Contribution of Hydrogen Bonding to Lipid–Lipid Interactions in Membranes and the Role of Lipid Order: Effects of Cholesterol, Increased Phospholipid Unsaturation, and Ethanol†

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Received December 14, 1992; Revised Manuscript Received January 27, 1993

ABSTRACT: It is proposed that increased phospholipid unsaturation in membranes and perturbation by agents such as ethanol weaken interlipid hydrogen bonding involving water and that the process is independent of effects on lipid order. To investigate this, the rates of phospholipid desorption, as a measure of the strength of interlipid interactions, from "donor" lipid vesicles was determined. This was accomplished using (7-nitrobenzo-2-oxa-1,3-diazole-4-yl)aminohexanoate (C_6-NBD) labeled phospholipids, the rate of desorption being followed from changes in fluorescence with time. The rates of desorption of the NBD-phospholipids from phosphatidylcholine (PC) donor vesicles was in the order phosphatidylcholine (PC) > phosphatidylserine (PS) > phosphatidylethanolamine (PE), the slower rates in the PS and PE reflecting direct interlipid hydrogen bonding. For PC, the interlipid hydrogen bonding was restricted to the "hydration layer", the network of hydrogen-bonded water molecules extending between phospholipid head groups. The rate of C_6-NBD-PC desorption was elevated with higher levels of donor PC *sn*-2 unsaturation, due the increased head group spacing weakening the lipid–lipid interactions that occur via the hydration layer. Ethanol also increased the rate of NBD-phospholipid desorption from donor PC vesicles in the order PC > PS > PE, showing that PC interactions, here limited to the weaker hydrogen-bonded water molecule network, were more susceptible compared to stronger, direct interlipid hydrogen bonds involving PE and PS. The relative magnitude of the ethanol-induced increase in the desorption rate was amplified with higher levels of donor lipid *sn*-2 unsaturation. Cholesterol had little effect on the rate of phospholipid desorption. This indicates that its hydrogen bonding to adjacent carbonyls is weak and further, since cholesterol strongly orders lipids, lipid order has little or no influence on surface hydrogen bonding. Therefore a weakening of the hydrogen-bonded network of interlipid water molecules, rather than effects on lipid order, appears to responsible for the effects on phospholipid desorption. The results indicate that the effects of unsaturation and ethanol and other related membrane perturbants on membrane function may involve a perturbation of membrane surface hydrogen bonds.

Water has a primary role in the formation and maintenance of cell membrane architecture the basic lipid bilayer structure being an obligatory response of phospholipids exposed to an aqueous environment. A knowledge of the finer details of hydration at the molecular level, involving hydrogen bonding at the aqueous–lipid bilayer interface, is of interest due to its potential in the control of biological processes, mediated at the level of the cell membrane [see reviews on hydration by Crowe and Crowe (1984) and Rand and Parsegian (1989)].

While a structural role of hydrogen bonding in proteins, often involving water, is well recognized [see reviews by Teeter (1991) and Saenger (1987)], the recognition of a similar importance of hydrogen bonds in lipid bilayer structure has been relatively recent [reviewed by Brockerhoff (1982) and Boggs (1987)]. Several types of hydrogen bonding are possible, a direct lipid–lipid hydrogen bonding, for example, between the amine group on PE[1] or PS and an oxygen atom of the phosphate group of any other phospholipid class (including PC) (Boggs, 1987). Also the carbonyl oxygens are available for hydrogen bonding.

Hydrogen bonding may also occur between any two phospholipid classes, involving a bridging by one or more water molecules. Such water molecules participate in a hydrogen-bonded network that extends between the lipids, termed the "hydration layer" (Prats et al., 1987; Teissie et al., 1990). A number of previous studies have established the existence of water molecules hydrogen bonded to phospholipids (Small, 1967; Ladbrooke & Chapman, 1969; Finer & Darke, 1974; Jendrasiak & Hasty, 1974; Newman & Huang, 1975; Ueda et al., 1986; White et al., 1987; Tsai et al., 1987; Sen & Hui, 1988). The number of "bound" water molecules per phospholipid is ~7–20 waters/phospholipid. This head group solvation is an important element in the formation of lipid bilayers, as it serves to partially offset the large electrostatic repulsion between charged head groups. The orientation of

† This work was supported by Public Health Service Grants AA08022, AA07215, AA07186, and AA07465.

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[1] Abbreviations: BSA, bovine serum albumin; DPH, 1,6-diphenyl-1,3,5-hexatriene; EYPC, egg phosphatidylcholine; k_{off}, apparent first-order rate constant for phospholipid desorption; LUV, large unilamellar vesicles; NBD, (7-nitrobenzo-2-oxa-1,3-diazole-4-yl)aminohexanoate; NBD-PC, 1-palmitoyl-2-(NBD)hexanoyl-PC; NBD-PE, 1-palmitoyl-2-(NBD)hexanoyl-PE; NBD-PS, 1-palmitoyl-2-(NBD)hexanoyl-PS; PC, phosphatidylcholine; PDPC, 1-palmitoyl-2-docosahexaenoylphosphatidylcholine; PE, phosphatidylethanolamine; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; POPE, 1-palmitoyl-2-oleoylphosphatidylethanolamine; POPS, 1-palmitoyl-2-oleoylphosphatidylserine; PS, phosphatidylserine; Rho-PE, *N*-(lissamine Rhodamine B sulfonyl)dipalmitoyl-L-α-phosphatidylethanolamine.

0006-2960/93/0432-3714$04.00/0

water molecules at a bilayer surface is controlled by the nature of lipids. Thus the lipid bilayer surface modifies the effective dipole moment of water normal to the surface and thus the membrane dipole potential. This dipole potential is considered to drop exponentially in a direction normal to the plane of the bilayer (McIntosh et al., 1991).

The potential in allosteric regulation by such a perturbed water layer around proteins and lipid bilayers was discussed in a recent article (Rand, 1992) in connection with evidence that such water may play an important part in hemoglobin structure/function (Colombo et al., 1992). With respect to the structural role of water in membranes, there have been a series of landmark studies on the properties of water that exist between two closely apposing bilayers [see, for example, LeNeveau et al. (1976), McIntosh and Simon (1986), Rand et al. (1988), Ragavan et al. (1992), Rand (1992), Simon et al. (1992), and a review by Rand and Parsegian (1989)] that are the key to understanding the features of the hydration layer. For instance, a "hydration pressure" exists which opposes the approach of bilayers and which is proposed, from theoretical analyses, to be due to the polarization and orientation of the interbilayer water [e.g., Marcelja and Radic (1976)]. This same hydrogen-bonded water molecule network extends between head groups in the plane of the bilayer.

An important goal in investigating hydrogen bonding in membranes is to determine the contribution to protein–lipid interactions and therefore to function. However, before this can be accomplished there are a number of gaps in our basic knowledge that need to be filled. For instance, it is not known if effects on lipid order are manifest as modifications to membrane surface hydrogen bonding. Cholesterol is a major membrane component and has a well known lipid ordering effect in cell membranes [see review by Yeagle (1985)]; however, while there is some evidence for an ability to participate in surface hydrogen bonding (Brockerhoff, 1974; Yeagle et al., 1975; Huang, 1976), whether this actually occurs remains in debate (Bush et al., 1980; Choi et al., 1991). With regard to phospholipid unsaturation, which varies considerably in cell membranes [reviewed by Stubbs and Smith (1984), Spector and York (1985), McMurchie (1988), and Quinn et al. (1989)], while its effects on lipid order are gradually becoming clear, the impact on membrane surface hydrogen bonding remains unknown.

Ethanol, by intercalating between lipids, induces a disordering effect that weakens van der Waals interactions between acyl chains. Traditionally, the focus has been on this aspect of the lipid bilayer perturbing effects of both ethanol and related compounds, often in terms of its ability to "fluidize" or disorder membrane lipids [reviewed by Hoek and Rubin (1990), Little (1991), and Dietrich et al. (1989)]. Recently, a number of studies have implicated a disruption of direct interlipid hydrogen bonds as playing an important role in the action of inhalation anesthetics [see, for example, DiPaolo and Sandorfy (1974), Hobza et al. (1981, 1982), Brockerhoff and Box (1986), Chiou et al. (1990a,b), and reviews by Curatola et al. (1991), Ueda (1991), and Urry and Sandorfy (1991)]. This provokes the question of whether this may apply to anesthetics in general, and to ethanol in particular. So far, very little has appeared on the subject, and while some evidence has begun to emerge (Chiou et al., 1990a,b), it is not known if ethanol perturbs cell membranes by competing for direct interlipid hydrogen bonds or if it competes for hydrogen-bonded water in the hydration layer.

One approach to studying membrane surface hydrogen bonding is to examine the contribution to overall lipid–lipid interactions, which will also include hydrophobic and electrostatic interactions. It has been shown that this may be accomplished by measuring the rate of desorption of a phospholipid from a membrane [Gardam et al. (1989), Gardam and Silvius (1990) and Shin et al. (1991), as recently reviewed by Brown (1992)] and with this approach, it has been shown that hydrogen bonding contributes significantly to lipid–lipid interactions involving PE. To accomplish this, since the rate of phospholipid desorption is normally slow and difficult to measure, one acyl chain may be replaced with a fluorophore attached to a shorter acyl chain, this greatly increasing the rate of desorption. The desorption process can then be easily followed from changes in fluorescence, as the fluorophore-labeled phospholipid is desorbed into a different environment such as another vesicle, as previously described (Nichols & Pagano, 1982; Gardam et al., 1989; Gardam & Silvius, 1990; Shin et al., 1991). Also by this means, the desorbed lipid may be distinguished from the lipids that make up the membrane from which it is desorbed (the "donor lipid"), both of which may then be independently varied.

In the present study, using the above technique, it was found that cholesterol had only marginal effects on phospholipid desorption, in spite of its potential to hydrogen bond to phospholipid carbonyls and it strong lipid ordering effect. Thus it was concluded modifications to lipid order are unlikely to have much impact on hydrogen bonding at the head group region of membranes. It was further found that increased phospholipid unsaturation in the *sn*-2 acyl chain and addition of ethanol increased the rate of phospholipid desorption, which was considered to be due to a weakened membrane surface hydrogen bonding. These effects could have considerable influence on cell functioning should they be manifest at the protein–lipid interface as would appear likely to occur.

EXPERIMENTAL PROCEDURES

Materials. All phospholipids were obtained from Avanti Polar Lipids (Birmingham, AL); NBD-hexanoic acid was from Molecular Probes (Eugene, OR). BSA was from Sigma (St. Louis, MO). All other chemicals used were of analytical grade and were obtained from Fisher Scientific (Malvern, PA).

Phospholipids, especially those highly susceptible to oxidation, such as the polyunsaturated phosphatidylcholines, were handled so as to minimize oxidation as previously confirmed (Stubbs et al., 1981; Cox et al., 1992). This involved using vesicle preparations within a few hours of preparation, keeping vesicles at 4 °C in the dark, etc. Previously, these handing procedures have been found to result in no detectable oxidation product formation as monitored by thin layer chromatography, conjugated diene tests, and gas–liquid chromatography analysis of the fatty acyl composition of vesicles after experimental handling.

Preparation of Vesicles. LUV were prepared by first evaporating the chloroform from the required quantities of solutions, consisting of a mixture of the donor vesicle phospholipid and C_6-NBD-phospholipid (1 mol % of the donor phospholipid) with a stream of nitrogen. The lipids (50 μM final concentration) were then codispersed to form multilamellar vesicles by vortexing in 10 mM Tris-HCl buffer, pH 7.4, with 150 mM NaCl. LUV (100-nm diameter) were then made by the extruction technique using either a Lipex extruder (Lipex Biomembranes, Inc., Vancouver, BC) as previously described (Hope et al., 1985) or an Avestin Lipsofast Extruder (MM Developments, Ottawa, Canada) also as previously described (MacDonald et al., 1991); both techniques gave the same results.

Measurement of Desorption Rates. The desorption rates of the C_6-NBD-phospholipids were measured by a previously described procedure (Gardam et al., 1989; Gardam & Silvius, 1990; Shin et al., 1991). Briefly, the fluorescence intensity at 530 nm, obtained upon excitation at 470 nm, was determined using a PTI Alphascan fluorimeter (Photon Technology Instruments, Princeton, NJ). Desporption of the NBD probe from vesicles, initiated by the addition of BSA (65 μM), was measured at 25 °C (unless otherwise specified) and the fluorescence intensity decrease recorded as a function of time. Where the effects of ethanol, or other additives, were under study, the required quantity was added before the BSA. This method was found to be inappropriate in studies involving cholesterol, since it was also desorbed by BSA. In this case, acceptor vesicles of the same phospholipid type as the donor vesicles were used (including cholesterol) but including N-Rho-PE (1 mol %). The rate of phospholipid desorption was measured from the quenching of the NBD fluorescence as the C_6-NBD-phospholipid exchanged from the donor into the acceptor vesicles, as described previously (Nichols & Pagano, 1982). Without cholesterol, BSA and acceptor vesicles gave identical results. For both the BSA and the donor vesicles, the C_6-NBD-phospholipid probe concentration was 1% of the total phospholipid concentration, so that each C_6-NBD-phospholipid could be assumed to be surrounded by donor phospholipid, ensuring that the predominant interactions affecting desorption were between the donor and fluorophore-labeled phospholipid. The mechanism of C_6-NBD-phospholipid probe transfer from vesicles to acceptors involves the diffusion of phospholipid probe monomers through the aqueous phase (Nichols, 1985), and the apparent observed first-order rate constant, k_{off}, for the desorption of the C_6-NBD-phospholipid was that for the rate-determining step. The rate of desorption of the C_6-NBD-phospholipid was zero- and first-order for BSA and C_6-NBD-phospholipid concentrations, respectively.

Calculation of Rate Constant and Free Energy. The fluorescence intensity, $F(t)$, as a function of time, t, was fitted to an integrated first-order rate equation by means of linear regression analysis:

$$\ln[F(t) - F_{inf}] = \ln(F_0 - F_{inf}) - k_{off}t \quad (1)$$

where F_0 and F_{inf} are the initial and final fluorescent intensities, respectively. Comparisons of the free energies of transfer of C_6-NBD-phospholipid probes from vesicles to BSA for different systems were made by relation to a "reference" condition, consisting of POPC vesicles containing 1 mol % C_6-NBD-PC, using the following equation:

$$\Delta(\Delta G) = \Delta G_1 - \Delta G_2 = -RT \ln(k_1/k_2) \quad (2)$$

where ΔG_1, k_1 and ΔG_2, k_2 are the free energies of transfer and rate constants for the desorption of C_6-NBD-phospholipid probes for the reference and systems with which it was being compared. This holds as long as the on rates for the two systems are equal and the back desorption are negligible. For a diffusion-limited adsorption process, generally assumed in such systems, the first condition is probably correct. Again, the process should be independent of the acceptor concentration and first order with respect to the donor concentration, and there should be no differential interaction between donor and acceptors of the two systems. A direct substitution of rate constant for the equilibrium constant for the exchange of the C_6-NBD-phospholipid between LUV and BSA is possible, since initially the system is not in equilibrium, the desorption rate being much greater than the rate of back exchange.

Table I: Desorption Rates for NBD-Phospholipids[a]

donor lipid		control	+ethanol (200 mM)	+ethylamine (50 mM)
POPC				
	C_6-NBD-PC	2.55 ± 0.11	3.22 ± 0.11	6.88 ± 0.20
		2.66 ± 0.12[b]	3.60 ± 0.15[b]	6.59 ± 0.90[b]
		8.79 ± 0.20[c]	11.5 ± 0.32[c]	ND[d]
		0.86 ± 0.20[e]	1.02 ± 0.20[e]	ND
	C_6-NBD-PE	0.84 ± 0.02	1.15 ± 0.16	5.51 ± 0.30
		0.83 ± 0.01[b]	1.18 ± 0.01[b]	5.44 ± 0.51[b]
	C_6-NBD-PS	1.48 ± 0.04	1.87 ± 0.06	6.25 ± 0.30
		1.44 ± 0.01[b]	1.78 ± 0.01[b]	6.21 ± 0.60[b]
		8.77 ± 0.20[c]	9.54 ± 0.23[c]	ND
		1.11 ± 0.04[e]	1.42 ± 0.06[e]	ND
		1.21 ± 0.06[f]	1.76 ± 0.04[f]	ND
PDPC				
	C_6-NBD-PC	3.70 ± 0.21	4.70 ± 0.29	11.1 ± 0.12
		3.54 ± 0.55[b]	4.93 ± 0.40[b]	18.1 ± 1.20[b]
		13.6 ± 0.20[c]	17.6 ± 0.35[c]	ND
POPS				
	C_6-NBD-PC	0.34 ± 0.01	0.49 ± 0.01	ND
	C_6-NBD-PE	0.42 ± 0.02	0.65 ± 0.02	ND
	C_6-NBD-PS	0.53 ± 0.01	0.63 ± 0.01	ND

[a] Results are the first order rate constants, k_{off} ($\times 10^{-2}$ s^{-1}), for the desorption of C_6-NBD-labeled phospholipids from donor phospholipid vesicles at 25 °C (pH 7.4, 150 mM NaCl) and are the mean of three independent measurements (±SD). [b] Donor vesicles included 25 mol % cholesterol. [c] 37 °C. [e] 1 M NaCl. [d] ND, not determined. [f] pH 4.0.

Measurement of the Local Dielectric Constant for C_6-NBD-PC. To determine the local dielectric constant at the region of the NBD fluorophore attached to PC in POPC bilayers a previously described method was used with adaptations (Epand & Leon, 1992). Briefly, emission spectra were obtained for NBD-hexanoic acid (3.75 μM) in a series of solvents of known dielectric constant (Handbook of Chemistry and Physics), from which a calibration graph relating dielectric constant to the emission maxima for NBD-hexanoic acid in each solvent was constructed (see results in Figure 5). The emission spectrum of C_6-NBD-PC in POPC bilayers was then recorded, and the emission maximum was used to obtain the local dielectric constant for the NBD fluorophore in the bilayer. The effect of the addition of ethanol on the dielectric constant was determined by measuring the shift in the fluorescence emission maxima as a function of ethanol concentration.

RESULTS

The rates of desorption of C_6-NBD-labeled phospholipids from bilayers with differing lipid compositions are shown in Table I. The factors contributing to the control of the desorption rate were separated by adopting the following strategy. Variations were made in the C_6-NBD-phospholipid class (i.e., using PC, PE and PS) to distinguish between head group interactions. The effect of cholesterol in the donor vesicles was determined, since it is known to have pronounced lipid ordering effects (Yeagle, 1985) and could therefore be used to test for effects mediated by altered lipid ordering. Also with cholesterol there was the possibility of hydrogen bonding with the carbonyl region of the phospholipid head group region. The effect of donor vesicle phospholipid *sn*-2 unsaturation was also determined. Lastly, by measuring the effect of ethanol in combination with these variables, its ability to perturb membrane surface hydrogen bonding was ascertained.

Variation of Phospholipid Class. A typical data set, showing the change of fluorescence intensity with time, due to the desorption of C_6-NBD-PC from POPC vesicles to BSA, is shown in Figure 1. From these data, a first-order rate

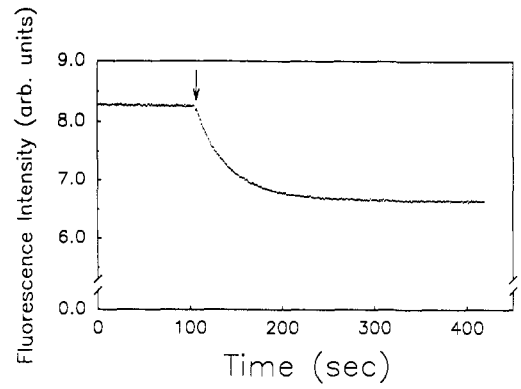

FIGURE 1: Example of a typical data set obtained for the desorption of C_6-NBD-PC from POPC initiated by the addition of BSA shown by the arrow. Details are as described under Experimental Procedures.

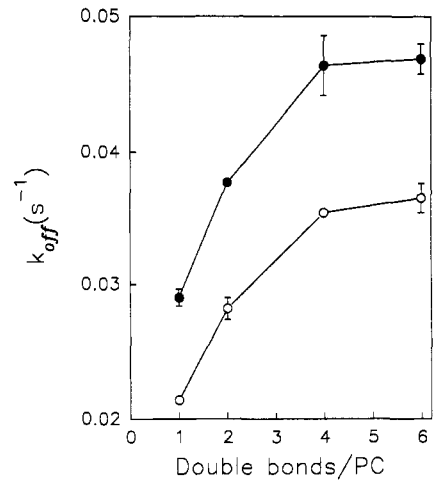

FIGURE 2: Effect of increasing the *sn*-2 unsaturation of PC donor vesicles on k_{off}, in the absence (open circles) and presence (filled circles) of ethanol. Vesicles were composed of POPC, EYPC, PAPC, and PDPC. EYPC was taken to contain an average of two double bonds/PC molecule (Smith & Jungalwala, 1981). Other details are described under Experimental Procedures.

constant, k_{off}, was obtained. This was obtained from the initial slope, since, as previously noted (Shin et al., 1991), the single-exponential fit deviated somewhat at longer times. The desorption rates of C_6-NBD-labeled PC, PE, and PS from POPC and POPS vesicles are shown in Table I. A dependence of the C_6-NBD-phospholipid desorption rate (k_{off}) from POPC donor vesicles on phospholipid head group type was revealed, following the trend C_6-NBD-PC > C_6-NBD-PS > C_6-NBD-PE. The much slower rate of desorption for C_6-NBD-PE as compared to C_6-NBD-PC has been shown previously with a different fluorophore label (Gardam et al., 1989) and was ascribed to direct interlipid hydrogen bonding. The free energy contribution of this hydrogen bonding, between C_6-NBD-PE and neighboring donor vesicle PC, was calculated from the ratio of the desorption rates of C_6-NBD-PE compared to that for C_6-NBD-PC from PC donor vesicles. This was estimated to be ~−700 cal/mol, which is in moderate agreement with that reported previously using a different system but similar approach (Gardam et al., 1990).

The value of k_{off} obtained for C_6-NBD-PS from POPC vesicles was found to be greater than that for C_6-NBD-PE, despite the potential for PS to participate in two direct interlipid hydrogen bonds, via its amine and carboxyl groups, although the effect may have been due to repulsion between the negatively charged carboxylate moieties. The rate of desorption of C_6-NBD-PS decreased on lowering the pH to 4, due to a decreased concentration of the deprotonated carboxylate form, suggesting that the faster rate of desorption of C_6-NBD-PS, compared to C_6-NBD-PE, may be due to repulsion between the deprotonated carboxylate groups (see Table I). Thus, the decrease in the rate may have been due to an increase in hydrogen bonding involving the protonated carboxylate form. In general, regarding the effect of ionic strength, while measurements were made at a moderate salt concentration (150 mM NaCl), some were performed at high salt concentrations (1 M), and a reduced rate of desorption was found (see Table I). However, even at this level the relative effects of elevated unsaturation and ethanol on the rate of desorption were maintained (see below), indicating that charge–charge interactions are not important in the effects of ethanol.

When POPS was used instead of POPC as the donor lipid, slower rates of desorption compared to POPC were found for each of the C_6-NBD-phospholipids. Again the rate of desorption of C_6-NBD-PS from POPS vesicles was greater than that for C_6-NBD-PE. The rates of desorption of C_6-NBD-PS from POPC were faster than the opposite case of C_6-NBD-PC from POPS. Since the strength of direct interlipid hydrogen bonds is a function of their rate of exchange (Boggs, 1987), which is proportional to the rate of head group motion and which is slower for PS, these results are consistent with the slower rate of motion of PS donor lipid head groups compared to that for PC donor lipids (Seelig & Seelig, 1980). From Table I it can be seen that increased temperature results in an increased rate of phospholipid desorption. The magnitude of the effect was close to that seen in similar systems [e.g., Nichols (1985), Fullington et al. (1990), and Jones and Thompson (1990)]; further, the activation energy for the desorption process, 18 kcal/mol as determined in this study, closely corresponds to that of 17 kcal/mol determined also for C_6-NBD-PC desorption (Nichols, 1985).

Effect of Cholesterol. It is known that cholesterol, a major component of lipid membrane bilayers, strongly increases lipid order and therefore van der Waals interactions between acyl chains in liquid crystalline phospholipid bilayers [see, for example, Ladbrooke et al. (1968) and the review by Yeagle (1985)] due to its rigid planar ring structure. The results revealed (Table I) that cholesterol (25 mol %), in either donor-POPC or PDPC vesicles, only resulted in marginal, if any, effects on the rate of NBD-phospholipid desorption.

Effect of Phospholipid Unsaturation. Increasing phospholipid unsaturation disorders the lipid bilayer [see, for example, Stubbs et al. (1981)] and also spaces the head groups [see, for example, Demel et al. (1972)], leading to an increased rate of head group (rotational) motion (Ghosh, 1988). To investigate the effect of varying unsaturation on membrane surface hydrogen bonding, the desorption rate of C_6-NBD-PC was measured from donor vesicles composed of PC of increasing *sn*-2 unsaturation. POPC, PAPC, and PDPC, which have 1, 4, and 6 cis double bonds/PC in the *sn*-2 acyl chain. The natural phospholipid mixture EYPC was also included, this having an average of two double bonds/PC, calculated from the fatty acid composition (Smith & Jungalwala, 1981). The results, shown in Figure 2, reveal a marked increase in the desorption rate of C_6-NBD-PC on increasing the level of *sn*-2 unsaturation.

Effect of Ethanol. The effect of ethanol was to increase the value of k_{off} for all C_6-NBD-phospholipid and donor vesicle phospholipid combinations tested, as shown in Table I. The effects of the ethanol were not affected by the nature of the acceptor, whether it was BSA or made from vesicles of the same lipid as the donor vesicles (see Experimental Procedures).

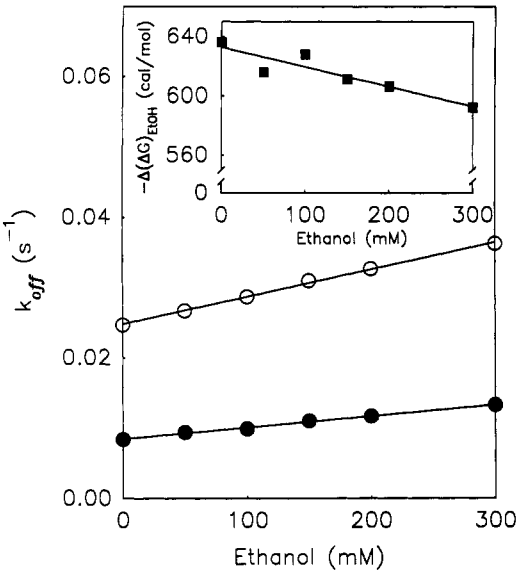

FIGURE 3: Effect of increasing ethanol concentration on values of k_{off} obtained for C_6-NBD-PC (open circles) and C_6-NBD-PE (filled circles) from POPC donor vesicles. (Inset) The same data replotted in terms of the free energy change occurring due to ethanol. Details are as described under Experimental Procedures. Free energies were calculated according to eq 2.

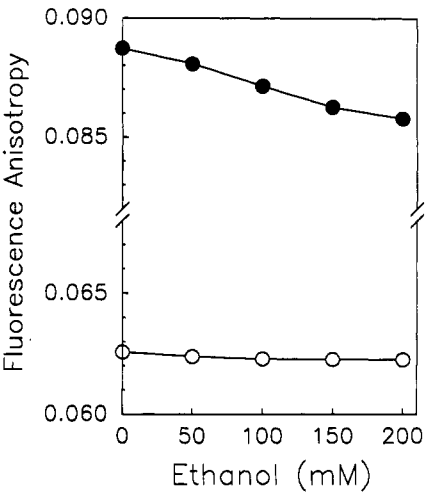

FIGURE 4: Effect of increasing ethanol concentration on the fluorescence anisotropy of DPH in POPC (filled circles) and in PDPC vesicles (open circles). Details are as described under Experimental Procedures.

The dependencies of the rates of desorption of C_6-NBD-PC and C_6-NBD-PE from POPC bilayers on ethanol concentration are shown in Figure 3. The sensitivity of the C_6-NBD-PC desorption rate to the effects of ethanol was approximately 2-fold greater than that found for the desorption of C_6-NBD-PE. This result is also presented in terms of the free energy change, calculated from the ratio of desorption rates obtained for C_6-NBD-PC and C_6-NBD-PE at each ethanol concentration (Figure 3, inset). A possible complication that needs to be considered is whether the location of the NBD-probe relative to the membrane surface is affected by ethanol, a result that would complicate data interpretation, especially in the dielectric constant experiment (see below). The fact that the NBD fluorophore, due to its relative polarity, locates as near to the membrane surface as possible, constained only by its attachment to the acyl chain from leaving the bilayer altogether, tends to argue against this, although such an effect cannot be ruled out. Ethanol by weakening head group interactions could conceiveably facilitate this to some extent, unless, as is likely, the acyl chain is already maximally constrained in allowing the NBD fluorophore access to the membrane surface.

In the presence of cholesterol, ethanol (and ethylamine, see also below) had the same effect on the desorption rates as observed in the absence of cholesterol. The effect of cholesterol (25 mol %) in POPC bilayers is to increase the fluorescence anisotropy of DPH by 0.078, indicating a relatively large increase lipid order. This compares with the much smaller *decrease* of 0.002 for 100 mM ethanol. Thus if the ethanol effects on desorption had been due to lipid order changes, then a marked reduction in the desorption rate should have occurred with cholesterol. The finding that this did not occur is evidence that lipid order changes did not influence the desorption rate and therefore to not contribute to the ethanol effect.

The increase in the desorption rate of C_6-NBD-PC from donor vesicles with increasing *sn*-2 unsaturation was found to be amplified in the presence of ethanol (Figure 2). If the amplification had been due to an increased ability of ethanol to disorder PDPC with increasing unsaturation, assuming that lipid order had an influence on the desorption rate of the C_6-NBD-phospholipids, then a greater effect of ethanol on the DPH fluorescence anisotropy would be expected with PDPC compared to POPC. On the contrary, the results shown in Figure 4 indicate that the magnitude of the disordering effect of ethanol is *greater* for POPC than for PDPC, which is in contrast to the *amplified* increase in the desorption rate induced by ethanol with increasing *sn*-2 unsaturation (Figure 2). Also the magnitude of the effects of ethanol on lipid order was small, compared with the effects on the desorption rate. These results again indicate that the increase in the desorption rate by ethanol may be unrelated to effects on lipid order.

To further distinguish between the perturbation of head group and van der Waals interactions by ethanol, the effect of ethylamine on the desorption rates of C_6-NBD-PC from POPC and PDPC was investigated. Ethylamine is cationic at neutral pH and unlike ethanol does not partition into the bilayer interior. Therefore it cannot directly affect hydrophobic interactions. To confirm this, the fluorescence anisotropy of DPH in POPC bilayers was determined in the presence of ethylamine and ethanol. While with 50 mM ethanol there was a distinct, although small, disordering effect, ethylamine at the same concentration had no such effect (results not shown). Compared to the effect of ethanol, ethylamine was found to have a much greater effect on the desorption rates for each of the C_6-NBD-phospholipid probes (see Table I), in agreement with its greater potency as a hydrogen bond donor. Again, the lack of an effect of ethylamine on lipid order, but pronounced effect on desorption, argues against effects on desorption being due to effects of ethanol on lipid order.

Ethanol Effects on Hydration Below the Head Group Region. Ethanol by intercalating between lipid head groups could increase the penetration of water into the lipid bilayer. To examine this possibility, the effect of ethanol on the dielectric constant at the locality of the bilayer where the NBD fluorophore residues was determined. This location has been shown to be at a depth in the bilayer corresponding to the glycerol backbone (Chattopadhyay & London, 1988). In the absence of ethanol, the dielectric constant was 38, taken from the calibration plot in Figure 5, whereas the value in the presence of ethanol was 44. Comparison of these values with the dielectric constant for pure ethanol of 24 (Handbook of Chemistry and Physics) suggested that on balance water was

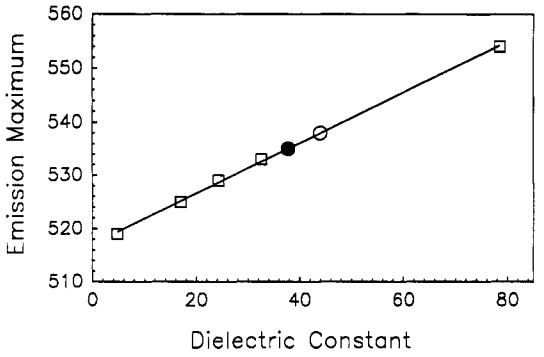

FIGURE 5: Calibration graph (open squares) relating the fluorescence emission maxima from spectra obtained for NBD-hexanoic acid in chloroform, butanol, ethanol, methanol, and water, respectively, in order of increasing dielectric constant. Also plotted are values obtained for C_6-NBD-PC in POPC bilayers in the presence (open circle) and absence (filled circle) of ethanol. Details are as described under Experimental Procedures.

increasing at the depth probed by the C_6-NBD-PC upon addition of ethanol.

DISCUSSION

Previous studies employing the lipid desorption technique have implicated hydrogen bonding as being an important force involved in the interaction of phospholipid head groups in lipid bilayers (Gardam et al., 1989, 1990; Shin et al., 1991). Such hydrogen bonding includes that occurring not only directly between adjacent lipids but also via the hydration layer. In the present study, the contribution of surface hydrogen bonding to lipid–lipid interactions was investigated with respect to the effects of cholesterol and *sn*-2 unsaturation and effects of the membrane perturbant ethanol. It was found that cholesterol had little effect on the rate of phospholipid desorption, in spite of its strong lipid-ordering ability, whereas perturbing effects of unsaturation and ethanol on lipid–lipid interactions involving hydrogen bonds were found, in particular those involving water.

The strength of any interlipid hydrogen bonding will be closely related to head group spacing, a function of cross-sectional molecular area, the rate of motion of the phospholipid head group, and potential influence from other regions of the membrane, as well as the presence external agents. Lipid head groups are free to undergo rotational motion, as governed by steric parameters and the strength of interlipid hydrogen bonding. Any weakening of interlipid hydrogen bonding will be reflected as a greater freedom of rotation of the head group leading to an increased rate of rotation, a property that therefore may be considered a useful diagnostic for a weakened hydrogen-bonding network (Ghosh, 1988). However, hydrogen bonds exchange extremely rapidly; therefore, for a single phospholipid, it may be more appropriate to consider the sum total or overall interaction with surrounding lipids, the strength of which is dependent on which direct and/or indirect hydrogen-bonding interactions are possible between the various phospholipid classes. For example, interactions between PC head groups are weaker than those between PE and PC, due the ability of the latter to form direct interlipid hydrogen bonds, as indicated by the observed slower desorption rate of C_6-NBD-PE compared to C_6-NBD-PC (Table I).

Evidence that PE can hydrogen bond with neighboring lipids arises from its slower rate of desorption compared to PC, as previously shown (Gardam et al., 1989; Gardam & Silvius, 1990; Shin et al., 1991). PS can also participate in direct interlipid hydrogen bonding, as illustrated by the generally slower desorption rates found for C_6-NBD-PS compared to C_6-NBD-PC (see Table I). However, the greater values of k_{off} obtained for C_6-NBD-PS, compared to C_6-NBD-PE, suggest that interlipid hydrogen bonds are weaker in the former. This behavior of C_6-NBD-PS may be due to electrostatic repulsions between carboxylate moieties of the PS and charges on neighboring head groups, which would have the effect of increasing head group spacing (Boggs, 1987) and therefore head group motional rate. This could potentially lead to the inhibition of both direct interlipid and indirect hydrogen bond formation via the hydration layer.

Cholesterol, apart from being a major membrane lipid component, is of interest for two reasons. First, it profoundly orders the hydrocarbon region of the membrane; secondly, it has the potential to hydrogen bond between the 3-β-hydroxyl group and an adjacent *sn*-1 carbonyl group. Previous studies have shown that cholesterol has negligible effect on the head group motional rates of phospholipids in the liquid crystalline phase (Ghosh, 1988; Milburn & Jeffrey, 1989; Han & Gross, 1991). Further, head group motion has been shown to be independent of acyl chain motional parameters (Milburn & Jeffrey, 1989). This could explain why the ordering induced by cholesterol presence had no effect on the rate of phospholipid desorption, if the latter effect is confined to the head group region. The implications from this would be that effects on lipid order in general are also unlikely to influence the rate of desorption of phospholipids. It follows that lipid order does not influence hydrogen bonding at the head group region although lipid order is a long-range effect and desorption is governed by local interactions. It may therefore be more correct to consider that it is the increase in van der Waals interactions between adjacent acyl chains, induced by the condensing effect of cholesterol (reflected by greater lipid order), that lacks influence on hydrogen bonding at the head group region. Another factor which must be considered is that cholesterol does itself occupy an appreciable area in the plane of the membrane, an effect that would tend to decrease dipole potential and possibly orient interfacial water. It would appear, however, that these effects on head group interactions, as reflected by the desorption measurements, are at best rather small. The lack of influence of cholesterol on phospholipid desorption would also imply that direct hydrogen bonding involving the hydroxyl group must at best be weak and of comparable strength to that in the hydration layer involving hydrogen-bonded water between phospholipid head groups. This is supported by the finding that cholesterol has been noted to have very little effect on membrane dipole potential or interbilayer hydration pressure (McIntosh et al., 1989).

Increasing the level of *sn*-2 unsaturation in PC increased the rate of phospholipid desorption. Lipid order is decreased with increased unsaturation. As shown, however, from the results with cholesterol and ethylamine, it would appear that this may not be responsible for the effect. The other effect of increasing lipid unsaturation is to decrease the strength of interactions between head groups due to the concomitant increase in head group spacing. From the above, this would appear to be sufficient to weaken interactions that occur via hydrogen-bonded water in the hydration layer. This being so, one would expect that the rate of head group rotation would also have increased. Very little has been reported on this subject; however, recently monounsaturated POPC and the diunsaturated DOPC were compared by ^{31}P NMR (Ghosh, 1988), the latter being found to have a greater rate of head group rotation in keeping with this argument.

Addition of ethanol increased the rate of phospholipid desorption in all instances examined. However, the increase was much greater for the desorption of PC from donor PC compared to situations in which PE or PS was involved. This would suggest that the contribution to the strength of interlipid interactions of direct hydrogen bonding is too strong for ethanol to have a significant effect. This observation is reasonable on thermodynamic grounds, since the value of the free energy of solvation of ethanol in water of −760 cal/mol (Tanford, 1980), being similar to the free energy of direct interlipid hydrogen bonding between C_6-NBD-PE and PC of ∼−700 cal/mol (calculated by comparison of the desorption rate of C_6-NBD-PE with that for C_6-NBD-PC; see Experimental Procedures), indicates this to be an energetically unfavorable process. By contrast, the desorption of C_6-NBD-PE, and C_6-NBD-PS from POPC was more susceptible to ethylamine compared to ethanol, suggesting that direct interlipid hydrogen bonding is more susceptible to this agent. This may be partly due to the positive charge carried by ethylamine promoting a location in the head group region. Also PE (and potentially PS) forms interlipid hydrogen bonds via the amine moiety (Boggs, 1987), which may be similar in strength and geometry to those between lipid and ethylamine. Again, according to the above arguments, the modest lipid disordering effect of ethanol and the lack of effect of ethylamine on lipid order would suggest that effects on lipid order are not involved in the process.

It was found that there was a reduction in the desorption rate of NBD-phospholipids with increasing ionic strength, an effect that has been previously observed (De Cuyper et al., 1983; Jones & Thompson, 1990), which was suggested to arise from less favorable water–lipid interactions inducing a decreased aqueous phase solubility of lipid monomers, a "salting out" effect, as also previously discussed (Tanford, 1980). The observation that effects of ethanol on the phospholipid desorption rate were independent of ionic strength indicates that these effects were not important in the mechanism of action of ethanol.

The increase in the desorption rate induced by ethanol could have involved a perturbation of hydrogen bonding in the hydration layer. When donor vesicle phospholipid *sn*-2 unsaturation was increased, the magnitude of increased rate of desorption by ethanol was amplified. One possibility might have been that ethanol was disordering the more unsaturated bilayer to an increased extent, thereby increasing the rate of desorption to a greater degree. However, this could be discounted, since, as shown in Figure 3, with increased unsaturation there was a *decreased* lipid disordering ability. The amplified ethanol effect with increased unsaturation could be due to increased head group spacing [see, for example, Demel et al. (1972)], weakening hydrogen bonding that occurs via the hydration layer, making it more susceptible to ethanol perturbation. Thus it would appear that ethanol acts by effectively competing for, and therefore displacing waters from, the hydrogen-bonded network of waters in the hydration layer, resulting in a reduction in its contribution to lipid–lipid interactions.

If the effect of ethanol is to weaken the hydrogen bonding in the hydration layer, spacing the lipid head groups, then as a consequence an increased water and ethanol penetration into the bilayer interior should accrue. The observation that the dielectric constant of the C_6-NBD fluorophore is greater than that for ethanol, while the former *increases* upon addition of ethanol to the bilayer, indicates that, on balance, the amount of water in the environment of the NBD fluorophore, rather than ethanol itself, increases as a result of ethanol addition. Since the location of the probe has been determined to be approximately 0.5 nm below the first methylene unit of the *sn*-2 acyl chain (Chattopadhyay & London, 1988), this indicates that ethanol increases the water penetration at this depth in the bilayer. Whether this water forms a continuum with the water in the hydration layer is uncertain, although this seems likely.

In summary, these studies provide evidence suggesting the importance of hydrogen bonding between phospholipids, which occur via the hydration layer. The contribution of this hydrogen bonding to overall interlipid interactions is influenced by phospholipid unsaturation, and therefore increasing head group spacing, and also by the presence of ethanol. That lipid order was not involved was shown by the lack of effect on desorption of cholesterol, which orders lipids, the considerable effect of ethylamine, which has no effect on lipid order, and the greater effect of ethanol on the desorption rate in PDPC, compared to POPC, in spite of the larger disordering effect of ethanol on the latter. The magnitude of the ethanol effect varies according to the phospholipid class and its level of unsaturation. This indicates that any compositionally distinct regions in cell membranes may be differently susceptible to ethanol perturbation of surface hydrogen bonding. These results may have important implications for the effect of ethanol on cell membranes and therefore on cell functioning. If these effects are also manifest at the protein–lipid interface, and there is no reason to suspect the contrary, then the ethanol-induced perturbation of hydrogen bonds could significantly alter the conformation and hence function of many membrane proteins, including receptors, ion channels, etc. Also, ethanol may affect the process of protein insertion into membranes by modifying hydrogen bonding. At this stage, the relationship between the perturbation of hydrogen bonding in the hydration layer and protein function is unknown, and it is toward this end that our present studies are being directed.

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