Review Letter

Lateral diffusion of lipids and proteins in bilayer membranes

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Current knowledge of lipid and protein diffusion rates in homogeneous, fluid, artificial bilayers is reviewed and the ability of current theory to account for these rates is evaluated as a reference for understanding lateral diffusion in biomembranes. Experimental evidence on the effects of increasing protein concentration on the lateral diffusion of membrane lipids and proteins is summarized to provide a qualitative description of the situation in biomembranes.

Lipid bilayers *Lateral diffusion* *Membrane proteins* *Fluorescent probes* *Photobleaching*

1. INTRODUCTION

Recent detailed experiments and theoretical work have made it possible to test various descriptions of the lateral diffusion of lipids and proteins in bilayer membranes (review [1,2]). Such an endeavor involves examining the fit of the explicit size and temperature dependences to the data, and this is best done with artificial bilayer membranes, where the most control over the experimental parameters and the most detailed knowledge about the physical system exist. Following the treatment of Nir and Stein [3] proposing two modes of diffusion in liquids, it seems reasonable to divide diffusive behavior in fluid bilayers into two regimes based on whether the molecular size of the diffusant is larger than or comparable to that of solvent, i.e., the lipids of the bilayer; these regimes may be separated by a transition region (see top of fig.1). For molecules comparable in size to the host phospholipids, free area or lattice models can be used to fit the data. For molecules large compared to the 'solvent' phospholipids (i.e., proteins), hydrodynamic models viewing the bilayer as a continuum can be used. This part of the paper deals with diffusion under 'ideal' conditions where lipid probe and protein are present in the bilayer in very dilute amounts. Next, the effects of increasing protein concentrations on the lateral diffusion of both lipids and membrane proteins are reviewed to begin an understanding of the 'non-idealities' caused by the higher amounts of protein found in biomembranes.

Abbreviations: DLPC, dilauroylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DOPC, dioleylphosphatidylcholine; egg PC, egg phosphatidylcholine; PE, phosphatidylethanolamine; NBD-phospholipid, *N*-(4-nitrobenzo-2-oxa-1,3-diazolyl)-conjugated to stated phospholipid; diI, used for the C_{18} and C_{16} forms of 3,3′-diacylindocarbocyanine iodide and 3,3′-dioctadecylindodicarbocyanine iodide; IMP, intramembranous particle; T, absolute temperature; T_m, gel-to-liquid crystalline phase transition temperature; k, Boltzmann constant; a_f, free area; η, viscosity

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2. DIFFUSANT COMPARABLE IN SIZE TO SOLVENT: LATERAL DIFFUSION RATES OF LIPID-LIKE MOLECULES IN SINGLE-COMPONENT, FLUID, ARTIFICIAL BILAYERS

As a background, it is instructive to mention developments in the description of transport properties in liquids. Success has been achieved using a quasicrystalline model of liquid structure. Such models yield transport properties which exhibit Arrhenius behavior with respect to temperature. However, the fluidity of associated liquids and many polymeric liquids display large departures from Arrhenius behavior and this stimulated renewed interest in various free volume approaches.

Transport properties such as viscosity of bulk fluids are intuitively expected to depend in some way on the free volume present. This expectation is founded on the empirical relationship discovered by Batschinski [4] and extended by Hildebrand [5], which demonstrated that in many non-associated liquids and their solutions, fluidity, self-diffusion and solute diffusion were all directly proportional to the relative volume expansion in these fluids. In fact, examination of transport properties over large temperature ranges showed that the dependence of these properties on relative volume expansion was not strictly linear [6].

Cohen and Turnbull [7] quantitatively considered the effect of free volume on diffusion in hard-sphere fluids. In their treatment, a spectrum of free volume sizes is formed by solvent density fluctuations. A test molecule can undergo a diffusive step if a free volume exists adjacent to it of a certain minimum size. The test molecule, although largely confined by the solvent cage, is assumed to possess a velocity given by the kinetic theory of gases and quickly moves into the adjacent 'hole'. The diffusive step is completed when another solvent fluctuation closes the free volume left by the test molecule before it can reenter this hole.

Macedo and Litovitz [8] combined the activated lattice diffusion approach and the Cohen-Turnbull free volume description to yield a two-parameter model capable of fitting a wide range of liquid transport data. The hybrid expression essentially recognized that a diffusive step of a test molecule requires both that an adjacent free volume of adequate size exist and that the molecule possess a minimum energy to break the attractive forces with its neighbors.

Less attention has been directed toward quantitatively accounting for the diffusion of lipids in bilayers. To begin, consider a two-dimensional square lattice in which the lipid lateral diffusion coefficient, D, is given by:

$$D = 1/4\nu\Lambda^2, \tag{1}$$

where ν is the jump frequency and Λ is the jump distance (i.e., the lattice spacing). This equation can be derived in an analogous way to computing the mean end-to-end distance of a random coil polymer. Recently, Pace and Chan [9] proposed a model for lipid lateral diffusion in which the rate limitation for lateral diffusion is assumed to be the separation of neighboring head groups allowing movement of the test lipid through the space created by the separation. The frequency, ν (eq.1), and activation energy for chain separations which are large enough to allow diffusion to occur can be calculated from theories of polymer motion. This calculation gives D values of the right order of magnitude. Unfortunately, over large temperature regions, Arrhenius plots of diffusion are not linear, giving rise to a departure of theory from experiment [14]. Pace and Chan [9] have noted that the temperature dependence of their calculated activation energy is not large enough to produce significant non-linearities in the Arrhenius plot.

Other investigators have studied the relationship of the lateral diffusion coefficient to the free area available in the bilayer. An adaptation of the Cohen-Turnbull equation to two-dimensional fluids by Galla and co-workers [10] is capable of fitting the lipid analog lateral diffusion data obtained by the photobleaching method [11,14]. MacCarthy and Kozak [12] have shown that this adaptation can be derived directly from cell theory in a form not requiring the specification of phenomenological constants yielding:

$$D = A\exp[\theta a^*/a_f(T)] \tag{2}$$

where a^* is close packed area per molecule and $a_f(T)$ is the mean free area per molecule at a given temperature, T, and is calculated as the increase in free area at the phase transition, plus the increase above the phase transition temperature (T_m) due to thermal expansion.

The preexponential factor, A, can take several forms. (1) According to Cohen-Turnbull theory, it involves the gas kinetic velocity of the diffusant within the cage defined by the surrounding solvent molecules and hence is dependent on $T^{1/2}$. Explicitly, the maximum jump frequency is given by $\nu_{max} = (\lambda/u)^{-1}$ where u is the velocity of the test molecule within its cage ($u = (2kT/m)^{1/2}$), m being the mass of the diffusant. (This maximum jump frequency must be multiplied by the probability of having free area $a_f \geqslant a^*$ and inserted into eq.1 to obtain the diffusion coefficient.) (2) Recent work [13,14] suggests that the pre-exponential factor should incorporate the fact that the lipid diffuses within its cage and is not in a vacuum but rather coupled to other lipids and the aqueous phase. In this treatment,

$$A = kT/f \quad (3)$$

where f is the translational frictional coefficient due to interaction of the diffusing particle with the adjacent aqueous layer and, for the case of diffusing lipid particle which only traverses one of the monolayers, with the opposing monolayer. Note that A is identified with the diffusion coefficient of the test molecule within its cage. (3) By analogy to bulk fluids [8], the diffusant can be required to have enough energy, E_a, to break the bonds with the neighboring lipids when an adjacent void of sufficient free area occurs. In this case, D is proportional to both the probability of having a requisite adjacent free volume as well as the energy to make the jump and

$$A = D_0 \exp - [E_a/RT], \quad (4)$$

A now having an exponential dependence on T.

The free area theory predicts an extremely steep dependence on the critical area for diffusion, a^*. Cohen-Turnbull theory [7] predicts that molecules occupying less area than the solvent will diffuse at the same rate as the solvent since a diffusive step of the test molecule is only completed when a solvent molecule jumps into the void left by the test molecule. Hence, in fig.1 the dotted line is horizontal at $r < 3.9$ Å. Of course this is an oversimplification since the solvent molecules move in a collective fashion to allow the rapid filling of voids smaller than the close-packed area of a solvent molecule. Nevertheless, data on single-chain diffusants [15] tend to support this prediction of the free volume theory. The generally untested nature of this size dependence is indicated by the broken line in fig.1.

However, the free area dependence of eq.2 can be tested by assessing the linearity of $\log D$ *vs* a_f^{-1} plots at constant temperature. Peters and Beck [16] elegantly demonstrated the validity of this relationship for monolayers at an air/water interface by measuring the translational diffusion of NBD-egg PE as a function of area per molecule in DLPC monolayers* (see fig.2A). This analysis can be extended to lateral diffusion in bilayers. In fig.2B, the lateral diffusion data of McCown et al. [18] is shown to be linear with reciprocal free area in the bilayer; in this case, free area was varied isothermally by changing the hydration of the bilayer in multibilayer specimens. Although the interbilayer spacing also changes with hydration, there is reason to believe, based on the similarity of elastic area compressibility data in single *vs* multibilayer samples, that lateral diffusion will not be especially sensitive to the changing interbilayer distance [18]. Free area can also be varied by changing temperature. In fig.2C the lateral diffusion coefficients of NBD-C_{12}PE in DLPC bilayers and NBD-C_{14}PE in DMPC bilayers are given as a function of free area in these bilayers. The free areas were calculated using the thermal expansion values quoted by Goodsaid-Zalduondo and Elson [11]. The linearity of the data in fig.2C indicates that the free area dependence dominates the temperature dependence in the pre-exponential factor of eq.2, over the small ranges of absolute temperature examined, and that larger temperature ranges would be required to examine the temperature dependence of the preexponential factor. Similar plots for NBD-egg PE diffusing in DLPC and DOPC bilayers also indicate a linear relationship between $\log D$ and a_f^{-1} [11]. It should be noted that the pressure dependence of the diffusion coefficient extracted from pyrene excimer measurements also suggests the validity of the free area theories [19].

* We note that Von Tscharner and McConnell [17] obtained a much weaker dependence of D for NBD-PE on area per molecule in DPPC monolayers transferred to glass substrates. However, the range of areas per molecule examined was more restricted than in the study of Peters and Beck [16].

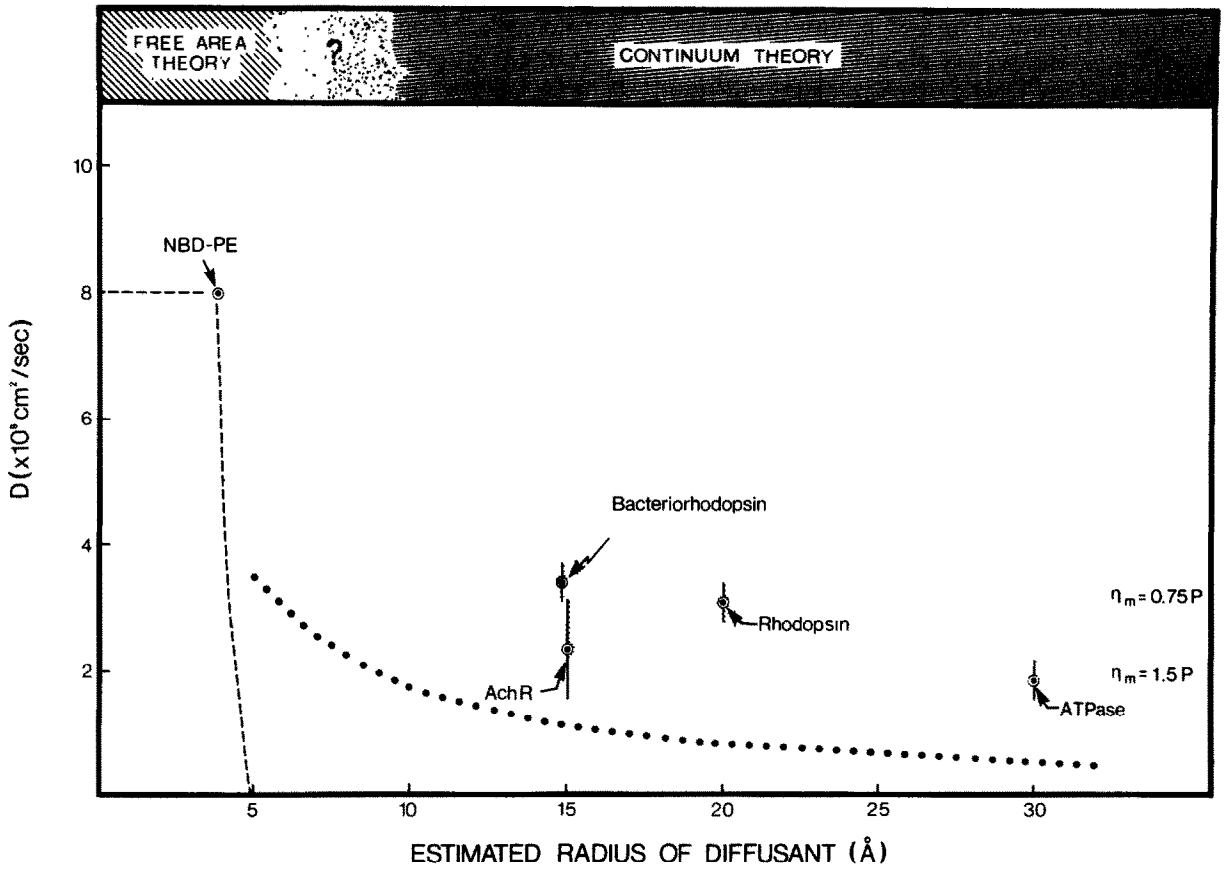

Fig.1. Diffusion coefficient as a function of diffusant radius in artificial DMPC bilayers at 36°C, with the exception of ATPase which was reconstituted in lipids from the sarcoplasmic reticulum. Bar at top shows theoretical regimes. The dotted line to the left shows the very steep dependence of D on size in the free area theory given by eq.2. The NBD-PE point is taken from data of Vaz et al. [14]. Experimental data are taken from Vaz et al. [30] (rhodopsin, acetylcholine receptor, and the ATPase from sarcoplasmic reticulum) and from Peters and Cherry [24] (bacteriorhodopsin). The shaded region gives the very weak dependence of D on size in the Saffman-Delbrück treatment (eq.6) for membrane viscosities (η_m) in the range 0.75–1.5 P. For reference, the beaded line gives the size dependence of the Stokes-Einstein equation ($D\alpha a^{-1}$) given a D value of 3.5×10^{-8} cm^2/s for a particle of radius $a = 5$ Å.

Free area descriptions have intuitive appeal to describing fluid transport; nevertheless, the agreement between theory and experiment (fig.2) is surprising in view of the origins of the theory (see [13], for critique). The free area theory was adapted from the parent free volume theory for hard-sphere rigid molecules, in which the motions of the test molecule are not influenced by the motions of the solvent molecules. Phospholipids, of course, have an attractive component to the intermolecular potential curves, and, when assembled into bilayers, undergo correlated motions. They also have internal degrees of freedom so that not all the free area developed is available for diffusion. That is, some of the free area in the liquid crystalline (fluid) phase is effectively filled by the variety of acyl chain conformers accessible to the molecule. However, the linearity of the curves in fig.2 suggests that this putative inaccessible free area is relatively small and/or constant when compared to the large free area developed at the phase transition. Finally, interactions of the diffusant head group with the bulk aqueous medium and terminal portion of the acyl chains with the opposing monolayer are neglected.

It is possible that some or all of these missing

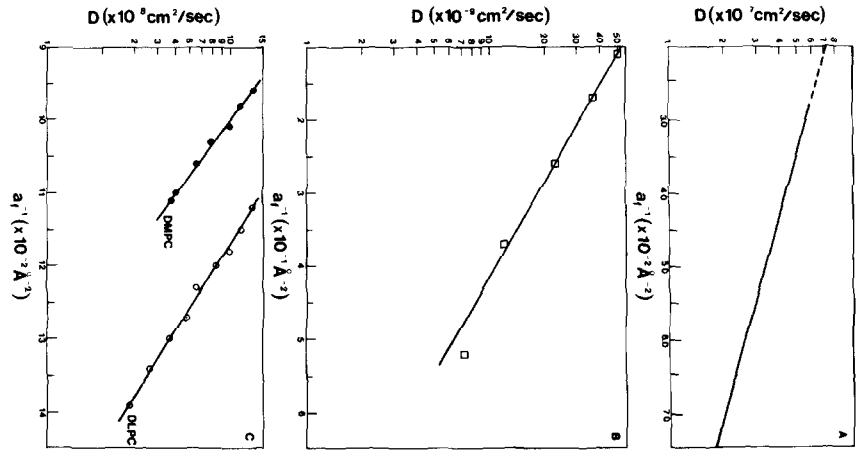

Fig.2. The dependence of lipid probe lateral diffusion coefficients on free area. (A) Data for NBD-egg PE in DLPC monolayers at the air/water interface. (Redrawn from Peters and Beck [16]). (B) Data for NBD-PE in egg PC bilayers in which area per molecule is varied by changing the hydration of the bilayer. (From the data of McCown et al. [18].) D at full hydration assumed to be 5×10^{-8} cm^2/s; close packed area at which diffusion ceases assumed to be 54 Å [18]. (C) Lateral diffusion of NBD-C_{14}PE in DMPC and NBD-C_{12}PE in DLPC measured at various temperatures above the phase transition temperature [14]. Free areas calculated according to the thermal expansion values quoted by Goodsaid-Zalduondo et al. [23].

features will have to be involved to explain one puzzling aspect of the data in fig.2C: at the same free areas, diffusion in DLPC is faster than in DMPC. The free area theory can be adapted to accommodate this feature of the data by adjusting the pre-exponential factor; however, given the similarity of the lipids, the justification for doing this is not obvious.

3. DIFFUSANT LARGER THAN SOLVENT: INTEGRAL PROTEIN DIFFUSION RATES IN SINGLE-COMPONENT, FLUID, ARTIFICIAL BILAYERS

The broken line in fig.1 indicates that, according to the free area theory, D decreases too rapidly with molecular size to account for the lateral diffusion of the larger membrane proteins. Fortunately, we can regard proteins as being large enough in most cases, so that the surrounding lipid solvent can be treated as a continuum. Motion is driven by random, fluctuating forces provided by unbalanced collisions with the solvent molecules and is resisted by the frictional forces inherent in a viscous solvent. The diffusion coefficient is given by $D = kT/f$, where k is the Boltzmann constant and f is the frictional coefficient. In the case of a spherical particle of radius a in a medium of viscosity η, $f = 6\pi\eta a$, resulting in the familiar Stokes-Einstein equation:

$$D = kT/6\pi\eta a \tag{5}$$

in which the diffusion coefficient is inversely proportional to both the radius of the diffusant, a, and the medium viscosity, η.

The continuum treatment was extended by Saffman and Delbrück [20,21] to diffusion in thin, viscous sheets and resulted in the following limiting equation:

$$D = (kT/4\pi\eta_m h)\ln[\eta_m h/\eta_w a - 0.5772] \tag{6}$$

where η_m and η_w are the viscosities of the membrane and bathing phases, respectively (with $\eta_w \ll \eta_m$), a is the radius of a cylindrical membrane diffusant, and h is the membrane thickness. This equation is seen to fit the data for membrane diffusants having larger radii ($r > 10$ Å) (see fig.1, solid line), confirming the remarkably weak size dependence predicted by eq.6. The range of protein radii examined has been extended by cross-linking of integral proteins. For example, the acetylcholine receptor protein diffusion was examined in soybean lipid bilayers as a monomer, covalently

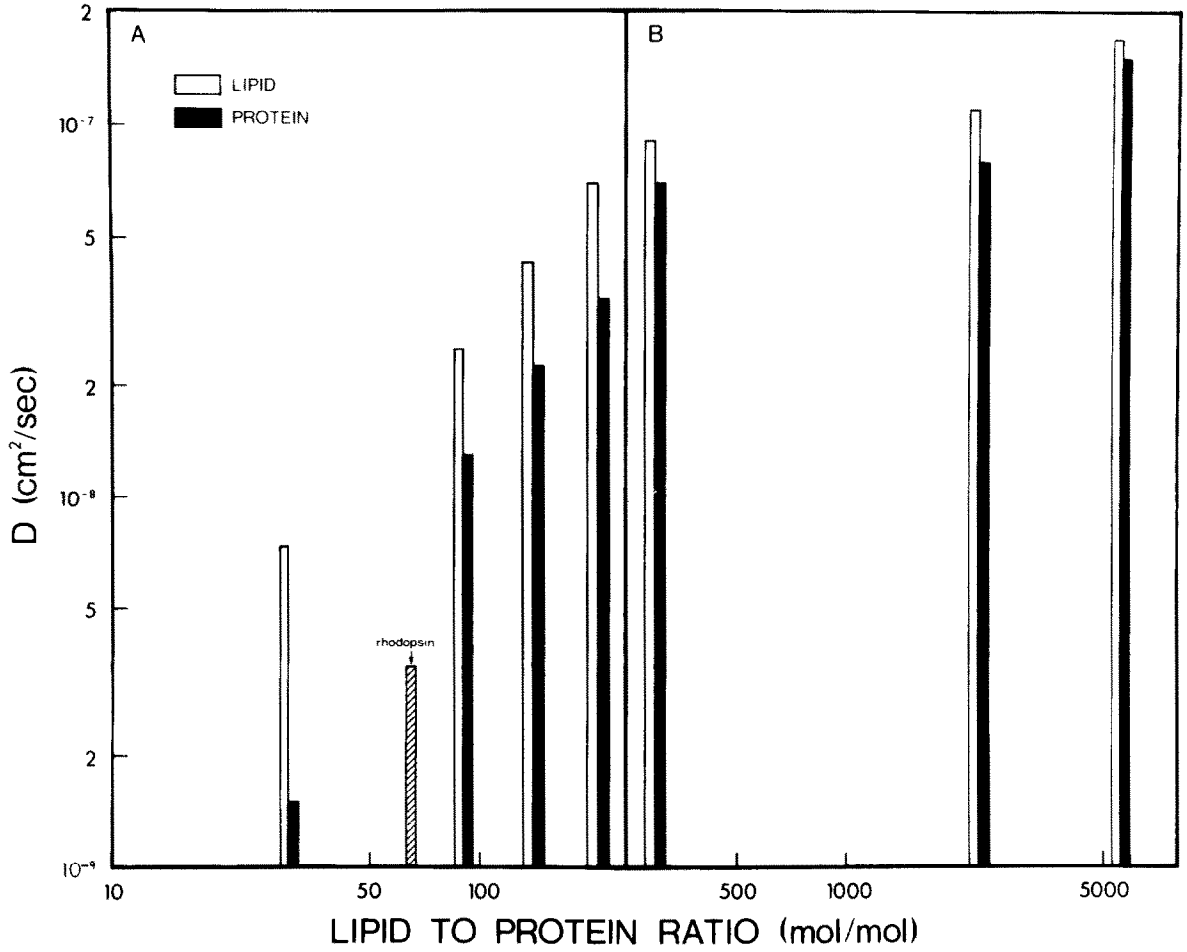

Fig.3. Effect of increasing protein concentration on lipid probe diffusion (open bars) and protein diffusion (filled bars) in bacteriorhodopsin-DMPC recombinants at the lipid:protein molar ratios indicated. (A) Data from Peters and Cherry [24]. Lipid probe was dioctadecyloxatricarbocyanine. Height of hatched bar represents D for rhodopsin in the intact rod outer segment membranes. (B) Data taken from Goodsaid-Zalduondo et al. [23]. Lipid probe was NBD-PE.

linked dimer, and covalently linked tetramer (Vaz, W.L.C. and Criado, M., in preparation). The translational diffusion coefficient for the 3 protein species was found to be the same within experimental error. In further work glycophorin diffusion was examined in DMPC bilayers in the presence and absence of wheat germ agglutinin (WGA) (Vaz, W.L.C., in preparation). WGA binds to the sialic acid residues in glycophorin and causes cross-linking of the protein. D for glycophorin in the presence of WGA was only 20% lower than D for this protein in the absence of the lectin. (It is important to note that the extension of the Saffman-Delbrück equation [22] for the situation that the bathing viscosities are comparable to the membrane viscosity is not required.*) The dependence of this equation on bilayer viscosity has not been tested.

4. TRANSITION REGION

Fig.1 indicates a transition region between the diffusive behavior described by the free area and

*The extended Saffman Delbrück equation would be required if the dimensionless parameter $\epsilon \geqslant 0.1$. Since $\epsilon = (2\eta_w/\eta_w)(a/h)$ and η_m is thought to exceed $10\eta_w$, the extended form is not required.

continuum theories. At this time, it is not clear into which regime important integral proteins with small diameter membrane spanning or inserting domains, such as glycophorin or cytochrome b_5, respectively, would fall.

5. EFFECT OF HIGHER PROTEIN CONCENTRATION ON LATERAL DIFFUSION

The previous section dealt with protein diffusion in the limit of very small concentrations of protein. Biomembranes, of course, have larger concentrations of proteins and recent data allow one to examine qualitatively the effect of protein concentration on both lipid and protein diffusion. Investigations have been performed by Goodsaid-Zalduondo et al. [23] and Peters and Cherry [24] who have reconstituted bacteriorhodopsin with lipids to form recombinants having a defined lipid:protein ratio. The effect of protein on lipid analog diffusion rates is given in fig.3 for two different fluorescent lipid probes (open bar). Essentially, the two groups worked in different regimes of lipid:protein ratio: dilute (panel B) and more concentrated protein (panel A). However, there were some quantitative discrepancies at either limit of lipid to protein ratio: Goodsaid-Zalduondo et al. [23] found complete immobilization of bacteriorhodopsin at a lipid:protein ratio of 74, in contrast to the sluggish diffusion of the protein depicted in fig.3A at a lipid:protein ratio of 30. In addition, the NBD-PE D value in pure DMPC (no protein limit) was larger by a factor of 2.5 in the study of Goodsaid-Zalduondo et al. [23] than was the dioctadecyloxatricarbocyanine D value measured by Peters and Cherry [24] in pure DMPC bilayers. The results show that increasing protein concentration gradually reduces the lipid diffusion coefficient in DMPC-bacteriorhodopsin recombinants.

It can be inferred from studies on fibroblast [25] and erythrocyte [26] membranes that lipid probe diffusion is retarded by proteins in cell membranes. In both of these studies, lipid probe diffusion in the intact membrane was compared to that found in pure lipid bilayers made by extracting the plasma membrane lipids from the respective cells, with the result that the diffusion coefficient of the lipid probe was increased by a factor of 4 by removing the membrane proteins. Similarly, Chazotte et al. [28] have recently fused lipid vesicles with inner mitochondrial membranes, resulting in a demonstrated dilution of membrane proteins. Increasing the lipid content by up to 700% increased the diI and the NBD-PE diffusion coefficients by a factor of about 3.

Additional evidence for protein effects on lipid lateral diffusion can be garnered by correlating the diffusion coefficients of the lipid analog, diI, to the concentration of integral proteins in several systems. We take the intramembranous particle (IMP) density obtained from freeze-fracture electron microscopy as a measure of the effective integral membrane protein concentration. The correlation of lipid diffusion rate to protein concentration is given in table 1 and again shows how increasing protein diminishes the diI diffusion coefficient with the maximum retardation occurring in the inner membrane of the mitochondrion.

One can apply the archipelago theory of Saxton [27] to these data under the following assumptions. Firstly, the protein domains are assumed not to interact with the lipid probes and to be impermeable to them. Secondly, we assume the proteins diffuse so slowly in the biomembranes that they are 'seen' by lipids to be effectively immobile. Finally, we take an average effective IMP diameter, realizing that these normally span a range of diameters in typical biomembranes. However, our purpose is only to assess crudely the applicability of percolation theory ([27], fig.2; $r = 0$ case) to explain the reduction of the diI diffusion coefficient caused by the presence of increasing concentrations of IMPs. While percolation theory does account for the reduction in lipid probe diffusion coefficients in protein-rich biomembranes such as the erythrocyte membrane and the inner mitochondrial membrane (table 1, columns 4–6; see also [26]), it fails to predict the magnitude of the reduction seen in the fibroblast and the lymphocyte plasma membrane. There are a number of obvious possible reasons for this. It may be that lipid diffusion in the protein free bilayer is not as rapid as $5 \times 10^{-8}\,cm^2/s$. Secondly, freeze-fracture may not be detecting all of the relevant membrane proteins, i.e., those which diffuse slowly enough to be impediments to lipid diffusion. It is known, for example, that capping of Con A and PHA receptors, as well as surface Ig and H2 and θ antigens by the appropriate ligands does not alter the IMP distribution [37]. (Simply taking a larger mean IMP diameter will

Table 1

Effects of membrane proteins on dil diffusion

Membrane	Surface 'IMP' density (number/μm^2)[a]	D for dil at 25°C (cm^2/s)	(D/D_F) expt.[b]	Area fraction of fluid lipid[c]	(D/D_f) theory[d]	Ref.[e]
Fluid DMPC	–0–	60×10^{-9}	–	–	–	[15]
Bilayer of fibroblast PM lipids	–0–	40×10^{-9}	–	–	–	[25]
Lymphocyte PM	350–700	17×10^{-9}	0.34	0.97–0.98	~0.9	[31,32]
Fibroblast PM	~850	10×10^{-9}	0.20	0.96	~0.8	[25,33]
Erythrocyte PM	~4200	8×10^{-9}	0.16	0.81	0.2	[34,35]
Inner mitochondrial membrane	~4300	5×10^{-9}	0.10	0.81	0.2	[28,36]

[a] Total of both fracture faces
[b] Assuming D_F (no protein) = 5×10^{-8} cm^2/s
[c] Assuming a mean IMP diameter of 75 Å
[d] [27], fig.2; $r = 0$ curve
[e] First reference: diffusion constant; second reference: surface IMP density estimate

not improve the fit of theory to experiment nearly enough.)

The effects of protein crowding on its own self-diffusion rate have also been examined in the bacteriorhodopsin-DMPC recombinant system [23,24]. The results are given in fig.3 as solid bars and show that bacteriorhodopsin self-diffusion is retarded as more protein is added to the membrane. Peters and Cherry [24] point out that rhodopsin lateral diffusion data in the frog retinal disc membrane fit on this graph (hatched bar). This may represent close to a lower limit of the lipid:protein ratio in fluid biomembranes; the even lower value of lipid:protein ratio in the bacteriorhodopsin-DMPC system may be in a regime where protein self-aggregation severely retards the diffusion rates [23,24].

It is important to note that in many biomembranes, protein lateral diffusion is even slower than the lower limit predicted by protein crowding. This fact implicates structures peripheral to natural membranes, e.g., the cytoskeleton and glycocalyx, in regulating the lateral diffusion and distribution of membrane proteins [29].

6. SUMMARY AND CONCLUSIONS

(1) It seems reasonable to consider the lateral diffusion of dilute solutes in lipid bilayers as divided into two size regimes according to whether (i) the solute is comparable in size to the 'solvent' lipid molecules or (ii) the solute is large compared to the solvent.

(2) For lipid-like molecules having dimensions similar to the lipids in the bilayer an expression adapted from the Cohen-Turnbull free volume theory for liquid diffusion fits available lateral diffusion data over limited temperature regions.

(3) For molecules larger compared to lipids, such as some integral membrane proteins, the solvent bilayer can be treated as a continuum and the theory of Saffman and Delbrück describes the weak dependence of D on protein radius, when the proteins are present in dilute concentrations.

(4) At this time, it is not clear where the breakpoint between the two regimes should be placed. For instance, is the lateral diffusion of proteins with relatively small diameter membrane spanning or penetrating segments best described by the free area or the continuum approach?

(5) When proteins are present in higher concentrations in both artificial and natural membranes systems, there is a definite reduction in both the lipid and protein lateral diffusion coefficients which is directly dependent on the amount of protein in the membrane.

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