Calibration of the Parallax Fluorescence Quenching Method for Determination of Membrane Penetration Depth: Refinement and Comparison of Quenching by Spin-Labeled and Brominated Lipids†

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Received November 13, 1991; Revised Manuscript Received March 13, 1992

ABSTRACT: We previously introduced the "parallax" method, which uses fluorescence quenching by spin-labeled lipids in order to measure the depth of molecules within a membrane [Chattopadhyay, A., & London, E. (1987) *Biochemistry 26*, 39–45]. In this report the accuracy of this method is established by comparison of spin-label quenching to that obtained using brominated lipids. To accomplish this, the fluorescent molecules used were a fatty acid labeled with a carbazole buried deeply within the acyl chain region of the membrane, an acyl-Trp with the Trp residue residing near the polar membrane region, and cytochrome b_5, which has Trp residues in its membrane-inserted region. The depths calculated from the amount of bromine quenching agreed with those determined using parallax analysis. This indicates that the depth reported by parallax analysis is accurate and that the spin labels reside very close to their predicted locations in the membrane. Furthermore, there was good agreement when parallax analysis was applied both to quenching by brominated and spin-labeled molecules, suggesting that the analysis is valid in both cases. The effect that different distributions and motions of fluorophores and quenchers would have on parallax analysis was also examined. For uniform distributions of quenchers or fluorophores over a range of depths, it was found that the analysis reports the average fluorophore depth. In addition, experimental data suggest that motional effects do not significantly alter the measured depths. This is consistent with the motions during the short excited state lifetime of the fluorophores being relatively small and/or relatively isotropic.

How far a specific site on a molecule is from the membrane surface or bilayer center is an important parameter in the study of membrane structure. A fluorescence-quenching technique to determine this membrane penetration depth of fluorophores was developed in our laboratory (Chattopadhyay & London, 1987). This method involves analyzing fluorescence quenching by quenchers attached to phospholipids at defined positions. It was found that the data could be analyzed by the use of simple algebraic expressions and thereby allow for the direct determination of membrane penetration depth. We named this approach the parallax method. A variety of NBD-labeled[1] lipids were studied using this method. The NBD groups were often found to be located near the surface. The polar nature of the NBD group provided an energetic rationale for its localization. Further work on the dependence of NBD fluorescence on polarity, the aqueous quenching of NBD groups by cobalt, and the determination of the apparent pK_a of the NBD groups all corroborated the results obtained from the parallax depth assay (Chattopadhyay & London, 1988; Chattopadhyay, 1990). The parallax assay has also been used to locate Trp residues in membrane-inserted acetylcholine receptor, diphtheria toxin, and the influenza hemagglutinin fusion peptide (Chattopadhyay & McNamee, 1991; Jiang et al., 1991; Clague et al., 1991).

In this report the validity of the method is further examined. For this purpose, a series of brominated lipids developed by Holloway and co-workers was used (Markello et al., 1985). A particular advantage to these probes is that in bilayers formed from brominated lipids the depths of the bromine atoms have been determined using X-ray diffraction (McIntosh & Holloway, 1987; Weiner & White, 1991). We found that the depths determined from spin-label quenching using parallax analysis agreed with those determined from the relative strength of the quenching obtained from the different brominated lipids.

In addition, parallax analysis was extended to take into account the effects of spatial and temporal distributions of fluorophore and quencher. These concerns are important as there is some motion on the fluorescence time scale, and recent studies have shown that molecular groups occupy a distribution of depths (Weiner & White 1991; Weiner et al., 1991). The extended analysis and experimental results show that the parallax analysis would give accurate results over a wide range of conditions.

Experimental Procedures

Materials. Spin-labeled and brominated PCs, rhodamine-PE, NBD-PE, DOPC, and POPC were purchased from Avanti

† Supported by NIH Grant GM 31986.

[1] Abbreviations: 2-, 6-, 9-, or 12-AS, 2-, 6-, 9-, or 12-(9-anthroyloxy)stearic acid; 16-AP, 16-(9-anthroyloxy)palmitic acid; 4,5-, 6,7-, 9,10-, or 11,12-BrPC, 1-palmitoyl-2-(4,5-, 6,7-, 9,10-, or 11,12-dibromostearoyl)-*sn*-glycero-3-phosphocholine; 11-CU,11-(9-carbazole)undecanoic acid; di-9,10-BrPC, 1,2-di(9,10-dibromo)stearoyl-*sn*-3-glycerophosphocholine; DOPC, 1,2-dioleoyl-*sn*-3-glycerophosphocholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; 5- or 12-SLPC, 1-palmitoyl-2-(5- or 12-doxyl)stearoyl-*sn*-glycerocholine; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; TOE, DL-tryptophan octyl ester hydrochloride; DMSO, dimethyl sulfoxide; TLC, thin-layer chromatography; ESR, electron spin resonance; Tempol, 4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy; PCA, 2,2,5,5-tetramethyl-3-pyrroline-1-oxyl-3-carboxylic acid; GC/MS, gas chromatography/mass spectroscopy; MLV, multilamellar vesicles; SUV, small unilamellar vesicles.

0006-2960/92/0431-5312$03.00/0

Polar Lipids. DMPC was purchased from Calbiochem. Anthroyloxy-labeled fatty acids, 11-CU, and PCA were purchased from Molecular 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, Triton X-100, and *Crotalus adamanteus* venom were purchased from Sigma. Cytochrome b_5 was a gift from Peter Holloway (University of Virginia). It was over 90% pure as judged from SDS gel electrophoresis.

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 a phosphate-sensitive spray (Dittmer & Lester, 1964) and charred. TLC of the fluorescent fatty acids and TOE was done in *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 lipid, the intensities of doubly integrated ESR spectra of spin-labeled lipids were compared to that of two standard spin labels, Tempol and PCA (Chattopadhyay & London, 1987). Duplicate samples containing spin-labeled lipids or a known concentration of standards dissolved in DMSO at approximately 0.2 mM were placed in 50-μL capillary tubes. We used samples of over 50 μL placed so as to ensure filling of the ESR sample cavity. DMSO was chosen because it was found that integrated intensities were insensitive to oxygen dissolved in DMSO. 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 0.9 ± 0.05.

Standard Curves for Determining Spin-Label Content by Fluorescence Quenching. We have developed a fluorescence spin-label assay using the quenching of NBD-PE by spin-labeled lipids to derive a standard curve. This method should be of use to workers who do not have access to an ESR spectrometer. Samples were prepared containing spin-labeled PC (previously assayed by ESR and phosphate assays) and DOPC (a total of 160 nmol of these two lipids) plus 1.6 nmol of NBD-PE,[2] all dissolved in chloroform. They were dried as usual (see below) and then hydrated with 1.5 mL of 10 mM sodium phosphate buffer and 150 mM NaCl, pH 7.0, by vigorous vortexing for 50 s to form MLVs. Occasional samples that did not disperse well despite vortexing were discarded. Fluorescence was measured with an excitation wavelength of 469 nm and an emission wavelength of 533 nm. The excitation and emission slit widths were both 2.5 mm (4.5-nm band-pass). Fluorescence intensity was obtained from the average of three 15-s readings. Background samples without NBD-PE gave negligible fluorescence (much less than 1%). In agreement with previous results (Chattopadhyay & London, 1987), it was found that the dependence of ln (F/F_0) on spin-label concentration is linear, as predicted by eq 1 (see Theory). Linear least-squares analysis gave slopes of −4.43 and −3.17 for the 5-SLPC and 12-SLPC curves, respectively.[3] To determine the spin-label concentration, one can measure F/F_0 in MLV containing SLPC, DOPC, and (1%) NBD-PE and determine the actual spin label concentration by substitution into eq 1 and making use of the slopes given above (e.g., ln $F/F_0 = -3.17c$ for 12-SLPC).

Analysis of Acylation Position. To check the acylation position of labeled fatty acids, phospholipase A_2 digests were performed on spin-labeled and brominated lipids essentially as described in Kates (1986). Lysolipid and fatty acid were separated by TLC in $CHCl_3/CH_3OH/H_2O$ (65:25:4 v/v), and their positions were identified by their R_f in parallel samples that were subjected to charring. The fatty acids were extracted (3×) from the silica gel with the TLC solvent, and the lysolipid was extracted (3×) with $CHCl_3/CH_3OH/H_2O$ (20:20:2 v/v). To separate the extraction solvent from the gel, the samples were centrifuged at low speed. Lysolipid derived from spin-labeled lipid was analyzed by phosphate assay and ESR, respectively. The lysolipid and fatty acid extracts from the brominated lipid were subjected to methanolysis to form fatty acid methyl esters (Kates, 1986) and then analyzed by GC/MS (Hewlett Packard 5890A gas chromatograph with Hewlett-Packard 5970 series mass selective detector). The amount of brominated and unbrominated fatty acid methyl esters in the lysolipid and fatty acid methanolysis products was then determined by comparing the area of the GC/MS peaks for each of these to standard curves derived from the methanolysis products of the parent brominated phospholipids.

In those samples in which complete phospholipase digestion was obtained, about 70–80% of the labeled chain was found in the 2-position, as found in earlier studies (Chattopadhyay & London, 1987). In the case of 6,7-BrPC and 5-SLPC, digestion was not complete. The interference with digestion by the labels, due to their close juxtaposition to the carboxyl group on the 2-position chain, is likely to be responsible for this. In these cases the total of the undigested labeled lipids (assessed by TLC) plus label found in the digested lipid also suggested that 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).

Quantitation of Bromine Content of Brominated Lipids. The number of bromines per brominated phospholipid was analyzed using the gold chloride method (Tietz, 1976) modified for use with brominated lipids (Markello et al., 1985; P. Holloway, personal communication). Dried lipids or bromide standards (NaBr) containing 0.22–2.2 μmol of bromine were mixed with 200 μL of 5% potassium *tert*-butoxide (Aldrich) in methanol, tightly capped to minimize evaporation, and then incubated for 1 h at 110–120 °C. Next, samples were redried and 200 μL of methanol was added to each. Then 300 μL of 30% HNO_3, 100 μL of 3% Triton X-100, and 600 μL of methanol were added. The samples were then vortexed until clear, and 30 μL of gold chloride ($AuNaCl_4 \cdot 2H_2O$) (5.9 mg/mL) was added to each. After 5 min, absorbance was measured at 440 nm. The bromine to phosphate ratios for the 6,7-BrPC, 9,10-BrPC, and 11,12-BrPC were 1.98, 2.54, and 1.81 bromines per phospholipid, respectively (expected value 2).

Since bromination dramatically affects lipid density, sucrose density centrifugation was also used to assess the degree of

[2] NBD-PE concentration may be determined from ϵ(470 nm) = 18 300 or ϵ(480 nm) = 16 300 L mol^{-1} cm^{-1}.

[3] Using these data, calculated NBD depths were about 1–2 Å closer to the center of the bilayer for NBD-PE and NBD-PCs than were found in our previous study. This may partly reflect an improved technique for calibration of ESR intensity.

Table I: Comparison of Spin-Labeled and Brominated Lipid Quenching and Calculated Depth of Fluorescent Molecules[a]

	z_{cf} (Å)		F/F_0				
			SLPC		BrPC		
	spin label	bromine	5-	12-	6,7-	9,10-	11,12-
TOE pH 4.5	13.5	11.7	0.40	0.59	0.14	0.23	0.43
11-CU pH 5	6.9	7.5	0.35	0.30	0.31	0.17	0.22
11-CU pH 10	8.8	8.4	0.32	0.31	0.24	0.16	0.23
cyt b_5	11.1	10.9	0.64	0.72	0.18	0.25	0.40

[a] F/F_0 refers to the fluorescence intensity in the presence of quencher-containing membranes divided by the intensity in the presence of membranes without quenchers. z_{cf} was calculated from eq 3. 5, 12, 6,7, 9,10, and 11,12 refer to the corresponding spin-labeled and brominated lipids. For calculations, the average bromine distances from the bilayer center based on X-ray diffraction were taken to be 10.8, 8.3, and 6.3 Å for the 6,7-BrPC, 9,10-BrPC, and 11,12-BrPC, respectively (McIntosh & Holloway, 1987). For the parallax calculation using brominated lipids, the average of depths calculated using the 6,7-, 9,10-BrPC pair and the 6,7-, 11,12-BrPC pair is reported. It is not practical to calculate depth using the 9,10- and 11,12-BrPC pair because for quenchers closely spaced in depth small errors in the experimentally determined degree of bromination will overwhelm differences due to depth. This would also be true for spin-labeled lipids closely spaced in depth. The distances from the center of the bilayer for the 5-SLPC and 12-SLPC were estimated to be 12.15 and 5.85 Å, respectively (Chattopadhyay & London, 1987). Samples of TOE and 11-CU were prepared as described in the legend to Figure 2. SLPC membranes contained 15 mol % spin-labeled lipids. BrPC membranes contained 100% brominated lipid. The reported depths calculated from eq 3 and F/F_0 values are the average from samples with greater than 40 μM lipid. Cytochrome b_5 samples were prepared as described under Experimental Procedures.

lipid bromination. MLVs (2 mM) of the different lipids, containing 0.25% rhodamine PE to provide a visual marker, were prepared in water. Samples (20 μL) were mixed into 160–180-μL sucrose solutions and then centrifuged in an Airfuge (Beckman) at roughly 150000*g* for 3 h using an A-100 rotor. The approximate density of the lipid vesicles was determined by ascertaining whether the vesicles floated or pelleted in different sucrose solutions. The densities determined (or approximate w/v percent sucrose having the same density as lipid) were 1.01 (3.5%) DOPC, 1.13 (31%) for 6,7-BrPC, 1.11 (27%) for 9,10-BrPC-BrPC, 1.12 (28%) for 11,12-BrPC, and 1.23 (50%) for di-9,10-BrPC. The densities for the putative dibrominated 6,7, 9,10, and 11,12 species agree with the data of Markello et al. (1985) and are halfway between the results obtained for unbrominated lipid and the putative tetrabrominated di-9,10 species, as expected.

The combination of the results from the gold chloride and density assays indicate that the 6,7-, 9,10-, and 11,12-BrPC carried two bromines per molecule within 10–20%. Further information was obtained by the quenching of cytochrome b_5 by the various brominated lipids. The values shown in Table I are in close agreement with those of Markello et al. (1985), confirming both the degree of bromination and the proper location of the bromines on the fatty acyl chains. Small differences between our results and those of Markello et al. may be due to some fatty acid impurities we detected by GC/MS.

Preparation of Samples for Quenching Studies. Samples were prepared by mixing the desired quantities 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 then dried under N_2 at around 35 °C, except for the brominated lipids which were not warmed. They were then mixed well in a few drops of chloroform and redried. (We have had problems when ethanol was used.) Then they were 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. Generally, either duplicates or triplicates were prepared for each sample containing quencher and fluorophore. For depth measurements three types of samples were prepared: one which contains a lipid with a quencher group located at a shallow location in the membrane, a second containing a quencher group deeper in the membrane, and a third containing no quencher. Fluorescence background samples containing membranes without fluorophore were also prepared.

For cytochrome b_5 experiments using spin-labeled lipids, the sample preparation was as follows. SUVs were prepared by hydrating 6 μmol of total lipid (POPC with or without 10 mol % of either 5-SLPC or 12-SLPC) with 0.1 mM EDTA and 10 mM HEPES, pH 8.01, to form MLVs. These lipid samples were then freeze-thawed five times using a dry ice/acetone bath. To produce SUVs each sample was then extruded 10 times through two stacked 0.03-μm pore size polycarbonate filters in an extruder (Lipex Biomembranes, Vancouver, BC). [This produces SUVs of around 600-Å diameter (Mayer et al., 1986). Sonication was avoided because it seemed to degrade the spin-label under some conditions.] To a 1-mL sample of 1 mM lipid, 1 nmol of cytochrome b_5 was added (4.6 μL of 218 μM cytochrome b_5 in 10 mM Tris-acetate, 1 mM EDTA, pH 8.1). The samples were then incubated for 30 min before fluorescence was measured. (This lipid to protein ratio was found to result in essentially all the protein being bound to vesicles.) For the quenching of cytochrome b_5 using brominated lipids, SUVs were prepared by sonication. Samples containing cytochrome b_5 with POPC or 100% BrPCs were then prepared as above. In all cases, background samples were prepared without protein.

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 unless otherwise noted. Unless otherwise noted the excitation and emission slit widths were 2.5 and 5 mm, respectively. These correspond to band-passes of 4.5 and 9 nm. 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 cytochrome b_5, fluorescence was measured in a 4 mm by 10 mm path length semimicrocuvette, with the long path length aligned with the excitation beam. Fluorescence was measured at 295 nm excitation and 340 nm emission. The excitation and emission slit widths were set at 1.25 and 5.0 mm (band-pass 2.25 and 9 nm), respectively.

Samples were mixed by agitation just prior to measurement of fluorescence. This was necessary to eliminate variations due to vesicle settling. Fluorescence was averaged over three 15-s readings. In all cases, the intensity from background samples without fluorophore was subtracted. When necessary, before calculating depth from fluorescence, the amount of fluorescence emanating from probes not bound to the membrane was subtracted from the measured intensity. This value (which was always very small) could be approximated from the fluorescence in the absence of lipid and the percent of fluorophore that is membrane-inserted [which was derived

from the binding curves (see Results)]. The temperature of the samples was room temperature unless otherwise noted. Further details are given in the figure legends.

Fluorescence polarization measurements were made by inserting Glan–Thompson polarizers (Spex 1935B polarization kit) in the excitation and emission beams. The fluorescence intensity of the vertical and horizontal components was measured and after subtraction of background intensities polarization calculated by

$$P = [I_{vv} - I_{vh}(I_{hv}/I_{hh})]/[I_{vv} + I_{vh}(I_{hv}/I_{hh})]$$

Absorbance measurements were made using either a Gilford 250 or a Cary 17 spectrophotometer.

For studies of the effect of temperature on apparent depth of anthroyloxy groups, samples were prepared containing 300 nmol of lipid, either 15 mol % SLPC/85 mol % DOPC or 100% DOPC, mixed with 1.6 nmol of fluorophore (6-AS or 12-AS) and then hydrated with 1.5 mL of 150 mM NaCl and 10 mM sodium acetate, pH 5. To reduce experimental error, several samples were prepared, checked for reproducibility of fluorescence intensities, and combined before measuring the reported fluorescence. The samples were then placed in a cell holder under thermostatic control, and the temperature was increased to the temperature used. Sample temperature was measured with a digital thermometer in a cuvette containing water placed in the sample holder. Possible O_2 effects were evaluated by bubbling saturating O_2 or Ar into the samples, immediately stoppering, and then measuring fluorescence. At 24 °C replacement of air with these gases did not affect calculated depth, indicating that the amount of O_2 present did not influence the results.

Theory

Parallax analysis is based on the extension of the Perrin quenching equation (Perrin, 1924) to fluorophore and quencher in two different planes separated by a distance z but randomly distributed within each plane (Chattopadhyay & London, 1987). This equation has been used to analyze quenching that is static in the sense that there is no significant motional changes in the distances between fluorophores and quenchers during the excited state lifetime of the fluorophore. To describe the distance dependence of quenching, the "hard sphere" (or equivalently "critical separation") model is used, in which quenching is described by a step function characterized by a critical separation distance R_c. In this all-or-none model, if a fluorophore is within R_c of a quencher, there is complete quenching, and when a fluorophore is beyond R_c from any quencher, there is no quenching. For example, with fluorophore and quencher in a single plane fluorescence is extinguished in a circle of area πR_c^2 around each quencher. The utility of this critical separation model is that it can be rigorously shown that it results in a close approximation to the actual quenching over a wide range of conditions, even when quenching is not precisely all-or-none (Chattopadhyay & London, 1987). Furthermore, this approach allows for the reduction of the Perrin quenching equation to simple algebraic expressions. The conditions of validity of this model are discussed in Chattopadhyay and London (1987). Its extension to include the effects of fluorophore and quencher distributions and motions is described below.

Case 1: Fluorophore and Quencher Distributed at Two Different Depths, No Motion. This is the case covered in Chattopadhyay and London (1987). The quenching of a fluorophore in one plane (depth) within a membrane by a quencher in another parallel plane is given by[4]

$$F/F_o = e^{-\pi[(R_c^2 - z^2)^{1/2}]^2 C} = e^{-\pi R_c^2 C + \pi z^2 C} \quad (1)$$

when $z < R_c$ or $\ln (F/F_o) = -\pi R_c^2 C + \pi z^2 C$, where F is the fluorescence in a sample containing fluorophore and quencher, F_o is the fluorescence in a sample containing fluorophore but no quencher, z is the distance between the planes containing fluorophore and quencher, and C is the quencher concentration in molecules of quencher/Å^2 of membrane [calculated by [(mole fraction of quencher lipid in total lipid)/70 Å^2] (Chattopadhyay & London, 1987)]. In eq 1 the expression $(R_c^2 - z^2)^{1/2}$ gives the radius of the circular area quenched by each quencher within the plane containing the fluorophore. $F/F_o = 1$ when $z > R_c$.

If quenching is measured in two samples, one with a quencher placed at a shallow location in the membrane (quencher 1) and a second sample with a deeper quencher (quencher 2), then the ratio of fluorescence in the two samples is given by

$$\frac{F_1}{F_2} = \frac{e^{-\pi R_c^2 C + \pi z_{1f}^2 C}}{e^{-\pi R_c^2 C + \pi z_{2f}^2 C}} \quad (2)$$

where F_1 is fluorescence in the presence of the shallow quencher, F_2 is fluorescence in the presence of the deeper quencher, z_{1f} is the distance between the planes of the fluorophore and shallow quencher, and z_{2f} is the distance between the plane of the deeper quencher and fluorophore. This equation can be rearranged into a form in which the location of the fluorescent molecule can be determined if the locations of the quenchers are known:

$$z_{cf} = L_{c1} + [-\ln (F_1/F_2)/\pi C - L_{12}^2]/2L_{12} \quad (3)$$

where z_{cf} is the transverse distance from the plane of the bilayer center to the plane containing the fluorophore, L_{c1} is the transverse distance from the bilayer center to the shallow quencher, and L_{12} is the transverse distance between the depths of the shallow and deeper quenchers.

Case 2: Fluorophore Distributed Over a Range of Depths. Let us assume that fluorophores are evenly distributed over some range of depths for which $z < R_c$ and that within this range there is an equal contribution to fluorescence from every depth (in the absence of quencher). The value of F/F_o is the sum of the contributions from fluorophores at each of these depths. In this case quenching is given by the average value of F/F_o, the sum of fluorescence from fluorophores in each plane divided by the number of planes containing fluorophore. The appropriate equation is

$$F/F_o = \lim_{\Delta z \to 0} \sum_{i=z_a}^{z_b} \frac{\Delta z}{(z_b - z_a)} [e^{-\pi R_c^2 C + \pi z_i^2 C}] \quad (4)$$

where the fluorophore is distributed in all planes from its deepest location, where the distance to the plane of the quencher is z_a, to its shallowest, and where the distance from the plane of the quencher is z_b. Δz is the width of each slab containing fluorophore. The ratio of fluorescence in the presence of shallower and deeper quenchers is then

$$\frac{F_1}{F_2} = \lim_{\Delta z \to 0} \frac{\sum_{i=z_{1fa}}^{z_{1fb}} \frac{\Delta z}{z_{1fb} - z_{1fa}} (e^{-\pi R_c^2 C + z_{1fi}^2 C})}{\sum_{i=z_{2fa}}^{z_{2fb}} \frac{\Delta z}{z_{2fb} - z_{2fa}} (e^{-\pi R_c^2 C + z_{2fi}^2 C})} \quad (5)$$

where the subscripts 1 and 2 represent the parameters for the

[4] Here and in subsequent sections we are only considering cases in which fluorescence quenching by trans membrane leaflet quenchers is negligible [see Chattopadhyay and London (1987)].

shallow and deeper quencher, respectively, z_{1fa} and z_{1fb} are the distances from quencher 1 to the deepest and shallowest fluorophores, and z_{2fa} and z_{2fb} are the same parameters for quencher 2. Equation 5 can be solved numerically for fluorophore distributions of various widths taking into account that $F/F_o = 1$ where $z > R_c$ (see Results section). The F_1/F_2 values so obtained can be substituted into eq 3 to calculate an apparent z_{cf} which reveals how parallax analysis would be affected by fluorophore distributions.

Case 3: *Quenchers Over a Range of Depths*. If quenchers are spread evenly over a range of depths, then the observed quenching will be the product of quenching contributions from every level containing quencher. In this case fluorescence quenching is given by

$$\frac{F}{F_o} = \lim_{\Delta z \to 0} \prod_{i=z_a}^{z_b} e^{-\pi R_c^2 C' + \pi z_i^2 C'} \tag{6}$$

where the quencher is evenly distributed in all planes from $z = z_a$ to $z = z_b$ from the plane of the fluorophore and the concentration of quencher in each plane C' is given by the total concentration C divided by the number of planes: $C'(= \lim_{\Delta z \to 0} C/[(z_b - z_a)/(\Delta z)])$. Since

$$\prod_{i=z_a}^{z_b} e^{u_i} = e^{\ln(\prod_{i=z_a}^{z_b} e^{u_i})} = e^{\sum_{i=z_a}^{z_b} \ln e^{u_i}} = e^{\sum_{i=z_a}^{z_b} u_i}$$

it can be shown that

$$\frac{F}{F_o} = \lim_{\Delta z \to 0} e^{\sum_{i=z_a}^{z_b} (-\pi R_c^2 C' + \pi z_i^2 C')}$$

and therefore after substituting for C′ and going over to the limit at Δz goes to 0

$$F/F_o = e^{\int_{z_a}^{z_b} (-\pi R_c^2 C + \pi z^2 C)[dz/(z_b - z_a)]} = e^{-\pi R_c^2 C + 1/3\pi(z_a^2 + z_a z_b + z_b^2)C} \tag{7}$$

If the fluorescence in the presence of shallow and deeper quenchers is compared, then

$$\frac{F_1}{F_2} = \frac{e^{-\pi R_c^2 C + 1/3\pi(z_{1fa}^2 + z_{1fa} z_{1fb} + z_{1fb}^2)C}}{e^{-\pi R_c^2 C + 1/3\pi(z_{2fa}^2 + z_{2fa} z_{2fb} + z_{2fb}^2)C}} \tag{8}$$

where subscripts 1 and 2 refer to the shallower and deeper quenchers, respectively, where z_{1fa} and z_{1fb} are the distances from the fluorophore to the deepest and shallowest quencher 1 molecules, respectively, and z_{2fa} and z_{2fb} are the distances from fluorophore to the deepest and shallowest quencher 2 molecules. If the distributions of the shallow and deeper quenchers have the same widths and the distance between their average depths is given by L_{12}, then it can be shown that simplification of eq 8 gives the expression of eq 3 for average z_{cf}. Therefore, for two quenchers with equal widths of depth distributions, the parallax method will report the correct average z_{cf} when all quenchers are at depths $< R_c$ from the depth of the fluorophore. Where some quenchers are beyond this distance, eq 6 can be used to numerically calculate the quenching in the presence of deep and shallow quenchers distributed over a range of depths, once again taking into account that $F/F_o = 1$ where $z > R_c$. These values can be substituted into eq 3 to again generate apparent z_{cf} values which reveal how parallax analysis is affected by quencher distributions (see Results).

Case 4: *Lateral (Horizontal) Motion of Quenchers Relative to Fluorophores*. Fast motions occurring during the excited state lifetime of a fluorescent molecule can influence its quenching. Both fluorophore and quencher will move, but we can define total horizontal motion as the motion of quencher relative to a fluorophore molecule. Let us consider a simplified model in which fast motions occur that cause a quencher to isotropically sample all lateral locations within h angstroms of its average location. In this case, for each quencher molecule the radius of the circle quenched in the plane of the flurophore becomes $(R_c^2 - z^2)^{1/2} + h$ and so quenching will be given by

$$F/F_o = e^{-\pi[(R_c^2 - z^2)^{1/2} + h]^2 C} \tag{9}$$

and the ratio of fluorescence in the presence of shallow and deeper quenchers will be given by

$$\frac{F_1}{F_2} = \frac{e^{-\pi[(R_c^2 - z_{1f}^2)1/2 + h]^2 C}}{e^{-\pi[(R_c^2 - z_{2f}^2)^{1/2} + h]^2 C}} \tag{10}$$

This equation can be solved numerically for horizontal motions of various amplitudes and then used to calculate apparent z_{cf} by substitution in eq 3 (see Results).

Case 5: *Transverse (Vertical) Motion of Quenchers Relative to Fluorophores*. Another type of motions that could affect apparent depth would be fast transverse (vertical) motions. Both fluorophore and quencher will move, but we can define total vertical motion in terms of the motion of the quencher relative to a fluorophore molecule. Let us consider the simplified case in which quenchers sample all depths within some range $\pm v$ around their average depth during the excited state lifetime of a fluorophore. In this case, the closest approach of fluorophore and quencher becomes $z - v$, and for each quencher molecule the radius of the circle quenched in the apparent plane containing the fluorophore is $[R_c^2 - (z - v)^2]^{1/2}$ so quenching will be given by

$$F/F_o = e^{-\pi[[R_c^2 - (z-v)^2]^{1/2}]^2 C} = e^{-\pi R_c^2 C + \pi(z-v)^2 C} \tag{11}$$

and the ratio of fluorescence in the presence of shallow and deeper quenchers will be given by

$$\frac{F_1}{F_2} = \frac{e^{-\pi R_c^2 C + \pi(z_{1f} - v)^2 C}}{e^{-\pi R_c^2 C + \pi(z_{2f} - v)^2 C}} \tag{12}$$

This equation can be solved numerically for transverse motions of various amplitudes and then used to calculate apparent z_{cf} by substitution in eq 3 (see Results).

It should be noted that equations analogous to 11 and 12 would also apply even in the absence of vertical motion if quenching was dependent upon the distance between the edges of fluorophore and quencher molecules closest to one another. The parameter v would then be related to the size of the fluorophore. However, this behavior is unlikely because quenching appears to involve an electron exchange mechanism in which quencher and fluorophore orbital overlap is the relevant parameter (Green et al., 1990; Turro, 1978).

Case 6: *Isotropic Motion of Quenchers Relative to Fluorophores*. In the extension of cases 4 and 5 to fast isotropic motion, a molecule would sample all locations in three dimensions within some distance d from its average position. Therefore, fluorescence will be extinguished within a sphere of radius $R_c + d$. For each quencher molecule, the radius of the circle quenched in the plane of the fluorophore would be $[(R_c + d)^2 - z^2]^{1/2}$. Notice that the effect of isotropic motion is simply to extend the range of quenching such that the effective R_c value is larger. Therefore, eqs 1–3 are valid in this

[5] This could not be done to compare TOE binding in the presence of brominated lipids because the change in fluorescence upon lipid binding was too small to measure accurately for 9,10- and 11,12-BrPC.

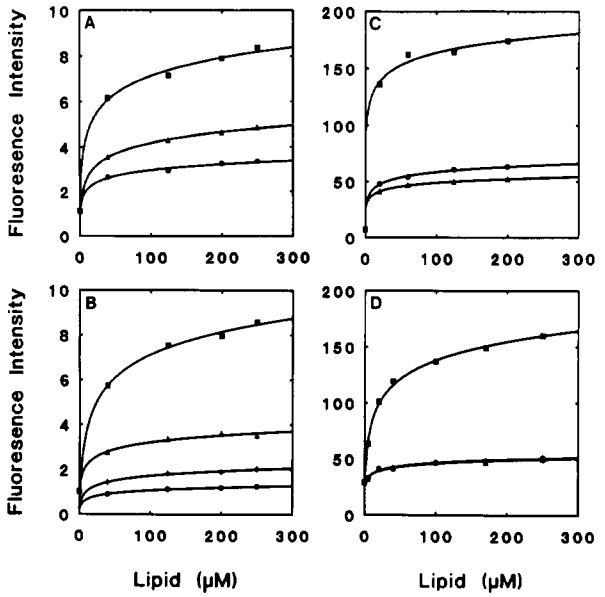

FIGURE 1: Binding of TOE and 11-CU to model membranes containing quencher lipids. (A) Binding of TOE to phospholipid vesicles containing spin labels. Samples were prepared containing 3 nmol of TOE mixed with various amounts of lipids and then dispersed in 2 mL of 10 mM acetate buffer and 150 mM NaCl, pH 4.5, to form MLV. Samples contained (■) DOPC, (●) 15 mol % 5-SLPC/85 mol % DOPC, or (▲) 15% 12-SLPC/85% DOPC. (B) Binding of TOE to phospholipid vesicles containing brominated lipids. Samples were prepared as above except using 100% brominated lipids. Samples contained (■) DOPC, (●), 6,7-BrPC, (+) 9,10-BrPC, or (▲) 11,12-BrPC. (C and D) Binding of 11-CU to phospholipid vesicles containing spin labels at pH 5 and 10. Samples containing 0.144 nmol of 11-CU mixed with various quantities of lipids were dispersed in 2 mL of either 150 mM NaCl and 10 mM sodium acetate, pH 5 (C), or 150 mM NaCl and 10 mM glycine, pH 10 (D). Samples contained (■) DOPC, (●) 15 mol % 5-SLPC/85 mol % DOPC, or (▲) 15% 12-SLPC/85% DOPC.

case, and isotropic motion does not affect the calculated z_{cf}.

RESULTS

Binding of Fluorescent Probes to MLV. In order to calculate depth accurately, fluorescence quenching must be measured in samples that have the same amount of bound fluorophore. Therefore, it was necessary to show that the presence of particular spin label or brominated lipids did not greatly alter fluorophore binding to membranes.

The enhancement of fluorescence intensity upon binding of fluorophores to membranes was used to establish binding curves for the fluorescent molecules used in this study. Figure 1A,B shows that the binding of TOE to MLVs composed of DOPC with and without quencher lipids is similar. In all cases binding is tight, probably being half-maximal at significantly less than 40 μM. Because the amount of fluorescence from bound fluorophore is reduced in the presence of quenchers, the similarity of binding is most easily seen when percent change in fluorescence vs lipid concentration is compared for different lipids (Figure 2A).[5] Figure 1C,D shows the MLV binding curves for 11-CU, and Figure 2B,C shows the curves on the percent change scale. Once again it is apparent that binding is very similar in all these cases.

To confirm that variations in binding did not affect the analysis, depths were calculated from the data in Figure 1 using eq 3. If binding is not affected by the presence of lipids carrying quencher probes, then depth should be independent of lipid concentration. Figure 3 shows that calculated depth does not vary significantly with lipid concentration for either TOE or 11-CU.

Comparison of Depths Determined by Brominated and Spin-Labeled Lipid Quenching. Figure 3 shows that there is agreement between depths obtained using the brominated lipids and spin-labeled lipids. This is shown more clearly in Table I in which depths and amounts of quenching are presented. When parallax analysis is applied to both spin label and brominated lipid quenching, there is very good agreement between the calculated depths to within 1–2 Å. The interesting difference in 11-CU depth at pH 5 and 10 represents a real shift in depth due to carboxyl ionization state and is discussed in the following paper (Abrams et al., 1992).

Just as important, the depths estimated by bromine quenching without use of parallax analysis also agree with spin-label quenching. For example, at pH 10 11-CU is most quenched by the 9,10-BrPC and equally quenched by the 6,7- and 11,12-BrPCs. This is consistent with a location halfway between the latter lipids. These results suggest that the 11-CU is very close to the 8.3-Å depth of the 9,10-BrPC and fit well with the calculated depths of 8.8 and 8.4 Å calculated using parallax analysis for spin-label and bromine quenching, respectively. In the case of 11-CU at pH 5, again the most quenching is by the 9,10-BrPC, but the 11,12-BrPC quenching is much greater than that of 6,7-BrPC. This locates the carbazole between the 9,10- and 11,12-BrPC but closer to the 9,10-BrPC. Therefore, at pH 5,11-CU appears to be in the range from 7.3 Å (halfway between 9,10- and 11,12-BrPC) to 8.3 Å (9,10-BrPC depth) from the center of the bilayer, which is within 1 Å of values calculated using parallax analysis. The observation that the 6,7-BrPC gives the strongest quenching of TOE and cytochrome b_5 Trp residues is also consistent with the results of parallax analysis. The additional data of Tennyson and Holloway (1986) that shows the quenching of cytochrome b_5 Trp by the 6,7-BrPC is stronger

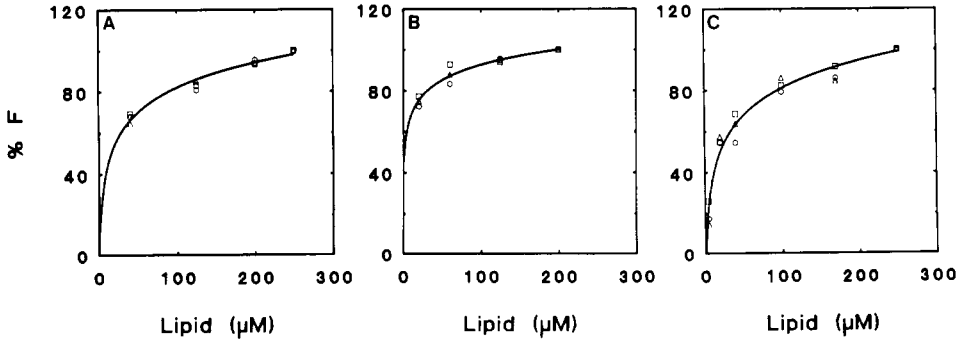

FIGURE 2: Normalized binding curves for TOE and 11-CU. Data of Figure 1 are plotted as the percent of maximum change in fluorescence vs lipid concentration. (A) TOE binding to spin-labeled vesicles: (□) DOPC, (○) 15 mol % 5-SLPC/85 mol % DOPC, or (△) 15% 12-SLPC/85% DOPC (B) 11-CU binding to spin-labeled vesicles at pH 5: (□) DOPC, (○) 15 mol % 5-SLPC/85 mol % DOPC, or (△) 15% 12-SLPC/85% DOPC. (C) 11-CU binding to spin-labeled vesicles at pH 10: (□) DOPC, (○) 15 mol % 5-SLPC/85 mol % DOPC, or (△) 15% 12-SLPC/85% DOPC.

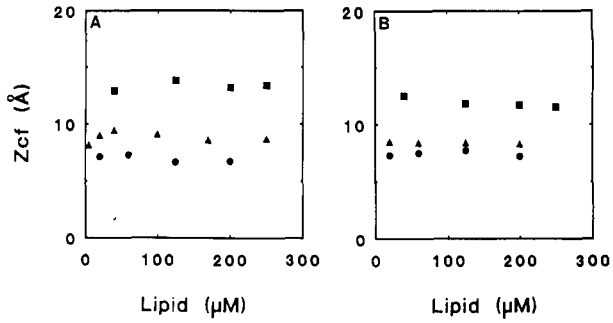

FIGURE 3: Depth vs lipid concentration. Depth was calculated from the data in Figure 2 using eq 3. (A) Depth calculated with spin-labeled lipids. (B) Depth calculated with brominated lipids. For the brominated lipid depth calculations, the plotted depths represent an average of depths obtained using the 6,7-BrPC, 9,10-BrPC pair and the 6,7-BrPC, 11,12-BrPC pair. Samples contained (■) TOE, pH 4.5, (▲) 11-CU, pH 10, or (●) 11-CU, pH 5. Samples of 11-CU in brominated lipid were prepared as the spin-label samples in Figure 2, except that 100% BrPCs were used.

Table II: R_c Values for the Fluorescent Molecules Used in This Study[a]

molecule	R_c (Å)	
	spin-label quenching	bromine quenching
TOE, pH 4.5	11.7	6.7
11-CU, pH 5	13.5	6.1
11-CU, pH 10	13.5	6.2
2-AS, pH 5	13.3	
6-AS, pH 5	14.4	
9-AS, pH 5	15.0	
12-AS, pH 5	15.7	
16-AP, pH 5	15.8	
2-AS, pH 10	13.4	
12-AS, pH 10	15.3	

[a] The depths in Table I were used to first calculate z_{1f} and z_{2f}. These parameters were then substituted into eq 1 to calculate R_c. Brominated lipid R_c values are the average of results obtained for the quenchers closer to the fluorophore. R_c values for the 12-AS and 16-AP may be slightly exaggerated by the presence of quenching from spin labels in the trans leaflet (Chattopadhyay & London, 1987).

than by 4,5-BrPC further localizes the Trp to the range 9.6 Å (halfway between 6,7- and 9,10-BrPC) to 11.6 Å (halfway between 4,5- and 6,7-BrPC) from the bilayer center. This also agrees with the depth obtained by parallax analysis.

Together these results indicate that parallax analysis is accurate within the range of depths occupied by our model fluorophores. Furthermore, since bromine positions have been determined by X-ray diffraction for the brominated lipids used here (McIntosh & Holloway, 1987), the agreement between bromine and spin-label quenching implies that the spin labels are at their predicted locations within the membrane. This is consistent with previous data on the approximate location of spin labels within membranes (Chattopadhyay & London, 1987, and references therein).

Table II shows the R_c values for spin-label and brominated lipid quenching of the fluorophores calculated from the quenching data. The R_c values for spin labels agree with those reported previously (Chattopadhyay & London, 1987). These values are not surprising in view of the recent demonstration that spin-label quenching does not even require contact between the fluorophore and quencher (Green et al., 1990). That the spin labels give larger R_c values than the brominated lipids is consistent with the stronger quenching of fluorophores by spin labels.

Apparent Depths Calculated by Parallax Analysis in the Absence of Motion When Fluorophores and Quenchers Are Restricted to Single Planes. In considering the factors that would affect the validity of parallax analysis, the assumptions that fluorophore and quencher can be treated as if they are at specific depths rather than distributed over a range of depths and that any motions they undergo do not affect the results must be examined. To do this, it is useful to examine theoretically what apparent z_{cf} would be found using parallax analysis (eq 3) under conditions in which fluorophore and quencher are distributed over a range of depths or moving significantly during the excited state lifetime of the fluorophore.

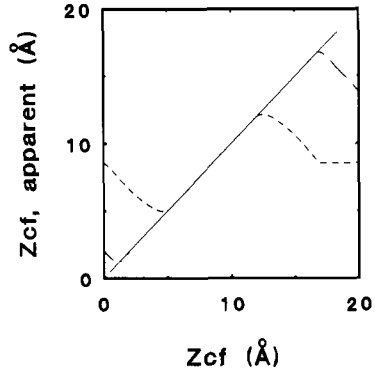

FIGURE 4: Effect of R_c on apparent fluorophore positions determined using parallax analysis. The abscissa is z_{cf}. The ordinate is apparent z_{cf} calculated from eq 3 for R_c equal to (---) 7 Å for quenchers at the positions of 6,7-BrPC and 11,12-BrPC with a quencher concentration of 0.0143 (100%) and for R_c equal to (– – –) 11 Å or (—) 14 Å for quenchers at the position of 5-SLPC and 12-SLPC and a quencher concentration of 0.00214 (15%).

First, it is useful to compare what would be the relationship between the actual depth of a fluorophore (z_{cf}) and its depth calculated from parallax analysis (z_{cf} apparent) in the absence of motional and distributional effects. This can be done by using eq 1 to calculate the quenching that would be observed at a specific quencher concentration and then substituting the result into eq 3. Figure 4 shows the relationship of these parameters. There is identity of z_{cf} apparent and z_{cf} as long as the fluorophore is within R_c of both of the quenchers used, as expected. When one is beyond R_c for one of the quenchers, eqs 1 and 3 are no longer valid (Chattopadhyay & London, 1987). If eq 3 is used to calculate z_{cf} apparent under such conditions, Figure 4 shows there will be an increasing deviation of z_{cf} apparent from z_{cf} as the distance between the fluorophore and quenchers increases.[6] At very large distances, where quenching by both quenchers become negligible, eq 3 will wrongly report the fluorophore to be halfway between the two quenchers used. For spin labels, which have an R_c of 11–15 Å, Figure 4 shows that these deviations should not be a significant problem over a wide range of depths. However, it is a more serious problem when using the parallax analysis with brominated lipids as quenchers due to their shorter R_c of 6–7 Å (see Table II). Therefore, the parallax analysis can only be used for brominated lipids when the bromines are close to the fluorophore depth.

Predicted Effects of a Distribution of Fluorophores and Quenchers Over a Range of Depths upon the Depths Measured by Parallax Analysis. One way to extend the parallax analysis is to take into account the fact that molecules in membranes do not take on a single depth but rather occupy a range of

[6] Although in a perfect all-or-none process incorrect use of eqs (1–3) could be detected by the absence of quenching in one or both quencher-containing samples, for a more realistic process the validity of the equations can begin to break down at a distance between fluorophore and quencher planes exceeding $0.85R_c$ and where F/F_0 is less than 1 (Chattopadhyay & London, 1987).

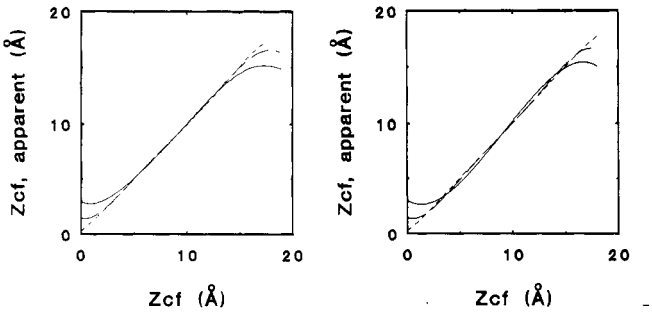

FIGURE 5: Effect of transverse (vertical) distributions of fluorophores and quenchers in membranes upon apparent fluorophore depth reported by parallax analysis. (Left) Apparent depth when quenchers are evenly distributed over a range of depths. Calculations are for quenchers at average positions equal to those estimated for 5-SLPC and 12-SLPC (see Table I), for a spin-label concentration of 0.00214 (15 mol %) and for R_c = 12 Å. The abscissa shows z_{cf}. The ordinate shows apparent z_{cf} for quenchers spread over a range of (- - -) 0 Å, (— — —) 5 Å, or (—) 10 Å. (Right) Apparent depth when fluorophore is evenly distributed over a range of depths. Apparent depth was calculated for fluorophore distributions centered at the average z_{cf}. Calculations are for quenchers at fixed positions equal to those estimated of 5-SLPC and 12-SLPC (see Table I), for a spin-label concentration of 0.00214 (15 mol %) and for R = 12 Å. The abscissa shows average z_{cf}. The ordinate shows apparent z_{cf} for fluorophore spread over (- - -) 0 Å, (— — —) 5 Å, or (—) 10 Å. The results of Weiner and White (1991) suggest that the values chosen for the distribution of fluorophore and quencher are realistic estimates for the range of molecular depth.

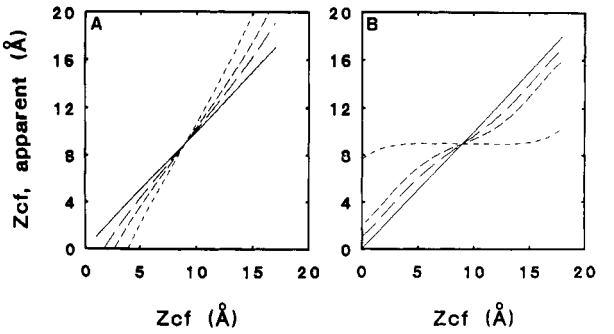

FIGURE 6: Effect of lateral (horizontal) and transverse (vertical) motions upon apparent positions of fluorophores determined by parallax analysis. (A) Effects of lateral motions. The abscissa shows z_{cf}. The ordinate shows apparent z_{cf} calculated from eqs 10 and 3 for fast lateral motions of (——) 0 Å, (— — — —) 2 Å, (- - -) 4 Å, and (- - -) 8 Å centered at the average lateral location of a molecule (i.e., 4 Å represents 2-Å excursions on either side of the average lateral position). (B) Effects of transverse motions. The abscissa shows average z_{cf}. The ordinate shows apparent z_{cf} calculated from eqs 12 and 3 for fast transverse fluorophore motions of (——) 0 Å, (— — — —) 2 Å, (- - -) 4 Å, and (- - -) 16 Å centered at the average transverse location of a molecule (i.e., 4 Å represents 2-Å excursions on either side of the average transverse position). Equations are solved for a spin-label concentration of 0.00214 and R_c = 12 Å for quenchers with an average position at the spin-label lipid depths.

depths. The very recent results of Weiner and White (1991) and Weiner et al. (1991) on the depth of double bonds and lipid-attached bromines have shown experimentally that such distributions occur in membranes. We also find that our fluorescent probes seem to distribute over a range of depths (Abrams et al., 1992). Therefore, the depth derived from parallax analysis must be some sort of average value.

To evaluate the relationship of fluorophore depth derived from parallax analysis to its actual average position, the critical separation model was extended to the cases of fluorophores and quenchers distributed over a range of depths (see Theory). The calculated results are shown in Figure 5.

The case of quenchers uniformly distributed over a range of depths is shown in Figure 5 (left). As noted under Theory, there is no difference between z_{cf} and z_{cf} apparent when all of the quenchers are within R_c of the fluorophore. When some of the quenchers are beyond R_c, there is the same type of deviation seen in Figure 4 when quenchers are restricted to a single depth (see Theory). The difference is that when the quenchers are spread over a range, the deviation is seen at smaller quenchers–fluorophore average distances, because there are cases where, even though the average quencher–fluorophore distance is within R_c, some of the quenchers are spread beyond R_c.

The case of fluorophores uniformly spread over a range of depths is shown in Figure 5 (right). Notice that the difference between z_{cf} (average) and z_{cf} apparent is negligible except at average distances close to the limit of the quenching range, where some fluorophores in a distribution will be beyond R_c, paralleling the results when quenchers are spread over a range of depths.

Predicted Effects of Motion upon Depth of Molecules Measured by Parallax Analysis. Motion during the time that a fluorophore is in the excited state could also affect quenching. One type of motion to consider is lateral (horizontal) motion. Lateral diffusion measurements would predict motions on the order of 2–4 Å over a typical 10-ns lifetime. Figure 6A shows the effect such motions would have on parallax analysis (see Theory). It shows that as a result of lateral motion fluorophores would appear to be farther apart than they really are.

Transverse (vertical) motions can also affect apparent depth (see Theory). The effects such motions would have are illustrated in Figure 6B. It shows that the effect of transverse motion is to suppress differences in location between fluorophores. At distances far from the quenchers, it is interesting to note that the fluorophores appear to be closer to the quencher than their real locations by the amount of the vertical motion.

In a real membrane motion is undoubtedly some superposition of lateral and transverse motions. If the lateral and transverse components of motion are equal (motion close to isotropic), then their effects on apparent depth will cancel out (see Theory). Of course, totally isotropic motion in the membrane is unlikely, but the small local motion likely to dominate during the excited state lifetime may come sufficiently close to isotropic to cancel out most motional effects. In any case, it is probable that there is a considerable cancellation of motional effects by the combination of lateral and transverse motions.

Experimental Evidence Concerning Effects of Motion and Molecular Distributions upon Measured Depths. In order to experimentally evaluate the effects of motion on depth, the temperature dependence of the apparent depth of membrane-inserted 6-AS and 12-AS was measured at pH 5. A significant increase in motion of these probes during the excited state lifetime was obtained by increasing the temperature from 15 to 50 °C. This was demonstrated by the decrease in fluorescence polarization from 0.134 at 15 °C to 0.095 at 50 °C for the anthroyloxy moiety of membrane-inserted 6-AS.

If, for example, increased transverse (vertical) motion dominated, it would result in increased encounters between an AS probe and the quencher farthest from it, while having little effect on (or possibly slightly decreasing) encounters between an AS probe and a quencher at the same depth. Therefore, increased transverse motions at higher temperatures should cause the 6-AS probe to appear deeper, due to increased encounters with the 12-SLPC, and the 12-AS probe to appear shallower, due to increased encounters with 5-SLPC. As a result, higher temperature should have brought the apparent depths of 6-AS and 12-AS probes closer to one another.

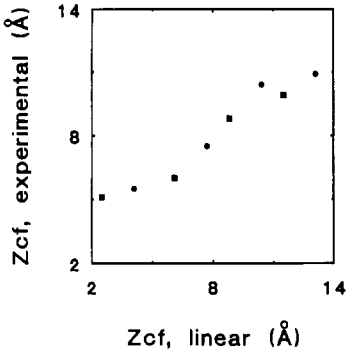

FIGURE 7: Comparison of the location of anthroyloxy groups determined experimentally using parallax analysis with positions derived assuming a linear depth distribution. Experimental data is from Abrams et al. (1992). (■) Anthroyloxy probes at pH 5; (●) anthroyloxy probes at pH 10. To calculate the "expected" linear locations, it was assumed that the 9-AS experimental positions were correct and that the other anthroyloxy probes would differ by 0.9 Å multiplied by the number of fatty acyl carbon atoms between attachment site and fatty acyl 9-carbon. This is based on the difference of 0.9 Å in depth between adjacent carbon atoms on the fatty acyl chain of a phospholipid (Chattopadhyay & London, 1987). The data for 2-AS were not included as it could be very unlikely that the nonpolar anthroyloxy group would be at the same position as the 2-carbon on the 2-position acyl chain, which is at the polar region adjacent to the glycerol (Pearson & Pascher, 1979). Values for 12-AS and 16-AP may be slightly shallower than those shown due to the presence of a small amount of quenching from spin-labels in the trans leaflet (Chattopadhyay & London, 1987).

This result was not obtained experimentally. Instead, only a very small (and perhaps insignificant) increase in depth for both fluorophores was found as temperature increased. The measured z_{cf} found for the anthroyloxy group of 6-AS was 10.3 Å at both 15 and 25 °C and 9.8 Å at 50 °C. The corresponding values for 12-AS were 6.1 Å at 15 °C, 5.8 Å at 25 °C, and 5.3 Å at 50 °C. Thus, the apparent distance between the two fluorophores (4.2–4.5 Å) remained constant or increased slightly as temperature increased.

The conclusion that measured depth is not strongly affected by motions is supported by the results obtained with a series of anthroyloxy-labeled fatty acids. In this case, it is possible to compare the experimentally determined depths of the anthroyloxy group (Abrams et al., 1992) to the behavior predicted for dominating lateral or transverse motions. Figure 7 shows the behavior of measured depth vs depth predicted by assuming that the position of the anthroyloxy groups reflects that of the fatty acyl carbon to which they are attached.[7] The results do not follow the behavior predicted for excess lateral or transverse motion in Figure 6. Instead, the slope of the graph of z_{cf} experimentally calculated vs predicted is 1 for the central range of depths, consistent with the presence of only small and/or largely isotropic motions.

Another question is whether the distribution of fluorophore and quenchers over a range of depths is affecting the experimentally determined depths. Figure 5 shows that spreading of fluorophore and quenchers over a range of depths would qualitatively mimic the experimentally observed anthroyloxy behavior. In this case the anthroyloxy groups at the extremes of the fatty acyl chain appear to be closer to the center of the chain than would be predicted if there were a linear dependence of depth upon anthroyloxy attachment position. In addition, preliminary results with lipids carrying quenchers in the polar headgroup region suggest that for 2-AS there is indeed a distortion in apparent fluorophore position where the fluorophore and quenchers are far from one another (unpublished observations). However, another explanation is that the distribution of anthroyloxy groups is really not linearly dependent on depth. It does seem unlikely that the bulky anthroyloxy groups would locate exactly at the depths that the carbon to which it is attached would take on in an unlabeled fatty acyl chain (Abrams et al., 1992). For example, the 16-AP molecule has a shorter acyl chain than the other AS probes and is also unlike them in that it has a terminal anthroyloxy group. It could have a tendency to bend upward.

Furthermore, it is unlikely that effects due to depth distributions strongly influence the results obtained for fluorophore depth in most of the cases studied in this report. As Figure 4 shows, if a fluorophore is in the region where the accuracy of the equations break down for bromine quenching but within the valid region for spin-label quenching, then the spin-labeled and brominated quenchers will report different depths. The effect of molecules distributing over a range of depths is to cause the breakdown to occur at distances closer to the quenchers than would otherwise be observed. Therefore, the agreement between brominated and spin-label quenching in Table I is consistent with the fluorescent molecules being within the regions in which parallax analysis is accurate and where distortions due to depth distributions do not occur.

DISCUSSION

Comparison of Brominated and Spin-Labeled Lipid Quenching. The agreement of brominated lipid quenching with the depths calculated from spin-label quenching demonstrates the validity of parallax analysis. However, it should be emphasized that we have mainly examined the accuracy of parallax analysis for fluorophores within the hydrocarbon region of the membrane. We do not yet know how far beyond this region the analysis can be extended. Preliminary results with a spin label attached to the polar headgroup of a phospholipid suggest that it will be possible to make accurate measurements throughout the headgroup region. The agreement of spin-label and brominated lipid quenching also indicates that the spin labels are located very close to the previously predicted locations in the membrane (Chattopadhyay & London, 1987, and references therein). In addition, this agreement suggests that the somewhat bulky and polar spin-label groups do not seriously perturb depth.

The conclusions of (Ellena et al., 1988) that the spin-label groups are a couple of angstroms more shallow than expected are not relevant to our experiments, because they involved measurements on spin-labeled fatty acids. Spin-labeled fatty acids have depths dependent on the state of ionization of the carboxyl group (Abrams et al., 1992, and references therein) and also are unlikely to occupy positions exactly the same as those attached to phospholipids (Abrams et al., 1992).

The agreement of spin-label and brominated lipid quenching of cytochrome b_5 supports the shallower Trp location reported in Markello et al. (1985), Everett et al. (1986), and Tennyson and Holloway (1986) rather than the deeper location found by Kleinfeld and Lukacovic (1985). The implicit assumption that the anthroyloxy energy transfer acceptors used in the latter study were restricted to one leaflet may have been the cause of this discrepancy. It was later shown that anthroyloxy fatty acids rapidly flip-flop between leaflets (Storch & Kleinfield, 1986), and the presence of acceptors in both leaflets would make the Trp seem anomalously deep due to trans leaflet quenching (Chattopadhyay & London, 1987).

It should be noted that our values of R_c for brominated lipids are slightly smaller than those recently calculated by Bolen

[7] Unfortunately, unlike spin labels, bromines do not significantly quench anthroyloxy groups (data not shown), so they cannot be used to corroborate spin-label results.

and Holloway (1990) using a peptide that locates a Trp near the center of the bilayer. This is partly due to the fact that, for the peptide, quenching by brominated lipids in both leaflets was not considered. In addition, the value of Bolen and Holloway may be a slight overestimate if the Trp residue is not exactly at the center of the bilayer.

Models Used To Estimate the Effect of Fluorophore and Quencher Motions and Depth Distributions. Previous studies have shown that there is a dominant static component involved in spin-label quenching in membranes in most cases as shown by the temperature independence of quenching and the exponential dependence of quenching on membrane quencher concentration (London & Feigenson, 1981; East & Lee, 1982). Nevertheless, the possibility of a significant motional component of quenching must also be considered. In this report, we presented a model modifying the Perrin approach to account for motional effects upon quenching. It represents an approximation because it assumes the most extreme case of all-or-none quenching in which quenching is solely determined by closest approach of fluorophore and quenchers and that all molecules diffuse to the same degree. However, this model is useful because it defines the maximal effects that may be expected to arise from motion. Furthermore, it should be a reasonable approximation to the real situation, since spin-label quenching is strongly distance dependent (Chattopadhyay & London, 1987), so that the amount of quenching of a fluorophore during its closest approach to the quencher is weighted especially heavily. Nevertheless, it will be desirable to develop a more sophisticated model of motions in the future.

The commonly used Stern–Volmer equation (Eftink & Ghiron, 1976) was not chosen here to evaluate the motional component of quenching for two reasons. The first arises from the fact that the Stern–Volmer approach uses the all-or-none quenching model in which the rate of quenching is zero until fluorophore and quencher approach to within a specific distance (which defines a collision) where it becomes large. In deriving parallax analysis, we specifically demonstrated that for static quenching the Perrin equations based on this all-or-none approach apply even for realistic distance dependencies of quenching (Chattopadhyay & London, 1987). However, it is not clear that the all-or-none approach can be used with the Stern–Volmer analysis in membranes where the degree of motion is small (see below). Second, the Stern–Volmer approach is applicable where diffusion is isotropic and thus does not lead to predictions of the different effects of lateral and vertical motions, as the model in this report does.

Interestingly, experimental results for anthroyloxy depths did not show an influence that could be attributed to motion. It is not clear whether the lack of motional effects arises because motions are relatively small during the short excited state lifetime or because motion is largely isotropic during this period. The lack of thermal effects upon quenching may suggest that there are no very large vertical motions during the time a fluorophore is in the excited state. The recent brief report of vertical motion detected by fluorescence quenching (Wardlaw et al., 1987) depended on the assumption that spin-label quenching requires the collision of fluorophore and quencher and the assumption that lifetime reductions in the presence of quencher are the signature of collisional quenching. However, since spin-label quenching involves electron exchange, contact between the fluorophore and quencher (i.e., at the distances of the van der Waals radii) is not necessary for quenching (Green et al., 1990). Furthermore, *lifetime reduction due to totally static quenching will occur in the absence of any collisions.* For any realistic quenching process, there will be fluorophores at distances from quenchers at which they are only partially quenched. This partial quenching will give rise to a lifetime-shortened population even when there is no motion. This fact is usually not recognized because it is not predicted by the assumption of all-or-none quenching. Such partial quenching effects would explain why Wardlaw et al. found "collisional" lifetime shortening by spin labels even in the solid-like gel lipid phase.

In addition to a model for motion, a model was developed to account for the effect of the distribution of fluorophores and quenchers over a range of depths on parallax analysis. Again, this model is simplified in the sense that a uniform distribution of molecules over a range of depths was chosen rather than a Gaussian distribution. However, this should only have a significant influence where an attempt is made to recover the precise range of molecular distributions from quenching data. Since this could only be done if one has first precisely determined the average position of the fluorophore by an independent method, the necessary data for such a detailed analysis do not exist at present.

Acknowledgments

We acknowledge the helpful suggestions of Maja Matic and the kind gift of some of the brominated lipids and cytochrome used in this study from Peter Holloway. We thank Dale Deutsch for generous help with the GC/MS experiments.

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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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Received November 13, 1991; Revised Manuscript Received March 13, 1992

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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0006-2960/92/0431-5322$03.00/0