Stone, W. L., Farnsworth, C. C., & Dratz, E. A. (1979) *Exp. Eye Res. 28*, 387-397.
Vaz, W. L. C., Clegg, R. M., & Hallmann, D. (1985) *Biochemistry 24*, 781-786.
Watts, A., Volotovski, I. D., & Marsh, D. (1979) *Biochemistry 18*, 5006-5013.
Watts, A., Davoust, J., Marsh, D., & Devaux, P. F. (1981) *Biochim. Biophys. Acta 643*, 673-676.

Parallax Method for Direct Measurement of Membrane Penetration Depth Utilizing Fluorescence Quenching by Spin-Labeled Phospholipids†

Amitabha Chattopadhyay and Erwin London*
Department of Biochemistry, State University of New York at Stony Brook, Stony Brook, New York 11794-5215
Received June 20, 1986; Revised Manuscript Received September 23, 1986

ABSTRACT: This report describes a method suitable for determining the depth of a wide variety of fluorescent molecules embedded in membranes. The method involves determination of the parallax in the apparent location of fluorophores detected when quenching by phospholipids spin-labeled at two different depths is compared. By use of straightforward algebraic expressions, the method allows calculation of depth in angstroms. Furthermore, the analysis can be extended to quenching by energy-transfer acceptors or brominated probes under appropriate conditions. Application of the method to quenching of 7-nitro-2,1,3-benzoxadiazol-4-yl (NBD)-labeled lipids by spin-labeled lipids located at three different depths is demonstrated in model membranes. It is shown that the calculated depths of the NBD groups are self-consistent to the extent that they are the same no matter which two spin-labels have been used in a particular experiment. In addition, the calculated depth is independent of spin-label concentration in the membrane within ±1 Å, ruling out major effects due to spin-label perturbation. The quenching experiments show that the location of the NBD group in head-group-labeled phosphatidylethanolamine is at the polar/hydrocarbon interface and that of an NBD label on the "tail" of cholesterol is deeply buried, as expected. Unexpectedly, NBD labels placed at the end of fatty acyl chains of phosphatidylcholines are also near the polar/hydrocarbon interface. Presumably, the polarity of the NBD group results in "looping" back to the surface of the NBD groups attached to flexible acyl chains.

One of the most important questions in the study of biological membrane structure is the membrane penetration depth, i.e., how far a molecule or a specific site within a molecule is from the membrane surface. Knowledge of the precise depth of a molecule or group should help define the conformation and topology of fluorescent probes and membrane proteins. Fluorescence has been one of the most widely used techniques to determine depth. Most studies have made use of dipole–dipole (Förster) energy transfer [e.g., see Shaklai et al. (1977), Koppel et al. (1979), Fleming et al. (1979), Dewey & Hammes (1980), Sklar et al. (1980), Baird & Holowka (1985), Holowka et al. (1985), Kleinfeld (1985), Kleinfeld & Lukacovic (1985), Davenport et al. (1985), and Hasselbacher et al. (1986)]. However, the analysis of depth in this way has proven to be somewhat complex. Other approaches have involved use of fluorescence quenching by spin-labels [see reviews by London (1982) and Blatt & Sawyer (1985)] or by brominated probes (Kao et al., 1978; Markello et al., 1985; Jain et al., 1985). Spin-labels have the advantage of being quenchers of a wide range of fluorophores, while brominated groups on lipids are relatively small, and probably the least perturbing of the quenchers (East & Lee, 1982). However, attempts to determine precise depth by spin-labels or brominated probes have tended to be more qualitative because, unlike energy transfer, theoretical expressions describing the distance dependence of quenching by these latter probes have been lacking. In this study, we have derived relatively simple mathematical expressions that are applicable to all types of quenching, including that by energy-transfer acceptors. The equations obtained allow straightforward and direct determination of membrane depth in angstroms by comparing the quenching obtained with quenchers at two different depths, i.e., by the apparent degree of parallax in fluorophore position as viewed by quenchers at two different depths.

The method has been tested by examining the location of the fluorescent groups in a series of 7-nitro-2,1,3-benzoxadiazol-4-yl (NBD)[1]-labeled lipids using quenching by spin-labels. Various NBD-labeled lipids have been commonly used as fluorescent lipid analogues [e.g., see Nichols (1985) and Pagano & Sleight (1985)], and closely related probes have

† This work was supported by National Institutes of Health Grant GM 31986. A preliminary version of some of the results in this report was presented at the 30th Annual Meeting of the Biophysical Society, San Francisco, CA, Feb 9-13, 1986.
* Address correspondence to this author.

[1] Abbreviations: NBD, 7-nitro-2,1,3-benzoxadiazol-4-yl; 5 spin-labeled PC, 1-palmitoyl-2-(5-doxyl)stearoyl-*sn*-glycero-3-phosphocholine; 10 spin-labeled PC, 1-palmitoyl-2-(10-doxyl)stearoyl-*sn*-glycero-3-phosphocholine; 12 spin-labeled PC, 1-palmitoyl-2-(12-doxyl)stearoyl-*sn*-glycero-3-phosphocholine; 6-NBD-PC, 1-palmitoyl-2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]caproyl]-*sn*-glycero-3-phosphocholine; 12-NBD-PC, 1-palmitoyl-2-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)-amino]dodecanoyl]-*sn*-glycero-3-phosphocholine; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; NBD-cholesterol, 25-(NBD-methylamino)-27-norcholesterol; DOPC, dioleoyl-*sn*-glycero-3-phosphocholine; Tempol, 4-hydroxy-2,2,6,6-tetramethylpiperidinyl-1-oxy; TPC, 2,2,5,5-tetramethyl-3-pyrroline-1-oxyl-3-carboxy acid; TLC, thin-layer chromatography; ESR, electron spin resonance.

0006-2960/87/0426-0039$01.50/0

been used as photoaffinity labeling phospholipids (Montecucco et al., 1985). The results of quenching studies strongly suggest that the method can be used to accurately pinpoint the depths of fluorescent groups.

Experimental Procedures

Materials. Spin-labeled PCs, NBD-labeled phospholipids, and DOPC were purchased from Avanti Polar Lipids. NBD-cholesterol and TPC were purchased from Molecular Probes. Tempol was purchased from Aldrich. *Crotalus adamanteus* venom was purchased from Sigma. Lipids were checked for purity by TLC on silica gel plates (Adsorbosil Plus, Alltech Associates, Deerfield, IL) in chloroform/methanol/water (65:35:5 v/v), unless otherwise noted. Spin-label PCs gave one spot with a phosphate-sensitive spray (Dittmer & Lester, 1964). Upon charring the TLC plates, we sometimes observed a small amount of a high-R_f contaminant, not initially present in the spin-label solutions. This contaminant does not contain any spin-label groups detectable by ESR, and its source is unknown to us. NBD-labeled lipids were virtually pure when detected by their color or fluorescence. The R_f values of the labeled PCs were very similar to that of DOPC. However, the 6-NBD-PC was distinguishable from the 12-NBD-PC by a slightly lower R_f. NBD-PE had a higher R_f than the corresponding PCs. NBD cholesterol had an R_f of about 0.3 in chloroform/methanol/water (65:35:1 v/v).

The concentrations of the spin-labeled PCs and DOPC were checked by phosphate assay (Bartlett, 1959) subsequent to total digestion (Morrison, 1964). To calculate the actual number of spins per spin-labeled lipid, the doubly integrated ESR intensity of approximately 0.2 mM solutions of each of the spin-labeled PCs in organic solvent was compared to the average of two low molecular weight nitroxides used as standards, Tempol and TPC. Duplicate samples were prepared. ESR spectra were recorded on a Varian E-4 spectrometer using 50-μL glass capillary sample tubes. Samples were flushed with argon. Spectra were recorded at a power of 2 mW to avoid differential saturation of the ESR signals. The ratio of spins per molecule found was 0.65, 0.75, and 0.75 for the 5, 10, and 12 spin-labeled PCs, respectively.

The dependence of the shape of the spin-label ESR signal upon the site of spin-label attachment to the fatty acyl chain was examined in multilamellar vesicles of 1:99 spin-labeled PC/DOPC (mol/mol). Spectral shape agreed with that previously reported in each case (Marsh, 1981), confirming the identity of the different spin-label PCs.

To check the acylation position of the spin-labeled PCs, phospholipase A_2 digests of these lipids were prepared by using *C. adamanteus* venom as previously described (London & Feigenson, 1981a). To calculate the percentage of spin-labeled fatty acid at each position, doubly integrated ESR signal intensities in the lyso-PC and fatty acid fractions were compared. It was found that for the 5, 10, and 12 spin-labeled PCs, 75%, 67%, and 85% of the spin-label was esterified to the expected 2-position fatty acid, consistent with previous results (London & Feigenson, 1981a).

Methods. To prepare samples for depth measurement, coarse multilamellar dispersions of lipid containing fluorophore and quencher were prepared. In general, NBD lipid (1.6 nmol) was combined with 160 nmol of the desired mixture of DOPC and a spin-labeled PC. A few drops of chloroform were added and mixed well, and the samples were then dried under a stream of N_2 while warming gently (35 °C). After further drying under a high vacuum for 30 min, 1.5 mL of 10 mM sodium phosphate/150 mM NaCl, pH 7, was added, and then each sample was vortexed for 50 s to disperse the lipid (London & Feigenson, 1981b). Single samples were prepared for each data point, except that duplicates were prepared for samples lacking spin-label.

Fluorescence was measured at room temperature with a Spex 212 Fluorolog spectrofluorometer operating in the ratio mode and using 1-cm path-length quartz cuvettes. Background intensity in samples in which the NBD lipid was omitted was less than 1% of sample values and was subtracted from all reported values. Inner filter effects were negligible. Excitation and emission slits with a nominal band-pass of 2.25 nm were used. The excitation wavelength used was 469 nm for all of the NBD lipids. The emission wavelength used was 533 nm for all except NBD-cholesterol and NBD-PE for which the emission was collected at 531 nm.

Theory

The Perrin quenching equation (Perrin, 1924), used to analyze static quenching of randomly distributed fluorophores and quenchers (Birks, 1970), can be extended to analysis of quenching in membranes. Such quenching is static in most common cases (London & Feigenson, 1981a; London, 1982; East & Lee, 1982). Consider fluorophore and quencher groups randomly distributed on a plane, as on one surface of a biological membrane. By substitution of the area of an infinitesimal ring for the volume of an infinitesimal shell used in derivation of the three-dimensional case, the two-dimensional Perrin equation for static quenching (eq 1) can be immediately stated. In this equation, $f(r)/f_0$ equals the ratio of

$$F/F_0 = e^{-[\int_0^\infty 2\pi r(1-[f(r)/f_0])dr]C} \quad (1)$$

fluorescence intensity of a single fluorophore in the presence of a quencher at distance r to that in the absence of the quencher, and F/F_0 is the ratio of total fluorophore fluroescence in the presence of randomly distributed quenchers to that in the absence of quenchers. The concentration of quencher in molecules per unit area is C. [Assuming the usual surface area of 70 Å^2 per lipid (Lewis & Engelman, 1983) gives concentration as (mole fraction of quencher lipid in total lipid)/70 Å^2.]

The model most commonly used to describe quenching is the hard-sphere approximation (Birks, 1970) in which quenching is described by a step-function characterized by a critical separation R_c, such that $f(r)/f_0 = 0$, for $r \leq R_c$, and $f(r)/f_0 = 1$, for $r \geq R_c$. Substitution into eq 1 yields

$$F/F_0 = e^{-[\int_0^{R_c} 2\pi r dr]C} = e^{-\pi R_c^2 C} \quad (2)$$

The expression πR_c^2 represents a circle around each quencher within which fluorescence is completely extinguished, exactly analogous to the familiar sphere of quenching derived in the three-dimensional case.

This approach can be used to estimate quenching even when the hard-sphere model is only approximately valid. For example, for quenching of a donor by an acceptor via dipole–dipole energy transfer, $f(r)/f_0$ equals $[1 + (R_0/r)^6]^{-1}$. Substitution into eq 1 and solution of the integral yield eq 3 where

$$F/F_0 = e^{-(2\pi/(3(3)^{1/2}))\pi R_0^2 C} \quad (3)$$

R_0 is the donor–acceptor distance giving 50% energy transfer. If we define R_c' as the value of R_c in eq 2 which gives the same amount of quenching as R_0 in eq 3, then $R_c' = (2\pi/(3\sqrt{3})^{1/2}) \times R_0 = 1.10R_0$. In Figure 1A, fluorescence quenching calculated from eq 3 is compared to the actual theoretical quenching due to dipole–dipole energy transfer in two dimensions calculated by the method of Wolber and Hudson (1979). The agreement is very good up to high quencher

concentrations. The discrepancy at high concentration is due to the implicit assumption in the Perrin analysis that the probability of being quenched by a particular quencher is independent of the presence of other nearby quenchers, which is strictly true only for hard-sphere quenching. Nevertheless, the initial slope given by the Perrin analysis for energy transfer is correct, and there is only a 1% error even at $F/F_0 = 0.5$, so energy transfer is nearly exponential in C, as noticed previously (Sklar et al., 1980).

For determination of fluorophore depth, one must solve the case in which fluorophore and quencher are located in different planes separated by a distance which we will call the difference in vertical distance or depth, z, adopting the definition of Kleinfeld (1985). Since z is then the closest possible approach of fluorophore and quencher, it becomes the lower limit of the integral in eq 2, and hard-sphere quenching is then given by eq 4 and 5. The comparison of the prediction of the z dependence of quenching by the hard-sphere model with the results derived for energy transfer (Wolber & Hudson, 1979; Dewey & Hammes, 1980) reveals an important point. As shown in Figure 1B, the agreement of the hard-sphere and exact energy-transfer solutions is very good up to $z/R_c \sim 0.9$. Within this region, determination of z using the Perrin analysis should be valid (within a few percent) for any type of quenching with an r^6 or higher power (i.e., more hard-sphere-like) dependence on r which includes spin-label quenching (see Results). This justifies use of the hard-sphere approximation.

$$F/F_0 = e^{-\pi R_c^2 C + \pi z^2 C} \quad (z \leq R_c) \tag{4}$$

$$F/F_0 = 1 \quad (z > R_c) \tag{5}$$

A second restriction that must be considered is the existence of a minimum closest allowed lateral approach of fluorophore and quencher, which we define as x. The closest fluorophore–quencher approach in this case is $(z^2 + x^2)^{1/2}$, and substitution into eq 2 yields

$$F/F_0 = e^{-\pi R_c^2 C + \pi z^2 C + \pi x^2 C} \quad (\sqrt{z^2 + x^2} \leq R_c) \tag{6}$$

Solving eq 6 for z requires knowledge of R_c and x, and a superior alternative is to compare the results of two quenching experiments, which eliminates both R_c and x as variables. This is the method employed in this report. In this type of experiment, two sets of samples are prepared. One set contains a fluorophore, a lipid labeled with quencher at one depth, and an unlabeled lipid. The other set contains the same fluorophore, a quencher lipid labeled at a different depth, and the unlabeled lipid. The ratio of fluorescence intensity in a sample from one set to that in a sample from another set is then given by eq 7 if equal concentrations of quenchers are present in the two samples. Using 1 and 2 as subscripts denoting the shallower and deeper quenchers, respectively, F_1 is the fluorescence in the presence of quencher 1, F_2 is the fluorescence in the presence of quencher 2, z_{1F} is the difference in shallow quencher and fluorophore depth, z_{2F} is the difference in deeper quencher and fluorophore depth, and L_{21} is the difference of the two quenchers in depth. These parameters are illustrated in Figure 4. [Note that all terms denoted L refer to the depths of the quenchers which can be accurately estimated, and thus represent "known" values (see below).] Cancelling out, substituting $L_{21} + z_{1F}$ for z_{2F}, and rearranging yield

$$\frac{F_1}{F_2} = \frac{F_1/F_0}{F_2/F_0} = \frac{e^{-\pi R_c^2 C + \pi z_{1F}^2 C + \pi x^2 C}}{e^{-\pi R_c^2 C + \pi z_{2F}^2 C + \pi x^2 C}} \tag{7}$$

$$z_{1F} = \left(\frac{1}{-\pi C} \ln \frac{F_1}{F_2} - L_{21}^2 \right) \Big/ 2L_{21} \tag{8}$$

Once z_{1F} is known, fluorophore distance from the center of the bilayer can be calculated from

$$z_{cF} = z_{1F} + L_{cl} \tag{9}$$

where L_{cl} is the distance from the center of the bilayer to the shallow quencher. (In this case, the subscript c refers to the bilayer center.) Also, note that once z_{1F} is known, R_c can be calculated for lipid probes from eq 4, because x will generally be negligible for small probes attached to lipids (even if x is assumed to be zero when x really equals $0.5R_c$, substitution into eq 6 shows the error in R_c will only be 14%).

A more complicated situation occurs when quenchers in both leaflets (monolayers) of the bilayer are close enough to quench a fluorophore. This is described in the Appendix.

An important value needed for the calculation of depth is the distance of the spin-labels from the bilayer center (i.e., L_{cl} and L_{c2}). As described under Discussion, considerable data justify placement of the spin-labels at the same depth as that which would be occupied by the fatty acyl carbon atom to which the spin-label is attached if it were in an unlabeled chain. In turn, the position of the carbon atoms in an unlabeled chain has been determined by a series of X-ray, NMR, and neutron diffraction studies, which have revealed the conformation of the polar head group, including an asymmetric disposition of the start of the fatty acyl chains (Hitchcock et al., 1974; Seelig & Seelig, 1974; Pearson & Pascher, 1979; Büldt et al., 1979), and the approximate spacing in depth between adjacent carbon atoms, derived directly (Oldfield et al., 1978; Zaccai et al., 1979) and through the increase in bilayer thickness with an increase in chain length (Caffrey & Feigenson, 1981; Lewis & Engelman, 1983). These studies indicate that for the lipids used in this report the hydrocarbon thickness, if defined to be the distance between the 2-position chain carbonyl carbons in opposite leaflets, is about 30 Å and there is a spacing of 0.9 Å between adjacent fatty acyl carbon atoms. Using these values, we can immediately calculate the distances of the spin-label groups from the bilayer center (see Figure 4).

Results

Figure 1C compares quenching by a spin-labeled lipid[2] to the predictions of the hard-sphere and energy-transfer (r^6) models. Clearly, spin-label quenching is intermediate, suggesting a higher than r^6 dependence of quenching upon distance. (The logarithmic scale in Figure 1C greatly exaggerates the difference between these models, as shown by comparison to Figure 1A.) This means that the Perrin analysis gives an excellent approximation of spin-label quenching and is suitable for determination of depth within the boundaries described under Theory.

Figure 2 shows the experimental quenching curves for various NBD lipids. Notice that in the case of NBD-cholesterol the 12 spin-labeled PC gives the strongest quenching, whereas the 5 spin-labeled PC gives the strongest quenching of the other NBD lipids. At very high spin-label concentration, the quencher behavior of NBD-cholesterol in 12 spin-labeled PC appears to be anomalous. We are not sure whether this

[2] Notice that the actual concentration of spin-label groups is calculated from the concentration of spin-labeled lipid and the ratio of spins per labeled lipid molecule, because the spin-labeled lipid includes some molecules without the spin-label group (see Experimental Procedures). It is the concentration of spin-label groups that is substituted into the quenching equations.

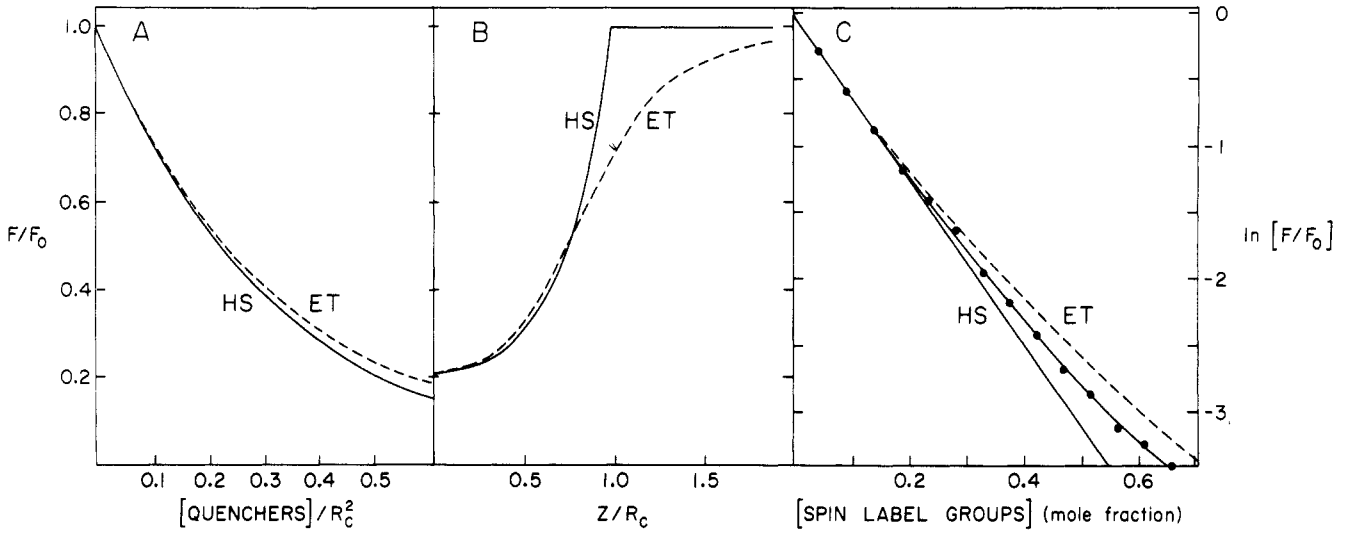

FIGURE 1: Comparison of hard-sphere (HS) and energy-transfer (ET) quenching. (A) Dependence of fluorescence quenching on quencher concentration. HS, calculated from eq 3 (—). ET, calculated for dipole–dipole energy transfer by the method of Wolber and Hudson (1979) using their equation (17) and $R_c = 1.1R_0$ (---). (B) Dependence of fluorescence quenching on minimum allowed separation between fluorophore and quencher. HS, calculated from eq 4 and 5 with $C = 0.5/R_c^2$. ET, calculated by the method of Wolber and Hudson (1979) and Dewey and Hammes (1980) approximant $A_{2,3}$ for $R_c = 1.1R_0$ ($C = 0.46/R_0^2$). (C) Comparison of quenching data of NBD-PE by 5 spin-labeled PC to the theoretical predications of Figure 1A, plotted on a logarithmic scale. HS and ET curves with initial slopes identical with the experimental data are shown.

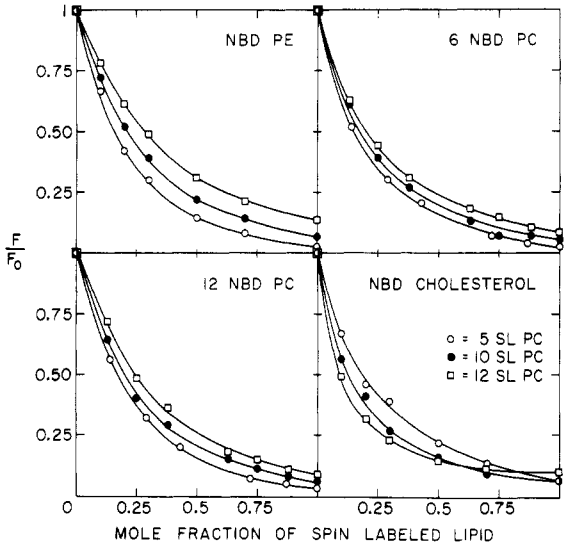

FIGURE 2: Fluorescence quenching of NBD-labeled lipids incorporated into aqueous multilamellar vesicles containing one of the three spin-labeled PCs and DOPC. The spin-labels used were 5 (○), 10 (●), and 12 (□) spin-labeled PC, respectively. The abscissa is the mole fraction of spin-labeled lipid in the lipid mixture. Notice that this is the total spin-labeled PC fraction and includes molecules that do not carry an unpaired spin (see text). The ordinate is the ratio of fluorescence in the presence (F) and absence (F_0) of the spin-labeled lipid. The fluorophore to lipid ratio was 1:100 (mol/mol). See Experimental Procedures for details of sample preparation.

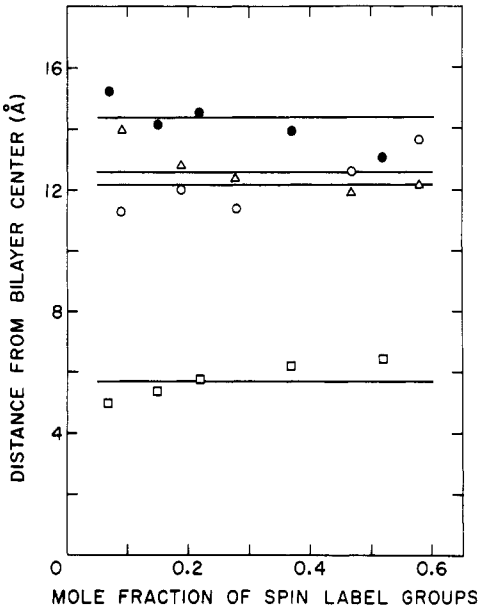

FIGURE 3: Effect of spin-label group concentration on apparent depth of (□) NBD-cholesterol, (○) 6-NBD-PC, (△) 12-NBD-PC, and (●) NBD-PE. Each point represents an average calculated from depth measured with all three pairings of spin-labeled lipids. In this figure, the abscissa shows the actual fraction of spin-label groups, rather than the total spin-labeled lipid. The lines correspond to the average depth of each NBD group (Table I). Experimental conditions are as in Figure 2.

represents a few percent experimental error, which becomes a problem at very high levels of quenching, or an artifact due to some structural perturbation in NBD-cholesterol-12 spin-labeled PC vesicles. Table I gives the depths of the NBD groups determined from these quenching curves. NBD-PE has the shallowest group, the NBD-PC's groups are almost as shallow (with no significant difference between 6- and 12-NBD-PCs), and the NBD-cholesterol has a deeply buried NBD group. The calculated depth is largely independent of which two spin-labels' quenching is analyzed. This internal self-consistency reinforces the reliability of the method and suggests that estimated spin-label depths used are probably reasonably accurate (see Discussion). In a control experiment (not shown), quenching of NBD-PE in coarse multilamellar vesicles was compared to that in small unilamellar vesicles prepared by a rapid 75-fold dilution of lipids in ethanol with buffer (Kremer et al., 1977). The depth calculated in the latter case was identical within 0.1 Å with that calculated in the multilamellar vesicles, as expected.

Table I also shows the R_c determined for spin-label quenching of NBD groups is 11.5–12 Å, which is very similar to that for quenching of other fluorescent probes, calculated by a slightly different method (London & Feigenson, 1981a).

Figure 3 shows the dependence of the apparent depth upon the concentration of spin-label groups in the membrane.[2] All

Table I: Depth of NBD Labels

fluorescent molecule	spin-labeled PC pair used for quenching analysis	calcd distance from bilayer center, z_{cF} (Å)	eq used	av z_{cF} (Å)[a]	R_c (Å)[b]
NBD-PE	5–12	14.3	8, 9		
	5–10	14.5	8, 9	14.2	12.0
	10–12	13.7	8, 9		
6-NBD-PC	5–12	12.5	8, 9		
	5–10	13.1	8, 9	12.2	11.4
	10–12	10.9	8, 9		
12-NBD-PC	5–12	12.9	8, 9		
	5–10	13.5	8, 9	12.6	11.5
	10–12	11.5	8, 9		
NBD-cholesterol	5–12	6.2 (6.4)	11,[c] 9; (8, 9)[d]		
	5–10	5.2 (7.6)	11,[c] 9; (8, 9)[d]	5.7 (5.8)	
	10–12	5.8 (3.4)	11,[c] 9; (8, 9)[d]		

[a] These average depth values are used in Figure 4. Calculated depths have not been adjusted for the minor scrambling of fatty acyl chains (see Experimental Procedures). It is possible that the calculated distance from the bilayer center should be decreased by 1 Å to account for this. [b] Calculated from eq 4 by substituting values of z_{1F} and F_1/F_0 and averaging. Average R_c values were also calculated for the individual spin-labeled PCs in the same way. The values obtained were 12.0, 11.5, and 11.4 Å for 5, 10, and 12 spin-labeled PC, respectively. [c] Assuming R_c equals 12 Å. [d] NBD-cholesterol is at the boundary of the region where use of eq 8 or eq 11 is appropriate, and eq 8 and 11 give similar results. However, the variation in apparent depth using different spin-label pairs and concentrations was in general greater using eq 8.

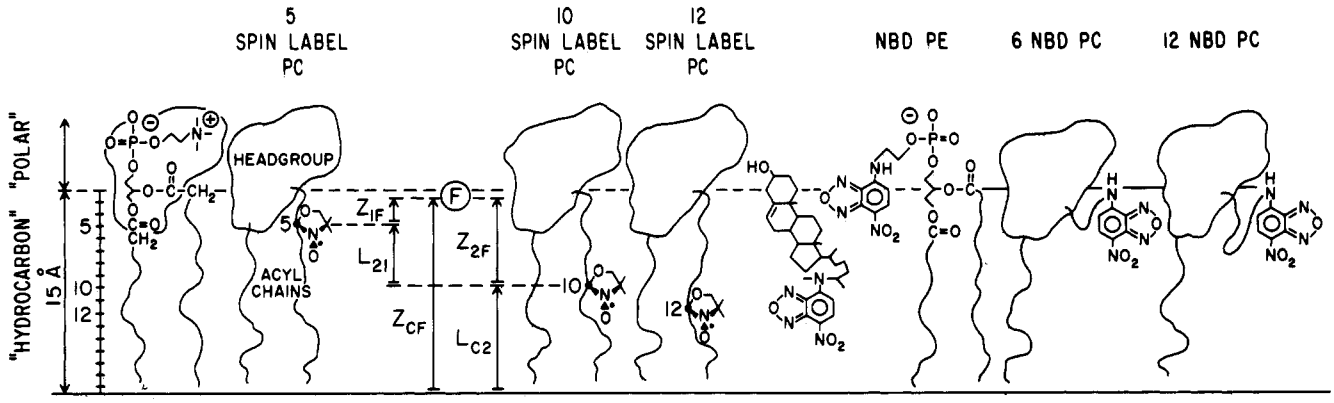

FIGURE 4: Schematic diagram of half of the membrane bilayer showing the depth of spin-labeled PCs and of NBD-labeled lipids as measured by quenching (Table I), and the parameters describing depth. The horizontal line at the bottom indicates the center of the bilayer. Parameters are shown for an experiment in which quenching of a hypothetical fluorophore at position F by the 5 and 10 spin-labeled PCs is compared (see text). Notice that distances are defined such that positive values are directed toward the surface of the leaflet containing the fluorophore being considered. The scale on the left shows the depth of the carbons on the 2-position acyl chain. An assumed distance of 0.45 Å from the final carbon to bilayer center is shown. The estimated distances from the spin-label to the bilayer center for the 5, 10, and 12 spin-labeled PCs are 12.15, 7.65, and 5.85 Å, respectively (see text).

the values fall within ±1 Å of the average, strongly suggesting that perturbation of membrane structure by high concentrations of spin-labels does not affect fluorophore depth. The small variations observed could largely be a result of an error in measured spin-label concentration, which can only be determined to ±10% (see Experimental Procedures and Discussion).

Figure 4 shows a summary of the apparent locations of the NBD groups in the labeled lipids used. The apparent depths of the NBD group in NBD-PCs and NBD-PE place them at the polar/hydrocarbon "boundary". The NBD group attached to the cholesterol tail is buried in the hydrocarbon region of the bilayer.

Discussion

The spin-label fluorescence quenching approach has several advantages as a method to measure depth, including the following: (1) it is experimentally and analytically straightforward to use; (2) it yields a numerical value for depth (in angstroms) rather than just a relative location; (3) it requires only small amounts of both fluorescent molecules and lipids; and (4) it is widely applicable to reconstituted systems because, unlike other types of quenchers, spin-labels can quench virtually all types of fluorophores, including tryptophans (Green et al., 1973; London, 1982). Furthermore, since quenching by spin-labels and brominated lipids in membranes is static unless unusually long-lived fluorophores (greater than 20–50 ns) are used (London & Feigenson, 1981a; London, 1982; East & Lee, 1982), the method is very insensitive to fluorescence properties such as lifetime and quantum yield, and therefore, it is usually unnecessary to measure these parameters. Since the analysis is useful for measurement of distances up to $0.9R_c$ (see Theory), and R_c is about 10–12 Å for many fluorophores [Table I and London & Feigenson (1981a)], quenchers centrally placed in fatty acyl chains can be used to locate fluorophores at any depth within a membrane leaflet. It should be noted that since the analysis itself is general, its extension to longer range energy-transfer experiments should allow localization of sites outside the bilayer.

Nevertheless, there are certain limits and precautions that must be considered. One consideration is that an average depth is obtained. It can be presumed that the depths obtained represent at least an average over a couple of angstroms, arising from a distribution of depths of fluorophores and spin-labels. Another important concern is the exact depth of the spin-label groups. Clearly, the accuracy of these values

will limit the accuracy of the value of fluorophore depth determined. Fortunately, several lines of evidence, described below, justify placement of the spin-labels close to the corresponding position expected in unlabeled PC (see Theory and Figure 4). First, it has been shown by ESR that the hydrocarbon chain of a spin-labeled fatty acyl group is on the average roughly perpendicular to the membrane surface, strongly suggesting an orientation identical with natural chains (Hubbell & McConnell, 1969), which in turn would place the spin-labels at the expected depths corresponding to those of unlabeled chains. Second, as the spin-label is attached progressively further from the carboxyl end of the fatty acyl chain, both the rate of spin-label reduction by hydrophilic ascorbate (Schreirer-Muccillo et al., 1976) and the polarity of the spin-label environment, as judged by hyperfine splitting (Griffith et al., 1974; Fretten et al., 1980), decrease in a smooth and monotonic order. This implies a regular progression in spin-label depth vs. attachment site in the expected direction. Third, studies have shown spin-labels roughly occupy the expected depth by their effect on the NMR signal of unlabeled lipid acyl chains (Godici & Landsberger, 1974). Fourth, our results show that the apparent fluorophore depth is independent of which depth spin-labels are used. If there was an error in the calculated depth in any one of the spin-labels, this agreement would not be observed. At the very least, this latter observation strongly suggests that the ratio of the distance between the 5- and 10-position labels to that between the 10- and 12-position labels must be correct. Taken together, these independent lines of evidence suggest that the uncertainty in estimating spin-label depth cannot be large, perhaps a couple of angstroms or less. Although this is not ideal, it should be pointed out that the uncertainty of probe location has always been an implicit limitation of photo-cross-linking and energy-transfer studies too. In fact, an important use of spin-label depth determination should be to help calibrate such probes. Nevertheless, to increase resolution, it may be desirable to calibrate spin-label depth more precisely. We plan to approach this by comparison of quenching by brominated and spin-labeled lipids. A series of lipids with brominated fatty acyl chains has been developed for fluorescence quenching studies (Markello et al., 1985). Since bromines are not polar, it is very likely that they will remain close to the depths expected for unlabeled chains, and X-ray studies confirm this (Lytz et al., 1984; R. E. McIntosh, S. A. Simon, and P. W. Holloway, personal communication).

A more minor concern arises from the definition of distance between spin-label and fluorophore. We do not know if distances between the van der Waals boundaries or transition dipoles will apply. Figure 4 has been drawn assuming the distance is that between the center of the free radical π orbital and the fluorescent group.

Orientation effects on quenching also deserve comment. A consequence of the small local motions of fluorophores and spin-labels is that orientation is likely to be randomized and unlikely to have a strong influence on quenching, just as orientation effects tend to be averaged out in energy transfer. This is reinforced by two other considerations. First, R_c is very insensitive to the spin-label used (Table I). Since orientation effects on the degree of quenching would appear as an effect on the apparent R_c (cf. energy transfer where R_0 is a function of the orientation factor κ^2), the similarity of R_c for all three quenchers and fluorophores used (Table I) implies orientation effects cannot be critical. Second, the analysis involves comparison of quenching by two probes. Only differences in orientation effects for the two different spin-labels used could influence the apparent depth. The agreement in measured depth when three different spin-labels are compared implies that no such differences are present.

It should also be noted that the concentration of spin-label should be calculated as carefully as possible. An error of 10% between calculated and actual values, which is conceivable, could lead to an error of ±0.5–1 Å according to sample calculations (not shown). In place of calculating spin-label concentration by ESR, which is not available to all, we suggest that one can substitute calculation of spin-group concentration by comparing the amount of quenching of a NBD lipid to the amount observed in this report.

These studies show that NBD probes attached to fatty acyl chains via an amino linkage move to the interface. Preliminary results suggest that the NBD amino group is uncharged at neutral pH (not shown). Therefore, we presume the polarity of the nitrogen- and oxygen-rich NBD group is responsible for its localization at the polar/hydrocarbon interface. The unexpected depth of this group when attached to acyl chains means that it must be used in analogues of natural lipids with caution. In addition, the quenching results show attachment to the tail of cholesterol results in deep burial of the NBD group. The most obvious interpretation is that the rigidity of the sterol rings prevents the NBD from approaching the surface or that the methyl group on the NBD reduces its hydrophilicity. We conclude that spin-label quenching combined with the determination of depth by analysis of dual-quenching experiments represents a powerful tool for investigation of membrane structure.

APPENDIX

Analysis of depth is more complicated when deep quenchers (close to the center of the membrane) are used to study deeply buried fluorophores. Quenchers in one leaflet can then quench a fluorophore in the opposite leaflet, which we call quenching arising from the trans leaflet.[3] The most important case of trans leaflet quenching occurs when only the deepest of the two quenchers used is close enough to fluorophores in the opposite leaflet to quench them. In this case, the ratio of fluorescence in the presence of the shallow quencher 1 to that in the presence of the deeper quencher 2 is given by eq 10.

$$\frac{F_1}{F_2} = \frac{e^{-\pi R_c^2 C + \pi z_{1F}^2 C + \pi x^2 C}}{(e^{-\pi R_c^2 C + \pi z_{2F}^2 C + \pi x^2 C})(e^{-\pi R_c^2 C + \pi z_{2tF}^2 C + \pi x_{2tF}^2 C})} \quad (10)$$

The subscript t refers to the appropriate value for the quenchers in the leaflet trans relative to the fluorophore. Thus, z_{2tF} equals the vertical distance from the deep quencher in the leaflet opposite the fluorophore to the fluorophore, which also equals $2L_{c2} + z_{2F}$. For lipid probes, the term x_{2tF}, the minimum lateral approach of fluorophore to deep quenchers in the opposite leaflet, is zero because there is no restriction on lateral approach for molecules in opposite leaflets. Cancelling out terms from eq 10, substituting, and rearranging yield eq 11

$$z_{1F} = -2L_{21} - 2L_{c2} \pm \sqrt{\frac{-1}{\pi C} \ln \frac{F_1}{F_2} + 2L_{21}^2 + 4L_{21}L_{c2} + R_c^2} \quad (11)$$

where L_{c2} is the distance from the center of the bilayer to the deep quencher. In this case, it is necessary to know R_c to derive

[3] This has an important implication. Earlier methods of evaluating depth from spin-label quenching have assumed that the fluorophore is closest to the depth of the quencher that gives the most quenching. In the presence of strong trans quenching, this is not always true.

z_{1F}, but since R_c is generally between 10 and 12 Å for many probes (London & Feigenson, 1981a; Table I) and since we find the value of z_{1F} obtained is not overly sensitive to the choice of R_c, this is not a critical problem. An important question is when to use eq 8 and when to use eq 11 to calculate z_{1F}. The answer is that if the depth analyzed by using eq 8 gives $z_{2tF} < R_c$, then trans leaflet quenching must be occurring to some degree and eq 11 should be used. If one chooses relatively shallow spin-labels, which can not significantly quench beyond the leaflet in which they reside, the need to use eq 11 can be largely avoided.

A final case occurs when both very deep quenchers and fluorophores are used. In this case, trans quenching will arise from both deeper and shallower quenchers. By analogy to the approach in eq 10 and 11, one can derive eq 12 in this case.

$$\frac{1}{\pi C} \ln \frac{F_1}{F_2} = 2(L_{21}^2 + 2L_{21}L_{C2}) \qquad (12)$$

Fluorescence is not a function of fluorophore depth in this case. The reason is that as fluorophore depth varies, loss of quenching by quenchers in one leaflet is balanced by the gain in quenching from the other leaflet. Use of at least one shallow spin-label avoids having experiments fall into this case.

Registry No. DOPC, 4235-95-4; NBD-PE, 92605-64-6; 6-NBD-PC, 91992-01-7; 12-NBD-PC, 105539-26-2; NBD-cholesterol, 105539-27-3; 5 spin-labeled PC, 66642-40-8; 10 spin-labeled PC, 105539-28-4; 12 spin-labeled PC, 55402-86-3.

REFERENCES

Baird, B., & Holowka, D. (1985) *Biochemistry 24*, 6252.

Bartlett, G. R. (1959) *J. Biol. Chem. 234*, 466.

Birks, J. B. (1970) *Photophysics of Aromatic Molecules*, pp 341–347, Wiley-Interscience, London.

Blatt, E., & Sawyer, W. H. (1985) *Biochim. Biophys. Acta 822*, 43.

Büldt, G., Gally, H. Y., Seelig, J., & Zaccai, G. (1979) *J. Mol. Biol. 134*, 673.

Caffrey, M., & Feigenson, G. W. (1981) *Biochemistry 20*, 1949.

Davenport, L., Dale, R. E., Bisby, R. H., & Cundall, R. B. (1985) *Biochemistry 24*, 4097.

Dewey, T. G., & Hammes, G. G. (1980) *Biophys. J. 32*, 1023.

Dittmer, J. C., & Lester, R. L. (1964) *J. Lipid Res. 5*, 126.

East, J. M., & Lee, A. G. (1982) *Biochemistry 21*, 4144.

Fleming, P. J., Koppel, D. E., Lau, A. L. Y., & Strittmatter, P. (1979) *Biochemistry 18*, 5458.

Fretten, P., Morris, S. J., Watts, A., & Marsh, D. (1980) *Biochim. Biophys. Acta 598*, 247.

Godici, P. E., & Landsberger, F. R. (1974) *Biochemistry 13*, 362.

Green, J. A., Singer, L. A., & Parks, J. H. (1973) *J. Chem. Phys. 58*, 2690.

Griffith, O. H., Dehlinger, P. J., & Van, S. P. (1974) *J. Membr. Biol. 15*, 159.

Hasselbacher, C. A., Preuss, D. K., & Dewey, T. G. (1986) *Biochemistry 25*, 668.

Hitchcock, P. B., Mason, R., Thomas, K. M., & Shipley, G. G. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 3036.

Holowka, D., Conrad, D. H., & Baird, B. (1985) *Biochemistry 24*, 6260.

Hubbell, W. L., & McConnell, H. M. (1969) *Proc. Natl. Acad. Sci. U.S.A. 64*, 20.

Jain, M. K., Rogers, J., Simpson, L., & Gierasch, L. M. (1985) *Biochim. Biophys. Acta 816*, 153.

Kao, Y. J., Soutar, A. K., Hong, K. Y., Pownall, H. J., & Smith, L. C. (1978) *Biochemistry 17*, 2689.

Kleinfeld, A. M. (1985) *Biochemistry 24*, 1874.

Kleinfeld, A. M., & Lukacovic, M. F. (1985) *Biochemistry 24*, 1883.

Koppel, D. E., Fleming, P. J., & Strittmatter, P. (1979) *Biochemistry 18*, 5451.

Kremer, J. M. H., Esker, M. W. J. v. d., Pathmamanoharan, C., & Wiersema, P. H. (1977) *Biochemistry 16*, 3932.

Lewis, B. A., & Engelman, D. (1983) *J. Mol. Biol. 166*, 211.

London, E. (1982) *Mol. Cell. Biochem. 45*, 181.

London, E., & Feigenson, G. W. (1981a) *Biochemistry 20*, 1932.

London, E., & Feigenson, G. W. (1981b) *Biochim. Biophys. Acta 649*, 89.

Lytz, R. K., Reinert, J. C., Church, S. E., & Wickman, H. H. (1984) *Chem. Phys. Lipids 35*, 63.

Markello, T., Zlotnick, A., Everett, J., Tennyson, J., & Holloway, P. W. (1985) *Biochemistry 24*, 2895.

Marsh, D. (1981) in *Membrane Spectroscopy* (Grell, E., Ed.) pp 51–142, Springer-Verlag, Berlin.

McConnell, H. M. (1976) in *Spin Labeling* (Berliner, L. J., Ed.) pp 525–560, Academic Press, New York.

Montecucco, C., Schiavo, G., & Tomasi, M. (1985) *Biochem. J. 231*, 123.

Morrison, W. R. (1964) *Anal. Biochem. 7*, 218.

Nichols, J. W. (1985) *Biochemistry 24*, 6390.

Oldfield, E., Meadows, M., Rice, D., & Jacobs, R. (1978) *Biochemistry 17*, 2727.

Pagano, R. E., & Sleight, R. G. (1985) *Science (Washington, D.C.) 229*, 1051.

Pearson, R. H., & Pascher, I. (1979) *Nature (London) 281*, 499.

Perrin, F. (1924) *C.R. Hebd. Seances Acad. Sci. 178*, 1978.

Schreirer-Muccillo, S., Marsh, D., & Smith, I. C. P. (1976) *Arch. Biochem. Biophys. 172*, 1.

Seelig, A., & Seelig, J. (1974) *Biochemistry 13*, 4839.

Shaklai, N., Yguerabide, J., & Romney, H. M. (1977) *Biochemistry 16*, 5585.

Sklar, L. A., Doody, M. C., Gotto, A. M., & Pownall, H. J. (1980) *Biochemistry 19*, 1294.

Wolber, P. K., & Hudson B. S. (1979) *Biophys. J. 28*, 197.

Zaccai, G., Büldt, G., Seelig, A., & Seelig, J. (1979) *J. Mol. Biol. 134*, 693.