# Synthesis and properties of a highly fluorescent derivative of phosphatidylethanolamine

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**Abstract** Properties of *N*-4-nitrobenzo-2-oxa-1,3-diazole-phosphatidylethanolamine (NBD-PE), prepared by alkylation of the free amino group of phosphatidyl-ethanolamine with 4-chloro-7-nitrobenzofurazan (NBD-Cl) are described. The alkylated product containing this fluorochromic group was purified by silicic acid chromatography and had a nitrogen/phosphorus ratio of 3.96 (4.0 theoretical). The NBD-PE was sparingly soluble in distilled water but freely soluble in organic solvents. Both the UV-visible absorption spectrum and the uncorrected fluorescence excitation spectrum of NBD-PE in absolute ethanol showed maxima at approximately 330 and 460 nm, while the fluorescence emission spectrum showed a single peak at 525 nm. Fluorescent intensity and emission maximum wavelength of NBD-PE are strongly dependent on the dielectric constant of the solvent. The fluorescent intensity of NBD-PE in absolute ethanol was directly proportional to its concentration from 1 ng/ml to approximately 3 $\mu$g/ml. This compound can be incorporated into phosphatidylcholine–dicetyl phosphate–cholesterol liposomes, rat brain synaptosomal particulate fraction, and human lymphocyte membranes. Incorporation of the derivative into rat liver mitochondrial membranes was facilitated by a postmitochondrial pH 5.1 supernatant fraction. These data indicate that NBD-PE might be a unique and effective probe for the phospholipid regions of membranes.

**Supplementary key words** fluorescent probes · phospholipids

Since the original observation by Newton (1), on the enhancement of fluorescence by 1-toluidinonaphthalene-8-sulfonate (1,8-TNS) introduced into bacterial membranes, fluorescent molecules have been extensively used to study membrane phenomena such as enzyme activity (2–6), transport (7), ligand binding (8–11), and anatomical distribution of various metabolites (12–14). Since most extrinsic probes are structurally unrelated to naturally occurring membrane constituents, the interpretation of data obtained on the interaction of such probes with biological membranes can often be difficult (15). The use of fluorescent derivatives of naturally occurring membrane components, such as dansylated phosphatidylethanolamine (16), or site-directed probes, such as *N*-dansylaziridine (17), does not completely resolve this problem, but we feel that such approaches may be more appropriate, particularly in the study of membrane phenomena. Consequently, we wish to describe the synthesis and certain properties of a highly fluorescent analog of phosphatidylethanolamine, and to offer evidence that suggests its utility as an extrinsic membrane probe.

## MATERIALS AND METHODS

### Chemicals and reagents

NBD-Cl was purchased from Aldrich Chemical Company, Milwaukee, WI. Egg phosphatidylethanolamine, phosphatidylcholine, dicetyl phosphate, and cholesterol were obtained from Avanti Biochemicals, Inc., Birmingham, AL. BRIJ-35 was purchased from Sigma Chemical Company, St. Louis, MO. All other reagents were obtained from commercial sources and were of the highest available purity.

### Synthesis of *N*-4-nitrobenzo-2-oxa-1,3-diazole-phosphatidylethanolamine (NBD-PE)

The phosphatidylethanolamine used in the synthesis of NBD-PE was transphosphatidylated with egg phosphatidylcholine according to the method of Yang, Freer, and Benson (18). Into a separatory funnel (Teflon stopcock) was added 1.0 g (1.21 mmol) of phosphatidylethanolamine in 10 ml of chloroform, 800 mg (4.0 mmol) of NBD-chloride in 70 ml of chloroform, and 0.28 ml (0.20 mmol) of triethylamine. After incubation at room temperature for 1 hr, the reaction was stopped by the addition of 40

Abbreviations: NBD, *N*-4-nitrobenzo-2-oxa-1,3-diazole; PE, phosphatidylethanolamine; DNS-PS, dansylated phosphatidylserine; BRIJ-35, 23-lauryl ether; ANS, 1-anilino-8-naphthalene sulfonic acid.

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ml of methanol and 20 ml of water. The bottom layer was separated and washed twice with the same volume of upper phase solvents (19). The solvent was then evaporated and the residue was taken up in chloroform–methanol–ammonia 80:20:2. Purification of the NBD-PE derivative was then effected in two stages, using two chromatographic columns, each having an internal diameter of 2.5 cm, and packed with 40 g of silica gel. The derivative was loaded onto the first column using a chloroform–methanol–ammonia 80:20:2 solvent, and was eluted with two column volumes of the loading solvent. The fractions were then pooled, the solvent was evaporated, and the residue was dissolved in a second solvent system consisting of benzene–methanol 49:1. This solution was then loaded onto the second column and the lipid was eluted with the benzene–methanol solvent mixture. A dark, unidentified band was first eluted with four column volumes of benzene–methanol 49:1. Then the NBD-PE was eluted with two column volumes of benzene–methanol 9:1. The fractions containing the purified NBD-PE were taken to dryness, and then dissolved in 100 ml of hexane containing 1% absolute ethanol. The solution was filtered, and the nitrogen and phosphorus content was determined. The reaction proceeded with approximately 70% yield, based on a mol wt of 905, and the nitrogen/phosphorus ratio (20, 21) was 3.96 (4.0 theoretical). The product exhibited a single spot on thin-layer chromatography using chloroform–methanol–water 65:25:4, chloroform–methanol–ammonia 75:25:4, or chloroform–methanol–acetic acid–water 25:15:4:2 and then visualizing by UV, molybdate spray, or charring. The solution containing the NBD-PE derivative was then stored at −20°C until used.

### Spectroscopic determinations

In all cases, aliquots of the derivative in hexane–ethanol solution were dried by evaporation under reduced pressure. The residue was then taken up in the various solvents.

Absorption measurements were made on a Beckman Acta C-III double beam, recording spectrophotometer. Fluorescence was measured using a Farrand Mark-I spectrofluorometer equipped with a 15OW xenon source, adjustable excitation and emission slits, and a W + W-1100 recorder. Experiments were done in a 3.0-ml quartz cuvette with a path length of 1.0 cm. Excitation spectra were obtained by setting the emission monochromator at 550 nm and automatically scanning the excitation spectrum from 300 to 500 nm. Emission spectra were recorded by maintaining the excitation monochromator at the excitation maximum and automatically scanning the emission spectrum. In cases where membranes or liposomes were used, the absorbance of the samples was less than 0.1 absorbance units to preclude the possibility of inner filter effects. Additional experimental details, such as solvent conditions, fluorochrome concentration, and pH, are given in the legends to figures and tables.

### Preparation of rat brain synaptosomal particulate fraction

Rat brain synaptosomal particulate fractions were prepared by a modification of the method of Cotman and Matthews (22). For a typical experiment, six adult, male Sprague-Dawley rats were killed by guillotine decapitation, and the brains were quickly removed and homogenized in 90 ml of cold 0.32 M sucrose in a glass homogenizer with a loose fitting motor-driven Teflon pestle. After centrifugation at 1,000 *g* for 10 min, the supernatant $S_0$ was decanted, and the pellet $P_0$, consisting of nuclei and cell debris, was rehomogenized in 45 ml of cold 0.32 M sucrose and centrifuged as before. The supernatant from this step was combined with $S_0$ and sedimented at 10,000 *g* for 20 min. The crude synaptosomal pellet $P_2$ obtained from this step was resuspended by homogenization in 45 ml of cold 0.32 M sucrose and layered onto discontinuous Ficoll–sucrose gradients consisting of 5 ml of 13% and 10 ml of 6% (w/v) Ficoll in 0.32 M sucrose. The gradients were placed in a SW 25 rotor and centrifuged at 65,000 *g* for 45 min in a Beckman L50 ultracentrifuge. The synaptosome band that sedimented at the 6%–13% Ficoll–sucrose interface was removed by aspiration, resuspended by homogenization in 40 ml of 0.32 M sucrose, and centrifuged at 20,000 *g* for 20 min. The synaptosomal pellets $P_3$ were resuspended in a small volume of 0.32 M sucrose and lysed by homogenization in 5 mM Tris-HCl, pH 8.0. After stirring on ice for 45 min, the lysis mixture was centrifuged at 11,500 *g* for 20 min, yielding a pellet $P_4$ enriched in plasma membranes. This pellet was resuspended in 0.32 M sucrose, stored at 4°C, and aliquots were taken for subsequent experiments. All experiments were performed on freshly prepared membrane fractions.

### Enzymatic assays on rat brain fractions

Cytochrome-*c* oxidase activity was measured as described by Duncan and Mackler (23), and lactase dehydrogenase activity was determined as described by Kornberg (24). $Na^+,K^+$-ATPase activity was determined by measuring the inorganic phosphate released from ATP during a 1-hr incubation at 37°C with ali-



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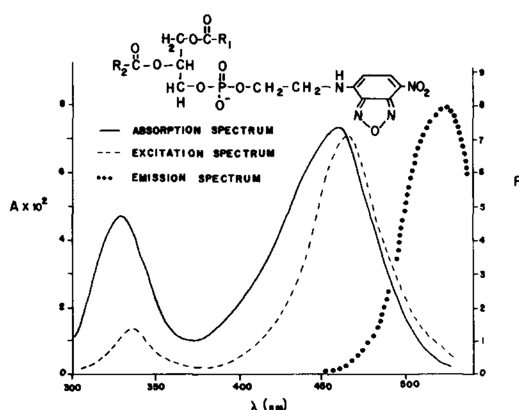

**Fig. 1.** Ultraviolet-visible absorption spectrum (——), uncorrected fluorescence excitation (– – –) and emission (· · ·) spectra of NBD-PE in absolute ethanol. The abcissa is given as wavelength in nm, while the left-hand ordinate is in absorbance units. The right-hand ordinate is shown as relative fluorescence. The concentration of NBD-PE was 1 μg/ml. Fluorescence spectra were recorded using 20-nm excitation and 5-nm emission slits.

quots of the fractions obtained during the preparation of rat brain synaptosomal particulate fraction. Reaction mixtures contained 2 mM $MgCl_2$, 120 mM NaCl, 15 mM KCl, 50 mM Tris-HCl, pH 7.4, 2 mM disodium ATP, and ±5 mM ouabain. Phosphate was determined according to the method of Bartlett (20). Protein was measured by the method of Lowry et al. (25) using lyophilized, fat-free, bovine serum albumin as standard.

### Preparation of rat liver mitochondria and pH 5.1 supernatant (phospholipid exchange protein)

A crude rat liver mitochondria and pH 5.1-supernatant fraction were prepared as described by Zilversmit (26).

*Lymphocytes.* Purified human lymphocytes were prepared as described by Boyum (27).

*Preparation of Liposomes.* Multilamellar liposomes were prepared according to the method of Bangham, Standish, and Watkins (28). Aliquots of phosphatidylcholine, dicetylphosphate, cholesterol, and NBD-PE, in chloroform were pipetted into a 50-ml round-bottom flask, such that the molar ratio of these components was 63:18:9:0.02, respectively. The chloroform was then evaporated under reduced pressure until the lipids covered the walls of the flask with a dry, uniform film. Ten ml of 0.145 M NaCl was then added, and the flask was gently agitated on a wrist action shaker until the opaque lipid film has been freed from the walls of the flask. This dispersion was equilibrated for 1 hr at room temperature before use. A control suspension, containing only 0.02 μmol of NBD-PE was prepared as above.

### Incorporation of NBD-PE in membrane fractions

Aliquots (5 ml) of suspensions of synaptosomal particulate fraction or lymphocytes were mixed with 5 ml of a solution of NBD-PE (0.5 μg/ml) in 0.1 M Tris-HCl, pH 7.4. After mixing, the membranes were sedimented at 10,000 *g* for 10 min, and the resulting pellet was resuspended by homogenization in 0.1 M Tris-HCl, pH 7.4, allowed to stir on ice for 30 min, and centrifuged as before. This process was repeated once more, and the final membrane pellet was resuspended by homogenization in 10 ml of 0.1 M Tris-HCl, pH 7.4. One hundred-μl aliquots of the final membrane suspensions were diluted to 3.0 ml in the fluorometer cuvette with Tris-HCl, pH 7.4, before determination of excitation and emission spectra.

In other experiments, 10-μl aliquots of synaptosomal particulate fraction or human lymphocyte suspensions were added to the fluorometer cuvette containing 3.0 ml of 0.2 μg/ml NBD-PE in 0.1 M Tris-HCl, pH 7.4. In all cases, uncorrected excitation and fluorescence emission spectra were recorded in the presence and absence of the membrane or lymphocyte suspensions.

### Assay of phospholipid transfer activity

The reaction mixture for measuring the incorporation of NBD-PE into rat liver mitochondria contained 2 mg of mitochondrial protein, 0.02 μmol of NBD-PE, 0.5 mmol of Tris-HCl, pH 7.4, and 0–200 μg of pH 5.1-supernatant protein. Samples were incubated at 37°C for 1 hr with gentle agitation. The reaction was terminated by placing samples in an ice bath for 10 min, followed by centrifugation at 15,000 *g* for 5 min. The supernatants were decanted, allowed to come to room temperature, and the absorbance (460 nm) and the relative fluorescence at 535 nm (excitation at 465 nm) were determined. Blanks containing only NBD-PE and exchange protein were run concomitantly to correct for possible interaction between NBD-PE and exchange protein. The percent exchange of NBD-PE was then calculated from the differences between the absorbances or relative fluorescence in the supernatant fractions in the presence and absence of exchange protein.

## RESULTS

**Fig. 1** shows the structure, UV-visible absorption spectrum, uncorrected fluorescence excitation spectrum, and emission spectrum of NBD-PE in absolute ethanol. The absorption and excitation spectra exhibited maxima around 340 and 450 nm, while



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the emission spectrum exhibited a single maximum at 525 nm. **Fig. 2** shows that the relative fluorescence of NBD-PE increased rapidly with increasing dielectric constant from approximately 2.0–6.0, reached a maximum at approximately 10.0, and slowly decreased thereafter. Conversely, the emission maximum wavelength (excitation at 465 nm) decreased (blue-shifts) with increasing dielectric constant until approximately 9.0, after which further increases in solvent dielectric constant increased the emission maximum (red-shift).

Fluorescence data on NBD-PE incorporated into phosphatidylcholine, dicetyl phosphate, and cholesterol liposomes are summarized in **Table 1.** When these components were included in the liposome preparation the relative fluorescence of NBD-PE was enhanced over control preparations containing only NBD-PE. The excitation (465 nm) and emission (535 nm) maximum wavelengths, however, were the same in both cases.

**Table 2** gives the distribution of lactic dehydrogenase cytochrome-*c* oxidase, and $Na^+$,$K^+$-ATPase specific activities in the fractions obtained during the isolation of the synaptosomal particulate fraction. The highest $Na^+$,$K^+$-ATPase specific activity observed in fraction $P_4$ is consistent with the results of other investigators, and indicates that this fraction is enriched in synaptic plasma membranes. Since we did not employ a density gradient step after lysing the intact synaptosomes, the relatively high specific activity of cytochrome-*c* oxidase in this fraction is probably the result of contamination by intrasynaptosomal mitochondria.

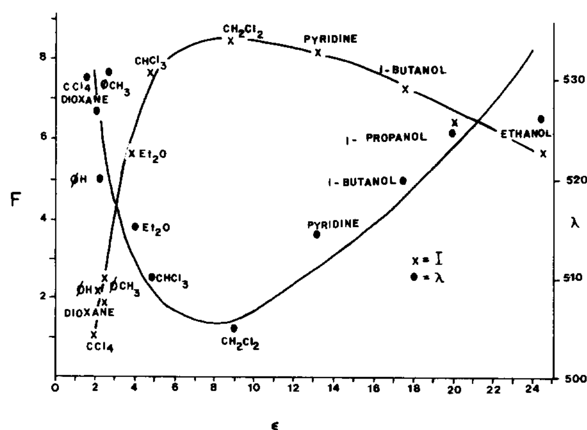


**Fig. 2.** Dependence of emission maximum wavelength (●) and relative fluorescence (X) of NBD-PE on solvent dielectric constant of the indicated solvents at 25°C. The left- and right-hand ordinates refer to relative fluorescence and emission maximum wavelength, respectively. Experiments were performed using 20-nm excitation and 5-nm emission slits. The concentration of NBD-PE was 1 μg/ml in all cases.

TABLE 1. Fluorescence data on NBD-PE incorporated into liposomes comprised of phosphatidylcholine, dicetyl phosphate, and cholesterol

| | Liposome Composition A. | Control B. |
|---|---|---|
| | 63 μmol phosphatidylcholine<br>18 μmol dicetyl phosphate<br>9 μmol cholesterol<br>0.02 μmol NBD-PE | 0.02 μmol NBD-PE |
| Excitation wavelength | 465 nm | 465 nm |
| Emission wavelength | 535 nm | 535 nm |
| Relative fluorescence (%) (535 nm) | 100[a] | 2 |

[a] Relative fluorescence arbitrarily set at 100%.

Experiments were performed as described in the Methods section. The solvent was 0.145 M NaCl. All experiments were done at room temperature. Excitation and emission slits were 10 and 2 nm respectively.

The uncorrected fluorescence emission spectrum of NBD-PE in the presence and absence of an aliquot of rat brain synaptosomal particulate fraction is shown in **Fig. 3*A*.** In this case, the solvent was 0.1 M Tris-HCl, pH 7.4. These data indicate that the relative fluorescence of NBD-PE in the presence of the membranes was increased approximately twofold over the fluorochrome in buffer, but that the emission maximum wavelength was the same in the presence and absence of the membranes. Similar results were obtained when NBD-PE was mixed with an aliquot of human lymphocytes under identical experimental conditions, as shown in Fig. 3b. These experiments were then repeated, using 0.145 M NaCl (unbuffered) as solvent with identical results, i.e., a twofold increase in relative fluorescence for NBD plus membranes or lymphocytes but no shift in emission maximum.

TABLE 2. Distribution of marker enzymes in rat brain fractions

| Fraction | LDH[a] | CYT-*c*. OX.[a] | $Na^+$, $K^+$-ATPase[b] |
|---|---|---|---|
| Homogenate | 0.098 | 0.014 | 0.432 |
| $P_1$ | 0.110 | [c] | 0.504 |
| $S_1$ | 0.221 | 0.028 | 0.954 |
| $P_2$ | 0.144 | 0.012 | 0.444 |
| $S_2$ | 0.398 | 0.003 | 0.078 |
| $P_3$ | 0.055 | 0.012 | 1.44 |
| Mitochondria[d] | 0.117 | 0.162 | 0.216 |
| $P_4$ | 0.066 | 0.125 | 2.84 |
| $S_4$ | 0.872 | 0.005 | 1.63 |

[a] μmol substrate utilized $min^{-1}$ $mg^{-1}$.
[b] μmol $P_i$ formed 60 $min^{-1}$ $mg^{-1}$.
[c] Undetectable.
[d] Mitochondrial pellet from the Ficoll–sucrose density gradient.
Experiments were performed as described in the Methods section, and all results represent the average of two separate determinations.



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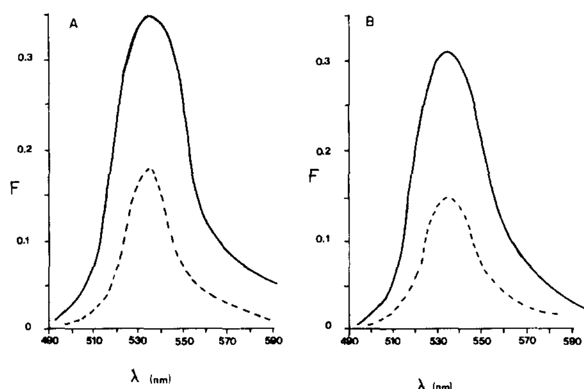


**Fig. 3.** *A*, Uncorrected fluorescence emission spectra (excitation 465 nm) of NBD-PE in the presence (——) and absence (– – –) of an aliquot of synaptosomal particulate fraction containing 0.1 mg protein/ml. The abcissa is expressed as wavelength in nanometers, while the ordinate is the relative fluorescence in arbitrary units. Excitation and emission slits were 10 and 2 nm, respectively. The concentration of NBD-PE was 1 $\mu$g/ml, and the solvent was 0.1 M Tris-HCl, pH 7.4. *B*, Uncorrected fluorescence emission spectrum (excitation 465 nm) of NBD-PE in the presence (——) and absence (– – –) of an aliquot of human lymphocytes containing approximately $6 \times 10^5$ cells/ml. All other conditions were identical with those of Fig. 3*A*.

Since preliminary experiments suggested that the presence of a small amount of nonionic detergent could effect enhancement of fluorescence and a shift in the emission maximum of NBD-PE in Tris-HCl, pH 7.4, the above experiments were repeated, including 0.01% BRIJ-35 in all steps. The results are shown in **Fig. 4*A*** and ***B***. The data indicate that when 0.01% BRIJ-35 was included in the solvent, the relative fluorescence of NBD-PE in the presence of synaptosomal particulate fraction and human lymphocytes was increased approximately 20-fold over NBD-PE in detergent plus buffer. The emission maximum of NBD-PE in the presence of synaptic plasma membranes and 0.01% BRIJ-35 was shifted from 535 to 525 nm, while the emission maximum in the presence of human lymphocytes showed a somewhat larger shift, from 535 to 520 nm.

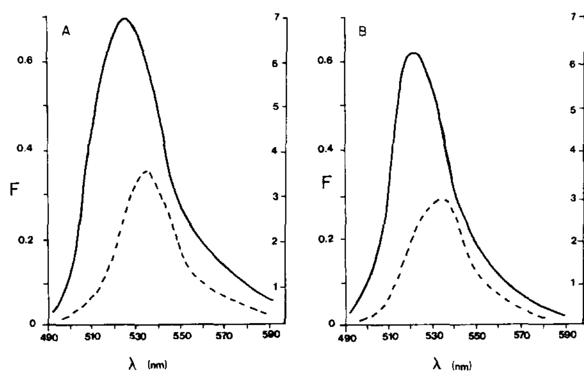


**Fig. 4.** *A*, Effects of 0.01% BRIJ-35 on the uncorrected fluorescence emission spectrum (excitation 465 nm) of NBD-PE in the presence (——) and absence (– – –) of an aliquot of synaptic plasma membrane fraction containing 0.1 mg protein/ml. Conditions were the same as Fig. 3*A* except that the solvent was 0.1 M Tris-HCl, pH 7.4, containing 0.01% (v/v) BRIJ-35. The left- and right-hand ordinates refer to the relative fluorescence of NBD-PE in the absence and presence of synaptic plasma membranes, respectively. *B*, Uncorrected fluorescence emission spectrum (excitation 465 nm) of NBD-PE in the presence (——) and absence (– – –) of an aliquot of human lymphocytes. Conditions were the same as Fig. 4*A*.

Very little, if any of the relative fluorescence of the synaptosomal particulate lymphocyte pellet after mixing with NBD-PE was lost in the two subsequent washings. This observation holds true in the presence and absence of 0.01% BRIJ-35. These data are summarized in **Table 3.**

NBD-PE will spontaneously incorporate into rat liver mitochondrial membranes in a manner analogous to lymphocytes or synaptosomal membranes, with an increase in relative fluorescence but no shift in emission maximum wavelength. However, incorporation of the molecule into rat liver mitochondrial membranes is markedly stimulated by crude phospholipid exchange protein, as indicated in **Fig. 5.** Experiments were carried out as described in the Methods section. The data indicate that in the absence of the pH 5.1-supernatant fraction, very little NBD-PE was incorporated in the mitochondrial fraction. However, increasing amounts of the exchange protein stimulated incorporation of NBD-PE up to approximately 13% over control.

## DISCUSSION

The results presented in this study have indicated that NBD-PE might be used as a fluorescent mem-

TABLE 3. Retention of NBD-PE by synaptosomal particulate fractions

| Fraction | Relative Fluorescent Intensity Recovery (% Control) |
|---|---|
| $P_1$ | 100 (3)[a] |
| $P_2$ | 97 (3) |
| $P_3$ | 95 (3) |
| $P_1'$ | 100 (4) |
| $P_2'$ | 95 (4) |
| $P_3'$ | 98 (3) |

[a] All results are expressed as the average values from the number of separate experiments shown in parentheses. The samples were excited at 465 nm, and emission was observed at 535 nm for $P_1$–$P_3$ and 525 nm for $P_1'$–$P_3'$. The relative fluorescence of the first pellet in both cases was arbitrarily set at 100%.

Experiments were performed as described in the Methods section. $P_1$ refers to the pellet of the synaptosomal particulate fraction after incubation with NBD-PE in 0.1 M Tris-HCl, pH 7.4. $P_2$ and $P_3$ refer to the same pellets after two successive washings via rehomogenization in the buffer and recentrifugation. $P_1'$, $P_2'$, and $P_3'$ refer to analogous pellets of synaptic plasma membranes treated as above, including 0.01% (v/v) BRIJ-35 in all steps.



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brane probe. The derivative is relatively simple to prepare and purify, and is stable for approximately 6 months when stored at −76°C. The variation in fluorescent intensity of NBD-PE in absolute ethanol was linear over a wide concentration range, and the fluorescent characteristics of the molecule are apparently quite sensitive to solvent dielectric constant. Consequently, it should act as an excellent indicator of membrane conformational changes. It is interesting to note that, in solvents of very low dielectric constant, the relative fluorescence of NBD is small while the emission maximum wavelength is fairly high. This observation is in contrast with the behavior of most other fluorescent probes such as ANS (1-anilino-8-naphthalene sulfonic acid.) One might speculate that in these solvents the fatty acid side chains of NBD-PE are oriented outward toward the solvent, while the polar head groups with the attached nitrobenzodiazole moieties are oriented inward, facing each other. Thus, the microenvironment of the fluorochrome would actually be much more polar than one would expect and might account for the observations. Since polar groups in close proximity would be in an energetically unstable configuration, more of these groups might re-orient outward toward the solvent as solvent dielectric constant is increased. Such behavior could account for the increase in relative fluorescence and the decrease in emission maximum wavelength observed between solvent dielectric constants of 2–9. Once a majority of the polar groups have re-oriented outward toward the solvent ($\epsilon > 10$), the fluorescence properties of NBD-PE are what one would expect from most other fluorescent probes.

When considering the data on the interaction of NBD-PE with synaptosomal particulate fraction and lymphocytes, the question naturally arises as to whether the molecule is actually incorporated into these membranes, or whether the observed effects are due to interactions on the membrane surface. Direct binding studies, such as fluorescence titration, are complicated by the fact that the emission maximum wavelength of NBD-PE (in the absence of detergent) in the presence and absence of the membranes is apparently identical, while binding studies in the presence of detergent are also subject to serious complications.

In a study on the interaction of dansylated phosphatidylserine (DNS-PS), Harris and Stahl (29) reported that simple mixing of DNS-PS micelles with microsomes did not result in incorporation of the DNS-PS into the membrane matrix. However, these investigators later reported that incubation of synaptosomes with DNS-PS in the presence of the chaotropic agent $NaClO_4$, followed by gradient centrifugation and dialysis, achieved incorporation of the probe in all gradient fractions, including synaptosomal plasma membranes (30). Harris and Stahl (30) also noted that the gradient distribution of the $NaClO_4$-treated synaptosomal material was similar to that described by Whittaker, Michaelson, and Kirkland (31) for osmotically shocked synaptosomes. We therefore wondered whether a milder technique for disrupting the synaptosomes, such as osmotic lysis, might yield a membrane fraction that would allow the incorporation of a somewhat different fluorescent phospholipid. Since we observe an increase in relative fluorescence of NBD-PE in the presence of synaptosomal particulate fraction or lymphocytes that is not significantly decreased by two subsequent washings, even in the presence of 0.01% BRIJ-35, we infer that NBD-PE is incorporated into these membranes. The position of the fluorescent moiety on the polar head group of the phospholipid would suggest that, if the NBD-PE is incorporated into these membranes, the fluor is most likely oriented toward the outer membrane surface. Thus, ligands that primarily interact with the polar head of membrane phospholipids might be expected to exert an effect on the fluorescent properties of membrane-bound NBD-PE.

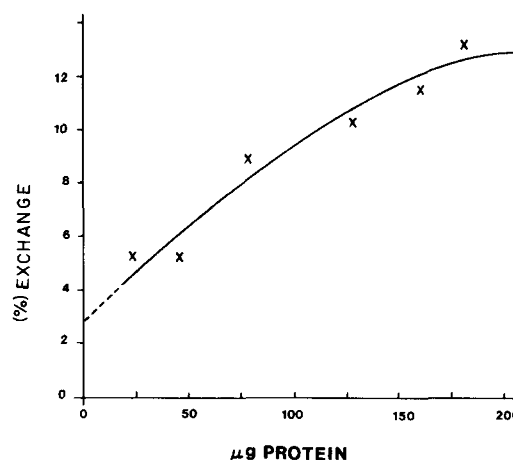


**Fig. 5.** Incorporation of NBD-PE into rat liver mitochondria in the presence and absence of a crude phospholipid exchange protein fraction. The percent exchange of NBD-PE incorporation was determined as described in the Methods section, and is shown on the ordinate. The abscissa gives the amounts of crude exchange protein added in μg. The data points (×) represent the average of two separate determinations.

We have also recently synthesized 1-acyl-2-($N$-4-nitrobenzo-2-oxa-1,3-diazole)-aminocaproyl phosphatidylcholine, an NBD derivative of phosphatidylcholine, in which the fluorescent moiety is attached to the $\beta$ carbon of PC, and thus might extend further into the bilayer than the fluor of NBD-PE. Comparing the fluorescent properties of these two



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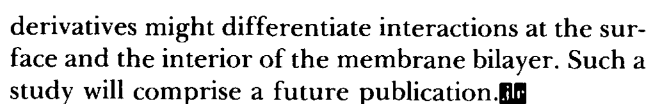

derivatives might differentiate interactions at the surface and the interior of the membrane bilayer. Such a study will comprise a future publication.

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