Control of the Depth of Molecules within Membranes by Polar Groups: Determination of the Location of Anthracene-Labeled Probes in Model Membranes by Parallax Analysis of Nitroxide-Labeled Phospholipid Induced Fluorescence Quenching†

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ABSTRACT: The location of anthracene-labeled molecules incorporated into model membranes was measured by fluorescence quenching. The depth of the anthracene group was calculated from the degree of quenching by lipids carrying a nitroxide at different depths, using the parallax analysis (Chattopadhyay & London (1987) *Biochemistry 26*, 39−45). A series of anthracene derivatives was examined in order to determine what polar functional groups would anchor at the membrane surface, and at what depth anchoring would occur. An anthracene with only a methyl group was not anchored at the membrane surface, but derivatives with polar or charged groups did anchor near the membrane surface as demonstrated by a shallower anthracene depth. Based on anthracene depths, protonated primary amine, secondary amine, and hydroxyl groups appear to be located 15−16 Å from the center of the membrane. A quaternary amino locates more shallowly, at 18 Å from the bilayer center. A protonated carboxyl group is slightly deeper, at 14 Å from the center of the bilayer. Ester groups are found to be weakly anchoring, having a location dependent on the structure of the molecule to which they are attached. In methyl 9-anthracenepropionate, the ester group is located about 13 Å from the bilayer center. Anthracene esters attached to cholesterol or cholesterol esters showed various depths. An anthracene ester attached to the tail of cholesterol was located 1−6 Å from the center of the bilayer for a cholesterol derivative, but at 12 Å from the bilayer center for a cholesterol oleate derivative. These studies show that a single polar group is sufficient to anchor molecules at the membrane surface and that small changes in chemical structure can greatly influence the depth of a molecule in membranes. Overall, there was a close correlation between the location of those polar groups which are found in lipids and the same polar groups when linked to anthracene. This approach may make it possible to predict the depth of molecules in membranes from their chemical structure.

One of the fundamental questions involved in the study of membrane structure and function is how chemical and structural factors control the depth of molecules in membranes. Many molecules of biological interest interact with membranes. For example, a wide variety of hydrophobic and amphipathic compounds such as alcohols, anesthetics, and organic solvents block voltage-gated ion channels, bind to membrane bound enzymes, or bind to membrane bound receptors (Jain, 1988). What determines the location of these molecules within the bilayer is largely unknown, although critical for understanding their function. In addition, details of the influence of hydrophobic and polar amino acid side chains in controlling penetration of proteins into membranes are unknown. Understanding the features that control the degree of penetration of molecules in lipid bilayers should be very useful for the design of membrane-inserting molecules of various types.

Our group has developed a fluorescence quenching technique, called the parallax analysis, that can determine the depth of fluorophores in membranes (Chattopadhyay & London, 1987; Abrams et al., 1992; Abrams & London, 1993). This method involves the measurement of the fluorescence quenching induced by phospholipids derivatized with nitroxide (spin-)label quenchers located at different depths in the bilayer. The ratio of fluorescence intensity in the presence of a shallow quencher to that in a separate sample with a deep quencher is substituted into an algebraic equation to calculate fluorophore depth at the angstrom level of resolution (Chattopadhyay & London, 1987; Abrams & London, 1993). Using this method, the membrane location of NBD,[1] anthroyloxy, and carbazole groups attached to fatty acids has been determined in membranes (Chattopadhyay & London, 1987; Abrams et al., 1992; Abrams & London, 1993). The method has also been applied by several groups to study the orientation and location of polypeptides and

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[1] Abbreviations: AM, 9-methylanthracene; AMAM, 9-[(methylamino)methyl]anthracene; AMC, 9-anthracenylmethyl 3β-hydroxy-22,23-bisnor-5-cholenate; AMC acetate, 9-anthracenylmethyl 3β-acetoxy-22,23-bisnor-5-cholenate; AMC oleate, 9-anthracenylmethyl 3β-(*cis*-9-octadecenoyloxy)-22,23-bisnor-5-cholenate; AMOH, 9-anthracenemethanol; APA, 9-anthracenepropionic acid; APAC, [3-(9-anthracenyl)propyl]ammonium chloride; APAM, methyl 9-anthracenepropionate; APTAB, *N*-(9-anthracenylpropyl)trimethylammonium bromide; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; ESR, electron spin resonance; MLV, multilamellar vesicles; NBD, 7-nitro-2,1,3-benzoxadiazol-4-yl; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; PE, phosphatidylethanolamine; 5- or 12-SLPC, 1-palmitoyl-2-(5- or 12-doxyl)stearoyl-*sn*-glycero-3-phosphocholine; SUV, small unilamellar vesicles; Tempocholine, 4-[*N*,*N*-dimethyl-*N*-(2-hydroxyethyl)ammoniumyl]-2,2,6,6-tetramethylpiperidine-1-oxyl; TempoPC, 1,2-dioleoyl-*sn*-glycero-3-phosphotempocholine; TLC, thin-layer chromatography.

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proteins inserted into membranes (Chattopadhyay & McNamee, 1991; Clague et al., 1991; Chung et al., 1992; Ulbrandt et al., 1992; Palmer & Merill, 1994; Matsuzaki et al., 1994; Jones & Gierasch, 1994; Rodionova et al., 1995).

This report attempts to answer the question: where do polar groups locate in membranes? To do this, the depth of a series of 9-substituted anthracene probes attached to various functional groups has been determined using the parallax analysis. We find that a wide variety of polar functional groups locate near the membrane surface. Interestingly, changes both in the chemical structure of the functional group and in the molecule to which the functional group is attached affect a functional group's depth.

EXPERIMENTAL PROCEDURES

Materials. 9-Methylanthracene (AM), 9-anthracenemethanol (AMOH), and 9-[(methylamino)methyl]anthracene (AMAM) were purchased from Aldrich. 9-Anthracenepropionic acid (APA), *N*-(9-anthracenyl)propyltrimethylammonium bromide (APTAB), 9-anthracenylmethyl 3β-hydroxy-22,23-bisnor-5-cholenate (AMC), 9-anthracenylmethyl 3β-acetoxy-22,23-bisnor-5-cholenate (AMC acetate), and 9-anthracenylmethyl 3β-(*cis*-9-octadecenoyloxy)-22,23-bisnor-5-cholenate (AMC oleate) were purchased from Molecular Probes. [3-(9-Anthracenyl)propyl]ammonium chloride (APAC) was a gift from Professor C. V. Kumar (University of Connecticut). The purity of all these anthracene probes was checked by TLC on silica gel plates, and the percent purity was determined by measuring the relative fluorescence intensities of the main spot and that of the impurity. Almost all of these compounds were relatively pure (>99%) and were used as received except for 9-anthracenemethanol, which was recrystallized from chloroform (final mp 165−167 °C) after which it was also >99% pure. AMC oleate was found to be 95% pure and was used without further purification.

Nitroxide-labeled PCs and DOPC were purchased from Avanti Polar Lipids. The purity of the phospholipids was also checked by TLC on silica gel plates as described previously (Abrams & London, 1993). No impurities were detected on the lipids after the plates were sprayed with 40% sulfuric acid and charred. The concentration of phospholipids was determined by phosphate assay (Bartlett, 1959) subsequent to total digestion (Morrison, 1964). The actual nitroxide content on the nitroxide-labeled lipids was calculated from the intensities of the doubly integrated ESR spectra as described previously (Chattopadhyay & London, 1987). Alternatively, nitroxide concentration was assayed with fluorescence quenching of anthroyloxy fatty acids by determining the percent of uncalibrated nitroxide-labeled lipid that had to be incorporated into MLV to give the same quenching as an ESR-calibrated sample with 15% nitroxide-labeled lipid (Abrams & London, 1993). The ratio of nitroxide groups to lipid was generally found to be in the range 0.8−0.9.

Preparation of Methyl 9-Anthracenepropionate (APAM). Methyl 9-anthracenepropionate was prepared following a condensation reaction of 9-anthracenepropionic acid and methanol as described in Furniss et al. (1978). The methyl ester was extracted into ether, and the unreacted acid was removed by shaking with 1 N NaOH. The ether solution was collected, and the solvent was evaporated under vacuum. The residue was collected, and a yellow solid was obtained: mp 65−67 °C; ^{1}H NMR ($CDCl_3$) δ 2.79 (2H, t), 3.74 (3H, s), 3.97 (2H, t), 7.50 (4H, m), 8.0 (2H, d), 8.2 (2H, d), 8.37 (1H, s).

Preparation of Samples for Binding Studies. MLV samples containing increasing concentrations of phospholipid and a constant concentration of anthracene probe (2 μM) were prepared as described previously (Abrams & London, 1993). The dried lipid/fluorophore mixtures were hydrated with 1 mL of 10 mM sodium acetate/150 mM NaCl, pH 5.0. (pH 5.0 was used to ensure that ionizable fluorescent probes would be protonated.) MLV samples containing the fluorophore were prepared in duplicate. Single background samples without the fluorophore were prepared similarly. Background intensity was generally negligible (<1% of sample intensity).

Preparation of Samples for pH Titration. Solutions were prepared containing 2 μM APA or APAC in the presence of 200 μM DOPC. SUV samples were prepared by dissolving the dried lipid/fluorophore mixture in 20 μL of ethanol and then adding 2 mL of 150 mM NaCl/5 mM glycine/5 mM acetic acid/5 mM NaH_2PO_4 buffer, pH 5. The solutions were titrated with small aliquots of 1 M acetic acid to bring them to lower pH prior to the start of the fluorescence measurements in which pH was titrated upward.

Preparation of Samples for Fluorescence Quenching Studies. MLV samples containing the fluorophore and lipids were prepared essentially as described previously (Abrams & London, 1993). For depth measurements, MLV with four different lipid compositions were prepared: one containing DOPC and the other three containing 85 mol % DOPC and 15 mol % of one of the spin-labeled PC's. The final total lipid concentration of these solutions was 200 μM except for AMOH, APAC, APTAB, and AMAM, where 500 μM of total phospholipid was used. In all cases, the final concentration of the fluorophore was 2 μM, and the samples were dispersed in 1 mL of 10 mM sodium acetate/150 mM NaCl, pH 5.0. Duplicates were prepared for each sample. Single background samples without the fluorophore were prepared similarly. Background intensity was generally negligible (<1% of sample intensity). Some quenching experiments were done with SUV. Similar depths were obtained (not shown).

Fluorescence Measurements for Binding Studies. Samples were placed in a 1 cm path length quartz cuvette, and their fluorescence intensity was measured with a Spex 212 Fluorolog spectrofluorimeter operating in the ratio mode. The excitation and emission slits were set between 1.0 and 2.5 mm (1.7−4.3 nm bandpass) so as to prevent saturation of the photomultiplier tube. The following excitation wavelengths were used for anthracene probes: 365 nm for AM, AMOH, and AMC oleate; 366 nm for APA and AMC; 367 nm for AMAM and AMC acetate; 368 nm for APAC; 369 nm for APAM and APTAB. In all cases, emission was monitored at 415 nm.

Samples were vortexed before taking the fluorescence intensity reading to eliminate variations due to MLV settling. The fluorescence intensities were found to be stable over time in all cases. Fluorescence was measured at room temperature and averaged over three 6 s readings. The fluorescence intensity from the duplicate MLV samples containing the fluorophore was averaged, and the intensity of the background samples without fluorophore was subtracted.

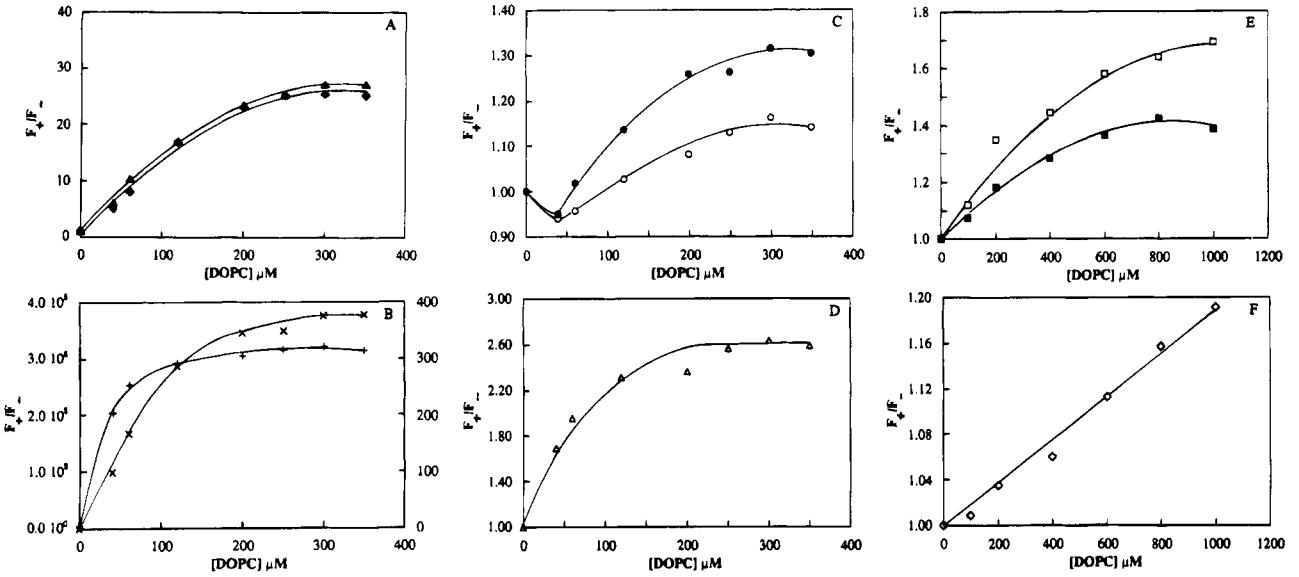

FIGURE 1: Binding of anthracene probes to model membrane vesicles. F_+/F_- is the ratio of fluorescence in the presence of DOPC vesicles to that in its absence. For details see Experimental Procedures. (A) AM (▲) and AMC oleate (♦). (B) AMC (×) and AMC acetate (+). (C) APAC (●) and APA (○). (D) APAM (△). (E) APTAB (□) and AMOH (■). (F) AMAM (◇).

Fluorescence vs pH. The solutions were placed in a 1 cm quartz cuvette, and the excitation wavelength was set to 368 nm (2.5 mm slit) for APAC and 366 nm for APA (0.5 mm slit, 0.86 nm bandpass). For both probes, the fluorescence emission was measured at 420 and 410 nm (using a 5.0 mm slit, 8.6 nm bandpass for APAC and 0.5 mm slit for APA). For titrating pH, successive aliquots of 1 M NH_4-OH or 1 M NaOH were then used to increase the pH of the solutions and fluorescence was remeasured. The time between successive fluorescence measurements was about 5 min.

Fluorescence Quenching Experiments. The same fluorimeter settings were used as described above except for the emission wavelengths, which were 411 nm for AMOH; 412 nm for AMC; 414 nm for APAM and APTAB; 415 nm for AM, APA, APAC, AMAM, AMC acetate, and AMC oleate.

The same procedure was followed for taking the fluorescence readings as described above. The fluorescence intensities in the presence of the quenchers were substituted into the parallax equation:

$$z_{cf} = L_{c1} + ((-1/\pi C) \ln(F_1/F_2) - L_{12}^2)/2L_{12}$$

(Chattopadhyay & London, 1987; Abrams & London, 1993) to calculate the anthracene depth. z_{cf} is the distance of the fluorophore from the center of the bilayer, L_{c1} is the distance from the center of the bilayer to the shallow quencher, C is the quencher concentration in molecules per unit area, F_1 is the fluorescence in the presence of the shallow quencher, F_2 is the fluorescence in the presence of the deeper quencher, and L_{12} is the transverse distance between the depths of the shallow and deeper quenchers.

To calculate the depth of functional groups, it was necessary to account for the methylene spacer between anthracene and the functional group. It was assumed that the methylenes are 0.9 Å apart in depth, as previously found for acyl chain on lipids, and anthroyloxy probes (Oldfield et al., 1978; Zaccai et al., 1979; Lewis & Engelman, 1983; McIntosh & Holloway, 1987; Wiener & White, 1992; Abrams & London, 1993). There are two possible average orientations for the anthracene moiety, one in which the bond between the spacer and the anthracene 9-carbon is parallel to the acyl chains (as shown in Figure 3), and the other in which it is perpendicular. For our calculations, the average of these orientations (45° tilt) was assumed to be present. (If the orientation of this bond is exclusively parallel to the acyl chains, then the functional group depths are 1.5 Å shallower than reported in Table 1.) The distance of the functional group from the bilayer center (Z_{group}) is then estimated to be the distance of the center of anthracene from the center of the bilayer plus 2 Å for the difference in depth between the center of the anthracene and start of the spacer (the methylene that is attached to the 9-carbon of anthracene) plus 0.9 Å times the number of methylene groups (including the methylene attached to the 9-carbon of anthracene) in the spacer.

RESULTS

Binding of Anthracene Probes to Model Membranes. The amount of phospholipid required to establish binding of the fluorescent probes to model membranes was determined by the increase in fluorescence intensity upon binding. In Figure 1 the ratio of anthracene fluorescence at 415 nm in the presence of DOPC (F_+) relative to that in its absence (F_-) is shown as a function of DOPC concentration. The amount of lipid required to obtain 50% binding is in the range of 30−125 μM for most of the probes.[2] The binding of AMOH, AMAM, and APTAB was significantly weaker. To obtain 50% binding of AMOH to DOPC, 200 μM lipid was required. AMAM and APTAB bind to DOPC even more weakly, with binding incomplete even at 1000 μM DOPC. Because of light scattering problems, binding of these probes at higher DOPC concentrations was not studied. However, fluorescence quenching data allow us to estimate that these probes are 50% bound to lipid at about 600 μM (see below).

[2] The binding curves of APAC and APA show an initial decrease in the fluorescence intensity at low vesicle concentration followed by a gradual increase in intensity. This type of decrease in fluorescence is probably due to self-quenching when the probe is at a high concentration within the membrane (i.e., a high density within the bilayer) and has been seen with other fluorophores (Burke & Tritton, 1984; Hoekstra et al., 1984; London, 1986).

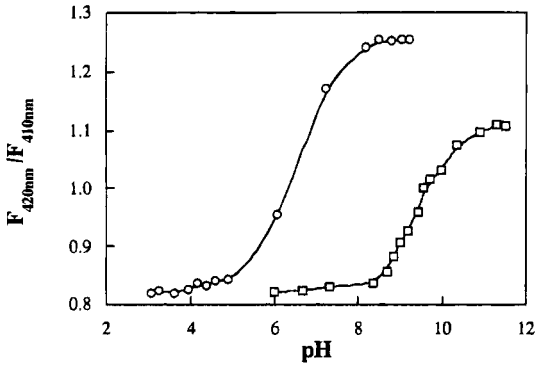

FIGURE 2: Effect of increasing pH on the fluorescence of ionizable anthracence probes in the presence of 200 μM DOPC (SUV). The ratio of emission intensity at 420 nm to that at 410 nm is shown on the ordinate. (○) APA. (□) APAC.

For the ionizable probes, it was important to determine whether binding of the ionized or protonated form of the molecules was being measured. To examine this point, the pK_a of the ionizable probes APA and APAC was determined by measuring fluorescence vs pH. As shown in Figure 2, the fluorescence shows a sigmoidal dependence on pH, and the pK_a, which is the midpoint of the curve, is at 6.5 for APA and 9.4 for APAC. This means that at pH 5 we are examining the protonated forms of these molecules. This should also be true for AMAM because its secondary amine should have a pK_a close to that of the primary amine of APAC. Attempts to examine the binding of APA and APAC at high pH failed due to weak binding to model membranes for APA, and unstable fluorescence intensity in the case of APAC.

Depth of Anthracene Probes in Model Membranes. We next determined the location of a series of anthracene probes within the lipid bilayer using fluorescence quenching. Anthracene probes were chosen containing polar functional groups linked to the fluorophore through a spacer.

Table 1 shows the amount of quenching of various anthracene probes with three different quenchers, lipids labeled with a nitroxide at a shallow, medium, or deep location in the bilayer (Abrams & London, 1993). The absolute level of quenching is correlated with the degree of probe binding to the bilayers. All of the more tightly bound derivatives can be quenched 70−80% by at least one of the nitroxide-labeled lipids. Quenching is less (40−60%) for the more weakly binding AMOH, AMAM, and APTAB molecules. For these molecules, a significant amount of fluorescence arises from molecules dissolved in aqueous solution and is therefore not quenched.

By simple inspection of the **relative** amount of quenching by the different nitroxide-labeled lipids, one can determine whether a fluorophore has a deep or shallow location in the bilayer. For example, the anthracene on AMC cholesterol is quenched the most by the deep 12-SLPC and, therefore, must be close to the center of the bilayer. The anthracene on AMAM and AMOH is quenched almost equally by the shallow and deep quenchers and, therefore, must be close to a depth halfway in between these two quenchers.

To obtain more precise values for fluorophore depth, the parallax analysis of fluorescence quenching can be used (Chattopadhyay & London, 1987; Abrams & London, 1992, 1993). This method makes use of the ratio of quenching by two nitroxide-labeled lipids having the nitroxide at different depths to calculate a precise fluorophore depth (Abrams & London, 1993). Previous studies have calibrated this method and demonstrated the accuracy of the depths to be approximately within 1 Å (Abrams & London, 1992, 1993). Differences in depth of 1 Å or less may not be significant. The depth of the series of 9-substituted anthracene derivatives calculated by the parallax analysis is shown in Table 1 and schematically summarized in Figure 3. Several interesting observations arise from this analysis.

Table 1: Fluorescence Quenching Values and Calculated Membrane Location of 9-Substituted Anthracene Probes (z_{cf}) and Attached Functional Groups

compd	F_{TC}/F_0	F_5/F_0	F_{12}/F_0	z_{cf}[a]	Z_{group}[d] (group)
AM	0.310	0.221	0.207	8.2	
APA	0.394	0.321	0.348	10.0	14 (−COOH)
APAM	0.374	0.273	0.283	9.4	13 (−$COOCH_3$)
APAC	0.434	0.304	0.355	10.8	15.5 (−NH_3^+)
APTAB	0.512	0.399	0.568	13.3[b]	18 (−$N(CH_3)_3^+$)
AMAM	0.745	0.604	0.723	12.5[b]	15.5 (−$NH_2^+CH_3$)
AMOH	0.576	0.530	0.599	12.7[b]	15.5 (−OH)
AMC	0.565	0.405	0.213	1.4[c]	
AMC acetate	0.398	0.288	0.253	7.5	
AMC oleate	0.341	0.286	0.341	12.6	

[a] The values shown are the average of two independent experiments, each with duplicate samples. The agreement between these experiments was generally within 0.5 Å. Previous studies have shown that accurate depth values are obtained when the spin-label quenchers are closest to the fluorophore. Thus, the pair of spin-label quenchers which quenches most is used to calculate depth (z_{cf}) using the equation in Abrams and London (1993). However, in some instances the degrees of quenching of TempoPC and 12-SLPC were indistinguishable within experimental error (less than 5% difference in fluorescence intensity). In such cases the average of the depths obtained with TempoPC/5-SLPC and 5-SLPC/12-SLPC was used to calculate z_{cf} (see Abrams and London, 1993). [b] Depths calculated after correction for fluorophore in aqueous solution (see Results). The amount of fluorescence coming from the fluorophore in solution is calculated by subtracting 0.3 from the F/F_0 value for the strongest quencher. The amount of fluorescence coming from solution is then subtracted from the F/F_0 values, and the resulting values are substituted into the z_{cf} equation. The depths calculated from raw and corrected F/F_0 values were very similar. In addition, depths determined from experimental F/F_0 values in control experiments, where lipid concentration was varied to vary the amount of bound probe, showed little concentration dependence of z_{cf}. [c] This value is the lower limit to the distance of the fluorophore from the bilayer center. Transleaflet quenching may be a problem if a molecule is located 1.4 Å from the bilayer center (Chattopadhyay & London, 1987). The modified parallax equation (Chattopadhyay & London, 1987) for taking transleaflet quenching into account gives a z_{cf} of 6.5 Å, which may be considered an upper limit for the distance from the center of the bilayer (calculated using a R_c of 14.7 Å calculated from the quenching of AMC acetate and AMC oleate and eq 4 in Chattopadhyay and London, 1987). [d] See Experimental Procedures for calculation. The subscript TC refers to TempoPC, 5 to 5SLPC, and 12 to 12 SLPC.

The first observation is that 9-methylanthracene (AM) is located about 8 Å from the center of the bilayer. Since the hydrophobic section of the bilayer extends about 15 Å from the bilayer center (Wiener & White, 1992), a molecule spread over all depths should have an apparent average depth of 7−8 Å. Therefore, the depth of AM, which lacks any polar group, is consistent with its distribution over various depths within the hydrophobic core of the membrane.

Table 1 also shows that the depth of anthracene depends on the polar group and the length of the methylene spacer between the polar group and anthracene. For anthracene derivatives with attached polar groups, the anthracene depth is 1−5 Å shallower than for 9-methylanthracene. This implies that their polar groups must anchor at or near the membrane surface such that the anthracene depth is altered. Importantly, from the anthracene depth and the length of

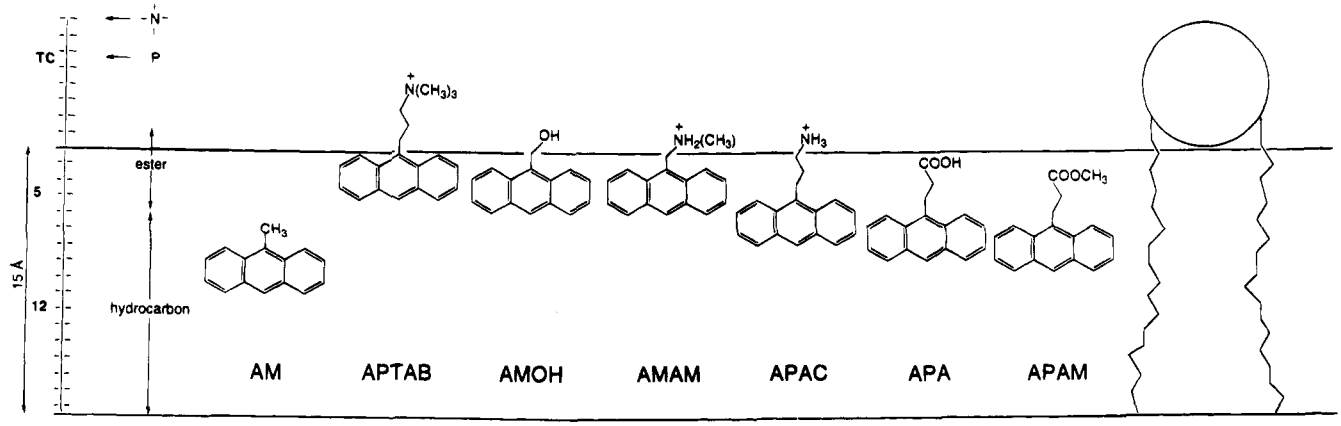

FIGURE 3: Schematic summary of the depth of anthracene probes. The depth of the center of the anthracenes corresponds to the values shown in Table 1. The location of the functional groups of a phospholipid, as estimated in the study of Wiener and White (1992), is shown on the left. The distances of the spin-labels from the bilayer center for the Tempo, 5, and 12 spin-labeled PC's are 19.5, 12.15, and 5.85 Å, respectively, as shown on the left (Chattopadhyay & London, 1987; Abrams & London, 1993).

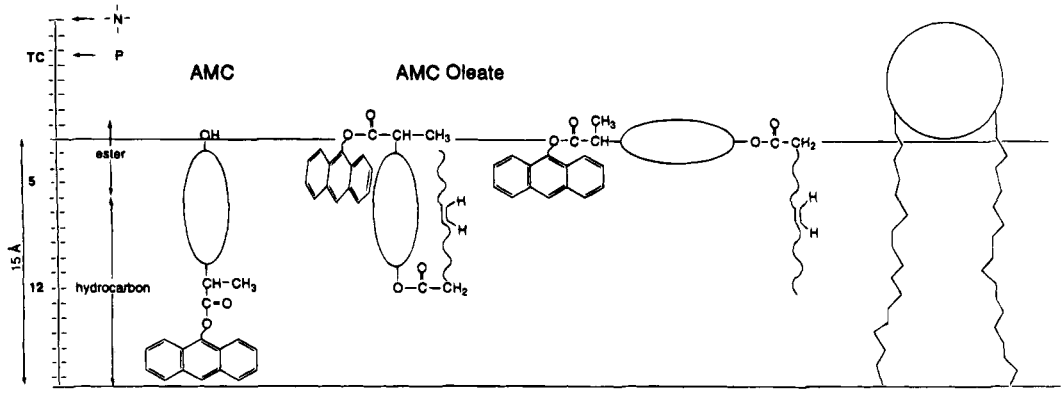

FIGURE 4: Schematic summary of the depth of anthracene labeled cholesterol and cholesterol esters. See Figure 3 for details. Notice that there are at least two possible orientations for AMC oleate. The location of AMC acetate may be an average of AMC and AMC oleate locations. Sterol rings (shown as elipse), and remainder of AMC molecules not shown to scale.

the spacer between anthracene and the functional group, the distance of the functional group from the bilayer center (Z_{group}) can be estimated (see Experimental Procedures).

Depth of Anthracene Probes in Model Membranes: Charged Amino Groups. The shallowest depths are seen for charged amino groups. From the depths of AMAM and APAC, it appears that protonated primary and secondary amino groups are at the same depth, 15−16 Å from the center of the bilayer. Despite having the same charge, quaternary amino groups are at an even shallower location, at 18 Å from the bilayer center. This difference is interesting in view of its correlation with other measures of amine location in membranes (see Discussion).

Depth of Anthracene Probes in Model Membranes: Uncharged Polar Groups. Based on the depth of AMOH, an −OH group appears to locate at the same depth as primary and secondary amino groups, about 15−16 Å from the bilayer center. Depth measurements on APA indicate a slightly deeper location for an uncharged carboxyl group of 14 Å from the bilayer center. The depth of ionized APA could not be determined because, as noted above, its binding to bilayers was too weak at higher pH.

To test whether an ester group is polar enough to anchor on the membrane surface, we synthesized and determined the depth of APAM in model membranes. APAM was slightly deeper than APA, giving an ester depth of about 13 Å.

Depth of Anthracene Probes in Model Membranes: Cholesterol and Cholesterol Ester Derivatives. We found the depth of an ester group to be dependent on the structure of the remainder of the molecule. Of all the probes that were investigated, the anthracene of AMC is located deepest in the membrane, between 1.4 and 6.5 Å from the bilayer center (Table 1). Presumably, the β-hydroxy of cholesterol is anchored to the membrane surface such that the cholesterol tail, to which anthracene is attached, is deeply located, thus overcoming the tendency of the ester adjacent to anthracene to anchor at the membrane surface (Table 1 and Figure 4). In contrast, the anthracene of AMC oleate is at a shallow location (12.6 Å) in the bilayer, while the anthracene of AMC acetate is at an intermediate location between AMC and AMC oleate. There are two possible models for the orientation of AMC oleate that are consistent with these data. As shown in Figure 4, one possibility is that the sterol orientation has inverted, and the second is that both ester groups are anchored at the surface in AMC oleate. The AMC acetate may take on a mixture of AMC and AMC oleate orientations, or some other intermediate location.

Distribution of Anthracene Probes at Different Depths. The distribution of molecules over a range of depths is another important aspect of their location in membranes (Wiener & White, 1991; Wiener et al., 1991; Abrams & London, 1992). The parallax analysis, which gives the average fluorophore distance from the bilayer center, is largely independent of the width of fluorophore distribution, and it cannot be used to measure depth distributions (Abrams & London, 1992). Nevertheless, the data in Table 1 do suggest that different anthracene probes are distributed over

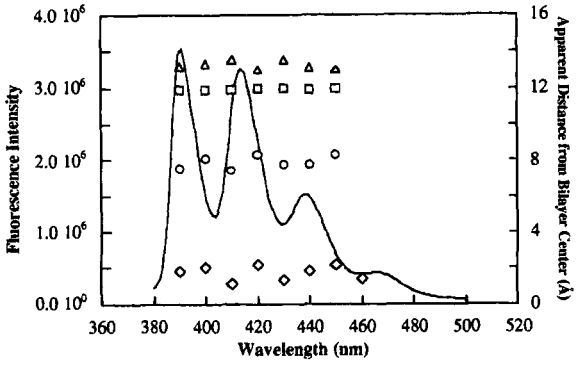

FIGURE 5: The effect of emission wavelength on the apparent distance of anthracene probes from the center of the bilayer (z_{cf}). The wavelength dependence of the apparent depth is shown for APTAB (△), APA (□), AMC acetate (○), and AMC (◇). For comparison, the emission spectrum of 2 μM APA in 200 μM DOPC (MLV) is shown superimposed on the z_{cf} values. Similar emission spectra were obtained for the other anthracene probes, with shifts in the peak positions of no more than 1−2 nm.

different ranges of depth. This is seen most easily for probes of similar average depth. For example, AMOH and AMAM have similar average depth, but the maximum difference in quenching between two nitroxides ($\Delta F/F_0$) is 0.14 for AMAM but only 0.07 for AMOH. This suggests that AMOH may be distributed over a wider range of depths than AMAM, suppressing the differences between quenching as a function of depth. Table 1 also shows a similar difference between APA and APAC quenching, suggesting APA is more widely distributed in depth. Under some conditions differences in the distribution of two fluorophores could lead to small differences in their apparent z_{cf} that are not real. Extending quenching studies to obtain distribution information will be a major challenge in the future (see Discussion).

The Dependence of Apparent Depth on Emission Wavelength. Previous studies using the parallax analysis have shown that the apparent depth of anthroyloxy-labeled fatty acids, which contain an ester group directly attached to the anthracene, is dependent on the emission wavelength (Abrams et al., 1992; Abrams & London, 1993). For each fluorescence peak, at longer emission wavelengths, the fluorescence is dominated by a population of solvent-relaxed excited state fluorophores that have moved closer to the membrane surface, probably due to their increased polarity in the excited state (Werner & Hoffman, 1973; Matayoshi & Kleinfeld, 1981; Demchenko & Shcherbatska, 1985; Abrams & London, 1993). To determine whether this is a common feature of other anthracene derivatives, we investigated the apparent depth dependence of APA, APTAB, AMC, and AMC acetate on emission wavelength (Figure 5). Figure 5 shows a slight dependence on the apparent depth of AMC and AMC acetate on the emission wavelength with shallower depths at the red end of each emission peak. This is consistent with the solvent relaxation phenomenon seen with the anthroyloxy probes. In contrast, the depths of APA and APTAB do not show this pattern. There are several possible explanations for this difference in behavior. One is that the anthracene excited states in APA and APTAB are not polar enough to move toward the surface. A second is that they are already in a sufficiently polar location in the ground state so that they do not move toward a more polar location after excitation. The third is that their solvent relaxed populations do not give significantly red shifted fluorescence relative to the unrelaxed populations and so cannot be detected.

DISCUSSION

Polar Groups Tend To Locate at the Membrane Surface, but Anchoring Location Depends on the Nature of the Polar Group. These studies show that several different polar functional groups tend to anchor at or close to the polar regions of the membrane. In each case a single polar group is sufficient to induce anchoring. However, the depth of anchoring is not always the same. In general, charged groups are more shallow than uncharged polar groups. This is in agreement with the difference between the deeper location of the protonated carboxyl group relative to its ionized form as seen previously (Abrams & London, 1993).

However, even similar charged groups do not necessarily anchor at the same depth. Comparison of the depth of APAC, AMAM, and APTAB shows that a quaternary $-N(CH_3)_3^+$ group is located about 2.5 Å at a shallower location on the membrane surface than the primary ($-NH_3^+$) or secondary ($-NH_2^+CH_3$) amino groups. This result is in agreement with other properties of these groups. For example, crystal data show the quaternary ammonium of PC is farther from the membrane interior than the primary amine of PE, the P−N dipole in PE being inclined by 15° with respect to the bilayer surface (Hitchcock et al., 1974), while in PC this dipole is tilted at an average angle of 22° (Pearson & Pascher, 1979; Wiener & White, 1992). In addition, it has been observed that ammonium ions bind to membranes much more strongly than tetramethylammonium ions (Eisenberg et al., 1979). Both of these observations are consistent with quaternary amino groups seeking a more polar environment than primary amino groups. We do not know what causes this difference, but it may be important that the quaternary amino group is unable to participate in any type of hydrogen bonding. The somewhat deeper location of ester groups as compared to amino groups also is analogous to the relative depth of these groups in phospholipid molecules themselves (Wiener & White, 1992). The fact that aspects of the actual structural behavior of lipids within membranes can be mimicked with simple model compounds is an indication that it may be possible to derive rules that allow prediction of depth within membranes.

Anchoring Propensity Depends on Chemical Context. Despite these results, it does not seem likely that a polar group on a molecule seeks a specific depth independently of the structure of the remainder of the molecule. This is shown most clearly by the behavior of molecules containing an ester group. Although the ester has surface anchoring properties in APAM, the anthracene ester of AMC has a deep location. This variability is confirmed by the behavior of the series of anthroyloxy fatty acids, in which the anthroyloxy ester group takes on a depth close to that of the fatty acyl carbon atom to which it is attached[3] (Chalpin & Kleinfield, 1983; Abrams & London, 1993). Furthermore, the observation that cholesterol esters and triglycerides tend to occupy the nonpolar core of lipoproteins also indicates ester functions do not always seek a polar location. The difference in ester location in AMC, AMC acetate, and AMC oleate also indicates the extent to which chemical context can strongly influence the location of a polar group.

[3] On the other hand, a weakly surface seeking behavior may explain the apparent 2 Å difference between the depth of −COOH groups attached to anthroyloxy and anthracene probes. In the former case, the polarity of the anthroyloxy ester may cause it to take a slightly shallower depth in the membrane, resulting in an overestimate of −COOH distance from the bilayer center.

Thus, it is clear that the surface seeking properties of a polar group depend on the structure of the molecule to which it is attached. This issue may be important for understanding membrane protein structure. Many of the functional groups studied in this report (hydroxy, carboxyl, amino) are found on amino acid residues. The results of this report indicate that even a single residue carrying a polar group on the lipid-facing surface of a transmembrane helix would tend to increase the energy of its transmembrane orientation. However, we cannot yet predict how many of such polar residues in a single helix would result in a change in helix orientation. Therefore, it will be of great importance to quantitate the membrane surface seeking propensities of different polar groups by determining the relative sensitivity of their anchoring properties to their chemical environment. Only then will it be possible to predict the depth of molecules in membranes.

Comparison to Previous Results in Literature and the Accuracy of Depths Determined by the Parallax Analysis. As noted above, the difference in depth between primary and quaternary amino groups determined by nitroxide quenching is in agreement with a number of observations in the literature. The depths observed in this report are also consistent with a number of other previous studies. In the study of Sanson et al. (1976), the relative location of carboxyl, ester, and hydroxy groups attached to fatty acids was inferred from the amount of motion of a spin-label attached to the acyl chain. In agreement with our study, these investigators found that hydroxy and protonated carboxyl groups anchored at a shallower depth than an ester group. Using emission wavelength data, Waggoner and Stryer (1970) found that an ester could anchor a simple anthroyloxy derivative near the membrane surface, but did not if the anthroyloxy ester group was attached to a fatty acyl chain. This agrees with our results on the relative sensitivity of ester anchoring to chemical context noted above, and our previous studies of anthroyloxy fatty acid localization within membranes (Abrams & London, 1992; Abrams et al., 1992). Overall, although the studies using other methods do not pinpoint depth as precisely as the parallax analysis, their agreement with the quenching results reinforces our previous studies establishing the reliability of the parallax analysis approach. Nevertheless, the inability of the parallax analysis to determine the distribution of fluorophore over a range of depths (see Results) remains an important limitation. Preliminary efforts to incorporate such information into quenching studies have been made (Ladokhin, 1993). Extension of such approaches to nitroxide quenching is an important goal.

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