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# The location of fluorescence probes with charged groups in model membranes

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**Abstract**

The location of commonly used charged fluorescent membrane probes in membranes was determined in order to: (1) investigate the relationship between the structure of hydrophobic molecules and their depth within membranes; and (2) aid interpretation of experiments in which these fluorescent probes are used to examine membrane structure. Membrane depth was calculated using parallax analysis, a method in which the quenching induced by lipids carrying a nitroxide group at different locations in the membrane is compared. Shallow locations were found for xanthene dyes (fluorescein, eosin, Texas Red and rhodamine) both in free form and when attached either to the headgroup of phospholipids or long hydrocarbon chains. The exact structure of the xanthene and the nature of its linkage to lipid had only a modest effect on membrane location, which ranged between 19 and 24 Å from the center of the bilayer in a charged state. Thus, the location of these fluorophores largely reflects their intrinsic properties rather than the nature of the groups to which they are attached. Furthermore, cationic and anionic xanthene derivatives had similar depths, indicating the type of charge does not have a large effect on depth. Consistent with this conclusion, shallow locations were also found for other hydrocarbon chain-linked cationic (acridine orange and styrylpyridinium) and anionic (coumarin, anilinonaphthalenesulfonic acid (ANS), and toluidinylnaphthalenesulfonic acid (TNS)) charged probes. These all located at 16–18 Å from the bilayer center. We conclude that both anionic and cationic molecules that are otherwise hydrophobic predominantly occupy shallow locations within the polar headgroup region of the bilayer no matter how hydrophobic the molecule to which they are linked. This depth is significantly shallower than that occupied by most previously studied uncharged polar molecules that locate near the membrane surface. Consistent with this conclusion, a 2–4 Å deeper location was found for xanthene probes with no net

Abbreviations: ANS, 1-anilinonaphthalene-8-sulfonic acid; DDAF, 5-(*N*-dodecanoyl)aminofluorescein; DHDASP, 4-(4-dihexadecylaminostyryl)-*N*-methylpyridinium; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; ESR, electron spin resonance; fluorescein-PE, *N*-(5-fluoresceinthiocarbamoyl)-1,2 dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine; HDAE, 5-(*N*-hexadecanoyl)aminoeosin; HDAF, 5-(*N*-hexadecanoyl)aminofluorescein; HDANS, 2-(*N*-hexadecyl)-aminonaphthalene-6-sulfonic acid; HDHC, 3-hexadecanoyl-7-hydroxycoumarin; MANS, 2-(*N*-methylanilino)naphthalene-6-sulfonic acid; MLV, multilamellar vesicles; NBD, 7-nitro-2,1,3-benzoxadiazole-4-yl; ODAO, octadecyl acridine orange; ODF, octadecyl fluorescein; ODRB, octadecyl rhodamine B; OG, *n*-octyl β-D-glucopyranoside; PE, phosphatidylethanolamine; PSA, 1-pyrenesulfonic acid; rhodamine-PE, *N*-(lissamine rhodamine B sulfonyl)-1,2 dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine; 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))ammonium-2,2,6,6-tetramethylpiperidine-1-oxyl; TempoPC, 1,2-dioleoyl-*sn*-glycero-3-phosphotempocholine; Texas Red-PE, *N*-(Texas Red sulfonyl)-1,2 dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine; TLC, thin-layer chromatography; TNS, 2-(*p*-toluidinyl)naphthalene-6-sulfonic acid

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charge. In other experiments, methods to avoid chemical reactions that can distort the measurement of depth by fluorescence quenching were developed. 0005-2736/98/$ – see front matter 

*Keywords:* Fluorescence quenching; Nitroxide; Parallax analysis; Membrane structure; Ionization

## 1. Introduction

Fluorescence spectroscopy is a powerful method to examine the structure and function of biological membranes. The depth of a probe in a membrane is important to the interpretation of such experiments, but has often been difficult to determine. Thus, the relationship between chemical structure and membrane location has generally been difficult to define. In this study, parallax analysis, a fluorescence quenching method, was used to identify the membrane location of a series of fluorescent probes in order to define the relationship between chemical structure of charged molecules that are membrane bound and their location. Parallax analysis is a technique in which the amount of quenching induced by nitroxide-labeled phospholipids located at different depths in the bilayer is used to measure fluorophore depth [1,2]. In a series of studies, our group has shown that the method can specify average fluorophore depths at the 1–2 Å level of resolution [1–4], and we have used it to measure the depth of anthracene, anthroyloxy, carbazole, indole, NBD and phenol groups in membranes [1–6]. The method has also been used by several groups to study the orientation and location of polypeptides and proteins inserted into membranes [7–15].

In this study, parallax analysis has been applied to a series of commonly used membrane probes, including derivatives of fluorescein, rhodamine, eosin, aromatic sulfonic acid probes (such as ANS and TNS), coumarin, acridine orange, and styrylpyridinium. These probes have been applied to questions of membrane structure and function in well over 100 studies [16].

## 2. Materials and methods

### 2.1. Materials

ANS, TNS, HDAE, HDAF, DDAF, ODF, HDANS, MANS, ODRB, DHDASP, ODAO, HDHC, PSA, fluorescein-PE, rhodamine-PE, and Texas Red-PE were purchased from Molecular Probes. Eosin Y, rhodamine B, and fluorescein were purchased from Aldrich. OG was purchased from Sigma. Purity of the fluorophores were checked by TLC and (except for the fluorescent phospholipids, see below) all gave a single fluorescent spot. A solvent system composed of $CHCl_3$/methanol in varying ratios was used for all the non-phospholipid fluorophores. The identity of the fluorescent probes was confirmed by their excitation and emission spectra which corresponded closely to expected values [16]. The purity of the phospholipids was checked by TLC on silica gel plates using chloroform/methanol/water (65:25:4, v/v) as the developing solvent. Rhodamine-PE and fluorescein-PE showed minor contaminants, with the former about 98% pure and the latter $>96\%$ as judged by measuring the relative fluorescence intensities of the main spot and impurities after extraction with ethanol. The identity of the fluorescent probe-labeled phospholipids was confirmed by their emission and excitation spectra, which corresponded to expected values. Stock solutions of the fluorescent-labeled phospholipids were prepared in ethanol and their concentration was determined by measuring the absorbance of the fluorophore using the reported molar extinction coefficient ($M^{-1}$ $cm^{-1}$) at the appropriate wavelengths [16]: for fluorescein-PE at 497 nm, 75 000; rhodamine-PE at 563 nm, 73 000; Texas Red-PE at 584 nm, 100 000.

TempoPC, 5-SLPC, 12-SLPC and DOPC were purchased from Avanti Polar Lipids. The concentration of phospholipids was determined by phosphate assay subsequent to total digestion [3] or by dry weight. The actual nitroxide content on the nitroxide-labeled lipids was calculated from the intensities of the doubly integrated ESR spectra as described previously [1]. Alternatively, nitroxide concentration was assayed from the fluorescence quenching of 2- and 12-anthroyloxy fatty acids by determining the % of uncalibrated nitroxide-labeled lipid that had to be incorporated into multilamellar vesicles (MLV) to

give the same quenching as an ESR-calibrated sample with 15% nitroxide-labeled lipid [2].

### 2.2. Preparation and fluorescence measurements of samples for lipid binding studies

Model membranes vesicle samples were prepared with a fixed concentration of fluorophore (0.5 μM for ODAO, fluorescein, rhodamine B and eosin Y, 1 μM for the hydrocarbon-linked xanthene dyes, DHDASP and PSA; 2 μM for the other aromatic sulfonic acids, and HDHC) and various concentrations of DOPC. The mixtures of DOPC and fluorophore in organic solvent were dried under $N_2$, resuspended in 20 μl ethanol, vortexed, diluted with 980 μl buffer, and then vortexed briefly. Ten mM Na phosphate, 150 mM NaCl pH 7 was used except for HDAE, ODF, HDAF, DDAF, eosin Y, rhodamine B where 10 mM glycine, 150 mM NaCl, pH 10 was used (in order to fully ionize carboxyl and/or hydroxyl groups (see below)), and rhodamine B and fluorescein for which 10 mM glycine, 150 mM NaCl pH 3 was used. Duplicate samples with fluorophore and single background samples lacking fluorophore were prepared.

The solutions were placed in a 1 cm semi-micro quartz cuvette and their fluorescence intensity measured with a Spex 212 Fluorolog Spectrofluorimeter operating in ratio mode. The excitation and emission slits were set between 0.2 and 2.5 mm (0.85–10.6 nm bandpass) so as to prevent saturation of the photomultiplier tube. The excitation and emission wavelength settings (ex:em) for each fluorophore (in nm) was as follows: ODF (490:521), HDAF (483: 521), DDAF (500:520), ODRB (559:583), HDAE (527:542), eosin Y (524:542), rhodamine B at pH 3 (564: 583), rhodamine B at pH 10 (553:574), fluorescein (433:515), HDANS (352:418), MANS (315: 423), ANS (373:481), TNS (320:444), PSA (346: 375), DHDASP (467:560), HDHC (426:456), and ODAO (496:521). For all samples, two to three 10 s readings were taken and then averaged. Background intensities were subtracted from reported values.

### 2.3. pH Titration: sample preparation and fluorescence measurements

Single vesicle samples containing 200 μM DOPC and 1–2 μM fluorophore in a final volume of 1 ml pH 5 buffer (10 mM acetate, 150 mM NaCl) were prepared as described above. The solutions were titrated down to pH 1.5 with 1 M HCl then titrated upwards with small aliquots of 0.1–1 N NaOH. Control experiments demonstrated pH equilibration across the bilayer after NaOH addition is rapid (S. Lew and E. London, unpublished observations). After each addition of NaOH, fluorescence intensity was measured as described above. Excitation was measured at the same wavelengths as above, with the exception of DDAF, for which excitation at 492 nm was used. Emission was followed at two wavelengths in order to detect spectral shifts. The emission wavelengths used were: ODF (511, 521), HDAF (505, 516), DDAF (512, 519), ODRB (583, 598), HDAE (542, 552), HDANS (418, 430), MANS (410, 421), ANS (481, 511), PSA (375, 391), DHDASP (540, 560), HDHC (457, 470), and ODAO (521, 541).

### 2.4. Preparation of samples for quenching experiments

Vesicles prepared by ethanol or octylglucoside dilution were used for the quenching experiments with the non-phospholipid probes. For vesicles prepared by ethanol dilution, DOPC or mixtures of 15 mol% nitroxide labeled PC[2] with DOPC were combined with fluorophore in ethanol. The final concentration of lipid was 200 μM, except for PSA (500 μM lipid), ANS (500 μM), TNS (600 μM), rhodamine B (500 μM, pH 3; 800 μM, pH 10), eosin Y (800 μM) and fluorescein (1000 μM)[3]. The final concentration of fluorophore was as used in the binding experiments (see above). The mixtures were dried under $N_2$, resuspended in 20 μl ethanol and then vortexed. Next

[2] The nitroxide-labeled lipid contains some molecules that lack a nitroxide group. The amount of nitroxide-labeled lipid is adjusted to give 15% nitroxide-containing molecules and 85% other lipid molecules (DOPC plus inactive 'quencher').

[3] These concentrations were chosen to obtain as complete a binding as possible. For ANS, MANS, TNS, and PSA experiments in which a second lipid concentration 2–4 fold higher or lower than these values were performed to confirm that depth did not depend on lipid concentration (not shown). $z_{cf}$ values at the second concentration were within 0.5–1 Å of those reported in the tables (not shown).

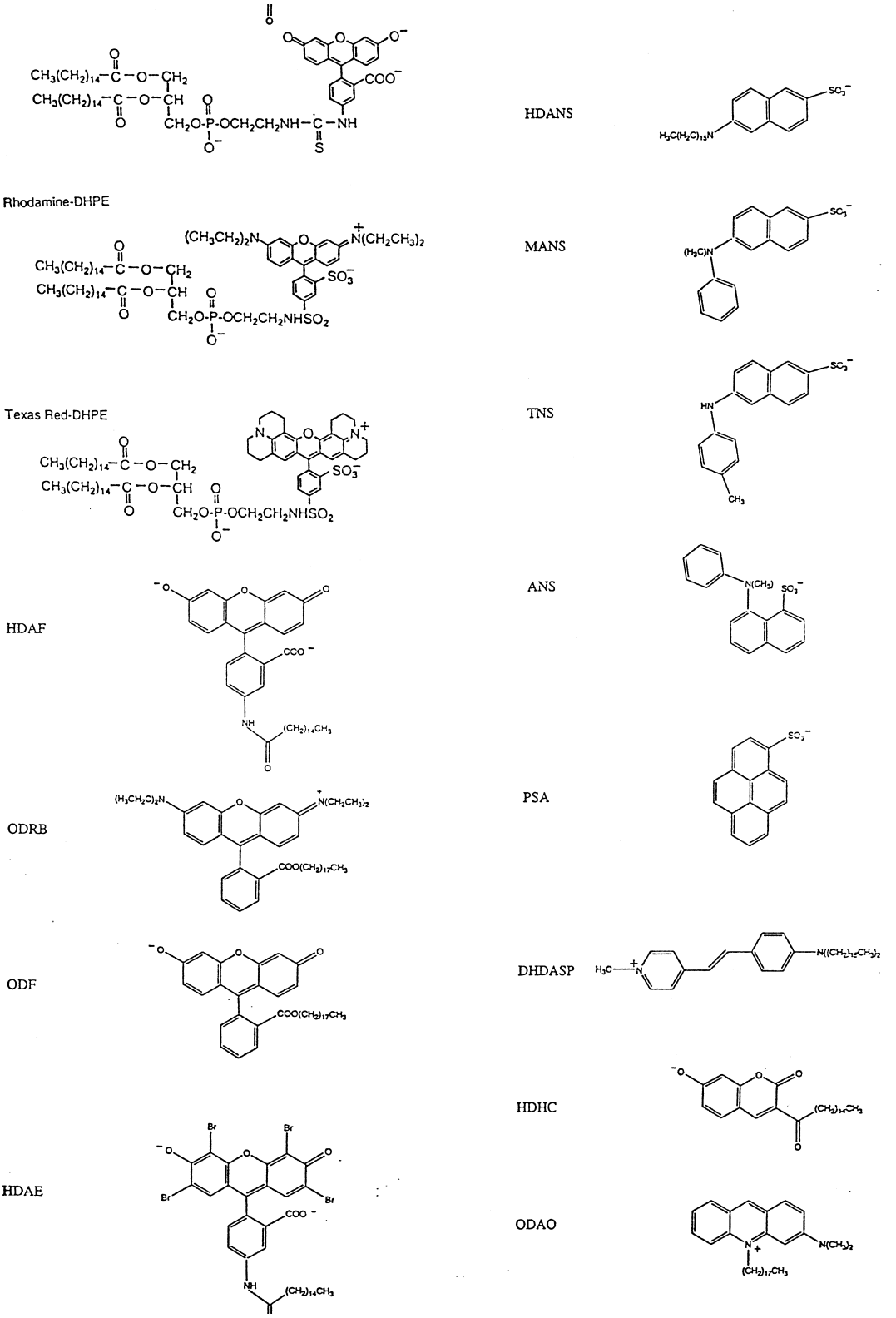


Fig. 1. Chemical structure of the probes used in this study. The ionization states shown are those predominating at pH 7 and above. The free xanthene dyes (not shown) differ from the derivatives shown in the figure in that they lack both the hexadecyl and amide groups of HDAE, HDAF or the octadecanol attached to ODRB.

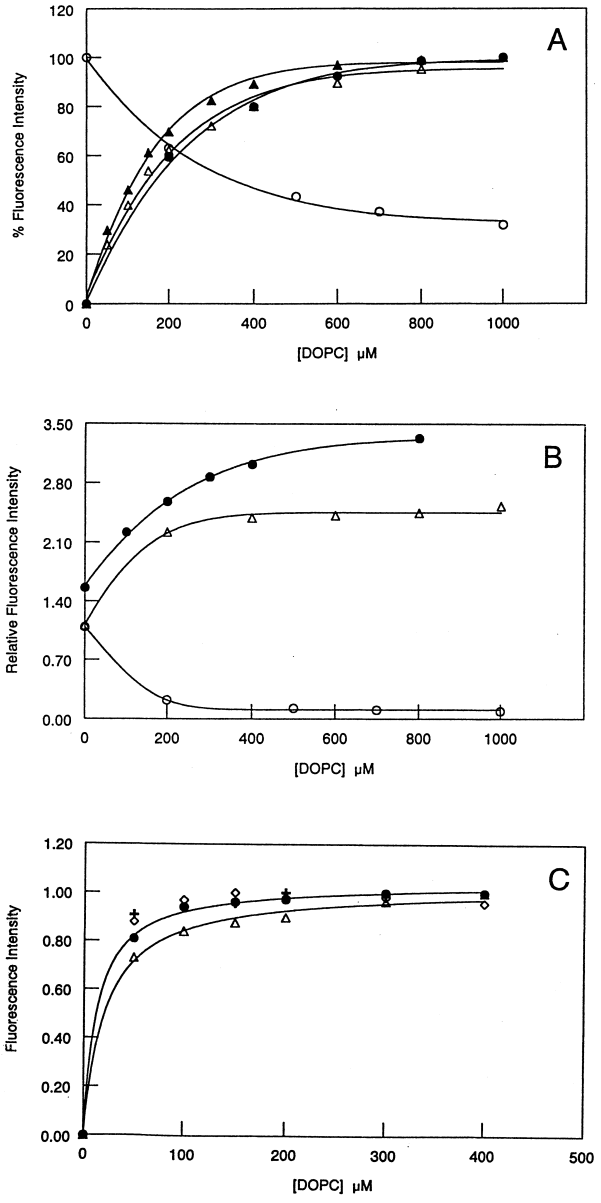


Fig. 2. Binding of probes to DOPC vesicles. (A) Effect of DOPC concentration on the fluorescence of (▲) ANS at pH 7, (△) TNS at pH 7, and (●) MANS at pH 7. The effect of lipid concentration on fluorescence is shown normalized to % of maximal intensity. The change in % fluorescence intensity for rhodamine B at pH 10 was similar to that for these compounds in this panel. Also shown is the effect of 15 mol% TempoPC/85 mol%DOPC concentration on the fluorescence of (○) PSA, at pH 7. (B) Effect of DOPC concentration of the fluorescence of (●) eosin Y at pH 10, (△) rhodamine B at pH 3, and (○) fluorescein at pH 3. (C) Effect of DOPC concentration on fluorescence of (●) DDAF at pH 10, (△) DHDASP at pH 7, (+) HDAE at pH 10, and (◇) ODRB at pH 7. The effect of lipid concentration on fluorescence is shown normalized to the fractional change in fluorescence intensity. The change in fluorescence intensity profile for HDAF, ODF, and HDHC (all at pH 10), and ODAO and HDANS (both at pH 7) was similar to the compounds in this panel. The actual maximal fluorescence enhancement in the presence of DOPC vesicles relative to in buffer was: 65-fold for ODF, 2.1-fold for HDAF, 2.7-fold for DDAF, 74-fold for ODRB, 372-fold for HDAE, 13-fold for HDANS, 280-fold for MANS, 270-fold for ANS, 590-fold for TNS, 4800-fold for DHDASP, 1000-fold for HDHC, 360-fold for ODAO, 2.1-fold for eosin Y, 2.3-fold for rhodamine B (pH 3) or 1.6-fold (pH 10), 0.08-fold for fluorescein. Little or no effect of DOPC on fluorescence was seen for PSA. ←

980 μl buffer was added and vortexed briefly. Ten mM Na phosphate, 150 mM NaCl pH 7 was used for all the compounds except for rhodamine B, HDAE, ODF, HDAF, DDAF, eosin Y and HDHC where 10 mM glycine, 150 mM NaCl, pH 10 was used; fluorescein and rhodamine B where 10 mM glycine, 150 mM NaCl pH 3 was used; and ODF where 10 mM Na acetate, 150 mM NaCl pH 4.6–5 was used.

For vesicles prepared by octyl glucoside dilution, lipids were combined with 1 μmol OG in ethanol and dried under $N_2$. Next, the desired amount of fluorophore and octylglucoside were mixed in ethanol, dried, and dissolved in water to give an OG concentration of 50 mM. Then 20 μl of this solution was added to the dried lipid and mixed thoroughly. The final volume was then brought to 1 ml by adding 980 μl buffer and the samples vortexed briefly. The final concentration of OG was 2 mM. The final concentration of lipid and fluorophore used was the same as in ethanol dilution vesicles.

In general, three quenching experiments were run for each probe, two with ethanol dilution vesicles and one with OG dilution vesicles. (For the free xanthene dyes only two experiments using the ethanol dilution method were performed.) In each experiment duplicate samples containing fluorophore and lipid were prepared along with corresponding backgrounds containing only lipid. $z_{cf}$ values (see below) generally were reproducible within 0.5 Å when individual experiments were compared.

That intact vesicles were formed by the octyl glucoside and ethanol dilution procedures was confirmed by dithionite reduction of DOPC vesicles with trace NBD-PE. The fractional reduction of

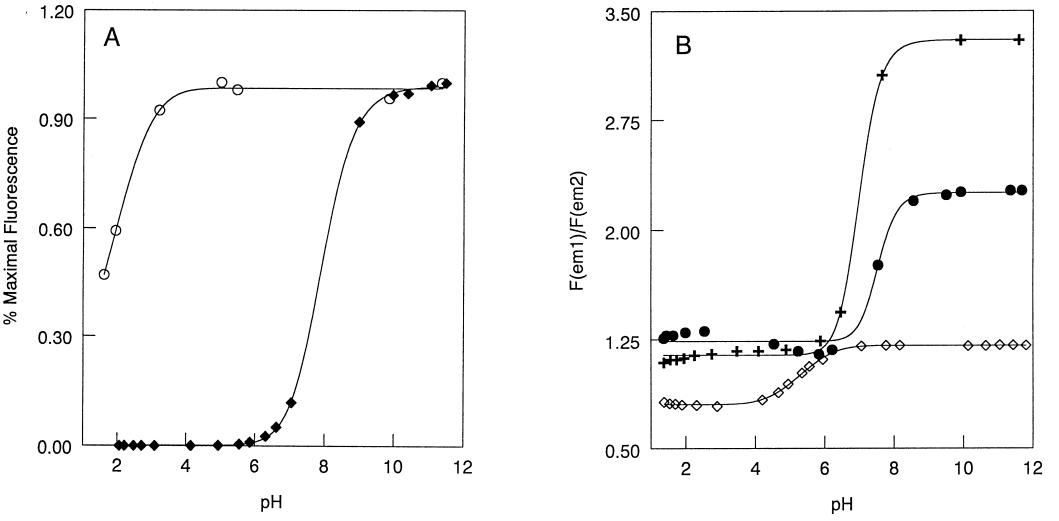


Fig. 3. Ionization of probes in the presence of 200 μM DOPC. (A) Effect of pH on fluorescence intensity of: (○) HDANS (for emission at 457 nm); (◆) HDHC (for emission at 418 nm). (B) Effect of pH on ratio of intensity at different emission wavelengths (see Section 2 for wavelengths used): (◇) HDAE, (●) HDAF and (+) ODF. At the pH values used in the quenching studies, fluorescence arises almost entirely from SUV-bound probes in the presence of 200 μM DOPC.

NBD-PE corresponded to that expected for intact unilamellar vesicles [17]. The amount of reduction with octyl glucoside vesicles (50%) suggested they were somewhat larger than SUV.

### 2.5. Preparation of phospholipid probe samples for depth measurements

Vesicles prepared by octylglucoside dilution were also used for the depth measurements with the fluorescent-labeled phospholipids. Mixtures of OG and fluorophore and lipid were prepared as above and hydrated with 10 mM Na acetate, 150 mM NaCl pH 6.6–7 for Texas Red-PE and rhodamine-PE or 10 mM glycine, 150 mM NaCl pH 3 or pH 10 for fluorescein-PE. The remainder of sample preparation was as described above. In each experiment samples were prepared in duplicate. The average of duplicate experiments is reported. The final concentration of the fluorescent-labeled phospholipids was: 0.05 μM for fluorescein-PE, 0.20 μM for Texas Red-PE, and 0.4 μM for rhodamine-PE. The final total lipid concentration was 100 μM.

Single background samples without the fluorophore were prepared similarly except the fluorophore/OG mixture was replaced by 20 μl of buffer.

For the phospholipid probes, calculated $z_{cf}$ values derived from vesicles made by the octylglucoside procedure were again very similar to that observed when vesicles were prepared by the ethanol dilution procedure (not shown).

### 2.6. Measurement of nitroxide quenching and calculation of depth

Fluorescence of the non-phospholipid probes was measured in 1 cm semimicro quartz cuvettes as described for the binding studies above, with the exception of ODF, for which emission was monitored at 524 nm (pH 5) or 538 nm (pH 10). After fluorescence was measured, 100 μl aliquots were removed and diluted nine-fold with absolute ethanol. Fluorescence intensities were then measured on these samples to check for reactions that might have occurred during sample preparation or in the vesicles. In most cases the ethanol-dissolved samples with or without nitroxide-labeled lipid gave about equal intensities (within 5%). For a few compounds the ethanol-dissolved samples with quenchers were 10–30% more fluorescent than without quencher, suggesting a bleaching reaction destroying the fluorophore in the absence of quencher (see Section 3. In these cases, $F/F_o$ values were corrected to what they would have been in the absence of reaction calculated by dividing raw $F/F_o$ values by the ratio: (intensity in ethanol-dissolved sample with 15% nitroxide-labeled lipid/in-

Table 1
Quenching of xanthene dye probes by nitroxide-labeled lipids[a,b]

| Fluorescent probe | $F_{tc}/F_o$ | $F_5/F_o$ | $F_{12}/F_o$ | $z_{cf}'$ | $z_{cf}$ |
|---|---|---|---|---|---|
| Octadecyl fluorescein (ODF) pH 5 | 0.34 | 0.54 | 0.66 | 20 | 20 |
| | 0.34 | 0.52 | 0.62 | 20 | |
| Octadecyl fluorescein (ODF) pH 10 | 0.18 | 0.40 | 0.54 | 24 | 23.5 |
| | 0.22 | 0.44 | 0.56 | 23 | |
| Hexadecylaminofluorescein[c] (HDAF) pH 10 | 0.22 | 0.40 | 0.46 | 22 | 21.5 |
| | 0.26 | 0.43 | 0.51 | 21 | |
| Dodecylaminofluorescein (DDAF) pH 10 | 0.28 | 0.46 | 0.56 | 20.5 | 20.5 |
| | 0.31 | 0.49 | 0.59 | 20.5 | |
| Octadecyl rhodamine B (ODRB) pH 7 | 0.43 | 0.58 | 0.67 | 19 | 19 |
| | 0.49 | 0.63 | 0.69 | 18.5 | |
| Hexadecylaminoeosin (HDAE) pH 9.5 | 0.31 | 0.62 | 0.62 | 23 | 22.5 |
| | 0.36 | 0.65 | 0.64 | 22 | |
| Fluorescein pH 3 | 0.76 | 0.86 | 0.88 | 17.0 | 17.0 |
| Rhodamine B pH 10 | 0.80 | 0.86 | 0.88 | 16.5 | 16.5 |
| Rhodamine B pH 3 | 0.58 | 0.75 | 0.80 | 18.5 | 18.5 |
| Eosin Y pH 10 | 0.47 | 0.62 | 0.72 | 18.5 | 18.5 |
| Fluorescein-PE pH 10 | 0.25 | 0.41 | 0.47 | 21 | 21 |
| Fluorescein-PE pH 3 | 0.72 | 0.81 | 0.79 | 17 | 17 |
| Rhodamine-PE pH 7 | 0.45 | 0.64 | 0.69 | 19.5 | 19.5 |
| Texas Red-PE pH 7 | 0.49 | 0.71 | 0.73 | 19.5 | 19.5 |

[a] $F_{TC}/F_o$, $F_5/F_o$ and $F_{12}/F_o$ are $F/F_o$ values which give the ratio of fluorescence intensity in vesicles containing 15 mol% TempoPC, 5-SLPC, or 12-SLPC, respectively, to that in DOPC vesicles lacking nitroxide-labeled lipid. Where two sets of values are given, the top line of values are for vesicles prepared by ethanol dilution, and the bottom line, unilamellar vesicles prepared by octylglucoside dilution.
[b] $z_{cf}'$ is the depth for each individual type of preparation. $z_{cf}$ is the average of the $z_{cf}'$ values.
[c] HDAF depth was unaffected by the presence of 11 mM EDTA.

tensity in ethanol-dissolved vesicles with DOPC alone).

The fluorescence in samples containing fluorescent phospholipids was measured similarly. The excitation and emission wavelengths (ex:em) used were (in nm): (457:518) for fluorescein-PE at pH 3 and (505:525) at pH 10, (590:609) for Texas Red-PE, and (571:590) for rhodamine-PE. The excitation and emission slits were set between 1.25 and 5.0 mm (2.25–9 nm bandpass) so as to prevent saturation of the photomultiplier tube. Samples were vortexed and then fluorescence intensity was measured at room temperature and averaged over three 6 s readings. The fluorescence intensity from duplicate samples containing the fluorophore was averaged, and the intensity of the background samples without fluorophore was subtracted. (Background intensity was less than 3% of the sample intensity.)

Using the corrected $F/F_o$ values the distance of fluorophores from the center of the bilayer was calculated using the parallax equation [1,2]:

$$z_{cf} = L_{c1} + (-\ln(F_1/F_2)/\pi C - L_{21}^2)/2L_{21}$$

where $z_{cf}$ is the distance of the fluorophore from the center of the bilayer, $F_1$ is the fluorescence intensity ($F/F_o$) in the presence of the shallow quencher (quencher 1), $F_2$ is the fluorescence intensity ($F/F_o$) in the presence of the deeper quencher (quencher 2), $L_{c1}$ is the distance of the shallow quencher from the center of the bilayer, $L_{21}$ is the distance between the shallow and deep quenchers, and the $C$ is the concentration of quencher in molecules/Å$^2$. The quenching by the pair of quenchers (i.e. nitroxide-labeled lipid) that quench the most (i.e. the TempoPC/5-SLPC pair or 5-SLPC/12-SLPC pair) is used to calculate $z_{cf}$ [2]. The values used for the distances of the nitroxide group from the bilayer center were 5.85 Å for 12-SLPC, 12.15 Å for 5-SLPC, and 19.5 Å for TempoPC [1,2].

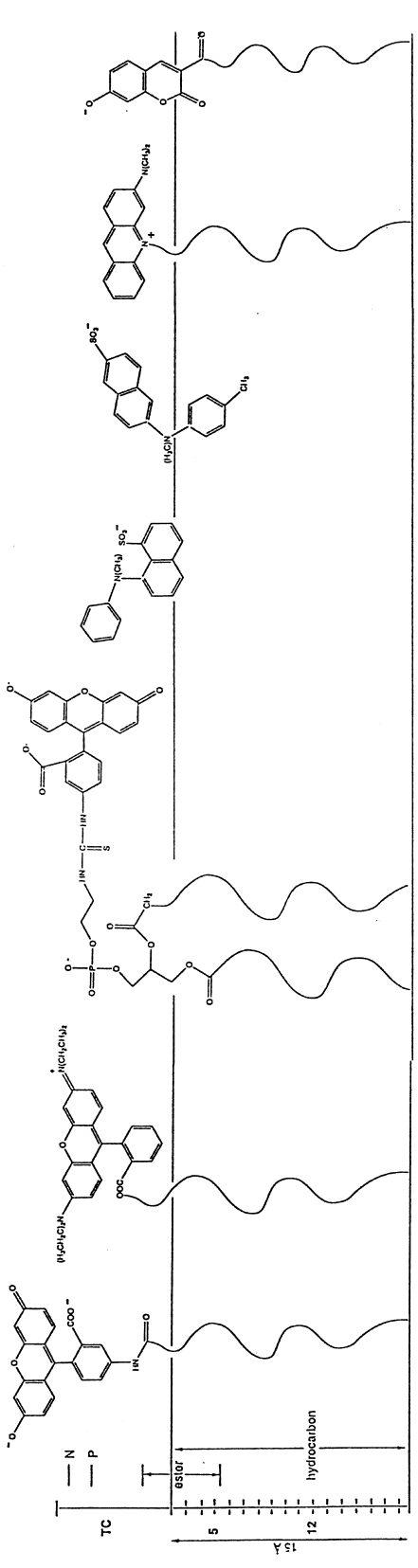


Fig. 4. Schematic figure illustrating the depth of hydrocarbon and acyl linked fluorescent probes. Identity of probes is given in Fig. 1. The location of the phospholipid functional groups is based on the study of Wiener and White [35]. The positions of the nitroxide spin-labels from the bilayer center are shown at the left.

### 2.7. Preparation of MLV

To examine the effect of vesicle preparation procedure on chemical reaction between fluorophore and nitroxide-labeled lipids MLV containing fluorophores were prepared as previously described [2]. MLV samples contained identical amounts of fluorescent-labeled lipid and spin-labeled PC as in ethanol dilution vesicles (see above).

## 3. Results

### 3.1. Binding of fluorescent probes to model membranes

The behavior of a variety of charged fluorescent probes (Fig. 1) was examined. Since such probes can have a significant solubility in aqueous solution, the conditions under which they would bind to model membrane vesicles had to be determined[4]. Fig. 2 shows the binding of the probes to model membrane unilamellar vesicles, as monitored by the change in fluorescence intensity in the presence of increasing concentrations of lipid. In general, there was an increase in fluorescence upon binding to vesicles composed of DOPC, although there was a decrease upon fluorescein binding to vesicles at pH 3. Pyrenesulfonic acid did not show a significant change in intensity in the presence of DOPC vesicles. To evaluate its binding to model membranes, quenching of its fluorescence upon binding to 85% DOPC/15% TempoPC vesicles was measured[5]. For almost all probes, the change in probe fluorescence (and thus probe binding) was near maximal in the range 100–500 μM lipid. Lipid concentrations for subsequent experiments were based on this information.

### 3.2. Ionization of model membrane bound fluorescent probes

Since many of the probes used in this study have

[4] This was not done for the phospholipid-linked probes, which should be as water insoluble as unlabeled phospholipids.

[5] Previous studies have shown that the presence of 15% nitroxide-labeled lipid does not influence fluorophore binding [3].

Table 2
Quenching of hydrophobic sulfonic acid probes by nitroxide-labeled lipids

| Fluorescent probe | $F_{tc}/F_o$ | $F_5/F_o$ | $F_{12}/F_o$ | $z_{cf}'$ | $z_{cf}$ |
|---|---|---|---|---|---|
| Hexadecyl aminonaphthalene (HDANS) pH 7 | 0.23 | 0.23 | 0.33 | 15.5 | 15.5 |
| | 0.27 | 0.26 | 0.36 | 15.5 | |
| Methylanilinonaphthalenesulfonate (MANS) pH 7 | 0.36 | 0.35 | 0.42 | 15.5 | 16.0 |
| | 0.38 | 0.40 | 0.40 | 16.0 | |
| Anilinonaphthalenesulfonate (ANS)[a] pH 7 | 0.30 | 0.39 | 0.51 | 18 | 18.0 |
| | 0.37 | 0.45 | 0.53 | 18 | |
| Toluidinyl naphthalenesulfonate (TNS) pH 7 | 0.31 | 0.32 | 0.47 | 16 | 15.5 |
| | 0.40 | 0.38 | 0.48 | 15 | |
| Pyrenesulfonate (PSA) pH 7 | 0.34 | 0.38 | 0.39 | 17.0 | 17.0 |
| | 0.35 | 0.39 | 0.40 | 17.0 | |

[a]ANS depth was unaffected by the presence of 11 mM EDTA.

properties (including fluorescence) that depend on their ionization state, changes in their ionization were monitored by the pH dependence of fluorescence (Fig. 3). Fluorescein derivatives, hexadecylaminoeosin and hexadecanoylhydroxycoumarin showed a prominent ionization with a p$K_a$ around 5–8. Therefore, the depth of these dyes was generally studied either significantly above pH 7 to obtain the fully ionized form, or at low pH to examine the protonated form. Octadecyl rhodamine B, octadecyl acridine orange, dihexadecylaminostyrylpyridinium, and all the sulfonic acid derivatives (not shown except for hexadecylaminoapthalenesulfonate) exhibited a p$K_a$ at 2 or below, and their depth was generally examined at pH 7.

### 3.3. Quenching of fluorescent probes by nitroxide-labeled lipids

The quenching of the fluorescent probes was measured in unilamellar vesicles containing shallow (TempoPC), medium (5-SLPC), or deep (12-SLPC) nitroxide-labeled PCs. The relative amount of fluorescence quenching obtained from the different nitroxide-labeled lipids was used to calculate fluorophore depth using the parallax equation (see Section 2). In several cases quenching data were obtained from vesicles prepared by different procedures to see if the method of vesicle preparation would effect the results (see tables). No significant effect of preparation method on depth was observed.

### 3.4. Depths of anchored xanthene dyes

The xanthene dyes include rhodamine, fluorescein, Texas Red and eosin derivatives. The location of several hydrocarbon-anchored xanthene dyes was examined first (Table 1 and schematic illustration in Fig. 4). In all cases, the fluorescent xanthene moieties of hydrocarbon-attached fluorescein, rhodamine and eosin were found to be located in the polar headgroup region of the bilayer, 18–24 Å from the bilayer center. The length of the alkyl chain to which the xanthene dye was attached seemed to have little effect on depth. This is shown by the behavior of the hexadecylaminofluorescein and dodecylaminofluores-

Table 3
Quenching of miscellaneous hydrocarbon-linked fluorophores by nitroxide-labeled lipids

| Fluorescent probe | $F_{tc}/F_o$ | $F_5/F_o$ | $F_{12}/F_o$ | $z_{cf}'$ | $z_{cf}$ |
|---|---|---|---|---|---|
| Dihexadecanoylaminostyryl pyridinium (DHDASP) pH 7 | 0.68 | 0.82 | 0.86 | 17.5 | 17.5 |
| | 0.74 | 0.88 | 0.90 | 17.5 | |
| Hexadecanoyl hydroxycoumarin (HDHC) pH 10 | 0.15 | 0.16 | 0.22 | 17 | 17 |
| | 0.15 | 0.16 | 0.31 | 17 | |
| Octadecyl acridine orange (ODAO) pH 7 | 0.44 | 0.56 | 0.67 | 18 | 18 |
| | 0.45 | 0.58 | 0.64 | 18 | |

cein, which have alkyl tails differing in chain length by four carbons, yet located within 1 Å of each other. Phospholipid-anchored xanthenes also located within the polar headgroup region of the bilayer (17–21 Å from the bilayer center). Therefore, the structure of the hydrophobic anchor (alkyl vs. phospholipid) has little effect on the membrane location of these probes.

### 3.5. Location of free xanthene dyes in membranes

The behavior of the hydrocarbon and phospholipid-linked xanthene dyes raised the question of whether the depth of the xanthene group was influenced by its attachment to a hydrophobic anchor. To examine this, the behavior of free xanthene dyes was examined. Fluorescein, rhodamine B, and eosin Y were all able to bind to zwitterionic PC model membranes (Fig. 2), suggesting they have some degree of hydrophobic interaction with membranes. Their depth in the membrane bound state was then measured. They all located in the polar region of the bilayer (17–19 Å from the bilayer center), suggesting the lack of attachment to an anchor had little effect on depth.

### 3.6. Effect of probe ionization state and other environmental factors on depth

Additional experiments demonstrated ionization state affects xanthene probe depth. For octadecyl fluorescein depth changed from 20 Å from the center of the bilayer in the protonated state at pH 5, to 23–24 Å in the ionized, anionic form at pH 10[6]. A similar result was found for fluorescein-PE (Table 1). At pH 10, the ionized, anionic form of fluorescein-PE was located 21 Å from the bilayer center but its depth decreased to 17 Å at pH 3 where the fluorescein group is protonated[7]. Interestingly, an opposite effect of pH was observed for rhodamine B, which located 2 Å more deeply at pH 10 than at pH 3. This difference probably reflects the fact, in contrast to fluorescein, that rhodamine B is cationic (+1) at low pH, and uncharged in the deprotonated state

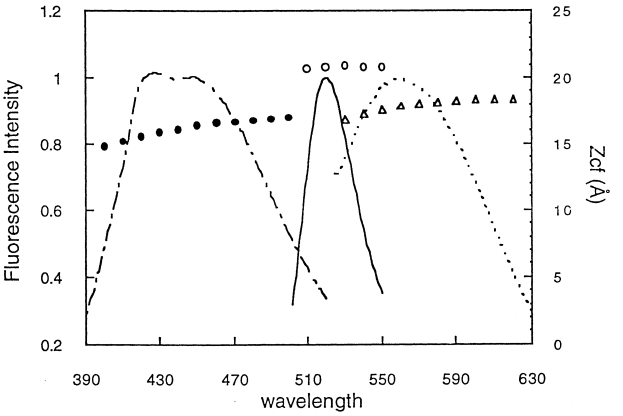


Fig. 5. Dependence of apparent depth of fluorophores on wavelength. Top panel: emission spectra (lines) and measured $z_{cf}$ values (points) for (-·-·-, ●) MANS, (———, ○) DDAF, and (....., △) DHDASP. Relative fluorescence is shown normalized to a value of 1 at the wavelength of maximum intensity.

at high pH. Thus, in all cases the loss of charge on a membrane-bound molecule induced movement to a deeper depth. Combined with our previous results using fluorescent fatty acids [2], it appears that in general for molecules that have ionizable groups localized in the polar headgroup region there can be a 2–4 Å deeper location in the uncharged state (see Section 4.

The effects of ionic strength and lipid composition were also examined. No significant change in depth (i.e. 1 Å or less) was found for ionized fluorescein-PE in membranes containing 50 mol% of the anionic lipid DOPG relative to that in 100 mol% PC (data not shown). In addition, the absence of 150 mM NaCl in the buffer also had no significant effect on fluorescein-PE depth (data not shown). These results suggest that lipid charge and ionic strength may have little influence on the depth of a charged fluorophore.

### 3.7. Depth of aromatic sulfonic acid and other probes

Aromatic sulfonic acids are another important group of membrane probes. This group includes

[6] This experiment could not be done for free fluorescein, which did not bind strongly to DOPC vesicles at high pH.

[7] Since the final ionization of model membrane bound fluorescein occurs at about 7.5 [18] it should be in the dianion form at pH 10. Its ionization state at pH 3 (monoanion or un-ionized) is difficult to determine, as its fluorescence is similar in these states [19]. This is not an issue for octadecylfluorescein which has a blocked carboxyl and so only one ionizable group.

the frequently used probes anilinonaphthalenesulfonate (ANS) and toluidinylnaphthalenesulfonate (TNS). Table 2 shows aromatic sulfonic acids locate at 16–18 Å from the bilayer center, again well within the polar headgroup region. These molecules could be oriented with their charged sulfonic acid group anchored at a shallow location, and their non-polar aromatic portion extending towards the hydrocarbon part of the bilayer.

The depth of several additional hydrocarbon chain linked fluorophores were also examined (Table 3). The cationic probes octadecyl acridine orange and dihexadecylaminostyrylpyridinium, and the anionic hexadecyl hydroxycoumarin also all occupy shallow locations with $z_{cf}$ = 17–18 Å.

### 3.8. Wavelength dependence of depth

The $z_{cf}$ values given above were determined measuring fluorescence emission near the emission wavelength maximum. We previously found the measured $z_{cf}$ value can be a function of the wavelength at which fluorescence emission is measured [2,3,5]. This wavelength dependence is a complex combination of several factors [2,3,20–22].

The dependence of depth on emission wavelength was also examined for a number of the probes used in this report. As in previous studies, at most only a weak dependence of depth on emission wavelength was found. This was not only true for dodecylaminofluorescein, dihexadecylaminostyrylpyridinium and methylanilinonaphthalenesulfonate as shown in Fig. 5, but also hexadecylaminoeosin, octadecyl acridine orange, hexadecanoyl hydroxycoumarin, fluorescein-PE and octadecyl rhodamine B (not shown).

### 3.9. Avoiding reactions that can distort fluorescent quenching

The membrane probes examined in this study are chemically complex, and in early experiments we detected chemical reactions that affected fluorescence by dissolving the vesicles in ethanol in order to abolish nitroxide-induced fluorescence quenching effects. It was found that significant intensity differences persisted after ethanol solubilization between samples containing nitroxide-labeled lipids and those containing only unlabeled lipids in MLV samples. In addition, wavelength shifts in fluorescein and rhodamine absorbance and emission spectra were observed after dissolving nitroxide-containing MLV samples. The degree of shifting was different for each of the nitroxide-labeled lipids. These changes suggested some sort of chemical reaction was occurring. We determined that the reactions causing these changes largely occurred during the relatively long co-incubation of fluorophore and nitroxide-labeled lipid in organic solvent that was part of the MLV preparation (not shown). Therefore, all subsequent experiments were done in unilamellar vesicles[8].

It was also found that (over several hours) time-dependent reactions would occur subsequent to vesicle preparation. In some cases there was a bleaching of fluorescence which was greatest in samples with nitroxide-labeled phospholipids (e.g. with hexadecylhydroxycoumarin), whereas in other cases there was a bleaching which was greatest when only unlabeled lipid was present (e.g. with hexadecylaminoeosin). Again, in some cases, wavelength shifts were observed. To avoid artifacts due to these reactions, quenching measurements were made immediately after sample preparation, and when necessary, appropriate ethanol solubilization controls were performed (see Section 2.6).

Interestingly, there was no evidence of reactivity for the probes we had studied previously (data not shown) in the vesicles appropriate to those studies, including carbazole, indole, and phenol groups in SUV [6], and anthracene and anthroyloxy derivatives in MLV[9] [1,2,5]. Presumably, the lack of reaction reflects a lack of reactive groups on most of these probes.

[8] In addition, use of unilamellar vesicles avoids the possibility that quenchers in one bilayer quench a fluorophore in an adjacent bilayer. This was of particular concern in this study because the fluorophores used locate at the surface of the vesicles.

[9] In the case of NBD, some bleaching reaction was observed, but only in samples lacking nitroxide that had been dried during sample preparation for a prolonged period (over 1 h). This should not have affected earlier results.

## 4. Discussion

### 4.1. Aromatic charged probes locate at the polar surface of membranes

The charged aromatic molecules examined in this study were found to locate at the polar surface of membranes (Fig. 4). Presumably, this localization occurs because they are aromatic molecules, which gives them significant hydrophobic character and an affinity for the bilayer, but have polar and charged groups prohibit their deep burial within the hydrocarbon region of the bilayer. These results suggest deep burial of a charged group in a membrane would require attachment to even more hydrophobic molecules than those tested in this study, or perhaps interaction with a buried counterion.

The precise location of the charged group itself (e.g. the sulfonic acid group on ANS) can only be estimated from our studies because in many cases the probe molecule should be able to orient with its charged group at a shallower location than the remainder of the molecule. Nevertheless, combining the results of this study with our earlier ones [2,5] strongly suggests a charged group will rarely be closer than 16–18 Å from the center of the bilayer.

Differences in the chemical structure of these molecules had only a modest affect on depth. An example of this is the similar depth found for the different xanthene dyes (eosin, fluorescein, rhodamine, Texas Red) despite the wide variety of different substituents on the xanthene rings (Fig. 1). In addition, the presence of a membrane anchoring hydrocarbon chain or lipid had little effect on depth. This suggests that in general their depth is determined by their intrinsic polarity, and is not strongly influenced by the nature of the molecule to which they are attached. Interestingly, the type of charge on a membrane probe does not seem to be critical for depth. There was no significant difference between the anionic and cationic probes. This observation could have general significance. Extrapolating to amino acid residues would suggest that placing anionic or cationic residues at the ends of hydrophobic sequences would have similar effects on their location in membranes. We plan to examine this proposal directly with transmembrane helix-forming peptides [15].

It should be noted that the average $z_{cf}$ values we observed here may not fully represent the distribution of these probes in the bilayer. For a fluorophore depth having a single average depth, quenching should drop gradually as the difference in fluorophore and quencher depth increases [1]. However, when a fluorophore has separate shallow and deep populations this pattern may not be observed [33]. In an extreme case, the shallowest and deepest nitroxides quench more than the intermediate one [34]. There is evidence of such subpopulations for a few of the probes examined in this study. For example, hexadecylaminoeosin shows quenching by the deep nitroxide of 12-SLPC about as strong as that by the medium depth nitroxide 5-SLPC despite its overall shallow depth as shown by the very strong TempoPC quenching. This suggests there is a small subpopulation of deep eosin groups in addition to the predominant shallow population. Several other probes show similar behavior. Nevertheless, it can be shown that when there is only a small subpopulation the depth obtained by the parallax analysis is very close to the true average $z_{cf}$ [33].

### 4.2. The effect of charge on depth

The most significant difference in depth was observed between the charged and uncharged forms of the fluorescent probes. Previous studies with anthroyloxy-, carbazole-, and nitroxide-labeled fatty acids have shown that protonation of a fatty acid carboxyl group causes a shift to a location a few angstroms deeper location in the bilayer [2,3,23,24]. The uncharged state was also moderately deeper than the charged one for the probes in this study, suggesting that such behavior is general. The fact that the change is only 2–4 Å in all cases probably reflects the fact that even the uncharged state an ionizable group is polar and thus tends to seek the membrane surface, although to some lesser degree. This conclusion is consistent with the observations that a number of uncharged polar probes have a slightly deeper location than observed for charged probes [5,6] (Fig. 4). On the other hand, it should be noted highly polar probes such as NBD can occupy a location about as shallow as charged species [3,25].

### 4.3. Comparison of quenching results to previous studies

There is strong evidence that parallax analysis of quenching provides accurate depths under many conditions. There is good agreement of depths obtained by parallax analysis for anthroyloxy fatty acids, NBD, Trp and Tyr analogs and anthracene derivatives with information obtained, often much more indirectly and/or at a lower level of resolution, by other methods [1–6]. Recently, the accuracy of the method has also been calibrated in some detail with a transmembrane helical peptide [15].

The shallow depths found by parallax analysis for the probes in this report are also consistent with other properties that give indirect information about their membrane location. A shallow location for dihexadecylaminostyrylpyridinium and hexadecylhydroxycoumarin probes has been inferred from their spectral properties [26–28]. The emission properties of the octadecyl analog of the hexadecylaminonaphthalenesulfonate probe, octadecylnaphthyl-2-amino-6-sulfonic acid (ONS), indicated a location in the polar region of membranes [29]. The locations of ANS and ONS have also been reported to be near the polar headgroup from NMR and diffraction measurements, although it should be noted it was necessary to use very high concentrations of these probes in the membrane [30,31]. Furthermore, the ability of this class of probes to accurately report the electrostatic potential near the membrane surface also indicates their location must be close to the surface [32].

Lipid-attached rhodamine has also been found to occupy a very shallow location in a study of NBD to rhodamine energy transfer [25]. That study found a rough range for the depth of a lipid-attached rhodamine that was even shallower (31–55 Å from the bilayer center) than we report here. As the authors pointed out, only the former value is consistent with the physical dimensions of the probe molecule. However, even the lower limit of 31 Å would place the rhodamine outside the bilayer proper, and is not consistent with the observation of strong binding of free rhodamine to membranes, which instead suggests a location somewhere well within the bilayer. (This tendency should be even more pronounced for lipid-anchored rhodamine.) Furthermore, in the energy transfer calculations it was necessary to make the assumption that donor and acceptor could not approach each other laterally closer than 20–25 Å in order to obtain the 31 Å value. This assumption is hard to justify. Therefore, it seems most likely some variable causes an overestimate of fluorophore distance from the bilayer center in the energy transfer measurement. On the other hand, when a fluorophore is far away from the quenchers used to calculate depth, the distance of the fluorophore to the furthest quencher used in the depth calculation can be underestimated [2,3]. For the shallowest fluorophores studied here we cannot rule out that possibility the measured $z_{cf}$ slightly underestimates distance from the bilayer center.

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