures far from the transition temperature, and because previous results (Scarlata et al., 1984) and subsequent computer simulations show the enthalpy change is primarily due to the broadness of the transition rather than the magnitude of the values of *b* we feel the assumption is valid. This point is exemplified in the case of eggPE where, as the amount of probe is raised from 0.1% to 1% mol % ov 11-AU, the *b* values and the transition temperature are unaffected but the transition is visibly broadened and the enthalpy almost halved.

We can compare the *b* enthalpy values determined here to the macroscopic phase transition enthalpies determined by calorimetry (Table 4). In DMPC, if a one-state transition was assumed, the *b*-enthalpy was close to the calorimetric one although the fit was very poor (Fig. 3 *a*). A good fit was obtained assuming two transitions, however, the enthalpy change is then much greater than detected by calorimetry and cannot be accurately fit. Thus, while this analysis may yield lower enthalpies if the transition region is broad, very sharp transition cannot be resolved due to the accuracy of the data.

In eggPE the enthalpy change was greatly reduced when the probe concentration was raised indicating that an increase in impurity concentration lessens the cooperativity of the transition and hence the enthalpy. No enthalpy changes were observed by calorimetry for the bilayer to hexagonal phase transition of *cis*-unsaturated phosphatidylethanolamines model membranes of which eggPE is mostly comprised, although this transition has been observed by various spectroscopic techniques (for a review see Gruner et al., 1985). How can a substantial enthalpy change be detected by this spectroscopic analysis and not be calorimetry? Similar behavior has been observed for the protein *d*-amino-oxidase by Sturtevant and Mateo (1978) where the authors verified a reported fluorometric ΔH for the protein transition (Massey et al., 1965) but could not detect an enthalpy change calorimetrically. The discrepancies between the spectroscopic and calorimetric enthalpies can only mean that the microscopic ΔH as seen by changes in fluorophore environment does not reflect the true enthalpy change but rather relates to cooperative unit of the transition (Edsall and Gutfreund, 1983). Thus, by raising the concentration of contaminant, we are reducing the size of the cooperative unit. Also, the *b*-enthalpy may be dependent on the probe giving different membrane microstates. Previous studies on the DPPC gel to liquid crystal transition indicate that microviscosity changes are greatest towards the center of the bilayer (Thulborn and Sawyer, 1978). Experiments

are underway to find the factors that contribute to *b* enthalpy.

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