

Fluorophore Environments in Membrane-Bound Probes: A Red Edge Excitation Shift Study

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ABSTRACT: A shift in the wavelength of maximum fluorescence emission toward higher wavelengths, caused by a shift in the excitation wavelength toward the red edge of the absorption band, is termed the Red Edge Excitation Shift (REES). This effect is mostly observed with polar fluorophores in motionally restricted media such as very viscous solutions or condensed phases. In this paper, we report the red edge excitation shift of a membrane-bound phospholipid molecule whose headgroup is covalently labeled with a 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD) moiety. When incorporated into model membranes of dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), the NBD-labeled phospholipid (NBD-PE), exhibits a red edge excitation shift of 10 nm. In addition, fluorescence polarization of NBD-PE in membranes shows both excitation and emission wavelength dependence. The nonpolar membrane probe 1,6-diphenyl-1,3,5-hexatriene (DPH) does not show red edge excitation shift in model membranes. The lifetime of NBD-PE in DOPC vesicles was found to be dependent on both excitation and emission wavelengths. These wavelength-dependent lifetimes are correlated to the reorientation of solvent dipoles around the excited-state dipole of the NBD moiety in the membrane. The magnitude of the red shift in the emission maximum for NBD-PE was found to be independent of temperature, between 12 and 54 °C, and of the physical state (gel or fluid) of the membrane. Taken together, these observations are indicative of the motional restriction experienced by this fluorophore in the membrane. Red edge excitation shift promises to be a powerful tool in probing membrane organization and dynamics.

Organized molecular assemblies such as membranes can be considered as large cooperative units with characteristics very different from the individual structural units which constitute them. Fluorescence spectroscopy has been one of the principal techniques to study organization and dynamics of biological and model membranes because of its suitable time scale, noninvasive nature, and intrinsic sensitivity (Radda, 1975; Lakowicz, 1980, 1981). For a fluorophore in a bulk nonviscous solvent, the dipolar relaxation of the solvent molecules around the fluorophore in the excited state is much faster than the fluorescence lifetime. The fluorescence decay rates and the wavelength of maximum emission, in such a case, are therefore usually independent of the excitation wavelength. However, these parameters do show excitation wavelength dependence if the dipolar relaxation of the solvent molecules is slow in the excited state, such that the relaxation time is comparable to or longer than the fluorescence lifetime (Chen, 1967; Fletcher, 1968; Galley & Purkey, 1970; Rubinov & Tomin, 1970; Castelli & Forster, 1973; Itoh & Azumi, 1975; Demchenko, 1982; Lakowicz & Keating-Nakamoto, 1984). Such a shift in the wavelength of maximum emission toward higher wavelengths, caused by a shift in the excitation wavelength toward the red edge of the absorption band, is termed the red edge excitation shift (REES).¹ This effect is mostly observed with polar fluorophores in motionally restricted media such as very viscous solutions or condensed phases, that is, when the fluorophore mobility relative to the surrounding matrix is considerably reduced. Thus, REES serves as an indicator of fluorophore environment.

The origin of the red edge effect lies in the change in fluorophore-solvent interactions in the ground and excited states, brought about by a change in the dipole moment of the fluorophore upon excitation, and the rate at which solvent

molecules reorient around the excited-state fluorophore (Galley & Purkey, 1970; Itoh & Azumi, 1975; Macgregor & Weber, 1982; Demchenko, 1982, 1986, 1988a; Lakowicz & Keating-Nakamoto, 1984; Demchenko & Ladokhin, 1988). For a polar fluorophore, there exists a statistical distribution of solvation states based on their dipolar interactions with the solvent molecules in both the ground and excited states. Since the dipole moment (magnitude as well as direction) of a molecule changes upon excitation, the solvent dipoles have to reorient around this new excited-state dipole moment of the fluorophore, so as to attain an energetically favorable orientation. This readjustment of the dipolar interaction of the solvent molecules with the fluorophore essentially consists of two components: first, the redistribution of electrons in the surrounding solvent molecules because of the altered dipole moment of the excited-state fluorophore, and second, the physical reorientation of the solvent molecules around the excited-state fluorophore. The former process is almost instantaneous, i.e., electron redistribution in solvent molecules occurs at about the same time scale as the process of excitation of the fluorophore itself (10^{-15} s). The reorientation of the solvent dipoles, however, requires a net physical displacement. It is thus a much slower process and is dependent on the restriction to their mobility as offered by the surrounding matrix. More precisely, for a polar fluorophore in a bulk

¹ Abbreviations: ANS, 1-anilinonaphthalene-8-sulfonate; DMPC, dimyristoyl-*sn*-glycero-3-phosphocholine; DOPC, dioleoyl-*sn*-glycero-3-phosphocholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; DPPC, dipalmitoyl-*sn*-glycero-3-phosphocholine; MLV, multilamellar vesicle; NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; NBD-AHA, 6-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]hexanoic acid; NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; Patman, 6-palmitoyl-2-[[[2-(trimethylammonio)ethyl]amino]methyl]naphthalene chloride; PC, phosphatidylcholine; REES, red edge excitation shift; TLC, thin-layer chromatography; TNS, 6-(*p*-toluidinyl)-2-naphthalenesulfonic acid; ULV, unilamellar vesicle.

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nonviscous solvent, this reorientation occurs at a time scale of the order of 10^{-12} s, so that all the solvent molecules completely reorient around the excited-state dipole of the fluorophore well within its excited-state lifetime, which is typically of the order of 10^{-9} s. Hence, irrespective of the excitation wavelength used, all emission is observed only from the solvent-relaxed state. However, if the same fluorophore is now placed in a viscous medium, this reorientation process is slowed down to 10^{-9} s or longer. Under these conditions, excitation by progressively lower energy quanta, i.e., the excitation wavelength being gradually shifted toward the red edge of the absorption band, selectively excites those fluorophores which interact more strongly with the solvent molecules in the excited state. These are the fluorophores around which the solvent molecules are oriented in such a way as to be more similar to that found in the solvent-relaxed state. Thus, the necessary condition for giving rise to REES is that a different average population is excited at each excitation wavelength and, more importantly, that the difference is maintained in the time scale of fluorescence lifetime. As discussed above, this requires that the dipolar relaxation time for the solvent shell be comparable to or longer than the fluorescence lifetime, so that fluorescence occurs from various partially relaxed states. This implies a reduced mobility of the fluorophore with respect to the surrounding matrix.

Application of the red edge effect to systems of biological relevance has so far been restricted mainly to indole, tryptophan, and other fluorescent probes in viscous solvents and when bound to proteins (Demchenko, 1982, 1985, 1988a,b; Lakowicz & Keating-Nakamoto, 1984; Macgregor & Weber, 1986; Bismuto et al., 1987; Demchenko & Ladokhin, 1988; Albani, 1992; Wasylewski et al., 1992). Demchenko and Ladokhin (1988) have investigated the excitation-wavelength-dependent changes in the fluorescence emission spectra of indole and tryptophan in glycerol and glucose glasses. These authors observed a significant red shift of the emission maximum upon excitation at the red edge of the absorption profile of the fluorophore (Demchenko, 1988b; Demchenko & Ladokhin, 1988). The fluorescence probe TNS shows no such red edge excitation effect in methanol or dioxane (Lakowicz & Keating-Nakamoto, 1984) but shows REES when bound to proteins such as apomyoglobin, β -lactoglobulin, β -casein, the human α_1 -acid glycoprotein, and the serum albumins (Demchenko, 1982; Lakowicz & Keating-Nakamoto, 1984; Albani, 1992). REES of the tryptophan fluorescence of the intact eye lens (Rao et al., 1989), and of native and mutant cytochrome b_5 (Ladokhin et al., 1991), has also been recently reported.

A direct consequence of highly organized molecular assemblies such as membranes is the restriction imposed on the mobility of their constituent structural units. It is well known that interiors of biological membranes are viscous, with the effective viscosity comparable to that of light oil (Cone, 1972; Poo & Cone, 1974). It is thus of interest to investigate whether the phenomenon of REES takes place in such assemblies. In this paper, we report the observation of red edge excitation effect of NBD-PE in model membranes of dioleoyl-*sn*-glycero-3-phosphocholine (DOPC).

The membrane is a highly anisotropic medium largely confined to two dimensions. The hydrophobic membrane interior cannot be mimicked by any isotropic organic solvent. Order parameter calculations from magnetic resonance studies reveal that while the center of the bilayer is nearly isotropic, the upper portion, only a few angstroms away towards the surface of the membrane, is highly ordered (Seelig, 1977;

Perochon et al., 1992). This not only results in the anisotropic behavior of the constituent lipid molecules but also, more importantly, the environment of a probe molecule becomes very much dependent on its location (depth) in the membrane. Our study is thus all the more relevant in regard to membrane organization, because the precise orientation and location of NBD-PE in the membrane is known (Chattopadhyay & London, 1987, 1988; Pagano & Martin, 1988; Chattopadhyay, 1990; Mitra & Hammes, 1990; Wolf et al., 1992). NBD-labeled lipids are widely used as fluorescent analogues of native lipids in biological and model membranes to study a variety of processes [for a review, see Chattopadhyay (1990)]. In NBD-PE, the NBD label is covalently attached to the headgroup of a phosphatidylethanolamine molecule. The NBD group in NBD-PE is located at the membrane interface. This region of the membrane has unique motional and dielectric characteristics distinct from both the bulk aqueous phase and the hydrocarbon-like interior of the membrane (Ashcroft et al., 1981; Stubbs et al., 1985; Perochon et al., 1992) and plays an important role in functional aspects such as substrate recognition and activity of lipolytic enzymes (El-Sayed et al., 1985). Application of the red edge effect to probes whose location in membranes are well defined could provide useful information regarding the structural organization and dynamics in specific regions of the membrane.

Some of the results presented here have been previously reported in preliminary form (Chattopadhyay, 1991).

MATERIALS AND METHODS

DOPC was purchased from Avanti Polar Lipids (Birmingham, AL). DPPC, DMPC, egg PC, and DPH were purchased from Sigma Chemical Co. (St. Louis, MO). NBD-PE was from Molecular Probes (Eugene, OR). Lipids were checked for purity by TLC on silica gel plates in chloroform/methanol/water (65:35:5 v/v/v). All phospholipids gave one spot with a phosphate-sensitive spray and on subsequent charring (Dittmer & Lester, 1964). NBD-PE was found to be pure when detected by its color or fluorescence. Concentrations of lipids were determined by phosphate assay subsequent to total digestion by perchloric acid (McClare, 1971). DMPC was used as a standard to assess lipid digestion. Solvents used were of spectroscopic grade.

Two kinds of vesicles were used. All experiments involving steady-state fluorescence measurements were done using multilamellar vesicles of DOPC containing 1% (mol/mol) NBD-PE or DPH, as described previously (London & Feigenson, 1981; Chattopadhyay & London, 1987). In general, 160 nmol of DOPC in chloroform was mixed with 1.6 nmol of the probe in chloroform. A few drops of chloroform were added and mixed well, and the samples were dried under a stream of nitrogen while being warmed gently ($\sim 35^\circ\text{C}$). After further drying under a high vacuum for at least 3 h, 1.5 mL of 10 mM sodium phosphate and 150 mM sodium chloride buffer, pH 7.0–7.2, was added, and each sample was vortexed for 3 min to disperse the lipids. Background samples were prepared the same way except that the probes were omitted. All experiments were done with multiple sets of samples, and average values of fluorescence and polarization are shown in the figures. The spectral shifts obtained with different sets of samples were identical in most cases. In other cases, the values were within ± 1 nm of the ones reported. For experiments in which NBD-PE and DPPC were used, everything was done as above, except that swelling of lipids in the buffer was done above the phase-transition temperature of DPPC.

For experiments in which fluorescence lifetimes were measured, unilamellar vesicles (ULV) of DOPC labeled with 1% (mol/mol) NBD-PE were prepared by the ethanol injection method (Batzri & Korn, 1973; Kremer et al., 1977). For this, 1280 nmol of DOPC and 12.8 nmol of NBD-PE were dried together. The dried lipids were then dissolved in ethanol, to give a final concentration of about 40 mM lipid in ethanol. This ethanolic lipid solution was then injected into 10 mM sodium phosphate and 150 mM sodium chloride buffer, pH 7.0–7.2, while vortexing, to give a final concentration of 0.85 mM lipid in the buffer. In addition, we prepared DOPC ULVs containing 160 nmol of lipid labeled with 1% NBD-PE by the ethanol injection method, as described above. These vesicles were used as a control for the steady-state fluorescence experiments in which emission maxima were monitored as a function of excitation wavelength.

Steady-state fluorescence measurements were performed with a Hitachi F-4000 spectrofluorometer using 1-cm path-length quartz cuvettes. Slits with a nominal bandpass of 3 nm were used for measurement of emission maxima as a function of excitation wavelength. For polarization measurements, excitation and emission slits with a nominal bandpass of 5 nm were used. All experiments, unless mentioned otherwise, were done at 23 °C. Background intensities of samples in which fluorophores were omitted were subtracted from each sample spectrum to cancel out any contribution due to scattering artifacts. Polarization measurements were performed using a Hitachi polarization accessory. Polarization values were calculated from (Chen & Bowman, 1965)

$$P = \frac{I_{VV} - GI_{VH}}{I_{VV} + GI_{VH}} \quad (1)$$

where I_{VV} and I_{VH} are the measured fluorescence intensities with the excitation polarizer vertically oriented and the emission polarizer vertically and horizontally oriented, respectively. G is the grating correction factor and is equal to I_{HV}/I_{HH} . The polarization values reported in Figures 3 and 7 are the average of several measurements, and all individual data points are within 3% of the reported values.

Fluorescence lifetimes were calculated from time-resolved fluorescence intensity decays using a Photon Technology International (London, Western Ontario, Canada) LS-100 luminescence spectrophotometer in the time-correlated single photon counting mode. This machine uses a thyatron-gated nanosecond flash lamp filled with nitrogen as the plasma gas (15 ± 1 in. of mercury vacuum) and run at 22–25 kHz. Lamp profiles were measured at the excitation wavelength using Ludox as the scatterer. To optimize the signal-to-noise ratio, a minimum of 5000 photon counts were collected in the peak channel. All experiments were performed using slits with a nominal bandpass of 8 nm or less. The sample and the scatterer were alternated after every 10% acquisition to ensure compensation for shape and timing drifts occurring during the period of data collection. The data stored in a multichannel analyzer was routinely transferred to an IBM PC/AT computer for analysis. Intensity decay curves were fitted as a sum of exponential terms:

$$F(t) = \sum_i \alpha_i \exp(-t/\tau_i) \quad (2)$$

where α_i is a preexponential factor representing the fractional contribution to the time-resolved decay of the component with a lifetime τ_i . The decay parameters were recovered using a nonlinear least-squares iterative fitting procedure based on the Marquardt algorithm (Bevington, 1969). The program

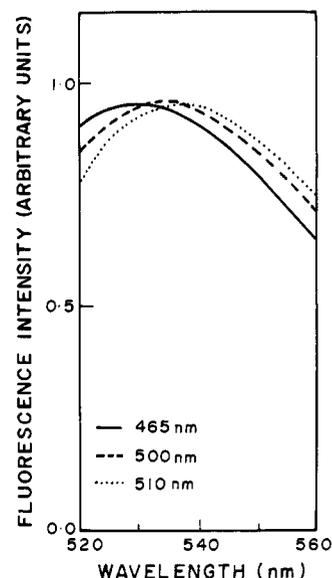


FIGURE 1: Fluorescence emission spectra of NBD-PE in multilamellar vesicles of DOPC at different excitation wavelengths. The excitation wavelengths were 465 (solid line), 500 (dashed line), and 510 (dotted line) nm. All spectra are intensity-normalized at the emission maximum. Fluorophore to lipid ratio was 1:100 (mol/mol). See Materials and Methods for other details.

also includes statistical and plotting subroutine packages (O'Connor & Phillips, 1984). The goodness of the fit of a given set of observed data and the chosen function was evaluated by the reduced χ^2 ratio, the weighted residuals (Lampert et al., 1983), the autocorrelation function of the weighted residuals (Grinvald & Steinberg, 1974), the runs test (Hamburg, 1985), and the Durbin-Watson parameters (Durbin & Watson, 1950, 1951). A fit was considered acceptable when plots of the weighted residuals and the autocorrelation function showed random deviation about zero with a χ^2 value not more than 1.5. Mean (average) lifetimes ($\langle \tau \rangle$) for a double-exponential decay of fluorescence was calculated from the decay times and preexponential factors using (Lakowicz, 1983)

$$\langle \tau \rangle = \frac{\alpha_1 \tau_1^2 + \alpha_2 \tau_2^2}{\alpha_1 \tau_1 + \alpha_2 \tau_2} \quad (3)$$

RESULTS

The fluorescence emission spectra of NBD-PE in DOPC vesicles obtained using different excitation wavelengths are shown in Figure 1. This figure shows dependence of the emission spectrum on the excitation wavelength for NBD-PE. As shown in the figure, upon excitation at 465 nm, the maximum of fluorescence emission² occurs around 530 nm, as has been observed previously (Chattopadhyay & London, 1988). Changing the excitation wavelength to 500 nm and then to 510 nm causes a progressive shift in the wavelength of maximum emission from 530 to 537 nm. Such dependence of the emission spectra on the excitation wavelength is characteristic of the red edge effect. Our present observation of such an effect in model membranes containing NBD-PE indicates that this membrane-bound fluorophore is in an

² We have used the term maximum of fluorescence emission in a somewhat wider sense here. In every case, we have monitored the wavelength corresponding to maximum fluorescence intensity, as well as the center of mass of the fluorescence emission. In most cases, both these methods yielded the same wavelength. In cases where minor discrepancies were found, the center of mass of emission has been reported as the fluorescence maximum.

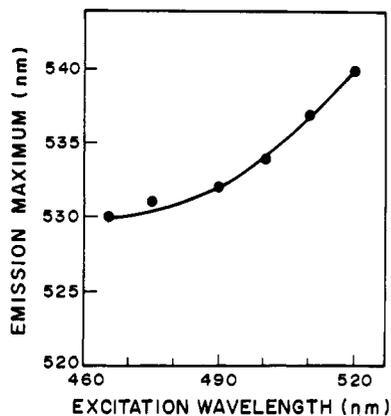


FIGURE 2: Effect of changing excitation wavelength on the wavelength of maximum emission for NBD-PE in DOPC vesicles. All other conditions are as in Figure 1.

environment where its mobility is considerably restricted. Since the NBD group in NBD-PE is known to be located at the membrane interface (Chattopadhyay & London, 1987, 1988; Mitra & Hammes, 1990; Chattopadhyay, 1990; Wolf et al., 1992), such a result would directly imply that this region of the membrane offers considerable restriction to the reorientational motion of the solvent dipoles around the excited-state fluorophore.

The change in fluorescence emission maxima of NBD-PE in DOPC vesicles as a function of excitation wavelength is shown in Figure 2. This figure shows that as the excitation wavelength of NBD-PE is changed from 465 to 520 nm, the emission maximum is shifted from 530 to 540 nm, which corresponds to a REES of 10 nm. Observation of such a shift in the wavelength of maximum emission with a change in excitation wavelength implies that the NBD moiety in NBD-PE is in an environment where its mobility is considerably reduced.

In a control study (not shown), we repeated the above experiment using DOPC ULVs containing 1% NBD-PE made by the ethanol injection method, keeping the lipid concentration the same. The results obtained were identical with the ones reported here. In another control study, instead of using a pure synthetic lipid (DOPC) for making vesicles, egg PC was used. In this case also, we did not find any difference with our reported values.

In addition to the shift in emission maximum on red edge excitation, fluorescence polarization is also known to be dependent on excitation wavelength in viscous solutions (Weber, 1960a,b; Lynn & Fasman, 1968; Weber & Shinitzky, 1970; Valeur & Weber, 1977a,b, 1978; Lakowicz, 1983, 1984; Demchenko, 1986; VanderMeulen et al., 1990). Valeur and Weber (1977b, 1978) observed an increase in polarization with increasing excitation wavelength for aromatic fluorophores such as naphthylamines. These results were interpreted as indicating a larger apparent molecular volume, which in turn was explained as the result of an out-of-plane electronic transition, which requires more displacement of solvent than an in-plane transition. They suggested that these out-of-plane transitions are selectively excited upon red edge excitation. Weber and Shinitzky (1970) had previously suggested that such an effect could be interpreted as the failure of self energy transfer. Lakowicz (1984) proposed an alternate explanation for the same observation. According to this proposition, there is a decreased rotational rate of the fluorophore in the relaxed state, because of strong dipolar interactions with the surrounding solvent molecules. On red edge excitation, a selective excitation of this subclass of fluorophores occurs. Because of

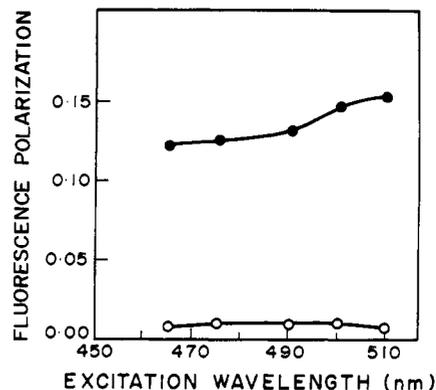


FIGURE 3: Fluorescence polarization of NBD-PE as a function of excitation wavelength in chloroform (O), and in DOPC vesicles (●). Polarization values were recorded at 531 nm. Fluorophore to lipid ratio was 1:100 (mol/mol) in DOPC vesicles. The concentration of NBD-PE in chloroform was 1.1 μ M. See Materials and Methods for other details.

Table I: Lifetimes of NBD-PE in DOPC Vesicles as a Function of Excitation Wavelength^a

excitation wavelength (nm)	α_1	τ_1 (ns)	α_2	τ_2 (ns)	χ^2
465	1.0	7.4			1.1
480	0.6	7.5	0.4	3.7	1.2
490	0.4	6.9	0.6	1.7	1.2
500	0.1	4.5	0.9	0.7	1.2
510	1.0	1.4			1.5

^a Emission wavelength 530 nm.

strong interactions with the polar solvent molecules in the excited state, one may expect these "solvent-relaxed" fluorophores to rotate more slowly, thereby increasing the polarization.

The excitation polarization spectra (i.e., a plot of steady-state polarization vs. excitation wavelength) of NBD-PE in model membranes of DOPC and in chloroform are shown in Figure 3. The polarization of NBD-PE in chloroform remains essentially invariant over the range of excitation wavelengths. On the other hand, polarization in the membrane changes upon alteration of the excitation wavelength, with a sharp increase occurring toward the red edge of the absorption band. Such an increase in polarization upon red edge excitation for peptides and proteins containing tryptophans (Weber, 1960b) as well as other aromatic fluorophores (Valeur & Weber, 1978), especially in media of reduced mobility such as propylene glycol at low temperatures, has been reported before. This reinforces our previous conclusion that the NBD group in NBD-PE is in a motionally restricted region in the membrane.

As has been discussed earlier, the origin of the red edge effect lies in differential extents of solvent reorientation around the excited-state fluorophore, with each excitation wavelength selectively exciting a different average population of fluorophores. Since fluorescence lifetime is known to be sensitive to excited-state interactions, differential extents of solvent relaxation around a given fluorophore could be expected to give rise to differences in its lifetime. Table I shows the lifetimes of NBD-PE in DOPC vesicles as a function of excitation wavelength, keeping the emission wavelength fixed at 530 nm. In order to obtain fluorescence decays with good signal-to-noise ratios, these experiments were carried out using ULVs with a final concentration of 0.85 mM lipid in the buffer, as opposed to 0.11 mM in case of the steady-state

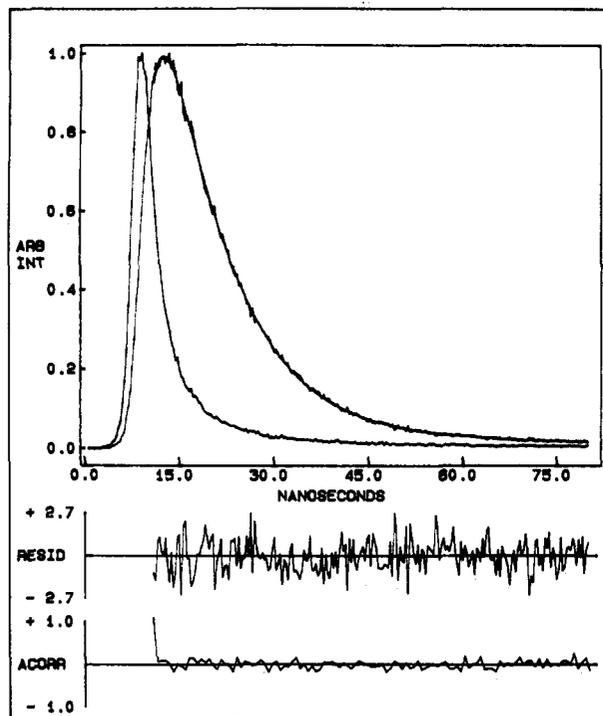


FIGURE 4: Time-resolved fluorescence intensity decay of NBD-PE in ULVs of DOPC when excited at 465 nm. Emission was monitored at 530 nm. The sharp peak on the left is the lamp profile. The relatively broad peak on the right is the decay profile, fitted to a monoexponential function. The two lower plots show the weighted residuals and the autocorrelation function of the weighted residuals. Fluorophore to lipid ratio was 1:100 (mol/mol). See Materials and Methods for other details.

experiments. In these experiments as well, the DOPC to NBD-PE ratio was maintained at 100:1 (mol/mol). The vesicles used were ULVs in order to avoid scattering artifacts that would be produced by MLVs at comparable concentrations. In order to check whether these ULVs prepared using a higher DOPC concentration behaved the same way as the MLVs of lower DOPC concentration which have been used for the steady-state experiments, the steady-state REES experiments were repeated with these samples. The results obtained were identical in both cases.

As can be seen from Table I, when NBD-PE in DOPC vesicles is excited at its mean excitation wavelength, i.e., 465 nm, the decay fits to a monoexponential function, with a lifetime of 7.4 ns. The decay profile with its monoexponential fitting and the various statistical parameters used to check the goodness of the fit are shown in Figure 4. The lifetime of the NBD group in NBD-AHA has previously been reported to be about 7–10 ns in various aprotic solvents (Lin & Struve, 1991). Lifetime of the NBD moiety in the case of dilauroyl- and dimyristoyl-NBD-PE incorporated in vesicles of egg PC has also been reported to be 6–8 ns (Arvinte et al., 1986). In water, however, this lifetime reduces to about 1 ns, which has been attributed to hydrogen-bonding interactions between the fluorophore and the solvent (Lin & Struve, 1991). Thus, the lifetime obtained by us is in agreement with these authors.

When the excitation wavelength was gradually shifted toward the red edge of the absorption band (keeping the emission wavelength constant at 530 nm), it was found that the decays so obtained could no longer be fitted to a single exponential. When the excitation wavelength was shifted from 465 to 480 nm, the major component of the lifetime was still about 7.4 ns. However, a minor component with a much shorter lifetime (3.7 ns) appeared. On changing the excitation

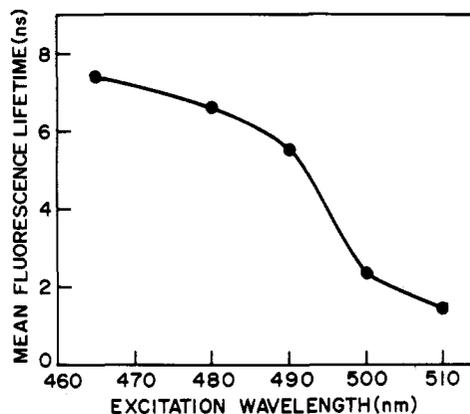


FIGURE 5: Mean fluorescence lifetime of NBD-PE in DOPC vesicles as a function of excitation wavelength. Emission wavelength was kept constant at 530 nm. Mean lifetimes were calculated from Table I using eq 3. Fluorophore to lipid ratio was 1:100 (mol/mol). See Materials and Methods for other details.

wavelength further to 490 nm and then to 500 nm, there was an overall decrease in the lifetimes of both the components, and the relative contribution of the short-lived component increased. Finally, when the sample was excited at 510 nm, the long-lived component totally disappeared, and the decay again fitted to a monoexponential function, this time with a lifetime of 1.4 ns. The mean fluorescence lifetime of NBD-PE was calculated using eq 3 and plotted as a function of excitation wavelength in Figure 5. As shown in the figure, there is a steady decrease (~80%) in the mean lifetime with increasing excitation wavelength from 465 to 510 nm. Such a decrease in the mean lifetime of a fluorophore with increasing excitation wavelength in an environment of restricted mobility has been previously reported (Castelli & Forster, 1973; Conti & Forster, 1974; Valeur & Weber, 1978; Demchenko, 1985).

This marked shortening of the lifetime of the NBD moiety at the red edge of the absorption band could be rationalized as follows. When the NBD group is excited at the mean excitation wavelength (i.e., 465 nm), the majority of the fluorophores will emit at 530 nm (the steady-state fluorescence emission maximum at 465-nm excitation), and will have a certain mean lifetime (7.4 ns in this case) associated with them. However, if the same fluorophore is now excited at the red edge of the absorption band, keeping the emission wavelength still at 530 nm (which is now no more the emission maximum), the lifetime will be shortened because of the preselection of only those fluorophores which have emitted early, and hence the solvent reorientation has not been allowed to shift the emission wavelength to the average (steady-state) maximum of fluorescence emission for that excitation wavelength.

Figure 6 shows the lifetimes of NBD-PE in DOPC vesicles as a function of emission wavelength, keeping the excitation wavelength constant at 465 nm. All decays corresponding to different emission wavelengths could be fitted to monoexponential functions. As can be seen from this figure, the lifetime of the excited state increases by about 11% with increasing emission wavelength from 530 to 560 nm. Such an observation has been previously reported for fluorophores in environments of restricted mobility (Ware et al., 1971; Easter et al., 1976, 1978; Badea et al., 1978; Lakowicz & Cherek, 1980; Matayoshi & Kleinfeld, 1981; Lakowicz et al., 1983a,b; Demchenko & Shcherbatska, 1985). Such increasing lifetimes across the emission spectrum may be interpreted in terms of solvent reorientation around the excited-state fluorophore as follows. Observation at shorter wavelengths of emission selects for

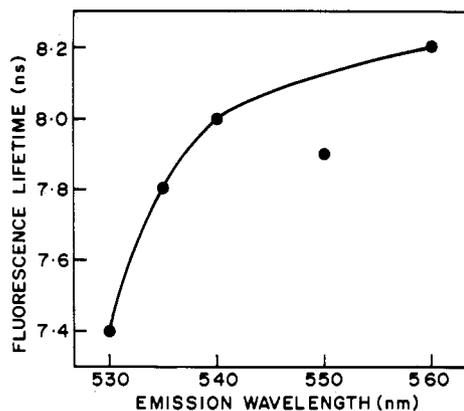


FIGURE 6: Fluorescence lifetime of NBD-PE in DOPC vesicles as a function of emission wavelength. Excitation wavelength used was 465 nm. All other conditions are as in Figure 5.

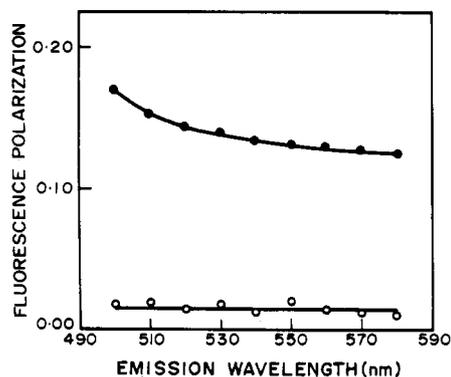


FIGURE 7: Fluorescence polarization of NBD-PE as a function of emission wavelength in chloroform (O) and in DOPC vesicles (●). The excitation wavelength was 465 nm. Fluorophore to lipid ratio was 1:100 (mol/mol) in DOPC vesicles. The concentration of NBD-PE in chloroform was 1.1 μ M. See Materials and Methods for other details.

unrelaxed fluorophores. Their lifetime is shorter because this population is decaying, both at the rate of fluorescence emission at the given excitation wavelength and by decay to longer (unobserved) wavelengths. In contrast, observation at the long-wavelength (red) edge of the emission selects for the relaxed fluorophores, which have spent enough time in the excited state to allow increasingly larger extents of solvent reorientation.

Such longer-lived fluorophores, which are also those which emit at higher wavelengths, should, in principle, have more time to rotate in the excited state, giving rise to lower polarization. Figure 7 shows the variation in steady-state polarization of NBD-PE in DOPC vesicles and in chloroform as a function of wavelength across its emission spectrum. As seen from the figure, while polarization values do not show any significant variation over the entire emission range in chloroform, there is a considerable decrease in polarization with increasing emission wavelength in the case of membrane-bound NBD-PE. The lowest polarization is observed toward the red edge where the relaxed emission predominates. Similar observations have previously been reported for other membrane-bound fluorophores (Matayoshi & Kleinfeld, 1981; Lakowicz et al., 1983b; Demchenko & Shcherbatska, 1985; Sommer et al., 1990).

In order to study the effect of temperature on the extent of REES in membranes, experiments were done with DOPC vesicles labeled with 1% NBD-PE at different temperatures. The results of this study are presented in Table II, which shows the emission maxima as a function of excitation

Table II: Effect of Changing Excitation Wavelength on the Wavelength of Maximum Emission of NBD-PE in Multilamellar Vesicles of DOPC at Different Temperatures

excitation wavelength (nm)	emission maximum (nm)		
	12 °C	23 °C	54 °C
465	529	530	530
475	530	531	531
490	531	532	532
500	534	534	534
510	537	537	537

Table III: Effect of Phase Transition on the Wavelength of Maximum Emission of NBD-PE in Multilamellar Vesicles of DPPC

excitation wavelength (nm)	emission maximum (nm)	
	gel phase (23 °C)	liquid crystal phase (54 °C)
465	530	529
475	530	530
490	532	532
500	534	534
510	537	537
515	538	538

wavelength for NBD-PE at 12, 23, and 54 °C. As is apparent from the table, although there is a slight variation of the emission maxima in some cases, there is no significant change with temperature in the magnitude of REES in this temperature range. Similar results were obtained by others with TNS-labeled DOPC vesicles (Lakowicz & Keating-Nakamoto, 1984). Table III shows the effect of phase transition on the wavelength of maximum emission of NBD-PE in MLVs made of DPPC (phase transition temperature 41 °C) containing 1% NBD-PE. Measurements were done at temperatures well below (23 °C) and above (54 °C) the phase transition temperature. No appreciable change in the emission maximum was observed with the variation in the physical state of the membrane. Such phase-independent relaxation behavior for DMPC vesicles labeled with TNS has been previously reported (Lakowicz & Keating-Nakamoto, 1984). Taken together, these results indicate that there is no major change in the extent of REES of NBD-PE either with a temperature change in the range mentioned or with phase transition. Furthermore, these studies show that the average environment of NBD-PE, as monitored by the absolute position of its emission maximum at a particular excitation wavelength, remains unaffected by these changes. These results are not particularly surprising in view of the fact that changes in membrane organization brought about by phase transition (or temperature) are largely restricted to the fatty acyl region of the membrane (Lentz et al., 1976) and are not sensed by the NBD moiety, which is linked to the headgroup in the case of NBD-PE and is located at the membrane interface (Chattopadhyay & London, 1987, 1988; Pagano & Martin, 1988; Chattopadhyay, 1990; Mitra & Hammes, 1990; Wolf et al., 1992). It has been previously reported that the rates of solvent relaxation for probes that are not deeply buried in the hydrocarbon interior of the membrane are more or less independent of temperature (Lakowicz et al., 1983a; Lakowicz & Keating-Nakamoto, 1984). In addition, small molecular motions of the headgroup region of membranes, which are responsible for spectral relaxation, have been shown not to be affected in a major way by phase transition (Lakowicz et al., 1983a; Mellier, 1989). This is in contrast to the behavior of the amphiphilic membrane probe Patman, which displays temperature-dependent REES in DOPC vesicles, probably

because of the deeper location of its fluorophore in the membrane (Lakowicz et al., 1983b).

An important criterion for REES to occur is that the fluorophore should be polar; that is, it should have a permanent dipole moment in the ground state. For a totally nonpolar fluorophore, there will be no change in dipole moment upon excitation, and the process of solvent relaxation becomes irrelevant, since it is the change in dipole moment that triggers the solvent relaxation. This was confirmed by monitoring the effect of changing excitation wavelength on the wavelength of maximum emission for the nonpolar fluorescence probe DPH in DOPC vesicles (not shown). We found no change in the emission maximum of DPH as the excitation wavelength was varied from 358 to 400 nm. In other words, DPH in DOPC vesicles does not exhibit any REES. This gives further support to our interpretation of the shift in the emission maxima as a function of excitation wavelength for NBD-PE in the membrane, based on differential extents of fluorophore-solvent dipolar interactions in the ground and excited states.

DISCUSSION

The phenomenon of REES as applied to membranes and its use to monitor environments of membrane-bound fluorophores have been the focus of this report. We have shown that NBD-PE, when incorporated in model membranes of DOPC, shows REES. Very little literature exists about the application of REES in membranes. Previous reports in which excitation wavelength dependence of emission maxima of the fluorescent probes ANS or TNS in model membranes of egg PC or DOPC was investigated indicated no appreciable red shift (Lakowicz & Keating-Nakamoto, 1984; Demchenko & Shcherbatska, 1985). These results could be rationalized on the basis of the location of these probes in the membrane. Both these probes are charged at neutral pH and, therefore, access the phospholipid headgroups from the external aqueous phase. This implies that, for these probes, the immediate environment will be the aqueous phase adjacent to the headgroup, where solvent relaxation is extremely fast, and as such, no red edge effect can be expected. However, REES has been reported in the case of DOPC vesicles labeled with Patman, an amphiphilic phase-sensitive membrane probe (Lakowicz et al., 1983b). Because of the fatty acyl chain, this molecule partitions very well into the membrane. The choice of a suitable probe is thus of considerable importance in designing membrane-active molecules capable of exhibiting REES. It is desirable that the probe be able to intercalate with normal components of membranes, i.e., the phospholipids, and that the fluorescent portion of the molecule be suitably embedded in the membrane. In this regard, the probe we chose for this work is appropriate, because it is an amphiphilic phospholipid molecule that is known to strongly partition into the membrane. Also, it is important that the chosen probe have a unique location in the membrane and not a distribution of locations. This would help correlate the extent of REES with a specific fluorophore environment. Our knowledge about the location of the fluorescent moiety in this molecule in its membrane-bound form (Chattopadhyay & London, 1987, 1988; Pagano & Martin, 1988; Chattopadhyay, 1990; Mitra & Hammes, 1990; Wolf et al., 1992) is thus useful. As mentioned earlier, the NBD moiety in NBD-PE is located at the interfacial region of the membrane, a region characterized by unique motional and dielectric characteristics (Ashcroft et al., 1981; Stubbs et al., 1985; Perochon et al., 1992) different from the bulk aqueous phase (experienced by charged aqueous

probes such as ANS and TNS) and the more isotropic hydrocarbon-like deeper regions of the membrane. This specific region of the membrane is also known to participate in intermolecular charge interactions (Yeagle, 1987) and hydrogen bonding through the polar headgroup (Boggs, 1987; Gennis, 1989; Shin et al., 1991). These structural features which slow down the rate of solvent reorientation have previously been recognized as typical features of solvents giving rise to significant red edge effects (Itoh & Azumi, 1975). Furthermore, previous electrophoretic measurements have shown that the NBD group in NBD-PE is uncharged at neutral pH in the membrane (Chattopadhyay & London, 1988). This would ensure that the NBD group does not project into the external aqueous phase. This is advantageous since the ability of a fluorophore to exhibit red edge effects could very well be dependent on its location in the membrane.

While preparing this paper, we came across a very recent paper (Gakamsky et al., 1992) in which REES of membrane-bound 1-phenyl-naphthylamine has been reported. However, this probe suffers from the same drawback as discussed above; i.e., it has a distribution of locations instead of a unique location in the membrane.

Fluorescence studies in membranes should be carried out with caution because of possible REES effects in membranes. Red edge effects should be taken into consideration if membrane-bound fluorophores are excited at the red edge of the absorption spectra rather than at the absorption maxima. Failure to do so in studies such as energy transfer in membranes could lead to significant errors. It has been very recently observed by one of us that there is a wavelength-dependent variation in the measured location (depth) of fatty acyl attached probes (Abrams et al., 1992). In general, it was found that there is a decrease in the apparent depth of the fatty acyl attached probes both at longer excitation wavelengths and at longer emission wavelengths.

The ability of a membrane-bound probe to exhibit REES thus depends on a number of factors such as its polarity, as well as the effective polarity of its immediate environment, and its fluorescence characteristics (e.g., lifetime). All these properties of a membrane-bound fluorophore are a function of its location in the membrane. The extent of REES could thus be related to the location of a fluorophore in the membrane (S. Mukherjee and A. Chattopadhyay, unpublished observations). REES appears to be a potentially powerful tool to study membrane organization and dynamics.

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