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Determination of the Location of Fluorescent Probes Attached to Fatty Acids Using Parallax Analysis of Fluorescence Quenching: Effect of Carboxyl Ionization State and Environment on Depth†

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ABSTRACT: In this report, parallax analysis of fluorescence quenching (see the preceding paper in this issue) was used to determine the location (depth) of anthroyloxy and carbazole probes attached to model membrane inserted fatty acids. A monotonic increase in depth was found as the number of carbon atoms between the attachment site of the probe and the fatty acyl carboxyl group is increased. It was also found that depth is sensitive to pH, with an increase in probe depth upon protonation of the fatty acid carboxyl group of around 0.5-2.5 Å, depending on probe location and identity. This result shows that carboxyl protonation causes an increase in depth all along a fatty acid chain. In addition, it indicates that parallax analysis is very sensitive to small changes in depth. At a given pH, no significant change in probe depth was observed in vesicles containing anionic phospholipid or at various ionic strengths, suggesting these parameters do not strongly regulate fatty acyl chain location. It was also found that there is a decrease of the apparent depth of each of the fatty acyl attached probes both at longer excitation wavelengths and at longer emission wavelengths. This is consistent with there being a distribution of depth for each fluorophore, with shallower fluorophore dominating the fluorescence at red-shifted wavelengths. Solvent relaxation effects also appear to contribute to this wavelength dependence.

Fatty acids labeled with spectroscopic reporter groups have proven to be useful membrane probes. They have been used to measure the dynamic properties of the lipid bilayer through anisotropy measurements [e.g., Tilley et al. (1979), Vincent et al. (1982), and Vincent and Gallay (1984)]. They have also been used to learn about the properties of membrane-bound fatty acids themselves [e.g., Reboiras and Marsh (1991), Barratt and Laggner (1974), Sanson et al. (1976), and Von Tscharner and Radda (1981)]. In addition, fatty acid attached reporter groups have found use as probes of the structure of membrane-bound proteins and peptides and other small molecules through their use as energy transfer partners or fluorescence quenchers [e.g., Voges et al. (1987), Haigh et al. (1979), Sikaris et al. (1981), Kleinfeld (1985), Kleinfeld and Lukacovic (1985), and Mitra and Hammes (1990)].

In this report, we studied the location of membrane-inserted fluorescent probes attached to fatty acids both to further evaluate the sensitivity and accuracy of parallax fluorescence quenching analysis (Chattopadhyay & London, 1987; Abrams & London, 1992) and to examine how environmental conditions affect the location of fatty acids within a membrane. We chose to concentrate on the anthroyloxy series of probes because many of their properties have been characterized, and some information on their location in the membrane has been obtained [e.g., Werner and Hoffman (1973), Podo and Blasie (1977), Thulborn and Sawyer (1978), Matayoshi and Kleinfeld (1981), Chalpin and Kleinfeld (1983), Eisinger and Flores (1982), and Eisinger and Flores (1983)]. Using fluoresence quenching by spin-labeled phospholipids, we find that the anthroyloxy groups take on a graded series of depths depending on their site of attachment to the fatty acyl chain and that anthroyloxy depth is affected by the state of ionization of the fatty acid carboxyl group. A dependence of depth on emission and excitation wavelengths used has also been found. This

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reflects the distribution of the fluorescent probes over a range of depths. The results of these studies demonstrate the utility and sensitivity of parallax analysis.

Experimental Procedures

Spin-labeled PCs,[1] DOPG, and DOPC were purchased from Avanti Polar Lipids. 11-CU-PC was a generous gift of John Silvius (McGill University). DMPC was purchased from Calbiochem. The anthroyloxy-labeled fatty acids (2-AS, 6-AS, 9-AS, 12-AS, and 16-AP) and 11-CU were purchased from Molecular Probes. All other chemicals were reagent grade.

To check their purity about 100 nmol of each phospholipid was chromatographed 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 lipids showed no impurities after the plates were sprayed with phosphate-sensitive spray (Dittmer & Lester, 1964) and charred. 11-CU-PC gave a single fluorescent spot on TLC. TLC of the fatty acids was done in *n*-hexane/methanol/ethyl ether/acetic acid (80:25:20:1 v/v) as the solvent system. No fluorescent impurities were observed in the fluorescent fatty acid samples.

The concentration of phospholipids was determined by phosphate assay (Bartlett, 1959) subsequent to total digestion (Morrison, 1964). To calculate the actual number of spins per spin-labeled lipid, the intensities of doubly-integrated ESR spectra of the lipids and spin standards were measured and compared as described in the preceding paper.

To check the acylation position of the spin-labeled fatty acyl chain, phospholipase A_2 digestion was carried out on the spin-labeled lipids as described in the preceding paper. To check the position of the carbazole carrying acyl chain on 11-CU-PC, phospholipase A_2 digestion was carried out as described for the spin-labeled lipids. Essentially complete digestion was obtained. The digestion products were separated on TLC in chloroform/methanol/water (65:25:4 v/v). Visual inspection under UV irradiation showed the fatty acid spot was clearly much more intensely fluorescent than the lyso-PC spot. For more quantitative analysis, the lyso-PC was extracted (3×) from the TLC plate material using the solvent chloroform/methanol/water (20:20:2 v/v), and the fatty acid was extracted (3×) using the 65:25:4 TLC solvent system. The samples were then centrifuged at low speed to separate the extraction solvent from the gel. The extracts were dried and redissolved in ethanol. The carbazole present in the fatty acid and the lysophopholipid samples was quantitated by fluorescence. It was found that 97% of the carbazole was attached to the 2-position fatty acyl chain.

MLV samples were prepared and fluorescence measured as described in the preceding paper (Abrams & London, 1992) and in the figure legends. In the case of DMPC vesicles, samples were prepared using buffer at 30 °C. Unless otherwise noted, the fluorescence of samples containing anthroyloxy groups was measured at an excitation wavelength of 365 nm and an emission wavelength of 461 nm. The fluorescence of samples containing carbazole was measured at 294-nm excitation and 350-nm emission. Excitation slits were set at 2.5 mm (band-pass 4.5 nm) and emission slits were set at 5.0 mm (band-pass 9 nm) unless otherwise noted. Fluorescence was averaged over at least 15 s for each sample reading. Intensities were found to be stable over time. Background samples lacking fluorophore were prepared in all experiments, and their fluorescence intensity was subtracted from reported values.

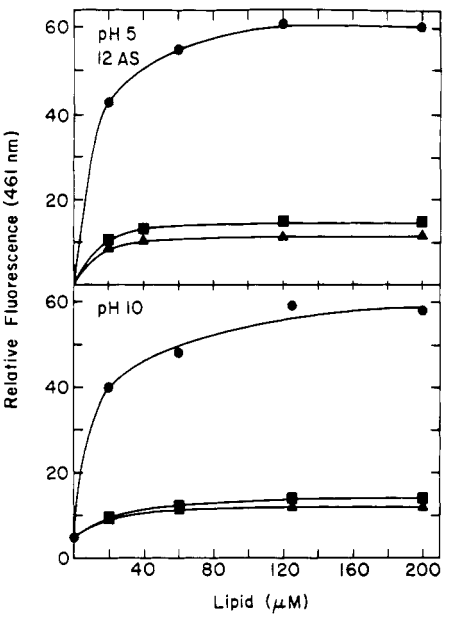

FIGURE 1: Binding of 12-AS to model membranes. (Top) 12-AS fluorescence vs lipid concentration at pH 5. (Bottom) 12-AS fluorescence vs lipid concentration at pH 10. The MLV samples contained mixtures of 1.6 nmol of 12-AS and various amounts of phospholipids suspended in 1.5 mL of 10 mM acetate and 150 mM NaCl, pH 5, or 10 mM glycine and 150 mM NaCl, pH 10. Samples contained (●) DOPC, (■) 15 mol % 5-SLPC/85 mol % DOPC, or (▲) 15 mol % 12-SLPC/85 mol % DOPC.

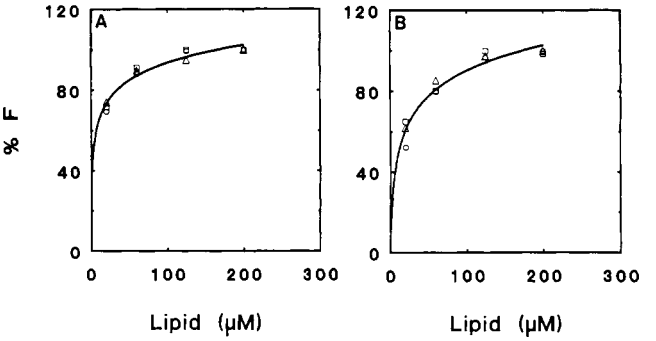

FIGURE 2: Normalized binding curves for 12-AS. The data of Figure 1 are here plotted as the percent of maximum change in fluorescence vs lipid concentration. (A) pH 5. (B) pH 10. (□) DOPC, (○) 15% 5-SLPC/85% DOPC, (△) 15% 12-SLPC/85% DOPC.

Results

Binding of Fluorescent Fatty Acids to Model Membranes. In order to calculate depth accurately, fluorescence quenching must be compared for samples that have the same amount of bound fluorophore. To verify that this is in fact the case, the effect of the presence of spin labels on fluorophore binding to membranes was examined. The enhancement of fluorescence intensity upon binding of fluorophores to membranes was used to establish binding curves for the anthroyloxy- and carbazole-labeled fatty acids. Figure 1 shows the binding of 12-AS to model membranes both at pH 5, where the carboxyl group is protonated, and at pH 10, where the carboxyl group

[1] Abbreviations: 2-, 6-, 9-, or 12-AS, 2-, 6-, 9-, or 12-(9-anthroyloxy)stearic acid; 16-AP, 16-(9-anthroyloxy)palmitic acid; 11-CU, 11-(9-carbazole)undecanoic acid; 11-CUPC, 1-palmitoyl-2-[11-(9-carbazole)]undecanoyl-*sn*-glycero-3-phosphocholine; DOPC, 1,2-dioleoyl-*sn*-3-glycerophosphocholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DOPG 1,2 dioleoyl-*sn*-glycero-3-phosphoglycerol; PC, 1,2 diacyl-*sn*-glycero-3-phosphocholine; PG, 1,2 diacyl-*sn*-glycero-3-phosphoglycerol; 5- or 12-SLPC, 1-palmitoyl-2-(5- or 12-doxyl)stearoyl-*sn*-glycero-3-phosphocholine; TLC, thin-layer chromatography; ESR, electron spin resonance; NMR, nuclear magnetic resonance; MLV, multilamellar vesicles; SUV, small unilamellar vesicles.

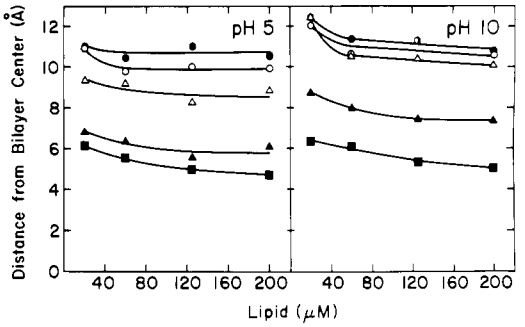

FIGURE 3: Effect of lipid concentration on apparent depth of the anthroyloxy groups of anthroyloxy fatty acids in membranes. Experimental details are as described in the legend to Figure 1 for 12-AS. Depth (z_{cf}, the distance from the bilayer center) was calculated at each lipid concentration using eqs 8 and 9 of Chattopadhyay and London (1987). (Left) pH 5. (Right) pH 10. (●) 2-AS, (○) 6-AS, (△) 9-AS, (▲) 12-AS, (■) 16-AP. Values shown have not been corrected for anthroyloxy fluorescence arising from unbound anthroyloxy probes. The correction is only significant at 20 μM lipid.

Table I: Depth of Anthroyloxy Groups of Anthroyloxy Fatty Acids at pH 5 and 10[a]

	z_{cf} (Å)		F/F_o 5-SLPC		F/F_o 12-SLPC	
molecule	pH 5	pH 10	pH 5	pH 10	pH 5	pH 10
2-AS	10.7	11.2	0.310	0.299	0.357	0.360
6-AS	9.9	10.9	0.255	0.263	0.275	0.308
9-AS	8.8	10.4	0.237	0.238	0.232	0.268
12-AS	6.0	7.5	0.246	0.233	0.190	0.206
16-AP	5.1	5.5	0.260	0.263	0.186	0.195

[a] z_{cf} is distance of the fluorophore from the bilayer center.[3] Depth was determined from the ratio of fluorescence in various samples containing 1.07 μM AS or AP and between 60 and 200 μM mixed 15 mol % 5- or 12-SLPC/85 mol % DOPC in pH 5 or 10 buffers (see Figure 1) using eqs 8 and 9 of Chattopadhyay and London (1987). The average depth values for the different lipid concentrations are given. Corrections for the fluorescence of fluorophores in solution were not significant at the lipid concentrations used.

is unprotonated. At a given pH, the binding curves are similar in the presence and absence of spin labels, with binding being half-maximal at around 10–15 μM. That the binding is in fact similar is more clearly illustrated in Figure 2, where the normalized curves of percent maximum fluorescence vs lipid concentration are displayed. Similar results, not shown here, were found for all of the other anthroyloxy-labeled fatty acids in 11-CU.

Effect of Attachment Position of Anthroyloxy Group to Its Depth in the Membrane. Figure 3 shows the apparent depth of the anthroyloxy groups of the anthroyloxy-labeled fatty acids plotted vs lipid concentration. In general, the depths determined for lipid concentrations at and above 60 μM are consistent to within about 1 Å of each other. As expected, the most anomalous values are found at the lowest lipid concentration (20 μM) where binding is most incomplete. Table I summarizes the quenching-determined depths of the anthroyloxy groups of the membrane-inserted anthroyloxy fatty acids at pH 5 and 10. Let us first consider probe locations at any single pH. Table I shows that the anthroyloxy groups occupy a graded series of depths at each pH.[2] This is consistent with

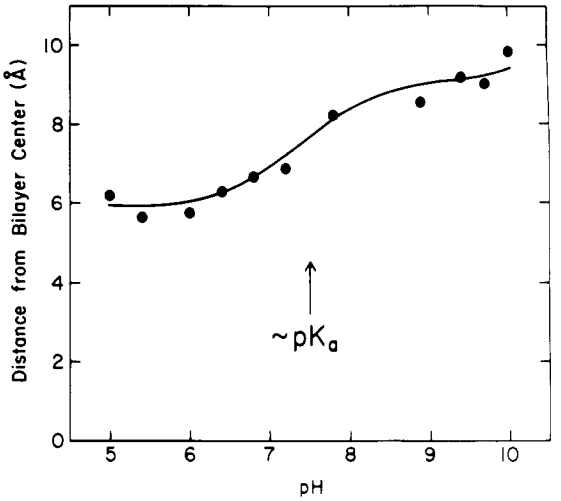

FIGURE 4: Effect of pH on the depth of the carbazole group of 11-CU. Samples containing 10 mL of MLVs were prepared from mixtures of 0.72 nmol of 11-CU and 400 total nmol of 15 mol % 5- or 12-SLPC/85 mol % DOPC suspended in 150 mM NaCl, 5 mM glycine, 5 mM acetic acid, and 5 mM NaH_2PO_4 that had been titrated to pH 5.0 with NaOH (total Na concentration, 158 mM). All samples were titrated from pH 5 to 10 by adding small aliquots of 1 M NaOH, measuring fluorescence after each addition (the acetic acid and chloride movements allowing pH equilibration across the membranes). The time between readings was 7–8 min. Depth was calculated at each pH as in Figure 3.

a structure in which the fatty acid carboxyl groups are anchored at the polar interface and the fatty acyl chains orient such that their terminal methyl groups are the part of the fatty acid chain closest to the center of the bilayer. Such an orientation is consistent with that inferred from a number of studies (see Discussion). The anthroyloxy group appears to be a little deeper for 2-AS, and somewhat shallower for 16-AP, than expected on the basis of a linear extension of the acyl chain. Possible reasons for this behavior are considered in the Discussion.

Effect of Carboxyl Ionization on the Depth of the Fatty Acid Attached Probes. The effect of the state of carboxyl ionization on the depth of fluorophores attached to fatty acids was also examined. Since the pK_a of membrane bound fatty acids is around 7, pH 5 was chosen for measurement of the depth of the protonated form of the fatty acids and pH 10 for the unprotonated form. It can be seen in Table I that ionization of the carboxyl groups of the fatty acids causes the fluorescent groups to shift to a shallower location in the bilayer. This observation demonstrates the sensitivity of the depth assay to small changes in depth. To ensure that the small changes observed in the more extreme locations were not experimental artifacts, measurements were repeated at pH 5 and 10 using a single set of samples. This was accomplished by first preparing depth-quenching samples of 2-AS, 9-AS, and 16-AP at pH 10. After measurement of the fluorescence to determine depth, the pH was adjusted to 4.8 by the addition of acetic acid, which is membrane permeable, and the fluorescence was remeasured. The depths calculated from these samples, at both low and high pH, were found to be very similar to those where

[2] It should be noted that Table I also makes the point that it is difficult to estimate depths by using a single quencher with a series of similar fluorophores. This is illustrated by the lack of a clear correlation of differences between F/F_o values with anthroyloxy depth for the 5-SLPC. The reason is that there are differences between different fluorophores other than depth, such as fluorescence lifetime, that affect the amount of quenching. This problem is not encountered when using a single fluorophore with a series of quenchers.

[3] The z_{cf} values in Table I may be slight underestimates due to trans leaflet quenching, which is quenching due to spin labels in the leaflet opposite to the one in which a specific fluorophore molecule is located (Chattopadhyay & London, 1987). Although it is theoretically possible to correct for this effect (Chattopadhyay & London, 1987), we find the correction is difficult to apply because it involves the use of the critical separation model, which is less reliable at the large distances involved, and an R_c value is needed.

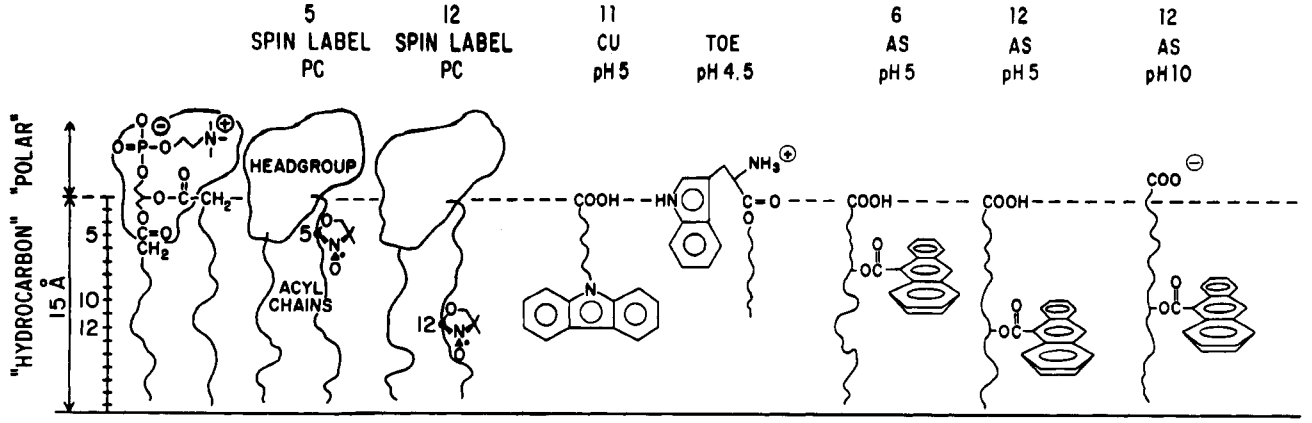

FIGURE 5: Schematic diagram illustrating the depths of some of the fluorophores and quenchers used in these studies.

samples had been prepared independently at each pH. The ability of the fatty acid to shift to a deeper location after decreasing the pH to 4.8 also showed that the decreased depth at pH 10 was not an artifact due to high pH induced lipid degradation.

The dependence of depth on the ionization of the carboxyl group was explicitly shown by pH-dependent depth measurements over the titration range for the carboxyl group of membrane bound 11-CU, a carbazole-labeled fatty acid. Figure 4 shows that for the carbazole fatty acid a change in carbazole depth to a shallower location at high pH occurs with a midpoint near pH 7.5. This is consistent with the change being due to fatty acid carboxyl ionization. The change in depth of the carbazole group appears to be somewhat larger than for the anthroyloxy groups at a similar depth, but it should be noted that in some experiments the change in carbazole depth with pH was not quite so large (Abrams & London, 1992).

Effect of Other Environmental Factors on Fatty Acyl Depth. In order to examine what physical factors might influence the membrane location of fatty acids, the effects of ionic strength and lipid chemical structure on the depth of the anthroyloxy group of 9-AS were examined. In one experiment, anthroyloxy depth in membranes containing 50% of the anionic lipid PG was measured at pH 10. Since the fatty acid carboxyl group will be ionized under these conditions [stearic acid in DMPG membranes has been reported to have a pK_a of 8.0 (Sankaram et al., 1990)], such samples allow the evaluation of the effect of electrostatic repulsions between the phospholipids and fatty acid carboxyl groups on depth. Table II shows that with membrane vesicles composed to 50 mol % PG and 50 mol % PC there is at most a very small effect of ionic strength on depth over the range 0–750 mM NaCl. In addition, the anthroyloxy depth in membranes of 50% PG is similar to that obtained with 100% PC. These results suggest that electrostatic interactions between the fatty acid carboxyl and the lipid phosphate do not greatly influence fatty acyl depth.

The depth of the anthroyloxy group of 9-AS was also examined in DMPC, a lipid which makes thinner bilayers than DOPC (Lewis & Engelman, 1983) and which does not contain double bonds. When the depth was measured for 9-AS incorporated into DMPC vesicles at pH 5, an apparent depth of 8.2 Å from the center of the bilayer was found as compared to the 8.8 Å obtained using DOPC vesicles. This indicates that the depth of the anthroyloxy group is not very different in the two lipids. However, due to the change in bilayer thickness the spin-label groups themselves may be at somewhat different depths in the DMPC membranes than they are in DOPC membranes. Since the spin-label locations are uncertain, the precise location of the anthroyloxy group in DMPC cannot be specified.

Table II: Ionic Strength and Lipid Effects on Depth of Anthroyloxy and Carbazole Groups Attached to Lipids[a]

sample	z_{cf} (Å)
9-AS in 50% DOPG/35% DOPC/15% SLPC, pH 10	
0 NaCl	10.6
150 mM NaCl	10.4
750 mM NaCl	10.2
9-AS in 85% DOPC/15% SLPC, pH 10	
150 mM NaCl	10.4
9-AS in 85% DMPC/15% SLPC, pH 5	8.2
9-AS in 85% DOPC/15% SLPC, pH 5	8.8
11-CU in 85% DOPC/15% SLPC, pH 5	6.9
11-CU in 85% DOPC/15% SLPC, pH 10	8.8
11-CU-PC in 85% DOPC/15% SLPC, pH 5	7.6

[a] Lipid compositions are given in mol %. Samples contained 0.072 μM CU probe or 1.07 μM 9-AS incorporated in MLV containing a total of 200 μM nonfluorescent lipids. Depth was calculated from spin-labeled lipid quenching as described in the footnote to Table I.

To compare the membrane location of a fatty acid with that of the fatty acyl chain of a phospholipid, the depth of a carbazole group attached to a fatty acid was compared to that of the same group attached to a fatty acyl chain of a phospholipid. Table II shows that the location of the carbazole group attached to a fatty acid is similar to that obtained when it is attached to the 2-position chain of a phospholipid.

In Figure 5, we schematically summarize the depth of the anthroyloxy, carbazole, and Trp probes studied, under various conditions, in this report and the preceding paper (Abrams & London, 1992).

Effect of Emission and Excitation Wavelength on Apparent Depth of Fluorescent Groups. Figure 6 shows the effect of emission wavelength on the depth obtained for anthroyloxy groups of MLV-incorporated anthroyloxy fatty acids. In every case when fluorescence is measured at longer wavelengths, the fluorophore appears to be closer to the membrane surface. The range of measured depths does not appear to be significantly dependent on the position of attachment of the anthroyloxy group or the state of carboxyl ionization. This wavelength dependence of depth indicates that there is a distribution of anthroyloxy groups that emit at slightly different wavelengths and are located at different depths (see Discussion).

It should be pointed out that the apparent range of measured depths is very likely to represent only a lower limit to the actual range of fluorophore depth distribution because of the extensive overlap of the emission spectra of these fluorophores in different environments. In this regard, it should also be noted that we have seen a slightly larger emission wavelength de-

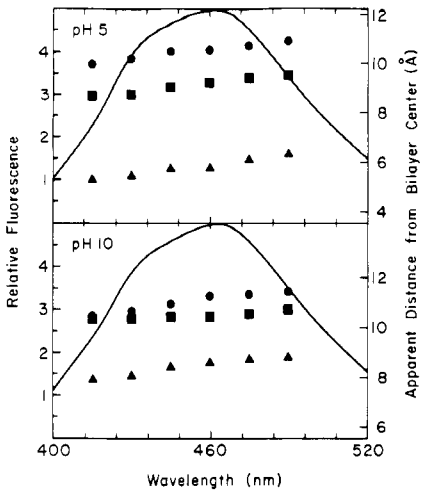

FIGURE 6: Effect of emission wavelength on apparent anthroyloxy depth. (Top) pH 5. (Bottom) pH 10. The emission spectra (solid lines) of 6-AS incorporated into DOPC MLV at the indicated pHs are superimposed for reference. The emission spectra of the other anthroyloxy fatty acids are similar, except for being red- or blue-shifted by a few nanometers. The emission wavelength dependence of calculated distances from the bilayer center (z_{cf}) are shown for (●) 6-AS, (■) 9-AS, and (▲) 12-AS. They were calculated as in Figure 2. Excitation was set at 365 nm. Samples contained MLV composed of 1.6 nmol of AS and 300 nmol of 15 mol % 5- or 12-SLPC/85 mol % DOPC suspended in 1.5 mL of either 150 mM NaCl and 10 mM acetate, pH 5, or 150 mM NaCl and 10 mM glycine, pH 10.

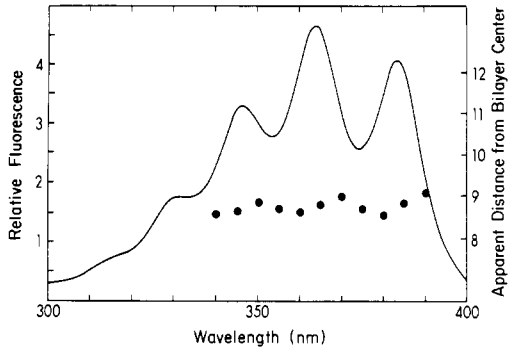

FIGURE 7: Effect of excitation wavelength on apparent anthroyloxy depth for 9-AS at pH 5. Samples were prepared as in Figure 6. Fluorescence was measured at various excitation wavelengths, and the emission wavelength was fixed at 461 nm. Depth (●) was calculated as in Figure 3. The excitation spectrum of 9-AS incorporated into DOPC MLV (solid line) is superimposed on the depth results.

pendence of depth for 11 CU-PC and a much larger dependence for a PC containing a deeply locating indolyl group (data not shown) at the end of the 2-position fatty acyl chain (a gift of Dr. J. Silvius). This may be a result of either a wider distribution of depths for these fluorophores and/or increased spectral resolution of the shallow and deep fluorophore populations.

The effect of excitation wavelength on depth measurements was also examined. Figure 7 shows the effect of excitation wavelength on the depth of the anthroyloxy group of 9-AS incorporated into MLV. For each individual excitation peak, the measured depth is shallower at the red end than it is at the blue end, again indicating heterogeneity in depth.

Discussion

Comparison of Anthroyloxy Positions Determined by Parallax Analysis to Literature Data. There have been several studies that have examined the location of the fatty acid anthroyloxy probes in membranes (Podo & Blasie, 1977; Thulborn & Sawyer, 1978; Chalpin & Kleinfeld, 1983; Eisenger & Flores, 1983). Most of this work has only resulted in conclusions about the relative locations of the anthroyloxy groups. Comparison of these studies to our results is further complicated by differences in the samples and conditions used. For example, most studies were conducted at about pH 7, where fatty acids are in a mixture of ionized and unionized forms. However, notwithstanding differences in experimental conditions, there is fundamental agreement between the previous studies and this report in that the anthroyloxy groups have been found to locate at a graded series of depths in the bilayer and that this series is monotonically dependent on the number of carbon atoms between the fatty acid carboxyl and the anthroyloxy group.

In one study, using red blood cell membranes, actual values for the location of the anthroyloxy groups have been reported (Eisinger & Flores, 1983). These results cannot be directly compared to ours for a number of reasons. The erythrocyte membranes are structurally quite different from our model membranes. For example, they contain considerable amounts of cholesterol and proteins. The fact that the transleaflet flip-flop of anthroyloxy fatty acids appears to be slow in erythrocyte membranes (Eisinger & Flores, 1983) but fast in model membranes (Storch & Kleinfeld, 1986) is an indication that these differences do affect fatty acid behavior. In addition, the study of Eisenger and Flores makes complex assumptions concerning the approach of their quenching hemoglobin molecules to the inner surface of the erythrocyte membrane. Nevertheless, their results parallel ours in that the anthroyloxy groups are found to occupy a graded series of depths.

The range of depth we observe for the anthroyloxy groups attached at different positions is somewhat less spread out than would be predicted if there was a linear dependence of anthroyloxy depth on attachment position. One can ask whether this is simply an experimental artifact. We think this is unlikely to be the case. First, the assumption that depths will be a simple reflection of attachment position is unrealistic. The anthroyloxy group has almost as many carbon atoms as the entire fatty acyl chain, and it is unlikely that its location would only reflect the energetic influences of the acyl chain. In addition, the predicted artifacts in apparent depth arising from different motions do not predict the anthroyloxy behavior found (see the preceding paper). Therefore, the nonlinear distribution of anthroyloxy groups is likely to be, at least in part, a real physical phenomenon. For example, in the case of the 16-AP the shorter acyl chain and terminal position of the anthroyloxy group may allow it to curl somewhat toward the membrane surface relative to its "expected" position.

On the other hand, we would like to caution that it is still possible that there are some limitations in parallax analysis that are acting as sources of error. Preliminary results using a spin label attached to the polar headgroup suggest that the anthroyloxy group of 2-AS is actually at a shallower location than that found here using the spin-label quenchers deeper in the membrane (unpublished observations). Therefore, it may be that errors arising when the quenchers are too far from the fluorophores may have an effect in some cases (see the preceding paper). Nevertheless, combining the results of the preceding paper and the agreement of the anthroyloxy results with literature data, it is clear that parallax analysis is a powerful method to obtain useful depth information.

The Effect of the Ionization of Fatty Acids on Depth. This study has also shown that carboxyl ionization decreases fatty acid depth in membranes. Previous ESR work had suggested that such a movement occurred upon ionization as judged by pH-induced changes in the order parameter of nitroxide groups

on spin-labeled fatty acids [e.g., Barratt and Laggner (1974) and Sanson et al. (1976)]. Here, it is directly demonstrated that a change in depth occurs, and, in addition, the magnitude of this movement is revealed. Furthermore, the results show that the movement occurs throughout the fatty acyl chain.

That these small changes in depth can be detected shows that parallax analysis is very sensitive to small changes in depth. This conclusion is strengthened by its ability to distinguish the depths of the various closely spaced anthroyloxy probes, despite the large size of the anthroyloxy group.

One question that arises from the pH dependence of fatty acyl depth is why ionization decreases the depth of fatty acyl chains. This is likely to be a result of the ionized carboxyl group tending to lower its energy by seeking a more polar region.

The Wavelength Dependence of Depth. When fluorescence was measured at longer emission and excitation wavelengths, the depth of the molecules was found to be nearer to the membrane surface. Although in the case of the anthroyloxy groups the effect on depth is small, in some cases the spectral dependence of depth was found to be larger. Therefore, it is important to consider the choice of wavelengths in a depth experiment. In general, depth should be measured near λ_{max}, where it would be most representative of the average depth.

There are two explanations for the wavelength dependence of depth which suggest themselves to us. One involves the anthroyloxy groups occupying a range of depths. This is no doubt true, in view of the recent results that describe depth distributions in membranes (Wiener et al., 1991, Wiener & White, 1991). Since anthroyloxy fluorescence is sensitive to the polarity of its environment (Waggoner & Stryer, 1970; Werner & Hoffman, 1973), it is likely that the emission spectrum from the shallower molecules, being closer to the polar region of the membrane, are red (long wavelength) shifted relative to the deeper molecules. As a result, the red end of the spectrum in our samples will tend to be dominated by shallower molecules and the blue (short wavelength) end by deeper molecules, as reflected in the measured depths.

A second contribution may come from the movement of the fluorophores toward the membrane surface during the time thay are in the excited state. This could occur because the excited state of the anthroyloxy groups is considerably more polar than its ground state (Werner & Hoffman, 1973). Because in membranes anthroyloxy groups that have been in the excited state longest tend to emit at longer wavelengths (due to slow solvent relaxation) (Matayoshi & Kleinfeld, 1981), the fluorescence at long wavelengths would tend to emanate from molecules that had moved to a shallower location. A similar proposal was made by Demchenko and Shcherbatska (1985). At present, it is difficult to separate the wavelength dependence due to the distribution of fluorophores at different depths from that due to a possible movement of these molecules while in their excited states.

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