Effects of Size of Macrocyclic Polyamides on Their Rate of Diffusion in Model Membranes

Cuihua Liu, Alison Paprica, and Nils O. Petersen
Department of Chemistry, University of Western Ontario, London, Ontario N6A 5B7, Canada

ABSTRACT A series of homologous amphiphilic molecules with surface areas in the range of 0.3 nm^2 to 3.0 nm^2 were prepared and used to investigate the diffusion in model dimyristoylphosphatidylcholine membranes as a function of temperature. The diffusion behavior of smaller molecules can be described by the interfacial viscosity limited free area theory promoted by Vaz and his co-workers, and that of the larger molecules can best be modeled by a recent interpretation of the theoretical description proposed by Evans and Sackmann. The experimental data show that the rate of diffusion is controlled by the size of the molecules at the interface of the lipid membrane, and provide evidence for a view of the membrane as a hydrodynamic triple layer with a low-viscosity central layer encased by two more viscous, yet fluid, layers.

INTRODUCTION

The lateral transport of membrane components in the plane of the lipid bilayer is of intrinsic physicochemical interest (Clegg and Vaz, 1985; Adam and Delbrück, 1968) and of biological and biochemical interest because many membrane-linked, multimolecular biochemical reactions may be diffusion controlled (Hackenbrock, 1981; Koppel, 1982). However, an important consequence of the two-dimensional fluid nature of lipid membranes is the unusual size dependence of the lateral diffusion coefficient on the particle radius (Sackmann, 1996). There are several theoretical studies on the size dependence of the diffusion coefficients in both three- and two-dimensional systems that are based on quite different models (Galla et al., 1979; Hildebrand, 1971; Saffmann and Delbrück, 1975; Hughes et al., 1982; Evans and Sackmann, 1988; Tamm, 1991). Vaz and his co-workers have experimentally investigated the size dependence of phospholipids and of large integral membrane proteins, with radii in the plane of the membrane that are 1.0 nm or greater (Vaz et al., 1982a,b, 1984, 1985a,b; Edidin, 1989; Johnson et al., 1996). Both theoretical and experimental work indicates that it is reasonable to divide diffusive behavior in fluid bilayers into two regimes, as originally proposed by Nir and Stein (1971), based on whether the molecular size of the diffusant is comparable to or larger than that of the solvent, i.e., the lipids of the bilayer. For molecules comparable in size to the host phospholipid with a surface area of ~0.65 nm^2, a free area model based on free volume models in three-dimensional fluids (Cohen and Turnbull, 1959; Galla et al., 1979; Hildebrand, 1971) can be used to fit the data. For molecules large compared to the "solvent" phospholipid (i.e., proteins) with surface areas greater than ~3 nm^2, continuum hydrodynamic models can be employed (Saffmann and Delbrück, 1975; Hughes et al., 1982; Evans and Sackmann, 1988; Tamm, 1991). It is not yet certain what description to use when the size of diffusant falls in the intermediate region between the diffusive behaviors described by free area or continuum theories. It is therefore important to establish whether the free area theory can be extended to molecular areas exceeding 0.65 nm^2 or whether the hydrodynamic description can be exploited at molecular areas smaller than ~3 nm^2.

To address this question, we have synthesized two series of intermediate-sized macrocyclic polyamide amphiphiles with and without a fluorescent nitrobenzoxadiazole (NBD) label (Paprica and Petersen, 1994). Their surface areas were determined by monolayer techniques to span the transition region between 0.3 nm^2 and 3 nm^2 discussed above. We have used the NBD-labeled amphiphiles to determine their diffusion coefficients in dimyristoylphosphatidylcholine (DMPC) model membranes by fluorescence photobleaching measurements at various liquid crystalline-phase temperatures (Petersen et al., 1986; Axelrod, 1985; Edidin et al., 1976; Axelrod et al., 1976). These data show clearly that the transition to behavior described best by the hydrodynamic model occurs at surface areas as low as 1 nm^2 and provide further evidence for the use of the hydrodynamic models for diffusions of molecules other than lipids.

MATERIALS AND METHODS

Materials

DMPC was purchased from Fluka (Ronkonkoma, NY). Nitrobenzoxadiazole (NBD)-labeled phosphatidylethanolamine (NBD-PE), was obtained from Avanti Polar Lipids (Alabaster, AL). Both were used without further purification. Other NBD-labeled macrocyclic polyamide amphiphiles were prepared by P. A. Paprica (Paprica and Petersen, 1994). The structures of these compounds are shown in Fig. 1. The compounds are 1-(nitrobenzoxadiazoyl)-4-lauroyl-1,4-diazacyclohexane (N_2L[NBD]), 1-(nitrobenzoxadiazoyl)-4,7-dilauroyl-1,4,7-triazacyclononane (N_3L_2[NBD]), 1-(nitrobenzoxadiazoyl)-4,7,10-trilauroyl-1,4,7,10-tetraazacyclododecane (N_4L_3[NBD]), 1-(nitro-

Received for publication 18 April 1997 and in final form 18 August 1997.

Address reprint requests to Dr. Nils O. Petersen, Department of Chemistry, University of Western Ontario, London, ON N6A 5B7, Canada. Tel.: 519-661-3122; Fax: 519-661-3022; E-mail: scinop@uwoadmin.uwo.ca.

Dr. Paprica's present address is Pharmaceutical Development Lab, Astra Pharma Inc., Mississauga, ON L4Y 1M4, Canada.

0006-3495/97/11/2580/08 $2.00

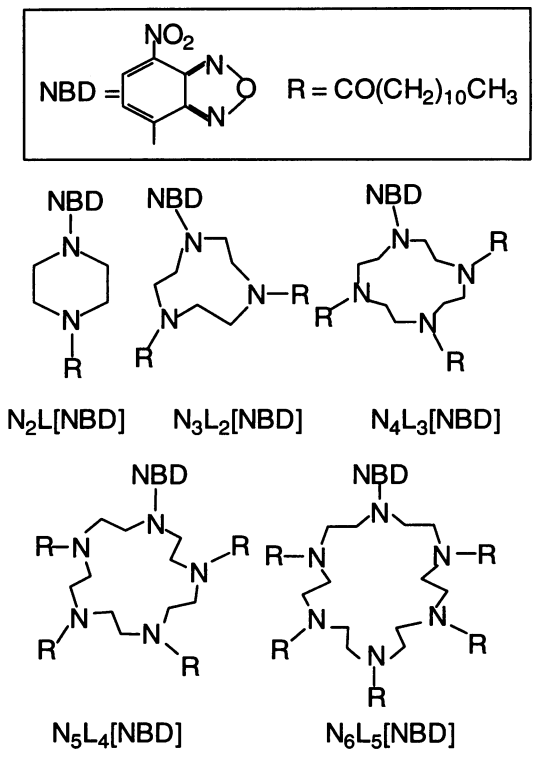

FIGURE 1 The structures of NBD-labeled macrocyclic polyamides.

benzoxadiazoyl)-4,7,10,13-tetralauroyl-1,4,7,10,13-pentaazacyclopentadecane (N_5L_4[NBD]), and 1-(nitrobenzoxadiazoyl)-4,7,10,13,16-pentalauroyl-1,4,7,10,13,16-hexaaza-cyclooctadecane (N_6L_5[NBD]). They were repurified, and their purity was confirmed by thin-layer chromatography before use. All solutions of DMPC and NBD-labeled compounds were prepared with glass-distilled chloroform.

Sample preparation

DMPC lipid vesicles were prepared by a modification of the method of Balcolm and Petersen (1993). Typically 2 mg of DMPC was mixed with the NBD probe at a probe-to-lipid molar ratio of 1/1000 or less in chloroform solution and allowed to stand for 30 min at room temperature. A sample of 200 μl of this solution was deposited as a single drop in the center of a 22-mm-diameter clean (sonified in ethanol for 30 min) and dry glass coverslip heated to 70°C. The chloroform was allowed to evaporate at 70°C, and the sample was dried under house vacuum at room temperature for at least 2 h. The sample was then heated to 70°C for 5 min, and a 200-μl drop of doubly distilled deionized water was placed on top of the dry lipid. A clean, dry 18-mm-diameter glass coverslip was gently placed on top of the water droplet. The two coverslips, forming a glass/water sandwich, were put in an oven at 70°C for 12–15 min. After the sample was cooled, the edge of the coverslips was sealed with paraffin wax to prevent further evaporation of water. The samples were incubated overnight in the dark at 40°C. Finally, the samples were cooled slowly in the oven over a period of several hours and resealed. The lipid vesicles were large, with diameters in the range of 10–100 μm. Only symmetrical, circular, or elliptical vesicles were selected for diffusion measurements.

Diffusion measurement

Diffusion measurements were performed with a home-built spot fluorescence photobleaching recovery instrument (Petersen et al., 1986). A Coherent Inc. Innova 70 4-W argon ion laser was used as the illumination source. The laser was operated in light regulation mode, with an output of ~50 mW at 476.5 nm. Timing of the bleach and monitor beams was controlled by a locally designed computer control unit interfaced to a Zenith computer. The microscope used was a Zeiss Universal model fitted for epiillumination. Florescent emission was detected by using an RCA 31034A photomultiplier tube that was fitted to the top of the optical column and cooled with dry ice. Data files were saved in ASCII format, then transferred to a VAX or CRAY mainframe computer, where the fluorescence recovery curves were fit and the diffusion coefficients were extracted from the recovery curve of best fit. The objective lens was a 40× water immersion lens with a numerical aperture of 0.75. A laser diameter beam of 1.2 μm was produced at the focal plane of the microscope by the combination of a 140-mm focusing lens and the 40× objective lens. The intensity of the bleach and monitor beams was attenuated with neutral density filters of 0.6–1.4 O.D.

Samples were inverted and mounted in a hollow copper holder that contained an axially symmetrical hole in the center, which was covered with an 18-mm glass coverslip and sealed with silicone sealant. The holder was filled with water, covering the sample, and a light film of thermally conducting grease was applied to the bottom. The temperature was regulated with a Cambion Bipolar temperature controller (Cambion Division of Midland Ross, Brampton, ON) with a microscope stage subassembly. The temperature of the sample was monitored by dipping a small 100-kΩ thermistor into the water and calculating the temperature from a resistance versus temperature calibration curve provided by the manufacturer (Yellow Springs Instrument Co.). The temperature control is accurate to within ±0.5°C.

RESULTS

The lateral diffusion coefficients of five NBD-labeled macrocyclic polyamides (N_2L[NBD], N_3L_2[NBD], N_4L_3[NBD], N_5L_4[NBD], N_6L_5[NBD]), and NBD-PE as a control for lipid-diffusion, were determined by photobleaching experiments in DMPC multilamellar vesicles at different temperatures (27–38°C) in the liquid-crystalline phase. The results are shown in Table 1. The mean values of the diffusion coefficients were determined with a standard error of the mean of 4–10% at a 95% confidence level. For all NBD-labeled molecules and at all temperatures, the mobile fraction, X_m, was close to unity, which allowed for multiple experiments on the same vesicle. This mobile fraction also confirms that homogeneous incorporation of the probes in the bilayer was achieved. The molar ratio of fluorescent probe/lipid for all diffusion measurements was in the range of 1/1000 to 1/1500. This concentration is low enough for the sample to behave as a nearly ideal system and high enough to allow for good signal-to-noise ratio in the collection of experimental data. The mobile fraction of unity also attests to a nearly ideal mixing of the probe and the lipids in the liquid-crystalline phase.

Fig. 2 shows the correlation of diffusion in fluid DMPC model membranes with the surface areas of NBD-labeled macrocyclic polyamides at 30 mN/m of surface pressure (Paprica and Petersen, 1994). This value of surface pressure is the best estimate available for describing the surface pressure in each monolayer of a bilayer membrane (Seeling, 1987; Jones and Chapman, 1995). It is very clear that the rate of lateral diffusion depends on molecular area in the region from 0.3 nm^2 to 3 nm^2. It is also seen that the relative effect of the size of the diffusing molecule is almost inde-

TABLE 1 Summary of diffusion and surface area measurements of NBD-labeled amphiphiles

NBD-probe	A^* (nm^2)	E_a[#] (kJ/mol)	D[§] (n[¶]) (μm^2/s)				
			27°C	30°C	32°C	35°C	38°C
NBD-PE	0.51 ± 0.05	36.4 ± 1.8	6.5 ± 0.6 (32)	7.3 ± 0.4 (35)	8.8 ± 0.7 (34)	9.1 ± 0.6 (38)	11.1 ± 0.6 (34)
N_2L[NBD]	0.30 ± 0.03	37.2 ± 1.5	6.8 ± 0.5 (32)	7.3 ± 0.5 (30)	9.2 ± 0.4 (37)	9.4 ± 0.6 (37)	11.5 ± 0.8 (30)
N_3L_2[NBD]	0.77 ± 0.07	37.4 ± 2.0	6.3 ± 0.3 (40)	7.2 ± 0.8 (32)	8.6 ± 0.6 (35)	8.7 ± 0.6 (36)	11.0 ± 0.8 (38)
N_4L_3[NBD]	1.00 ± 0.01	35.2 ± 4.0	5.2 ± 0.3 (34)	5.9 ± 0.5 (36)	6.3 ± 0.4 (32)	6.8 ± 0.6 (35)	8.8 ± 0.5 (35)
N_5L_4[NBD]	1.56 ± 0.16	23.4 ± 4.0	5.0 ± 0.3 (38)	5.4 ± 0.3 (35)	5.7 ± 0.4 (38)	5.8 ± 0.2 (37)	7.2 ± 0.6 (34)
N_6L_5[NBD]	2.24 ± 0.22	26.1 ± 4.8	4.6 ± 0.2 (39)	5.0 ± 0.2 (36)	5.3 ± 0.3 (34)	5.4 ± 0.3 (38)	6.9 ± 0.2 (39)

*Surface area ± SD measured by Langmuir film balance technique under a surface pressure of 30 mN/m (Paprica and Petersen, 1994).
#Apparent activation energy determined with Arrhenius plot. The correlation coefficients of linear regression least-squares analysis of the experimental points for NBD-PE, N_2L[NBD], N_3L_2[NBD], N_4L_3[NBD], N_5L_4[NBD], N_6L_5[NBD] are 0.96, 0.94, 0.95, 0.95, 0.88, 0.87, respectively. The uncertainty is estimated from the variation in E_a, obtained from systematic recovery of one of the five data points in the fit.
§Values of diffusion coefficients measured by a photobleaching technique at lipid liquid-crystalline temperatures, were given as mean ± SD.
¶The number of measurements.

pendent of temperature. These experiments indicate that the diffusion of smaller molecules with areas comparable to that of the lipid is faster than that of larger molecules. The rate of diffusion for NBD-PE (as in Table 1) is almost identical to literature values (Vaz et al., 1982a,b, 1984, 1985a,b; Edidin, 1989; Johnson et al., 1996).

The lateral diffusion of NBD-labeled molecules in fluid DMPC bilayers was studied in the temperature range from 27°C to 38°C. There is no phase transition in the bilayer of DMPC in this temperature range. For the lipid probe (NBD-PE), the Arrhenius plot is linear and yields an apparent activation energy of 36.4 kJ/mol, which compares favorably to 36.8 kJ/mol reported by Vaz (Vaz et al., 1982a). The diffusion coefficients of the five other NBD-labeled amphiphiles also exhibit linear Arrhenius plots, with the apparent activation energies shown in Table 1. The apparent activation energy for the smaller molecules (N_2L[NBD], N_3L_2[NBD], N_4L_3[NBD]) is very close to that of the lipid probe. In contrast, the apparent activation energy for the larger molecules (N_5L_4[NBD], N_6L_5[NBD]) is about two-thirds of that value. These results provide an indication that the diffusive mechanism differs as the size of the molecule changes.

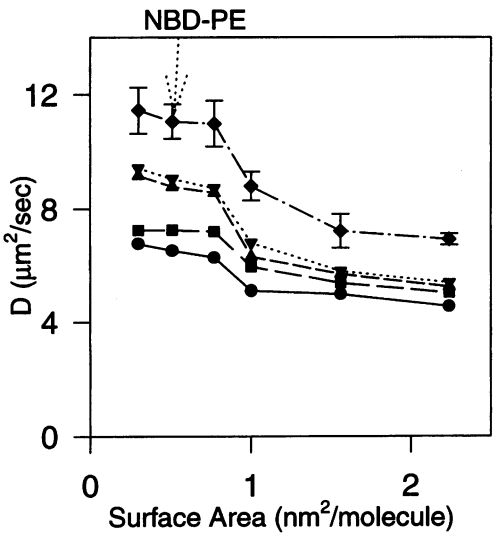

FIGURE 2 Dependence of the lateral diffusion coefficients of NBD-labeled amphiphiles as a function of their surface areas at five temperatures, 27°C (●), 30°C (■), 32°C (▲), 35°C (▼), and 38°C (◆). The reference molecule (NBD-PE) used as the standard control is indicated by the arrow. For simplicity the set of standard errors is shown only for measurements at 27°C.

Vaz and co-workers (Vaz et al., 1985a,b) have treated the diffusion of NBD-PE as typical of self-diffusion of lipids, because it has approximately the same surface area as DMPC. The surface area of lipids is reported to be ~0.65 nm^2 for a variety of saturated acyl chain lengths at corresponding lipid crystalline phase temperatures of lipids (Lewis and Engelman, 1983). Our values of the surface areas of the NBD-labeled molecules, N_2L[NBD], NBD-PE, and N_3L_2[NBD], are thus comparable to that of DMPC. Moreover, both NBD-PE and N_3L_2[NBD] contain two acyl chains. Although the latter is two methylene segments shorter than the chains in DMPC, the acyl chain length has been found to not affect the diffusion coefficient (Vaz et al., 1985a). Vaz has argued that the diffusion behavior of lipid-like molecules can be expected to be described best by a free area theory of lipid self-diffusion. To confirm this expectation, we used the interfacial viscosity limited free area theory (Vaz et al., 1985a,b), developed from the free-volume theory of Cohen and Turnbull (Cohen and Turnbull, 1959; Grest and Cohen, 1981) for diffusion in liquid, glass-forming materials. This model takes into account the viscous drag forces experienced by a particle in a membrane due to its contact with water at the aqueous interface and with the ends of the lipid molecules of the opposing monolayer at the bilayer midplane. In this model, the diffusion coefficient is given by Eq. 1, where k is Boltzmann's constant, T is the temperature in Kelvins, f is the translational friction coefficient resulting from drag forces at the membrane-water interface and at the bilayer midplane, T_m is the lipid bilayer main phase

$$D = \left(\frac{kT}{f}\right)\exp[(-\gamma a^*)/(a_0[\beta + \alpha_a(T - T_m)])] \quad (1)$$

transition temperature in Kelvins, γ is a numerical factor that accounts for the overlap of free area (γ has values between 0.5 and 1.0), a^* is the critical free area, a_0 is the van der Waals area per lipid molecule, $a_0\beta$ is the free area at T_m, and α_a is the lateral thermal expansion coefficient in the liquid-crystalline phase. Using Eq. 1, we calculated the diffusion coefficients expected for molecules with surface areas up to 0.8 nm^2; these results are shown as the dotted line in Fig. 3. We assume that $\gamma a^*/a_0 = 0.4$, $\alpha_a = 2.3 \times 10^{-3}$ K^{-1}, $\beta = 0.148$, and for the DMPC bilayers, $T_m = 23.9$°C (Galla et al., 1979; Vaz et al., 1985a,b). The friction coefficient was considered to contain two terms, $f = f_1 + f_2$, where f_1 is due to the interaction of the lipid polar headgroup with the aqueous phase at the bilayer-water interface, and f_2 is due to the interaction of the acyl chain ends of the lipid with the other half of the bilayer. Generally, friction coefficients have the form $f_i = C_i\mu_i$, where C_i is a constant that is equal to 4π times a radius of a particle and μ_i is the viscosity of corresponding liquid. Thus $f_1 = 4\pi\mu_W R$, where R is the protruding radius of the particle, and μ_W is the viscosity of water, which is itself a function of temperature. There is no good estimate for f_2, because it is difficult to pick a value for the radius of the acyl chain end, which is embedded within the bilayer and is not treated well as a sphere. Here the value for f_2 was used as an adjustable parameter in the model calculation of the diffusion coefficients shown in Fig. 3. The values of f_2 used are shown in the caption of Fig. 3 and are found to have a value of $\sim 4 \times 10^{-11}$ Ns/m (Vaz et al., 1985a). The diffusion coefficients calculated from Eq. 1 with these listed values are in good agreement with the experimentally determined diffusion coefficients at all temperatures (Fig. 2). This appears to be an excellent description of diffusion for small molecules with areas as large as 0.8 nm^2.

The hydrodynamic property of diffusing molecules with a radius much greater than 1 nm has been described by the Saffmann and Delbrück model (Saffmann and Delbrück, 1975). The relevant equation for lateral diffusion of a cylinder in a thin viscous sheet, such as for an integral membrane protein (bacteriorhodopsin and rhodopsin) in a lipid bilayer, is given by Eq. 2. In this equation, h is the thickness of the viscous sheet, which is equal to the height of the cylinder,

$$D = \frac{kT}{4\pi\mu_M h}\left[\ln\left(\frac{\mu_M h}{\mu_w R}\right) - 0.5772\right] \qquad (2)$$

μ_M and μ_w are the viscosities of the membrane and the surrounding aqueous solution, respectively, and R is the radius of the diffusing cylinder. Equation 2 has been tested experimentally and has been found to describe adequately the lateral diffusion of some large membrane proteins (area > 3 nm^2) that span the phospholipid bilayer completely (Vaz et al., 1984; Peters and Cherry, 1982), and for which D depends only weakly on the radius of the particle.

The Saffmann-Delbrück model is strictly applicable only to molecules that span a symmetrical membrane entirely. Evans and Sackmann have extended the hydrodynamic approach (Evans and Sackmann, 1988) by treating the diffusing particle as a cylinder inserted into an asymmetrical phospholipid bilayer. Thus the bilayer is a pseudo-two-dimensional fluid continuum, but with different boundary conditions above and below the diffusant. The lateral diffusion is expressed by

$$D = \frac{kT}{4\pi\eta_m} \times \left[\frac{\epsilon^2}{4} \times \left(1 + \frac{b_1}{b_2}\right) + \epsilon \times \frac{K_1}{K_0}\right]^{-1} \qquad (3)$$

where $\eta_m = \mu_M h$ is the two-dimensional membrane viscosity, h is the height of the diffusing particle, K_0 and K_1 are modified Bessel functions, ϵ is the dimensionless particle radius given by Eq. 4, and b_1, b_2, b_3 are coefficients of friction caused by drag against the viscous surrounding. Equations 3 and 4 were derived originally to describe the diffusion of a cylinder:

$$\epsilon = R\left(\frac{b_2 + b_3}{\eta_m}\right)^{1/2} \qquad (4)$$

in a membrane which is supported by a solid substrate, and there b_1 and b_3 are the viscous coefficients of friction of diffusant with the substrate and water, respectively, and b_2 reflects the drag between the two monolayers. The Evans-Sackmann model was subsequently used by Tamm to describe the diffusion of a cylinder inserted into only one monolayer of the bilayer (Tamm, 1991). In this interpretation, the frictional coefficients refer to the drag at the aqueous interface (b_3), at the interior of the bilayer (b_2), and at the bottom (b_1) of the diffusant. The approach by Tamm can be applied to our experiments, because our NBD-labeled amphiphiles are not long enough to span the whole bilayer membrane, and therefore they are asymmetrically embedded in the bilayer. Conceptually, this hydrodynamic model is related to the size and shape of the diffusing molecule, the viscosity of the continuous membrane sheet and the interfacial aqueous phase, and the mutual friction of diffusant with the interior of the membrane and with the interface of membrane. For cylindrical molecules inserted partially into one monolayer of a membrane, Eqs. 3 and 4 should still be valid.

The diffusion coefficients were calculated according to Eq. 3 for molecular surface areas greater than 1 nm^2 and are shown in Fig. 3 as solid lines. The parameters used for this theoretical calculation are listed in the caption for Fig. 3. The membrane viscosity (μ_M) is set to be ~ 1 Poise, and the viscosity of water (μ_w) is generally ~ 0.01 Poise (Levine, 1988). (The crystal structure of rubrene (Bergmann and Herlinger, 1936) and the calculation of molecular dimensions following the procedure of Edward (1970) provide an effective radius of rubrene of 0.5 nm. The diffusion coefficient of 5.8×10^{-12} m^2/s of rubrene in DMPC at 29°C (Balcom and Petersen, 1993) yields, by application of the Stokes-Einstein equation, a value of membrane viscosity of 1.15 Poise at 29°C.) Both of these values vary with temperature in a predictable manner. The value for the frictional

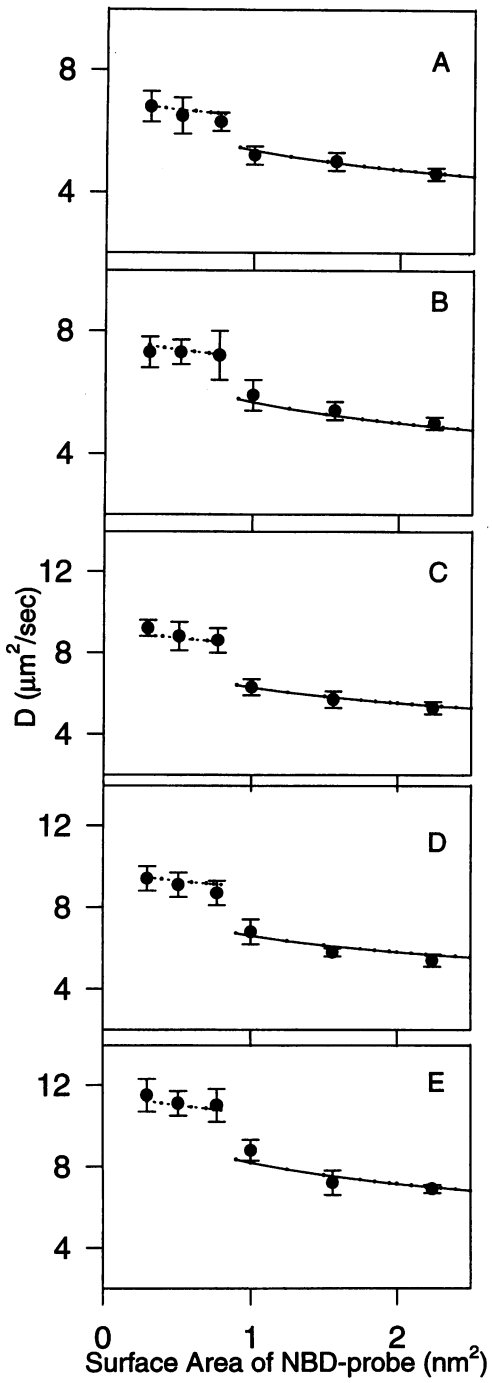

FIGURE 3 Comparison of measured values (●) of lateral diffusion coefficients for NBD-labeled cyclic polyamide amphiphiles with calculated values. The model calculations use the modified free area theory (Eq. 1) for small molecules (·····) and the Evans-Sackmann hydrodynamic model (Eq. 3) for larger molecules (——). The error bar represents the standard error of the mean of the measurements. The simulations were performed with the following fixed parameters: $\gamma a^*/a_0 = 0.4$, $\beta = 0.148$, $\alpha_a = 2.3 \times 10^{-3}\ K^{-1}$, $T_m = 23.9°C$, h = 1.05 nm, $b_1 = 3 \times 10^6$ Ns/m^3, $b_1/b_2 = 1/2.5$. The adjustable parameters were μ_M, μ_w, f_2. These were set to obtain the best apparent fit for each temperature as follows: (*A*) 27°C, $\mu_w = 0.0088$ Poise, $\mu_M = 1.25$ Poise, $f_2 = 4.35 \times 10^{-11}$ Ns/m; (*B*) 30°C, $\mu_w = 0.00808$ Poise, $\mu_M = 1.1$ Poise, $f_2 = 4.4 \times 10^{-11}$ Ns/m; (*C*) 32°C, $\mu_w = 0.00765$ Poise, $\mu_M = 1.05$ Poise, $f_2 = 4.0 \times 10^{-11}$ Ns/m; (*D*) 35°C, $\mu_w = 0.00728$ Poise, $\mu_M = 1.0$ Poise, $f_2 = 4.2 \times 10^{-11}$ Ns/m; (*E*) 38°C, $\mu_w = 0.007$ Poise, $\mu_M = 0.78$ Poise, $f_2 = 3.9 \times 10^{-11}$ Ns/m.

coefficient between layers of the bilayer membrane, b_2, has been measured to be 7.5×10^6 N s/m^3 or smaller (Merkel et al., 1989), which is 2.5 times the value of b_1. The frictional coefficient between the aqueous phase on the membrane and diffusant, $b_3 \simeq \mu_w^2/\eta_M$, is on the order of 10^3 to 10^4 Ns/m^3 (Evans and Sackmann, 1988). The height of the diffusant was assumed to be 1.05 nm. (Theoretical calculations, experimental studies of chain packing, and conformational statistics in lipid bilayers are consistent with an estimate of 1.0 nm for the height of our NBD-labeled macrocyclic polyamides containing lauroyl groups (Lewis and Engelman, 1983; Fattal and Ben-Shaul, 1994; Lu et al., 1995).) For the calculations of diffusion coefficients at all temperatures, we employed the same condition, $h = 1.05$ nm, $b_1 = 3 \times 10^6$ Ns/m^3, $b_1/b_2 = 1/2.5$. The calculation at each temperature was adjusted for the temperature dependence of the viscosity of water and of membranes. Consequently, it was found that experimental data were described very well by the Evans-Sackmann model.

It is important to reiterate that the calculations shown in Fig. 3 use the same values for each set of parameters at all temperatures, and it is therefore the explicit temperature dependence of these parameters (f_2, μ_M, μ_w) that accounts for the variation in diffusion coefficient from 27°C to 38°C. For example, the known temperature dependence of the viscosity of water (μ_w) was used in these calculations (Levine, 1988). The temperature dependence of the viscosity of membrane (μ_M) is less well characterized, but the values used here follow a trend quite similar to that of water, as might be expected. The variation in f_2 is too small to provide a good sense of the temperature dependence, but it is decreasing slightly, reflecting the interdependence of f_2 and μ_M. The calculations are internally consistent and their compatibility with the experimental data for all the probes at all temperatures provides the strongest evidence for the applicability of an Evans-Sackmann-type model as used here. It also should be noted that there appears to be a discontinuity in both the experimental data and in the theoretical calculations at molecular areas between 0.8 nm^2 and 1 nm^2. This now defines the transition region between free area and hydrodynamic descriptions of diffusion in model membranes. It should be recognized that there are experimental errors associated with the area measurements and that the absolute area values reflect the choice of 30 mN/m as the bilayer surface pressure. Whereas the absolute areas may be open to some uncertainty and interpretation, the relative areas are determined very well. Thus the data presented here provide the best description of the area dependence of molecular diffusion in membranes available to date. Moreover, these molecules are designed as a homologous series to ensure that they have similar chemical, physical, and structural properties. The molecular modeling suggests that these molecules contain circular rings that are rigid and fairly incompressible (Paprica and Petersen, 1994) and therefore vary in size but not in shape.

DISCUSSIONS

We have found that the lateral diffusion of NBD-labeled macrocyclic polyamide amphiphiles is dependent on the size and shape of these molecules in the liquid-crystalline phase of model membranes. The surface areas of the NBD-labeled amphiphiles span the transition region of interest from 0.3 nm^2 to 3 nm^2. Now it is very clear that the diffusion behavior of these intermediate-size molecules can be described by different models. The diffusion behavior of small lipid-like molecules (surface area < 1 nm^2, radius $<$ 0.56 nm) with dimensions similar to those of the lipids in the bilayer can be described by an expression of interfacial viscosity limited free area theory (Vaz et al., 1985a), whereas that of larger molecules (surface area ≥ 1 nm^2, radius ≥ 0.56 nm) is much slower than lipid self-diffusion and can be fitted by a continuum hydrodynamic model recently developed by Tamm (1991), based on the Evans and Sackmann model (Evans and Sackmann, 1988). We also observed that the apparent activation energies of diffusion for smaller molecules (surface area < 1 nm^2) are similar to those of lipid molecules, but the apparent activation energies of diffusion for larger molecules (surface area ≥ 1 nm^2) are smaller than those of lipids. The peculiar observation that N_4L_3[NBD] moves slowly but has a high apparent activation energy may reflect the fact that this molecule is situated in the middle of the transition region.

It is evident that the hydrodynamic description is appropriate for the larger molecules, even though they span only part of the membrane. In fact, it appears that the diffusion is controlled only by the size of the molecule in the region of the membrane closet to the aqueous interface. The question remains why there is an apparent discontinuity in the diffusion behavior at molecular areas above 1 nm^2. For smaller molecules, density flunctuations in the interface region can generate free area (or volume), thereby permitting the small molecules to move in a diffusive process along the surface. The relatively large apparent activation energy suggests that the temperature dependence of the fluctuations in the equilibrium density is quite large. On the other hand, for large molecules, the fluctuations in the equilibrium density are not the only constraint on the diffusive motion. Rather, the frictional forces generated when the molecules move past one another appear to dominate and cause a reduction in the observed diffusion rate. The smaller apparent activation energy indicates that these frictional forces, which are interpreted as the viscosity, are much less sensitive to temperature.

The current data show that the diffusion coefficient is sensitive to a change in size of a molecule at the surface of the membrane. This is in marked contrast to the observations by Balcom and Petersen (1993) that the diffusion coefficient of amphiphilic molecules is insensitive to a change in size in the interior of the membrane. In that work, it was shown that the diffusion behaviors of derivatives of citronellol, solanosol, and dolichols were identical, even though the volume of the molecule in the interior was increased by more than an order of magnitude. Thus increasing the area at the surface affects the diffusion, whereas increasing the volume in the interior does not. Still, molecules such as tetracene and rubrene, which are entirely in the interior of membrane, exhibit a Stokes-Einstein type dependence on diffusion, i.e., the diffusion depends on the size (Balcom and Petersen, 1993). These observations suggest that the surface interface region of the bilayer membrane is more restrictive to diffusion than is the middle of the bilayer. The best view of the membrane may then be as a triple layer (Fig. 4), a region close to the aqueous interface that is more restrictive to diffusion, and the middle region, which is more fluid and less restrictive to diffusion. In this view, any molecule penetrating into the more rigid interface region (all of those studied in this work and the isoprenol derivatives studied earlier) will move laterally in a manner controlled by this region, irrespective of the size of the molecule in the aqueous region or in the middle region. Thus lipid-anchored proteins and dolichols share the feature that there are large bulky groups extruding into regions of lower viscosity, where the additional drag minimally influences the diffusion. Moreover, molecules residing only in the interior experience a more fluid environment, and diffusion may be more rapid than that of the host lipid, as exemplified by tetracene (Balcolm and Petersen, 1993).

It is intriguing to speculate that the high-viscosity region of the triple-layer membrane corresponds to the region with large acyl chain order parameters. This region is ~8–10 carbons deep, corresponding to a ~1.0-nm thickness (coincidentally, the height used to fit the diffusion data). In that case, the more fluid middle region would correspond to the

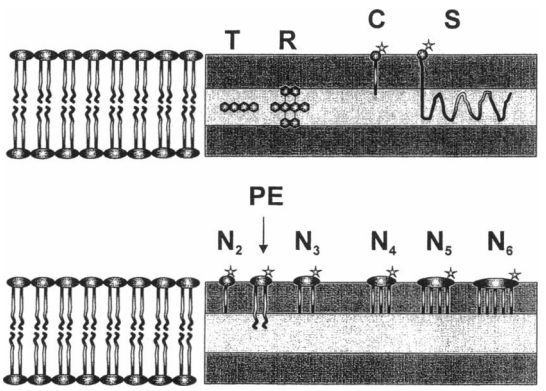

FIGURE 4 Diagrammatic representation of the "triple layer" model for a membrane. The darker region represents the ~1-nm-thick viscous region close to the surface, and the lighter region represents the 1–1.5-nm-thick fluid region in the hydrophobic interior. A series of molecules are depicted: T, tetracene; R, rubrene; C, NBD-labeled citronellol; S, NBD-labeled solanosol; PE, NBD-labeled phospholipid analog; N_n, NBD-labeled macrocycle with n nitrogens in the ring (see text). The diffusion data suggest that C, S, PE, N_2 (N_2L[NBD]), and N_3 (N_3L_2[NBD]) have the same diffusion coefficient because they are comparable in size to the lipid molecule, and the bulk in the interior is of no consequence. The diffusion rates of N_4 (N_4L_3[NBD]), N_5 (N_5L_4[NBD]), and N_6 (N_6L_5[NBD]) are slower because their size in the viscous region of the membrane is larger than that of the host lipid.

region with relatively small chain order parameters in the layer where the two monolayers meet. This region is 6–8 carbons deep for each monolayer corresponding to a 1–2-nm-thick middle layer. This region is sufficiently large to accommodate large hydrophobic chain segments, such as in dolichol, without impeding the diffusion significantly.

Thinking about a membrane functionally as a triple layer may be useful from a number of perspectives. Diffusion of lipids and proteins will differ mostly because the latter span two viscous regions. Diffusive transport across the membrane will be dominated by penetration through the ~1.0-nm-thick layers at each surface. Once in the middle region of the membrane, the lateral diffusion would be facile and perhaps more rapid than escape across the other, more viscous region.

In summary, we predict that an amphiphile that resides partly in the membrane region closest to the surface will exhibit lateral diffusion at the rate of the lipids if it is small (surface area < 1 nm^2), or will be slightly slower if it is large (surface area > 1 nm^2). The size of the molecule in the aqueous region or in the hydrophobic interior is of little consequence, because the viscous drag will be less. Any hydrophobic molecule that resides exclusively in the interior may diffuse more rapidly than the solvent (lipid), but will exhibit a dependence on size similar to that observed in three-dimensional diffusion. Any molecule that spans both bilayers should be reduced in diffusion by a factor of 2 relative to a molecule of comparable cross-sectional area present only in one monolayer; this prediction has yet to be tested rigorously. Finally, molecules larger than 1 nm^2 are adequately described by the hydrodynamic models, provided that the effective height is understood to be the thickness of the viscous layers.

The work was supported by an operating grant from the Natural Sciences and Engineering Research Council (NSERC), Canada (NOP), and a NSERC postgraduate fellowship (PAP).

REFERENCES

Adam, G., and M. Delbrück. 1968. Reduction of dimensionality in biological diffusion processes. *In* Structural Chemistry and Molecular Biology. A. Rich and N. Davidson, editors. W. H. Freeman and Company, San Francisco and London. 198–222.

Axelrod, D. 1985. Fluorescence photobleaching techniques and lateral diffusion. *In* Spectroscopy and the Dynamics of Molecular Biological Systems. P. Bayley and R. Dale, editors. Academic Press, London. 163–176.

Axelrod, D., D. E. Koppel, J. Schlessinger, E. Elson, and W. W. Webb. 1976. Mobility measurement by analysis of fluorescence photobleaching recovery kinetics. *Biophys. J.* 16:1055–1069.

Balcom, B. J., and N. O. Petersen. 1993. Lateral diffusion in model membranes is independent of the size of the hydrophobic region of molecules. *Biophys. J.* 65:630–637.

Bergmann, E., and E. Herlinger. 1936. The structure of rubrene and some remarks on the "ortho-effect." *J. Chem. Phys.* 4:532–534.

Cherry, R. J., and R. E. Godfrey. 1981. Anisotropic rotation of bacteriorhodopsin in lipid membranes. *Biophys. J.* 36:257–276.

Clegg, R. M., and W. L. C. Vaz. 1985. Translational diffusion of proteins and lipids in artificial lipid bilayer membranes. A comparison of experiment with theory. *In* Progress in Protein-Lipid Interactions. A. Watts and J. J. H. H. M. DuPont, editors. Elsevier Science Publishing, Amsterdam. 173–229.

Cohen, M. H., and D. Turnbull. 1959. Molecular transport in liquids and gases. *J. Chem. Phys.* 31:1164–1169.

Derzko, Z., and K. Jacobson. 1980. Comparative lateral diffusion of fluorescent lipid analogues in phospholipid multibilayers. *Biochemistry.* 19:6050–6057.

Edidin, M. 1989. Rotational and lateral diffusion diffusion of membrane proteins and lipids. *Curr. Top. Membr. Transp.* 29:91–127.

Edidin, M., Y. Zagyansky, and T. J. Lardner. 1976. Measurement of membrane protein lateral diffusion in single cells. *Science.* 191: 466–468.

Edward, J. T. 1970. Molecular volumes and the Stokes-Einstein equation. *J. Chem. Educ.* 47:261–269.

Evans, R., and E. Sackmann. 1988. Translational and rotational drag coefficients for a disk moving in a liquid membrane associated with a rigid substrate. *J. Fluid Mech.* 194:553–561.

Fattal, D. R., and A. Ben-Shaul. 1994. Mean-field calculations of chain packing and conformational statistics in lipid bilayers: comparison with experiments and molecular dynamics studies. *Biophys. J.* 67:983–995.

Frey, S., and L. K. Tamm. 1990. Membrane insertion and lateral diffusion of fluorescence-labeled cytochrome c oxidase subunit IV signal peptide in charged and uncharged phospholipid bilayers. *Biochem. J.* 272: 713–719.

Galla, H. J., W. Hartmann, U. Theilen, and E. Sackmann. 1979. On two-dimensional passive random walk in lipid bilayers and fluid pathways in biomembranes. *J. Membr. Biol.* 48:215–236.

Grest, G. S., and M. H. Cohen. 1981. Liquids, glasses and the glass transition: a free-volume approach. *Adv. Chem. Phys.* 48:455–525.

Hackenbrock, C. R. 1981. Lateral diffusion and electron transfer in the mitochondrial inner membrane. *Trends Biochem. Sci.* 6:151–154.

Hildebrand, J. H. 1971. Motions of molecules in liquids: viscosity and diffusivity. *Science.* 174:490–493.

Hughes, B. D., B. A. Pailthorpe, L. R. White, and W. H. Sawyer. 1982. Extraction of membrane microviscosity from translational and rotational diffusion coefficients. *Biophys. J.* 37:673–676.

Johnson, M. E., D. A. Berk, D. Blankschtein, D. E. Golan, R. K. Jain, and R. S. Langer. 1996. Lateral diffusion of small compounds in human stratum corneum and model lipid bilayer systems. *Biophys. J.* 71: 2656–2668.

Jones, M. N., and D. Chapman. 1995. Micelles, Monolayers and Biomembranes. Wiley-Liss, New York.

Koppel, D. E. 1982. Measurement of membrane protein lateral mobility. *Tech. Life Sci. B4/I.* B4 25:1–25.

Levine, I. 1988. Physical Chemistry. McGraw-Hill, New York.

Lewis, B. A., and D. W. Engelman. 1983. Lipid bilayer thickness varies linearly with acyl chain length in fluid phosphatidylcholine vesicles. *J. Mol. Biol.* 166:211–217.

Lu, D., I. Vavasour, and M. R. Morrow. 1995. Smoothed acyl chain orientational order parameter profiles in dimyristoylphosphatidylcholine-distearoylphosphatidylcholine mistures: a ^{2}H-NMR study. *Biophys. J.* 68:574–583.

Merkel, R., E. Sackmann, and E. Evans. 1989. Molecular friction, and epitactic coupling between monolayers in supported bilayers. *J. Physique.* 50:1535–1555.

Nir, S., and W. D. Stein. 1971. Two modes of diffusion in liquids. *J. Chem. Phys.* 55:1598–1603.

Paprica, P. A., and N. O. Petersen. 1994. Monolayers of polylauroyl macrocyclic polyamides and nitrobenzoxadiazole (NBD) labeled polyauroyl macrocyclic polyamides at the air-water interface. *Langmuir.* 10:4630–4636.

Peters, R., and R. J. Cherry. 1982. Lateral and rotational diffusion of bacteriorhodopsin in lipid bilayers: experimental test of the Saffman-Delbrück equations. *Proc. Natl. Acad. Sci. USA.* 79:4317–4321.

Petersen, N. O., S. Felder, and E. L. Elson. 1986. Measurement of lateral diffusion by fluorescence photobleaching recovery. *In* Handbook of

Experimental Immunology. D. M. Weir, editor. Blackwell, Edinburgh. 24.1–24.3.

Sackmann, E. 1996. Supported membranes: scientific and practical applications. *Science.* 271:43–48.

Saffman, P. G., and M. Delbrück. 1975. Brownian motion in biological membranes. *Proc. Natl. Acad. Sci. USA.* 73:3111–3113.

Seelig, A. 1987. Local anesthetics and pressure: a comparison of dibucaine binding to lipid monolayers and bilayers. *Biochim. Biophys. Acta.* 899: 196–205.

Tamm, L. K. 1991. Membrane insertion and lateral mobility of synthetic amphiphilic signal peptides in lipid model membranes. *Biochim. Biophys. Acta.* 1071:123–148.

Vaz, W. L. C., R. M. Clegg, and D. Hallmann. 1985a. Translational diffusion of lipids in liquid crystalline phase phosphatidylcholine multibilayers. A comparison of experiment with theory. *Biochemistry.* 24:781–786.

Vaz, W. L. C., M. Criado, V. M. C. Madeira, G. Schoellmann, and T. M. Jovin. 1982a. Size dependence of the translational diffusion of large integral membrane proteins in liquid-crystalline phase lipid bilayers. A study using fluorescence recovery after photobleaching. *Biochemistry.* 21:5608–5612.

Vaz, W. L. C., Z. I. Derzko, and K. A. Jacobson. 1982b. Photobleaching measurements of the lateral diffusion of lipids and proteins in artificial phospholipid bilayer membranes. *Cell Surface Rev.* 8:83–135.

Vaz, W. L. C., F. Goodsaid-Zalduondo, and K. Jacobson. 1984. Lateral diffusion of lipids and proteins in bilayer membranes. *FEBS Lett.* 174: 199–207.

Vaz, W. L. C., D. Hallmann, R. M. Clegg, A. Gambacorta, and M. DeRosa. 1985b. A comparison of the translational diffusion of a normal and a membrane-spanning lipid in Lα phase 1-palmitoyl-2-oleoylphosphatidylcholine bilayers. *Eur. Biophys. J.* 12:19–24.