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Theoretical studies of phospholipid-glycophorin bilayer membranes using electron spin resonance and fluorescent probes

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We have calculated the average value of the order parameter of a spin labelled lipid hydrocarbon chain in a DMPC bilayer containing a concentration c, of glycophorin, for a temperature above the main lipid phase transition temperature. We use the results of differential scanning calorimetry together with the results of other calculations to evaluate the parameters involved. To determine the orientation of the spin label, which is located near the glyceride backbone, we use the rotation isomeric model of hydrocarbon chains and allow for rocking and rotation of the chain. Our results are in good agreement with recent measurements and enable us to say that between about 200 and 1300 lipid molecules can be under the large glycophorin polar group 'umbrella' depending upon its conformation. In the case where this polar group adopts a 'pancake' conformation with about 1300 lipid molecules under it, we find that about 750–800 of them are perturbed and experience a reduced effective lateral pressure. We have calculated the average order parameter of a diphenylhexatriene (DPH) molecule under the same conditions as above, using the parameters determined there. We have used this calculation to predict the value of r_∞ that should be observed as a function of glycophorin concentration at $T = 30°C$. The predicted curve displays an unusual shape not observed in other lipid-protein bilayer membranes.

Introduction

Recently there have been studies carried out on the thermodynamics of glycophorin in DMPC bilayer membranes. These included differential scanning calorimetry (DSC), energy transfer, electron spin resonance (ESR) and fluorescence recovery after photobleaching (FRAP). The unusual behaviour of the energy transfer measurements as a function of glycophorin concentration was explained as a consequence of conformational changes occurring in the glycophorin polar group. Specifically Ruppel et al. [1] proposed that at low glycophorin concentration, c, the polar segment, which accounts for approx. 80% of the molecular weight of the molecule, adopts a pancake-like conformation in which it lies approximately in a plane parallel to the membrane surface and adjacent to it. As c increases, because of packing constraints, the fraction of glycophorin molecules whose polar groups can adopt the pancake-like conformation decreases, and the polar groups of the others are oriented outwards from the plane of the membrane. Although this model appeared to completely account for the unusual behaviour of the energy transfer results, no explanation was given of the unusual behaviour of the shape of the DSC curves, the equally unusual behaviour of the

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transition enthalpy, ΔH, and the behaviour of the 'order parameter' as deduced from ESR measurements, all as a function of glycophorin concentration.

In a previous paper a model which built upon the proposal of Ruppel et al. [1] was constructed. The intention was to understand in detail the reasons for the behaviour of the DSC and ΔH data as a function of c, to construct a phase diagram and to make some predictions. The essential assumption of the model was that when the polar group of a glycophorin molecule is in its pancake-like conformation (designated as the 'down' or D state) it interacts either directly or indirectly, with lipid polar groups in its neighbourhood. This interaction is assumed to weaken the forces which bring the bilayer into existence. Because these forces can be represented as giving rise to an effective lateral pressure, Π, in the plane of the bilayer, acting on the lipid hydrocarbon chains (Marčelja 1974), then the interaction between a glycophorin in its D state and the lipid molecules in its neighbourhood was represented as decreasing the lateral pressure upon the hydrocarbon chains of those lipid molecules. It was found that the polar group of a glycophorin molecule which was oriented out of the plane of the bilayer (designated as the 'up' or U state) did not interact significantly in this way with lipid molecules.

Computer simulation studies were carried out on this model and it was found that a decrease in Π by approx. 10% (from about 30 dyn/cm to about 26 or 27 dyn/cm) could account for all of the DSC and ΔH observations. The picture which emerged was: Each glycophorin polar group excludes other glycophorin molecules from an area around its α-helical core due to hard-core or similar interactions. These polar groups conformations are 'umbrellas' over lipid molecules in the bilayer under them. Under the U umbrella no perturbation to the bilayer occurs while under part of the D umbrella a lateral pressure reduction by about 3 or 4 dyn/cm occurs. At low concentrations the bulk lipid consists of unperturbed lipids with dynamical patches of perturbed lipids. As c increases the perturbed lipids eventually form a percolating cluster and become in turn the bulk lipids. At a concentration, $c = c_1$, this effect is maximal with all of the bilayer plane essentially covered by glycophorin polar groups in their D states. As c increases beyond c_1 the fraction of glycophorin molecules in their U states, f_u, increases rapidly and the bulk lipid, composed of perturbed lipids, becomes diluted by dynamical patches of unperturbed lipids. As c increases f_u approaches unity and these patches of unperturbed lipids become, in turn, a percolating cluster with the unperturbed lipids becoming once again the bulk lipids, diluted by dynamical patches of perturbed lipids as well as glycophorin alphahelical cores which are now numerous enough to have an effect upon the thermodynamics.

Here we will apply the results of this model to understand the dependence of the 'order parameter', as measured by ESR, upon c, and to predict the results to be expected from measurements of r_∞ and the steady-state polarization of the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH). We shall confine ourselves to the case where the temperature, T, is greater than the pure lipid main transition temperature, T_c, because we do not wish to become embroiled in complications arising from the coexistence of two phases. In the next section we shall outline a simplified model based upon the results of the work of MacDonald and Pink [3] and derive equations for the quantities of interest.

Theory

(a) Calculation of the average 'order parameter'

Consider a phospholipid bilayer membrane which has been reconstituted to contain N_p glycophorin molecules. The α-helix of each protein possesses a cross-section area in the plane of the bilayer equivalent to that of n_c phospholipid hydrocarbon chains. Let the total number of lipid molecules making up the bilayer be $N - n_c N_p$ with half of them forming one sheet of the bilayer. The concentration of proteins (mole fraction of proteins) is defined to be

$$c = N_p/(N - (n_c - 1)N_p) \tag{1}$$

We assume that half of the proteins are oriented in the opposite direction through the bilayer to that of the other half. Each protein possesses two polar groups, one of molecular mass of approx. 26

kDa and the other of approx. 3900 Da, and an α-helical core of molecular mass of approx. 3300 Da [4]. Each of the polar groups is larger than the α-helix and we assume that the smaller one projects onto the plane of the bilayer an excluded area shape inside of which other glycophorin molecules cannot normally penetrate. This 'umbrella' of the smaller polar group is assumed to cover $n_t - n_c$ lipid hydrocarbon chains. The larger polar group is assumed to have two predominant conformational states: U and D as described in the Introduction. In each of these states, this polar group projects onto the plane of the bilayer an area, from which other glycophorin molecules are excluded. In these areas $n_U - n_c$ and $n_D - n_c$ lipid hydrocarbon chains can be accommodated. When the larger polar group of a protein is in its U state then it is assumed not to perturb any of the $(n_U - n_c)/2$ lipid molecules under it. When it is in its D state, however, then it is assumed that $xn_D/2$ ($x \leqq 1$) lipid molecules are perturbed by the, direct or indirect, interaction due to the proximity of the larger polar group to the lipid/water interface of the bilayer. Finally, around each isolated α-helical core of a protein, n_A lipid molecules can fit on both sides of the bilayer.

It has been shown that the interaction between the larger polar group and the bilayer yields the result that isolated glycophorin molecules are in their D state [3] despite the fact that their U state is entropically favoured [5]. We assume, therefore, that they make a transition to their U states only because of packing considerations when they are sterically prevented from being in their D states. Accordingly, if N_U and N_D are the number of proteins in their U and D states, on one side of the bilayer then

$$N_D = N_p/2,\ N_U = 0 \text{ if } (n_D + n_t)N_p/2 < N,$$

or otherwise

$$\begin{aligned} N_D &= [N - (n_u + n_t)N_p/2]/(n_D - n_U) \\ N_U &= [(n_D + n_t)N_p/2 - N]/(n_D - n_U) \end{aligned} \tag{2}$$

Consider now a lipid hydrocarbon chain to which is attached a nitroxide spin label. At a given temperature, if the labelled molecule is inserted into a bilayer membrane containing glycophorin, it will report the existence of three environments: A 'free' lipid environment in which it will have an order parameter $\langle S\rangle_F$, and two 'perturbed' lipid environments: Under part of a protein polar group in its D state and adjacent to an α-helical core, for which the order parameters are $\langle S\rangle_D$ and $\langle S\rangle_A$, respectively, where $S = (3\cos^2\psi - 1)/2$ and ψ is the angle that the N → O group makes with a perpendicular to the plane of the bilayer. The average order parameter measured is then

$$\begin{aligned} \langle S\rangle = \langle S\rangle_F + [x(n_D - n_c)N_D(\langle S\rangle_D - \langle S\rangle_F) \\ + n_A N_p(\langle S\rangle_A - \langle S\rangle_F)]/(N - n_c N_p) \end{aligned} \tag{3}$$

We must thus calculate $\langle S\rangle_F$, $\langle S\rangle_D$ and $\langle S\rangle_A$.

If, instead of a spin labelled hydrocarbon chain, we are using a fluorescent probe such as DPH displaying average values of its order parameter, $\langle S_D\rangle_F$, $\langle S_D\rangle_D$ and $\langle S_D\rangle_A$ in the three environments, where $S_D = (3\cos^2\theta - 1)/2$ and θ is the angle between the long axis of the molecule and the bilayer perpendicular, then the average value of the order parameter, $\langle S_D\rangle$, is given by Eqn. 3 with the appropriate replacements.

(b) Calculation of spin label order parameters for environments F and D

We shall be concerned here with calculating the order parameter of a spin label which is attached near the glyceride backbone, when the lipid bilayer membrane is in its fluid phase for temperatures, T, greater than T_c, the main lipid phase transition temperature. We shall outline the method and apply it to the probe used by Ruppel et al. [1]. This was a spin label (12,3) and the upper part of the chain near the glyceride backbone is shown extended in Fig. 1a. The dots represent the remainder of the chain which will contain a number of *gauche* bonds appropriate to a fluid phase. We assume that the chain can twist into a number of rotationally isomeric states as shown in Fig. 1b to h. The remainder of the chain is shown as a sequence of dots and we assume that these represent essentially identical conformational states. In this case they may be ignored in our calculation. We also assume that the chain, as a whole, can rotate about a long axis and that this long axis can reorient with respect to the bilayer

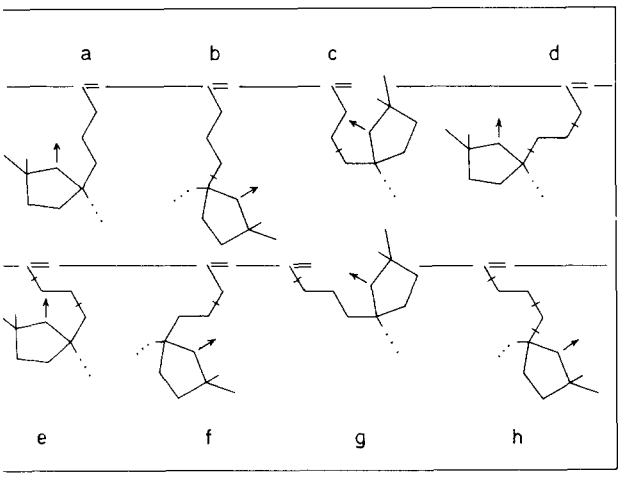

Fig. 1. The rotational isomeric states of the four C-C bonds of a hydrocarbon chain nearest to the glyceride backbone with a nitroxide spin label attached as shown. A dash crossing a C-C bond indicates a *gauche* conformation at that bond. The dots indicate the remainder of the hydrocarbon chain and it is assumed that this remainder is sufficiently long so that it possesses conformational states essentially independent of the eight states shown here, when the chain is in a fluid phase at $T > T_c$. Here, the states which are rejected are: c and e (for steric reasons), g (protrudes into the polar region) and h (very highly excited).

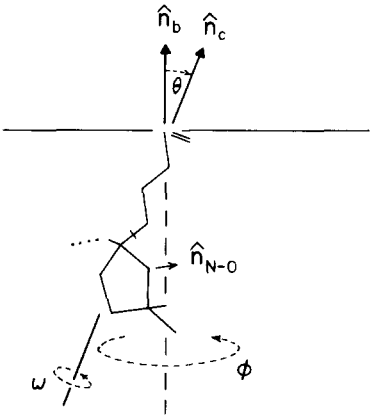

Fig. 2. The angles defining the orientation of the nitroxide spin label. The confirmation shown is (b) of Fig. 1. $\hat{n}_b$ is a unit vector normal to the local plane of the bilayer, $\hat{n}_c$ is a unit vector along the average long axis as shown and $\hat{n}_{N\text{-}O}$ is a unit vector between N and O on the spin label. θ is the angle between $\hat{n}_b$ and $\hat{n}_c$, ω is the angle for rotations around $\hat{n}_c$ and ϕ is the angle describing rotations around $\hat{n}_b$.

normal, as shown in Fig. 2 for the case of Fig. 1b. Here $\hat{n}_b$ and $\hat{n}_c$ are unit vectors representing the normal to the bilayer and the axis of the hydrocarbon chain respectively, with $\hat{n}_b \cdot \hat{n}_c = \cos\theta$. The angles ω and ϕ are those swept out as the chain rotates around $\hat{n}_c$ and $\hat{n}_b$, respectively, and the angle between $\hat{n}_b$ and the unit vector $\hat{n}_{N\text{-}O}$ is ψ. Evidently ψ is determined by the conformational state of the chain and the angles θ and ω. The Hamiltonian operator for a chain, from which all its thermodynamic properties can be calculated is assumed to be

$$\mathscr{H} = \sum_k [\Pi A(k, \theta) + E(k)] \tag{4}$$

Here k identifies the conformational state of the chain, $E(k)$ is the energy associated with the number of gauche bonds shown in Fig. 1 and $A(k, \theta)$ is an effective cross sectional area of the chain given by Ref. 6,

$$A(k, \theta) = A_0 L_0 / L(k) \cos\theta \tag{5}$$

Here, $L(k)$ is the effective length of the portion of the chain shown in Fig. 1 so that $L(k)\cos\theta$ is its projection onto $\hat{n}_b$. A_0 is the cross sectional area of an extended chain, ≈ 20.4 Å^2 and L_0 is the length, in C-C bond units, of the most extended segment in Fig. 1. This occurs for Fig. 1a so that $L_0 = 5$. The assumption underlying Eqn. 5 is that on the scale shown here the density is essentially constant. This is a somewhat stronger assumption than Marčelja's original assumption that the density is constant on the average throughout the bilayer.

We have calculated the partition function, Z, and the order parameter, $\langle S \rangle_\alpha$,

$$Z = 2\pi \sum_k e^{-\beta E(k)} D(k) \int_0^{\pi/2} e^{-\beta \Pi_\alpha A_0 L_0 / L(k) \cos\theta} \sin\theta \, d\theta$$

$$\langle \cos^2\psi \rangle = \frac{\pi}{Z} \sum_k e^{-\beta E(k)} D(k)$$

$$\times \int_0^{2\pi} [(3\cos^2\psi(k) - 1)\cos^2\theta + (1 - \cos^2\psi(k))]$$

$$\times e^{-\beta \Pi_\alpha A_0 L_0 / L(k) \cos\theta} \sin\theta \, d\theta \tag{6}$$

Here $\beta = (k_B T)^{-1}$ where k_B is Boltzmann's constant and T is the absolute temperature, $D(k)$ is the degeneracy of the k-th conformational state of a hydrocarbon chain, $\psi(k)$ is the angle between

$\hat{n}_{N\text{-}O}$ and $\hat{n}_b$ (see Fig. 2) in the k-th state and Π_α is the effective lateral pressure acting on the chain when it is either 'free' ($\alpha = F$) or in the perturbed region under a 'down' protein polar group ($\alpha = D$).

Finally, we note that Figs. 1c and e are probably sterically unlikely while Fig. 1g has the spin label at the interface between the acyl chain and water regions and Fig. 1h not only has a large area but is very highly excited with three *gauche* bonds. Accordingly, we have omitted them and have retained only the four remaining states in the sum over k in Eqn. 6. These four states are listed in Table I.

The integrals in Eqn. 6 can be represented as exponential integrals and must be integrated numerically. We have found it satisfactory to use an integration procedure involving Jacobi polynomials [7]. We have chosen to integrate θ from 0 to $\pi/2$, and not from 0 to π, because we have assumed that the long axis of the hydrocarbon chain will not penetrate into the water region. We have evaluated the integrals for $\Pi_F = 30$ dyn/cm, the approximate lateral pressure inside an unperturbed phosphatidylcholine bilayer [8], as well as for $\Pi_D = 27$ dyn/cm and 26 dyn/cm. Previously [3] we found that the perturbation under a down glycophorin polar group reduces the effective lateral pressure to approx. 26 or 27 dyn/cm. Finally, we evaluated the integrals at $T = 303.2$ K (30°C) in order to compare our results with those of Ruppel et al. [1]. We found the following results:

$$\Pi_F = 30 \text{ dyn/cm}, \langle S \rangle_F = 0.545$$

$$\Pi_D = 26 \text{ dyn/cm}, \langle S \rangle_D = 0.518 \quad (7)$$

The result of $\langle S \rangle_F = 0.545$ is about 7% smaller than the value of 0.584 reported by Ruppel et al. [1] using a spin-labelled fatty acid I(12,3). However, our calculation compares favourably with a value of 0.547 reported for a (10,3)-labelled lipid by Knowles et al. [9], though the closeness of agreement is possibly fortuitous.

TABLE I

STATES OF THE UPPER PORTION OF THE HYDROCARBON CHAIN

State, k	$E(k)$ [a]	$D(k)$ [b]	$L(k)$ [c]	$\cos \psi(k)$ [d]
a	0	1	5	1.0
b	0.45	2	4	0.5
d	0.90	2	4	1.0
f	0.45	2	3	0.5

[a] In units of 10^{-13} erg. $0.45 \cdot 10^{-13}$ erg is the approximate energy of a *gauche* bond.

[b] Each *gauche* bond has two conformations. An approximately planar kink such as d has only two states: $g^+ tg^-$ and $g^- tg^+$, the other two having a very high energy.

[c] The length is in units of C-C bonds along a perpendicular to the bilayer plane.

[d] We have represented C-C-C bonds as having an angle of 120° and the *gauche* bonds involving a rotation of 180° for simplicity. The only angles occurring here for $\psi(k)$ are thus 0 and 60°.

(c) Calculation of DPH order parameters for environments F and D

In this section we wish to calculate $r = r_0 \langle S_D \rangle^2$ [10–13] as well as the steady-state polarization,

$$P = 3(r_0 + r_\infty K)/[2 + r_0 + (2 + r_\infty) K]$$

$$K = \tau_F/\tau_c, \; r_0 = 0.39 \quad (8)$$

where K is the ratio of the fluorescence lifetime to the DPH molecule rotational correlation time [11,12]. It is true that a calculation or measurement of r_∞ is free from ambiguities associated with uncertainties in the relaxation times, and that it is sufficient to confine oneself to a consideration only of r_∞. Nonetheless, measurements are made of P, and therefore we have calculated it based upon the assumption that K is a constant independent of glycophorin concentration at a fixed temperature. We have, however, calculated what would be the effect if K changed with concentration and this is discussed below. We treat the molecule as an effectively rigid rod, because it is considered to fluoresce only in its linear conformation [14], which makes an angle θ with the bilayer normal. Again using the assumption of constant density in the bilayer and taking the cross-sectional area of the molecule to be about equal to that of an extended lipid hydrocarbon chain, $A_0 = 20.4$ Å^2, the Hamiltonian is

$$\mathcal{H} = \Pi A_0 / \cos \theta \quad (9)$$

However, the length of the DPH molecule, L_{DPH}, is less than the thickness of the bilayer. Accordingly, for a given angle θ, there is a range of

distances, along the normal to the bilayer, inside the membrane where the molecule can be located. This is shown in Fig. 3 where the thickness of the bilayer is $2a$. For a given angle θ the centre of the molecule can be located anywhere along the z-axis between $-(a - L_{DPH}/2)$ and $(a - L_{DPH}/2)$. Since, however, the molecule is entirely hydrophobic, it is not constrained to be located in, for example, one half of the bilayer, but could lie in the middle and spanning both sheets. The possibility that the effective lateral pressure that it experiences depends upon the 'depth' at which it is located in the bilayer should be considered since one might expect that this pressure is lower near the centre of the bilayer than near its boundaries. Rather than guess at some functional form of effective lateral pressure dependence upon depth in the bilayer, we have chosen simply to define a region near the centre of the bilayer in which the DPH molecule will be oriented perpendicular to the bilayer normal. This is shown in Fig. 3 where

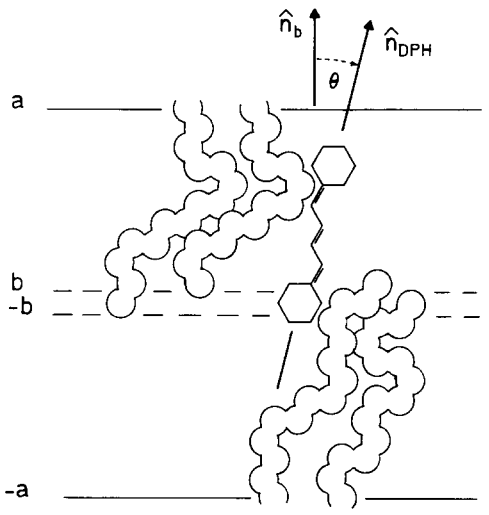

Fig. 3. Schematic diagram of the crosssection through a DMPC bilayer in which is embedded a DPH molecule. The unit vector $\hat{n}_b$ is normal to the local plane of the bilayer, $\hat{n}_{DPH}$ is a unit vector along the long axis of the DPH molecule, and θ is the angle between these two vectors. The thickness of the hydrophobic region of the bilayer is $2a$, and there is a region at the centre of the bilayer in which the DPH probe will lie perpendicular to $\hat{n}_b$ when its centre of mass lies between $-b$ and $+b$. Our calculations show that if $a \approx 11.5$ Å (hydrophobic region of the bilayer ≈ 23 Å thick for DMPC) then $b = 0.047$ Å.

we define a region from $-b$ to b in which the molecule is preferentially oriented perpendicular to the unit vector $\hat{n}_b$. The partition function and average value of $\cos^2\theta$ is then

$$Z = 2\pi\int_0^{\pi/2} e^{-\beta\Pi_a A_0/\cos\theta}[2(a-b) - L_{DPH}\cos\theta] \times \sin\theta\, d\theta + 4\pi b$$

$$\langle\cos^2\theta\rangle = \frac{2\pi}{Z}\int_0^{\pi/2} e^{-\beta\Pi_a A_0/\cos\theta}[2(a-b) - L_{DPH}\cos\theta] \times \cos^2\theta \sin\theta\, d\theta \qquad (10)$$

By direct calculation we find that $L_{DPH} \approx 12$ Å, and that from the constant density assumption and the requirement that a melted chain possesses a cross-section area of ≈ 30 Å^2 we obtain that $a \approx 11.5$ Å. For $\Pi_F = 30$ dyn/cm and $T = 30°C$ we find if we choose $b = 0$ we obtain $\langle S_D\rangle_F = 0.386$. If we use a value of $K = 8$ [12] then we obtain $P = 0.137$ which is close to the value of $P = 0.125$ reported by Hoffmann et al. [15]. This value of $\langle S_D\rangle_F$, however, gives a value for $r_\infty = r_0\langle S_D\rangle_F^2$ of 0.0587 which is significantly larger than the value of 0.0385 reported by Lakowicz et al. [16]. In order to obtain that value for r_∞ we find that we must choose $b = 0.0472$ Å which gives $P = 0.125$ if we choose $K = 6.254$ [15]. Accordingly, we chose those values for b and K, and find

$$\Pi_F = 30 \text{ dyn/cm},\ r_0\langle S_D\rangle_F^2 = 0.0385,\ P_F = 0.125$$

$$\Pi_D = 26 \text{ dyn/cm},\ r_0\langle S_D\rangle_D^2 = 0.0358,\ P_D = 0.122 \qquad (11)$$

We see that the proportional change in the DPH steady-state polarization is smaller than that in the ESR order parameter.

(d) Estimation of order parameters for environment A

We have not been able to evaluate $\langle S\rangle_A$ for the spin label, or $\langle S_D\rangle_A$ for DPH from a calculation. This is not surprising since there is evidence that the values are due to steric interactions with the protein hydrophobic surface [15]. Accordingly we have attempted to estimate them from experiment. Because such an interaction with the protein hydrophobic surface results in an 'immobilized' ESR spectrum characteristic of a gel-like state, it is

reasonable that $\langle S \rangle_A$ is greater than about 0.75.

In the case of DPH we make use of an analysis of the concentration dependence of P in DMPC bilayer membranes containing gramicidin. It seems plausible that the hydrophobic core of glycophorin and gramicidin will induce about equal order into a DPH molecule adjacent to them. From previous calculations [15] it was found that for DMPC-gramicidin bilayers, $r_0 \langle S_D \rangle_A^2 = 0.165$ (parameter B of Eqn. 6 evaluated for case (b)).

That $\langle S_D \rangle_A$ for DPH is substantially smaller than $\langle S \rangle_A$ for the (12,3) spin label is in accord with our result, above. There we found that $\langle S_D \rangle_F = 0.314 < \langle S \rangle_F = 0.545$ and that $\langle S_D \rangle_D = 0.303 < \langle S \rangle_D = 0.518$. Clearly, in this case, it is reasonable that a nitroxide spin label located near to the glyceride backbone of a lipid would display a larger value of its order parameter than would a DPH molecule, which measures an average order parameter across the bilayer.

Results and Discussion

We have used the calculated or estimated order parameters in Eqn. 3. We must, however, choose values for n_c, n_A and n_t as well as values for n_U, n_D and x in order to evaluate N_D as a function of N_p and N. The first three parameters are determined by what we know of the structure of glycophorin. The cross sectional area of a glycophorin α-helix (mol. mass = 3300 Da) is equivalent to about 6 or 7 lipid hydrocarbon chains, around which about 12 lipid hydrocarbon chains can fit on either side of the bilayer. Accordingly, we choose $n_c = 7$ and $n_A = 12$. The smaller polar group (mol. mass = 3900 Da) would form an 'umbrella' over about one lipid chain layer around the α-helix so that we obtain $n_t = 19$.

It was proposed from earlier measurements [1] and found from calculations [3] that the abrupt change in slope of the transition enthalpy, ΔH, with concentration, c, occurring at $c \approx 0.0008$ indicates that essentially the entire bilayer surface is covered by glycophorin polar groups in their D state. This result tells us that $n_D \approx 2600$ lipid hydrocarbon chains which means that each polar group in its 'pancake' conformation has associated with itself an area of the plane of the bilayer made up of about 1300 lipid molecules. This number is not that which would be deduced from the transition enthalpy versus concentration curve by extrapolating ΔH to zero. The approximate value of x, namely, the fraction of those n_D lipid hydrocarbon chains which experience the reduced lateral pressure, Π_D, due to the perturbation brought about by the down polar group is obtained from the results of [3]. It was pointed out in [1] that the heating DSC curves (Fig. 1a) broadened as c increased from 0 to 0.0007 and then narrowed as c increased further to 0.0014.

It was shown in Ref. 3 that this unusual behaviour is predicted by the model as long as $x \approx 0.5$–0.6. That is, not all of the 1300 lipid molecules 'under' (in some sense) the polar group in its pancake conformation experience the reduced lateral pressure, but about 700 of them appear to do so. This number of 700 lipid molecules is that which would be deduced from the extrapolation of ΔH to zero. It is somewhat smaller than the value of about 1000 reported by Sackmann et al. [2], but rather larger than the number of about 300 lipid molecules deduced from the ΔH vs. c curve of Ruppel et al. (Ref. 1, Fig. 8). However, the latter is deduced from a straight line drawn through only two points, one of which is the $c = 0$ value of ΔH, and more data may change this number. For our purposes, we chose $x = 0.6$.

The last parameter is n_U, the number of lipid hydrocarbon chain 'under' the 'umbrella' of a glycophorin polar group in its up conformation. Grant and McConnell [17] and Van Zoelen et al. [18] report measurements using lipid : protein ratios of 120 : 1 and down to about 220 : 1, respectively. If these numbers represent the maximum number of glycophorin molecules that could be incorporated into the bilayer then they imply that n_U lies between 240 and about 440. Sackmann et al. [2] suggest that for $c \gtrsim 0.01$ a partial collapse of the bilayer might explain some of their results so that the smallest lipid : protein ratio for an intact bilayer is $\gtrsim 100$ so that $n_U \gtrsim 200$. We are thus constrained to use a value of n_U lying between about 200 and 450.

Fig. 4 shows the results of our calculation of the ESR spin label order parameter, as a function of glycophorin concentration in DMPC at $T = 30\,°C$ for various choices of the parameters $\langle S \rangle_A$

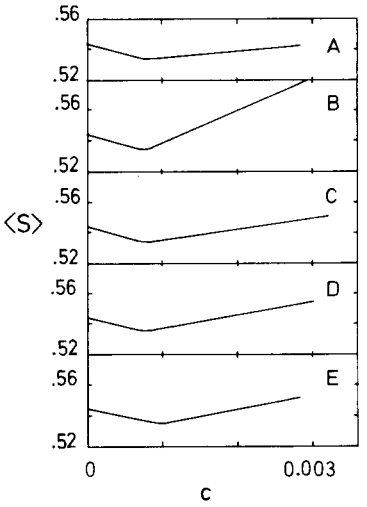

Fig. 4. Calculated ESR order parameter, $\langle S \rangle$ (Eqn. 3), of a spin label attached as shown in Fig. 1, as a function of glycophorin concentration, c, in a DMPC bilayer membrane at 30°C. The values of the parameters ($\langle S \rangle_A$, n_D, n_U) are: A: (0.85, 2600, 100). B: (0.85, 2600, 1300). C: (0.85, 2600, 400). D: (1.00, 2600, 400). E: (0.90, 2000, 500). Case D uses the correct numbers, deduced from DSC data, for n_D and n_U and is closest in accord with the measurements of Ref. 1. The pressure experienced by lipids unperturbed by the glycophorin polar group is 30 dyn/cm, while that experienced by perturbed lipids is 26 dyn/cm. $\langle S \rangle_A$ is the spin label order parameter when it is adjacent to a glycophorin α-helix, and n_D and n_U are the number of lipid hydrocarbon chains under the 'umbrella' of a glycophorin polar group in its D ('down') and U ('up') states, respectively.

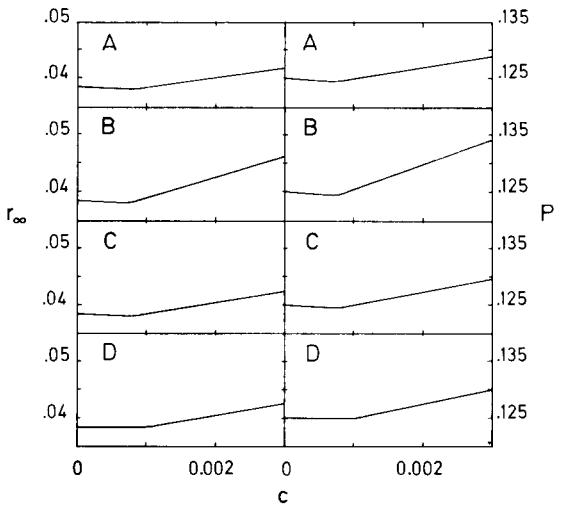

Fig. 5. Predicted r_∞ and steady-state polarization, P, of a DPH molecule in a DMPC bilayer at 30°C containing a concentration, c, of glycophorin. The former is more reliable since the value of P shown here depends upon the assumption that K is a constant whereas the value of r_∞ is independent of K. The values for (n_D, n_U) are: A: (2600, 100). B: (2600, 1300). C: (2600, 400). D: (2000, 500). From the results of our calculations, we predict that the curve for r_∞ of (C) should be observed for this system.

and n_U. A comparison of these results with the measurements of Ruppel et al. [1] (Fig. 2 (a)) shows that the best agreement is with $\langle S \rangle_A \approx 1.0$ and $n \gtrsim 400$ and shown in Fig. 4D. The only difference is that the experimentally measured minimum at $c \approx 0.0008$ may be more rounded than that of our calculations. This probably arises because our model possesses no cooperativity at all in modelling the protein polar group change of state between the D and U states. In Fig. 4 we can see that a value of $n_U = 100$ is too small to give the increase in $\langle S \rangle$ reported for $c > 0.0008$ [1], while $n_U \approx 1000$ is too large.

With these results we are now able to predict the glycophorin concentration dependence of r_∞ and the steady-state polarization of DPH in DMPC for $T > T_c$, and the results are shown in Fig. 5. Following the analysis above and the results of Fig. 4, where the case $n_U = 400$ appears to agree best with experiment, we predict that r_∞ for DPH will follow the curve of Fig. 5C but having a somewhat more rounded minimum at $c = 0.0008$. Other cases, corresponding to Fig. 4A, B and E are shown for comparison. Fig. 5 also shows the steady-state polarization for $K = 6.254$. We have also calculated how P changes if $K = 5.0$ or $K = 12.0$. In the case that $n_u = 400$ which we predict should be observed unless quenching occurs, we find that $P(c)$ takes on the following values for the indicated concentrations

$K = 5.0 \qquad P(0.003) = 0.143$

$K = 12.0 \qquad P(0.003) = 0.100$

These values should be compared with the values of $P(0.003) = 0.1295$ shown in Fig. 5C. It can be seen that changes in K make non-negligible changes in P and that a more reliable parameter is r_∞ [13]. At such low concentrations of glycophorin, however, where $c < 0.003$, K may not change significantly.

Our results, then, can be summarized as follows:

(i) We have developed models in order to calculate the order parameter of a nitroxide spin label attached near the glyceride backbone of a phospholipid, and to calculate the order parameter of a DPH molecule in a lipid bilayer. The former makes use of the rotation isomeric model of lipid hydrocarbon chains together with the rocking and rotation of the chain as a whole, while the latter allows the possibility that the DPH molecule can lie parallel to the plane of the bilayer between the two lipid sheets.

(ii) The decrease in the ESR order parameter, $\langle S \rangle$, as c increases from zero can be entirely understood by the model [3] which assumes that a fraction of the lipid molecules under the umbrella of a protein with its polar group in a pancake conformation [1] (state D) experience an effective lateral pressure reduced by approx. 13% from that which they experience otherwise in the bilayer.

(iii) The number of lipid molecules under a glycophorin polar group in its D state is approx. 1300, while the number under the polar group in its U state is approx. 200 molecules. The fraction of the former which experience a reduced effective lateral pressure of $\Pi_D \approx 26$ dyn/cm is approx. 0.6 so that about 750 to 800 lipid molecules experience the reduced pressure under the 'down' polar group. The remaining molecules experience an effective lateral pressure of $\Pi \approx 30$ dyn/cm.

(iv) We have predicted the curve of r_∞ as a function of glycophorin concentration, c, for the fluorescent probe DPH, at $T = 30°C$ (Fig. 5C). We have also calculated the steady-state polarization under the assumption that τ_F/τ_c does not change significantly with protein concentration, and this may be true for c lying in the low-concentration range of 0 to 0.003. As long as significant quenching of the probe does not occur and as long as the probe is distributed like the lipid molecules, the measured curve of r_∞ should look like our prediction of Fig. 5C, though it may display some slight curvature due to cooperativity in U to D and D to U transitions of the glycophorin polar group.

The number of lipid molecules calculated to be perturbed by a protein polar group in its D state, about 780, is between the values of about 300 [1] and about 1000 [2] reported experimentally. At first sight this number may appear to be large since this is only approx. 60% of the total number of lipid molecules, about 1300, deduced to be under the polar group in its D state. Ruppel et al. [1] have argued that about 400 lipid molecules would be 'covered' by the area calculated from an effective radius defined by the average mean-squared length on a plane of a single-stranded polymer possessing no side-chains. In the case of glycophorin, of course, there are side-chains so that the area so-defined might be larger. Furthermore, the large polar group of glycophorin possesses up to 32 negative charges and any repulsion that they give rise to would contribute to increasing the effective area under such a polar group.

We have assumed that the spin-labelled probes are randomly distributed throughout the lipid 'sea'. In other lipid-protein bilayers for $T > T_c$ there is no evidence that any significant phase separation occurs, and there seems no compelling reason to consider that possibility here.

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