Journal of Fluorescence, Vol. 5, No. 1, 1995

Fluorescence Techniques for Probing Water Penetration into Lipid Bilayers

Christopher D. Stubbs,[1] Cojen Ho,[1] and Simon J. Slater[1]

Received September 27, 1994; accepted September 27, 1994

Fluorescence spectroscopy can be used as a highly sensitive and localized probe for hydration in lipid bilayers. Water associates with the head-group region, where it participates in an interlipid network of hydrogen bonds. Deeper in the bilayer, water is contained within acyl-chain packing defects. Fluorescence methodology is available to probe both the interstitial and head-group hydration in lipid bilayers, and results are in good agreement with other techniques. Using fluorescence spectroscopic approaches, cholesterol is shown to dehydrate the acyl-chain region, while hydrating the head-group region. Membrane proteins appear to increase acyl-chain hydration at the protein–lipid interface. Overall fluorescence spectroscopic techniques may be most effective in studying the water content of lipid bilayers and especially of biological membranes.

KEY WORDS: Fluorescence; hydration; DPH; lipid bilayer; fluorescence lifetime.

INTRODUCTION

Water has a primary role in the formation and maintenance of cell membrane architecture. The potential in allosteric regulation by a "perturbed water layer" around proteins and lipid bilayers was discussed in a recent article [1], where evidence was presented that such water may play an important part in hemoglobin structure/function [2] and lipid bilayers (reviewed, e.g., in Ref. 3). It has been stated that hydration in membranes "provides a fast and inexpensive regulatory mechanism for lipid membranes to adapt their characteristics, at least locally and transiently, to new requirements" [4].

A number of studies have established that water hydrogen bonds directly to phospholipids and participates in the lipid bilayer structure (see, e.g., Refs. 4–10). These water molecules within the head-group region participate in a hydrogen-bonded network that extends between the lipids that has been termed the "hydration layer" [11,12]. This hydration layer arises from a "bound" hydration shell of 9–20 waters per phospholipid [3]. 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. Direct interlipid hydrogen bonding will also occur between some lipids, for example, between the NH_3^+ of PE and a phosphate oxygen of another lipid [13].

Considerable insight into the structural role of water in membranes has come from a series of studies on the properties of water that exist between two closely apposing bilayers (see, e.g., Refs. 1, 3, and 14–18) that are the key to understanding the features of the hydration layer. For instance, a "hydration pressure" exists which opposes the approach of bilayers, which is proposed, from theoretical analyses, to be due to the polarization and orientation of the interbilayer water (e.g., Ref. 19) due to the head-group dipoles.

The lipid bilayer of cell membranes contains water not only within the lipid head-group region, but also between the fatty-acyl chains as "interstitial hydration" [20–25]; see Fig. 1. This means that overall, as the dis-

[1] Department of Pathology and Cell Biology, Thomas Jefferson University, Philadelphia, Pennsylvania 19107.

1053-0509/95/0300-0019$07.50/0

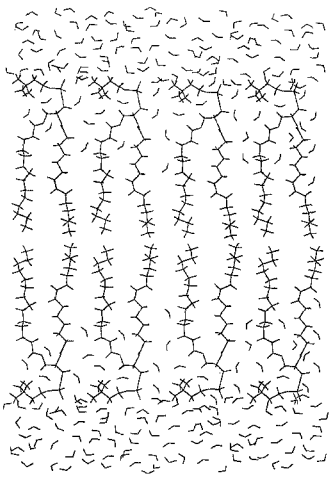

Fig. 1. Diagram of a lipid bilayer showing water molecules at the membrane surface and within both the head-group and acyl-chain regions.

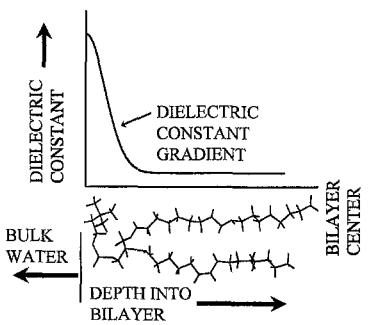

Fig. 2. Diagram illustrating the dielectric constant gradient.

tance from the surface increases, the water content decreases, following a so-called "dielectric constant gradient" from a dielectric constant at the membrane surface of ~70 to ~5 at the bilayer center [26,27]. The steepness of the gradient depends on the degree and depth of the water penetration (see Fig. 2) and is at its steepest at the head-group region, where only a small shift of the gradient may potentially result in a large change in the dielectric constant. This could be sensed by an adjacent portion of a membrane protein and be translated into a functional effect.

Recent studies have shown that the introduction of small peptides, consisting of three amino acids, can cause a shift of water deeper into the bilayer [28]. However, the possibility of hydration at the protein–lipid hydrophobic interface of transbilayer membrane proteins has hardly been explored, although the possibility of water occurring at the relatively hydrophobic surfaces of proteins is not entirely unknown [29]. Recently, using fluorescence spectroscopic approaches, it was shown that water may indeed exist at the membrane protein–lipid interface [30].

Much of the basic properties of the hydration layer are not yet understood. For instance, it is not known whether the extended hydrogen-bonded network extends down into the bilayer to include interstitial water, i.e., water trapped in acyl-chain packing defects, or whether it even extends right across the bilayer. The effects of varied phospholipid unsaturation have also been hardly explored and the details of the relationship of lipid order, acyl-chain packing properties, and hydration are still only vaguely understood. The hydration layer obviously contributes significantly to the stability of the bilayer and to the energetics of lipid–lipid interactions. It is therefore axiomatic that altered hydration properties are going to have a significant influence on protein–lipid interactions and therefore on protein function. Further, evidence that membrane perturbation, for instance, by alcohols and anesthetics, may proceed by a disruption of hydration is steadily accumulating (see reviews in Refs. 31–33). However, many of the details of these interesting possibilities remain to be worked out.

There are a number of techniques that have been used to reveal the presence and properties of water within the lipid bilayer. Neutron diffraction has been used for a number of years and remains a powerful technique, since it provides a profile of the degree of water penetration across the bilayer (see, e.g., Refs. 21 and 28). The disadvantages of the technique are the requirement of a neutron source, long data acquisition times, and the poor stability of the thin films of lipids that are required for the experiments. Information has also been obtained by electron spin resonance and electrical capacitance techniques [20,22–25,34]. Several methods have been used to determine directly the number of water molecules that are associated with a single phospholipid; for example, Sen and Hui [35] published a method using inverted micelles in hexane and radiolabeled water, although there is the drawback that usually these approaches cannot be performed with a bilayer. It is also possible to obtain information on the penetration of water into lipid bilayers using time-resolved fluorescence spectroscopy [27,36–42], and lipid-fluorophore desorption techniques [43]. While the interpretation of the fluorescence spectroscopic approaches relies heavily on the information provided by other approaches, there is the great advantage that measurements can be performed rapidly on small amounts of material and therefore on biological samples. The following sections discuss fluorescent techniques for the study of hydration in lipid bilayers, except for the use of Laurdan, which is dis-

cussed by T. Parasassi and E. Gratton elsewhere in this issue.

EFFECTS ON FLUOROPHORE INTENSITY/ EMISSION MAXIMA

The excited-state lifetime is shorter for an increased content of water in the excited-state solvent cage through collisional quenching. Thus a shorter fluorescence lifetime or lower fluorescence intensity is a useful indicator of increased water in the region where the fluorophore resides. In general, fluorescence approaches to the study of hydration depend on this effect and have suffered from the disadvantage that other factors besides water may contribute to a decrease in the fluorescence lifetime/ intensity. With some limitations, only the deuterium isotope exchange approach enables the presence of water to be inferred with any reliance (see below). Other fluorescence approaches require at least some initial knowledge of the system, or require that other confirmatory techniques must be used.

The excited-state lifetime, and therefore the observed fluorescence emission maxima of a fluorophore, are a function of the molecular polarizability of the solvent cage and therefore of its dielectric constant. Thus the dielectric constant can be used as an index of the activity of water in the solvation shell of the fluorophore, from which the relative degree of hydration in the locality of interest may be evaluated. To assess the dielectric constant of a region in the bilayer, a fluorophore is first selected that is known to locate in this locality, for example, at the head-group region. The emission maxima of the fluorophore is then determined for a range of organic solvents for which the dielectric constant is known (from standard tables). A plot is then constructed of dielectric constant against emission maxima. The emission maxima for the fluorophore in the membrane of interest can then be determined and the dielectric constant of the fluorophore location then read from the calibration plot. This approach has been used in a number of laboratories (e.g., see Refs. 43 and 44 and see article by R. M. Epand in this issue).

The main advantage of the above method is that it is relatively straightforward to obtain the dielectric constant/emission plots and to determine the value for the membrane of interest. It suffers from two problems. First, it is often not possible to know with the required degree of certainty the exact location of a fluorophore in the membrane. Second, it is important that the fluorophore is in a monomeric form in the organic solvents used to set up the calibration plot. Thus some phospholipid-labeled fluorophores may form inverted micelles in organic solvents, and the emission spectra of the fluorophore will then not be solely governed by the solvent, but by features of the inverted micelle structure, invalidating the approach. However, this can be circumvented if a portion of the fluorophore-labeled phospholipid is used. For example, for dansyl-labeled-PE, dansyl lysine could be used, and for NBD-labeled phospholipids, a short-chain NBD-fatty acid might be used. However, micellar aggregation may still be a problem with this approach. Another limitation is that the dielectric constant in the head-group region is anisotropic, due to the alignment of water dipoles, whereas in the solvent this will not be the case, so the method is somewhat semiempirical.

Recently the above approach was used in a study of the effect of ethanol on lipid bilayers [43]. A question that is difficult to resolve with ethanol and similar hydrophobic perturbants is whether hydration is increased or decreased as a result of the presence of these agents in the membrane. For instance, ethanol, by intercalating between lipid head groups, could increase the amount of interstitial water present in the lipid bilayer, or ethanol itself may enter this region with the amount of water remaining constant, or even decreasing. To distinguish between these possibilities, the effect of ethanol on the dielectric constant at the locality of the bilayer where the NBD fluorophore resides was determined. This location has been shown to be at a depth in the bilayer corresponding to the glycerol backbone [45]. In the absence of ethanol, the dielectric constant from a calibration plot [43] was 38, whereas the value in the presence of ethanol was 44. Comparison of these values with the dielectric constant for pure ethanol of 24 [46] suggested that, on balance, water rather than ethanol was increasing at the depth probed by the C_6-NBD-PC (*1*-palmitoyl-*2*-*N*-(7-nitrobenzo-2-oxa-1,3-diazole-4-yl)aminohexanoyl-PC) upon addition of ethanol, the main effect of which may have been at the surface, in terms of a head-group spacing effect. Thus if ethanol had entered into the locality of the NBD fluorophore or if the water content had decreased, then the dielectric constant should have decreased.

DEUTERIUM ISOTOPE EXCHANGE

This method is based on a solvent isotope effect on the fluorescence quantum yield, and is especially useful in the study of hydration in the locality of the fluorophore [47,48]. The basic principle is that when H_2O is replaced by D_2O it reduces the nonradiative decay rate

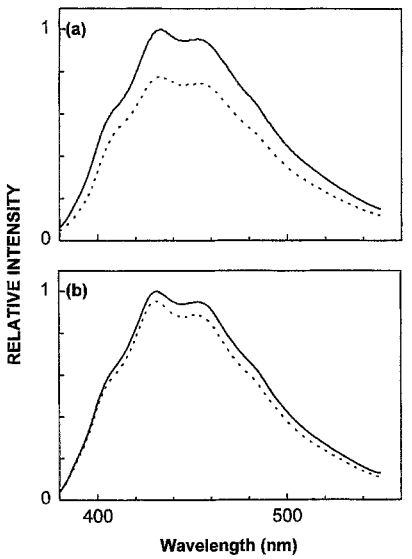

Fig. 3. Fluorescence emission spectra for TMA-DPH in *1*-palmitoyl-2 oleoyl-PC (POPC) bilayers, with and without cholesterol in either D_2O or H_2O buffers. (a) POPC/gramicidin in D_2O (—) or H_2O (···) buffer; (b) with cholesterol. Note that in D_2O the fluorescence emission is enhanced compared to H_2O, showing that the fluorophore is accessible to water. (from Ref. 30, with permission.)

(i.e., proton transfer) of the fluorophore during the excited-state lifetime and consequently increases the fluorescence quantum yield. Thus if the D_2O:H_2O fluorescence intensity ratio is greater than unity, then the fluorophore is in contact with water. On the contrary, if the fluorophore is shielded from water, then the ratio should equal one. This method conveniently allows routine measurements of water penetration into [30] or across the lipid bilayer [49]. The fluorophore should have an exchangeable proton; examples include aminonaphthalene trisulfonic acid (ANTS), dimethyl-amino-naphthalenesulfonate (dansyl), and trimethylammonium-diphenylhexatriene (TMA-DPH) and tryptophan. Using the intrinsic tryptophan of a protein offers an approach for examining the question of hydration at the protein–lipid interface [30] or in other regions of membrane proteins, although the presence of large numbers of tryptophan residues in membranes limits the usefulness of this approach except for defined model systems.

An example spectrum is shown in Fig. 3 showing the dehydrating effects of cholesterol. Using this approach, it was possible to determine the D_2O/H_2O fluorescence intensity ratio for a variety of phospholipid compositions, as shown in Table I. The trend for increased hydration, shown using TMA-DPH, follows the DPH-PC lifetime data, as discussed below. Again protein is shown to hydrate the protein–lipid interfacial region, indicating that the protein introduced more water into the locality probed by the TMA-DPH. The gramicidin tryptophans are known to locate near the lipid head-group region and are in contact with the surrounding lipids [50,51]. Again the D_2O:H_2O fluorescence intensity ratio was greater than unity, indicative of water in this region, i.e., at the protein–lipid interface.

FLUORESCENCE LIFETIME AND LIFETIME DISTRIBUTIONS

The fluorescence lifetime of a suitably placed fluorophore can be a useful indicator of the relative amount of water in the region of the membrane in question. DPH is an example of an environmentally sensitive fluorophore, and it has been used as such to explore the water content of lipid bilayers and membranes [26,27,30,39]. As with other fluorescence approaches, it is useful to have some starting knowledge of the system. For instance, it is known that cholesterol reduces the water content of lipid bilayers (e.g., see Ref. 22). Thus the modulatory effect of other lipids on the ability of cholesterol to dehydrate the phospholipid acyl-chain region could be investigated in terms of the effects on the fluorescence lifetime of a suitably located fluorophore in the acyl-chain region.

It is important not to disregard the fundamental value of the fluorescence lifetime center in considering the water content of a lipid bilayer. Thus fluorophores at *fixed* positions across the bilayer, while not probing across the dielectric constant gradient, can still be used to reveal the varied hydration at the position in question without recourse to fluorescence lifetime distributional analysis. This approach has been used in the study of cholesterol and phosphatidylcholine unsaturation, for instance, using DPH-type fluorophores [52,53]. It may be possible to "map" hydration effects by the use of a range of fluorophores considering only the fluorescence lifetime center. The decrease of fluorescence lifetime of DPH with increased unsaturation [52,53] and its increase with cholesterol (see, e.g., Refs. 30, 53, and 54) would appear to be due mostly to the altered level of interstitial or interchain water. Table II lists data from this laboratory showing the lifetime of DPH-PC in phospholipid bilayers of varying composition. For DPH-PC, the DPH is tethered to the *sn-2* position of PC so that its location is precisely known. It can be seen that with increased unsaturation or protein addition, the lifetime decreases. Cholesterol, by contrast, increases the lifetime of DPH-PC, in keeping with its known bilayer acyl- chain de-

Table I. Fluorescence Intensity Ratios (D_2O/H_2O)[a] for TMA-DPH and dansyl-PE in Vesicles of Varying Lipid Composition[b]

	TMA-DPH[c]	dansyl-PE[d]
1-palmitoyl-*2*-oleoyl-PC	1.14 ± 0.01[e]	1.15[f]
1-palmitoyl-*2*-linoleoyl-PC	—	1.19[f]
1-palmitoyl-*2*-arachidonoyl-PC	1.19 ± 0.02[e]	1.20[f]
1-palmitoyl-*2*-docosahexaenoyl-PC	1.23 ± 0.02[e]	1.23[f]
dioleoyl-PC	1.25[f]	1.19[f]
dilinoleoyl-PC	1.27[f]	1.22[f]
dilinolenoyl-PC	1.35[f]	1.23[f]
1-palmitoyl-*2*-oleoyl-PC + cholesterol (25%)	1.05 ± 0.02[e]	1.19[f]
1-palmitoyl-*2*-oleoyl-PC + gramicidin (10%)	1.23[e]	—
1-palmitoyl-*2*-arachidonoyl-PC + gramicidin (10%)	1.26[f]	—
1-palmitoyl-*2*-docosahexaenoyl-PC + gramicidin (10%)	1.29[f]	—
1-palmitoyl-*2*-oleoyl-PC + brain-PS (10%)	1.16 ± 0.03[e]	—
1-palmitoyl-*2*-oleoyl-PC + brain-PS (10%) + apocytochrome C (10%)	1.15 ± 0.05[e]	—

[a]The fluorescence intensities were separately determined in D_2O and H_2O buffers.
[b]All data were collected at 37°C.
[c]Trimethyl-ammonium DPH.
[d]6-dimethylaminonaphthalene sulfonate phosphatidylethanolamine.
[e]Ref. [30].
[f]Unpublished data.

Table II. Fluorescence Lifetime[a] of DPH-PC in Vesicles of Varying Lipid Composition[b]

	τ(-protein)	τ(+protein)[c]
1-palmitoyl-*2*-oleoyl-PC	7.41 ± 0.04[d]	7.21 ± 0.04[d,e]
1-palmitoyl-*2*-linoleoyl-PC	7.33 ± 0.08[g]	—
1-palmitoyl-*2*-arachidonoyl-PC	7.23 ± 0.04[h]	—
1-palmitoyl-*2*-docosahexaenoyl-PC	7.08 ± 0.04[h]	—
dioleoyl-PC	6.75 ± 0.06[g]	—
dilinoleoyl-PC	6.67 ± 0.08[g]	—
dilinolenoyl-PC	6.52 ± 0.05[g]	—
1-palmitoyl-*2*-oleoyl-PC + cholesterol (25%)	8.15 ± 0.13[d]	7.57 ± 0.10[d]
1-palmitoyl-*2*-oleoyl-PC + *1*-palmitoyl-*2*-oleoyl-PE (40%)	7.47[g]	—
1-palmitoyl-*2*-oleoyl-PC + brain-PS (10%)	7.29 ± 0.19[h]	7.02[h,i]
1-palmitoyl-*2*-arachidonoyl-PC + brain-PS (10%)	7.05[h]	6.89[h,i]
1-palmitoyl-*2*-docosahexaenoyl-PC + brain-PS (10%)	6.79[h]	6.48[h,i]

[a]The major fluorescence lifetime center (τ, sec. × 10^{-9}) from biexponential or bimodal Lorentzian distributional analyses.
[b]All data were collected at 37°C.
[c]Protein:phospholipid = 1:10 (molar).
[d]Ref [30].
[e]Gramicidin.
[g]Unpublished data.
[h]Ref [42].
[i]Apocytochrome C.

hydrating property. When the probe is at the head-group region, for example, for dansyl-PE, the effect of increasing unsaturation again is to decrease the fluorescence lifetime; however, cholesterol now also decreases the lifetime, indicating that its small "head-group" volume cannot completely "fill" the space available. Interestingly, a plot of lipid order, determined from time-resolved anisotropy measurements also with DPH-PC, when plotted as a function of the fluorophore lifetime, shows a strong linear relationship over a broad range of lipids, which includes phosphatidylcholine with varied *sn-2* unsaturation or di-(*sn-1,2*) unsaturation, PE, and

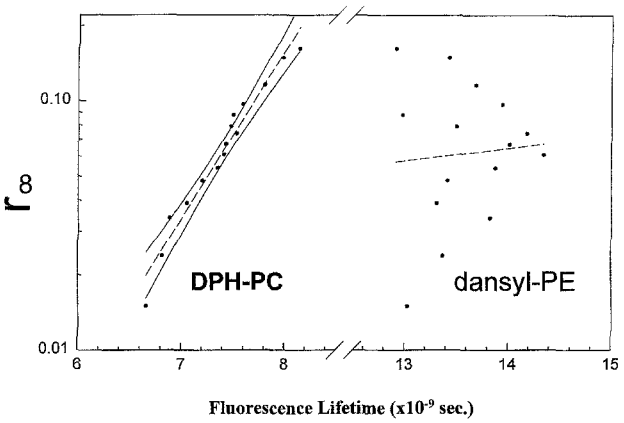

Fig. 4. Relationship of lipid order with bilayer hydration. The plot on the left shows that there is a strong correlation between the order determined from time-resolved fluorescence anisotropy data (r_∞) for DPH-PC and the major lifetime center for DPH-PC, the latter being sensitive to interstitial hydration (dashed line is correlation, 0.96; solid lines show 95% confidence limits; data for a wide range of lipid bilayer compositions, including varying *sn-2* unsaturation, *sn-1,2* diunsaturation, and PE and cholesterol content, taken from published and unpublished measurements). By contrast, the right plot shows that the fluorescence lifetime of dansyl-PE, reporting on head-group hydration, does not correlate with lipid order, again from DPH-PC data.

cholesterol (see Fig. 4). By contrast, when the fluorophore (in this case dansyl attached to PE) is located at the lipid head-group region, the correlation between hydration (now at the head-group region) and lipid order is no longer seen (see Fig. 4). This suggests that hydration at the lipid head-group region is a function of hydrogen bonding and is strongly orientation dependent. By contrast, interstitial hydration appears to be a function of acyl-chain order.

If an ensemble of fluorophores locates at various depths into the lipid bilayer, then individual fluorophores will locate and decay from the excited state with a rate dependent on the depth, i.e., on the dielectric constant. Thus a range of decay rates will accrue. A faster decay rate will result from a fluorophore located more toward the bilayer surface, where the water content is higher and a slower decay rate will result nearer to the bilayer center. Analysis of the fluorescence decay as a discrete or (single) exponential process fails to reveal this type of information, termed fluorophore "environmental heterogeneity." A number of laboratories have recently explored alternative methods of analysis of the decay of the excited state allowing investigation of environmental heterogeneity [27,36–40,55–65].

Several types of fluorophore have been used to explore environmental heterogeneity in lipid bilayers, although DPH has been the probe of choice. DPH and TMA-DPH detect environmental heterogeneity by sampling ("vertically") across restricted regions of the dielectric constant gradient and laterally in the bilayer, whereas DPH-PC only samples lateral heterogeneity, since it cannot vertically sample [37,38,42]. The latter property makes it useful in the study of environmental heterogeneity introduced by proteins in natural membranes since it has been shown that environmental heterogeneity, detected by DPH-PC, can be entirely attributed to the protein–lipid interface, whereas DPH detects heterogeneity, both from the dielectric constant gradient and the protein–lipid interface [37,38,42]. Regarding analysis of the fluorescence decay in environmental heterogeneous systems, continuous fluorescence lifetime distributions, e.g., Gaussian or Lorentzian, are commonly employed [66]. The *full-width at half-maximum* of the distribution will then relate to the *diversity* of fluorophore environments. This can then be used as a tool to explore environmental heterogeneity in membranes. This will occur provided the DPH probes across the dielectric constant gradient, *within the excited-state lifetime*, as demonstrated [27]. However, the factors governing the *width* of the distribution are complex and interrelated [39], and must be carefully considered if a meaningful interpretation of data is to be achieved. These include the rate of exchange of the fluorophore solvent cage (basically the rate of lipid motion), the intrinsic fluorescence lifetime (dependent on the molecular properties of the fluorophore itself), the location in the membrane together with shape and charge factors, etc.

Using the fluorescence lifetime distribution approach, it was first shown that, while above the gel–liquid phase-transition temperature (T_C) (e.g., for dipalmitoylphosphatidylcholine) the fluorescence lifetime distributional width was ~0, below T_C a width was found indicating a range of decay rate [27]. In this case, the environmental heterogeneity below T_C may be due to the slower rate of exchange of the fluorophore excited-state solvent cage (slower rate of lipid motion), packing defects in the gel-phase lipids, and an altered fluorophore location. We have studied bilayers of single species of phosphatidylcholine with varying *sn-2 cis* unsaturation and again found that DPH reports environmental homogeneity above T_C [37]. However, in *mixed*-PC species bilayers, such as egg-PC, even above T_C, analysis of the decay as a fluorescence lifetime distribution revealed distributional width indicative of environmental heterogeneity [39]. This shows that the acyl chains must be sufficiently mismatched to introduce packing defects in the bilayer in which water molecules may reside. Thus, increased acyl-chain unsaturation tends to increase "in-

terstitial water'' and/or it moves the dielectric constant gradient deeper into the bilayer.

In the case of cholesterol, the effect is to increase the fluorescence lifetime [30,54], and to reduce the *range* of decay rates available to a fluorophore such as DPH [30,57,65,67]. The increased lifetime may be attributed to the cholesterol rigid planar structure ''straightening'' the acyl chains, reducing the interstitial water trapped in packing defects. The reduced water in the excited-state solvent cage of the fluorophore will then lengthen its excited-state lifetime. This effect, and the more ordered packing reducing the ability of the DPH to ''sample'' nearer the head-group region, would effectively reduce the range of dielectric constant probed and result in a reduced fluorescence lifetime distributional width.

The effect of membrane proteins on the fluorescence lifetime and fluorescence lifetime distribution in lipid bilayers has been documented in a number of studies from this laboratory [30,36–39,42]. Basically the effect is to reduce the fluorescence lifetime center and to broaden the distributional width. The former effect could be attributed to the presence of water at the protein–lipid interface, as shown elsewhere [30]. The latter effect would appear to be due to the greater diversity of fluorophore environments available at the protein–lipid interface as compared to the lipid bilayer without protein. However, cholesterol, while narrowing the distributional width of DPH in lipid bilayers without protein, has the opposite effect in the presence of a protein [37]. In bilayers of single species of phosphatidylcholine with varying *sn-2* unsaturation, the fluorescence decay of DPH-PC is essentially homogeneous. However, in the presence of a model membrane protein we have recently shown that a distributional width appears (i.e., environmental heterogeneity increases) and, further, that it increases with unsaturation. This may be due to the fact that although unsaturation increases packing defects into the bilayer that accommodate water, DPH-PC, being unable to sample across the dielectric constant gradient, reports a homogeneous environment. However, with a protein it would appear that the heterogeneous surface may, together with the unsaturated acyl chains, introduce *lateral* heterogeneity, an effect that would be detected as a range of decay rates for DPH-PC, as found [38,42]. The protein effect on fluorescence lifetime heterogeneity could originate from at least three different causes: the direct protein–lipid or protein–fluorophore contact, probe rotational heterogeneity, and water penetration (fluorophore detects a range of dielectric constants). Several model proteins, including gramicidin, apocytochrome C, cytochrome b_5, and bacterhodopsin [30,36–38,42], have been shown to introduce environmental heterogeneity into the lipid bilayer, as detected by the appearance of a broad distributional width for DPH or DPH-PC. Also, when membranes from rat-liver microsomes were prepared with DPH-PC incorporated, a considerably broadened distributional width was recovered which was not present in vesicles of total extracted lipids [37], again showing that environmental heterogeneity arises from the protein. Interestingly, compared to model systems with a single protein, there was a greater environmental heterogeneity (for DPH-PC) in microsomal membranes [37], an effect probably due to the large variety of multi-spanning proteins resulting in multiple fluorophore environments.

THE PHOSPHOLIPID DESORPTION TECHNIQUE

Hydrogen bonds exchange relatively rapidly, and in a lipid bilayer it is appropriate to consider the overall interactions between a lipid and neighboring molecules. This will be dependent on the lipid class distribution, since, for example, interactions between PC head groups are weaker than those between PE or PS, due the ability of the latter to form direct interlipid hydrogen bonds [13]. For PC, only water–water and lipid–water hydrogen bonding via the associated hydration layer is possible. Hydrogen bonding is ''directional'' and is a function of both the spatial orientation and the potentials of the participating donor/acceptor dipoles. Thus, hydrogen bonding will be a function of both the spacing of head groups, a static quantity, and dynamic contributions such as the rate of segmental motion of the phospholipid dipoles [68–70]. A greater rate of segmental motion of the lipid head groups will be reflected in a weakening of interlipid hydrogen bonding, so that, conversely, the rate is a useful diagnostic for a weakened hydrogen bonding network [68].

One approach to studying interlipid hydrogen bonding involving water is to examine the contribution to overall lipid–lipid interactions, which also include hydrophobic, van der Waals, and electrostatic interactions. This may be accomplished by measuring the rate of desorption of a phospholipid from a membrane (reviewed in Ref. 71; also see Refs. 72–74). The rate of phospholipid desorption is normally slow and difficult to measure; however, if one acyl chain is replaced with a fluorophore attached to a shorter acyl chain, the magnitude of the van der Waals interactions between acyl chains is decreased, and the rate of desorption increased. The desorption process can then be easily followed from

Table III. Correlations Between the Effects of Varying Lipid Bilayer Composition on C_6–NBD–PC Desorption Rate (k_{off}), Rotational Correlation Time,[a] and dansyl-PE Lifetime[b,c]

Modification	k_{off}[d]	ϕ[a]	τ[b]
Varied sn-*2*-acyl[f]	↑	↑	↓
Varied sn-*1,2*-diunsaturation[f]	↑[e]	↑	↓
sn-*2*-acyl unsaturation compared to sn-*1,2*-diunsaturation	↔	↔	↓
Cholesterol[g]	↔	↔	↓
PE[g]	↔	↔	↓
Effect of 200 mM ethanol	↑	↑[e]	↓

[a]Rotational correlation times from ^{31}P and ^{1}H NMR-refs [68,70].
[b]dansyl-PE fluorescence lifetime from Table II.
[c]↔ no change; ↑ increase; ↓ decrease.
[d]Ref [43].
[e]Slater and Stubbs (unpublished).
[f]Effect of increasing either *sn-2*-acyl unsaturation with *1*-palmitoyl-*2*-linoleoyl-PC, *1*-palmitoyl-*2*-arachidonoyl-PC, and *1*-palmitoyl-*2*-docosahexaenoyl-PC, or *sn-1,2*-acyl unsaturation with *1,2*-dioleoyl-PC, *1,2*-dilinoleoyl-PC, *1,2*-dilinolenoyl-PC.
[g]Effect of 25 mol% cholesterol or 50 mol% PE on C_6-NBD-PC desorption rate from PC vesicles.

changes in fluorescence, as the fluorophore-labeled phospholipid is desorbed into a different environment such as another vesicle (e.g., see Refs. 72–74). 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. A dependence of the pseudo-first-order rate constant k_{off} for C_6-NBD-PC desorption from PC donor vesicles following the trend C_6-NBD-PC > C_6-NBD-PS > C_6-NBD-PE is observed [43]. The much faster rate of C_6-NBD-PC desorption has been attributed to the relatively weak water–water hydrogen bonding between PC head groups via the hydration layer compared to direct interlipid hydrogen bonding possible for PE and PS [43,72].

How structural changes in regions of the bilayer distal from the head groups effect head-group interactions is poorly understood. Increasing phospholipid unsaturation decreases lipid order [75], increases the rate of head-group segmental motion [68], and increases head-group spacing [76]. The desorption rates of C_6-NBD-PC from PC donor vesicles increase with the level of PC *sn-2* unsaturation [43]. Lipid order is not directly responsible for the effect, since the presence of cholesterol (25 mol %) in vesicles comprised of PC, which has a large lipid ordering effect in cell membranes (reviewed in Ref. 77), has only a marginal effect on the rate of C_6-NBD-PC desorption [43]. Also, ethanol, which disorders lipid bilayers, *potentiates* the effect of increased unsaturation, while the effect of the alcohol on lipid order *decreases* with unsaturation [43]. Thus modifications in the acyl-chain region on rates of NBD-labeled phospholipid desorption are mediated primarily by the modulation of head-group interactions.

The negligible effect of cholesterol compared to the potentiating effect of increasing PC unsaturation on phospholipid desorption rates [43] cannot be explained on the basis of head-group spacing since *both* increase this parameter (see, e.g., Ref. 76). Further, as shown in Table III, there is no apparent correlation between the degree of water penetration into the head-group region, assessed from dansyl-PE lifetime measurements, and desorption rate. However, previous studies have shown that cholesterol has a negligible effect on the motional rates of the phosphocholine moiety of phospholipids in the liquid crystalline phase (see, e.g., Refs. 68–70), while increased unsaturation (and ethanol) leads to an increases this parameter [43]. This suggests that phospholipid desorption rate and therefore hydrogen bonding between head groups correlate with the dynamic properties of the head group, rather than static properties, such as head-group spacing (Table III). Support for this hypothesis comes from the finding that the desorption rates of fluorophore-labeled PC from vesicles comprised of PC of increasing *sn-2* unsaturation are identical to those of *sn-1,2*-diunsaturated lipids containing the same total number of double bonds per PC (C. D. Slater and S. J. Stubbs, unpublished results; see Table III). This agrees with the observation that the head-group motional rates are also similar [68], and shows a lack of a correlation between lipid desorption rates and head-group spacing, since the cross-sectional molecular area of *sn-1,2*-unsaturated PC is *greater* than that of the corresponding *sn-2*-unsaturated PC containing the same number of double bonds (see, e.g., Ref. 76). Also, the presence of PE in vesicles composed of PE and PC (1:1 molar) has no effect on the desorption rate of C_6-NBD-PC (Table III). This again correlates with an observed negligible effect of PE on the motional freedom of the phosphocholine moiety, as assessed from ^{31}P and ^{1}H NMR spin–lattice relaxation times [68,70]. Thus, since hydrogen bonds are "directional" and require that acceptor and donor have the correct spatial geometry, it is likely that the strength of water–lipid hydrogen bonding will be a function of the rate of motion of the phosphocholine moiety. Further, since phospholipid desorption is a function of the strength of water–lipid and water–water hydrogen bonds in the hydration layer [43], then this parameter can be considered a useful diagnostic of the motional properties of the phospholipid head group.

SUMMARY AND PERSPECTIVES

In summary, fluorescence spectroscopic approaches add considerably to the armory available for the study of hydration in lipid bilayers and cell membranes. The main advantages of fluorescent methods are sensitivity and the fact that probes may be precisely located. A disadvantage is that it is not always possible to assign unambiguously effects to hydration changes without recourse to other approaches. Membrane proteins are known to undergo subtle changes in conformation, for example, ion channel gating mechanisms, interactions of various extrinsic membrane proteins with the lipid bilayer, such as protein kinase C, etc. Small changes in hydration at the protein–lipid interface could provide the energy to drive or modulate such conformational modifications. With the advances in site-directed mutagenesis one may foresee probes on membrane proteins being used to assess the role of hydration in these effects. Another area that should see development in the short term is the use of fluorescent approaches to increasing understanding of the role played by altered hydration at the membrane surface in the effects of perturbation such as occurs in drug and anesthetic interactions with membranes.

ACKNOWLEDGMENTS

Part of this work was supported by U. S. Public Health Service grants AA08022, AA07215, AA07186, and AA07465.

REFERENCES

1. R. P. Rand (1992) *Science* **256**, 618.
2. M. F. Colombo, D. C. Rau, and V. A. Parsegian (1992) *Science* **256**, 655–659.
3. R. P. Rand and V. A. Parsegian (1989) *Biochim. Biophys. Acta* **988**, 351–376.
4. G. C. Newman and C. Huang (1975) *Biochemistry* **14**, 3363–3370.
5. D. M. Small (1967) *J. Lipid Res.* **8**, 551–557.
6. B. D. Ladbrooke and D. Chapman (1969) *Chem. Phys. Lipids* **3**, 304–356.
7. E. G. Finer and A. Darke (1974) *Chem. Phys. Lipids* **12**, 1–16.
8. G. L. Jendrasiak and J. H. Hasty (1974) *Biochim. Biophys. Acta* **337**, 79–91.
9. I. Ueda, H. S. Tseng, Y. Kaminoh, S. M. Ma, H. Kamaya, and S. H. Lin (1986) *Mol. Pharmacol.* **29**, 582–588.
10. A. Sen and S. W. Hui (1988) *Chem. Phys. Lipids* **49**, 179–184.
11. M. Prats, J. F. Tocanne, and J. Teissie (1987) *Eur. J. Biochem.* **162**, 379–385.
12. J. Teissie, M. Prats, A. LeMassu, L. C. Stewart, and M. Kates (1990) *Biochemistry* **29**, 59–65.
13. J. M. Boggs (1987) *Biochim. Biophys. Acta* **906**, 353–404.
14. D. M. LeNeveu, R. P. Rand, and V. A. Parsegian (1976) *Nature* **259**, 601–603.
15. G. L. Kirk, S. M. Gruner, and D. L. Stein (1984) *Biochemistry* **23**, 1093–1102.
16. R. P. Rand, N. Fuller, V. A. Parsegian, and D. C. Rau (1988) *Biochemistry* **27**, 7711–7722.
17. K. Raghavan, M. Rami Reddy, and M. L. Berkowitz (1992) *Langmuir* **8**, 233–240.
18. S. A. Simon, T. J. McIntosh, A. D. Magid, and D. Needham (1992) *Biophys. J.* **61**, 786–799.
19. S. Marcelja and N. Radic (1976) *Chem. Phys. Lett.* **42**, 129–130.
20. O. H. Griffith, P. J. Dehlinger, and S. P. Van (1974) *J. Membr. Biol.* **15**, 159–192.
21. D. L. Worcester and N. P. Franks (1976) *J. Mol. Biol.* **100**, 359–378.
22. S. A. Simon, T. J. McIntosh, and R. Latorre (1982) *Science* **216**, 65–67.
23. J. M. Smaby, A. Hermetter, P. C. Schmid, F. Paltauf, and H. L. Brockman (1983) *Biochemistry* **22**, 5808–5813.
24. S. A. Simon and T. J. McIntosh (1986) in *Methods in Enzymology*, Vol. 127, Academic Press, pp. 511–521.
25. A. Blume, W. Hubner, and G. Messner (1988) *Biochemistry* **27**, 8239–8249.
26. C. Zannoni, A. Arcioni, and P. Cavatorta (1983) *Chem. Phys. Lipids* **32**, 179–250.
27. R. Fiorini, M. Valentino, S. Wang, M. Glaser, and E. Gratton (1987) *Biochemistry* **26**, 3864–3870.
28. R. E. Jacobs and S. H. White (1989) *Biochemistry* **28**, 3421–3437.
29. M. M. Teeter (1991) *Annu. Rev. Biophys. Biophys. Chem.* **20**, 577–600.
30. C. Ho and C. D. Stubbs (1992) *Biophys. J.* **63**, 897–902.
31. I. Ueda (1991) in R. C. Aloia, C. C. Curtain, and L. M. Gordon (Eds.), *Drug and Anesthetic Effects on Membrane Structure and Function*, Wiley-Liss, New York, pp. 15–33.
32. D. W. Urry and C. Sandorfy (1991) in R. C. Aloia, C. C. Curtain, and L. M. Gordon (Eds.), *Drugs and Anesthetic Effects on Membrane Structure and Function*, Wiley-Liss, New York, pp. 91–131.
33. G. Curatola, G. Lenaz, and G. Zolese (1991) in R. C. Aloia, R. C. Curtain, and L. M. Gordon (Eds.), *Drug and Anesthetic Effects on Membrane Structure and Function*, Wiley-Liss, New York, pp. 35–70.
34. R. F. Flewelling and W. L. Hubbell (1986) *Biophys. J.* **49**, 541–552.
35. A. Sen and S.-W. Hui (1988) *Chem. Phys. Lipids* **49**, 179–184.
36. B. W. Williams, A. W. Scotto, and C. D. Stubbs (1990) *Biochemistry* **29**, 3248–3255.
37. C. Ho, B. W. Williams, and C. D. Stubbs (1992) *Biochim. Biophys. Acta* **1104**, 273–282.
38. C. Ho, B. W. Williams, M. B. Kelly, and C. D. Stubbs (1994) *Biochim. Biophys. Acta* **1189**, 135–142.
39. B. W. Williams and C. D. Stubbs (1988) *Biochemistry* **27**, 7994–7999.
40. C. D. Stubbs, B. W. Williams, and C. Ho (1990) *Proc. SPIE Spectrosc. Biochem.* **1204**, 448–455.
41. C. D. Stubbs and B. W. Williams (1992) in *Topics in Fluorescence Spectroscopy*, Vol. 3, Plenum Press, New York, pp. 231–271.
42. C. Ho, M. B. Kelly, and C. D. Stubbs (1994) *Biochim. Biophys. Acta* **1193**, 307–315.
43. S. J. Slater, C. Ho, F. J. Taddeo, M. B. Kelly, and C. D. Stubbs (1993) *Biochemistry* **32**, 3714–3721.
44. R. M. Epand and B. T. Leon (1992) *Biochemistry* **31**, 1550–1554.
45. A. Chattopadhyay and E. London (1988) *Biochim. Biophys. Acta* **938**, 24–34.
46. *Handbook of Chemistry and Physics* (1994), p. E-55.
47. L. Stryer (1966) *J. Am. Chem. Soc.* **88**, 5708–5712.
48. L. Lee and G. W. Robinson (1985) *J. Phys. Chem.* **89**, 1872–1875.
49. M. Kuwahara and A. S. Verkman (1988) *Biophys. J.* **54**, 587–593.

50. B. Cornell (1987) *J. Bioenerg. Biomembr.* **19**, 655–676.
51. B. A. Wallace (1990) *Annu. Rev. Biophys. Biophys. Chem.* **19**, 127–157.
52. M. Straume and B. J. Litman (1987) *Biochemistry* **26**, 5113–5120.
53. M. Straume and B. J. Litman (1987) *Biochemistry* **26**, 5121–5126.
54. K. Kinosita and A. Ikegami (1984) *Biochim. Biophys. Acta* **769**, 523–527.
55. M. L. Wratten, E. Gratton, M. van de Ven, and A. Sevanian (1989) *Biochem. Biophys. Res. Commun.* **164**, 169–175.
56. S. Wang, J. M. Beechem, E. Gratton, and M. Glaser (1991) *Biochemistry* **30**, 5565–5572.
57. R. Fiorini, E. Gratton, and G. Curatola (1989) *Biochim. Biophys. Acta* **1006**, 198–202.
58. T. Parasassi, G. De Stasio, A. Miccheli, F. Bruno, F. Conti, and E. Gratton (1990) *Biophys. J.* **35**, 65–73.
59. G. Zolese, E. Gratton, and G. Curatola (1990) *Chem. Phys. Lipids* **55**, 29–39.
60. T. Parasassi, G. Ravagnan, O. Sapora, and E. Gratton (1992) *Int. J. Radiat. Biol.* **61**, 791–796.
61. R. M. Fiorini, M. Valentino, E. Gratton, E. Bertoli, and G. Curatola (1987) *Biochem. Biophys. Res. Commun.* **147**, 460–466.
62. F. Schroeder, G. Nemecz, E. Gratton, Y. Barenholz, and T. E. Thompson (1988) *Biophys. Chem.* **32**, 57–72.
63. M. Valentino, M. Governa, E. Gratton, R. Fiorini, G. Curatola, and E. Bertoli (1988) *FEBS Lett.* **234**, 451–454.
64. T. Parasassi, F. Conti, E. Gratton, and O. Sapora (1987) *Biochim. Biophys. Acta* **898**, 196–201.
65. G. Nemecz and F. Schroeder (1988) *Biochemistry* **27**, 7740–7749.
66. J. R. Lakowicz, H. Cherek, I. Gryczynski, N. Joshi, and M. L. Johnson (1987) *Biophys. Chem.* **28**, 35–50.
67. E. F. Kalb, F. Paltauf, and A. Hermetter (1989) *Biophys. J.* **56**, 1245–1253.
68. R. Ghosh (1988) *Biochemistry* **27**, 7750–7758.
69. M. P. Milburn and K. R. Jeffrey (1989) *Biophys. J.* **56**, 543–549.
70. X. Han and R. W. Gross (1991) *Biochim. Biophys. Acta* **1063**, 129–136.
71. R. E. Brown (1992) *Biochim. Biophys. Acta* **1113**, 375–389.
72. M. A. Gardam, J. J. Itovitch, and J. R. Silvius (1989) *Biochemistry* **28**, 884–893.
73. M. A. Gardam and J. R. Silvius (1990) *Biochem. Soc. Trans.* **18**, 831–835.
74. T-B. Shin, R. Leventis, and J. R. Silvius (1991) *Biochemistry* **30**, 7491–7497.
75. C. D. Stubbs, T. Kouyama, K. Kinosita, and A. Ikegami (1981) *Biochemistry* **20**, 4257–4262.
76. R. A. Demel, W. S. M. Geurts van Kessel, and L. L. M. van Deenen (1972) *Biochim. Biophys. Acta* **266**, 26–40.
77. P. L. Yeagle (1985) *Biochim. Biophys. Acta* **822**, 267–287.