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Electron Spin Resonance and Steady-State Fluorescence Polarization Studies of Lipid Bilayers Containing Integral Proteins†

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ABSTRACT: We derive equations that describe changes in the steady-state fluorescence polarization of the probe 1,6-diphenyl-1,3,5-hexatriene (DPH) or in the spectrum of electron spin resonance (ESR) nitroxide spin-labeled lipid probes as a function of the intrinsic molecule concentration in lipid bilayer membranes. We make use of an assumption used by us in an earlier paper. The equations are independent of any membrane model. They are valid when a DPH probe or a spin-labeled chain is equivalent to an unlabeled lipid hydrocarbon chain only as far as their general space-filling properties are concerned. We consider cases where the bilayer is either in a single homogeneous phase or in a two-phase region. We apply our equations to analyze ESR data from delipidated sarcoplasmic reticulum membranes and from egg yolk phosphatidylcholine bilayers containing Ca^{2+}-ATPase, and DPH data from dipalmitoylphosphatidylcholine (DPPC) bilayers containing Ca^{2+}-ATPase, both for $T > T_c$. The following conclusions were derived: (i) Ca^{2+}-ATPase oligomers are "randomly" distributed, for the concentrations studied, in the fluid phase. (ii) There is no fixed stoichiometric ratio of "boundary" lipids and oligomers. (iii) Between $24k$ and $28k$ lipid molecules are able to surround each isolated oligomer composed of k Ca^{2+}-ATPase monomers. Finally, we apply our equations to analyze DPH studies on DPPC bilayers containing Ca^{2+}-ATPase for $T < T_c$. We find that the results reported are in accord with the predictions of the model. In the Appendix, we show that an analytical expression for probabilities used by us is in very good agreement with the results of computer simulation.

The question of what is measured by perturbing probes, such as nitroxide-labeled acyl chains used in electron spin resonance (ESR)[1] studies or the fluorescent probe DPH, when used in phospholipid bilayers containing integral proteins appears to still be unresolved. There is the view [e.g., see Marsh et al. (1982)] that the apparent discrepancy between ^2H NMR measurements and nitroxide-labeled ESR probes can be accounted for by a time-scale argument. Because such probes do report information on the static order parameter of the chain to which they are attached, it has been found necessary to bolster this with an additional picture of the state of lipid chains adjacent to an integral protein: Two competing effects have been suggested [e.g., see Jähnig (1980)] to explain results observed on the ^2H NMR time scale. These measurements make it appear as though lipid chains adjacent to a protein are essentially statically identical with chains in the absence of any protein, at the same temperature T, in some range of $T > T_c$. The suggested picture envisages hydrocarbon chains adjacent to an intergral protein to have fewer gauche bonds than those sufficiently far from the protein but to have a long-axis orientation that does not, on the average, coincide with the bilayer normal. It is argued that ^2H NMR will detect the combined motion but that ESR will detect only the reduction in gauche conformers. This view is not supported by recent infrared studies (Cortijo et al., 1982; Alonso et al., 1982). These studies show that for a number of intrinsic molecules,[2] the gauche "content" as measured by IR spectroscopy is essentially identical with that reported by ^2H NMR spectroscopy [e.g., see Jacobs & Oldfield (1981) and Seelig & Seelig (1980)]. The question as to what property is being reported by steady-state DPH fluorescence polarization or by nitroxide-labeled ESR probes thus remains unanswered.

Some years ago, a new interpretation of DPH steady-state fluorescence polarization measurements was made (Hoffmann et al., 1981). In this paper, we extend these ideas and present a number of new results. The original work came about because the observed steady-state polarization as a function of

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[1] Abbreviations: ESR, electron spin resonance; NMR, nuclear magnetic resonance; DPH, 1,6-diphenyl-1,3,5-hexatriene; T_c, pure lipid main phase transition temperature; PC, phosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; SR, sarcoplasmic reticulum; IR, infrared; c, intrinsic molecule concentration defined as (number of intrinsic molecules)/(number of intrinsic molecules + number of lipids); EYPC, egg yolk phosphatidylcholine; proxyl, 2,2-dimethylpyrrolidinyl-1-oxy.

[2] In Hoffmann et al. (1981), "intrinsic molecule" refers to cholesterol, gramicidin A, or integral proteins in a lipid bilayer. Here, although we shall refer to "integral proteins" and apply our equations accordingly, the most general equations should be, in addition, applicable to the cases of cholesterol and gramicidin A in lipid bilayers composed of a single kind of lipid. Readers should note the changes to be made when such a molecule is restricted to half of the bilayer.

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the intrinsic molecule[2] concentration, c, in DMPC for $T > T_c$ showed a monotonic increase which reached a maximum at some large value of c. Since it was generally believed that this quantity was reporting information about the static lipid order parameter, then this observation was not surprising in the case of cholesterol. In the cases of gramicidin A and cytochrome oxidase, however, such observations appeared not to be in accord with ^2H NMR studies (Rice & Oldfield, 1979; Rice et al., 1979).

The following assumption (postulate I) was made in order to understand these results: *The motion of a DPH molecule is significantly changed*[3] *by its proximity to at least one intrinsic molecule.* No conjecture need be made about the mechanism, but one possibility is simply a steric effect: DPH fluoresces in its linear configuration, and its motion may be sterically hindered by a protein which might not hinder a more flexible saturated hydrocarbon chain.

The observation that ESR measurements on cytochrome oxidase in DMPC for $T > T_c$ showed a similar behavior to the results of the DPH probe (Knowles et al., 1979) suggested a model based on the following assumption (postulate II): *The motion of a nitroxide-labeled hydrocarbon chain is significantly changed*[3] *by its proximity to at least one intrinsic molecule.* This may be because, for example, the label protrudes out from the chain in question, and steric hindrance, due to the proximity of, e.g., an integral protein, changes its motion in contrast to an unlabeled chain.

Previously, Hoffmann et al. (1981) combined postulate I with a model of the dependence of the lipid hydrocarbon chain order parameter upon the intrinsic molecule concentration (c) and an assumed dependence of τ_F/τ_c, the ratio of the fluorescence lifetime to the rotational correlation time, upon c. They showed that even though the lipid order parameter changed in a complicated way with c, the effect of cytochrome oxidase was to dominate the probe's behavior. Their results showed that, since the "random" distribution of proteins permitted protein–protein "contacts" this implied that there was no permanent "annulus" of lipids completely surrounding a protein, i.e., no fixed stoichiometric ratio of "boundary lipids" to protein.

Hoffmann et al. (1981) also used postulate II to show that the fraction of the ESR signal interpreted as indicating immobilized lipids ("fraction immobilized component") could be understood over the entire concentration range for the case of cytochrome oxidase in DMPC bilayers for $T > T_c$. The only parameter entering the calculation was the number of lipid molecules which could fit around an isolated protein in the bilayer.[4] It is noteworthy that the original interpretation of these data in terms of an annulus of immobilized lipids made use of only half of the measured points (Knowles et al., 1979).

It was pointed out by Hoffmann et al. (1981) that, if postulates I and II were shown to be correct, then this gave a method of discovering the size of the intrinsic molecule's perimeter and the existence of oligomers.

Here we extend the ideas presented earlier in the following ways: (i) We derive equations which are sufficiently general so as not to depend upon any model of lipid hydrocabon chain ordering, but depend only on postulates I and II. We consider the cases in which the system is either in a single homogeneous phase or in a two-phase region. (ii) We analyze ESR data on delipidated SR membranes (Jost & Griffith, 1978; Thomas et al., 1982) and show that an earlier analysis (Marsh & Watts, 1982) has underestimated the number of labeled lipids contributing to the "immobilized" component. We show that the ESR data and measurements using DPH steady-state fluorescence polarization (Gomez-Fernandez et al., 1980) to study Ca^{2+}-ATPase in DPPC bilayers are essentially in agreement. We also show that our model gives an excellent fit to data obtained very recently by Silvius et al. (1984) for Ca^{2+}-ATPase in EYPC. We conclude the following: (a) Ca^{2+}-ATPase oligomers are "randomly" distributed in a fluid phase for $T > T_c$. (b) There is no evidence for a fixed stoichiometric ratio of "boundary lipids" to Ca^{2+}-ATPase in the membranes studied. (c) Although labeled lipids or DPH molecules adjacent to Ca^{2+}-ATPase molecules are motionally perturbed, there is no evidence to suggest that unlabeled lipids are similarly perturbed. (iii) We give examples of the phenomena that might be observed if the two-component protein–lipid bilayer is in a two-phase region. In this case, we assume that the probes partition into the two phases as if they were unlabeled lipid chains. If this does not occur, then we outline the changes that might be expected. Our results are in general agreement with measurements on Ca^{2+}-ATPase in DPPC bilayers. Throughout this paper, it is to be understood that in a single phase the probes are assumed to be distributed like the unlabeled lipids. (iv) In the Appendix, we show that the probability equation which we use is in very good agreement with the results of computer simulations for a variety of "protein" sizes over a wide range of concentrations. Finally, we remark that similar results for cytochrome oxidase suggest that conclusions a–c under (ii) may be common to a variety of integral proteins in bilayer membranes.

Theory

In this section, we outline the steps needed to obtain the equations we want. We begin by representing the plane of half of the bilayer by a triangular lattice (also referred to as a hexagonal lattice), each site of which can be occupied either by a lipid hydrocarbon chain, which may be spin-labeled, or by a DPH molecule. Although these may have very different dynamic properties, this model simply reflects the fact that the cross-sectional areas of a labeled or unlabeled lipid chain or of a DPH molecule are similar when compared to that of an integral protein. An integral protein is repesented by a hexagon which occupies a number of lattice sites. This shape represents the average cross-sectional area of an integral protein in the hydrophobic region of the bilayer. If the distribution of integral proteins is "random", the probability that a DPH molecule or a labeled lipid chain is not adjacent to any integral protein is denoted by $p(M,c)$. Here, M is the maximum number of chains or DPH molecules which can fit around an isolated integral protein or oligomer in half of the bilayer. Evidently, for bilayer-spanning molecules, M is the maximum number of lipid molecules which can fit around an isolated integral protein (or an isolated oligomer) in the bilayer.

In our derivation of the equations which follow, we assume that the probes, whether they are DPH molecules or labeled lipid chains, are distributed like the unlabeled lipids. Thus, for example, we assume that the probes partition into two coexisting phases in amounts proportional to the amounts of unlabeled lipids in the two phases.

If this assumption is not valid, then the basis for the use of probes to deduce properties of unlabeled lipids is weakened. However, if the ratio of probes to unlabeled lipid molecules

[3] "Changed" compared to its motion when the intrinsic molecules are absent, i.e., in a pure lipid bilayer.

[4] In that paper, the cross section of the molecules was referred to as effectively cylindrical. In fact, the calculations were performed for a triangular lattice (as stated there) with the cross section of the molecules taken to be hexagons. In the Appendix here, we show that the equation used there is in very good agreement with the results of computer simulations involving hexagons on a triangular lattice.

is very low, then the probes are fairly certainly randomly distributed in a single phase for $T > T_c$.

Steady-State Fluorescence Polarization of DPH Probes as a Function of c. (A) Two-Phase Region. In a homogeneous phase n ($n = 1, 2$) containing a concentration c_n of intrinsic molecules, the steady-state intensities are

$$I_S(c_n) = I_S^L(c_n)p(M,c_n) + I_S^P(c_n)[1 - p(M,c_n)] \quad (1a)$$

$$S = \parallel \text{ or } \perp \quad (1b)$$

where we have allowed the possibility that the steady-state intensities of the probe when it is surrounded by lipids, $I_S^L(c_n)$, or is adjacent to at least one intrinsic molecule, $I_S^P(c_n)$, depend upon the concentration of intrinsic molecules in that phase. The fractions of lipid molecules in each of the two phases are

$$\frac{N_{L1}}{N_L} = \frac{(1 - c_1)(c_2 - c)}{(c_2 - c_1)(1 - c)} \quad (2a)$$

$$\frac{N_{L2}}{N_L} = \frac{(1 - c_2)(c - c_1)}{(c_2 - c_1)(1 - c)} \quad (2b)$$

where N_{L1} and N_{L2} are the numbers of lipid molecules in phases 1 and 2, respectively, $N_L = N_{L1} + N_{L2}$, and $c_1 < c < c_2$. If we assume that the DPH probes partition into the two phases proportional to the fractions of the lipid molecules in those phases, then the steady-state intensity averaged over the two phases is

$$I_S(c) = \frac{N_{L1}}{N_L}I_S(c_1) + \frac{N_{L2}}{N_L}I_S(c_2) \quad (3a)$$

$$S = \parallel \text{ or } \perp \quad (3b)$$

Using the equation relating the steady-state polarization, $P(c)$, to the intensities, $P(c) = [I_\parallel(c) - I_\perp(c)]/[I_\parallel(c) + I_\perp(c)]$ (Shinitzky & Barenholz, 1978), we get

$$P(c) = \frac{(1 - c_1)(c_2 - c)I_-(c_1) + (1 - c_2)(c - c_1)I_-(c_2)}{(1 - c_1)(c_2 - c)I_+(c_1) + (1 - c_2)(c - c_1)I_+(c_2)} \quad (4a)$$

$$I_\pm(c_n) = I_\parallel(c_n) \pm I_\perp(c_n) \quad (4b)$$

Let us now define the normalized polarization, $P'(c)$:

$$P'(c) = [P(c) - P(c_1)]/[P(c_2) - P(c_1)] \quad (5)$$

Then, a straightforward calculation gives

$$P'(c) = \frac{(1 - c_2)(c - c_1)I_+(c_2)}{(1 - c_1)(c_2 - c)I_+(c_1) + (1 - c_2)(c - c_1)I_+(c_2)} \quad (6)$$

where substitutions must be made for $I_+(c_1)$ and $I_+(c_2)$. For the particular case that $c_1 = 0$, as it might effectively be for some protein–lipid bilayers at $T < T_c$ [e.g., see Bienvenue et al. (1982) and Lookman et al. (1982)]

$$P'(c) = \frac{(1 - c_2)I_+(c_2)c}{c_2I_+(0) - c[I_+(0) - (1 - c_2)I_+(c_2)]} \quad (7)$$

which is of the form $Ac/(1 - Bc)$.

(B) One-Phase Region. When the system is in a single homogeneous phase as, for example, in some protein–lipid bilayers for $T > T_c$ (Gomez-Fernandez et al., 1980; Boggs et al., 1980), then the average steady-state intensity is

$$I_S(c) = I_S^L(c)p(M,c) + I_S^P(c)[1 - p(M,c)] \quad (8a)$$

$$S = \parallel \text{ or } \perp \quad (8b)$$

and the steady-state polarization is

$$P(c) = \frac{I_-^L(c)p(M,c) + I_-^P(c)[1 - p(M,c)]}{I_+^L(c)p(M,c) + I_+^P(c)[1 - p(M,c)]} \quad (9)$$

If $P(c)$ has been measured over some range $0 < c < c_m$, then we can calculate the normalized polarization:

$$P'(c) = [P(c) - P(0)]/[P(c_m) - P(0)] \quad (10)$$

Substituting eq 9 into eq 10, we obtain

$$P'(c) = \{\{I_+^P(c_m) + [I_+^L(c_m) - I_+^P(c_m)]p(M,c_m)\}/\{I_+^P(c) + [I_+^L(c) - I_+^P(c)]p(M,c)\}\}\{\{I_+^L(0)I_-^P(c) - I_-^L(0)I_+^P(c) + [I_+^L(0)[I_-^L(c) - I_-^P(c)] - I_-^L(0)[I_+^L(c) - I_+^P(c)]]p(M,c)\}/\{I_+^L(0)I_-^P(c_m) - I_-^L(0)I_+^P(c_m) + [I_+^L(0)[I_-^L(c_m) - I_-^P(c_m)] - I_-^L(0) \times [I_+^L(c_m) - I_+^P(c_m)]]p(M,c_m)\}\} \quad (11)$$

Now, in many cases it seems plausible that $I_S^P(c)$ will be independent of c since it is a property of the interaction between the probe and a single intrinsic molecule. $P'(c)$ then simplifies to become

$$P'(c) = \{\{I_+^P + [I_+^L(c_m) - I_+^P]p(M,c_m)\}/\{I_+^P + [I_+^L(c) - I_+^P]p(M,c)\}\}\{\{I_+^L(0)I_-^P - I_-^L(0)I_+^P + [I_+^L(0)[I_-^L(c) - I_-^P] - I_-^L(0)[I_+^L(c) - I_+^P]]p(M,c)\}/\{I_+^L(0)I_-^P - I_-^L(0)I_+^P + [I_+^L(0)[I_-^L(c_m) - I_-^P] - I_-^L(0)[I_+^L(c_m) - I_+^P]]p(M,c_m)\}\} \quad (12)$$

Finally, for many integral proteins, it appears plausible from ^{2}H NMR data (Jacobs & Oldfield, 1981; Seelig & Seelig, 1980) that $I_S^L(c)$ is also independent of c. We then get

$$P'(c) = \frac{I_+^P + (I_+^L - I_+^P)p(M,c_m)}{1 - p(M,c_m)} \frac{1 - p(M,c)}{I_+^P + (I_+^L - I_+^P)p(M,c)} \quad (13)$$

If c_m is sufficiently large so that $p(M,c_m)$ is so close to zero[5] that c_m can effictively be chosen as unity, then we obtain the most simple form of eq 11:

$$P'(c) = \frac{1 - p(M,c)}{1 - (1 - I_+^L/I_+^P)p(M,c)} \quad (14)$$

Electron Spin Resonance. Since many of the equations here will be similar to those given in the preceding sections, we shall not repeat them. The quantity of interest here is the fraction of the signal contributed by the "immobile" component. We denote the ESR spectrum, divided by the number of labeled chains, for a concentration c of intrinsic molecules as

$$S(c) = S_f f_f(c) + S_i f_i(c) \quad (15)$$

where S_f and S_i are the spectra due to a "free" and an immobile spin-labeled lipid chain, respectively. $f_f(c)$ and $f_i(c)$ are the fractions of free and immobile spin-labeled lipid chains, with $f_f(c) + f_i(c) = 1$. We wish to obtain expressions for these fractions.

(A) Two-Phase Region. In a homogeneous phase with concentration c_n of intrinsic molecules, the ESR spectrum is

$$S(c_n) = S_f(c_n)p(M,c_n) + S_i(c_n)[1 - p(M,c_n)] \quad (16)$$

where we have allowed S_f and S_i to depend upon the concentration. By using eq 2 and

$$S(c) = \frac{N_{L1}}{N_L}S(c_1) + \frac{N_{L2}}{N_L}S(c_2) \quad (17)$$

we obtain

[5] This is always possible for the model used here.

$$S(c) = \{[(1 - c_1)(c_2 - c)S_f(c_1)p(M,c_1) + (1 - c_2)(c - c_1)S_f(c_2)p(M,c_2)] + [(1 - c_1)(c_2 - c)S_i(c_1)[1 - p(M,c_1)] + (1 - c_2)(c - c_1)S_i(c_2)[1 - p(M,c_2)]]\}/[(c_2 - c)(1 - c)] \quad (18)$$

If S_f and S_i are independent of c, then we obtain for this case

$$f_i(c_1,c_2,c) = \{(1 - c_1)(c_2 - c)[1 - p(M,c_1)] + (1 - c_2) \times (c - c_1)[1 - p(M,c_2)]\}/[(c_2 - c_1)(1 - c)] \quad (19)$$

If $c_1 = 0$ (see comment between eq 6 and 7), then

$$f_i(0,c_2,c) = \frac{1 - c_2}{c_2}[1 - p(M,c_2)]\frac{c}{1 - c} \quad (20)$$

Note that with $c_2 = 1$ we find that $f_i(0,1,c) = 0$. This simply reflects the fact that for $c_2 = 1$ there are no lipids at all in phase 2 and therefore, by our assumption leading to eq 4 and 18, no probes in phase 2.

(*B*) *One-Phase Region.* In this case, we have simply

$$S(c) = S_f(c)p(M,c) + S_i(c)[1 - p(M,c)] \quad (21)$$

so that

$$f_i(c) = 1 - p(M,c) \quad (22)$$

Probabilities. In order to use the equations derived here, we must obtain an expression for $p(M,c)$, the probability that, if the distribution of integral proteins is "random", a DPH molecule or a labeled lipid chain is not adjacent to any integral protein. In the Appendix, we provide evidence from computer simulations with hexagons distributed on a triangular lattice that, at least to a very good approximation

$$p(M,c) = (1 - x)^M \quad (23)$$

Here $x = c$ if the integral proteins span the bilayer, or $x = c/(2 - c)$ if they are confined to half of the bilayer. If the molecules span the bilayer and k of them form an oligomer (a k-mer) in the bilayer, then $x = c/[k - (k - 1)c]$ and M is the maximum number of chains or DPH molecules which can fit around an isolated hexagonal oligomer in half of the bilayer. Thus, M is then the maximum number of lipid molecules which can fit around an isolated oligomer in the bilayer.

We have pointed out before (Hoffmann et al., 1981) that $p(M,c) = (1 - x)^M \approx e^{-Mx}$ when x is small. When x is large, $(1 - x)^M$ and e^{-Mx} may differ by orders of magnitude, but because they are both very small, they are both effectively zero as far as experimental resolution is concerned. Hence, for some practical purposes, $p(M,c) \approx e^{-Mx}$. When this is substituted into eq 14, we see the orgin of the exponential behavior of $P'(c)$ referred to by Hoffmann et al. (1981). For the case of cytochrome oxidase considered by Hoffmann et al. (1981), $1 - I_+^L/I_+^P \approx -0.025$ so that $P'(c) \approx 1 - (1 - x)^M \approx 1 - e^{-Mx}$.

Results and Discussion

Lipid Bilayers Containing Ca^{2+}-ATPase. In this section, we are concerned with bilayers in a homogeneous fluid phase. We use eq 14 to analyze the concentration dependence of DPH fluorescence polarization and eq 22 to study the ESR spectrum.

Figure 1A shows the data of Gomez-Fernandez et al. (1980) as a function of protein concentration in DPPC bilayers at 46 °C. In order to estimate the asymptotic value of the steady-state fluorescence polarization, we noted that the ESR immobilized fraction had a value of $\gtrsim 0.7$ when $c = 0.05$ (Figure 1B). We, therefore, took the normalized polarization at the highest concentration measured ($c = 0.05$) to be 0.75. With this choice, we found the asymptotic value to be $P(1) = 0.262$. Experimentally, $P(0) = 0.130$, and these two values gave $1 - I_+^L/I_+^P = -0.025$. We replotted the data of Gomez-Fernandez et al. (1980) as the normalized polarization $P'(c)$, and the six data points are shown in Figure 1A. Using eq 14, we found that the best fit, by eye, was the monomer curve with $M = 25$, or the dimer, trimer, and tetramer curves with $M = 50$, 75, or 100, respectively.

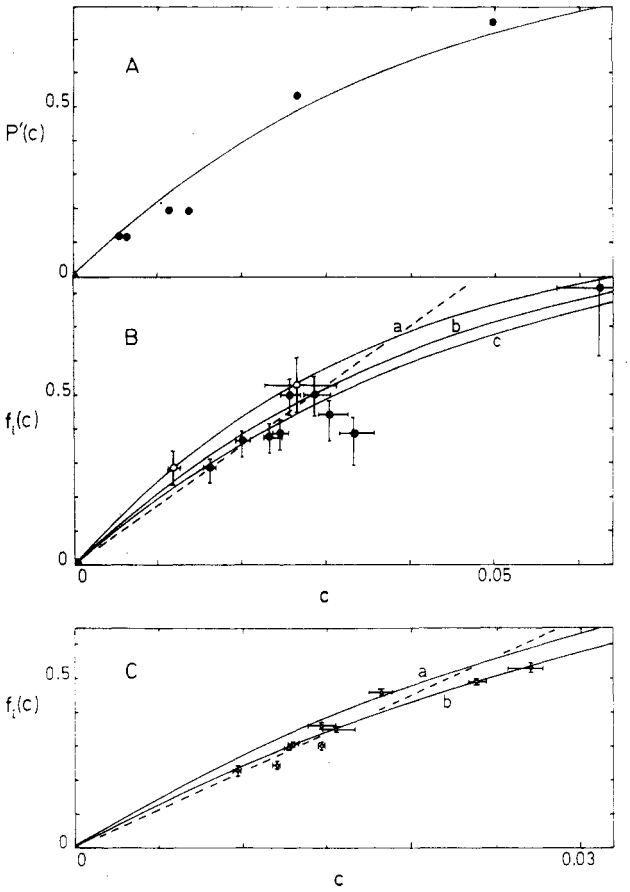

FIGURE 1: (A) DPH steady-state fluorescence polarization as a function of Ca^{2+}-ATPase concentration in DPPC at 46 °C: (●) data points of Gomez-Fernandez et al. (1980) replotted here (see the text). The solid line is a plot of eq 14 for monomers ($k = 1$) with $I_+^L/I_+^P = 1.025$ and $M = 25$. (B) ESR fraction-immobilized component, $f_i(c)$, as a function of Ca^{2+}-ATPase concentration in delipidated SR membranes: (●) data points of Jost & Griffith (1978); (○) data points of Thomas et al. (1982). The solid lines are plots of eq 22 for dimers ($k = 2$) with (a) $M = 56$, (b) $M = 48$, and (c) $M = 44$. The dashed line is a plot of eq 26, which follows from the assumption that each monomer is always surrounded by M lipid molecules, and which gives $M \approx 17$. The error bars associated with the data of Jost and Griffith are deduced from their Figure 16. The upper limits for those of their point at $c = 0.0625$ are $f_i \approx 0.89$ and $c \approx 0.075$. The most reliable values of f_i may be nearer to their upper limits (P. C. Jost, private communication). (C) ESR fraction-immobilized component, $f_i(c)$, as a function of Ca^{2+}-ATPase concentration in EYPC bilayers at 25 °C: (○) data points of Silvius et al. (1984) using a proxyl spin-label. The solid lines are plots of eq 22 for dimers with (a) $M = 64$ and (b) $M = 56$. The dashed line is a plot of eq 26 with $M = 22$.

Figure 1B shows the data of Jost & Griffith (1978) at $T = 24$ °C as a function of Ca^{2+}-ATPase concentration in delipidated SR membranes, together with two data points of Thomas et al. (1982). Equation 22 was used, and the results for dimers with $M = 44$, 48, or 56 are shown. These correspond to 22, 24, or 28 lipids per monomer, respectively, and the monomer ($M = 22$, 24, or 28) and tetramer ($M = 88$, 96, or 112) curves gave the same results. The best fit for the data of Jost and Griffith is 22 or 24 lipids per monomer, while the best fit for the data of Thomas et al. is 28 lipids per monomer. It should be noted that in an analysis of the data of Jost & Griffith (1978), Marsh & Watts (1982, Figure 13) concluded that ~17 lipid molecules per monomer were immobilized by the Ca^{2+}-ATPase.

In order to see why their analysis gives this low value not in agreement with other measurements, we observe that they require that the ratio of free to immobilized or boundary lipids, n_f/n_b, satisfy an equation of the form

$$n_f/n_b = A + B(n_{tot}/N_p) \quad (24)$$

where A and B are constants, $n_{tot} = n_f + n_b$, the total number of lipids, and N_p = the number of proteins. However, n_f/n_b must also satisfy the identity

$$n_f/n_b = -1 + n_{tot}/n_b \quad (25)$$

By comparing eq 24 and 25, we see that $A = -1$ and $n_b = (1/B)N_p$. Thus, each protein *always* has associated with it $1/B$ "bound" lipids. Call this number M; i.e., $n_b = MN_p$. Let N be the total number of molecules. Then $n_b = MNc$. Therefore, according to Marsh & Watts (1982), the fraction of immobilized component is

$$f_i^{MW}(c) = Mc/(1 - c) \quad (26)$$

This should be compared with our eq 22: $f_i(c) = 1 - (1 - c)^M$. The slope of eq 26 increases as c increases while that of eq 22 decreases with increasing c.

If one now tries to fit the data of Jost and Griffith in Figure 1B to eq 26, then we find that it possesses a value of $M \approx 17$. This is shown as a dashed line in Figure 1B, where it can be seen that the general agreement with the data points is not as good as that of the curves a–c. In our view then, what appears to be a small value of M deduced by Marsh & Watts (1982) follows directly from their assumption that each protein is surrounded by a (permanent) annulus of M bound lipid molecules.

Finally, Figure 1C shows very recent data of Silvius et al. (1984) at $T = 25$ °C as a function of Ca^{2+}-ATPase concentration in EYPC bilayer membranes using a 14-proxyl spin-label. The solid curves show eq 22 for dimers with $M = 64$ and $M = 56$ (32 and 28 lipid molecules per Ca^{2+}-ATPase monomer, respectively). The dashed line shows the plot of eq 26 for $M = 22$, and we consider that the data are best fit by the curve of eq 22 for $M = 56$ (28 lipids per monomer).

The conclusion, therefore, is that both DPH steady-state fluorescence polarization data and ESR spectroscopy using nitroxide-labeled lipid hydrocarbon chains are in accord with the following picture for the state of a lipid bilayer, at $T > T_c$, containing Ca^{2+}-ATPase: (i) the Ca^{2+}-ATPase oligomers are randomly distributed in the sense of eq 23, and there is no fixed stoichiometric ratio of boundary lipids and proteins. (ii) As far as static properties are concerned, there appears to be two dominant populations of probes, those adjacent to at least one Ca^{2+}-ATPase oligomer and those surrounded by unlabeled lipids or other probes. ^2H NMR and IR data, however, suggest that there is only one population of unlabeled lipids. (iii) A miximum number of $24–28k$ lipid molecules can fit around an isolated Ca^{2+}-ATPase k-mer.

Possible Effects of Phase Separation upon DPH Steady-State Polarization. Here we shall show the type of behavior to be expected if the bilayer is in a two-phase region on the phase diagram. This might occur, for example, for $T < T_c$. It must not be forgotten that the equations which we will use assume that the probes partition into the two phases in the same ratio as do the unlabeled lipids. Evidence from Ca^{2+}-ATPase in DPPC (Gomez-Fernandez et al., 1980) and lipophilin in DPPC (Boggs et al., 1980) using freeze–fracture electron microscopy, and from rhodopsin in DMPC (Bienvenue et al., 1982) using ^2H NMR, suggests that for some range of $T < T_c$ the bilayer is composed of two phases, an essentially pure lipid gellike phase and a protein-rich phase. Computer simulation studies (Lookman et al., 1982) suggest that this may be a phenomenon common to many integral proteins which appear not to introduce any significant static order into lipid hydrocarbon chains for $T > T_c$. The results of Bienvenue et al. (1982) and Lookman et al. (1982) show that for some range of $T < T_c$ the lipids in the protein-rich phase are statically disordered on the ^2H NMR time scale. In addition, the computer simulations suggest that as T falls below T_c those lipid hydrocarbon chains get more statically ordered, finally becoming "frozen" non cooperatively below ~−10 °C.

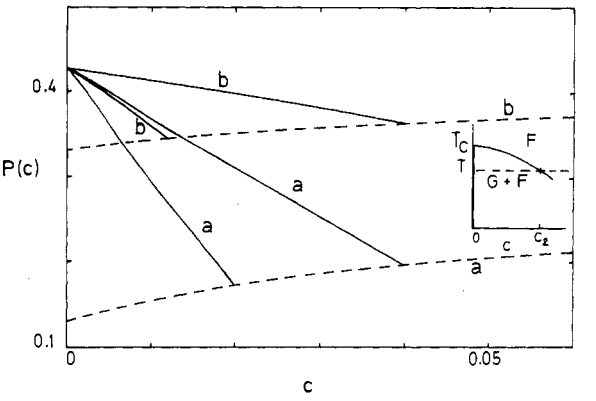

FIGURE 2: Calculated DPH steady-state fluorescence polarization in a two-phase region (G + F) of a phase diagram [see the inset and see Figure 4 of Lookman et al. (1982) and Figure 33 of Davis (1983)] at temperature T. In the two-phase region, the solid lines are followed as c increases. The solid lines meet the dashed lines at $c = c_2$. The dashed lines are for the single-phase region (F, inset), and for $c > c_2$, the dashed lines are followed. For both cases, $I_-^L(0)/I_+^L(0) = 0.429$ [from Gomez-Fernandez et al. (1980) for $T = 25$ °C] and $M = 25$. (a) $I_-^P/I_+^P = 0.235$, $I_-^L(c_2)/I_+^L(c_2) = 0.130$, and $c_2 = 0.02$ or 0.04. (b) $I_-^P/I_+^P = 0.385$, $I_-^L(c_2)/I_+^L(c_2) = 0.333$, and $c_2 = 0.012$ or 0.04.

To study the effects of phase separation, we have used eq 4 with $c_1 = 0$. We must now choose $I_\pm(0)/I_+(c_2)$ and $I_-(c_2)/I_+(c_2)$. The phase diagram is shown schematically in the inset of Figure 2 [compare Figure 4 of Lookman et al. (1982) and Figure 33 of Davis (1983)]. We are concerned with the behavior to be observed as c increases along the dashed line at temperature $T < T_c$.

Figure 2 shows two choices for the parameters above, for monomers with $M = 25$. In both cases, we chose $I_\pm^P$ to be independent of c. For the curve labeled a, we chose $I_-^L(c_2)/I_+^L(c_2) = 0.130$ and $I_-^P/I_+^P = 0.235$, which are near the values used for the previous cases. This choice is in keeping with a fluidlike phase with a protein concentration c_2. To calculate $I_-^L(0)/L_+^L(0)$, we used the results of Gomez-Fernandez et al. (1980) for Ca^{2+}-ATPase in DPPC at $T = 25$ °C. There, the value of $P(0)$ gives $I_-^L(0)/I_+^L(0) = 0.429$. The two solid curves in Figure 2 labeled a are for $c_2 = 0.02$ and $c_2 = 0.04$. They meet the dashed curve a, which is for a single homogeneous fluid (F) phase (as in Figure 1A) at those values of c_2. Thus, as c increases, the value of $P(c)$ measured follows one of the solid curves labeled a until $c = c_2$. $P(c)$ then follows the dashed curve a as c increases further. Curves b were obtained by choosing $I_-^L(c_2)/I_+^L(c_2) = 0.333$ and $I_-^P/I_+^P = 0.385$. This means that the proteins in question order DPH more than Ca^{2+}-ATPase. It also means that the lipids in the protein-rich F phase are more statically ordered at that temperature than in the F phase at a higher temperature, $T > T_c$. For these cases, we chose $c_2 = 0.012$ and $c_2 = 0.04$. As for the curves labeled a, the measured value of $P(c)$ would follow one of the solid curves labeled b as c increases until it reaches $c = c_2$. It would then follow the dashed curve b with increasing c.

The values of $P(c)$ at the high-c end of the dashed curves are determined by I_-^P/I_+^P while their $c = 0$ values are determined by $I_-^L(c_2)/I_+^L(c_2)$. If, as T decreases, $I_-^L(c_2)/I_+^L(c_2)$ increases, then the low-c portion of the dashed curves will move to higher values of $P(c)$. Since $I_-^L(c_2)/I_+^L(c_2)$ will probably increase faster with decreasing $T < T_c$ than $I_-^L(0)/I_+^L(0)$ (since, in the pure gel (G) phase lipid, the latter will be near its maximum value), then the total $P(c)$ curve observed will move upward and get shallower as T decreases. This has been observed by Gomez-Fernandez et al. (1980; Figure 5 for $T < T_c$).

Finally, one can ask the following question: What if the DPH probes do *not* partition into the two phases in the same ratio as the lipid molecules? This is easily answered. If the ratio of probes in the F phase to those in the G phase is *greater* than the ratio of unlabeled lipids in those phases, then it will be manifested *as if* c_2 is smaller than it actually is. Conversely, if the ratio is less, then a larger value of c_2 will be reported by $P(c)$ than actually exists.

Conclusions

We have extended a theory of the static behavior of DPH probes and nitroxide-labeled lipid hydrocarbon chains in bilayers of saturated phospholipids which contain integral proteins.[2] We have used this theory to analyze DPH fluorescence polarization measurements for Ca^{2+}-ATPase reconstituted in DPPC bilayers at 46 °C, as well as ESR nitroxide or proxyl spin-label measurements on delipidated SR membranes and on EYPC membranes containing Ca^{2+}-ATPase, both in the fluid phase.

The data fit curves of the form of eq 14 and 22. The ESR data thus appear not to fit eq 26, which it should if each oligomer were surrounded by an annulus of lipids. The following conclusions were obtained from our analysis: (i) Ca^{2+}-ATPase oligomers appear to be randomly distributed (in the sense of eq 23; see the Appendix) in the fluid phase for at least some temperatures greater than T_c. (ii) Thus, because oligomers can come into "contact" with each other at all concentrations, this implies that there is no permanent annulus of lipids associated with each Ca^{2+}-ATPase oligomer; i.e., each oligomer appears not to be surrounded by a permanent shell of lipids so that there is no fixed stoichiometric ratio of boundary lipid to protein. (iii) There appear to be two dominant environments for the probes studied here. These are (a) one in which the probes are adjacent to at least one protein and (b) one in which they are surrounded by lipids. There is no evidence to suggest that the motion of unlabeled lipid molecules is similar to that of the probes described here, in the presence of integral proteins. We propose that the motion of probe molecules is significantly changed due to the proximity of at least one protein, while unlabeled lipid hydrocarbon chains do not experience such a change in their motion. Thus, ^2H NMR, IR spectroscopy, ESR, and DPH steady-state fluorescence polarization studies are all in agreement with the picture that, as far as static properties are concerned, there is essentially a single population of unlabeled lipids in lipid bilayers, which contain integral proteins, in a fluid phase for $T > T_c$.

We have previously reported similar conclusions from a study of cytochrome oxidase in lipid bilayers (Hoffmann et al., 1981). The results of that paper and this work suggest that the conclusions reached may be features common to a wide variety of integral proteins in lipid bilayers.

We found that the measurements analyzed here are in accord with values of M corresponding to between 24 and 28 lipid molecules per Ca^{2+}-ATPase monomer. It should be

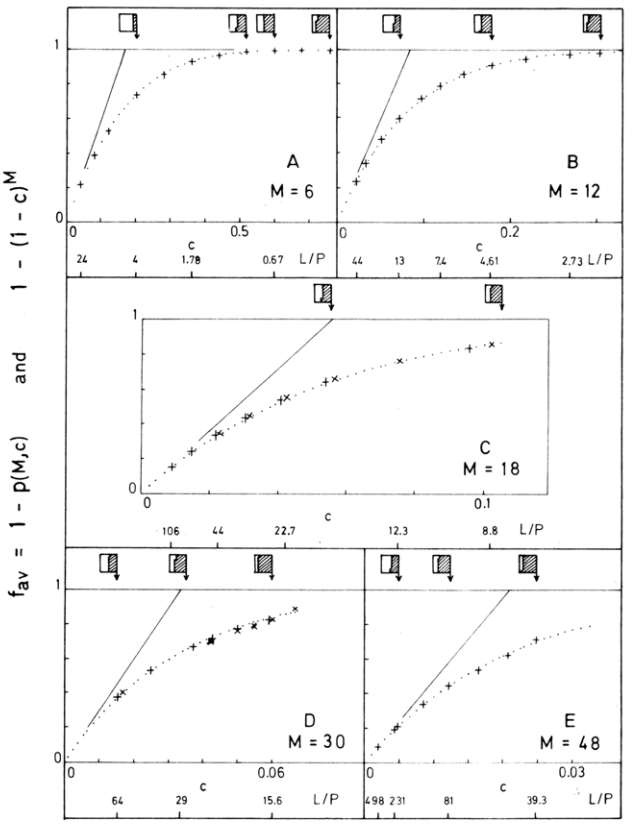

FIGURE 3: Calculation of the average fraction of sites, $f_{av} = 1 - p(M,c)$, adjacent to at least one hexagon as a function of the hexagon concentration on a triangular lattice. The abscissa shows both the concentration, c, of hexagons in units of mole fraction and the "lipid to protein" ratio, L/P, which is the ratio of the number of sites not occupied by hexagons to the number of hexagons. M denotes the maximum number of sites which can be adjacent to a hexagon, and $n_H = 1 + [M(M - 6)]/12$ is the number of sites covered by one hexagon. A hexagon center must occupy a lattice site, and hexagons may not overlap. N_s is the total number of lattice sites. The dots are a plot of $1 - (1 - c)^M$. The figures also show, diagrammatically, the fraction of the total area covered by the hexagons. (A) $M = 6$ ($n_H = 1$, the "hexagon" occupying a single site) for $N_s = 2500$ (+). (B) $M = 12$ ($n_H = 7$) for $N_s = 3600$ (+). (C) $M = 18$ ($n_H = 19$) for $N_s = 6400$ (+) and $N_s = 2500$ (×). (D) $M = 30$ ($n_H = 61$) for $N_s = 10000$ (+) and $N_s = 3600$ (×). (E) $M = 48$ ($n_H = 169$) for $N_s = 40000$ (+).

remembered that M is the maximum number of lipid chains or DPH molecules which can fit around an isolated oligomer in half of a bilayer, or the maximum number of lipid molecules fitting around an isolated oligomer in the bilayer. These numbers are not in accord with the conclusions of Marsh & Watts (1982), who analyzed the same measurements for the delipidated SR membranes and reported that 17 lipid molecules per monomer were immobilized. We have explained how this conclusion arises from their assumption of a fixed number of lipids forming an annulus around each protein. All of the data we know [see also Pink (1984)] are in accord with the theory originally presented by Hoffmann et al. (1981) and extended here.

Finally, we studied the behavior of DPH steady-state fluorescence polarization when the lipid–protein bilayer is in a two-phase region. In the examples of Figure 2, we assumed that $T < T_c$ and that one of the phases was a pure lipid gel phase, though the equations are not restricted to this case. The results that we obtained were in general agreement with those of measurements on Ca^{2+}-ATPase in DPPC bilayers (Gomez-Fernandez et al., 1980). We stress that in all the calculations here we have assumed that the probes partition into the two phases in the same ratio as the unlabeled lipid con-

centrations in those two phases, and we pointed out the change to be expected if this is not so.

Acknowledgments

We are grateful to Professor P. C. Jost, who kindly provided us with the original data on which Figure 16 of Jost & Griffith (1978) was based. Thanks also to Robin Speedy for his comments on the packing of hard disks. D.A.P. expresses his appreciation to Professor Rodney Biltonen and Professor Joseph Larner of the Department of Pharmacology, University of Virginia, for a very enjoyable vist during the Spring of 1983. D.A.P. also thanks Patricia Jost for raising questions concerning eq 23 and expresses his appreciation to her and Hayes Griffith, Johannes Volwerk, Robert Mazo, Gert Rempfer, and Mel Jackson for a stimulating and enjoyable visit to Eugene, OR, in February, 1984. Thanks also to S. McCabe for typing the manuscript.

Appendix

Computer Simulation of $p(M,c)$. Here we shall present the results of computer simulations to support the expression for $p(M,c)$ of eq 23. We shall first describe the lattice model of a lipid bilayer used here. We shall then describe how, by using computer simulations, we calculate numerical values of $p(M,c)$ for a given concentration c of hexagons on a triangular lattice, each isolated hexagon having M lattice sites adjacent to it. We shall perform the simulations for five sizes of hexagons in various concentrations and show that the simulation results are in good agreement with eq 23.

Every site of a triangular lattice represents either a (labeled or unlabeled) lipid hydrocarbon chain or a DPH molecule, or is part of a hexagon which represents the average cross section of an integral protein. The hexagons may not overlap. Hoffmann et al. (1981) referred to this cross-sectional area as effectively cylindrical, but it should have been described as hexagonal. The triangular lattice thus represents half of a bilayer. Let N_s be the total number of sites on one triangular lattice and N_p be the number of hexagons. Let M be the number of sites adjacent to and surrounding a single isolated hexagon. Then, the number of sites occupied by each hexagon is

$$n_H = 1 + 6 + 12 + \ldots + (M - 6) = 1 + [M(M - 6)]/12 \quad \text{(A1)}$$

The concentration of hexagons is defined

$$c = \frac{N_p}{N_p + (N_s - n_H N_p)} \quad \text{(A2)}$$

If the number of DPH probes is only a small fraction of the number of lipid chains, then this is also, effectively, the protein concentration in units of mole fraction.

For this model, because one of the components occupies only one site, then all sites are occupied independent of the hexagon concentration. In the case of a mixture of two sizes of hard disks, for example, holes can remain unfilled due to the packing properties of the disks. This cannot happen in the model used here.

Given a "central" site not occupied by a hexagon, let us consider the case where $M = 6$ so that the "hexagons" occupy single sites ($n_H = 1$). There are six sites around this central site which can be occupied by hexagon centers. If one of these sites is occupied by a hexagon center, then the central site will be adjacent to that hexagon. The probability that any one of the sites is not occupied by a hexagon center is $1 - c$. We can define a "random" distribution of hexagons to be an ensemble of configurations of hexagons on an infinitely large lattice, such that the probability that none of the six sites around the central site (above) is occupied by a hexagon center is

$$p(6,c) = (1 - c)^6 \quad \text{(A3)}$$

For hexagons occupying more than one site ($M > 6$, $n_H > 1$), it is clear that correlations in their position due to their packing can arise.[6] We can write the identity

$$p(M,c) = (1 - c)^M + [p(M,c) - (1 - c)^M] = (1 - c)^M + p_{corr}(M,c) \quad \text{(A4)}$$

where $p_{corr}(M,c)$ accounts for correlations not contained in $(1 - c)^M$. If these correlations are irrelevant, then $p(M,c)$ should be essentially given by

$$p(M,c) = (1 - c)^M \quad \text{(A5)}$$

In order to check this expression, we have carried out computer simulations on triangular lattices with N_s ranging from 2500 to 40 000 and with periodic boundary conditions. The simulation proceeds as follows: (i) Hexagons are distributed randomly (except at very high concentrations) on the lattice so that they do not overlap. (ii) Each hexagon is visited once and only once in one step (which defines a "step"). The order in which they are visited is determined by a random-number generator. When a hexagon is visited, one of the six directions in which it can be moved on the lattice by one lattice constant is randomly selected. If the hexagon can be so moved without overlapping another hexagon, then the move is carried out. If this is not possible, then the hexagon is not moved, and another hexagon is visited. When all hexagons have been visited once, the step has been completed. (iii) This procedure is carried out for a large number of steps (typically from 500 to 5000) in order to initialize the system so that it has a distribution characteristic of what one thinks of as random. Evidently, at high hexagon concentrations, the starting distribution, (i), can be very important. At high hexagon concentrations, some distributions cannot be reached from the starting distribution because of what amounts to an infinite energy barrier between them. (iv) After initialization, procedure ii is carried out for a large number of steps, typically from 1000 to 5000. At each step, the number of sites which are adjacent to at least one hexagon is recorded and an average, f_{av}, calculated at the end. We also recorded the mean square distances moved by the hexagons in order to see to what extent movement, and thus sampling of different distributions, occurs. If eq A5 is correct, then f_{av} should be equal to $1 - (1 - c)^M$.

Figure 3 shows the results of simulations for five sizes of hexagons with $M = 6$, 12, 18, 30, and 48. The last three would represent bilayer-spanning proteins of molecular weights greater than about 15 000, 45 000, and 120 000, respectively. The values of f_{av} together with points representing $1 - (1 - c)^M$ have been plotted as a function of c and the "lipid to protein" ratio, L/P. Also shown, diagrammatically, are the fractions of the total area covered by the hexagons. Figure 3A, for $M = 6$, simply shows that eq A3 is exact. In this case, between 100 and 1900 "hexagons" were used, far more than were used in the other simulations. In Figure 3B, for $M = 12$, we see that the agreement is good down to L/P values of $\sim M/5$, below which we did not go. In these cases, we used lattices of 50^2 and 60^2 or 61^2 sites, respectively. In panels C and D of Figure 3, for $M = 18$ and 30, respectively, the

[6] In the case of the packing of hard disks, some idea of the problems involved can be gained from recent work by, e.g., Berryman (1983) and Speedy (1983) and references cited therein.

agreement is good down to L/P values of $\lesssim M/2$, and we did not put in more hexagons to go below this ratio. In Figure 3E, for $M = 48$, we have not been able to go much below an L/P value of ~ 40 because of difficulties in placing the large hexagons on a lattice with 200^2 sites.

In panels C and D of Figure 3, we show results for lattices with 50^2 and 80^2 sites and with 60^2 and 100^2 sites, respectively. In figure 3D, we can see that use of the larger lattice gives f_{av} values closer to $1 - (1 - c)^M$ than those for the smaller lattice.

In all five figures, we have plotted Mc as a solid line. This is to indicate the slope as $c \rightarrow 0$. We can conclude the following: (i) The equation $p(M,c) = (1 - c)^M$, for an infinite lattice, is in good agreement with data obtained from simulations performed on large lattices, decorated with hexagons, for the range of L/P values shown. (ii) If the hexagons represent protein cross sections and the lattice sites lipid chains, then the L/P values studied here encompass essentially all of those studied experimentally. (iii) The difference between the behavior of $Mc/(1 - c)$, representing a model in which each protein is always surrounded by an annulus of M lipids, and the curve obtained when hexagon–hexagon (i.e., protein–protein) "contacts" prevent the formation of a lipid annulus around each protein is unambiguous.

Registry No. DPPC, 2644-64-6; ATPase, 9000-83-3.

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