Novel Spin-Labels for the Study of Lipid–Protein Interactions. Application to (Na^+,K^+)-ATPase Membranes

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ABSTRACT: The interactions of a series of spin-labeled fatty acids, in which the nitroxide ring is incorporated in different ways as an integral part of the hydrocarbon chain, with the (Na^+,K^+)-ATPase in membranes from *Squalus acanthias*, have been studied by electron spin resonance spectroscopy. The fatty acids are 2,4-, 2,5-, and 3,2-substituents of 2,2,5,5-tetramethylpyrrolidine-*N*-oxyl and belong to the class of minimal perturbation nitroxide probes. For all five fatty acid labels, a motionally restricted lipid component was observed in the ESR spectra of (Na^+,K^+)-ATPase membranes, in addition to the fluid component, which was found in the spectra of the extracted membrane lipids. The pH dependence of the motionally restricted spin-label population indicated a sensitivity in the selectivity of the lipid–protein interaction to the protonation state of the fatty acid. These results agree with those found previously for the conventional oxazolidine (doxyl) fatty acid and phospholipid spin-label derivatives [Esmann, M., Watts, A., & Marsh, D. (1985) *Biochemistry 24*, 1386–1393] and indicate that the motion of the lipid chains is significantly hindered by interaction with the protein, irrespective of the nature of the spin-label group.

Electron spin resonance (ESR)[1] spectroscopy of spin-labeled lipids has been used extensively to study lipid–protein interactions in membranes [for recent reviews, see Marsh (1985, 1988) and Devaux and Seigneuret (1985)]. Early controversies regarding the identification of motionally restricted lipid by spin-labels now have been largely resolved in favor of two-component ESR spectra (Watts et al., 1981; Ellena et al., 1983). Nevertheless, it is not clear to what extent the degree of motional restriction of the spin-labeled lipid by the protein, which gives rise to the second spectral component, is influenced by the detailed structure of the spin-labeled group. NMR experiments on lipid–protein interactions, using deuterium-labeled lipids, have indicated that the degree of motional restriction by many integral proteins may be relatively small (Seelig et al., 1982; Bloom & Smith, 1985). Among the possible exceptions is the myelin proteolipid protein (Meier et al., 1986). It will be noted, however, that the thermodynamic and structural conclusions regarding the nature of the lipid–protein interaction which have been reached by the spin-label method [see, e.g., Jost et al. (1973) and Brotherus et al. (1980, 1981)] are unaffected by the degree of motional restriction. This is because the method relies only on the spectral resolution of the lipids at the intramembranous surface of the integral protein and not on their dynamic properties per se (Marsh, 1983, 1985).

Most of the ESR spin-label experiments on lipid–protein interactions to date have been performed with doxyl-labeled lipids, analogues of 14-doxylstearic acid (14-SASL). In the equilibrium conformation of such fatty acids, the spin-label group projects out from the hydrocarbon chain, which may affect the degree of motional restriction induced by steric interactions with the intramembranous surface of the integral protein. A type of "minimal perturbation" spin-labeled fatty acid has been introduced by Lee et al. (1978), in which the nitroxide ring is incorporated as an integral part of the hydrocarbon chain. These azethoxyl spin-labels contained the nitroxide ring by attachment of the fatty acid chain at the 2 and 5 positions of the pyrrolidine ring [*n*-(2′,5′)FASL in the abbreviated nomenclature used here].

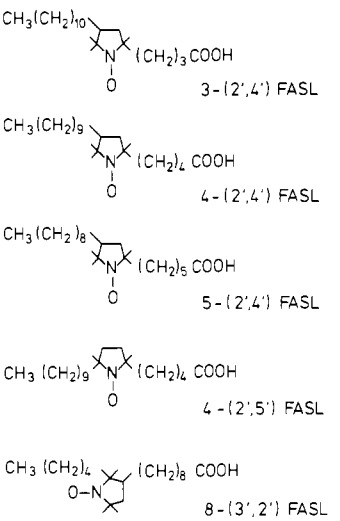

FIGURE 1: Structures of the pyrrolidine fatty acid spin-labels.

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[1] Abbreviations: 14-SASL, 14-(4,4-dimethyl-*N*-oxyoxazolidinyl)-stearic acid; 3-(2′,4′)FASL, 2,5,5-trimethyl-4-undecyl-2-(3-carboxypropyl)pyrrolidine-*N*-oxyl; 4-(2′,4′)FASL, 2,5,5-trimethyl-4-decyl-2-(4-carboxybutyl)pyrrolidine-*N*-oxyl; 5-(2′,4′)FASL, 2,5,5-trimethyl-4-nonyl-2-(5-carboxypentyl)pyrrolidine-*N*-oxyl; 4-(2′,5′)FASL, 2,5-dimethyl-5-decyl-2-(5-carboxybutyl)pyrrolidine-*N*-oxyl; 8-(3′,2′)FASL, 2,5,5-trimethyl-2-pentyl-3-(8-carboxyoctyl)pyrrolidine-*N*-oxyl; doxyl, 4,4-dimethyloxazolidine-*N*-oxyl; (Na^+,K^+)-ATPase, sodium and potassium ion activated adenosinetriphosphatase (EC 3.6.1.3); CDTA, *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid; $C_{12}E_8$, octaethylene glycol dodecyl monoether; Tris, tris(hydroxymethyl)aminomethane; ESR, electron spin resonance; NMR, nuclear magnetic resonance; SDS, sodium dodecyl sulfate.

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We have extended the previous studies on minimal perturbation labels by synthesizing a series of fatty acids in which the hydrocarbon chain is attached at various different positions to the pyrrolidine ring (Hideg & Lex, 1984), as shown in Figure 1. As can be seen from the figure, the original azethoxyl derivative, 4-(2′,5′)FASL, is included in this series. Besides their greater chemical stability compared with the doxyl labels, these new labels have several advantages for the study of lipid–protein interactions. The principal among these is the ability to compare labels with different steric properties and with different orientations of the nitroxide axes, which give different optimal sensitivities to the various modes of chain motion. The labels have been used to investigate lipid–protein interactions in (Na^+,K^+)-ATPase membranes from *Squalus acanthias*, a system that previously has been characterized in detail by us, using doxyl label derivatives (Esmann et al., 1985; Esmann & Marsh, 1985). All members of the series are found to display two-component ESR spectra in (Na^+,K^+)-ATPase membranes and single component spectra in the extracted membrane lipid, indicating that the hindering of the chain motion is not caused by a specific steric interaction of the doxyl nitroxide group. Both the doxyl and minimum perturbation probes indicate that the rotational correlation time of the spin-labeled chain motion is increased from the 1-ns to the 10-ns time regime on interacting with the intramembranous surface of the protein.

Materials and Methods

The pyrrolidine-based fatty acid spin-labels 3-(2′,4′)FASL, 4-(2′,4′)FASL, 5-(2′,4′)FASL, 4-(2′,5′)FASL, and 8-(3′,2′)-FASL were synthesized as described by Hideg and Lex (1984, 1986). The two stereoisomers were not specifically resolved, although examination of the synthetic routes involved indicates that they should yield predominantly the trans isomer. Detailed analysis of the ESR spectra of these labels in oriented model membrane systems has fully confirmed that they are characteristic of the trans isomer (T. Heimburg, K. Hideg, and D. Marsh, unpublished results). Additionally, comparison was made with a shorter chain analogue of the 5-(2′,4′)FASL label, for which the stereoisomers could be resolved. The trans isomer of 2,5,5-trimethyl-4-hexyl-2-(5-carboxypentyl)-pyrrolidine-*N*-oxyl yielded ESR spectra that were very similar to those reported here for the 5-(2′,4′)FASL label (Esmann et al., 1988).

(Na^+,K^+)-ATPase-rich membranes from the rectal gland of *Squalus acanthias* were prepared as described by Skou and Esmann (1979), but the treatment with saponin was omitted. The (Na^+,K^+)-ATPase constituted typically 50–70% of the protein (determined as the content of α- and β-subunits from SDS gel electrophoresis), and the specific activity ranged accordingly from 1100 to 1500 μmol of ATP hydrolyzed (mg of protein)$^{-1}$ h^{-1}. Since solubilization of these preparations in the detergent $C_{12}E_8$ yields an insoluble precipitate, presumably of peripheral proteins, and a supernatant which contains practically pure (Na^+,K^+)-ATPase (Esmann et al., 1979), it can be assumed that the (Na^+,K^+)-ATPase is the only integral protein in the membrane. Extraction of the membrane lipids and spin-labeling of the (Na^+,K^+)-ATPase membranes and extracted lipids were performed as described by Esmann et al. (1985). The buffer used for suspending and pelleting the ESR samples was 10 mM sodium phosphate, pH 5.7, or 10 mM Tris, pH 9.2.

ESR spectra were recorded on a Varian E-12 9-GHz spectrometer equipped with nitrogen gas flow temperature regulation. Further details of the ESR spectroscopy are given in Esmann et al. (1985). Spectral subtractions and data analysis were performed as described in Marsh (1982).

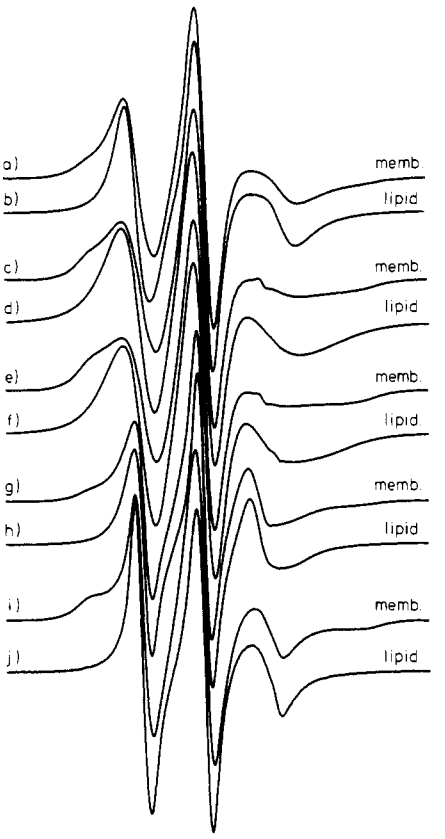

FIGURE 2: ESR spectra of the various pyrrolidine fatty acid spin-labels in (Na^+,K^+)-ATPase membranes from *Squalus acanthias* and in the extracted membrane lipids suspended in 10 mM sodium phosphate, pH 5.7. The upper spectrum of each pair is from the membranes and the lower spectrum from the extracted lipids. (a) 3-(2′,4′)FASL in membranes; (b) 3-(2′,4′)FASL in lipids; (c) 4-(2′,4′)FASL in membranes; (d) 4-(2′,4′)FASL in lipids; (e) 5-(2′,4′)FASL in membranes; (f) 5-(2′,4′)FASL in lipids; (g) 4-(2′,5′)FASL in membranes; (h) 4-(2′,5′)FASL in lipids; (i) 8-(3′,2′)FASL in membranes; (j) 8-(3′,2′)FASL in lipids. Total scan width = 100 G; T = 15 °C.

Results

ESR spectra of the different minimal perturbation fatty acid spin-labels in (Na^+,K^+)-ATPase membranes and in aqueous dispersions of the extracted membrane lipids are given in Figure 2. The upper spectrum of each pair is from the membranes and the lower spectrum of each pair is from the extracted lipids. As can clearly be seen, each membrane spectrum consists of two resolved components. One component is closely similar to the single component found in the extracted lipids. The other component, which is characterized by the broad peaks in the outer wings of the spectrum, is assigned to spin-labeled lipids interacting directly with the hydrophobic surface of the protein [cf. Marsh (1985) and Esmann et al. (1985)]. Although the fluid component spectra of the extracted lipids differ considerably between the various labels, corresponding to the different nitroxide orientations, the motionally restricted component in the membrane spectra has similar outer hyperfine splittings in each case. This indicates that, for the lipid population interacting directly with the intramembranous surface of the protein, the chain rotational modes to which the different labels are optimally sensitive all lie in the slow motional regime of conventional nitroxide ESR spectroscopy.

The temperature dependence of the ESR spectra of the 3-(2′,4′)FASL fatty acid spin-label in (Na^+,K^+)-ATPase membranes is given in Figure 3. It is seen that the fluid

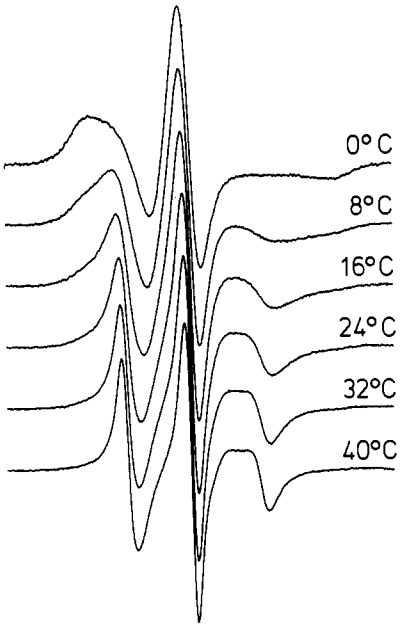

FIGURE 3: Temperature dependence of the ESR spectra of the 3-(2′,4′)FASL pyrrolidine fatty acid spin-label in (Na^+,K^+)-ATPase membranes from *Squalus acanthias*. Buffer: 0.1 M NaCl, 20 mM histidine, and 1 mM CDTA, pH 7.4. Total scan width = 100 G.

component, in the central regions of the spectrum, has a much steeper temperature dependence than the motionally restricted component in the outer wings. This illustrates the difference in chain rotational dynamics in the two lipid environments. The fluid component lies in the range of optimal motional sensitivity of nitroxide ESR spectroscopy, whereas the motionally restricted component lies in the slow motional regime [cf. Marsh (1988)]. Correspondingly, there is an optimal temperature range for resolution of the two components, and this can be chosen when the relative proportions are quantitated by difference spectroscopy. It will also be noted that this temperature dependence of the chain dynamics correlates with the differences seen between the spectra of the different positional isomers of the *n*-(2′,4′)FASL recorded at constant temperature in Figure 2.

Spectral subtractions and additions used in quantitating the two components for the membrane spectra of the 4-(2′,5′)-FASL fatty acid spin-label are shown in Figure 4. Subtraction of the lipid spectrum (Figure 4b, full line) from the membrane spectrum (Figure 4a, full line) yields a motionally restricted end point (Figure 4c, dotted line), which corresponds very closely to the motionally restricted spectrum obtained from dimyristoylphosphatidylcholine vesicles in the gel phase (Figure 4c, full line). The complementary subtraction of the motionally restricted gel-phase spectrum (Figure 4c, full line) from the membrane spectrum (Figure 4a, full line) yields a fluid spectrum (Figure 4b, dotted line), which corresponds very closely with the spectrum of the extracted lipids (Figure 4b, full line). Alternatively, addition of the motionally restricted gel-phase spectrum (Figure 4c, full line) to the extracted lipid spectrum (Figure 4b, full line) yields a two-component spectrum (Figure 4a, dotted line), which corresponds very closely to the original membrane spectrum (Figure 4a, full line). All three independent methods of quantitation yield consistent results (to within ±2%) for the fraction of motionally restricted lipid in the membrane spectrum, namely, a mean value of 44%.

The mean values for the fraction of motionally restricted lipid for the different fatty acid labels, both in their protonated and in their fully dissociated states, were obtained by the above methods and are given in Table I. Recently, similar prelim-

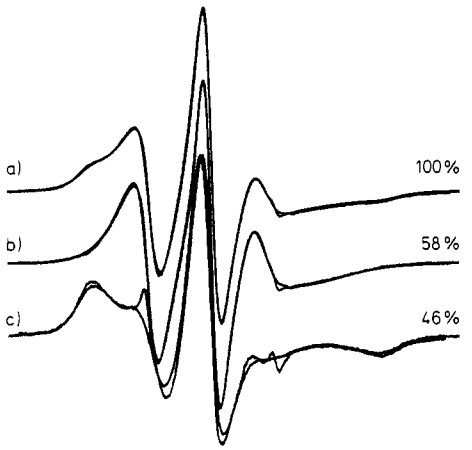

FIGURE 4: Spectral subtraction and addition with the fatty acid spin-label 4-(2′,5′)FASL in (Na^+,K^+)-ATPase membranes in 10 mM sodium phosphate, pH 5.7. Full lines are original spectra: (a) membranes at 5 °C; (b) extracted membrane lipids at 5 °C; (c) dimyristoylphosphatidylcholine vesicles at 3 °C (motionally restricted). Dotted lines are summed and difference spectra: (a) 56% lipid spectrum plus 44% motionally restricted spectrum; (b) membrane spectrum minus 42% motionally restricted spectrum; (c) membrane spectrum minus 54% lipid spectrum. Total scan width = 100 G.

Table I: Fraction of Motionally Restricted Lipid, *f*, for Spin-Labeled Fatty Acid Analogues in (Na^+,K^+)-ATPase Membranes at 10–15 °C, pH 5.7 and 9.2[a]

spin-label	pH 5.7	pH 9.2
3-(2′,4′)FASL	0.30	0.49
4-(2′,4′)FASL	0.30	0.40
5-(2′,4′)FASL	0.40	0.49
4-(2′,5′)FASL	0.42	0.51
8-(3′,2′)FASL	0.50	

[a] Values are means of those obtained from subtraction of the extracted lipid spectrum to yield a motionally restricted difference spectrum and from subtraction of a gel-phase dimyristoylphosphatidylcholine vesicle spectrum to yield a fluid difference spectrum.

inary results have been obtained with a shorter chain analogue of 5-(2′,4′)FASL, 2,5,5-trimethyl-4-hexyl-2-(5-carboxypentyl)pyrrolidine-*N*-oxyl, which yielded a value of $f = 0.49$ at pH 9.2, in good agreement with the results of Table I (Esmann et al., 1988). The shorter chain analogue, however, has the disadvantage that the partitioning into the membrane is less favorable, and the ESR spectra are complicated by a signal from the aqueous spin-label. For comparison, the values obtained previously with the doxyl fatty acid (14-SASL) in the same membrane system were $f = 0.22$ and $f = 0.43$ at pH 5.7 and 9.2, respectively (Esmann & Marsh, 1985).

Discussion

The results with a series of different minimal perturbation labels indicate that the spin-labeled lipids interacting directly with the intramembranous surface of the protein are motionally restricted on the 10-ns time scale, independent of the configuration of the spin-label ring in the lipid chain. Two distinct components are resolved in the membrane spectra of all the different fatty acid labels, including the 14-SASL doxyl label (Esmann & Marsh, 1985), in spite of the different motional sensitivity indicated by the spectra in the fluid bilayers of the extracted membrane lipids (see Figure 2). It thus seems highly unlikely that the motional restriction arises solely from the presence of the spin-label group but rather it reflects a real effect of the protein on the lipid-chain dynamics. In a more limited study of lipid–protein interactions, Lee et al. (1979) have similarly detected a motionally restricted component for the 10-azethoxyheneicosanoic acid spin-label in chromato-

phores from *Rhodopseudomonas sphaeroides*. These findings with ESR are not inconsistent with the observation of a single-component NMR spectrum in lipid–protein systems, since the exchange between the two lipid environments has been shown to be fast on the NMR time scale (Bloom & Smith, 1985; Seelig et al., 1982; Meier et al., 1987) but slow on the ESR time scale (Horváth et al., 1988; Ryba et al., 1987; East et al., 1985; Marsh, 1985).

Examination of Figure 1 and the ESR spectra of the different fatty acid labels in the extracted membrane lipids demonstrates the differential sensitivities of the various labels to particular modes of the chain motion [cf. Griffith and Jost (1976)]. The *n*-(2′,4′)FASL labels have the nitroxide *z* axis inclined at an angle of approximately 90° to the chain axis, display ESR spectra characteristic of *y*-axis rotation, and are optimally sensitive to rotation around the chain axis (Heimburg et al., unpublished results). The *n*-(2′,5′)FASL labels have the nitroxide *z* axis inclined at an angle of approximately 40° to the chain axis, display ESR spectra resembling *z*-axis rotation, and are more sensitive to chain fluctuations (Heimburg et al., unpublished results; Lee et al., 1979). The *n*-(3′,2′)-FASL labels have the nitroxide *z* axis inclined at an angle of approximately 60° to the chain axis, display ESR spectra more characteristic of *x*-axis rotation, and are most sensitive to rotation around the chain axis (Heimburg et al., unpublished results). On the other hand, the doxyl fatty acids, *n*-SASL, have the nitroxide *z* axis oriented approximately parallel to the chain axis, display ESR spectra characteristic of *z*-axis rotation, and are optimally sensitive to trans-gauche isomerization and chain axis fluctuations (Lange et al., 1985). The observation of a motionally restricted, protein-associated component in the ESR spectra of all the different labels indicates that the lipid–protein interaction causes a slowing down of all these modes of rotation of the spin-labeled chain. Roughly speaking, the rotational correlation time is increased by interaction with the protein, from values in the region of 1 ns for the fluid bilayer regions of the membrane to values of the order of 10 ns for the motionally restricted component [cf. Marsh (1988)].

The higher degree of association with the protein for the negatively charged form of the fatty acids at pH 9.2, than for the protonated form at pH 5.7, is in good agreement with previous results obtained with the doxyl fatty acid label. This latter label was shown to exhibit a pronounced pH titration for its selectivity of lipid–protein interaction in (Na^+,K^+)-ATPase membranes, with a pK_a of 8.0 (Esmann & Marsh, 1985). The magnitude of the change in the fraction of motionally restricted fatty acid is comparable for the different spin-labels, although the absolute values do differ considerably. On the whole, higher values of f are found for the labels studied here than were obtained previously for the doxyl label. These differences are greatest for the protonated form of the fatty acids, which may be related to different degrees of vertical movement of the different labels on protonation. It has been demonstrated previously that the doxyl fatty acids sink deeper into the membrane on protonation (Barratt & Laggner, 1974; Sanson et al., 1976). This effect may also lead to a somewhat different shift in the membrane pK_a for the various labels, which will further contribute to the observed differences in motionally restricted populations. In addition to the difference from the doxyl label, the smaller differences that are observed between the various pyrrolidine labels in Table I seem to indicate some sensitivity of the lipid–protein interaction to the detailed chain structure. This suggests that there might be a selectivity between lipids bearing saturated and unsaturated chains for interaction with the (Na^+,K^+)-ATPase.

In summary, the experiments with fatty acid labels in which the nitroxide group is incorporated in different ways into the hydrocarbon chain allow a detailed comparison between a series of minimal perturbation probes. The results indicate that a motionally restricted ESR spectral component is observed in lipid–protein systems, independent of the nature of the spin-label geometry and mode of chain linkage. It thus appears that the lipid chain motion is significantly retarded by interaction with the intramembranous surface of integral proteins, which offers a mechanism whereby the large-scale protein motions may be interfaced to the fluid lipid environment in which they are embedded.

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Articles

Interactions of Cationic Lipid Vesicles with Negatively Charged Phospholipid Vesicles and Biological Membranes†

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ABSTRACT: Lipid vesicles with a positive surface charge have been prepared by using mixtures of phosphatidylethanolamine (PE) and phosphatidylcholine (PC) together with low mole fractions of a cationic lipid analogue, 1,2-bis(oleoyloxy)-3-(trimethylammonio)propane (DOTAP). We have used measurements of vesicle aggregation, lipid mixing, contents mixing, and contents leakage to examine the interactions between these vesicles and similar vesicles that carry a negative surface charge. Mixtures of vesicles with opposite surface charges aggregate readily at physiological or lower ionic strengths, and the extent of this aggregation is enhanced for vesicles that contain high proportions of PE relative to PC. Mixing of lipids and aqueous contents can also be observed between such vesicles, particularly when the vesicles contain substantial proportions of PE. Surprisingly, these latter processes are strongly promoted by monovalent salts and do not proceed at very low ionic strengths. PE/DOTAP vesicles show substantial lipid mixing with negatively charged vesicles containing high proportions of phosphatidylcholine, as well as with human erythrocyte ghosts, on a time scale of a few minutes. These interactions are strongly promoted both by the presence of high levels of PE in the cationic vesicles and by the presence of complementary surface charges on the two membrane populations.

A number of studies in recent years have examined the fusion of lipid vesicles to model some aspects of the fusion of biological membranes (Liao & Prestegard, 1979; Papahadjopoulos et al., 1980; Wilschut et al., 1980, 1985; Uster & Deamer, 1981; Düzgünes et al., 1981a, 1985; Sundler & Papahadjopoulos, 1981; Sundler et al., 1981; Morris et al., 1985; Gagnê et al., 1985; Ellens et al., 1986, 1987a; Parente & Lentz, 1986; Leventis et al., 1986). Most studies of this sort have examined the interactions of charged lipid vesicles of like composition, which can be induced to aggregate and in some cases to fuse when electrostatic repulsions between vesicles are reduced through binding of counterions to the lipid surface (Lansmann & Haynes, 1975; Bentz & Nir, 1981; Düzgünes et al., 1981b; Rydhag et al., 1982; Ohki et al., 1982, 1984; Bentz & Düzgünes, 1985; Rupert et al., 1985; Carmona-Ribeiro et al., 1985). These systems are popular objects for study because the tendency of such vesicles to associate with one another can be readily controlled by varying the ionic composition of the medium.

A second possible means to promote interactions between lipid vesicles would be to mix two populations of vesicles with opposite surface charges, an approach that has not been widely employed to date. In some applications, this approach may offer significant advantages over the approach described above. Mixtures of vesicles with opposite surface charges may be useful, for example, to examine interactions between vesicles with different lipid compositions while suppressing interactions between vesicles of like composition, or to provide systems in which interactions between vesicles are not strongly dependent upon the binding of ions to the lipid surface, which may substantially alter the hydration and other properties of the surfaces involved.

In light of the considerations just noted, we have examined in this study the interactions between negatively and positively charged lipid vesicles, composed of neutral phospholipids as a majority component together with small amounts of anionic lipids or of a cationic lipid analogue, 1,2-bis(oleoyloxy)-3-(trimethylammonio)propane (DOTAP).[1] Our results indicate

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[1] Abbreviations: ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid disodium salt; 12-CPS-18-PC, 1-hexadecanoyl-2-[12-[[*N*-[4-[7-(dimethylamino)-4-methylcoumarin-3-yl]phenyl]carbamoyl]methyl]thio]-octadecanoyl]-*sn*-glycero-3-phosphocholine; 12-DABS-18-PC, 1-hexadecanoyl-2-[12-[*p*-[[*p*-(dimethylamino)phenyl]azo]-*N*-methylbenzenesulfonamido]octadecanoyl]-*sn*-glycero-3-phosphocholine; DOTAP, 1,2-bis(oleoyloxy)-3-(trimethylammonio)propane; DPX, *N,N'-p*-xylylenebis(pyridinium bromide); EDTA, ethylenediaminetetraacetic acid trisodium salt; LUV, large unilamellar vesicle(s); Mes, 2-(*N*-morpholino)ethanesulfonic acid sodium salt; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; PC, phosphatidylcholine (from egg yolk); PE, phosphatidylethanolamine (prepared by transphosphatidylation of egg yolk PC); PS, phosphatidylserine (dioleoyl); Rho-PE, *N*-(lissamine Rhodamine B sulfonyl)phosphatidylethanolamine; Tes, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid sodium salt; HPLC, high-performance liquid chromatography.

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