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Temperature, pressure and cholesterol effects on bilayer fluidity; a comparison of pyrene excimer/monomer ratios with the steady-state fluorescence polarization of diphenylhexatriene in liposomes and microsomes

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Pyrene excimer/monomer (E/M) ratios have been compared with the steady-state fluorescence polarization (P) of diphenylhexatriene (DPH) in multilamellar liposomes of dilaurylphosphatidylcholine and rat liver microsomes. The purpose was to use the well-understood properties of DPH to reveal the nature of bilayer fluidity which pyrene manifests as an E/M ratio. Reducing the temperature (from 37°C to 8°C), increasing the hydrostatic pressure (from 0.1 to 70 MPa), and, in liposomes, cholesterol enrichment (up to 0.30 mole fraction) separately decreased the E/M ratios and increased P. The pyrene membrane/buffer partition coefficient was affected by temperature but not by pressure, and in the case of cholesterol enrichment, it was assumed to be unaffected. Plots of P as a function of the E/M ratio showed the two to be closely correlated ($r = 0.99$ in liposomes and 0.96 in microsomes), independent of the treatment used to reduce fluidity. The apparent activation volume and enthalpy for excimer formation was calculated and compared with published data. Pyrene E/M ratios probably reflect the intermolecular volume (fluidity) of the outer region of the bilayer, which is reduced by a decrease in temperature and an increase in pressure and cholesterol. DPH reports the bilayer interior, which is similarly ordered by the experimental treatments. The regional distinction between the two probes, however, accounts for the divergence of E/M ratios and P, which has been reported in membranes enriched with fluidizing fatty acids.

Introduction

Fluorescence polarization spectroscopy has proved a particularly useful technique for determining membrane fluidity, i.e., the degree of structural ordering of lipid bilayers. The technique depends on understanding the photophysical properties, motional characteristics and location of the fluorescent probe in the bilayer. DPH (diphenylhexatriene) is one such probe, the steady-state fluorescence polarization (P) of which provides information on the structural order of bilayers. Pyrene is another probe whose steady-state emission intensity provides a rather less well characterised index of fluidity. The ratio of the excimer (dimer)/monomer fluorescence intensities has been regarded as a measure of the resistance

Abbreviations: E/M ratio, eximer/monomer ratio; DPH, diphenylhexatriene; P, steady-state fluorescence polarization; DLPC, dilaurylphosphatidylcholine; ANS, 8-anilinonaphthalene 1-sulphonate.

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which a bilayer offers to the lateral diffusion of the monomer [1]. Recently, two criticisms of this interpretation have been published. One involves the binding of pyrene to proteins in natural membranes [2], and the other rejects the view that the excimer/monomer (E/M) ratio is determined by the diffusion-controlled collisional frequency of the monomers, even in a pure phospholipid bilayer [3]. Instead, the E/M ratio may reflect the localised aggregation of pyrene, and hence fluidity, which may bear little relationship to lateral diffusion.

In order to evaluate pyrene as a fluorescent probe which can be used to measure membrane fluidity with inexpensive steady-state fluorescence equipment (in field laboratories and on research ships, for example [4,5]) this paper compares pyrene E/M ratios with the fluorescence polarization of DPH in two different membranes. Liposome bilayers of dilaurylphosphatidylcholine (DLPC) were used and their fluidity was altered by varying temperature, hydrostatic pressure and cholesterol content. Rat liver microsomes were also studied and their E/M ratios compared with DPH fluorescence polarization, both as a function of temperature and pressure. Particular attention has been given to the concentration-dependent nature of the pyrene E/M ratio and to the effect of pressure and temperature on the membrane/buffer partition coefficient of pyrene.

Methods

Multilamellar liposomes were prepared from DLPC (Koch Light) and cholesterol (Sigma) by vortex-mixing at 50 °C, 2.5 mg lipid/ml, 100 mM NaCl solution, made from triple distilled water. The suspension was stored at 5 °C for 24–72 h before dispersing to an absorbance at 540 nm of 0.1 immediately prior to use.

Liver microsomes were prepared from rats using established methods [6]. Each batch of microsomes was stored at −25 °C and used within 1 month. Unfrozen microsomes gave a slightly higher E/M ratio than frozen membranes, but the effect of temperature on the ratio was the same. The purity of the microsomes was checked by electron microscopy.

Membranes were suspended in a solution comprising 220 mM sucrose/22.8 mM Tris-HCl/1.07 mM EDTA/1 mM DL-dithiothreitol (pH 7.4) (37 °C) and were stored at −25 °C for no longer than 4 weeks prior to use. The protein and phospholipid contents of the microsomes were also determined [7–9]; the latter was 0.41 ± 0.039 μmol ($n = 4$) phosphate/mg protein.

Pyrene

Pyrene (Sigma) in ethanol was added to a suspension of DLPC liposomes at a ratio of 6 mmol pyrene per mol lipid. The mixture was equilibrated for at least 1 h at room temperature before use.

To a suspension of microsomes (0.2 mg protein or 80 nmol phospholipid phosphate per ml) at an absorbance at 540 nm of 0.1, pyrene was added in a solution of ethanol at a ratio of 25 mmol pyrene per mol phospholipid phosphate. The stirred suspension was equilibrated with pyrene for 1 h at room temperature. The final concentration of ethanol, as in the liposomes, was without effect. The E/M ratio in both types of membrane at 37 °C at normal pressure varied linearly with pyrene concentration with similar slopes. A 10% increase in the pyrene concentration in the suspension caused a 6% increase in the E/M ratio. The concentration of pyrene used was similar to that used by others and it ensured an adequate fluorescence intensity in all the experimental treatments, especially in the pressure vessel whose quartz windows (see below) significantly reduced the light reaching the photomultiplier in the spectrometer. Both types of membrane suspension were degassed in a vacuum after equilibration with pyrene. Samples were withdrawn from the bottom of the suspension and transferred to 1-cm light-path quartz cuvettes. The subsequent influx of atmospheric oxygen (which quenches the fluorescence) had negligible effect on the E/M ratio.

Fluorescence was measured with a Perkin Elmer LS3 spectrometer. Following the procedure cited in Ref. 10, excitation at 320 nm was used, with the excimer emission measured at 472 nm and the monomer emission at 392 nm. In addition, an excitation wavelength of 340 nm was used, with excimer emission at 480 nm and monomer emission at 390 nm. Although the two excitation wavelengths gave slightly different E/M ratios, there

was no significant difference between the two in the manner in which the E/M ratios varied with experimental treatment.

The pressure vessel required for the pyrene measurements comprised a square stainless steel block with a centre well and two cylindrical quartz windows of 8 mm diameter (Optical Works, Ealing, London) set at 90° to each other and sealed by the unsupported area method. The whole apparatus was aligned to the 'L' format light path of the spectrometer. Helium gas was used to pressurise the microsomal suspension which was contained in a bottle-shaped quartz cuvette of 8 mm diameter with a neck of 3 mm internal diameter and 7 mm length. The pressure of helium was controlled by either down-regulating a stock cylinder or intensifying the stock supply by means of a hydraulic pump [11]. The temperature of the pressure vessel was controlled to ± 0.2°C by water circulating through holes bored out of the steel block.

The helium which dissolved in the top of the suspension had no effect on the fluorescence measured in the light path some 7 mm below the meniscus, but it limited the sequence of pressures used. Decompression was limited to a maximum of 90% to avoid the possible release of gas bubbles in the light path. E/M ratios at normal atmospheric pressure were the same whether determined in the pressure vessel or in conventional cuvettes.

Artefacts

Hydrostatic pressure increases the activity of dissolved oxygen and might therefore initiate oxygen quenching [12]. Here it is assumed to be without effect because the p_{O_2} in the suspensions was very low.

Oil-water partition coefficients (K) vary with temperature and pressure according to the enthalpy and volume changes arising from the movement of the solute between the two solvents., i.e.,

$$\frac{d \ln K}{dT} = \frac{\Delta H}{RT^2} \text{ and } \frac{d \ln K}{dP} = \frac{-\Delta V}{RT}$$

Because the E/M ratio is dependent on the concentration of pyrene in the membrane, changes in the partition coefficient arising from the temperature or pressure treatment were measured (cholesterol is assumed to be without effect).

The effect of temperature on the partition of pyrene between DLPC liposomes and the 100 mM NaCl solution was determined by confining 8 ml of liposomes, labelled with pyrene, in a bag of Visking dialysis tubing immersed in 100 mM NaCl solution. Both the contents of the bag and the outer solution were stirred. The concentration of pyrene in the outer solution was determined at intervals over 4 h to equilibrium, at 37°C and 12°C, by measuring the fluorescence intensity of the monomer (excitation wavelength, 340 nm and emission wavelength, 390 nm). Appropriate checks were made on the binding of pyrene to, and the release of fluorescent compounds from, the Visking tubing. At 37°C, the fluorescence intensity of the liposome-free solution was 6% of that of the standard suspension of labelled liposomes, and at 12°C it was 2%. This means that cooling a suspension of liposomes from 37°C to 12°C increased the lipid/water partition coefficient, causing the E/M ratio to be slightly higher than would otherwise be the case.

The liposome suspension comprised approximately 0.005% lipid by weight. Assuming the lipid : water partition ratio for pyrene is 60000 [10] it may be calculated that the concentration of pyrene in the bilayer lipid was $1.5 \cdot 10^{-3}$ g/g and in the aqueous phase it was $2.5 \cdot 10^{-8}$ g/g. A reduction in the concentration of pyrene in the aqueous phase to one-third by lowering the temperature from 37°C to 12°C causes a 20% increase in the concentration of pyrene in the bilayer lipid. From the dependence of the E/M ratio on the concentration of pyrene in the liposome suspension (above) the effect of such a concentration change would be to increase the ratio by 12% (see Fig. 1). The effect of pressure on the partition of pyrene between DLPC liposomes and the aqueous phase was not directly measured, but was assumed to be similar to that of microsomes, i.e., negligible (see below).

The distribution of pyrene between microsomes and their aqueous phase was treated as a microsome/supernatant ratio. For present purposes, microsomes are defined as material which sediments at a given centrifugal force and the supernatant may contain particles of hydrophobic material, as indeed was noted in Ref. 3.

TABLE IA

EFFECT OF TEMPERATURE ON THE DISTRIBUTION OF PYRENE BETWEEN MICROSOMES AND THEIR SUPERNATANTS AT THE CENTRIFUGALLY GENERATED PRESSURE OF 79 MPa

The data are fluorescence intensities of high speed supernatants from microsomal suspensions. They were measured at atmospheric pressure and at the temperature of prior centrifugation and are expressed as mean percent (±S.D.) of the intensity of control microsomal suspensions measured at the matching temperature. Excitation wavelength, 320 nm; excimer emission, 472 nm, monomer emission, 392 nm (in parentheses; excitation wavelength of 340 nm; excimer 480 nm, monomer 390 nm). n, number of experiments.

	Fluorescence intensity (% of control)					
	37 °C		25 °C		13–17 °C	
Excimer wavelength	5.8±1.1	$n=4$	5.05±0.52	$n=4$	6.8±1.4	$n=5$
	(4.6±0.8)	$n=4$	(3.85±0.29)	$n=4$	(5.7±1.1)	$n=5$
Monomer wavelength	23.6±2.2	$n=4$	16.9±2.24	$n=4$	12.5±1.1	$n=5$
	(18.4±1.7)	$n=4$	(11.7±1.9)	$n=4$	(9.3±0.25)	$n=3$

TABLE IB

EFFECTS OF CENTRIFUGALLY GENERATED PRESSURE ON THE DISTRIBUTION OF PYRENE BETWEEN MICROSOMES AND SUPERNATANT

The data are fluorescence intensities of the supernatant, expressed as percent of the intensity of the microsomal suspension, each measured at 13 °C, normal pressure. The results of two experiments, a and b, are shown. Excitation wavelength, 320 nm;excimer emission, 472 nm, monomer emission, 392 nm (in parentheses: excitation wavelength 340 nm;excimer, 480 nm; monomer, 390 nm).

		Fluorescence intensity (% of control)	
		low speed, 10 MPa	high speed, 79 MPa
Excimer wavelength	a	5.7 (3.6)	7.3 (4.8)
	b	4.3 (3.0)	5.2 (3.9)
Monomer wavelength	a	3.0 (1.7)	3.7 (2.2)
	b	2.2 (1.4)	2.5 (1.8)

The effects of temperature and pressure on the fluorescence of pyrene in microsomes and their supernatants were determined by centrifuging the microsomes and measuring the fluorescence intensity in the supernatant at a standard temperature. The effect of varying the temperature was obtained by centrifuging suspensions at 30000 rpm at 37 °C and at a temperature range of 13–17 °C. The supernatants separated from the sedimented membranes at the high pressure generated by the combined action of the centrifugal force and the density of the suspending solution. This pressure was 79 MPa, calculated from $w^2\ \rho(x^2 - x^2o)/2$, in which w is the angular velocity in radians $\cdot\ s^{-1}$, ρ is density, and constant x is the distance from the bottom of the sample to the centre of rotation and o is the distance between the sample's meniscus and the centre of rotation [13]. Table IA shows the supernatants contained varying amounts of pyrene, whose fluorescence was between 5 and 23% of the corresponding emission from the original microsomal suspension. The temperature of centrifugation affected this significantly; the monomer intensity at 37 °C, 23.6% of the corresponding emission from the original microsomal suspension, declined to 12.5% at 13–17 °C, and the excimer intensity showed a negligible change over the same temperature range. The former implies that low temperature increases the microsomal/supernatant partition coefficient, at 79 MPa. It is assumed to similarly increase at normal pressure.

The effect of pressure at constant temperature on the partitioning of pyrene between microsomes and supernatant was determined by centrifuging at 30000 rpm for 1 h and at 10000 rpm for 18 h (which yielded a firm pellet). The supernatants thus obtained separated from the membranes at 79 MPa and 10 MPa, respectively. Because of the length of time involved, centrifugation was carried out only at 13 °C.

Table IB shows that the fluorescence intensity of the high speed, high pressure supernatant is

TABLE II

EFFECT OF TEMPERATURE ON THE LIGHT SCATTERING AND ENDOGENOUS FLUORESCENCE INTENSITIES OF UNLABELLED SUSPENSIONS OF MICROSOMES

Data are mean percent (±S.D.) of the intensity of control microsomal suspensions labelled with pyrene, at matching temperatures. Excitation wavelength, 320 nm; excimer emission, 472 nm; monomer emission, 392 nm (in parentheses: excitation wavelength, 340 nm; excimer, 480 nm; monomer, 390 nm).

	Fluorescence intensity (% of control)					
	37°C		25°C		13–17°C	
Excimer wavelength	10.0±1.9	$n=6$	9.2±1.5	$n=4$	7.75±4.2	$n=4$
	(7.5±1.8)	$n=4$	(6.7±1.7)	$n=4$	(7.2±1.3)	$n=5$
Monomer wavelength	5.0±1.05	$n=6$	6.5±2.5	$n=4$	3.1±0.33	$n=4$
	(3.8±0.5)	$n=4$	(3.9±1.1)	$n=4$	(2.4±0.32)	$n=5$

very similar to that of the lower speed, low pressure supernatant, and both are small in relation to the fluorescence obtained from the suspension. The conclusion is that 79 − 10 MPa i.e. 69 MPa pressure has no significant effect on the distribution of pyrene between microsomes and their supernatant at 13°C. This is assumed to apply over the temperature range used in the present study, and in DLPC liposomes.

The contribution which scattering and endogenous fluorescence from the microsomes made to the fluorescence intensities of pyrene-labelled suspensions was also examined as a function of temperature and pressure. Typically, the contribution was between 2 and 10%, depending on the excitation wavelength, but their temperature dependence was small (Table II) and their pressure dependence negligible (not shown).

TABLE III

COMBINED CORRECTION FOR THE EFFECTS OF TEMPERATURE ON THE PYRENE FLUORESCENCE OF MICROSOMES AND THEIR SUPERNATANTS (TABLE IA) AND ON THE SCATTERING AND ENDOGENOUS FLUORESCENCE OF MICROSOMES (TABLE II)

Data are mean percent of the intensity of control microsomal suspensions and are to be deducted from the measured intensities. The numbers are for excitation wavelengths of 320 nm, excimer emission 472 nm, monomer emission 392 nm (in parentheses: excitation wavelength, 340 nm; excimer, 480 nm, monomer, 390 nm).

	Fluorescence intensity (% of control)		
	37°C	25°C	13–17°C
Excimer	15.8 (12.1)	14.2 (10.5)	14.5 (12.9)
Monomer	28.6 (22.2)	23.4 (15.6)	15.6 (11.9)

Corrected E/M ratios were obtained from microsomes by deducting the excimer and monomer intensities given in Table III, expressed as percentages. Note that the corrections are for the combined temperature effects on (i) partitioning between microsomes and their supernatants (Table IA) and (ii) for the scattering and endogenous fluorescence (Table II). For example, the excimer correction at 37°C is −15.8%, comprising 5.8% partitioning (Table IA) and 10% scattering (Table IIA). The combined corrections for excimer and monomer fluorescence at 13–17°C were the same, i.e. no correction was required to calculate the required E/M ratio.

Corrected E/M ratios thus obtained were normalised to eliminate variation arising from minor errors in the concentration of pyrene used in different experiments. Thus, corrected E/M ratios were expressed as a percentage of the value obtained at 37°C under normal pressure (see Results).

Diphenylhexatriene

1,6-Diphenyl-1,3,5-hexatriene (DPH) (Aldrich, puriss grade) was added in a solution of tetrahydrofuran, to a suspension of membranes of an A_{540} of 0.1, to give a final concentration of 2 μM in the suspension. The fluorescence polarization of the suspension was measured with a Perkin-Elmer LS-3 spectrometer or, at high pressure, with a T-format spectrometer (Applied Photophysics) as previously described [5]. Measurements at atmo-

spheric pressure used conventional 1 cm light path quartz cuvettes, whilst those at high pressure used a round quartz cuvette sealed with a flexible plastic plug, and placed in the centre well of a stainless steel, high pressure vessel. This vessel was fitted with 4 cylindrical quartz windows of 8 mm diameter set at 90°, and each was sealed by the unsupported area principle. The cuvette was immersed in ethanol which transmitted the pressure generated by a hydraulic pump, via a water/ethanol piston-separator. Pressure was measured with a calibrated Bourdon tube gauge. The temperature of the pressure vessel was maintained constant (±0.1 C°) by water circulating through holes bored in the steel.

Errors in the DPH polarization measurements

Small variations in the concentration of DPH in the bilayer do not exert significant influence on polarisation values. Accordingly, there is no need to correct for changes in the membrane/buffer partition ratio of DPH. Unlabelled suspensions and the supernatant from suspensions labelled with DPH emitted negligible fluorescence. Polarization was calculated from $(I_{\parallel} - I_{\perp})/(I_{\parallel} + I_{\perp})$.

Results

Liposomes

The mean uncorrected E/M ratio at 37°C, normal pressure was 0.172 ± 0.025 (S.D.) ($n = 10$, excitation wavelength 340 nm, emission wavelengths 480 and 390 nm). The effects of low temperature, high pressure and cholesterol enrichment on the E/M ratios are shown in Fig. 1. Linear regressions are drawn in Fig. 1 (and in all subsequent figures) to enable selected data to be conveniently interpolated (see below). Lowering the temperature from 37 to 22.6°C reduced the E/M ratio to 66%. Increasing the pressure to 54 MPa, at 37°C, also reduced the E/M ratio to 66%, as did increasing the mole fraction of cholesterol to 19%.

The steady-state fluorescence polarization of DPH was increased by lowering the temperature or increasing the cholesterol content to the extent shown in Fig. 2. Polarization of DPH in liposomes was not measured as a function of pressure. However, from published data [14] for the pressure coefficient of DPH anisotropy in DMPC and DPPC liposomes at 20°C above their transition

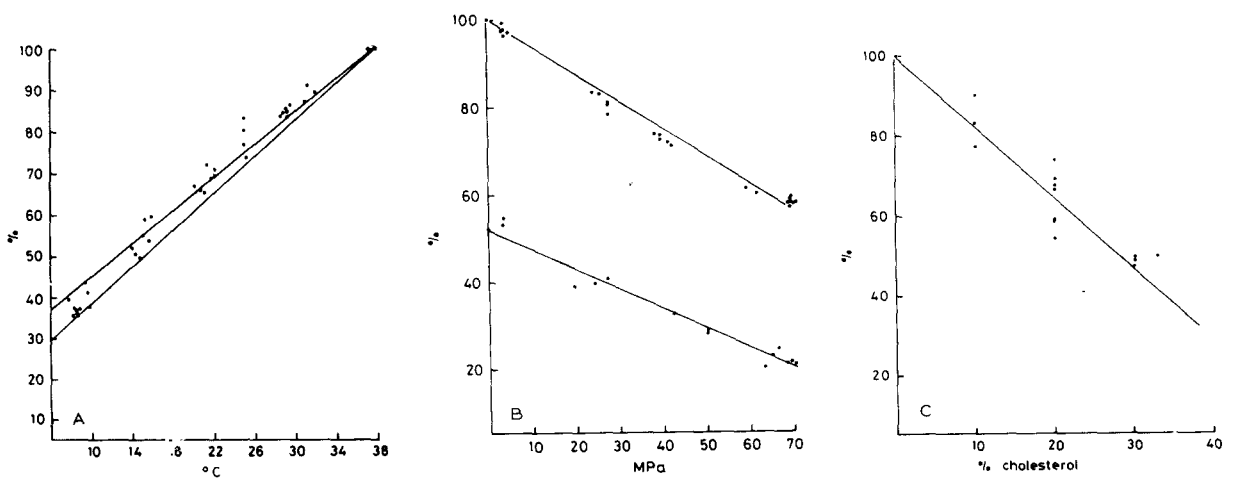

Fig. 1. Pyrene E/M ratio in DLPC liposomes, expressed as % of the ratio at 37°C, normal pressure (excitation 340 nm; emission 480 and 390 nm). Each point represents one determination. The lines represent linear regressions according to the constants given below. (A) E/M ratio as a function of temperature. $A = 23.754$, $B = 2.0836$, $r = 1.043$. 10 separate liposome suspensions. The upper line is the linear regression; the lower line shows the correction offsetting the consequences of low temperature increasing pyrene's membrane/buffer partition coefficient. (B) E/M ratio as a function of pressure. Upper line 37°C; $A = 98.88$, $B = -0.6087$, $r = 0.9947$. Lower line 15°C; $A = 52.57$, $B = -0.4702$, $r = 0.975$, for three separate liposome suspensions. (C) E/M ratio as a function of cholesterol, expressed as % mole fraction. $A = 99.74$, $B = -1.769$, $r = 0.982$, for seven liposome suspensions.

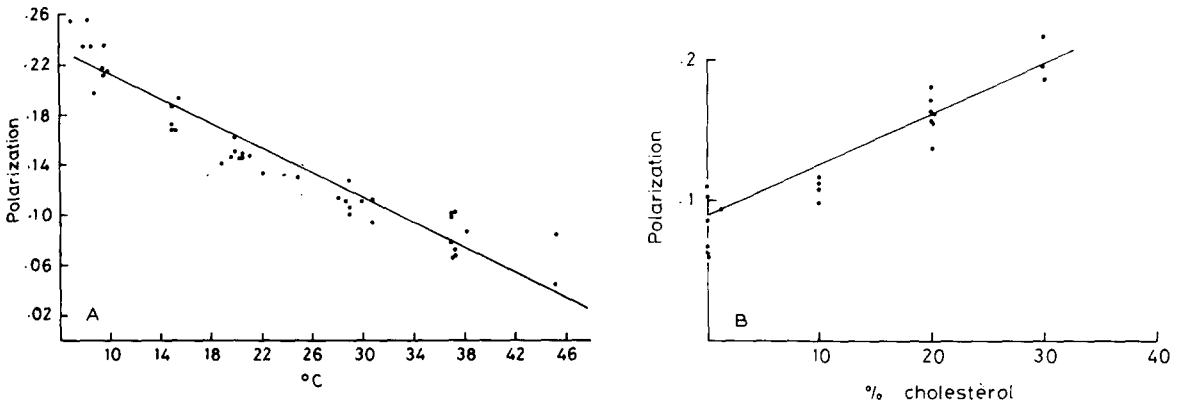

Fig. 2. Fluorescence polarization of DPH in DLPC liposomes as a function of (*A*) temperature and (*B*) cholesterol (% mole fraction) at 37 °C. Each point represents a single determination. The lines represent linear regressions, according to: (A) $A = 0.2584$, $B = 0.0048467$, $r = 0.9541$ (nine liposome suspensions); (B) $A = 0.0894$, $B = 3.4742$, $r = 0.9315$ (six liposome suspensions).

temperature, a value of $8.75 \cdot 10^{-4}$ anisotropy units/MPa may be estimated. Using this value, the polarization of DPH in DLPC liposomes, at 37 °C, at 50 MPa and 70 MPa is 0.142 and 0.165, respectively.

The correlation between the corrected *E/M* ratios and DPH polarization values is shown in Fig. 5. Arbitrarily widely spaced values of *E/M* and polarization were taken from the regressions in Figs. 1 and 2. For example, the data for

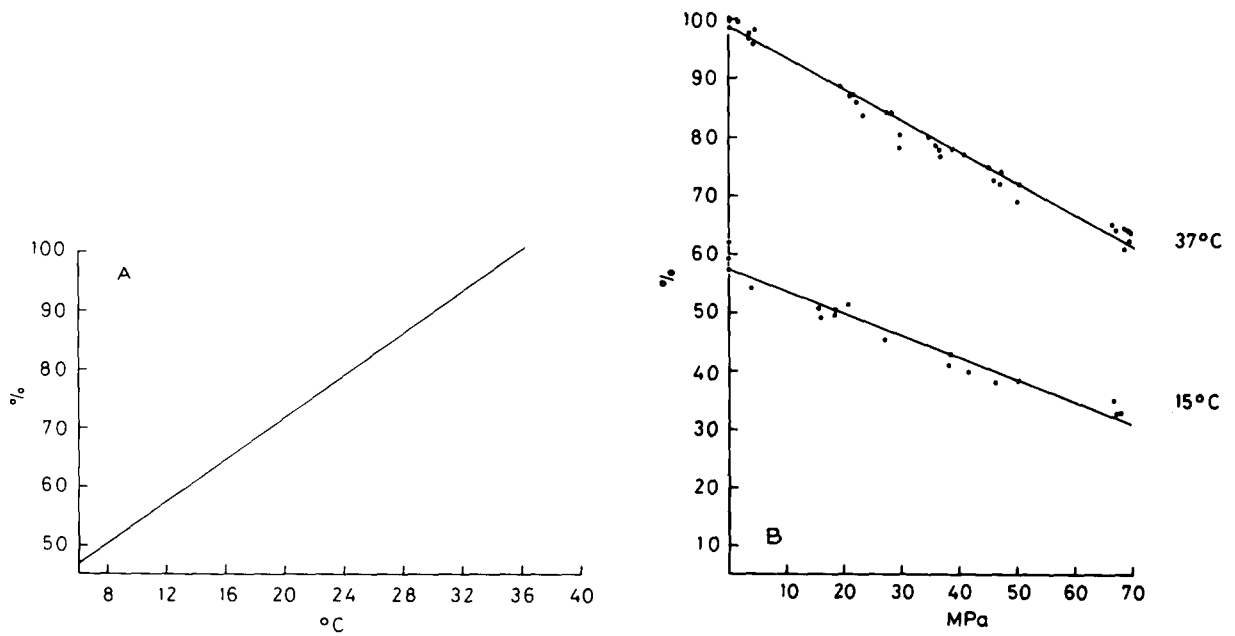

Fig. 3. Pyrene *E/M* ratios in microsomes as a function of temperature (*A*) and as a function of pressure (*B*) (excitation, 320 nm; emission, 472 and 392 nm). (A) Corrected *E/M* ratios, as percent of the ratio at 37 °C, as a function of temperature. The line represents the linear regression according to $A = 35.6369$, $B = 1.7919$, $r = 9898$ (16 microsome suspensions, 66 individual data points omitted for clarity). (B) Corrected *E/M* ratios, expressed as percent of the ratio at 37 °C, atmospheric pressure, plotted against presure. Each point is one determination. The lines represent linear regressions according to, at 37 °C, $A = 98.7215$, $B = -0.5360$, $r = 0.9932$ and at 15 °C, $A = 57.176$, $B = -0.3717$, $r = 0.979$ (five microsome suspensions).

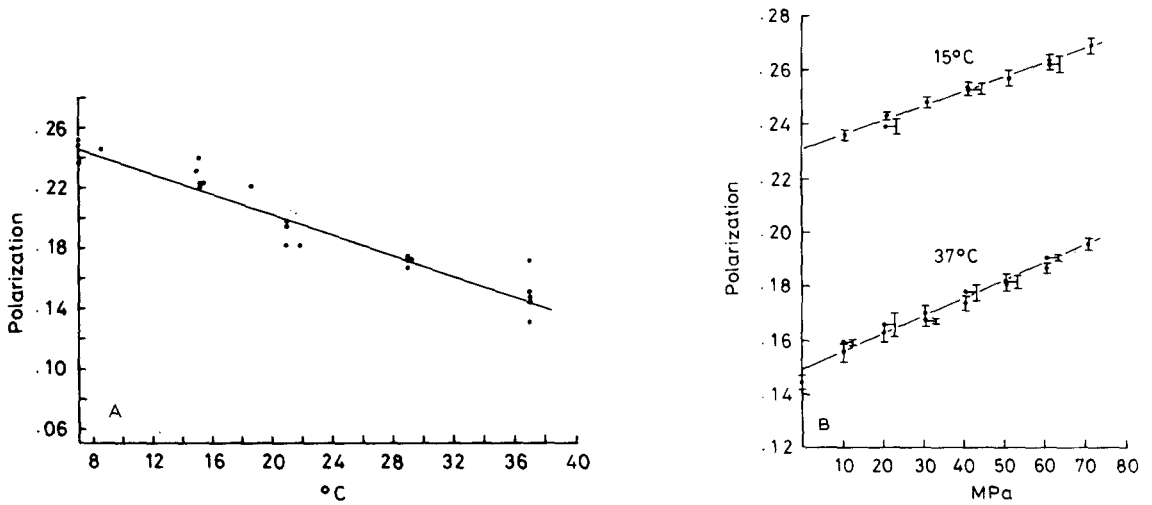

Fig. 4. Fluorescence polarization of DPH in microsomes as a function of temperature (A) and pressure (B). (A) Each point represents one determination.. The line is the linear regression according to $A = 0.2707$, $B = -0.00345$, $r = 0.9712$ (five microsome suspensions from three batches). (B) Each point represents the mean ± S.E. of three determinations on one batch of microsomes. The line is the linear regression according to $A = 0.231$, $B = 0.054$, $r = 0.984$, (15 °C); $A = 0.149$, $B = 0.065$, $r = 0.973$.

DLPC/30% cholesterol liposomes with a polarization of 0.19 (Fig. 2B) and an E/M ratio of 46% (Fig. 1C) is plotted as a single point in Fig. 5. Also DLPC liposomes (37 °C, normal pressure) with an E/M ratio of 100% and a polarization of 0.079 (Figs. 1a, 2a) acquired, at 70 MPa, an E/M ratio of 55% (Fig. 1B) and a polarization value of 0.165 [14], and this is plotted as two points. Fig. 5 shows that the correlation between the corrected E/M values and DPH polarization ($r = 0.991$) is independent of the means by which the liposome bilayer is ordered.

Microsomes

Using an excitation wavelength of 320 nm (emission 472, 392 nm) the mean corrected E/M ratio at 37 °C, normal pressure, was 0.317 ± 0.049 (19 separate determinations from a total of three batches of microsomes). The ratio was reduced to 66% by lowering the temperature to 16.8 °C or increasing the pressure at 37 °C to 60 MPa (Fig. 3). Excitation at 340 nm (emission 480, 390 nm) yielded a mean E/M ratio of 0.273 ± 0.04 ($n = 17$), with a similar temperature coefficient. Steady-state polarization varied linearly as a function of temperature and pressure (Fig. 4). The correlation between the E/M ratios and DPH polarization, each varied by pressure and temperature, is shown in Fig. 5, whose derivation is explained above. The correlation ($r = 0.961$) is independent of the means by which the fluidity of the microsomal membrane was varied.

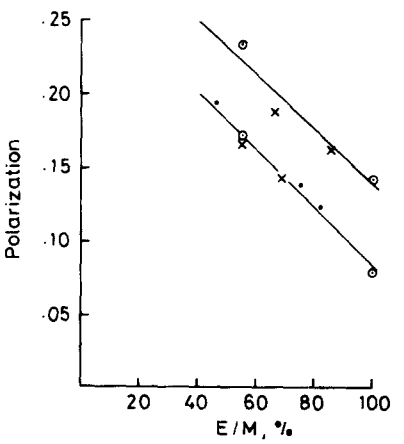

Fig. 5. Pyrene E/M ratios correlated with fluorescence polarization of DPH in DLPC liposomes (lower line) and microsomes (upper line) whose fluidity is varied by means of temperature, ⊙; pressure, ×; and cholesterol•. Data from previous figures and Ref. 14 (see text). The lower line represents a linear regression according to $A = 0.28057$, $B = 0.001975$, $r = 0.9911$. The upper line is according to $A = 0.3257$, $B = -0.00187$ and $r = 0.961$.

Discussion

The E/M ratio in the microsomes (37 °C, 340 nm, excitation wavelength) was 0.273 compared to 0.172 in the DLPC liposomes. This higher ratio is much less than would be expected from the 4-fold greater pyrene/lipid ratio in the microsomes (see methods). Therefore, excimer formation in the microsomes appears to be impeded, perhaps by the nature of the bilayer or by the binding of some of the pyrene to membrane proteins [2]. Nevertheless, the E/M ratio and DPH polarization vary with experimental treatment in much the same way in the two membranes, consistent with the notion that the amount of protein-bound pyrene in the microsomes is constant (Fig. 5).

The effects of temperature and pressure on the E/M ratios are similar to published data, in which the ratio has been interpreted as a measure of pyrene's lateral diffusion. For example, the E/M ratio as a function of temperature in DLPC liposomes, crab nerve, *E. coli* membranes, and, to a lesser extent, human erythrocyte membranes, are similar to the data here [15–18]. E/M ratios as a function of pressure have been reported for human erythrocyte membranes (31 °C, 0.1–80 MPa) and they also are comparable to those reported here [19].

Table IV summarises these data using activation parameters. Because the rate-limiting step in excimer formation remains to be clearly resolved (between the earlier view that it is determined by the diffusion-controlled collisional frequency of the monomers [1] and the recent view that it lies in a 'mixed lattice' mechanism [3]), Table IV refers only to apparent $\Delta V^{\ddagger}$ and $\Delta H^{\ddagger}$. It shows that the former are relatively large and increase with the order of the bilayer and the latter are relatively small and constant.

The reduction of E/M ratios and the increase in DPH polarization caused by the addition of cholesterol to DLPC liposomes (Fig. 1) is also similar to published data [15,19]. Although the present experiments did not vary the cholesterol content of microsomes, others [20] have shown that rat liver microsomes, enriched with cholesterol

TABLE IV

APPARENT $\Delta V^{\ddagger}$ AND $\Delta H^{\ddagger}$ FOR PYRENE EXCIMER FORMATION IN VARIOUS MEMBRANES

$\Delta V^{\ddagger}$ ml·mol^{-1} over 0.1–70 MPa	$\Delta H^{\ddagger}$ kcal·mol^{-1}, normal pressure (temperature)	Membrane bilayer (temperature range)	Reference
83 (40 °C) 0.1–15 MPa	4.19 (40 °C)	sonicated liposomes of DPPC + 0.17 mole fraction cholesterol (60–40 °C)	48 [b]
26 (39 °C)	6.65 (31 °C)	human erythrocyte, (31–14 °C)	18 [a]
27.9 (15 °C)	–	multilamellar liposomes DLPC	this paper
20.5 (37 °C)	4.36 (37 °C)	multilamellar liposomes DLPC (37–25 °C)	this paper
–	4.22 (37 °C)	sonicated liposomes DLPC (40–10 °C)	15 [a]
21.3 (15 °C)	–	rat liver microsomes	this paper
18.6 (37 °C)	3.33 (37 °)	rat liver microsomes (37–25 °C)	this paper
–	5.19 (37 °C)	sonicated liposomes, *E. coli* extracts (40–10 °C)	17 [a]
–	4.99 (37 °C)	crab nerves (40–10 °C)	16 [a]
10 (23 °C)	2.11 (23 °C) [b]	toluene solvent	49

[a] From published graphs.
[b] Calculated from E_a.

to increase DPH polarization by 0.095 units, simultaneously showed a reduction in E/M ratio of 52%. This is similar to the relationship shown in Fig. 5.

There is a close correlation between the E/M ratios and DPH polarization, each varied by temperature, pressure, and in the case of liposomes, by the addition of cholesterol (Fig. 5). This correlation is all the more interesting because other treatments, notably incorporation of fatty acids, cause E/M ratios and DPH polarization values to diverge (see below). The explanation probably lies in the different regions of the bilayer occupied by the probes.

DPH is rod shaped, 1.15 nm long with a sphere of 0.25 nm diameter at each end [21,22]. DPH partitions into gel and liquid-crystalline phases equally [23], and it exhibits multi-exponential lifetimes in bilayers, consistent with it occupying different microenvironments [24]. From comparisons with TMA-DPH and other techniques, it is now thought that a proportion of the DPH molecules align approximately parallel to the acyl chains in a phospholipid bilayer, but a significant fraction may be distributed parallel to the bilayer surface, probably between the two monolayers [24–27]. Thus, the steady-state fluorescence polarization of DPH in bilayers, particularly in heterogeneous bilayers in natural membranes, is the sum of varied contributions, which provides an overall measure of the structural order of the interior of the bilayer [28–31]. It is significant that DPH does not occupy the more polar, glycerol region of a phospholipid bilayer [32,27] and it only reports structural changes which occur there if they indirectly affect the more hydrophobic bilayer interior.

In contrast, pyrene is thought to partition into the outer, relatively polar regions of lipid bilayers [2,33–36]. In particular, in sarcoplasmic reticulum membranes there is evidence that pyrene occupies a region with a polarity similar to that of ethanol or butanol [37] and taken with other evidence [38], this means that pyrene does not partition in the hydrophobic core nor at the polar surface of a lipid bilayer. E/M ratios are sensitive to changes in the polarity of the probe's environment [39] and its local concentration. The question therefore arises, 'do the experimental variables affect the E/M ratio through these two factors?'. The intramolecular excimer probe, di(1-pyrenylmethyl)ether ($PyCH_2OCH_2Py$), which is similar to pyrene in its sensitivity to polarity, has been studied in sarcoplasmic-reticulum membranes where it showed no sign of experiencing a change in polarity when the membranes were enriched with cholesterol [37]. The temperatures and pressures used here are unlikely to change the polarity of pyrene's environment, and, like cholesterol, certainly decrease the bilayer fluidity in a way which should reduce the collisional frequency of pyrene, and hence reduce E/M. As for the variables altering the local concentration of pyrene (as distinct from the lipid/buffer partition coefficient), it seems unlikely that significant changes occur and here it is assumed that such changes are unimportant.

Therefore, in the absence of any pressing reason to the contrary, and in view of the clear correlation between E/M ratios and DPH polarization, only fluidity or its correlate, free volume, is invoked to interpret the E/M ratios.

Pyrene's shape, an oval disc of approximately 1 nm length, renders it an effective intercalating probe in the ordered regions. Excimer formation is enhanced in DPPC bilayers treated with ANS which binds close to the choline headgroups [36] and separates them. Pyrene-3-sulphonate excimer formation has been measured in mitochondrial membranes and found to respond to the same structural changes as ANS. Accordingly, the excimer formation of pyrene 3-sulphonate in bilayers has been interpreted as an indicator of intermolecular volume and probe mobility rather than of viscosity [40]. More recently other experiments have led to the idea that pyrene excimer formation in bilayers may not be rate-determined by lateral diffusion through the bilayer and that it probably arises from localised aggregates of pyrene [3]. The greater the free volume available for the pyrene, the higher the steady-state level of excimer formation.

A decrease in temperature, an increase in pressure and the addition of cholesterol to bilayers exert a common effect, namely a reduction in the lateral intermolecular distance or volume between the constituents of each leaflet of the bilayer. The anisotropic contraction of bilayers by cooling and

by hydrostatic compression have been measured and shown to be largely lateral [41,42]. The pressure equivalent of cholesterol enrichment in DLPC bilayers estimated here is 100 MPa≡0.38 mole fraction cholesterol. This is similar to the pressure equivalent of cholesterol enrichment of DOPC liposomes, 100 MPa≡0.44 mole fraction [43]. Cholesterol, as is well known, intercalates in the outer region of fluid bilayers, increasing the molecular packing density and decreasing the molecular motion [44,45]. Thus, all three treatments, low temperature, high pressure and cholesterol enrichment, could inhibit pyrene excimer formation by reducing the intermolecular volume and motion in the outer region of the bilayer, where pyrene resides. The interior of the bilayers subjected to the same treatments also undergoes an increase in packing and reduction in motion, which is shown using DPH.

The distinction made here between the regions of the bilayer, shown by using pyrene and DPH, explains the striking effect of oleic-acid enrichment of lymphocyte membranes to decrease DPH polarization without affecting the pyrene E/M ratio [46]. Comparable, differential effects on the pyrene E/M ratio and DPH polarization are also seen in microsomes enriched with unsaturated fatty acids [50]. The fatty acid probably intercalates the bilayer with little increase in the packing density of the outer region (as for example with alkanols in DPPC bilayers [47]) and its *cis*-unsaturated acyl chain exerts a well-known fluidizing influence in the bilayer interior.

In conclusion, the pyrene E/M ratio provides a simple, steady-state fluorescence method for quantifying changes in the fluidity of the outer regions of bilayers and it is particularly sensitive to the ordering effect of pressure. However, its dependence on membrane concentration and the associated corrections which arise are a disadvantage. It remains to be seen whether the E/M ratio is determined by the collisional frequency of localized [3] or laterally diffusing [1] monomers.

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