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PRESSURE DEPENDENCE OF PYRENE EXCIMER FLUORESCENCE IN HUMAN ERYTHROCYTE MEMBRANES

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The intensity of pyrene excimer fluorescence in human erythrocyte membranes and in sonicated dispersions of the membrane lipid (liposomes) was examined as a function of pressure (1–2080 bar) and temperature (5–40°C). Higher pressure or lower temperature decreased the excimer/monomer intensity ratios. A thermotropic transition was detected in both membranes and liposomes by plots of the logarithm of the excimer/monomer intensity ratio versus 1/K. The transition temperature of the membranes was 19–21°C at 1 bar and 28–31°C at 450 bar, a shift with pressure of approx. 20–22 K per kbar. Corresponding transition temperatures of the liposomes were 21°C at 1 bar and 33°C at 450 bar, a shift of approx. 27 K per kbar. The observed pressure dependence of the thermotropic transition temperature is similar to that reported for phospholipid bilayers and greatly exceeds that of protein conformation changes. In concert with the liposome studies the results provide direct evidence for a lipid transition in the erythrocyte membrane.

Human erythrocyte membranes undergo a thermotropic transition at approx. 15–20°C as indicated by a variety of techniques, including viscosity measurements [1], light scattering [2], fluorescence methods [2–4], electron spin resonance [5], nuclear magnetic resonance [6], Raman spectroscopy [7] and positron annihilation [8]. Although some authors have ascribed the transition to the membrane lipids [1,6,7], the evidence for this conclusion is not definitive, inasmuch as total lipid extracts of the membranes have not consistently shown the transition [4,6]. To examine this question we have studied the effects of pressure on the transition temperature** as monitored by pyrene excimer fluorescence in erythrocyte membranes and in liposomes of the membrane lipid [4]. Prior studies demonstrate that the thermotropic transition temperatures of phospholipid dispersions exhibit pressure dependences of approx. 20–30 K per kbar, whereas the corresponding pressure dependence of protein conformation transitions is only 2–7 K per kbar [9–14].

Venous blood was collected from normal adult donors into EDTA-coated tubes and the erythrocytes were separated from the buffy coat by low-speed centrifugation and washed three times with

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** The term 'transition temperature' is used in this report to denote the break point temperature observed in an Arrhenius plot Given the complexity of the erythrocyte membrane lipids, it is unlikely that the break point temperature observed corresponds to one thermotropic transition of the sort observed in liposomes of one, chemically defined phospholipid; rather, the break point temperature probably corresponds to either the onset or completion of a broad transition, as documented for other mammalian membranes

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ten volumes of an isotonic 'wash buffer' (30 mM sodium phosphate, pH 7.4, 117 mM NaCl and 2.8 mM KCl). Washed ghost membranes were prepared by the method of Dodge et al. [15], using 8 mM sodium phosphate of pH 7.4 for lysis. Membrane suspensions containing 0.2 mg membrane protein per ml 'wash buffer' were incubated with shaking in the presence of 25–30 μM pyrene (Aldrich Chemical Co., 99 + %) for 2 h at 37°C. Pyrene was added from a 10 mM stock solution in absolute ethanol and the final ethanol concentration did not exceed 0.3%. Thereafter the membranes were washed twice with 50 vol. of 'wash buffer' and finally suspended to their initial concentration in the same buffer. Fluorimetric estimation of the pyrene content [16] indicated membrane concentrations of 80–90 nmol/mg protein. Erythrocyte membrane lipids were extracted by the method of Folch et al. [17], suspended in 'wash buffer' to a concentration of 0.2 mg/ml and sonicated at 0°C under N_2 for 10 min in a Branson sonifier (Branson Sonic Power Co., Model 350) as previously described [16]. Pyrene was incorporated by co-sonication at a final concentration of 15–20 μM probe. At the pyrene concentrations attained in both membranes and liposomes corrections for light scattering were insignificant. Fluorescence spectra were obtained in a SPEX Fluorolog fluorimeter using a stainless steel cell (Union Giken Engineering, Hirakota, Japan) with excitation and emission windows of quartz and a light path of 10 mm [18]. Excimer and monomer fluorescence intensities were estimated from the peak heights at 480 nm and 386 nm, respectively, using an excitation wavelength of 340 nm. The cell pressure, generated through an intensifier by means of an oil hand pump, could be maintained constant up to 10 h and was monitored by a precision Burdon-type pressure gauge (Heise, model CM 23235). Temperature control of the sample cell was maintained via a circulating water bath. Although the temperature within the sealed sample compartment was not monitored directly, it was found to correspond to the temperature of the water bath within 0.5°C at extreme temperatures. Prior reports indicate that the temperature dependence of pyrene excimer fluorescence in media of relatively high viscosity, such as lipid vesicles and membranes, reflects changes in the rate of dimer formation [19–21]. Therefore, the logarithm of the excimer/monomer intensity ratio was plotted as a function of 1/K (Arrhenius plot).

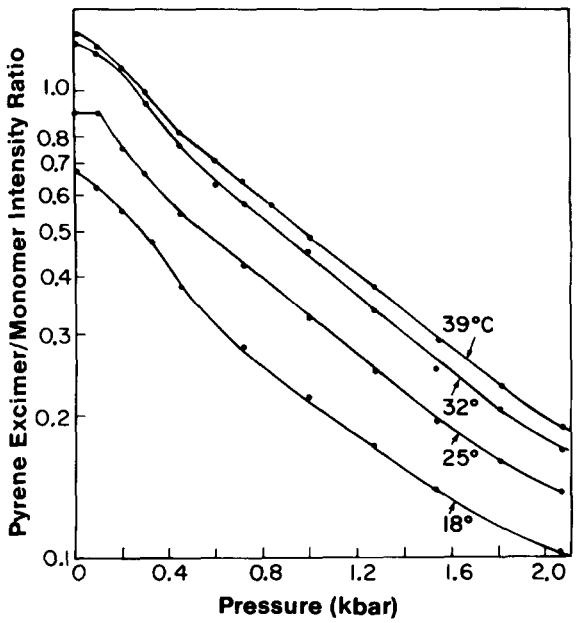

Fig 1 Effects of pressure on the pyrene excimer/monomer fluorescence intensity ratio in erythrocyte membranes studied at four different temperatures

The pressure dependence of pyrene excimer fluorescence in ghost membranes was studied in the temperature range 18–39°C and the results are illustrated in Fig. 1. At each temperature tested an increase in pressure from 1 bar to 2080 bar decreased the excimer/monomer intensity ratio by approx. 84–86%. To examine the temperature dependence of the excimer/monomer intensity ratio, membrane or liposome suspensions maintained at either 1 bar or 450 bar were cooled progressively from 40°C to 5°C. As illustrated in Fig. 2, a change or discontinuity in slope of the Arrhenius plot was observed for both membranes and liposomes at each of the pressures studied. The break points and apparent fusion activation energies are listed in Table I. At atmospheric pressure the intact membranes showed a transition temperature at 19–21°C, in agreement with prior observations [1–8]. Increase in pressure to 450 bar shifted the observed break point to 28–31°C, corresponding to an increase in transition temperature of 20–22 K per kbar (Table I). The liposome suspension gave

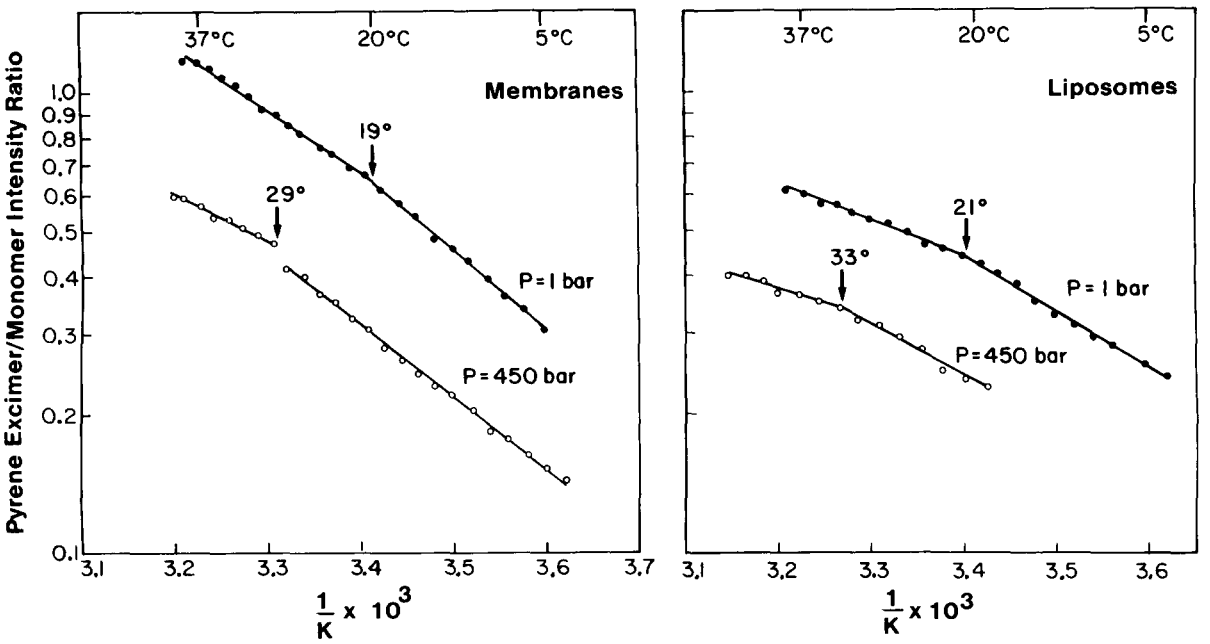

Fig 2. Temperature dependence of the pyrene excimer/monomer intensity ratio in erythrocyte membranes and in liposomes of the membrane lipid examined at 1 and 450 bar

comparable results, with break points at approx. 21°C and 33°C, respectively, at 1 and 450 bar (Fig. 2), corresponding to an increase in transition temperature of 27 K per kbar.

The pressure dependence of the thermotropic transition agrees well with that reported for phospholipid dispersions studied by light scattering [11], dilatometry [13], electron spin resonance [9,10], or fluorescence polarization [14], and it is considerably greater than the values reported for protein conformation changes [11]. In concert with the liposome studies (Fig. 2) the results clearly

TABLE I

EFFECTS OF PRESSURE ON THE THERMOTROPIC TRANSITION OF ERYTHROCYTE MEMBRANES AND LIPOSOMES

Values were obtained from the plots of the logarithm of the pyrene excimer/monomer intensity ratio versus 1/K, as illustrated in Fig. 2

Preparation	Pressure, P (bar)	Transition temperature, T_t (°C)	$\frac{\Delta T_t}{\Delta P}$ (K/kbar)	Apparent fusion activation energy (kcal/mol)	
				$T > T_t$	$T < T_t$
Membranes	1	21		5.1	6.4
	450	31	22	5.1	6.4
Membranes	1	19		6.4	7.8
	450	29	22	5.1	7.4
Membranes	1	19		6.4	6.9
	450	28	20	6.0	7.6
Liposomes	1	21		3.7	6.7
	450	33	27	2.8	5.1

demonstrate that the erythrocyte membrane transition is a lipid transition. The molecular events which underly this transition are unknown but apparently do not involve a highly cooperative liquid crystal-to-crystalline gel transition, inasmuch as differential scanning calorimetry has not consistently detected an enthalpic change [22]. Prior authors have suggested the occurrence of microstructural rearrangement of lipid domains [7,8] and a sudden change in free volume of the bilayer [8]. Whatever the precise mechanism, it is reasonable to suggest that at the transition temperature the erythrocyte membrane lipid undergoes a reordering which involves a notable change in entropy [23] but relatively little change in enthalpy, and that the pressure dependence of the change is similar to that of a phospholipid melting process. It is also noteworthy that the excimer/monomer intensity ratio of pyrene in the erythrocyte membranes and liposomes increased with temperature from 5°C to 40°C. These ratios are reported to decrease with temperature when the probe is examined in ionic detergents [18] or isotropic solvents [24], owing to the predominant effect of thermal dissociation of the excimer complex over the rate of lateral diffusion of the monomer. That no decrease in excimer/monomer ratio was noted at higher temperatures in our experiments indicates that in the relatively less fluid lipid environments of the erythrocyte membrane or liposomes the extent of excimer formation is controlled by the rate of lateral diffusion of pyrene monomers rather than by the rate of dissociation of the complex.

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