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MEASUREMENT AND INTERPRETATION OF FLUORESCENCE POLARISATIONS IN PHOSPHOLIPID DISPERSIONS

C. LINDSAY BASHFORD, CHRISTOPHER G. MORGAN and GEORGE K. RADDA

Department of Biochemistry, University of Oxford, South Parks Road, Oxford, OX1 3QU (U.K.)

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SUMMARY

An instrument that measures the temperature dependence of fluorescence polarisation and intensity directly and continuously is described. The behaviour of four fluorescent probes bound to a number of well characterised model systems was then examined. The motional properties of the probes were determined from the polarisation and intensity data and were found to be sensitive to the crystalline-liquid crystalline phase transitions in phospholipid vesicles of dimyristoyl and dipalmitoyl phosphatidylcholine. Binary mixture of dilauroyl and dipalmitoyl phosphatidylcholine show lateral phase separation and in this system the probes partition preferentially into the more 'fluid' phase. In systems that have been reported to contain 'short range order' or 'liquid clustering', such as dioleoyl phosphatidylcholine and liquid paraffin, the motion of the probes was found to have anomalous Arrhenius behaviour consistent with the idea that homogeneous phases were not being sampled. The significance of these findings for the interpretation of the behaviour of fluorescent probes bound to natural membranes is discussed.

INTRODUCTION

The mobility of the different components of a biological membrane is thought to be of fundamental physiological importance [1]. In particular, much attention has recently been paid to the idea that the physical state of the membrane lipids may be an important factor modulating the biological activities of membranes. Typically these studies have depended on the correlation of changes in biological activity and lipid fluidity in response to changes in an external variable. Two of the most widely used variables have been the lipid composition of the membrane and temperature.

A whole range of physical techniques has been used to monitor changes in lipid fluidity. The methods include differential scanning calorimetry [2], X-ray diffraction [3], nuclear magnetic resonance [4] and especially the use of electron spin

Abbreviations: ANS, 1-anilinonaphthalene-8-sulphonic acid; 12-AS, 12-(9-anthroyloxy)-stearic acid; 2-AP, 2-(9-anthroyloxy)-palmitic acid.

resonance [5] and fluorescent probes [6]. Recently fluorescence polarisation measurements have been shown to be particularly suited to the study of changes in the microviscosity of the environment of a fluorescent probe bound to a membrane [7–11].

X-ray, NMR and calorimetric measurements are non-perturbing techniques, whereas the two probe methods rely on the introduction of an 'impurity' into the membrane. The justification for using probe methods is that they can be readily extended to studies of complex membranes, as well as to simple models. The major limitations are that there must be some perturbation at least at the locus of the impurity and that in heterogeneous systems the probe may not necessarily be evenly distributed in the lipid phase. This investigation examines these two problems in well defined model systems that have been extensively studied by other methods. We also show that, by improving the accuracy of fluorescence polarisation measurements, interesting new information about lipid clusters may be obtained. This has important implications for fluorescence polarisation studies involving natural membranes. The desired accuracy is obtained by the use of a simple instrument that is capable of recording the temperature dependence of fluorescence intensity and polarisation directly and continuously.

MATERIALS AND METHODS

The instrument

The apparatus described here measures the parallel ($I_{||}$) and perpendicular ($I_{\perp}$) polarised components of the fluorescence emission simultaneously. These components are used for the continuous calculation of the fluorescence polarisation (p) where

$$p = \frac{I_{||} - I_{\perp}}{I_{||} + I_{\perp}}$$

and of the total fluorescence intensity defined by the function

$$I = I_{||} + 2I_{\perp}$$

The whole system is shown in Fig. 1.

The two photomultiplier tubes (Type EMI 9514) are used in an analogue mode. Correct functioning of the system depends on the linearity of the response of each photomultiplier tube and on the matching of the pair. The best matching was achieved by driving each photomultiplier from a separate, variable power supply. The linearity of the response of each tube was checked by the insertion of glass filters of known transmittance and plotting the output. The matching of the pair of photomultipliers was checked by following the output voltage of each as a function of the excitation slitwidth in the presence of a scattering sample. The maximum deviation over an output range of 0–400 mV was 2 mV at 400 mV. In fluorescence measurements the instrument was set up so that the emitted light intensity corresponded to about 200 mV. In this case changes in the intensity of the emitted light with temperature would not affect the output accuracy of the photomultipliers.

During the operation of the instrument it was necessary for the gains of the photomultiplier tubes to be balanced and to compensate for emitted light due to scattering by the sample [7]. Thus with the excitation polaroid horizontal, so that

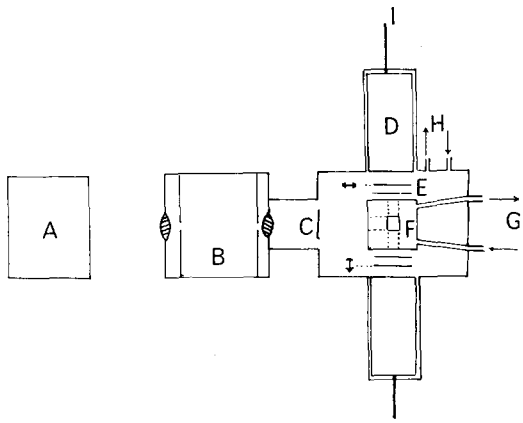

Fig. 1. Diagram of apparatus used for studies of polarisation as a function of temperature. A, 250 Watt xenon lamp; B, Zeiss prism monochromator; C, rotatable ultraviolet-transmitting polariser; D, photomultiplier tube; E, filters and fixed polarising filters. The plane of polarisation is indicated by an arrow in the figure. Filters were identical at each side of the instrument. F, thermostatted cuvette holder; G, water inlets; H, inlets for dry N_2 or air; I, leads from photomultiplier to the power supply and to preamplifier.

the fluorescence emission would be polarised in a plane perpendicular to the plane of polarisation of both the emission polaroids, and with zero backoff on the preamplifiers, the gain of each channel was adjusted to equalise the preamplifier outputs. In this configuration both photomultipliers receive emitted light of equal intensity. This procedure has the effect of compensating for any difference in filter transmission, When the gains were balanced the output signal was adjusted to about 200 mV, a level appropriate to the computer input, by adjusting the excitation monochromator

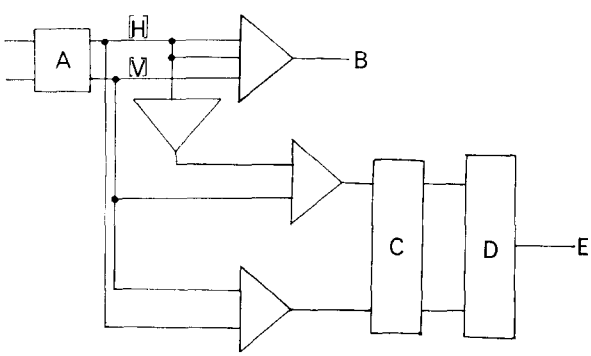

Fig. 2. Simplified diagram of analogue computer setup. Triangles represent fixed gain summing and inverting amplifiers. A, dual channel preamplifier with gain and backoff for each channel. The amplifier is non-inverting. B, analogue intensity output to chart recorder. The function is V+2H. C, block representing changeover switch and appropriate scaling and inverting amplifiers. The switch selects polarisation or its scaled inverse for output to the X–Y recorder. D, analogue divider unit with associated amplifiers giving variable gain and backoff on output. E, output to plotter. Not shown are backoff and gain controls nor scaling and sign changing amplifiers. V and H represent inputs from the vertically and horizontally polarised channels of the apparatus.

TABLE I

VARIATION OF APPARENT FLUORESCENCE POLARISATION WITH PHOTOMULTIPLIER OUTPUT

The polarisation of a standard fluorescent block was measured and the output signal of the photomultipliers varied by changing the excitation slitwidth at constant excitation wavelength.

Output signal (mV)	Apparent polarisation
300	0.2970
400	0.2940
500	0.2920
600	0.2905
700	0.2895

(B in Fig. 1) slitwidth. The excitation polaroid was now orientated to yield vertically polarised exciting light and the instrument was ready for use.

To correct for contributions caused by the scatter of the exciting light, a cuvette containing all the components of the experimental system but no fluorescent probe was placed in the instrument after the balancing of the gains. The output of each photomultiplier was then set to zero by subtraction of a voltage at the summing input. No change was made either in the excitation slitwidth or in the photomultiplier gains. The contribution from scattered light was never greater than 5 % of the total fluorescence emission. With the sample containing the fluorescent probe in place after correction for scatter, the signal outputs of the photomultipliers were processed in an EAL 380 analogue computer programmed as shown in Fig. 2. The multiply/divide unit in this computer is not essential for a calculation of this kind and a useful instrument can be built using committed gain instrumentation amplifiers. In this case a logarithmic divider should be used, as this has a wider dynamic range than the quarter-square type. Table I shows the change in apparent polarisation over a wide range of output intensity. This indicates that a change of output intensity of 50 % yields an error of only 2 % in the apparent polarisation.

The temperature of the sample cuvette was measured directly by a thermistor bead immersed in the solution above the light path. The bead was connected to an electric thermometer whose output was displayed on a meter and also on the *X*-axis of a Hewlett Packard MOSELEY 7035B X–Y recorder. The *Y*-axis of the recorder could be connected to the computer to monitor fluorescence polarisation, a scaled inverse of the fluorescence polarisation or the total fluorescence intensity. The absolute values of these parameters were read from the digital voltmeter attached to the computer.

Other fluorescence measurements

Initial fluorescence intensity and spectral measurements were made with a Hitachi Perkin-Elmer MPF-2A Spectrofluorimeter. Fluorescence lifetimes were measured with an Ortec nanosecond pulsed fluorimeter modified to include the nanosecond spectral source and optical system of Applied Photophysics Ltd., London. This system provides a high frequency flash of sufficient intensity to employ monochromators. In our measurements a grating monochromator was used at the excitation side and a combination of filters at the emission side.

Labelling of phospholipid vesicles

In the preparation of phospholipid dispersions, sufficient fluoresecnt probe in the form of an ethanolic solution was added to a chloroform solution of the required phospholipid. The organic solvent was evaporated first under N_2 and subsequently under vacuum. Water was added to the probe phospholipid residue and the mixture was sonicated under low power with a DAWE Soniprobe until optical clarity was obtained. This generally took about 10 min provided that the temperature of the sample was above that of the phospholipid phase transition. All sonication was carried out under an N_2 atmosphere. The final concentration of phospholipid was in the range 0.5–0.8 mM and the molar ratio of phospholipid to probe was never less than 300. After sonication samples were centrifuged at $10\,000 \times g$ for 10 min at room temperature to remove the titanium released by the sonicator probe. In experiments using organic solvents the fluorescent probes were dissolved directly to give the required final concentration.

The pH of the aqueous dispersions of phospholipids containing the fluorescent probes fell within the pH range 5.5–6.5 and was not significantly altered by the heating and cooling experiments described below. Buffers were not used, so that the results could be directly correlated with those of differential scanning calorimetry [15] and for comparisons with bleaching experiments in oriented lipid bilayers [30].

Chemicals

Phospholipids synthesised as described elsewhere [12] were the kind gift of Dr. B. de Kruijff and were at least 99 % pure as indicated by thin-layer chromatography. Liquid paraffin BP was obtained from Boots Ltd., Cornmarket Street, Oxford. 1-anilinonaphthalene-8-sulphonic acid (ANS) was obtained from K and K Laboratories and was recrystallised as the magnesium salt. *N*-phenyl-1-naphthylamine was from British Drug Houses Ltd., Poole, Dorset and was recrystallised from 60 % ethanol before use. 12-(9-anthroyloxy)-stearic acid (12-AS) and 2-(9-anthroyloxy)-palmitic acid (2-AP) were synthesised in this laboratory from anthracene-9-carboxylic acid and the corresponding hydroxy fatty acid by a mixed anhydride synthesis using trifluoroacetic acid anhydride [13]. The product was recrystallised three times from hexane and found to be free of fluorescent impurities. Water was twice distilled in an all glass apparatus and all other chemicals were of the highest purity commercially obtainable.

Calculation of probe rotation rate

The relationship between the motion of a chromophore and its fluorescence polarisation is expressed by the Perrin Equation [7, 14]:

$$\left(\frac{1}{p} - \frac{1}{3}\right) = \left(\frac{1}{p_o} - \frac{1}{3}\right)\left(1 + \frac{3\tau}{\rho}\right)$$

where p is the fluorescence polarisation, p_o the polarisation of a rigidly held, randomly orientated array of chromophores, τ the fluorescence lifetime and ρ the rotational relaxation time of the chromophore. The temperature dependence of the rate of chromophore rotation ($1/\rho$) can thus be calculated from the temperature dependence of p and τ. In this work we have assumed that the fluorescence lifetime has the same temperature dependence as the fluorescence intensity, thus [8]

TABLE II

CORRELATION OF FLUORESCENCE LIFETIME AND FLUORESCENCE INTENSITY WITH TEMPERATURE

Fluorescence intensities were measured for the probes 12-AS and 2-AP bound to sonicated aqueous dispersions of dipalmitoyl phosphatidylcholine. Probe concentration was 2 μM and lipid concentration 0.8 mM. Excitation was at 385 nm and emission was measured above 410 nm in the instrument described. Fluorescence lifetime measurements were made on the same samples in an Ortec nanosecond pulsed fluorimeter with excitation at 361 nm.

Temperature (°C)	Fluorescence intensity / Fluorescence lifetime	
	12-AS	2-AP
18.5	–	0.2196
22.9	0.2038	0.2155
27.1	0.2037	0.2109
32.5	0.2018	0.2094
37.5	0.2000	0.2119
42.6	0.1865	0.2016
46.5	0.1937	0.2139
48.3	0.1959	0.2201
Mean	0.1978	0.2129
Standard error	0.0024	0.0021

$$\frac{\tau_x}{I_x} = \frac{\tau_y}{I_y}$$

We have tested this assumption for the probes 12-AS and 2-AP bound to sonicated dispersions of dipalmitoyl phosphatidylcholine and Table II clearly indicates that τ_T/I_T is a constant within the accuracy of the fluorescence lifetime measurements. We have evaluated fluorescence lifetime from the relative fluorescence intensity, as the latter could be measured considerably more accurately over the temperature range employed. p_0 values of 0.5 were used for 12-AS and 2-AP and of 0.35 for *N*-phenyl-1-naphthylamine on the basis of some time-resolved polarisation measurements made in this laboratory. The value of p_0 for ANS was taken to be 0.4. The secondary plots of $\log_e (1000/\rho)$ against reciprocal absolute temperature presented here were calculated using the mean polarisation and intensity values obtained in a number of different determinations at intervals of 1 °C. Under these conditions the standard errors of the mean of each point were less than 2 %.

RESULTS

The temperature dependence of the fluorescence polarisation of several probes bound to a sonicated aqueous dispersion of dipalmitoyl phosphatidylcholine is shown in Fig. 3. The curves represent the direct output of the polarisation instrument and indicate the very favourable signal to noise ratio obtained in these experiments. We found that repeated heating and cooling of the same sample generated a family of superimposable curves with no detectable hysteresis effects. The plots in Fig. 3

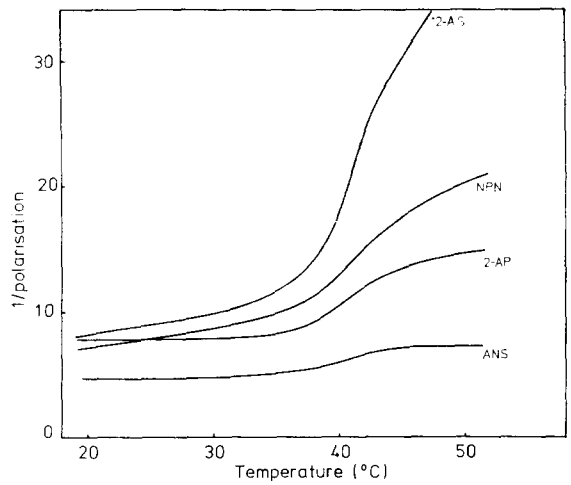

Fig. 3. Polarisation of fluorescent probes bound to dipalmitoyl phosphatidylcholine. The reciprocal fluorescence polarisation of probes bound to vesicles of dipalmitoyl phosphatidylcholine was followed as a function of temperature. In each case the concentration of probe was 2 μM and the concentration of lipid 0.7 mM. 12-AS and 2-AP were excited at 385 nm, ANS and 380 nm and *N*-phenyl-1-naphthylamine (NPN) at 350 nm. Fluorescence emission above 410 nm was analysed for the calculation of $1/p$. Transition temperatures determined from the first differentials were as follows: 12-AS = 40.8 °C; *N*-phenyl-1-naphthylamine = 41 °C; ANS = 41 °C; 2-AP = 40.5 °C.

are characteristically sigmoid and the midpoint of the change occurs around 40 °C in all cases. This temperature corresponds closely to that of the crystalline-liquid crystalline phase transition known to occur in fully hydrated dipalmitoyl phosphatidylcholine [15]. The first differentials of these primary and the later secondary plots give maxima at the temperatures indicated in the figure legends. These tempera-

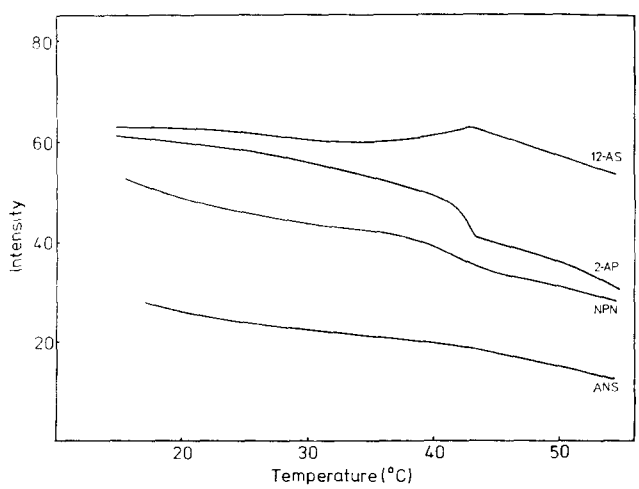

Fig. 4. Fluorescence intensity of probes bound to dipalmitoyl phosphatidylcholine. Conditions were the same as described in the legend to Fig. 3. Fluorescence intensity is in arbitrary units.

tures are all within the range of values of the transition temperatures observed by non-perturbing techniques. The magnitude of the change in fluorescence polarisation is the greatest for those probes, 12-AS and *N*-phenyl-1-naphthylamine, that occupy the hydrocarbon interior of a phospholipid bilayer [16]. The temperature dependence of the fluorescence intensity of the probes bound to the same system is shown in Fig. 4. Once again changes are also seen around the transition temperature, although they are generally smaller. The combinations of the polarisation and the intensity plots using the fluorescence lifetime at 25 °C (Table III) are shown as Arrhenius plots of the rate of chromophore rotation against reciprocal temperature in Fig. 5. The secondary plots indicate that the phase transition in dipalmitoyl phosphatidylcholine is reflected by changes in the apparent activation energy for the rotation of probes bound to the system.

TABLE III

FLUORESCENCE PROPERTIES OF PROBES BOUND TO DIPALMITOYL PHOSPHATIDYLCHOLINE AT 25 °C

The probes were bound to sonicated aqueous dispersions of dipalmitoyl phosphatidylcholine. Probe concentration was 3 μM and the lipid concentration 0.9 mM. Excitation and emission maxima are the uncorrected machine maxima obtained with a Hitachi Perkin-Elmer MPF-2A spectrofluorimeter. Lifetimes were measured on an Ortec nanosecond pulsed fluorimeter.

Probe	Excitation maximum (nm)	Emission maximum (nm)	Lifetime (ns)
ANS	380	476	9.0
N-phenyl-1-naphthylamine	347	416	7.0
12-AS	345, 365, 385	437	11.0
2-AP	345, 365, 385	444	9.8

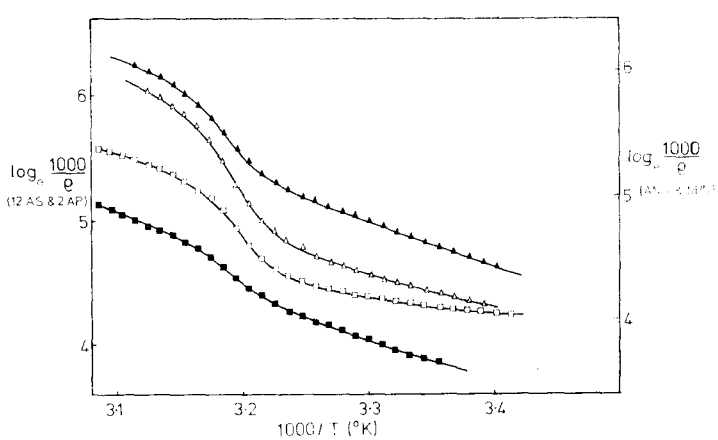

Fig. 5. Arrhenius plots of the rate of rotation of fluorescent probes bound to dipalmitoyl phosphatidylcholine. $1000/\rho$ was evaluated from the Perrin equation as described under Materials and Methods. Conditions as described in legend to Fig. 3. Symbols: 12-AS (△); 2-AP (□); *N*-phenyl-1-naphthylamine (▲); ANS (■). Transition temperatures determined from the first differentials: 12-AS = 41 °C; 2-AP = 40.5 °C; *N*-phenyl-1-naphthylamine = 41.2 °C; ANS = 41 °C.

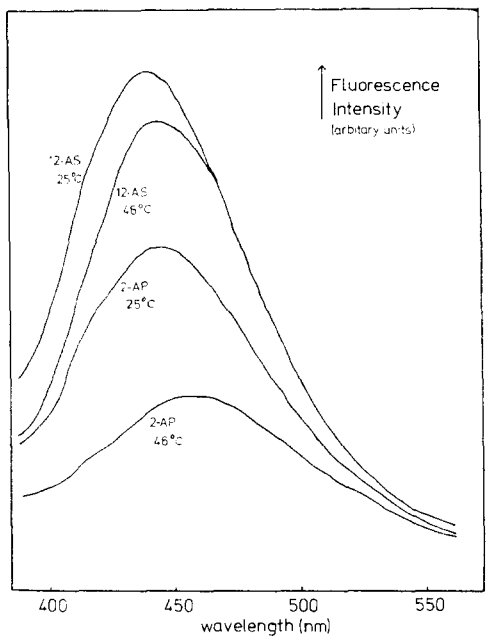

Fig. 6. Spectra of 12-AS and 2-AP bound to dipalmitoyl phosphatidylcholine. Conditions as described in legend to Fig. 3. Uncorrected machine spectra with excitation at 385 nm.

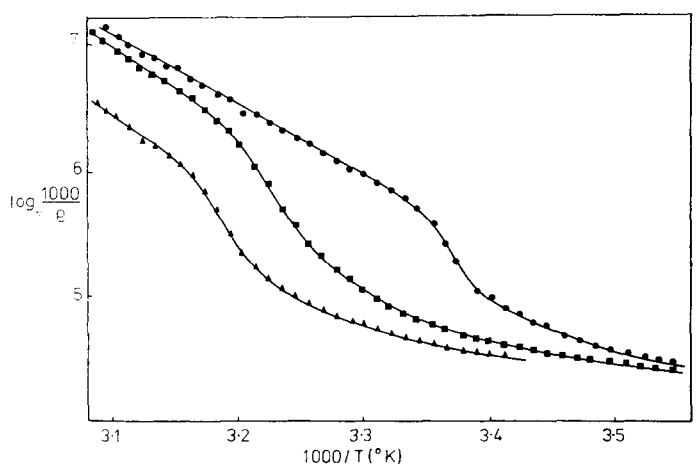

Fig. 7. Arrhenius plots of the rate of rotation of 12-AS bound to dipalmitoyl and dimyristoyl phosphatidylcholines and to an equimolar mixture of the two. Probe concentration 1.5 μM, lipid concentration 0.6 mM. Excitation at 385 nm, emission measured above 410 nm. 12-AS in dipalmitoyl phosphatidylcholine (▲), 12-AS in dimyristoyl phosphatidylcholine (●), 12-AS in equimolar mixture (■). Transition temperatures: dipalmitoyl phosphatidylcholine, 40.9 °C; dimyristoyl phosphatidylcholine, 23.5 °C; mixture, 36.5 °C.

The fluorescence emission spectra of the probes bound to dipalmitoyl phosphatidylcholine are all blue-shifted below the phase transition temperature compared to those obtained in 'fluid' phospholipids. Fig. 6 shows this change for the anthracene-based probes 12-AS and 2-AP bound to dipalmitoyl phosphatidylcholine. We report this observation, since fluorescence shifts are often interpreted in terms of changes in solvent 'polarity' yet increased constraint on solvent rearrangement can produce similar results [9, 17], as must be the case for our results.

Secondary Arrhenius plots of the probe 12-AS bound to dipalmitoyl phosphatidylcholine, dimyristoyl phosphatidylcholine and a binary mixture containing equimolar proportions of these lipids are shown in Fig. 7. Calorimetric evidence indicates phase transitions centred at 41.7 °C for dipalmitoyl phosphatidylcholine, 23.7 °C for dimyristoyl phosphatidylcholine [15] and a broader, intermediate transition for the binary mixture [18]. The correlation with the transition temperatures detected by our probe technique is good, confirming the validity of the method. In mixtures of dipalmitoyl and dilauroyl phosphatidylcholines lateral phase separation occurs and there are two distinct endothermic phase transitions at 8 and 38 °C [18]. The results of our experiments for 12-AS bound to this system are shown in Fig. 8. Since the probe is insensitive to the higher of the two transitions it is clear that in regions of lateral phase separation the probe is almost exclusively localised in the fluid phase. For technical reasons it proved impossible to make measurements below 4 °C and we were unable to follow the complete course of the dilauroyl phosphatidylcholine phase transition.

It has been suggested that vesicles of dioleoyl phosphatidylcholine exhibit lipid clustering below 30 °C [19]. In our experiments there is a small but experimentally

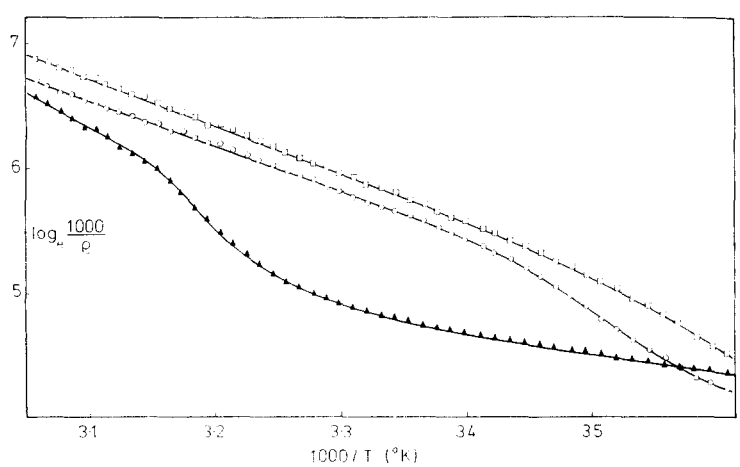

Fig. 8. Arrhenius plots of the rate of rotation of 12-AS bound to dipalmitoyl and dilauroyl phosphatidylcholines and in equimolar mixture of the two. Probe concentration 1 μM, lipid concentration 0.5 mM. Excitation at 385 nm, emission measured above 410 nm. 12-AS in dipalmitoyl phosphatidylcholine (▲), in dilauroyl phosphatidylcholine (□), in equimolar mixture (○). Transition temperatures: dipalmitoyl phosphatidylcholine, 40.6 °C; dilauroyl phosphatidylcholine, 4 °C; mixture, 9.7 °C.

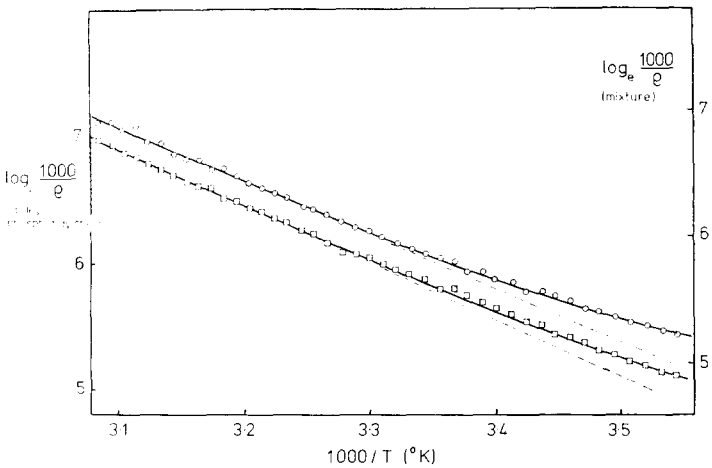

Fig. 9. Arrhenius plots of the rate of rotation of 12-AS bound to dioleoyl phosphatidylcholine and an equimolar mixture of dioleoyl and dipalmitoyl phosphatidylcholines. Probe concentration 2.5 μM, lipid concentration 0.75 mM. Excitation at 385 nm, emission measured above 410 nm. 12-AS in dioleoyl phosphatidylcholine (□), in equimolar mixture (○).

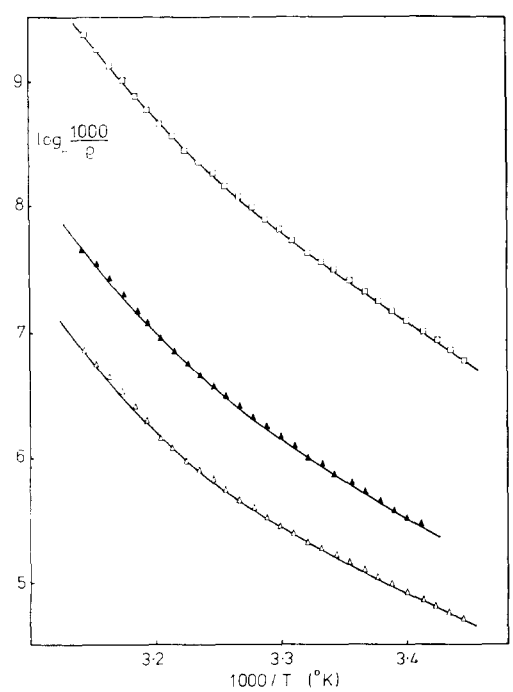

Fig. 10. Arrhenius plots of the rate of rotation of probes dissolved in liquid paraffin. Probe concentration 1 μM. Excitation at 385 nm for 12-AS and 2-AP and at 340 nm for *N*-phenyl-1-naphthylamine. Emission measured above 410 nm. 12-AS, (△), 2-AP (□), *N*-phenyl-1-naphthylamine (▲).

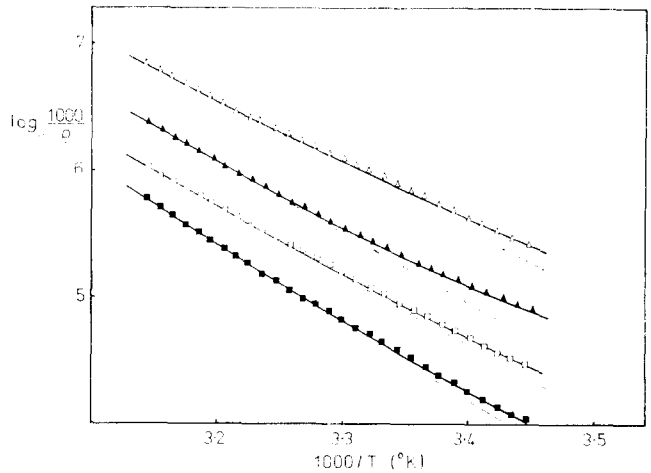

Fig. 11. Arrhenius plots of the rate of rotation of probes bound to egg lecithin. Probe concentration 2 μM, lipid concentration 0.8 mM. Excitation at 385 nm for 12-AS and 2-AP, 380 nm for ANS and 340 nm for *N*-phenyl-1-naphthylamine. Emission measured above 410 nm. 12-AS (△), 2-AP (□), *N*-phenyl-1-naphthylamine (▲), ANS (■).

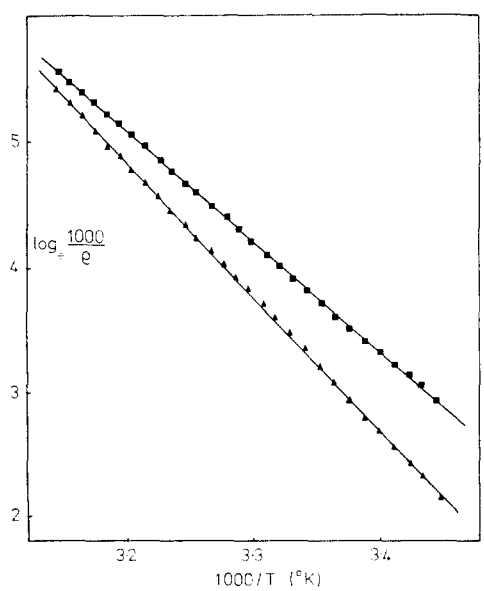

Fig. 12. Arrhenius plots of the rate of rotation of probes dissolved in glycerol. Probe concentration 2 μM. Excitation at 380 nm for ANS and 340 nm for *N*-phenyl-1-naphthylamine. Emission measured above 410 nm. ANS (■), *N*-phenyl-1-naphthylamine (▲).

significant deviation from linear Arrhenius behaviour in the low temperature region of the plot given in Fig. 9. The deviation commences at 30 °C and is enhanced by the presence of equimolar dipalmitoyl phosphatidylcholine. In mixtures of dioleoyl and dipalmitoyl phosphatidylcholines there is a broad second order transition that is completed around 30 °C [20], which could account for our observations. The curvature of the plots in Fig. 9 show that at the lower temperatures the rate of chromophore rotation is greater than would be expected on the basis of a simple Arrhenius temperature dependence. The existence of short-range order in hydrocarbon solvents, particularly the paraffins, has been proposed to explain the anomalous temperature-viscosity relationships found in these systems [21]. For this reason we examined the temperature dependence of different fluorescent probes dissolved in liquid paraffin. The results are indicated in Fig. 10 and show non-linear Arrhenius behaviour for all the probes in the temperature range used. From the point of view of hydrocarbon heterogeneity a reasonable analogy to liquid paraffin would be phospholipid vesicles composed of egg lecithin. Secondary plots for a number of probes bound to egg lecithin vesicles are shown in Fig. 11. The probes, 12-AS and *N*-phenyl-1-naphthylamine, bound most deeply in the hydrocarbon core of the vesicles show the greatest deviations from linearity commencing at about 35 °C.

When the probes are dissolved in an isotropic organic solvent that does not have anomalous temperature-viscosity relationships, the results indicate that the Arrhenius plots of rate of chromophore rotation against reciprocal temperature are linear (Fig. 12).

DISCUSSION

The validity of the probe approach in studies of natural and artificial membranes depends on three factors. Firstly, the location of the probe in the membrane must be known in order to relate the observed properties of the probe to the nature of its specific environment. Secondly, the introduction of the probe must not induce major perturbations in the system under investigation. Thirdly, the fluorescence properties of the probe must reflect its behaviour in the membrane phase.

The location of the probes used in these experiments when bound to phospholipid vesicles and natural membranes is indicated by their spectral properties. Thus their emission spectra and fluorescence lifetimes are consistent with their location in a hydrophobic environment and resemble the properties of the probes dissolved in organic rather than in aqueous solvents. The probes used were chosen to sample different regions of the phospholipid bilayer. ANS and 2-AP are thought to bind close to the polar-nonpolar interface and *N*-phenyl-1-naphthylamine and the anthracene group of 12-AS are located more towards the centre of the bilayer [16]. This assignment is supported by our observation that the motions of 12-AS and *N*-phenyl-1-naphthylamine are noticeably greater than those of 2-AP and ANS and reflect the 'flexibility gradient' found in phospholipid bilayers by magnetic resonance techniques [22].

The rate of rotation of the aromatic chromophores used here represents an average of several different types of motion [23] and both anisotropic rotation [24] and rapid oscillation within a limited range of angles [25] will contribute to the observed depolarisation of the fluorescence emission. However, the fact that the

rotation rate reflects the known chain and head-group mobilities of all the model systems used here implies that it can be taken as a qualitative measure of lipid fluidity. The local perturbation introduced by the probes means that the values of probe rotation obtained may not have absolute significance, but they can be used with confidence for comparative purposes in defined systems. We should add that one of the reviewers commented that p_0 may vary from instrument to instrument and sample to sample. Our experience is that the instrumental variation is relatively insignificant at defined monochromator bandwidths. Time-resolved anisotropy measurements also indicate that in phospholipid samples the same p_0 can be used in different systems. For these reasons it is preferable to express polarization measurements in terms of the Perrin equation, which makes allowance for the variation in fluorescence lifetimes.

The transition temperature determined by the probes bound to dipalmitoyl phosphatidylcholine is slightly lower than that found by a non-perturbing technique such as differential scanning calorimetry and the width of the transition is somewhat increased. The factors contributing to this observation are probably the local perturbations discussed above and the small size of the phospholipid vesicles which may lead to reduced cooperativity at the phase transition. The experiments with the other phospholipids described here clearly indicate, however, that the motion of a bound fluorophore is sensitive to the endothermic crystalline-liquid crystalline phase transition. The correlation of the transition temperatures determined for both pure and binary mixtures of phospholipids by calorimetry and by fluorescence polarisation is good (Figs. 7, 8 and 9).

Our observations are in contrast to a number of previous reports based on the interpretation of the fluorescence polarisation of probes bound to phospholipid dispersions. The polarisation of ANS fluorescence has been found to be insensitive to the phase transition in both dimyristoyl [26] and dipalmitoyl phosphatidylcholines [27] and the microviscosity of egg lecithin dispersions determined by probe methods was found to have linear Arrhenius behavior [8, 28]. These studies have relied on the independent measurement of the fluorescence parameters at each temperature, whereas our instrument provides a continuous output of the fluorescence parameters with temperature enabling us to detect the relatively small changes with much greater accuracy.

Recent studies have drawn attention to the fact that other types of thermal transition than the order-disorder phase transition may occur in phospholipid dispersions [19]. These transitions have been ascribed to the formation of lipid clusters or regions of short range order in the hydrocarbon phase of phospholipid membranes and in hydrocarbon solvents [19, 21]. We have found that the motional properties of fluorescence probes are sensitive to such transitions in well characterised model systems.

In binary lipid mixtures where solid-liquid phase separation occurs, our results clearly indicate that 12-AS preferentially partitions into the liquid regions of the bilayer. In these circumstances the chemical composition of the fluid regions will change continuously throughout the course of the phase separation and this could explain the observations that the probe mobility at low temperatures is higher than that expected from normal Arrhenius behaviour in mixtures of dioleoyl and dipalmitoyl phosphatidylcholines. In contrast, in single lipid systems (like dipalmitoyl phos-

phatidylcholine) there can be no such changes in composition, but within the temperature range of the melting we would expect preferential distribution of 12-AS in the liquid regions. This will mean that, unless lateral diffusion of the probe in the solid phase is rapid, the distribution of the probe in the final solid will be uneven. These arguments particularly apply for low lipid to probe ratios and in our experiments each lipid vesicle contains on the average only one or two probe molecules. The conclusions that 12-AS has a tendency to occupy the most fluid region of membranes preferentially has important implications for its use in studies of biological membranes.

The increased accuracy of our continuous polarisation measurements has enabled us to show deviations from linear Arrhenius behaviour in several systems. Most noticeably, this deviation is observed in dioleoyl phosphatidylcholine and liquid hydrocarbon, where other studies have been interpreted in terms of 'liquid clustering' or 'short range order' in the liquid phase [19, 21]. We also observe this type of anomaly in egg lecithin vesicles in which dioleoyl phosphatidylcholine is a major phospholipid component. In the saturated phospholipid dilauroyl phosphatidylcholine, however, the Arrhenius plot is strictly linear over the entire temperature range (40 °C) above the phase transition for this lipid. The possibility that 'liquid ordering' is responsible for the apparent increase in probe mobilities cannot be proved by our data. One possible explanation is that the higher than expected probe mobilities arise because concentration-dependent depolarisation of fluorescence [29] yields observed polarisation values that are too low. This mechanism requires the close approach of two or more probe molecules and could arise if the probe was segregating into relatively small regions of the bilayer by its exclusion from 'liquid clusters'. It seems that either interpretation of our data, regions of lower viscosity or concentration-dependent depolarisation, is consistent with the idea that the hydrocarbon core of several phospholipid systems does not represent a homogeneous phase even at temperatures well above that of the crystalline-liquid crystalline phase transition.

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