Extension of the Parallax Analysis of Membrane Penetration Depth to the Polar Region of Model Membranes: Use of Fluorescence Quenching by a Spin-Label Attached to the Phospholipid Polar Headgroup†

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ABSTRACT: The parallax method is a method by which the depth of fluorescent molecules within a membrane is calculated from the ratio of quenching induced by two spin-labeled phospholipids at different depths. In this report, the method is extended to measurements of depth in the polar headgroup region of the membrane through use of a lipid with a spin-label attached to the polar choline moiety. Quenching data indicate that the choline-attached nitroxide is close to 19.5 Å from the bilayer center, in good agreement with the choline location previously determined by diffraction measurements. By using quenching results obtained with this polar headgroup-labeled phospholipid, depths more accurate than those measured previously can be obtained for fluorophores in the polar region of the membrane. It appears that the most reliable results are obtained when depth is calculated from the quenching of the two spin-labels that quench a specific fluorophore most strongly. Applying this approach to a series of anthroyloxy-labeled fatty acids indicates that the depth of the anthroyloxy group is almost linearly related to the number of carbon atoms between it and the carboxyl group. The fatty acid carboxyl group itself is close to 18.6 Å from the bilayer center in the ionized form and 16 Å from bilayer center in the protonated form. This is close to the depth of the carboxyl groups on phospholipid fatty acyl chains. More accurate depths have also been obtained for 7-nitro-2,1,3-benzoxadiazol-4-yl (NBD) labeled phospholipids using the quenching of the choline-attached spin-label. The NBD group appears to locate about 19–20 Å from the bilayer center, in agreement with energy-transfer results (Wolf et al. (1992) *Biochemistry 31,* 2865–2873). Spin-label quenching has also been used to show that the anthroyloxy groups move toward the surface of the bilayer while in the excited state. The depth of anthroyloxy groups immediately after excitation appears to be given by the depth measured at the shortest emission wavelengths.

We have been developing a fluorescence quenching technique, called the parallax analysis, to determine the depth of specific fluorescent sites on molecules incorporated into model membrane vesicles. In this method, depth is determined by measuring the difference in fluorescence intensities in the presence of spin-label quenchers fixed at two different depths of the bilayer. The ratio of these two fluorescence intensities is then substituted into a simple equation that allows calculation of depth (Chattopadhyay & London, 1987). This method has proven useful for localizing proteins within the bilayer (Jiang et al., 1991; Chattopadhyay & McNamee, 1991; Clague et al., 1991; Ulbrandt et al., 1992; Palmer & Merrill, 1993) and for determining the depth and orientation of peptides with a series of single Trp substitutions (Chung et al., 1992). In previous studies, the accuracy of this method was established for fluorescent probes located in the acyl chain region of the bilayer. This was done by comparing the depth determined by brominated and spin-labeled quenchers (Abrams & London, 1992) and by showing that the method reports the graded series of depths expected for anthroyloxy groups on anthroyloxy-labeled fatty acids (Abrams et al., 1992).

This report describes the extension of the method to fluorescent groups located in the polar headgroup region of model membrane vesicles. To do this, the spin-labeled lipid phosphatidyltempocholine (TempoPC) (Kornberg & McConnell, 1971) has been used. TempoPC has its nitroxide attached to the choline group in the polar headgroup region. Results obtained by combining TempoPC quenching with that of lipids carrying acyl-chain-attached nitroxides were used to evaluate the depth of a number of fluorescent probes located near the membrane surface. In additional experiments, the suggestion from previous fluorescence (Demchenko & Shcherbatska, 1985) and quenching (Abrams et al., 1992) studies that fluorophores move toward the membrane surface in the excited state was examined. It was found that this movement does occur for anthroyloxy probes.

EXPERIMENTAL PROCEDURES

Materials. Spin-labeled PCs,[1] NBDPE, 12-NBDPC, and DOPC were purchased from Avanti Probe Lipids. DMPC was purchased from Calbiochem. Anthroyloxy-labeled fatty acids, 11-CU, and PCA were purchased from Molecular

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[1] Abbreviations: 2-, 6-, 9-, or 12-AS, 2-, 6-, 9-, or 12-(9-anthroyloxy)-stearic acid; 11-CU, 11-(9-carbazole)undecanoic acid; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; ESR, electron spin resonance; MLV, multilamellar vesicles; 12-NBDPC, 1-palmitoyl-2-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-phosphocholine; NBDPE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; PC, 1,2-diacyl-*sn*-glycero-3-phosphocholine; PCA, 2,2,5,5-tetramethyl-3-pyrroline-1-oxyl; 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; Tempol, 2,2,6,6-tetramethylpiperidine-1-oxyl; TempoPC, 1,2-dioleoyl-*sn*-glycero-3-phosphotempocholine; TLC, thin-layer chromatography; TOE, DL-tryptophan octyl ester.

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Probes. Tempol was purchased from Aldrich and then once recrystallized from chloroform–hexane (mp 72 °C). Recrystallization resulted in a 3% increase in its spin content per gram. TOE was purchased from Sigma.

The concentration of phospholipids was determined by phosphate assay (Bartlett, 1959) subsequent to total digestion (Morrison, 1964). DMPC was used as a standard to assess lipid digestion. Lipid purity of about 100 nmol of lipid was analyzed by TLC on silica gel plates [Adsorbosil Plus prekotes (no binder) soft layer plates, Alltech Associates, Deerfield, IL] in chloroform/methanol/water (65:25:4 v/v). The phospholipids showed virtually no impurities after the plates were sprayed with 40% H_2SO_4 and charred. (One of two batches of TempoPC had to be purified by TLC before use.) TLC of the fluorescent fatty acids and TOE was analyzed using *n*-hexane/methanol/ethyl ether/acetic acid (80:25:20:1 v/v). No fluorescent impurities were observed.

Assay of Spin-Label Content of Spin-Labeled Lipids. To calculate the actual spin content of the commercial spin-labeled lipids, the intensities of doubly integrated ESR spectra of spin-labeled lipids were compared to those of two standard spin labels, Tempol and PCA, dissolved in DMSO (Chattopadhyay & London, 1987; Abrams & London, 1992). Duplicate samples contained DMSO-solubilized spin-labeled lipids or a known concentration of the standards (close to 0.2 mM) were placed in 50-μL capillary tubes. Samples of over 50 μL were used so as to ensure filling of the ESR sample cavity. ESR spectra were obtained on a Varian E-4 spectrometer, interfaced for integration to a Spex Datamate computer. Spectra were acquired at 2 mW to avoid signal saturation. Ratios of spin to phosphate on the spin-labeled phospholipids were in the range 0.83 ± 0.06. We have preliminary indications that the TempoPC nitroxide is less stable than the doxyl nitroxides and should be reassayed regularly. (For those who wish to use the quenching method but do not have an ESR instrument available to calibrate spin concentration, it is possible to determine spin concentration by preparing vesicles with various percentages of uncalibrated spin–labels and identifying what percentage of uncalibrated spin-label gives quenching equal to that seen with the concentration-calibrated spin-labels used in this study.)

Analysis of Acylation Position for 5-SLPC and 12-SLPC. Acylation position analysis was performed as described by Abrams and London (1992). For acyl chain spin-labels, 70–80% of the label was in the 2-position. Calculated depths have not been corrected for the presence of quenchers on the 1-chain, which could decrease z_{cf} by up to 1 Å for the amount of scrambling observed (Chattopadhyay & London, 1987).

Preparation of Samples for Quenching Studies. Samples were prepared by mixing the desired volumes of solutions of the various lipids and fluorophores in organic solvents. (It should be emphasized that we find precise and accurate pipetting of organic solvents by positive displacement to be necessary for this step in order to obtain reproducible quenching measurements.) The samples were dried under N_2 at around 35 °C and then mixed well in a few drops of chloroform and redried. (We have had problems when ethanol was used.) Samples were then further dried under high vacuum for at least 1 h. Between 1 and 2 mL of the desired buffer was then added to each sample, followed by vortexing for 50 s to disperse the lipid and form MLVs.

In general, samples with a final total lipid concentration of 125 and 200 μM were made in each case. [This was done to check that lipid concentration did not influence depth (Abrams & London, 1992).] The buffers used were 10 mM sodium acetate/150 mM NaCl, pH 4.5, for TOE and 10 mM sodium phosphate/150 mM NaCl, pH 7, for NBD lipids. AS fatty acids and 11-CU samples were prepared in 1.5 mL of 10 mM glycine/150 mM NaCl, pH 10, and after fluorescence was measured, these samples were brought to pH 5 by the addition of 15 μL of 1.04 M acetic acid. The final concentrations of fluorophores were 11-CU, 0.072 μM; AS probes, NBDPE, and 12-NBDPC, 1.07 μM; and TOE, 1.5 μM.

Generally, either duplicates or triplicates were prepared for each sample containing quencher and fluorophore (quintuplets for TOE). Vesicles were prepared containing DOPC and spin-labeled phospholipids. For depth measurements, at least two types of samples were prepared: one containing a lipid with a quencher group and a second containing no quencher. Fluorescence background samples containing membranes without fluorophore were also prepared. The spin-label content of the vesicles was 15 mol %, except for the NBD lipids, in which cases 10 mol % was used.

Small unilamellar vesicles (SUVs) of similar compositions were prepared by the ethanol injection method (Batzri & Korn, 1973) as previously described (Jiang et al., 1991).

Fluorescence and Absorbance Measurements. Fluorescence was measured with a Spex 212 Fluorolog spectrofluorometer operating in ratio mode and using 1-cm path length quartz cuvettes. The excitation and emission slit widths were 2.5 and 5 mm, respectively, except when measuring the wavelength dependence of depth, where the slit widths for both excitation and emission were 2.5 mm. (Slit widths of 2.5 and 5.0 mm correspond to band-passes of 4.5 and 9.0 nm, respectively.) For TOE, the excitation wavelength was 280 nm and the emission wavelength was 335 nm. For 11-CU, the excitation wavelength was 294 nm and the emission wavelength was 350 nm. For the anthroyloxy fatty acids, the excitation wavelength was 365 nm and the emission wavelength was 461 nm. For NBD phospholipids the excitation wavelength was 469 nm and the emission wavelength was 533 nm.

Samples were mixed by agitation just prior to measurement of fluorescence. This was necessary to eliminate variations due to MLV settling. Fluorescence was measured at room temperature and averaged over three 15-s readings. In all cases, the intensity from background samples without fluorophore was subtracted. Depth values were then calculated from eq 1 (see below). The values at 125 and 200 μM lipid, which in all cases were very similar, were then averaged.

The coefficient of variation was calculated for several experiments. The maximum coefficient for fluorescence intensity was 5%, with the average being 2–3%. Sample calculations indicated that intensity errors resulting in a 10% change in F_1/F_2 (see below) could lead to an error of about 1 Å in z_{cf}.

THEORY

The distance of fluorophores from the center of the bilayer was calculated using the parallax equation (Chattopadhyay & London, 1987):

$$z_{cf} = L_{c1} + (-\ln(F_1/F_2)/\pi C - L_{21}^2)/2L_{21} \qquad (1)$$

where z_{cf} is the distance of the fluorophore from the center of the bilayer, F_1 is the fluorescence intensity in the presence of the shallow quencher (quencher 1), F_2 is the fluorescence intensity 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_{c2} is the distance of the deep quencher from the center of the bilayer, L_{21} is the distance between the shallow and deep quenchers ($L_{c1} - L_{c2}$), and the C is the concentration of quencher in molecules/Å^2.

This equation can be used to solve for the distance of the shallow quencher from the center of the bilayer when

Table I: Quenching of Fluorescent Probes by Spin-Labeled Phospholipids

fluorophore	F/F_0[a] quencher			z_{cf}' [b]			
	TempoPC	5-SLPC	12-SLPC	5-SLPC/12-SLPC	TempoPC/5-SLPC	z_{cf}	R_c
TOE, pH 4.5	0.519	0.407	0.589	13.5	13.5[c]	13.5	11.6
NBD-PE, pH 7	0.498	0.609	0.698	11.4	18.9	18.9	12.5
12-NBD-PC, pH 7	0.499	0.648	0.707	10.5	19.8	19.8	12.4
11-CU, pH 5	0.442	0.324	0.286	7.5	12.7	7.5	13.8
11-CU, pH 10	0.369	0.287	0.329	10.6	13.3	10.6[d]	13.7
2-AS, pH 5	0.310	0.310	0.357	10.7	15.8	15.8	13.7
6-AS, pH 5	0.289	0.255	0.275	9.9	14.6	12.3	14.4[e]
9-AS, pH 5	0.272	0.237	0.232	8.8	14.4	8.8	15.0
12-AS, pH 5	0.276	0.246	0.190	6.0	14.7	6.0	15.7
2-AS, pH 10	0.272	0.299	0.360	11.2	16.8	16.8	14.2
6-AS, pH 10	0.284	0.263	0.308	10.9	15.0	15.0	14.4
9-AS, pH 10	0.267	0.238	0.268	10.4	14.7	12.6	14.7
12-AS, pH 10	0.247	0.233	0.206	7.5	15.2	7.5	15.3

[a] F/F_0 is the ratio of fluorescence intensity in samples containing spin-label quencher to that in vesicles without spin-label. F/F_0 data for quenching of AS probes by 5- and 12-SLPC is from Abrams et al. (1992). [b] z_{cf}' values calculated from the quenching of the pair of quenchers indicated. z_{cf} is the best estimate for true distance from the center of the bilayer (in Å), determined from the strongest quenching spin-label pair. R_c calculated as described in Abrams and London (1992). See text for details. [c] Agrees with value from 5-SLPC/12-SLPC depth by definition, as value used to calibrate TempoPC nitroxide depth. [d] 11-CU values are slightly shallower than seen previously (Abrams & London, 1992), perhaps due to the higher 11-CU concentration used in this study. [e] Calculated from 5- and 12-SLPC data. The results were very similar when TempoPC and 5-SLPC data were used.

fluorophore depth is known. Rearranging eq 1 gives

$$L_{c1}^2 - 2z_{cf}L_{c1} + 2z_{cf}L_{c2} - L_{c2}^2 - (1/\pi C)(\ln[F_1/F_2]) = 0 \quad (2)$$

the solution of which for L_{c1} is

$$L_{c1} = z_{cf} \pm [(z_{cf} - L_{c2})^2 + (1/\pi C)(\ln[F_1/F_2])]^{1/2} \quad (3)$$

RESULTS

Depth of the Spin-Label Group of TempoPC. To use quenching by a spin-labeled lipid to locate the depth of a fluorescent molecule, the depth of the spin-label must be known. Although previous studies indicate that the choline-attached spin-label of TempoPC is in the polar headgroup region (Kornberg & McConnell, 1971), its precise location is not known. To locate the TempoPC nitroxide, its quenching of a fluorophore of known depth was measured, and the parallax equation was used to solve for quencher depth as the unknown. Potentially, any fluorophore previously localized could be used (Chattopadhyay & London, 1987; Abrams & London, 1992; Abrams et al., 1992). However, because the parallax analysis is most accurate when a fluorophore is close to the quencher being used (Chattopadhyay & London, 1987), it was desirable to choose a fluorophore that was not too far from TempoPC in depth. Trp octyl ester (TOE) was chosen for this purpose (Abrams & London, 1992).[2]

The depth of TOE tryptophan was first measured with 5- and 12-SLPC quenching, using multiple samples to get a precise value. The depth obtained for TOE was 13.4 Å from the center of the bilayer. We substituted this value, the depth of 5-SLPC, and the ratio of TempoPC to 5-SLPC quenching into eq 3. This calculation gave a depth for the Tempo nitroxide of 19.5 Å from the center of the bilayer. Since the Tempo group of TempoPC is attached to the choline part of the phospholipid, this result is in good agreement with the findings of Wiener and White (1992), who by using X-ray and neutron diffraction methodology located the choline group of DOPC at an average location of 21.9 Å from the center of the bilayer. The slightly deeper value we obtain for the TempoPC nitroxide could represent the tendency of the somewhat hydrophobic Tempo moiety to orient toward the more nonpolar region of the bilayer. Another factor is that under the conditions of complete hydration in our experiments, choline position is likely to be somewhat deeper than 21.9 Å (Wiener & White, 1992).

Evaluation of the Depth of Fluorophores Using Spin-Labels. Combining TempoPC quenching with 5- and 12-SLPC quenching, the depth of various fluorescent probes was determined. As noted above, the parallax analysis gives more accurate depth values when quenching by the pair of spin-labels closest to the fluorophore is used to calculate depth. The reason for this is that the parallax analysis is based on a model of the distance dependence of quenching that is very accurate at smaller differences in fluorophore and quencher depth but gradually becomes less accurate at larger differences in depth (Chattopadhyay & London, 1987). Therefore, for shallow fluorophores, the quenching of the shallower TempoPC/5-SLPC pair should allow accurate calculation of depth, and for deeper fluorophores, quenching by the deeper 5-SLPC/12-SLPC pair should be best. It is important to point out that it does *not* require prior knowledge of the depth of a fluorophore to know which pair of quenchers to use in calculating depth. Since the quenchers closest in depth to the fluorophore give the most quenching, they can easily be identified by inspection of the amount of quenching (see Table I).

Depth calculated using the pair of closest spin-labels was called z_{cf} (Table I). In rare cases where the levels of TempoPC and 12-SLPC quenching were indistinguishable within experimental error (less than a 5% difference in relative fluorescence intensity), the average of the depths calculated with the TempoPC/5-SLPC and 5-SLPC/12-SLPC pairs was considered likely to be a better estimate of depth than the results from an individual quenching pair, and thus the average value was defined as z_{cf}.

In one set of experiments, anthroyloxy depths were measured for the series of anthroyloxy-labeled fatty acids. The amount of spin-label-induced anthroyloxy quenching of 2-, 6-, 9-, and 12-AS is shown in Table I. In Figure 1, the calculated depths of the anthroyloxy groups (z_{cf}) shown in Table I are plotted versus the carbon number of their attachment position on the acyl chains. It appears that the anthroyloxy moiety may follow carbon attachment position in a roughly linear fashion, both when the AS fatty acid carboxyl group is in the ionized form at pH 10 and when it is in the protonated form at pH 5. The change in anthroyloxy depth when its attachment site is shifted

[2] The other possible choices, NBD-PE, 12-NBDPC, and 2-AS, were unsuitable, as they were quenched more strongly by TempoPC than the other spin-labels, indicating that they were significantly shallower than originally determined (see below).

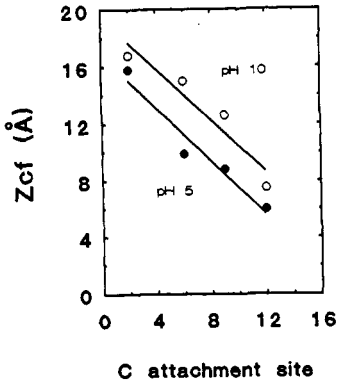

FIGURE 1: Anthroyloxy group depth vs carbon atom attachment site for anthroyloxy-labeled fatty acids incorporated into phosphatidylcholine vesicles. (●) Protonated carboxyl group, pH 5. (○) Ionized carboxyl group, pH 10. The lines are linear least-squares fits to the data.

along the acyl chain is roughly 0.9–0.95 Å per carbon atom moved. This is close to the difference in depth between carbon atoms in a unlabeled fatty acyl chain in the liquid crystal state (McIntosh & Holloway, 1987; Lewis & Engelman, 1983; Oldfield et al., 1978; Zaccai et al., 1979; Wiener & White, 1992).

The other striking observation concerning the AS probe is that in the ionized form the fatty acid chain appears to be about 2.5 Å more shallow than in the protonated form. By extrapolation of the lines in Figure 1 to the 1-carbon atom, the depth of the carboxyl group can be estimated. In the ionized form the carboxyl carbon appears to be at 18.6 Å from the bilayer center, and in the protonated form 16 Å from the bilayer center. These values are very close to the depth of the carboxyl groups on the acyl chains of phospholipids as judged by diffraction methods (Wiener & White, 1992).

The membrane location of the NBD moiety of NBDPE and NBDPC was also examined. In previous work using the 5-SLPC/12-SLPC quenching ratio, we found that the NBD groups of NBDPE and 12-NBDPC appeared to be located in the range 11.5–14 Å from the center of the bilayer (Chattopadhyay & London, 1987; Abrams & London, 1992). However, we now find TempoPC to be the strongest quencher of the three spin-labels (Table I), indicating that the NBD groups were considerably shallower than previously thought. Using the stronger quenching of the TempoPC/5-SLPC pair to calculate depth, the NBD groups of NBDPE and 12-NBDPC are found to be located at 18.9 and 19.8 Å from the center of the bilayer, respectively. These more accurate results are in agreement with a recent energy-transfer study of NBD depth (Wolf et al., 1992). This study placed the NBD groups at about the level of the phosphate of the phospholipid headgroup (Wiener & White, 1992).

In the multilamellar vesicles (MLV) used in these experiments, values for the depth of molecules in the polar headgroup region could potentially be influenced by the quenching of a fluorescent group located in one bilayer by a spin-label in an adjacent bilayer. However, control experiments showed that such interbilayer quenching was unlikely to be a factor in our results because the depths of NBD groups determined using SUVs were virtually identical to those found using MLVs. (In SUV, NBD distances from the center of the bilayer were 19.1 and 19.8 Å for NBDPE and 12-NBDPC, respectively).

Apparent Depth vs Emission Wavelength: Movement of Fluorescent Molecules toward the Membrane Surface when in Their Excited States. Previous studies showed that the anthroyloxy depth measured by the parallax analysis was slightly dependent on the emission wavelength, with depth being shallowest at longer wavelengths (Abrams et al., 1992). One explanation was that the anthroyloxy groups occupy a range of depths, with longer wavelength fluorescence coming from fluorophore close to the polar environment near the membrane surface. Another possibility was that the longer wavelength fluorescence arose from molecules that moved toward the membrane surface [due to the increased polarity of anthroyloxy groups in the excited state (Werner & Hoffman, 1973)] and then emitted fluorescence at longer wavelengths (primarily due to the slow structural relaxation of anthroyloxy groups in membranes[3]). Under such conditions, measurements at different wavelengths would allow time-resolved observation of the anthroyloxy group in its excited state.

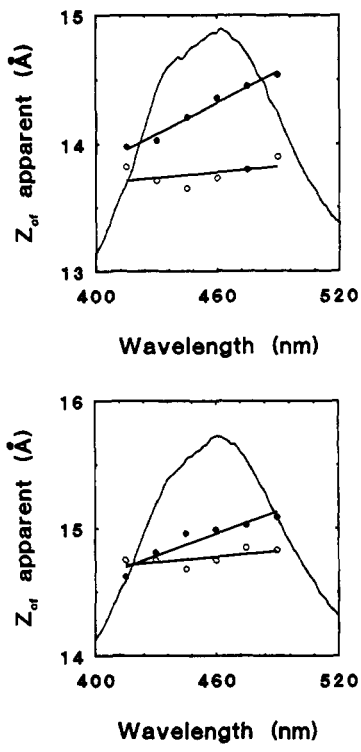

FIGURE 2: Apparent depth vs emission wavelength for 6-AS. Depths were determined from the quenching of vesicle-bound 6-AS by the TempoPC/5-SLPC pair at each of the wavelengths shown. (Top) Depths determined at pH 5 (○) with and (●) without a dopant at 15 mol % 12-SLPC in the vesicles. (Bottom) Depths determined at pH 10 (○) with an (●) without a dopant of 15 mol % 12-SLPC in the vesicles. Lines are linear least-squares fits. For reference, the emission spectra of 6-AS at the appropriate pH values are also shown.

To determine which of these alternatives was most important, the apparent depth of 6-AS was determined by measuring TempoPC and 5-SLPC quenching in vesicles doped with 12-SLPC and comparing the result to that determined as usual with no 12-SLPC. One effect of the deep 12-SLPC quencher is that it should preferentially quench the deeper subpopulation of 6-AS molecules. In that case, one would predict that 6-AS would appear to be shallower when depth was measured in the presence of 12-SLPC than in its absence. However, Figure 2 shows 6-AS appears *deeper* in the presence of 12-SLPC than in its absence.[4]

This result can be explained if 6-AS molecules move toward the surface while in the excited state. Spin-label quenching

[3] A shift of anthroyloxy fluorescence to longer wavelengths after being in the excited state for a period of time is thought to be a consequence of structural relaxation (Matayoshi & Kleinfeld, 1981). If the anthroyloxy groups move toward the surface, a combination of polarity- and relaxation-induced shifts to longer wavelengths should occur.

[4] Note that the apparent z_{cf} values determined by TempoPC/5-SLPC quenching are not the same as the z_{cf} values for this probe. In this experiment only the relative change in depth is being evaluated.

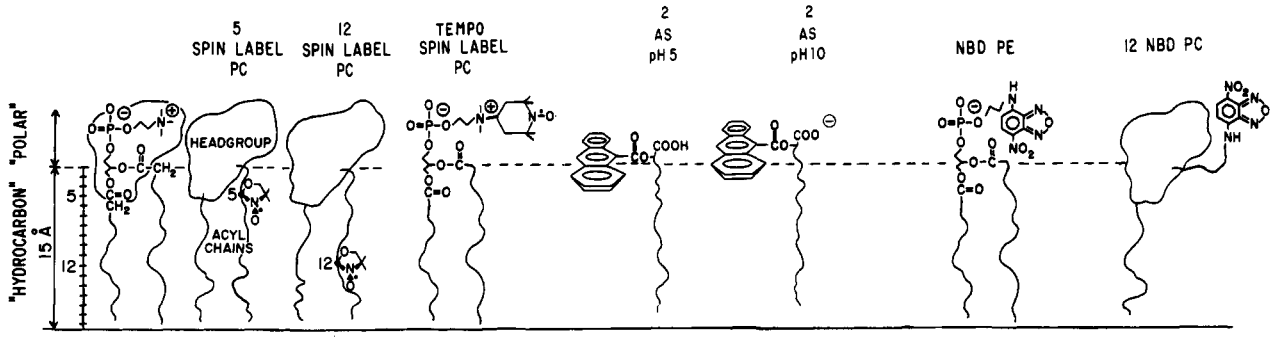

FIGURE 3: Schematic illustration of the membrane depths of some of the fluorophores and quenchers used in this study. The horizontal line at the bottom represents the bilayer center. The scale on the left shows the approximate positions of the carbon atoms of the 2-chain of a phospholipid.

shortens excited-state lifetime (Wardlaw et al., 1987; Green et al., 1990; Yeager & Feigenson, 1990; Matko et al., 1992), allowing less time for motion to occur.[5] The results in Figure 2 are consistent with decreased motion toward the surface due to the presence of 12-SLPC, making 6-AS molecules appear to be deeper in the membrane.

Supporting this conclusion, Figure 2 also shows that 6-AS depth in the absence of 12-SLPC at short wavelengths is almost the same as in the presence of 12-SLPC. At shorter wavelengths, the fluorescence of anthroyloxy groups is dominated by molecules that have not undergone structural relaxation, having been in the excited state only a short time (Matayoshi & Kleinfeld, 1981) and thus having not had enough time to move significantly.

DISCUSSION

Measurement of Depths for Shallow Fluorescent Probes. We have shown that the parallax analysis can be extended to the polar headgroup region of bilayers by the use of a quencher molecule attached to the choline group. The shallow depth of the 2-AS anthroyloxy groups and NBD groups found this way is supported by studies of anthroyloxy depth in erythrocytes using hemoglobins as an energy-transfer acceptor (Eisinger & Flores, 1983) and very recent studies of NBD-to-rhodamine energy transfer (Wolf et al., 1992), respectively. A schematic illustration of the position of these fluorophores is shown in Figure 3.

It is also interesting that it appears that there is near-linearity of anthroyloxy depth versus carbon atom attachment position. That the bulky anthracene groups appear to order in this way suggests that their interactions with their environment do not greatly influence the depth of the fatty acyl chain to which they are attached. This is in stark contrast to the behavior of the much more polar NBD group, which locates to the polar region of the membrane, even when attached to the end of a fatty acyl chain. Clearly, the effect of a fluorophore on fatty acyl conformation will depend on the particular fluorescent probe chosen. It will be our aim in future studies to find out how probe structure controls depth in additional cases.

Movement of Fluorescent Probes in the Excited State. We also found that fluorescent molecules in their excited state appear to move toward the membrane surface. This was proposed by Demchenko and Scherbatska (1985), who suggested movement to a more polar membrane environment as a possible explanation for why water-soluble fluorescence quenchers quench emission at early and late times after excitation to a different extent. However, they could not distinguish between preferential quenching of a subpopulation of molecules already at shallow depths when in the ground state and molecules that had moved in the excited state.

It is interesting that for the few nanoseconds that the excited state persists, there is enough time for movement toward the surface to occur. When using fluorescent groups to probe membrane structure, the possible influence of this motion on interpretation should be considered. By restriction of fluorescence measurements to the short-wavelength end of the fluorescence emission spectrum or by use of time-resolved spectroscopy, it should be possible to ascertain the ground-state location of the fluorescent probes when necessary.

Factors Influencing the Validity of the Parallax Analysis at Larger Fluorophore-to-Quencher Distances. One important issue to consider when using the parallax analysis is the range over which the equation used to calculate depth is valid. In the simplest case, the parallax equation should be valid for distances between quencher and fluorophore depth that are within 85–90% of the apparent critical quenching distance, R_c (Chattopadhyay & London, 1987). Given typical R_c values of 12–15 Å (Table I), this should be on the order of 9–12 Å for the probes used in this report.

However, results obtained using the AS probes suggest that for these groups the analysis is valid for only a shorter range. Comparison of the depth values obtained by the 5- and 12-SLPC pair and the TempoRC and 5-SLPC pair shows that there appears to be a problem when the difference in depth between the anthroyloxy group and one of the quenchers is over 5–6 Å. Some of this may be due to the spread of the anthroyloxy and spin-label groups over a range of depths in the membrane. This would result in an increase in the difference in depth between some quenchers and fluorophores such that they fall outside of the range when the parallax analysis is valid (Abrams & London, 1992). But this is not likely to be the full explanation for our results, since the spread of groups in membranes over the 8-Å range of depths found for methylene and bromine groups (Wiener & White, 1991; Wiener et al., 1991) does not appear to be nearly large enough to account for the quenching results (Abrams & London, 1992, and calculation not shown).

Another possibility is that there are complications arising from molecular motions occurring during the time the fluorophore is in the excited state. Such motions can influence apparent depth, but the pattern of apparent depths seen with the various anthroyloxy probes does not fit the pattern predicted for various types of motions (Abrams & London, 1992).

The nature of spin-label quenching is another factor to consider. The electron-exchange mechanism, which is believed

[5] That 12-SLPC quenching does reduce 6-AS motion was confirmed by fluorescence polarization. The polarization of 6-AS fluorescence was 0.177 in the presence of 15 mol % 12-SLPC and 0.135 in its absence (not shown). As 12-SLPC remains in the fluid state at room temperature (Chen & Gaffney, 1978), its effect on polarization cannot be due to its induction of a lipid-phase transition to the rigid gel state.

to dominate spin-label quenching, is dependent on the degree of spin-label and fluorophore orbital overlap, even though distances significantly beyond van der Waals radii can be involved (Green et al., 1990; Matko et al., 1992). Therefore, the size, shape, and orientation of fluorophore and quencher orbitals could influence the distance dependence of quenching. Quenching of fluorophores in one leaflet of the bilayer by quenchers in the other leaflet can also be a problem for deeply located fluorophores (Chattopadhyay & London, 1987). On the other hand, spin-label quenching is relatively insensitive to solvent polarity (Green et al., 1973, 1990), so any effects on quenching due to the differences in polarity at different depths in the bilayer should not be critical.

It is important to note that the problems with anthroyloxy groups may not exist or be as severe for other fluorophores. The observation that 5-SLPC/12-SLPC quenching located the Trp moiety of TOE at a shallower location than it did for anthroyloxy groups that are actually shallower than the TOE Trp group strongly suggests that the analysis remains valid for Trp moieties at distances considerably greater than for anthroyloxy probes. Furthermore, quenching is generally much more sensitive to distance for NBD, carbazole, and Trp groups than it is for anthroyloxy groups (Table I). This may indicate that depth measurements on anthroyloxy groups are affected by properties specific to that class of probes.

In any case, it is important to emphasize that problems due to a limited range of validity for the parallax equation can largely be avoided by using only data from the closer, strongest-quenching spin-label pair to determine depth. By the use of TempoPC in addition to 5-SLPC and 12-SLPC, the parallax analysis can be used to map fluorophore location over a wide range of depths.

Comparison of the Use of the Parallax Analysis for Determination of Depth from the Level of Quenching. It is worthwhile to compare depth determination by the parallax analysis to other quenching techniques. One alternate approach has been to measure quenching with a series of spin-label fatty acid quenchers at different depths and identify the depth of the fluorophore as closest to the depth of the spin-label giving the most quenching (reviewed in London (1982); Blatt & Sawyer, 1985). This method has had several limitations. First, a correction must be made for the amount of fatty acid that does not bind to the bilayer (Blatt & Sawyer, 1985). Second, spin-labeled fatty acids have a pK_a near 7 and are found at different depths depending on whether they are ionized or protonated (Barratt & Laggner, 1974; Sanson et al., 1976). Third, the actual nitroxide content of the spin-labeled fatty acid should be determined for each of the quenchers used, although this is rarely done. Without a nitroxide determination, the small differences in quenching seen for closely spaced spin-labels are hard to differentiate from effects due to different nitroxide concentrations.

Perhaps more important is that even with an extensive series of spin-labeled fatty acids (carrying nitroxides at 5, 7, 10, 12, and 16 positions), determination of the strongest-quenching spin-label allows accurate estimation of depth in only a limited number of cases. For example, if a fluorophore is quenched most by the 5-position nitroxide, it is only possible to say it could be anywhere above the level of the 6-carbon atom of a fatty acid. In general, the absence of spin-labels at depths between TempoPC and 5-SLPC would prevent examination of depth within the polar headgroup region at high resolution by this method. In contrast, the parallax analysis allows determination of depth at a high level of resolution over a wider range with fewer spin-label probes.

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