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Effect of integral proteins upon bilayer permeability to small ions

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Abstract. We have modelled the effect of integral proteins upon the permeability of a lipid bilayer membrane to small ions. Our intention is to predict the temperature- and protein concentration-dependence of the relative ionic diffusion coefficient if it is assumed that the ions diffuse entirely through the lipid regions of the membrane, and do not cross the bilayer either by moving through the protein or along its hydrophobic surface. We used models of lipid-protein bilayers that have been used successfully before, together with a modification of the (protein-free) permeability mechanism of Kanehisa, Tsong, Cruzeiro-Hansson and Mouritsen (KTCM). We used Monte Carlo techniques to simulate the thermodynamics and permeability of the membrane because such a calculation, which depends upon spatial and time correlations, cannot be done using a mean field approximation. We took the protein to be analogous to bacteriorhodopsin and calculated the relative diffusion coefficients, R, of small ions through a DMPC bilayer containing such proteins. We found that although there is only a small change in R when $T > T_m$, R can change by an order of magnitude, depending upon the protein concentration, for $T < T_m$. We showed that regions of largest lipid area fluctuations and, hence, compressibility occur (*i*) at the interfaces between regions (which may be very small and not macroscopic phases) of extended-chain and excited-chain lipids, thus showing the similarity between this model and the KTCM model, and (*ii*) in regions where the temperature is close to T_m but the lipids are prevented from becoming ordered because of the proximity of proteins which try to keep them in their excited-chain states. We have plotted lipid fluctuation and ionic permeability maps to show which regions of the membrane display the largest permeability, and we show how these regions change as a function of temperature.

Key words: Lipid-protein bilayers – Permeability – Computer simulation

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Introduction

An identification of the processes which determine the passive permeability of a lipid bilayer membrane to different molecules can lead to an understanding of the dynamics of such systems.

Although a substantial amount of work has been done on modelling diffusion of molecules through pure lipid bilayer membranes, no modelling has been done of the effect of integral membrane proteins upon small-ion permeability. An understanding of this effect is, however, an important question for two reasons: The first is the question of in which part of the membrane, if any, is the permeability most affected by the presence of the proteins. The second is how, if at all, can the permeability be changed by conformational or other changes in the proteins.

Here we will study one question only: In which part of the lipid bilayer membrane is the trans-bilayer permeability of small ions most affected by the presence of integral proteins, and how is the total permeability changed in the process. We shall assume that the proteins themselves do not directly contribute to the permeability in that ions do not traverse the bilayer via any part of the protein structure. Our calculation of the permeability as a function of temperature and protein concentration and a comparison with measurements can suggest whether, or to what extent, this assumption is true. Our calculations will be based on a modification of the successful model of Kanehisa and Tsong (1978) and Cruzeiro-Hansson and Mouritsen (1988), which we refer top as the KTCM model, by making use of a modification of the mechanism of ion permeability used by Doniach (1978) and Caille et al. (1980).

Our fundamental assumption is that the permeability via the lipid regions of the bilayer will depend upon its local compressibility and that regions of high permeability will coincide with regions of high compressibility. The (lateral) compressibility is related to fluctuations in the bilayer and, in the simple model used here, these are fluctuations in the lipid cross-section area. These can occur

anywhere in the bilayer where thermal fluctuations give rise to adjacent regions, which may be very small, composed of lipids in extended-chain and excited-chain states. In order to study the effect of integral proteins, we will consider the example of a compact region of such proteins in a lipid bilayer and show that a region of high compressibility occurs at the interface between extended-chain and excited-chain lipid layers surrounding the protein region. This result provides some evidence to support our assertion that the mechanism which we propose is essentially equivalent to the mechanism proposed in the KTCM model. We will find, however, that, because of the presence of proteins, when T is near T_m, the main (pure) lipid phase transition temperature, there are regions of large permeability that do not appear to be interfaces between such regions. As the KTCM model stands, however, it describes membrane permeability for a pure lipid bilayer and it must be modified to allow for a non-zero concentration of proteins. We will study this model by using computer simulation. Although a mean-field approximation would be adequate to deduce the general features of the phase diagram, it is necessary that we correctly take into account the correlations between protein positions and lipid states as well as the correlations between lipid states over a period of time. These correlations can be substantially different from those predicted by the use of a mean field approximation.

In the next section we will outline the model used, briefly describe the computer simulation techniques and present our modification of the KTCM model by making use of the local fluctuations in lipid area as an important mechanism determining bilayer permeability to small ions. In subsequent sections we will present the results of a variety of computer simulation studies, summarize our conclusions and outline other studies that could be carried out.

Theoretical model of a lipid – protein bilayer

In order to make a prediction about the trans-bilayer diffusion of small ions in the presence of integral membrane proteins we need (*i*) an adequate model of a lipid bilayer membrane containing integral proteins and (*ii*) a model of the processes which are thought to determine trans-bilayer diffusion. To address (*i*) we will use a model which has been used elsewhere (Pink et al. 1988) to calculate the thermodynamics of hydrogen bonded lipid bilayer membranes.

We represent the plane of one sheet of the membrane by a triangular lattice, each site of which can be occupied by a lipid molecule, here taken to be DMPC. Each hydrocarbon chain can be in one of two states, a ground state, g, or an excited state, e. The ground state, g, is obtained by performing a mean-field average over nine low-energy states which includes the all-trans conformation and conformations containing jogs and kinks (Pink et al. 1980). The hydrocarbon chain states of a lipid molecule are then a ground state, $G=\{g, g\}$, an excited state $E=\{e, e\}$ and intermediate states, $I=\{g, e\}$ and $\{e, g\{$, where $\{n, m\}$ denotes a pair of chains of a single molecule in states n and m respectively. These states have been used in a study of lipid bilayer membranes containing NPGS molecules (Pink et al. 1988) where they have been described. They are parameterized by an internal energy, E_n, a cross-section area, A_n, and a degeneracy, D_n $(n=G, I, E)$. The van der Waals interaction energy between the hydrocarbon chains of two such neighbouring molecules is $-J_0\,J$ (nm), and the interactions in the polar region, which bring the bilayer into existence, gives rise to a lateral pressure, Π, acting on the hydrocarbon chains. The Hamiltonian describing such interacting molecules is

$$\mathscr{H}_L=-\frac{-J_0}{2}\sum_{\langle ij\rangle}\sum_{nm}J(n\,m)\,\mathscr{L}_{in}\,\mathscr{L}_{jm}+\sum_i\sum_n\varepsilon_n\,\mathscr{L}_{in}$$

$$\varepsilon_n=E_n+\Pi A_n \qquad (n=G, I, E) \tag{1}$$

where $\langle ij\rangle$ indicates a sum over nearest neighbour sites i and j, and $\mathscr{L}_{in}$ is a projection operator for a lipid molecule at site i in state n. All the energies are taken from lipid models used elsewhere (Pink et al. 1988). The value of Π, deduced for PC bilayers, was taken as 30 dynes/cm (Georgallas et al. 1984).

We assume that the protein which we are modelling interacts with the lipids only via its hydrophobic trans-bilayer segments and that its polar segments do not interact with the lipid molecules so that they can be ignored. The projection, onto the plane of the bilayer, of the hydrophobic segment of an integral protein, spanning the bilayer, is represented as a hexagon with its centre and six vertices located at lattice sites. Sites occupied by hexagons may not be occupied by lipids, and a site may not be simultaneously occupied by two hexagons. The internal states of the proteins are ignored. That is, we are ignoring cases where temperature changes alone result in a protein change of state, in the range of temperature in which we are interested. The Hamiltonian describing protein-protein and protein-lipid interactions is

$$\mathscr{H}_p=\frac{-J_0}{2}\sum_{\langle kl\rangle}J_p(k\,l)\,\mathscr{P}_k\,\mathscr{P}_l-J_0\sum_{\langle il\rangle}\sum_n K_n(i\,l)\,\mathscr{L}_{in}\,\mathscr{P}_l \tag{2}$$

Here $\mathscr{P}_l$ is a projection operator for a protein (hexagon) with its centre at site l, $-J_0\,J_P(k\,l)$ is the interaction energy between two proteins with centres at sites k and l, and $-J_0\,K_n(i\,l)$ is the interaction energy between a protein, with its centre a site l, and a lipid in state n at site i. The symbols $\langle k\,l\rangle$ and $\langle i\,l\rangle$ indicate that the two proteins, or the lipid and the protein, are adjacent.

We chose all the $K_n(i\,l)=0\,(n=G, I, E)$ on the grounds that we are considering a protein for which there is no specific interaction between its hydrophobic surface and the lipid chains in any particular state. This point will be discussed later. We chose $J_p(k\,l)$ so that aggregation of proteins occurred, even in a fluid lipid phase, when the protein concentration became sufficiently high. In this way, and by choosing the hexagon size appropriately, we modelled a DMPC bilayer containing integral proteins similar to, in as far as their effect upon the lipid molecules are concerned, bacteriorhodopsin (Peters and Cherry 1982).

We were interested in calculating various thermal averages as functions of temperature, T, and lipid protein ratio, L/P, or protein concentration $x=N_p/(N_p+N_L)$ where N_p and N_L are the number of proteins and lipids in the bilayer. Using Monte Carlo simulation techniques we calculated the enthalpy, H, the average area per lipid molecule, $\langle A_L\rangle$, and the fluctuations in this average area, $(\Delta A_L)^2=\langle A_L^2\rangle-\langle A_L\rangle^2$. We also calculated the number of lattice bonds, $\langle PP\rangle$, connecting nearest neighbour sites occupied by two different proteins. These quantities helped us to deduce the phase diagram for the model. We also calculated thermodynamic quantities for each site in order to understand in which region of the membrane is the permeability highest as a function of temperature and L/P.

Monte Carlo simulation

The procedure for calculating equilibrium thermodynamic quantities has been described elsewhere (Pink 1984). We visited each molecule in one half of the bilayer in a random sequence: (*a*) If this molecule was a lipid then we attempted to change its state using the Glauber realization of the Metropolis algorithm. (*b*) If the molecule was a protein hexagon then we attempted to move it in a randomly chosen direction by one lattice constant. The lipid molecules that would be so displaced were moved to the lattice sites that would be left vacant if the hexagon was moved. To decide whether the attempted move took place, the Kawasaki realization of the Metropolis algorithm was used.

We were interested in calculating fluctuations in lipid states at each (lipid-occupied) site of the lattice. In order to do so we adopted the following procedure. In the course of a simulation run, at a fixed protein concentration and temperature, we carried out both procedures (*a*) and (*b*) for M_1 Monte Carlo steps. We then carried out only procedure (*a*) and kept the proteins fixed for M_2 Monte Carlo steps. The average cross-section area of each lipid, as well as the fluctuation in this area were calculated over the M_2 Monte Carlo steps. We then filled up average area and area fluctuation bins, $\{a(1),\ldots,a(p)\}$ and $\{f(1),\ldots,f(q)\}$ with the numbers of lipid molecules which possessed average areas and fluctuations appropriate to those bins. We then repeated this procedure a sufficient number of times in order to obtain reliable average numbers in the various bins.

In practice we used a lattice of 900 sites and we chose $M_1=100$, $M_2=5$ and repeated the procedure ten times. The choice of $M_1=100$ ensured that the proteins would move sufficiently between calculations of area and fluctuation bin occupancies, in order that we would sample different configurations. The choice of $M_2=5$ is essentially dictated by the characteristic time for a molecule to diffuse through a bilayer about 50 Å thick. The dominant contribution to transbilayer diffusion comes from areas of fluid lipids or areas undergoing sufficiently large fluctuations. The diffusion coefficient will thus be of at least the same order of magnitude as a lipid molecule diffusing in the plane of a lipid bilayer, viz. $\geq 2.5\times10^{-8}$ cm^2/s. If $r^2=\mathrm{D}t$ is, thus, about 2.5×10^{-13} cm^2, then we find that the time to diffuse through a bilayer is $t\lesssim10^{-5}$ s. Now, a lipid hydrocarbon chain undergoes $g\rightleftarrows e$ transitions on a time-scale of $\sim10^{-6}$ s. This is, therefore, the characteristic time scale of each Monte Carlo step which attempts to carry out such transitions. Accordingly, if we wish to find out the average lipid area, or fluctuations in this quantity, during the time that the ion takes to traverse the bilayer, we need to average over between 1 and 10 Monte Carlo steps. Our choice of $M_2=5$ is, therefore, a rough approximation. From a consideration of the average values which could be obtained, we chose $p=q=7$, and considered average area, $\langle A_L\rangle$, bins defined by the following ranges (in Å^2): 40 to 46, 46 to 50, 50 to 53, 53 to 55, 55 to 58, 58 to 62 and 62 to 68. In a similar way the ranges defining the area fluctuation, $(\Delta A_L)^2$, bins were chosen to be (in Å^4): 0 to 15, 15 to 25, 25 to 50, 50 to 80, 80 to 100, 100 to 120 and 120 to ∞.

Models of ionic permeability

There are two predominant models of small-ion trans-bilayer permeability. They are (*i*) the model of Kanehisa and Tsong (1978) which assums that permeability is highest through interfacial regions connecting areas of predominantly gel and fluid lipids; (*ii*) the model of Doniach (1978) which assumes that the energy barrier to permeability is proportional to the global lateral compressibility.

Cruzeiro-Hansson and Mouritsen (1988) simplified the model of Kanehisa and Tsong (1978) (KTCM model) and assumed that the probability $P(T)$ of an ion crossing the membrane could be written as

$$P(T)=a_b(T)\,p_b+a_c(T)\,p_c+a_i(T)\,p_i \tag{3}$$

where a_b, a_c and a_i are the fractions of the membrane area occupied by the bulk, the clusters and the interfaces, and p_b, p_c and p_i are the corresponding probabilities of ion transfer across the membrane. Cruzeiro-Hansson and Mouritsen assumed that the probabilities were temperature-independent, unlike Kanehisa and Tsong. The bulk was defined to be the equilibrium phase at the temperature of interest. Thus, the bulk was the gel (fluid) phase when $T<(>)\,T_m$, the main lipid hydrocarbon chain melting transition temperature. A cluster was defined as a connected set of at least 7 lipid molecules in states characteristic of the opposite phase to the bulk. An interface was a sequence of layers of molecules surrounding a cluster which was embedded in the bulk. Cruzeiro-Hansson and Mouritsen found that, for all practical purposes, the interface was one lipid layer thick.

Here we present a modification of the KTCM model and allow for the presence of integral membrane proteins in the lipid bilayer membrane. We begin by relating the number of ions diffusing through the bilayer, and thus leaving the liposome, at time t, $\mathrm{d}N(x,t,T)$ to the physical parameters of the system,

$$\mathrm{d}N(x,t,T)\propto c(x,t,T)\,v(T)\,A_L(x,T)\,P(x,T)\,\mathrm{d}t \tag{4}$$

where $c(x, t, T)=N(x, t, T)/V(x, T)$ is the concentration of ions at time t contained inside a liposome of volume $V(x, T)$ at temperature T, the membrane of which contains a concentration x, in mole fraction, of integral membrane proteins. $v(T)$ is average speed of an ion impinging upon the inside surface of the bilayer and is taken to be $v(T)=(k_B T/2\pi m)^{1/2}$, $A_L(x, T)$ is the area of the liposome surface due to the lipid molecules and $P(x, T)$ is the probability that an ion will diffuse through the bilayer after it hits it. We then relate $V(x, T)$ to the total surface area, $A(x, T)$, of the liposome, $V(x, T)=(A(x, T)^3/4\pi)^{1/2}/3$. We assume that we can write effectively, $A(x, T)=A_L(x, T)+A_p(x)$ where $A_p(x)$ is the total cross-section area, in the plane of the bilayer, of the bilayer-spanning proteins. With these relations we obtain,

$$dN(x, t, T) \propto N(x, t, T)\, D(x, T)\, dt$$
$$D(x, T)=C P(x, T)\, T^{1/2}\, A_L(x, T)/A(x, T)^{3/2} \tag{5}$$

where $C=3(2k_B/m)^{1/2}$. We then find that

$$f(x, t, T)=N(x, t, T)/N(0)=\exp(-D(x, T)\, t) \tag{6}$$

In practice, when $x=0$, it is the ratio $R(T; T_0)=\log(f(0, t, T))/\log(f(0, t, T_0))$, normalized at a preselected temperature T_0, which is measured (Cruzeiro-Hansson and Mouritsen 1988). Here we can choose to calculate the ratio $R(x, T; x, T_0)$ or $R(x, T; 0, T_0)$ defined as

$$R(x, T; x, T_0)=\frac{\log f(x, t, T)}{\log f(x, t, T_0)} \tag{7}$$
$$=\frac{A_L(x, T)\, A(x, T_0)^{3/2}\, T^{1/2}\, P(x, T)}{A_L(x, T_0)\, A(x, T)^{3/2}\, T_0^{1/2}\, P(x, T_0)}$$

and

$$R(x, T; 0, T_0)=\frac{\log f(x, t, T)}{\log f(0, t, T_0)} \tag{8}$$
$$=\frac{A_L(x, T)\, A_L(0, T_0)^{1/2}\, T^{1/2}\, P(x, T)}{A(x, T)^{3/2}\, T_0^{1/2}\, P(0, T_0)}$$

In order to be able to compare results for different concentrations of proteins, we will use the ratio (8).

Finally, we consider how to calculate the probability $P(x, T)$. We assume that a lipid molecule in a predominantly extended or excited state determines the local permeability in its immediate vicinity. In the absence, therefore, of any other mechanism, the probability of an ion traversing the bilayer, after it has penetrated it, is

$$P_0(x, T)=a_g(x, T)\, p_g+a_f(x, T)\, p_f \tag{9}$$

where a_g and a_f are the fractions of membrane surface covered by lipid molecules in predominantly extended or excited conformational states.

To include the possible effect of regions where the compressibility is higher than the average, we weight P_0 by a factor which contains the fraction of the membrane surface possessing sufficiently large fluctuations in the cross-section area of the lipid molecules, $F(x, T)$, to give the probability

$$P(x, T)=P_0(x, T)\,(1+\alpha F(x, T))/(1+\alpha) \tag{10}$$

Here α is a parameter which describes the importance of the membrane lateral compressibility to trans-bilayer diffusion, and $0 \leqq \alpha < \infty$.

The three quantities, $a_g(x, T)$, $a_f(x, T)$ and $F(x, T)$ are defined as

$$a_g(x, T)=N_g(x, T)/N_L(x)$$
$$a_f(x, T)=N_f(x, T)/N_L(x)$$
$$F(x, T)=n_F(x, T)/N_L(x) \tag{11}$$

where N_g, N_f and n_F are the numbers of lipid molecules defined to be in generally extended states, generally excited states or possessing sufficiently arge fluctuations respectively, and $N_L(x)$ is the total number of lipid molecules.

Referring to the average area bins defined at the end of the previous section, we have identified all lipid molecules in bins 1 to 3, possessing cross-section areas ranging from ~ 40 Å^2 to 53 Å^2, as contributing to a_g, the fraction of membrane surface covered by lipid molecules in predominantly extended-chain states, and those in bins 5 to 7 with areas ranging from 55 Å^2 to 68Å^2 as contributing to a_f, the fraction of membrane surface covered by predominantly excited-state lipid molecules.

In order to identify the fraction of the membrane surface undergoing sufficiently large fluctuations in the cross-section areas, we considered all possible combinations of lipid states which a molecule could sample in $M_2(=5)$ successive Monte Carlo steps. The values of $(\Delta A_L)^2$ obtained from these combinations ranged from 0 to ~ 138 Å^4. We required that a combination should possess at least one G and at least one E state in order that fluctuations be large enough so as to create sufficient free volume for a small ion to diffuse into. When all such combinations were listed we found that $(\Delta A_L)^2$ ranged from 80.6 to 138.2 Å^4, except for the combination GIIIE which possessed $(\Delta A_L)^2=57.6$ Å^4. Accordingly, we defined the quantity $F(x, T)$, the fraction of membrane surface possessing sufficiently large fluctuations (10), to be determined by the occupancies of area fluctuation bins 5 to 7 for which $(\Delta A_L)^2$ ranges from 80 Å^4 to ∞.

It is clear that the ratios $R(x, T; x, T_0)$ and $R(x, T; 0, T_0)$ are simply the ratios of the diffusion coefficients, $D(x, T)/D(x, T_0)$ and $D(x, T)/D(0, T_0)$ respectively. These diffusion coefficients characterize the entire vesicle. It will be useful to define an analogous quantity for a single lipid molecule at site i:

$$D(i, x, T)=C P(i, x, T)\, T^{1/2}\, A_L(i, x, T)/A(x, T)^{3/2} \tag{12}$$

Here $A_L(i, x, T)$ is the cross-section area of the lipid at site i and $P(i, x, T)$ is the probability of an ion traversing the bilayer in the neighbourhood of the lipid at site i, once it has entered the bilayer. In calculating $P(i, x, T)$, the quantities $a_g(x, T)$, $a_f(x, T)$ and $F(x, T)$ are now either 1 or 0. If the lipid molecule at i is in a predominantly extended-chain conformational state then $a_g=1$ and $a_f=0$, while if it is in a predominantly excited-chain state then $a_g=0$ and $a_f=1$. If it possesses sufficiently large cross-section area fluctuations, then $F=1$, and otherwise $F=0$. We

will make use of the ratio

$$R(i, x, T; 0, T_0) = \frac{N_L(0)\, A_L(i, x, T)\, A_L(0, T_0)^{1/2}\, T^{1/2}\, P(i, x, T)}{A(x, T)^{3/2}\, T_0^{1/2}\, P(0, T_0)} \quad (13)$$

in order to give an idea of which parts of the membrane are most permeable to small ions.

Results

We defined the interaction between two proteins to be proportional to the total number of nearest neighbour lattice bonds connecting sites on the edge of one hexagon with edge sites on another hexagon. We chose the proportionality constant equal to -0.8 (in units of 10^{-13} ergs, the energy units used here and elsewhere, e.g. Pink et al. 1980) which was about the same as we found to describe the (attractive) interaction between two neighbouring bacteriorhodopsin molecules (Pink et al. 1986).

Figure 1 shows histograms of the fraction of lipid molecules which possess areas, averaged over $M_2 = 5$ Monte Carlo steps, appropriate to the seven bins $a(i)$, $i = 1, \ldots, 7$, as described above. The fractions of lipid molecules which have their cross-section areas lying between A_i and $A_i + \Delta A_i$ $(i = 1, \ldots, 7)$ are denoted by $P_{\Delta A}(T)$. The distributions are for lipid: protein ratios, bearing in mind that each protein is assumed to span the bilayer, of ∞:1 (pure DMPC), 166 : 1, 76 : 1 and 31 : 1, and for a range of temperatures from $T = 1$ or 2 °C to $T = 45$ °C. These ratios effectively range over all protein concentrations of interest. It can be seen, that for any temperature, distributions divide into two sets: Those for which the three low-area bins, 40–46, 46–50 and 50–53 Å^2, are predominantly occupied, and those for which the three high-area bins, 55–58, 58–62 and 62–68 Å^2 are predominantly occupied. Bin 4, which is concerned with lipids with average areas between 53 and 55 A^2, is effectively always empty. These results justify our earlier division of the bins into those defining lipids in extended-chain states or excited states.

We chose $p_g = 0.01$, $p_f = 0.05$ and $\alpha = 50$ and calculated $R(0, T; 0, 63)$ for the case of pure DPPC bilayers. The results are shown in Fig. 2 (small solid circles) where a line has been drawn to aid the eye, and where a comparison has been made with the results of Papahadjopoulos et al. (1973) (larger open circles). Here, we have replotted their data normalized to the rate at 63 °C. The agreement is not unreasonable when the confidence limits of the data (not plotted here) is taken into account.

Figure 3 shows the phase diagram deduced for the system modelled here: a DMPC bilayer containing bilayer-spanning proteins about the size of bacteriorhodopsin and possessing an attractive interaction beween pairs of such proteins similar to that deduced for bacteriorhodopsin molecules. We used the temperature and concentration-dependence of the average number of nearest neighbour hexagon-hexagon bonds and the average area per lipid molecule to estimate the phase boundaries which are approximate. F is a homogeneous phase in which the

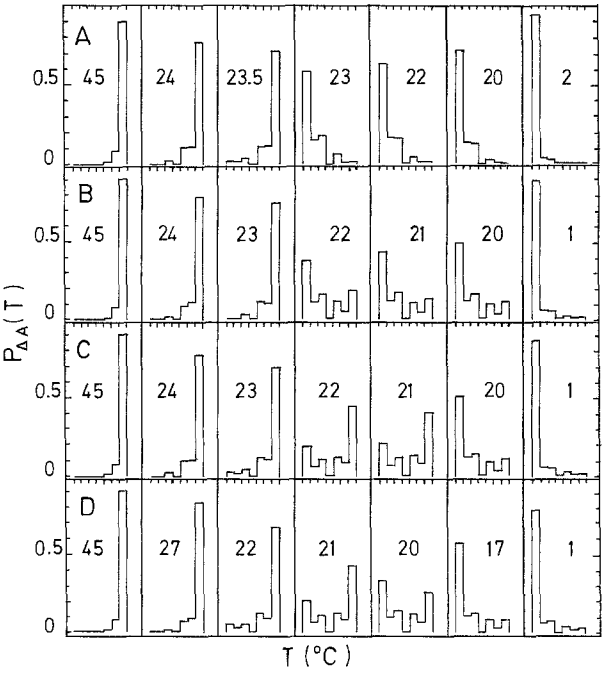

Fig. 1 A–D. Probabilities, $P_{\Delta A}(T)$, for finding a lipid molecule to possess a cross-section area lying between A_i and $A_i + \Delta A_i$ $(i = 1, \ldots 7)$. Averages are performed over 5 Monte Carlo steps and repeated ten times. For each histogram, reading from left to right, the area bins are defined by: $i = 1$, $A_1 = 40$, $\Delta A_1 = 6$; $i = 2$, $A_2 = 46$, $\Delta A_2 = 4$; $i = 3$, $A_3 = 50$, $\Delta A_3 = 3$; $i = 4$, $A_4 = 53$, $\Delta A_4 = 2$; $i = 5$, $A_5 = 55$, $\Delta A_5 = 3$; $i = 6$, $A_6 = 58$, $\Delta A_6 = 4$; $i = 7$, $A_7 = 62$, $\Delta A_7 = 6$, with all areas given in Å^2. The number associated with each histogram is the temperature (°C), and the protein concentrations (in mole fraction) are: **A** $x = 0$; **B** $x = 0.0060$; **C** $x = 0.0130$; **D** $x = 0.0313$

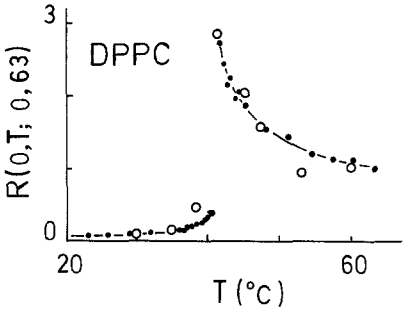

Fig. 2. $R(0, T; 0, 63)$; ((8), small solid circles) for pure DPPC for $p_g = 0.01$, $p_f = 0.05$ and $\alpha = 50$. The solid lines are a guide to the eye. Open circles are the experimental results of Papahadjopoulos et al. (1973). The numbers are normalized to the value at 63 °C

hexagons are randomly distributed and the lipid hydrocarbon chains are fluid, while G is a similar phase with the lipid chains in a generally extended state. P_F is a hexagon-rich phase in which the lipid chains are predominantly excited while P_1 is a hexagon-rich phase in which the lipid chains are in predominantly intermediate states with a smaller number in extended and excited states. Finally P_2 is a hexagon-rich phase in which the lipid chains are predominantly in their extended states. The dashed line drawn between $F + P_F$ and $G + P_1$ is the locus of a peak in the specific heat. Upon cooling from $F + P_F$, the specific heat rises relatively sharply and reaches a maximum at

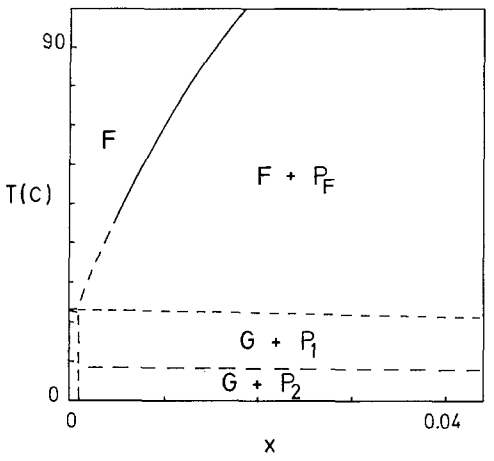

Fig. 3. Approximate phase diagram for the DMPC-protein system studied here. The phase boundaries were deduced from the statistics of protein clustering and the thermodynamic averages of the lipid variables. The dashed line separating $F+P_F$ and $G+P_1$ indicates the locus of the specific heat peak in this region, while that separating $G+P_1$ and $G+P_2$ indicates where fluctuations in protein-protein "contacts" have died away

the $F+P_F-G+P_1$ boundary. It then decreases to a value at the (dashed) $G+P_1-G+P_2$ boundary which suggests that the transition is essentially complete.

In order to calculate $R(x, T; 0, T_0)$ we must assign the probabilities p_g and p_f for this case of DMPC molecules. These probabilities are determined by calculating the probability that the ion will diffuse to the other side, after it has entered the bilayer. If we take as a simple model of such diffusion a particle undergoing a random walk along the axis of the hydrocarbon chains, i.e. essentially in one dimension, then we can formulate the following problem: Given a one-dimensional lattice extending from site 0 to site w. Let a particle start at site 1 and randomly walk from site to site. If it reaches site 0 then it is removed since we interpret this as returning to the interior of the liposome and failing to traverse the bilayer. If it reaches site w then it is removed since it has successfully traversed the bilayer. The probability, $p_s(w)$, of successfully traversing such a system is defined to be the number of times this occurs divided by the number of attempts made. We can also calculate the average "time", $\langle m_s\rangle_w$, taken to successfully traverse the bilayer. In this case it will be the average number of steps of the random walk required to do so. These two quantities possess the following solutions (see Appendix):

$$p_s(w)=1/w \qquad \langle m_s\rangle_w \sim w^2 \tag{14}$$

This dependence of $p_s(w)$ upon w is very much weaker than the exponential dependence conjectured by Georgallas et al. (1987); (7).

Accordingly, if the thicknesses of the gel and fluid bilayers of DPPC are w_g and w_f and if those of DMPC are w'_g and w'_f, then, since the density of a DMPC bilayer is essentially the same as for a DPPC bilayer and since it changes by less than 4% between the gel and fluid phases so that we take it be constant, it follows that

$$w_g/w'_g=w_f/w'_f \tag{15}$$

Thus, for the case of DMPC,

$$\begin{aligned} a_g p_g(w'_g)+a_f p_f(w'_f) &= a_g p_g(w_g)(w_g/w'_g)+a_f p_f(w_f)(w_f/w'_f) \\ &= (a_g p_g(w_g)+a_f p_f(w_f))(w_g/w'_g) \end{aligned} \tag{16}$$

If, therefore, we are comparing different results obtained by using one kind of lipid, we may use the numbers deduced for p_g and p_f from a fitting of the calculations to the data for DPPC (Fig. 2). We thus have no free parameters. Figure 4 shows calculated predictions of $R(x, T; 0, 45)$ for various values of protein concentration, x, at various temperatures, T, normalized to the corresponding value for pure DMPC at 45 °C, the highest temperature for which simulations were carried out. The solid points show the results of the calculation of small-ion diffusion for the mechanism being studied here. The lines are intended only as guides to the eye.

These are two points to note in Fig. 4. The first is that the temperature at which a maximum occurs in R decreases by about 2° for the range of x considered and coincides with the dashed line separating the two regions, $F+P_F$ and $G+P_1$. The second point is the slight decrease in R, for $T>T_m$, as x increases, but the substantial increase in R, by up to an order of magnitude, for temperatures lower than those at which the maximum occurs, as x increases.

Finally, we studied where, in the plane of the membrane and in the presence of proteins, were the fluctuations in lipid cross-section area, $(\Delta A_L)^2$, and the "relative diffusion coefficient", $R(i, x, T; 0, T_0)$ (13), largest. Recall that these quantities are defined at each lipid site, and are time-averages but not spatial averages. We stated above that the fluctuations should be largest at the interfaces between any regions composed predominantly of extended-chain and excited-chain lipids. These regions need not be macroscopic phases. Indeed, in the neighbourhood of a phase transition, microscopic regions of extended-chain and excited-chain lipids could give rise to large numbers of small interfaces. This is the physics of why an enhancement in permeability is observed at the main lipid phase transition temperature. On this basis we averred that this model, wherein large values of small-ion transbilayer diffusion were ascribed to large values of local fluctuations (instead of to large values of globally-averaged fluctuations (Doniach 1978; Caille et al. 1980)), was invoking essentially the same mechanism as that used in the KTCM model of Kanehisa and Tsong (1978) and Cruzeiro-Hansson and Mouritsen (1988). To study this mechanism in the presence of proteins we set up a model possessing a compact hexagonal region of protein sites, representing the protein rich phases P_F, P_1, or P_2, in a sea of lipids, kept the protein region fixed in space and studied the spatial dependence of the average lipid area and lipid area fluctuations as functions of temperature. We averaged these quantities over all lipids lying on a hexagonal "layer", l, concentric with the centre of the hexagon of protein sites. The number of protein sites corresponded to a concentration of $x\approx 0.08$. Figure 5 shows the results obtained for the average lipid area, $\langle A_L(l)\rangle$, and area fluctuation, $\langle \Delta A_L^2(l)\rangle$, as a function of hexagonal layer, l, from the edge of the protein hexagon, for four temperatures. For T

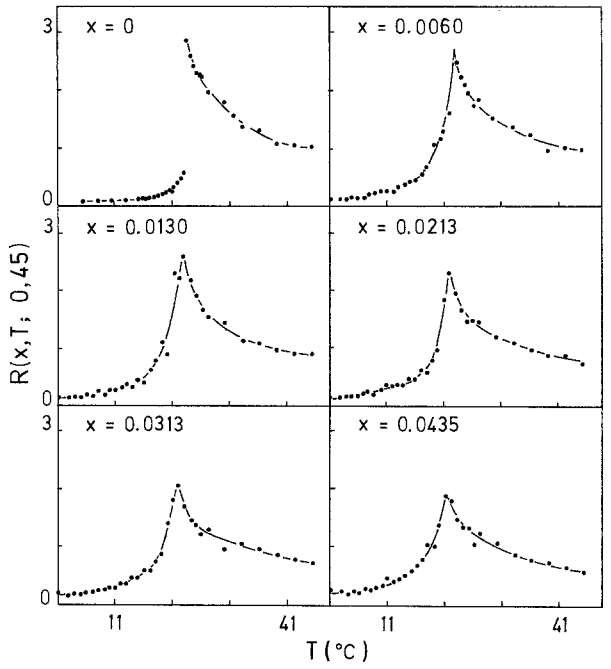

Fig. 4. Predicted values of $R(x, T; 0, 45)$ ((8); small solid circles) for the DMPC-protein system possessing the phase diagram of Fig. 3, for various protein concentrations x (mole fraction). The numbers are normalized to the value for $x=0$ at 45 °C. The solid lines are intended as a guide to the eye

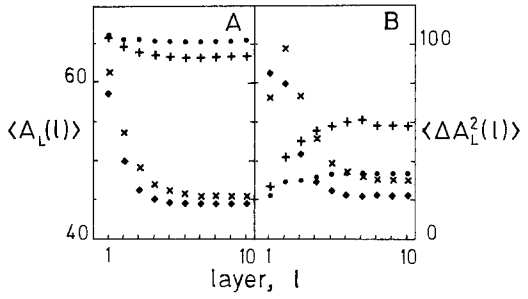

Fig. 5. **A** Lipid area, $\langle A_L(l)\rangle$, and **B** lipid area fluctuation, $\langle \Delta A_L^2(l)\rangle$, averages for concentric hexagonal layers, l, around a protein hexagon containing 91 sites. $l=1$ corresponds to the lipid layer adjacent to the hexagon. $T=29$ °C (●), $T=23$ °C (+), $T=20$ °C (×) and $T=17$ °C (◆). Averages were calculated over 500 Monte Carlo steps

well above T_m, the area depends only weakly upon l and the fluctuations are negligible. As T approaches T_m from above the protein region is seen to keep lipids adjacent to it in a melted state and keep their fluctuations at a minimum. The large fluctuations all occur at distances $l>3$ from the surface of the protein region.

At $T=20$ °C, at which the lipids sufficiently far from the hexagon are in a generally extended chain state, there is a melted region about one layer deep around the hexagon. A lipid molecule in this position possesses and average area of ~ 61 Å^2, while a molecule in the third layer from the perimeter of the hexagon possesses an area of ~ 49 Å^2. The second layer, made up of molecules with area ~ 54 Å^2, plays the role of an interface between a melted layer and a region of extended chains. It can be

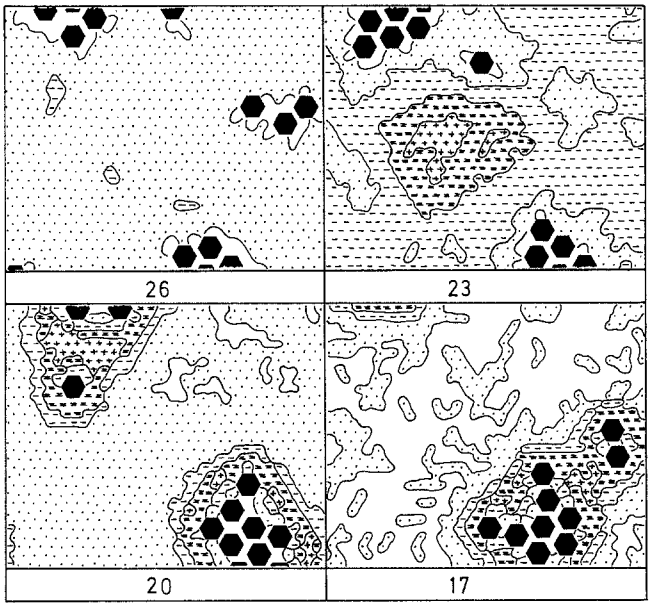

Fig. 6. Lipid area fluctuations for $x=0.006$ as a function of temperature. Proteins move and lipids change their state for 1500 Monte Carlo steps at a given temperature. Protein positions are then fixed and lipid area fluctuations are calculated over 500 steps. The temperature (°C) is given under each diagram. The fluctuation bins, describing a range of $\langle \Delta A_L^2\rangle$, shown are (in units of Å^4): 0 to 25 (blank); 25 to 50 (●); 50 to 80 (—); 80 to 100 (Z); 100 to 120 (+); 120 to ∞ (■)

seen in Fig. 5B that the maximum in the fluctuations occurs on this second layer, although substantial fluctuations take place on the first and third layers.

Finally, at $T=17$ °C, the second layer has become partially ordered with an area of ~ 50 Å^2 and the area per molecule in the first layer adjacent to the hexagon perimeter is ~ 59 Å^2. Large fluctuations now occur only on the first and second layers. These results confirm our earlier speculation that the greatest fluctuations occur at the interfaces between, possibly small, regions of extended-chain and excited-chain lipids. From Fig. 5B it can also be seen that as the temperature is decreased through T_m the regions of maximum fluctuations become closer to regions possessing a high concentration of proteins, until the largest fluctuations are occurring on lipid molecules adjacent to the proteins.

Figure 6 shows where lipid area fluctuations occur in the plane of the bilayer for $x=0.0060$ as a function of decreasing temperature. Here, the hexagons were allowed to move and the lipids allowed to change their state and come to equilibrium for 1500 Monte Carlo steps, after which the proteins were not allowed to move, but $\langle \Delta A_L^2\rangle$ was calculated for each lipid over 500 Monte Carlo steps. At $T=26$ °C there are few large fluctuations and in the neighbourhood of the proteins the fluctuations are less than the average. When $T=23$ °C however, there is a large area between protein-rich regions in which large fluctuations occur. We see here a case in which large fluctuations occur in a region which is not obviously an interface between extended-chain and excited-chain lipids. The fluctuations arise here because at 23 °C lipids far from a protein want to freeze, but the effect of the protein is to

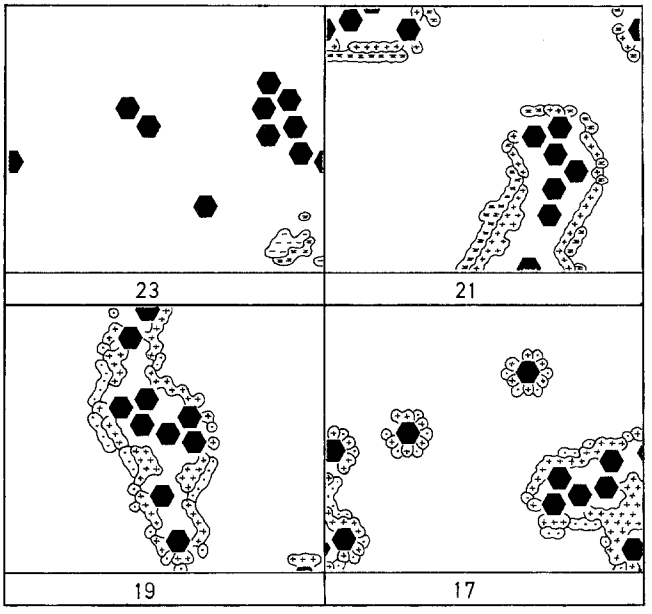

Fig. 7. $R(i, 0.006, T; 0, 45)$ as a function of temperature. Proteins move and lipids change their states for 1500 Monte Carlo steps, at a given temperature, after which the protein positions are fixed and R is calculated for all sites over 200 Monte Carlo steps. The temperature (°C) is given under each diagram. The bins, describing the ranges of R are: 0 to 5 (blank); 5 to 10 (•); 10 to 15 (:); 15 to 20 (—); 20 to 25 (Z); 25 to 30 (+); 30 to ∞ (■)

try to keep them in excited states. The equal importance of these competing effects gives rise to large fluctuations. In the neighbourhood of the proteins there are still only very small fluctuations occurring. At $T = 20°C$, the regions of large fluctuation have moved to the neighbourhood of the proteins, which still, however, keep the fluctuations in some of the lipids close to them relatively small. At $T = 17°C$, however, there are few areas of substantial fluctuations in the regions between protein-rich areas, and the largest fluctuations occur in the immediate neighbourhoods of the proteins. These results are analogous to what we found when using one large hexagonal protein patch, and shown in Fig. 5.

Figure 7 shows $R(i, x, T; 0, 45)$ and is a measure of the diffusion through the bilayer in the neighbourhood of a lipid at site i. Again we chose $x = 0.0060$ and decreased the temperature. The simulation was performed as above: The system was allowed to equilibrate at a given temperature for 1500 Monte Carlo steps, after which the proteins were not allowed to move. For a further 200 Monte Carlo steps all $R(i, x, T; 0, 45)$ were calculated. It can be seen that at 23 °C the largest diffusion takes place in a small region between small clusters of proteins. This is a consequence of the large fluctuations occuring far from proteins because of the competition between proteins, which want to keep the lipids in excited states, and the temperature of 23 °C at which pure lipids would freeze. At 21 °C the protein-rich phase is ringed by a high-diffusion band about two lipid layers away. As T is decreased to 19 °C and 17 °C these bands draw nearer to the proteins. The results are in accord with those of Fig. 6.

Conclusions

We have addressed the following question: What do theoretical models predict concerning the effect of integral membrane proteins upon the passive diffusion of small ions through a lipid bilayer membrane if the only mechanism considered is diffusion through the lipid regions but not diffusion via the proteins themselves. That is, we do not consider ion transfer through a protein or on a protein surface due to a specific interaction between the ion and the protein. We used a three-state model of lipid molecule hydrocarbon chain states, in which nine low-energy states of a single hydrocarbon chain were represented by a single effective ground state, obtained using a mean-field approximation. We modelled a particular kind of protein, viz. one which is about the size of bacteriorhodopsin and which appear to interact with lipids and aggregate in ways similar to that protein. We assumed that there were no interactions between the polar segments of the proteins and the lipids, and no specific interactions between a hydrocarbon chain and the surface of an integral protein. It seems plausible, however, that even if a protein does not interact specifically with a lipid molecule, it might change the effective degeneracy of the states available to the molecule because of the hard-core interaction between the lipid molecule and the protein surface. This, we feel, is a subject for a paper rather than a side issue in a study of trans-bilayer diffusion. A recent molecular dynamics study of the effect of the presence of an alpha helix upon hydrocarbon chains (Edholm and Johansson 1987) has not studied how the helix affects the distribution of states available to the hydrocarbon chains adjacent to them. This study has found, in accord with ^{2}H NMR studies, that the static "order parameters" of lipid chains adjacent to the helix differ little from those further away.

In order to calculate the permeability, we modified the theory of Cruzeiro-Hansson and Mouritsen (1988) by assuming that small ions move across those regions of a membrane where the local compressibility, as reflected in the lipid cross-sectional area fluctuations, is sufficiently large. We also modified their theory to take into account the presence of integral proteins spanning the lipid bilayer membrane. We (*a*) calculated the rate of diffusion of small ions out of liposomes containing such proteins and (*b*) studied through which regions of the membrane would the ions diffuse predominantly as a function of temperature.

Our conclusions are as follows:

(*a*) The normalized rate of small-ion diffusion out of liposomes, as a function of temperature and protein concentration, x, is shown in Fig. 4. The change in the calculated rate with x is most dramatic for temperatures slightly below T_m.

(*b*) The regions of greatest permeability depend upon the temperature. At high temperatures it is uniformly distributed except near the proteins where it is small. For $T \approx T_m^+$ the regions of greatest fluctuations lie approximately midway between protein clusters (Fig. 6, $T = 23$ °C) and these give rise to the largest permeabilities

(Fig. 7, $T = 23\,^{\circ}C$). Note that these regions do not occur at an interface between extended-chain and excited-chain lipids but exist because of the competition between the temperature, which is near T_m, and the presence of proteins which try to keep them in their excited states. As T decreases below T_m the regions of greatest permeability are in regions near to the proteins and become closer to them, eventually, disappearing, as T decreases further.

(*c*) By showing (Fig. 5) that some of the regions of largest fluctuations, which give rise to the greatest permeability, occur at interfaces between regions of excited-state lipids and extended-chain lipids in the bilayer, we have presented some evidence that the models of Kanehisa and Tsong, and Cruzeiro-Hansson and Mouritsen are effectively the same as the model used here. One should note, however, the exceptions to this remarked upon above. The model presented here recognizes that for small-ion permeability it is the local area fluctuations, and not the globally-averaged fluctuations used in earlier models (Doniach 1978; Caille et al. 1980), which affect trans-bilayer permeability.

If it is confirmed that this picture of small-ion permeability is correct than further work can usefully proceed towards modelling bilayer permeability to larger molecules and how this permeability can be controlled by the presence of molecules in the bilayer.

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Appendix

Label the sites 0, 1, ..., w and let the walker begin at site 1. Then if $p_s(w)$ is the probability of (successfully) walking to site w, without stepping on either site w or site 0 at any intermediate step, and if $p_f(w)$ is the probability of walking to site 0 in a similar fashion,

$$p_s(w) = p_s(w-1)\, p_f(w) = p_s(w-1)\,(1 - p_s(w)) \qquad \text{(A-1)}$$

which yields

$$p_s(w) = p_s(w-1)/(1 + p_s(w-1)) \qquad \text{(A-2)}$$

This is trivially iterated to give $p_s(w) = 1/w$.

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