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The thermal behaviour of phosphatidylcholine-glycophorin monolayers in relation to monolayer and bilayer internal pressure

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A thermodynamic relationship which allows calculation of the internal pressure (P_i) of a monolayer has been derived, viz: $P_i = T(\partial\pi/\partial T)_A - \pi$. Surface pressure ($\pi$) - area ($A$) isotherms were determined for dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC) and a mixture of the latter with 0.28 mol% glycophorin, an intrinsic membrane protein. The isotherms (10°C–42°C) were used to obtain values of $(\partial\pi/\partial T)_A$. Under conditions in which a monolayer is believed to most closely model a bilayer membrane (i.e. 37°C and $A = 0.6$ nm^2/molecule) internal pressures were: DOPC = 0.32 N m^{-1}; DPPC = 0.13 N m^{-1} and glycophorin / DPPC = 0.36 N m^{-1}. The results do not support some measurements on amphiphile solubility in natural membranes which had been interpreted as evidence of a large increase in internal pressure, due to the intrinsic membrane proteins.

Introduction

The concept of internal pressure has been evoked to explain the observation that some amphiphilic molecules have an extremely low solubility in natural membranes compared to that in phospholipid bilayers [1–3]. An explanation for this was that natural membranes have a large internal pressure as a consequence of the presence of intrinsic membrane proteins. This provoked much discussion [4–7] since it cast doubt on the validity of lipid monolayer and bilayer systems as adequate models of membrane phenomena. More recent studies do not confirm the difference between natural and artificial membranes [8–11], but still the question of whether or not protein can alter the internal pressure of a bilayer remains.

Internal pressure, as applied to bulk matter, can be precisely defined in thermodynamic terms and is a measure of the magnitude of intermolecular forces. For a pure system there will be one type of molecular interaction, which can be governed by several forces – electrostatic and electrodynamic (e.g. the van der Waals force). In a protein-lipid monolayer new interactions can arise between the protein, lipid, water and ions present. Most pertinent is the frequent observation that lipid molecules are immobilised around the hydrophobic regions of intrinsic proteins [12]. If the internal pressure in these lipid annuli is significantly different to than in the bulk lipid, and if the membrane contained a substantial amount of intrinsic protein, then obviously its internal pressure would not be the same as that of a lipid bilayer of similar lipid composition. Since the lipid in these annuli do not participate in chain melting, because they are already dissordered, then the presence of intrinsic membrane protein might lead to a lower internal pressure.

To our knowledge only one measurement has been made of the internal pressure in a cell membrane. In this the solubility of six gases in erythrocyte membranes were determined [12]. The measured Bunsen coefficients (α) were converted to a solubility parameter (δ) with the aid of a α vs. δ plot constructed from standard tables. The δ values for the gases were similar, and averaged at 10.3 ± 0.4 cal$^{1/2}$ cm$^{-3/2}$, leading to δ^2 values which correspond to 4380 atmospheres. By making the questionable assumption that the gases did not dissolve in the membrane protein it was argued

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that the minimum δ value for the bilayer was 8.7 $cal^{1/2}$ $cm^{-3/2}$, leading to a 28% reduction in internal pressure.

We report here measurements on phospholipid and phospholipid monolayers containing the intrinsic erythrocyte membrane protein glycophorin. These lead to estimates of internal pressure and enable the effect of protein upon this parameter to be judged.

Theory

In the bulk state internal pressure can be defined [13] as the partial derivative of internal energy (U) with respect to volume at constant temperature, i.e. $(\partial U/\partial V)_T$. For an ideal gas $(\partial U/\partial V)_T = 0$, because by definition such a gas has no intermolecular forces. For monolayers and bilayers we can define an internal pressure in an analogous fashion by replacing volume with area. From the First Law of Thermodynamics for a reversible change in internal energy, arising from changes in entropy (S) and area (A) at constant temperature (T) and surface pressure (π)

$$U = T\,dS - \pi\,dA$$

$$\therefore \left(\frac{\partial U}{\partial A}\right)_T = T\left(\frac{\partial S}{\partial A}\right)_T - \pi \qquad (1)$$

The entropy term in Eqn. 1 can be replaced directly with a partial differential in surface pressure which follows from a two-dimensional analogue of one of Maxwell's relationships [14], hence

$$P_i = T\left(\frac{\partial \pi}{\partial T}\right)_A - \pi \qquad (2)$$

where the two-dimensional analogue of internal pressure is represented by P_i. Thus from measurements of surface pressure as a function temperature at constant area P_i may be calculated.

Materials and Methods

Human erythrocyte membranes were isolated from out-dated transfusion blood by the method of Dodge et al. [16]. Glycophorin was isolated from the membranes by using a deoxycholate-phenol extraction procedure [17]. Purity of the glycophorin was assessed from PAS-stained and Coomassie-stained polyacrylamide gel electrophoretograms. These showed only the bands characteristic of the glycophorins [18,19]. No attempt was made to separate the different genetic variants since these are structurally very similar [20,21].

Synthetic L-α-dipalmitoylphosphatidylcholine (DPPC) and L-α-dioleoylphosphatidylcholine (DOPC) were obtained from the Sigma Chemical Co. (London), they were specified to be 99% and 98% pure, respectively. The purity of the DPPC was confirmed by high performance liquid chromatography. All other reagents were of analytical grade; water was triply distilled, with the penultimate distillation from an alkaline permanganate solution.

Surface pressure-area isotherms were measured using a Langmuir (Teflon) trough. To ensure good temperature control the trough was enclosed in a double-skinned steel cabinet through which thermostat water was circulated, this was also pumped through a glass serpentine coil in the substrate. From our previous work [22] it was established that glycophorin does not desorb, even at surface pressures of 30 mN m^{-1}, when spread on 1.6 M ammonium sulphate at pH 7. This substrate was used throughout this study and was purified immediately before each measurement by an activated charcoal procedure [23]. To form mixed monolayers the glycophorin was always spread first from a solution of 152.2 μg ml^{-1} in 0.1 M NaCl, 20 mM phosphate (pH 7). Lipids were spread from a 2:8 (v/v) ethanol-petroleum ether (b.p. 40–60°C) mixture at a concentration of 0.48 mg ml^{-1}.

For mixed monolayers the area per lipid molecule was calculated assuming that all the film area was available to them. The real area per lipid cannot be found by subtraction of the area occupied by the glycophorin, obtainable from its isotherm, because we have no clear idea of how the molecules in the mixed film pack together. However, as our internal pressure calculations are based on isotherm data above surface pressures of 15 mN m^{-1} we expect only the hydrophobic transmembrane segment of the protein to be at the interface. Since area of this region of the molecule is about 4 nm^2, then the 355 DPPC molecules present for each glycophorin would occupy, at 20 mN m^{-1}, an area of 170 nm^2. Thus our calculated areas per lipid molecule are overestimated by about 2.5%.

Results and Discussion

The erythrocyte membrane contains 250000–500000 copies of glycophorin, and has an area of 145 μm^2 exposed to the blood plasma [23–25]. Assuming a phospholipid area of 0.5–0.7 nm^2 then the lipid to glycophorin molar ratio will be in the range 1100–410:1 in one half of the membrane. Since there are other intrinsic proteins in the membrane the lipid to protein ratio will be somewhat lower than this. Calculation of an exact lipid:protein ratio, even for a well characterised membrane like the erythrocyte's, is not possible. Furthermore, since the lipid–protein interactions which are most likely to alter the internal pressure occur around the transmembrane regions, and the fraction of these regions available for interaction will be confounded by intra- and intermolecular protein asso-

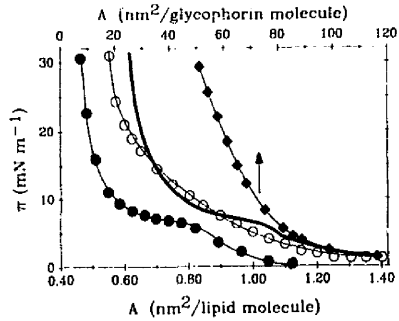

Fig. 1. Surface pressure (π)-area (A) isotherms for glycophorin (upper area axis, ♦), DPPC (●), and a 1:355 mixture of the two (○), together with a simulated curve for the mixed monolayer calculated from the π-A curves of the pure components assuming area additivity (———). All isotherms are at 20°C.

ciations [27,28], an exact ratio would be of little value. We have chosen a glycophorin:lipid ratio of 1·355.

Fig. 1 shows surface pressure–area isotherms for glycophorin, DPPC, a 1:355 mixture of the two and the simulated curve for the mixture assuming simple additivity of the component isotherms. The liquid expanded-liquid condensed (LE/LC) phase transition of the DPPC is still present in the simulated curve, whereas the measured curve shows that glycophorin–phospholipid interaction results in abolition of the phase transition. Differential scanning calorimetry on multilamellar liposomes incorporating glycophorin [28] has shown that the enthalpy of the gel-to-lamellar phase transition at 42.5°C (which has been regarded as analogous to the LC/LE transition in monolayers [30]) is reduced to a level consistent with the withdrawal of about 200 DPPC molecules per glycophorin. The extent of the glycophorin–lipid interaction in the monolayer at a molar ratio of 1:355 thus appears somewhat larger than in the bilayer. We have previously shown that glycophorin can immobilise up to 1250 DPPC at 30 mN m^{-1}, although this was at 20°C. At higher temperatures we would expect the number of DPPC immobilised to be less than this. On reducing the glycophorin to lipid molar ratio the LC/LE transition reappears [31].

Fig. 2 shows isotherms for mixed monolayers of glycophorin and DPPC (1:355) over the temperature range 10–42°C. For reference corresponding isotherms for DPPC and DOPC are shown in Fig. 3. The DPPC isotherms show the characteristic LC/LE phase transitions and are similar to those reported on other substrates [29,32]. The DOPC isotherms are slightly more expanded on 1.6 M ammonium sulphate than on a substrate of 0.1 M NaCl at 22°C [33]. Using the data in Figs. 2 and 3 plots of surface pressure versus temperature at constant area per molecule were made, Fig. 4. These were linear for glycophorin-DPPC, DOPC and

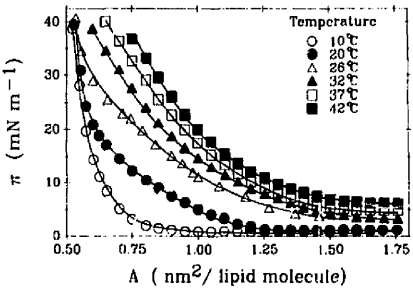

Fig. 2. Surface pressure (π) as a function of area per molecule (A) for mixed monolayers of glycophorin and DPPC at a molar ratio of 1:355 on 1.6 M ammonium sulphate (pH 7).

for DPPC above 22°C (except at areas below 0.65 nm^2/molecule). The slopes of the linear plots gave values of $(\partial\pi/\partial T)_A$ which are plotted as a function of A in Fig. 5. The data for DPPC spans the region of the LC/LE transition so that $(\partial\pi/\partial T)_A$ is lower than for the other two monolayers.

There have been several studies concerned with establishing the conditions (i.e. surface pressure/area per lipid) in a monolayer at the air/water interface which most closely correspond to those in a bilayer membrane [34–36]. From these we deduce that the behaviour of a bilayer system is very similar to that of a monolayer at surface pressures in the range 20–50 mN m^{-1}, with the higher figure being the more likely. The

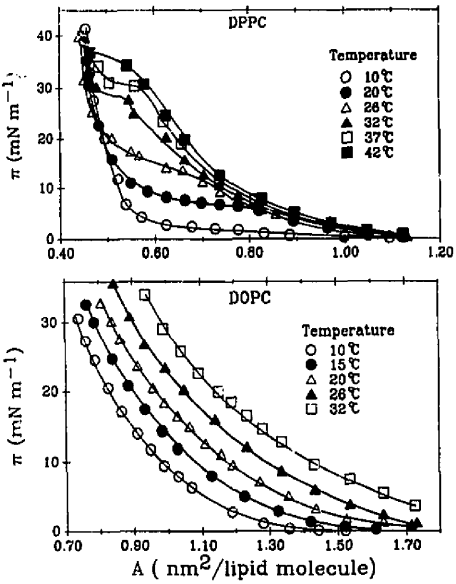

Fig. 3. Surface pressure (π) as a function of area per molecule (A) for DPPC (upper set) and DOPC (lower set) on 1.6 M ammonium sulphate (pH 7).

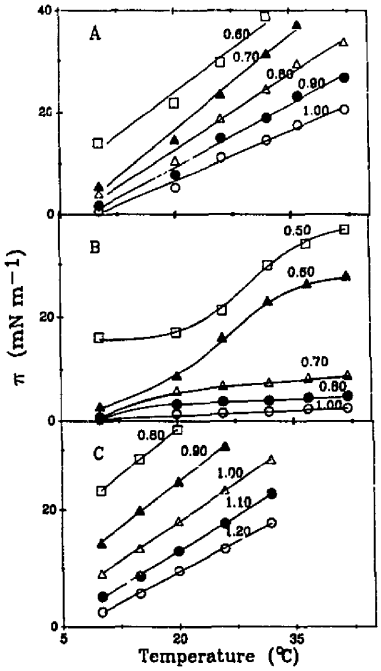

Fig. 4. Plots of surface pressure (π) against temperature at constant area for glycophorin/DPPC (1:355). A; DPPC, B; and DOPC, C. Numbers on each curve correspond to the area per phospholipid molecule in nm^2. Least-squares fitting has been carried out on data sets A and C.

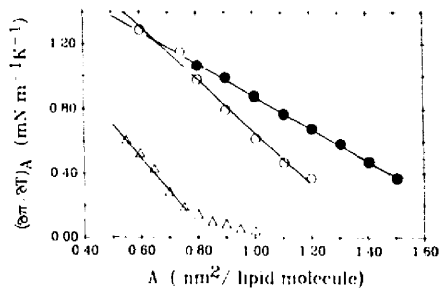

Fig. 5. Temperature coefficient of surface pressure $(\partial\pi/\partial T)_A$ as a function of area per molecule (A) for mixed monolayers of glycophorin and DPPC (○); DPPC (△) and DOPC (●) on 1.6 M ammonium sulphate (pH 7).

area per molecule found in phosphatidylcholine bilayers is in the range 0.50 to 0.70 nm^2 [37]. Table I shows values of $(\partial\pi/\partial T)_A$ and the corresponding values of P_i calculated from Eqn. 2. For DOPC the data had to be extrapolated to determine π at low values of A; although these values are uncertain this should not lead to serious errors in P_i as the $(\partial\pi/\partial T)_A$ term in Eqn. 2 predominates.

From Table I it is evident that the interaction between glycophorin and DPPC in mixed films leads to a substantial increase in internal pressure over that for pure DPPC monolayers. Since the LC/LE transition of DPPC leads to lower P_i values a better comparison may be that of a glycophorin-DPPC monolayer and a DOPC film in which there is no phase transition. In this case the internal pressures are of similar magnitude. To give some perspective to the values in Table I, if a film thickness of 1.7 nm is assumed then a P_i of 0.352 N m^{-1} corresponds to an internal pressure of 2070 atmospheres. At 25°C the internal pressure of octane and water are 2970 and 20000 atmospheres respectively. [38]. The octane value indicates that the calculated P_i of the monolayers is of the correct order of magnitude expected for a system consisting of mainly hydrocarbon chains. The approximate 30% lower monolayer P_i may be due to head group repulsion which will not occur in octane. Since the solubility of a compound is proportional to $|\delta_{solvent} - \delta_{solute}|$, then to account for the 10000-fold difference in amphiphile solubility between biomembrane and pure lipid bilayers [1,2], would require a large increase in the internal pressure of the lipid bilayer to be brought about by the protein. In this light even the increased internal pressure in the mixed film, with respect to the DPPC monolayer, would be expected to produce only a very

TABLE I

Internal pressure and related parameters in monolayers at 37°C

Surface pressures marked * were estimated by extrapolation.

Monolayer	$A = 0.5$ nm^2 molecule^{-1}			$A = 0.6$ nm^2 molecule^{-1}			$A = 0.7$ nm^2 molecule^{-1}		
	$\left[\frac{\partial\pi}{\partial T}\right]_A$ (mN m^{-1} K^{-1})	π (mN m^{-1})	P_i^A (N m^{-1})	$\left[\frac{\partial\pi}{\partial T}\right]_A$ (mN m^{-1} K^{-1})	π (mN m^{-1})	P_i^A (N m^{-1})	$\left[\frac{\partial\pi}{\partial T}\right]_A$ (mN m^{-1} K^{-1})	π (mN m^{-1})	P_i^A (N m^{-1})
DPPC	0.727 ± 0.024	34	0.191	0.515 ± 0.008	27	0.133	0.303 ± 0.007	15	0.079
DOPC	1.383 ± 0.085	100 *	≈ 0.33	1.282 ± 0.108	80 *	≈ 0.32	1.182 ± 0.130	64 *	≈ 0.30
Glycophorin + DPPC	1.455 ± 0.025	53	0.398	1.295 ± 0.020	44	0.357	1.135 ± 0.014	36	0.316

small decrease in amphiphile solubility. Thus there is no reason to believe that the presence of an intrinsic membrane glycoprotein in a monolayer gives rise to the exceptional increase in intermolecular cohesion required to explain these solubility measurements.

Given that the behaviour of a monolayer under appropriate conditions of surface pressure and area per molecule is a reasonable approximation to a bilayer membrane, as the literature suggests, then although we have found unequivocal evidence for glycophorin–phospholipid interactions (this work and Ref. 31) there is no evidence from this study that the presence of the intrinsic membrane protein significantly increases the intermolecular interaction in a bilayer membrane assessed from changes in the internal pressure, as here defined.

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