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Spectroscopic and ionization properties of *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-labeled lipids in model membranes

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The spectroscopic and ionization properties of various lipids labeled with the 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD) group have been studied in model membranes using fluorescence, absorbance and electrophoretic mobility measurements. Electrophoretic measurements show that the NBD group is uncharged at neutral pH. However, at high pH, hydroxyl addition or deprotonation occurs with a pK_a, depending upon conditions, of 11.5–11.8 for the NBD group of headgroup-labeled phosphatidylethanolamine (NBD-PE) and 11.1–11.5 for NBD labels placed at the end of one fatty acyl chain of a phosphatidylcholine (6-NBD-PC and 12-NBD-PC). This type of behavior is not observed in the case of a methylated NBD label placed in the flexible 'tail' of cholesterol (NBD-cholesterol). The similarity in pK_a for NBD-PE and NBD-PCs suggests that in these cases the NBD group is at a similar depth in the membrane. This was examined further by comparison of the fluorescence emission maximum of the NBD group in model membranes with that in solvents of varying polarity. The apparent polarity experienced by NBD groups in model membranes indicates that for NBD-PE and 12-NBD-PC they are located at the polar region whereas the NBD group of NBD-cholesterol is deeply buried in a nonpolar region of the membrane. This conclusion is supported further by fluorescence quenching experiments measuring NBD exposure to the aqueous quencher Co^{2+}. The results of this study confirm the tentative conclusions of our previous fluorescence quenching studies on the location of NBD groups in model membranes.

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Abbreviations: NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; 6-NBD-PC, 1-palmitoyl-2-(6-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl)-*sn*-glycero-3-phosphocholine; 12-NBD-PC, 1-palmitoyl-2-(12-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-dodecanoyl)-*sn*-glycero-3-phosphocholine; NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; NBD-cholesterol, 25-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)methylamino]-27-norcholesterol; DOPC, dioleoyl-*sn*-glycero-3-phosphocholine; MLV, multilamellar vesicle; SUV, small unilamellar vesicle; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulphonic acid); NBD-AHA, 6-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]hexanoic acid; NBD-MAP, 3-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)methylamino]propionitrile; PS, phosphatidylserine; PA, phosphatidic acid; PG, phosphatidylglycerol.

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Introduction

Ever since its introduction as a highly fluorescent moiety [1], the NBD group has been increasingly used to monitor the properties of biological membranes. Various phospholipids have been synthesized with the NBD group attached to the polar head group or to the nonpolar fatty acyl chain of the lipid [2–5]. These lipids are used as fluorescent analogues of native lipids in biological membranes to study a variety of processes, including spontaneous transfer of lipids between vesicles [6–10], membrane fusion by resonance energy transfer and related methods [11–17], lateral mobility of lipids by fluorescence recovery after photobleaching [18] and intracellular lipid transport in living cells [19,20]. Molecules that are structurally similar to these NBD lipids have also been used as photoaffinity-labeling phospholipids [21].

One of the concerns in many studies using NBD-labeled lipids is how well they mimic the behavior of native lipids. Recently, we have determined the membrane penetration depth of the NBD group for various NBD-labeled lipids in model membranes by means of a novel fluorescence quenching technique [22]. This 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. Our results indicated that the NBD group in head group-labeled NBD-PE is at the polar region of the membrane and that an NBD label on the 'tail' of NBD-cholesterol is deeply buried. In addition, NBD labels placed at the end of fatty acyl chains of phosphatidylcholines (6-NBD-PC and 12-NBD-PC) also appear to be near the polar region. Presumably, the polarity of the NBD group results in 'looping' back to the surface when NBD groups are attached to acyl chains. However, as the location of NBD groups is an important question, and the fluorescence quenching method is new, we strongly felt that it was necessary to confirm our tentative conclusions by independent methods. This report describes further studies on the spectroscopic and ionization properties of these lipids as determined by fluorescence, absorbance and electrophoretic mobility measurements. These properties confirm that the NBD groups on NBD-PE and NBD-PCs are at the polar region while that on NBD-cholesterol is deeply buried in the membrane. Another concern about NBD-labeled lipids is the charge of the NBD group. This report also shows that the NBD group is neutral at physiological pH.

Materials and Methods

Materials. The NBD-labeled phospholipids and DOPC were purchased from Avanti Polar Lipids (Birmingham, AL). NBD-cholesterol, NBD-AHA and NBD-MAP were from Molecular Probes (Eugene, OR). $CoCl_2 \cdot 6H_2O$ was obtained from J.T. Baker (Phillipsburg, NJ). All other chemicals used were reagent grade. NBD-labeled lipids and DOPC were checked for purity by TLC on Adsorbsil Plus silica gel plates (Alltech Associates, Deerfield, IL) in chloroform/methanol/water (65 : 35 : 5, v/v), except for NBD-cholesterol, for which chloroform/methanol/water (65 : 35 : 1, v/v) was used, as described previously [22]. DOPC gave one spot with a phosphate-sensitive spray and subsequent charring [23]. NBD-labeled lipids were virtually pure when detected by their color or fluorescence. Deacylated NBD-PE was prepared by mild alkaline hydrolysis of NBD-PE [24]. The NBD-labeled polar group was purified by TLC in methylene chloride/methanol/water (70 : 27 : 3, v/v). The major product (at least 80–90%) had an R_F of 0.2–0.3. It was extracted with methanol. Its concentration was estimated using $\varepsilon = 27\,000\ M^{-1} \cdot cm^{-1}$ at 470 nm [25] and its phosphate content was determined [26,27]. A phosphate to NBD ratio of approx. 1 was found, as expected.

Methods. Two kinds of vesicles were used. Multilamellar vesicles (MLV) of mixtures of DOPC and NBD-labeled lipids were prepared similarly to the methods described previously [22,28]. In general, lipids in chloroform were mixed, a few additional drops of chloroform were added, mixed well, and then the samples were dried under a mild flow of nitrogen while warming gently (35–40 °C). After further drying under a high vacuum for 30 min, 1.5 or 3 ml of the appropriate buffer were added to each sample. The samples were then vortexted for 50 s to disperse the lipid mixture. Small unilamellar vesicles (SUV) of mixtures of DOPC and NBD-labeled lipids were prepared by the ethanol injection method [29,30]. In

these samples lipids dissolved in ethanol were diluted 100-fold into the appropriate buffer. Further details of sample preparation for each experiment are given in the respective figure legends.

Fluorescence was measured at room temperature with a Spex 212 Fluorolog spectrofluorometer operating in ratio mode and using 1 cm pathlength quartz cuvettes. Excitation and emission slits with a nominal bandpass of 2.25 or 4.5 nm were used in all experiments. The excitation wavelength was 469 nm for all fluorophores containing the NBD group. Emission was collected at 531 nm, unless otherwise noted. For spectra, emission was scanned from 480 to 560 nm. Background intensity in samples in which NBD-labeled lipids were omitted was less than 1% of sample values. Inner filter effects were negligible, except in the quenching experiments using Co^{2+}. In these experiments fluorescence was corrected for the inner filter effect, as described previously [31,32].

Absorbance in 1 cm cuvettes was measured using Cary 17 or Gilford 250 spectrophotometers. For monitoring the rate of degradation of the NBD group at high pH (pH 13.0) by the time-dependent change in absorbance, samples were prepared as in the absorption spectra experiments (see Fig. 2) and the absorbance measured at 465 nm as a function of time. Also, a control sample was prepared in the same way at pH 7.3 with an identical lipid composition. The absorbance of this control sample at 465 nm was recorded at the beginning and the end of the experiment.

Electrophoretic mobility measurements were done with MLVs of DOPC containing 5 mol% NBD-labeled lipid. Details of these measurements have been already described [33,34]. The lipid mixtures in chloroform were vacuum dried in a glass round-bottom flask and MLVs were formed by adding buffer and mixing well. Electrophoretic mobilities were measured with Rank Brothers Mark I instrument (Bottisham, Cambridge, U.K.). Each measurement was done on 10 vesicles. These measurements were repeated in duplicate samples and the average of the two has been plotted in Fig. 3. Zeta potential was calculated from electrophoretic mobility u, according to the Helmholtz-Smoluchowski equation [34,35]:

$$\zeta = u\eta/(\varepsilon_r\varepsilon_0)$$

where ε_r is the dielectric constant of the aqueous solution, ε_0 is the permittivity of free space and η is the viscosity of the aqueous solution.

Results

The NBD-labeled lipids chosen in this study were NBD-PE, which has an NBD group attached to the ethanolamine of the head group, 6- and 12-NBD-PC, which have an NBD group attached to the final carbon of a 6- or 12-carbon long acyl chain at the 2-position, and NBD-cholesterol which has a methyl-NBD attached at the end of the flexible hydrocarbon chain.

The fluorescence properties of the NBD group can be used to follow its ionization [25]. Fig. 1 shows the effect of pH upon the fluorescence of NBD-labeled lipids. There is a plateau up to pH 11. At low pH the plateau continues down to at least pH 3 in all cases (not shown). Above pH 11, fluorescence intensity decreases by more than 20-fold for the NBD-labeled phospholipids. About half of this decrease is due to a decrease in absorbance at 469 nm (see Fig. 2) and the rest is due to a decrease in quantum yield. NBD-cholesterol shows only a small decrease in intensity at higher pH, probably due to a decrease in absorbance (see below) *. If the drop in NBD-labeled phospholipid fluorescence corresponds to ionization it should be reversible. This was tested by addition of acetic acid to SUV permitting fast equilibration of internal and external pH. Fig. 1C shows that the loss of NBD-labeled phospholipid fluorescence is indeed reversible. The apparent pK_a values ** derived from Fig. 1 are 11.5–11.8 for NBD-PE, 11.1–11.5 for 12-NBD-PC and 11.3 for 6-NBD-PC. The small variation in apparent pK_a is probably due to a weak dependence of pK_a on ionic strength and mol% NBD-labeled lipid in the membrane. If there is a difference in pK_a between NBD-labeled lipids in MLV (Fig. 1A and B) and those in SUV (Fig. 1C) it is also very small.

* The increased experimental error in the NBD-cholesterol curve is at least partly due to its relatively weak fluorescence (about 5-fold less than NBD-PE under experimental conditions). At neutral pH NBD-PE and NBD-PCs have similar intensities under the conditions used (NBD-PE is about 20% more fluorescent).

** All pK_a values reported are apparent pK_a.

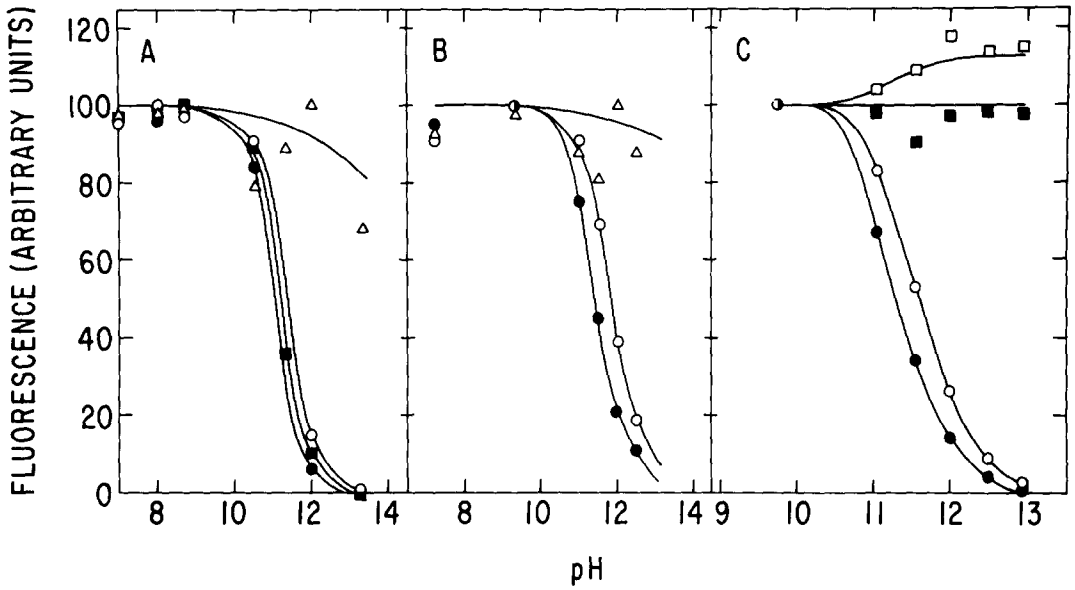

Fig. 1. Effect of pH on the fluorescence of the NBD group. (A) pH dependence of NBD fluorescence in MLVs composed of 99 mol% DOPC and 1 mol%: NBD-PE (○), 6-NBD-PC (■), 12-NBD-PC (●) or NBD-cholesterol (△). Samples contained 160 nmol total lipid in 1.5 ml of buffer. The buffers were: 10 mM phosphate/150 mM NaCl (pH 7.0), 10 mM Tris-chloride/150 mM NaCl (pH 8.0), 10 mM Tris-chloride/150 mM NaCl (pH 8.7), 10 mM Tris-chloride/150 mM NaCl (pH 10.5), 3.1 mM NaOH/150 mM NaCl (pH 11.3), 10 mM NaOH/150 mM NaCl (pH 12.0), or 200 mM NaOH/150 mM NaCl (pH 13.3). (B) Same experiment as in (A) except at lower ionic strength and with MLVs of 95 mol% DOPC and 5 mol%: NBD-PE (○), 12-NBD-PC (●) or NBD-cholesterol (△). Samples contained 160 nmol total lipid. The buffers were: 1 mM Pipes/10 mM NaCl (pH 7.2), 1 mM Tris-chloride/10 mM NaCl (pH 9.3), 1 mM KOH/10 mM NaCl (pH 11.0), 3.2 mM KOH/7.8 mM NaCl (pH 11.5), 10 mM KOH/1 mM NaCl (pH 12.0), and 32 mM KOH (pH 12.5). (C) NBD fluorescence reversibility upon acidification of high-pH samples. The fluorescence of SUVs of 95 mol% DOPC and 5 mol%: NBD-PE (○) or 12-NBD-PC (●) was measured. These samples contained 160 nmol total lipid in 3 ml of buffer. The buffers were: 10 mM Tris-chloride (pH 9.7), 1 mM KOH (pH 11.1), 3.2 mM KOH (11.5), 10 mM KOH (pH 12.0), 32 mM KOH (pH 12.5), and 100 mM KOH (pH 13.0). The pH was then decreased by adding 6, 19.2, 60, 192 and 600 μl of 1 M acetic acid, respectively, and fluorescence of the samples containing NBD-PE (□), or 12-NBD-PC (■) was remeasured immediately. After pH reversal all the samples had a pH of 5.2 ± 0.2. Values shown are corrected for dilution upon acidification.

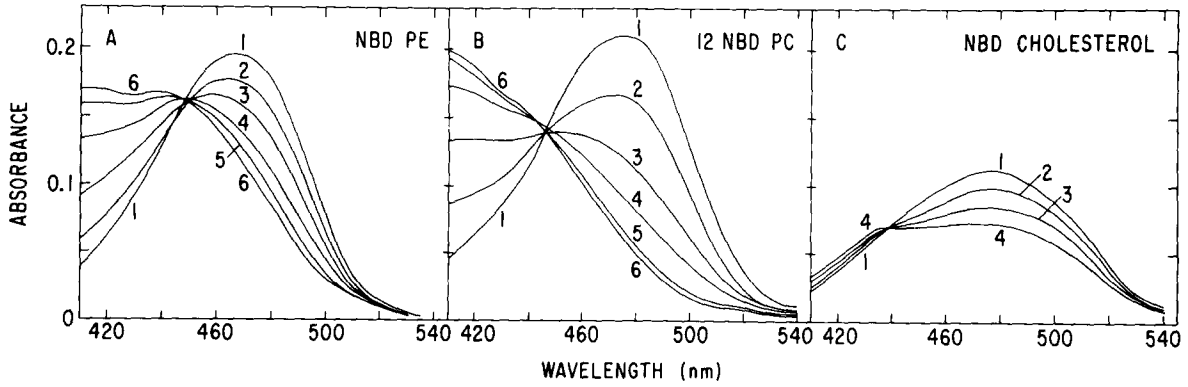

Fig. 2. Effect of pH on the absorption spectra of (A) NBD-PE and (B) 12-NBD-PC at: (1) pH 9.7, (2) pH 11.1, (3) pH 11.5, (4) pH 12.0, (5) pH 12.5 and (6) pH 13.0 and (C) NBD-cholesterol at (1) pH 9.7, (2) pH 11.1, (3) pH 12.0, and (4) pH 13.0. Samples contained SUVs of DOPC and 5 mol% NBD-labeled lipid at different pH made by rapid 100-fold dilution of an ethanolic solution containing DOPC (380 nmol) and NBD-labeled lipid (20 nmol) with 3 ml of buffer. The buffers were the same as in Fig. 1C. Background absorbance of vesicles without NBD-labeled lipid was subtracted. After recording the absorption spectra, pH of each sample was decreased by addition of 1 M acetic acid as described in Fig. 1 and spectra were recorded again. For NBD-PE and 12-NBD-PC this resulted in spectra superimposable with that at pH 9.7, when corrected for dilution (not shown).

Since the apparent ionization process detected by a fluorescence change may only reflect the behavior of the NBD group in the excited state, absorbance was also monitored as a function of pH to detect ionization. Fig. 2 shows the effect of pH on the absorption spectra of the NBD group. As the pH is increased from 9.7 to 13.0 there is a blue shift in the wavelength of absorbance maximum of NBD-PE and 12-NBD-PC with an approximate isosbestic point around 445 nm. There is also a small change in NBD-cholesterol absorbance at high pH. The reversibility of these changes was also determined. For NBD-PC and NBD-PE, the absorbance changes are totally reversible, but they are irreversible for NBD-cholesterol, suggesting the changes are due to chemical degradation in the latter case. These conclusions are reinforced by the kinetics of the absorbance changes at high pH. The change in absorbance at pH 13.0 is instantaneous for NBD-PE and NBD-PC, but gradual and somewhat variable ($t_{1/2}$ about 7 min) for NBD-cholesterol. For the NBD-labeled phospholipids the pK_a values derived from the changes in absorbance are close to 11.5, in agreement with the fluorescence results.

In control experiments the pK_a of water-soluble NBD groups was determined for approx. 1 μM solutions containing 150 mM NaCl. NBD-AHA was used as a water-soluble example of NBD group linked to an acyl chain. It had a pK_a of 10–10.2 by fluorescence, in agreement with previous results [25]. Water-soluble deacylated NBD-PE had a pK_a of 9.7–9.8. This similarity between a phosphorylated and non-phosphorylated NBD group may not be surprising as the amino pK_a of phosphorylated aliphatic amines is very similar to that of the corresponding ordinary aliphatic amines [36].

The preceding results show that there is an apparent ionization involving the non-methylated NBD groups near pH 11.5. However, they do not directly reveal the charge on the NBD group. To determine directly the charge of the NBD groups in membranes, the zeta potential, ζ, which is the potential at the hydrodynamic plane of shear, was measured. This was determined from the electrophoretic mobility of vesicles by applying the Helmholtz-Smoluchowski equation [34], as described under experimental procedures. The zeta

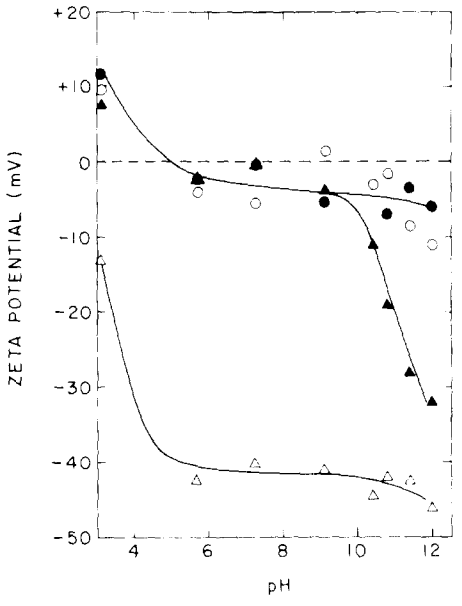

Fig. 3. Zeta potentials of MLVs formed from mixtures of 5 mol% NBD-PE (△), 12-NBD-PC (▲), NBD-cholesterol (○) in DOPC and pure DOPC (●) at various pH. Each sample contains 1.06 μmol DOPC and 53 nmol NBD-labeled lipid in 6–7 ml buffer. The buffers used are the same as in Fig. 1B, except that an additional buffer (0.32 mM NaOH/10.7 mM NaCl (pH 10.6)) was used.

potentials thus measured for MLVs containing 5 mol% 12-NBD-PC, NBD-PE or NBD-cholesterol and 95 mol% DOPC are plotted as a function of pH in Fig. 3. MLVs containing only DOPC were used as a control. It is clear from the figure that at neutral pH 12-NBD-PC, NBD-cholesterol and pure DOPC do not have any significant charge, while NBD-PE is negatively charged. For 5 mol% negative lipid in 0.01 M salt a zeta potential of -36 mV corresponds to 1 unit of charge per lipid molecule as judged by comparison to the results obtained with PS and PC [37]. Based on this value, the calculated charge per NBD-PE molecule at neutral pH is then -1. Therefore, to be consistent with the charge of NBD-PE and 12-NBD-PC the NBD group must be neutral at neutral pH. Fig. 3 also shows the effect of pH upon charge. For 12-NBD-PC the appearance of a negative charge at high pH can be observed directly via its effect on electrophoretic mobility. The pH at which a negative charge appears corresponds to the NBD pK_a, as determined by spectroscopic parameters. As expected, MLVs of pure DOPC and MLVs

containing NBD-cholesterol show only a small deviation from neutrality at increased pH. Curiously, NBD-PE shows no change in zeta potential at high pH, despite its pK_a being in this region. This is probably due to an exchange of a monovalent cation for the lost hydrogen ion (see Discussion). It should also be noted that there is the beginning of a change in zeta potential of MLVs at low pH, probably due to protonation of the phosphate groups on the lipids.

The similarity of NBD-PC and NBD-PE pK_a values suggest that their NBD groups are at a similar membrane depth (see Discussion). To investigate further the location of NBD groups in th emembrane the λ_{max} of fluorescence emission in model membranes was compared to that in organic

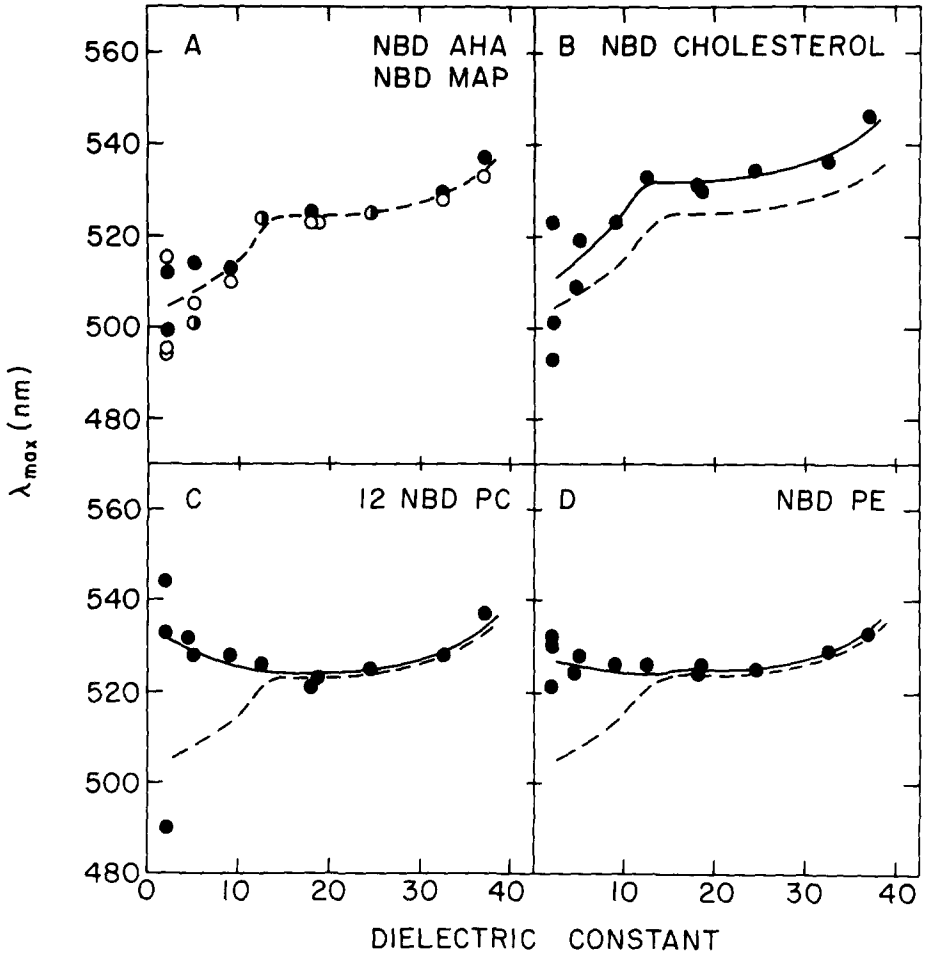

Fig. 4. Dependence of fluorescence emission maximum (λ_{max}) on solvent polarity for (A) NBD-AHA (●) and NBD-MAP (○), (B) NBD-cholesterol, (C) 12-NBD-PC and (D) NBD-PE. For convenience of comparison the 'standard' curve for the model compounds in (A) has been drawn in each case (– – –). Samples were prepared by dilution of a concentrated stock solution of NBD-labeled lipid or model compounds in ethanol with different solvents. The final NBD concentration was 1–2 μM and samples contained 0.1–0.2% ethanol (v/v). The solvents were (numbers in parenthesis indicate dielectric constants): *n*-hexane (1.89), 1,4-dioxane (2.21), carbon tetrachloride (2.24), diethyl ether (4.34), chloroform (4.81), methylene chloride (9.08), pyridine (12.3), 1-butanol (17.8), 2-propanol (18.3), ethyl alcohol (24.3), methyl alcohol (32.63) and ethylene glycol (37). The excitation wavelength was 469 nm. The fluorescence emission maxima for 1 mol% NBD-PE, 12-NBD-PC or NBD-cholesterol in MLVs with 99 mol% DOPC are 531, 535 and 522 nm, respectively.

solvents. In general, a blue shift in wavelength maximum is observed as a fluorophore is placed in increasingly nonpolar solvents. Therefore, λ_{max} should give a rough indication of the polarity of the environment around the NBD group. However, the behavior of the NBD group of NBD-PE in nonpolar solvents was reported to be anomalous in this regard [3], which was attributed to the formation of lipid aggregates in such solvents (see Discussion). We investigated this in detail by comparing the fluorescence of NBD model compounds in organic solvents to that of 12-NBD-PC, NBD-PE and NBD-cholesterol. The model compounds chosen were NBD-MAP, which has a methyl-NBD group like NBD-cholesterol, and NBD-AHA, which has a free NBD amino group like the NBD phospholipids. Fig. 4 shows the fluorescence λ_{max} for NBD lipids and model compounds in organic solvents of varying polarity plotted as a function of the dielectric constant of the medium. The 'scatter' in each curve suggests

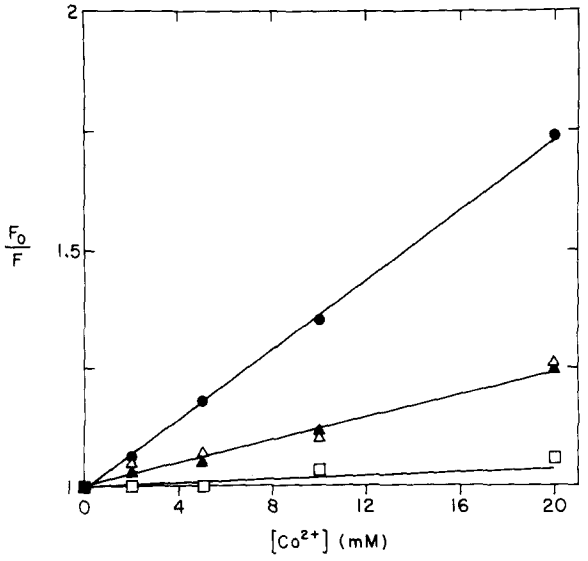

Fig. 5. Stern-Volmer plot of quenching of NBD fluorescence in SUVs by Co^{2+}. F_0 is the fluorescence in the absence of quencher and F is the fluorescence in presence of quencher. SUVs containing 1 mol% NBD-PE (●), 6-NBD-PC (△), 12-NBD-PC (▲) or NBD-cholesterol (□) were prepared by a rapid 115-fold dilution of an ethanolic solution containing DOPC (188 nmol) and NBD-labeled lipid (1.9 nmol) with 1.5 ml of 10 mM Tris-chloride/150 mM NaCl (pH 7.2) buffer containing varying amounts (0–20 mM) of Co^{2+}.

that λ_{max} is a function of more than just dielectric constant. Nevertheless, the behavior of the model compounds does show a progressive blue shift as dielectric constant decreases. In very nonpolar solvents anomalous behavior of fluorescence λ_{max} is observed for NBD-PE and 12-NBD-PC but not for NBD-cholesterol. Therefore, the λ_{max} curves for NBD-PE and 12-NBD-PC in organic solvents cannot be compared directly to λ_{max} values in model membranes to calibrate location. However, the λ_{max} curve for NBD model compounds can be used as a 'standard' curve in these cases. The fluorescence emission maxima for NBD-PE, 12-NBD-PC and NBD-cholesterol in MLVs of 1 mol% NBD-lipid and 99 mol% DOPC are 531, 535 and 522 nm, respectively. Comparison of λ_{max} values then shows that in model membranes NBD-PE and 12-NBD-PC are in a polar region of the membrane. NBD-cholesterol is in a nonpolar region, as judged by comparison of its behavior in membranes with either the model compounds or its own behavior in organic solvents. The results obtained by plotting λ_{max} against Kosower's Z-number, which is an empirical solvent polarity parameters [38,39], are very similar.

To examine further the location of NBD groups in membranes, fluorescence quenching experiments were performed with the aqueous quencher Co^{2+}. The paramagnetic cobaltous ion is water-soluble and is an efficient quencher of NBD fluorescence [40,41]. We measured the quenching of NBD fluorescence by Co^{2+} in SUVs made of 1 mol% NBD lipids and 99 mol% DOPC. The results of these experiments are plotted in Fig. 5 as a Stern-Volmer plot. The slope of such a plot (K_{SV}) is related to the degree of exposure of the NBD group to the aqueous phase. Apparent K_{SV} values are 36 M^{-1} for NBD-PE, 10 M^{-1} for 6-NBD-PC and 12-NBD-PC, and 2.5 M^{-1} for NBD-cholesterol. The higher the K_{SV}, the greater the degree of exposure, provided that there are no differences in fluorescence lifetime or differential electrostatic effects (see Discussion). On this basis, it is apparent that the NBD group in NBD-PE is the most exposed (most shallow) and that in NBD-cholesterol is the least exposed (most deep). This result is also in agreement with our reported depths for NBD groups. The NBD groups in 6- and 12-NBD-PC are at about the same depth and

fall between the two extremes, also in agreement with our previous quenching study, which indicated that they are at nearly identical depths, and slightly deeper than NBD-PE [22].

Discussion

The spectroscopic and ionization properties of NBD-labeled lipids have been the focus of this report. The ionization properties of the NBD group in aqueous solution were previously described by Meyers et al. [25] for NBD-AHA. Using NMR and fluorescence spectroscopy they observed that in solution the NBD group is neutral at pH 7, and apparently undergoes deprotonation of its amino group at higher pH. Others have reported that the change at high pH is due to hydroxyl addition to the NBD ring, forming a 'Meisenheimer adduct' [42]. The latter interpretation is supported by the observation that NBD-chloride, which does not have any amino group, exhibits a pK_a near pH 9.8 [43]. However, it should be emphasized that the equilibria involved in reversible hydroxylation and deprotonation are operationally indistinguishable, as shown by the equations below.

$$K_{\text{deprotonation}} = ([\text{NBD}^-][\text{H}^+])/[\text{NBD}]$$

$$K_{\text{hydroxylation}} = [\text{NBD}^-]/([\text{NBD}][\text{OH}^-])$$

$$= K_{\text{deprotonation}}/K_w, \text{ where } K_w = [\text{H}^+][\text{OH}^-]$$

In these equilibria NBD^- represents the anionic form of NBD whatever its chemical structure. An apparent K_a will exist, and will be equivalent to $K_{\text{deprotonation}}$ or $(K_{\text{hydroxylation}})(K_w)$, depending on which reaction occurs at high pH. Therefore, the arguments made below are independent of the nature of the change in NBD structure at high pH.

In this report we find the behavior of the NBD group on NBD-labeled lipids is quite similar to that of aqueous solutions of soluble NBD compounds, except that the pK_a for the phospholipids is about 1.5 pH units higher than in aqueous solution. Since a shift of about 2–3 pH units has been observed for the carboxyl group of free fatty acids incorporated into model membranes [44,45], and it is believed to be close to the surface, the magnitude of the pK_a shift also suggests that the NBD groups on NBD-labeled phospholipids are also near the surface. That there is a pK_a shift relative to that in water is not surprising, as the polar region is probably an environment more like a polar organic solvent than like water especially down at the level of the glycerol moiety, where quenching results indicate the NBD group residues [22].

Additional information can be derived from the similarity of the pK_a values for NBD-PE and NBD-PC. In NBD-PE, the NBD group is attached near the charged phosphate group, and so it must be in a relatively polar location. Therefore, its hydroxylation (or deprotonation), which results in a charged NBD group, should not be particularly unfavorable, whereas if the NBD group attached to PC were deeply buried in the hydrophobic acyl chain region, then one would expect its hydroxylation (or deprotonation) would be much more unfavorable, resulting in a much higher pK_a. However, the pK_a values of NBD-PC and NBD-PE are similar, even after correcting both for surface potential [46,47], which gives an intrinsic pK_a of about 11.0 for both NBD-PE and NBD-PC (calculation not shown), and for the small difference in intrinsic pK_a of the water-soluble analogs for these two types of NBD group. Therefore, it seems unlikely the NBD on NBD-PC is deeply buried. More rigorously, if we assume charged groups will not remain deeply buried in a membrane, equilibria describing the hydroxylation will then be:

$$\text{NBD}^0(\text{buried}) \overset{K_1}{\rightleftharpoons} \text{NBD}^0(\text{surface}) + \text{OH}^- \overset{K_2}{\rightleftharpoons} \text{NBD}^-(\text{surface})$$

The ionization of NBD group on PE is given by pK_2 if this group is always near the surface. That of a buried NBD group would be given by pK_2'' where $K_2'' = K_1K_2$ with $K_1 \leqslant 0.1$, predicting a large apparent pK_a difference. Again, no such difference between NBD-PE and NBD-PC ionization is observed, so the NBD group on PC is unlikely to be buried deeply. Identical arguments apply if deprotonation, rather than hydroxylation, occurs at high pH.

The electrophoretic mobility studies compliment the spectroscopic titrations. The change in absorbance and fluorescence properties of 12-NBD-PC correspond to the electrophoretically de-

tected change in vesicle charge. The similarity of 12-NBD-PC and NBD-PE spectroscopic changes indicates that the same ionization occurs for NBD-PE. However, the zeta potential of NBD-PE vesicles did not change around pH 11.5. A possible reason for this difference arises from the fact that while 12-NBD-PC goes from a neutral to anionic state, NBD-PE already has a negative charge below its ionization pH. Previous studies have shown that the zeta potentials of pure negative lipids (e.g., PS and PA) do not show a change in zeta potential upon a second ionization at high pH, probably due to the masking effects of surface exchange of Na^+ or K^+ for H^+ [48] (McLaughlin, S., personal communication). However, we cannot yet rule out the involvement of more complex changes.

The fluorescence emission properties of the NBD-labeled lipids also suggest a surface location for NBD groups attached to PC and PE. In addition, they show that comparison of NBD emission λ_{max} values in vesicles and organic solvents requires care, because of the tendency of the phospholipids to form inverted micelles. It is well established that hydrated phospholipids can form a reversed (inverted) micellar structure with a polar interior and a nonpolar outer surface in nonpolar solvents [49,55]. The polar interior can solubilize large amounts of water, forming what has been called a trapped 'water pool', and it has been observed that the polar core and trapped water can influence fluorescence λ_{max}, making a fluorophore residing in this region to appear to be in a polar environment, despite the fact that bulk solvent in nonpolar [50,51]. Furthermore, the effective polarities experienced by fluorophores in such systems is a sensitive function of the water concentration in the 'water pool' [49]. Previous investigators observed the anomalous λ_{max} behavior for NBD-PE and ascribed it to this type of phenomenon [3] *. Our comparison of NBD-labeled lipid and model compounds supports the occurrence of such inverted micelles both for NBD-PE and 12-NBD-PC, and also show that the difference between the behavior of model compounds and NBD-labeled phospholipids is significant only at low dielectric constants (below 15), suggesting that the formation of inverted micelles is significant only in the more nonpolar solvents. Clearly, the model compounds give a much better indication of the true effect of polarity on NBD fluorescence in these cases.

The Co^{2+} quenching is the final data that reports on the locations of the NBD groups. However, K_{SV} depends on fluorescence lifetime as well as quencher-fluorophore collision rate. If there is a significant difference in lifetimes between different NBD-labeled lipids, then interpretation of Co^{2+} quenching data becomes complicated. We are not aware of any published lifetime values for these lipids. However, preliminary experiments have shown that the lifetimes of 6-NBD-PC, 12-NBD-PC and NBD-PE in vesicles containing 4% NBD-labeled lipid are very similar (Homan, R. and Eisenberg, M., personal communication). Quenching may also depend on the degree of Co^{2+} binding to the NBD-labeled lipid. Therefore, another source of some concern is the electrostatic interaction between Co^{2+} and NBD-labeled lipids, since NBD-PE is negatively charged and the others are neutral at the experimental pH. In addition, the degree of quenching could be affected by differences between NBD fluorescence spectra for the various lipids. For these reasons, precise interpretation of aqueous quenching results is not possible.

Nevertheless, it is clear that both the spectroscopic and ionization properties of the NBD group indicate that the NBD group of NBD-PCs and NBD-PE is at the polar region of the membrane bilayer, and that it is deeply buried in the case of NBD-cholesterol. These results are in good agreement with our direct fluorescence quencing measurements of NBD group depth [22] and, therefore, they tend to confirm the quenching results and the validity of the quenching approach.

An important question raised by these results is why the NBD group attached to the end of a fatty acyl chain comes to the polar region of the bilayer. As we stated previously, one can only presume the polarity of the oxygen- and nitrogen-rich NBD group is responsible for this behavior [22]. In the case of NBD-cholesterol, either the rigidity of the sterol rings or the reduction of hydrophobicity due

* The λ_{max} values previously observed were slightly different in some solvents, presumably due to small differences in water content.

to the methyl group attached to NBD are possible reasons why it is deeper in the membrane. It is hard to predict whether other polar, covalently attached acyl chain probes would loop up in a manner similar to that observed with NBD-PCs. Spin-labels attached to free fatty acids can loop up to the surface, although not under all conditions [52,53]. Some photolabeling groups may also undergo 'looping' [54]. It appears that the possibility of 'looping' will have to be considered on a case by case basis.

Another important question concerning NBD-labeled lipids is whether they are acceptable analogues of natural lipids. We can make no blanket statement in this regard. These studies show that the charge on NBD-labeled lipids labeled in their acyl chains will be the same as the parent lipid under physiological conditions. On the other hand, the NBD group loops back to the surface in this case, giving a perturbed acyl chain conformation. To the degree that this property is a consequence of the polarity of the NBD group, it may be an unavoidable feature necessary to allow rapid incorporation of the probe into living cells. Furthermore, in many applications (such as fusion studies), the location of the NBD group should not always be critical. Therefore, use of NBD-labeled lipids is undoubtedly acceptable in many cases. Nevertheless, it is clear that care must be taken when using the NBD group as a probe, especially where acyl chain conformation is important.

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