

Fusion of Liposomes Containing a Novel Cationic Lipid, *N*-[2,3-(Dioleyloxy)propyl]-*N,N,N*-trimethylammonium: Induction by Multivalent Anions and Asymmetric Fusion with Acidic Phospholipid Vesicles[†]

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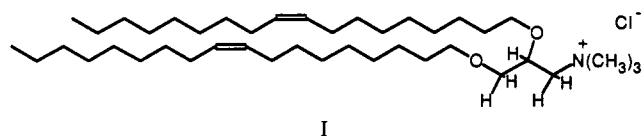
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ABSTRACT: The fusion behavior of large unilamellar liposomes composed of *N*-[2,3-(dioleyloxy)propyl]-*N,N,N*-trimethylammonium (DOTMA) and either phosphatidylcholine (PC) or phosphatidylethanolamine (PE) has been investigated by a fluorescence resonance energy transfer assay for lipid mixing, dynamic light scattering, and electron microscopy. Polyvalent anions induced the fusion of DOTMA/PE (1:1) liposomes with the following sequence of effectiveness: citrate > EDTA > phosphate, in the presence 100 mM NaCl, pH 7.4. Sulfate, dipicolinate, and acetate were ineffective. DOTMA/PC (1:1) vesicles were completely refractory to fusion in the presence of multivalent anions in the concentration range studied, consistent with the inhibitory effect of PC in divalent cation induced fusion of negatively charged vesicles. DOTMA/PE vesicles could fuse with DOTMA/PC vesicles in the presence of high concentrations of citrate, but not of phosphate. Mixing of DOTMA/PE liposomes with negatively charged phosphatidylserine (PS)/PE or PS/PC (1:1) vesicles resulted in membrane fusion in the absence of multivalent anions. DOTMA/PC liposomes also fused with PS/PE liposomes and, to a limited extent, with PS/PC liposomes. These observations suggest that the interaction of the negatively charged PS polar group with the positively charged trimethylammonium of DOTMA is sufficient to mediate fusion between the two membranes containing these lipids and that the nature of the zwitterionic phospholipid component of these vesicles is an additional determinant of membrane fusion.

The fusogenic properties of membranes composed of naturally occurring, negatively charged, or zwitterionic phospholipids have been well established (Nir et al., 1983a; Düzgüneş, 1985; Düzgüneş et al., 1987a; Bentz & Ellens, 1988; Wilschut & Hoekstra, 1986). Divalent cations, such as Ca²⁺ and Ba²⁺, are thought to induce fusion of membranes containing acidic phospholipids by partial surface charge neutralization and dehydration of the polar headgroups (Portis et al., 1979; Düzgüneş et al., 1981a; Hoekstra, 1982; Wilschut et al., 1985), leading to the formation of molecular packing defects which act as nucleation sites for membrane intermixing and fusion (Hui et al., 1981; Düzgüneş, et al., 1984). Fusion is initiated when a critical amount of divalent cation is bound per phospholipid molecule (Düzgüneş et al., 1981b; Bentz et al., 1983; Bentz & Düzgüneş, 1985). Recent experiments with trivalent La³⁺ have indicated the presence of long-lived intermediate intermediates during fusion (Bentz et al., 1988).

The recent synthesis of a novel, positively charged lipid, *N*-[2,3-(dioleyloxy)propyl]-*N,N,N*-trimethylammonium (DOTMA;¹ structure I), has enabled us to examine whether



multivalent anions exert fusogenic activity on membranes containing such lipids and whether these membranes fuse with negatively charged membranes. Liposomes containing DOTMA have been found to facilitate the transfection of cultured mammalian cells with recombinant DNA vectors to a greater extent than conventional transfection techniques (Felgner et al., 1987; Felgner & Ringold, 1989). The mechanism of this facilitation is not understood. We are currently investigating the use of DOTMA-containing vesicles to deliver other macromolecules, including proteins that regulate transcription, into cultured cells. Here, we have examined the fusion behavior of large unilamellar DOTMA/phosphatidylethanolamine (PE) and DOTMA/phosphatidylcholine (PC) vesicles, using a resonance energy transfer assay for lipid mixing (Struck et al., 1981; Rosenberg et al., 1983; Düzgüneş et al., 1987b). We have also investigated the structural transformation of the liposomes by freeze-fracture and thin-section

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¹ Abbreviations: DODAC, dioctadecyldimethylammonium chloride; DDAB, didodecyldimethylammonium bromide; LUV, large unilamellar vesicles; DOTMA, *N*-[2,3-(dioleyloxy)propyl]-*N,N,N*-trimethylammonium; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; Rh-PE, *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine; TES, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine.

electron microscopy and the increase in the size of the vesicles by dynamic light scattering.

MATERIALS AND METHODS

DOTMA was synthesized as described by Felgner et al. (1987) and kindly provided by Norman Dyson at Syntex Research. It was stored in ethanol under argon at -70°C . Bovine brain phosphatidylserine (PS), egg PC, PE prepared by transphosphatidylolation of egg PC, *N*-(lissamine rhodamine B sulfonyl)-PE (Rh-PE), and *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-PE (NBD-PE) were provided by Avanti Polar Lipids (Birmingham, AL) and stored in chloroform under argon in sealed ampules at -70°C . NaCl, NaHPO_4 , and Na_2SO_4 were from Fisher. EDTA and 2-[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid (TES) were from Sigma (St. Louis, MO). Sodium citrate was from Mallinckrodt. Water was distilled twice, the second time in an all-glass apparatus, and purified further in a Barnstead Nanopure system.

Large unilamellar vesicles (LUV) were prepared by reverse-phase evaporation followed by sequential extrusion through polycarbonate membranes (Nuclepore, Pleasanton, CA) of 200- and 100-nm pore diameter (Szoka et al., 1980; Düzgüneş et al., 1983). Extrusion through the 100-nm membrane was repeated four times. The size distribution of the vesicles was determined by dynamic light scattering in a Coulter N4 MD submicron particle analyzer. The mean diameters of the vesicles and the standard deviations were 187 ± 56 nm for DOTMA/PE/NBD-PE/Rh-PE (49:49:1:1) vesicles, 185 ± 51 nm for DOTMA/PE (1:1) vesicles, 183 ± 51 nm for DOTMA/PC vesicles, 134 ± 36 nm for PS/PC (1:1) vesicles, and 159 ± 41 nm for PS/PE (1:1) vesicles.

Three populations of vesicles were prepared for the resonance energy transfer assay for lipid mixing (Düzgüneş et al., 1985; Düzgüneş & Bentz, 1988): labeled vesicles, containing 1 mol % each of Rh-PE and NBD-PE, unlabeled vesicles, and vesicles to be used for the calibration of fluorescence to 100%, containing 0.2 mol % of each probe. Labeled vesicles were mixed with unlabeled vesicles at a 1:4 ratio, at a total lipid concentration of $50\ \mu\text{M}$ in 1 mL of 100 mM NaCl/5 mM TES, pH 7.4, at 25°C . The lipid concentration of the vesicle preparation was determined by phosphorus analysis of the phospholipid component of the mixed vesicles (Bartlett, 1959).

Fluorescence measurements were made in an SLM 4000 fluorometer. The excitation wavelength was 450 nm, and the emission wavelength was 520 nm, with the monochromator slits set at 4 nm. The fluorescence intensity of the labeled vesicles was taken as 0%. Maximal (100%) fluorescence (F_{max}) was set by suspending $50\ \mu\text{M}$ vesicles containing 0.2 mol % of each probe in the fluorometer cell. Complete mixing of the membranes of the labeled (1 mol % of each probe) and unlabeled vesicles would be expected to produce, ideally, vesicles containing 0.2 mol % of each probe (Düzgüneş et al., 1985). In the asymmetric fusion experiments, in which the fusion of DOTMA/PE vesicles with PS-containing vesicles was investigated, 100% fluorescence was again set by use of these vesicles. Dilution of the probe molecules from labeled vesicles into unlabeled vesicles results in a decrease of the efficiency of energy transfer from NBD to Rh, and hence, the fluorescence intensity of NBD increases. The probe dilution method has been shown to be insensitive to the mere aggregation of vesicles and to be a reliable indicator of lipid mixing during membrane fusion (Düzgüneş et al., 1987b). Fusion was initiated by injecting aliquots of concentrated stock solutions of either the anions or, in the cases of the asymmetric fusion experiments, of the unlabeled liposomes. The fluorometer

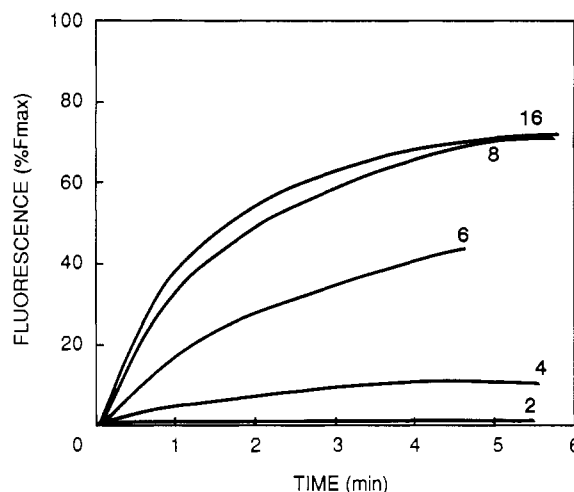


FIGURE 1: Time course of lipid mixing during fusion of DOTMA/PE (1:1) liposomes induced by EDTA, at the indicated concentrations (mM). Labeled vesicles, containing 1 mol % each of Rh-PE and NBD-PE and at a lipid concentration of 10 nmol, were mixed with 40 nmol of unlabeled vesicles in 1 mL of 100 mM NaCl/5 mM TES, pH 7.4, at 25°C . 100% F_{max} was set with 50 nmol of vesicles containing 0.2 mol % of each probe.

cuvette was stirred continuously and thermostated to 25°C .

For freeze-fracture electron microscopy, DOTMA/PE liposomes ($5\text{--}10\ \mu\text{mol}$ of lipid/mL) were treated with citrate (2 mM final concentration), 30% glycerol was added to the sample, and the mixture was frozen in freon cooled by liquid N_2 . The frozen sample was fractured in an RMC-Eiko (Model FD-5A) apparatus, shadowed with platinum/carbon, and viewed in a JEOL 100CX electron microscope. For thin-section electron microscopy, the liposomes were fixed in 0.1 M cacodylate buffer (pH 7.4) containing 0.8% glutaraldehyde and 0.7% osmium tetroxide for 1 h in ice (Hirsch & Fedorko, 1968), dehydrated in alcohol, and embedded in Epox (Ernest F. Fullam Co., Latham, NY).

RESULTS

Fusogenic Activity of Multivalent Anions. The effect of various multivalent anions on liposomes composed of DOTMA/PE (1:1) was investigated by a fluorescence resonance energy transfer assay for lipid mixing. The "probe dilution" method was utilized, in which the fluorescent molecules NBD-PE and Rh-PE are incorporated in one population of vesicles and their dilution into a 4-fold larger population of unlabeled vesicles during membrane fusion is observed as the increase in NBD fluorescence (Düzgüneş et al., 1985, 1987b). The time course of fluorescence development for DOTMA/PE vesicles upon addition of various concentrations of EDTA is shown in Figure 1. Lipid mixing was induced at a threshold concentration of 8 mM EDTA. The initial rate of lipid mixing increased as the anion concentration was increased (Figure 2).

The effect of two other anions, citrate and phosphate (Na salts), on the initial rate of lipid mixing is shown in Figure 2. Citrate was the most effective multivalent anion in inducing fusion of DOTMA/PE vesicles. Addition of Ca^{2+} did not induce any lipid mixing. However, if 10 mM phosphate was added after preincubation of the vesicles with 10 mM Ca^{2+} , rapid fusion was observed (Figure 3). The initial rate of fusion was faster than that obtained in the presence of 20 mM phosphate alone. This effect may be attributed to the formation of crystalline calcium phosphate complexes at the membrane surface (Fraley et al., 1980).

The fusogenic nature of multivalent anions precluded the

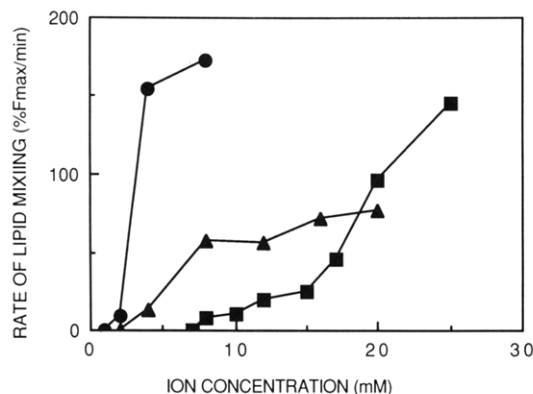


FIGURE 2: Initial rate of lipid mixing of DOTMA/PE (1:1) liposomes, given as the % F_{\max} /min, as a function of anion concentration: citrate (●), EDTA (▲), or phosphate (■). Other conditions were as in Figure 1.

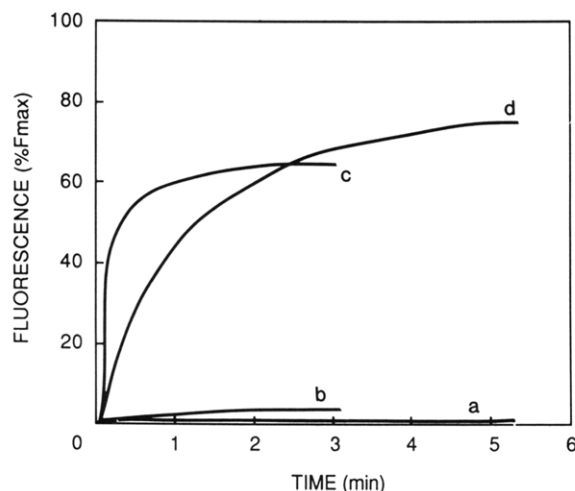


FIGURE 3: Time course of lipid mixing during fusion of DOTMA/PE (1:1) liposomes induced by 10 mM calcium (a), 10 mM phosphate (b), 10 mM phosphate with a 10-min prior incubation with 10 mM calcium (c), and 20 mM phosphate (d). Other conditions were as in Figure 1.

use of fluorescence assays for the intermixing of aqueous contents that employ encapsulation solutions such as terbium citrate or aminonaphthalenetrisulfonate. Stamatatos et al. (1988) have reported a similar problem with vesicles containing an analogue of DOTMA. To confirm that lipid mixing in the presence of anions was a result of membrane fusion, and not merely lipid transfer during aggregation of the vesicles or only the fusion of outer monolayers (Rosenberg et al., 1983; Ellens et al., 1985; Düzgüneş & Bentz, 1988), DOTMA/PE vesicles treated with citrate were observed with freeze-fracture and thin-section electron microscopy. As shown in Figure 4, the vesicles underwent extensive fusion, forming large multilayered vesicles.

The divalent anions sulfate and dipicolinate (Na salts) did not induce significant fusion at concentrations up to 40 mM (data not shown). Similarly, the monovalent anion sodium acetate was ineffective in mediating the fusion of DOTMA/PE vesicles.

Vesicles composed of DOTMA/PC did not exhibit any lipid mixing in the presence of up to 20 mM citrate or 40 mM phosphate (data not shown). However, they could be induced to fuse with DOTMA/PE vesicles in the presence of high concentrations of citrate, but not of phosphate (Figure 5). In these experiments the DOTMA/PE vesicles were labeled with NBD-PE and Rh-PE, and fusion was initiated by the injection of unlabeled DOTMA/PC vesicles into the fluorometer cell.

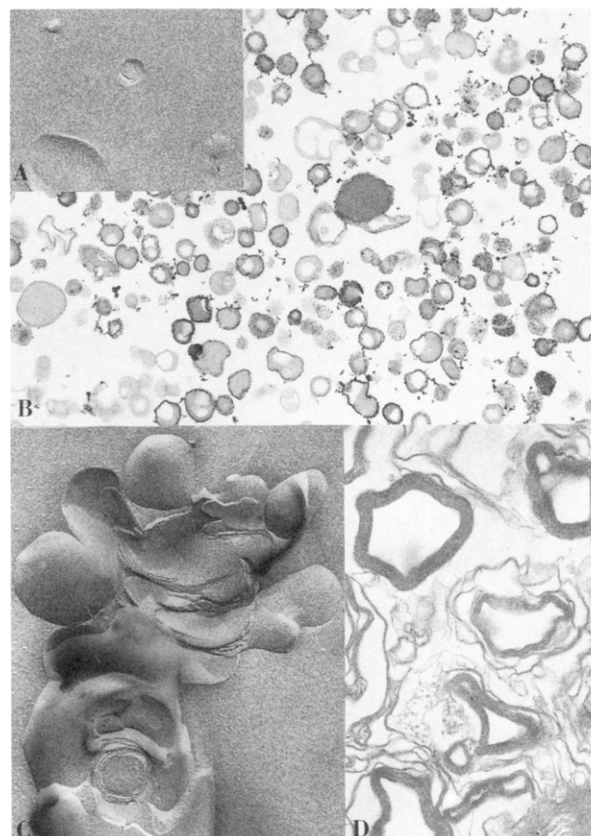


FIGURE 4: Freeze-fracture (A and C) and thin-section (B and D) electron micrographs of DOTMA/PE (1:1) liposomes in NaCl buffer (A and B) and DOTMA/PE liposomes treated with 10 mM citrate (C and D). The lipid concentration was 10 $\mu\text{mol/mL}$. The magnification was 24000 \times for (A) and (C) and 14000 \times for (B) and (D).

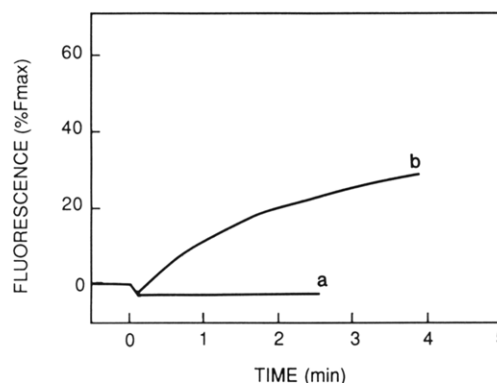


FIGURE 5: Time course of lipid mixing during fusion of labeled DOTMA/PE (1:1) liposomes with unlabeled DOTMA/PC liposomes induced by 20 mM citrate (b) or 40 mM phosphate (a). Other conditions were as in Figure 1.

Fusion of DOTMA-Containing Liposomes with Negatively Charged Phospholipid Vesicles. Because of the cationic nature of DOTMA, it was of interest to investigate whether liposomes containing this lipid could fuse with liposomes containing anionic lipids. Injection of unlabeled PS/PE (1:1) vesicles into a suspension of labeled DOTMA/PE vesicles resulted in rapid fusion, with the fluorescence intensity increasing up to about 30% F_{\max} . The fluorescence then plateaued (Figure 6). The vesicle suspension did not appear turbid at this stage and extensive aggregates did not form. Freeze-fracture electron microscopy revealed fused and aggregated vesicles (data not shown). Dynamic light scattering measurements indicated that the mean diameter (\pm SD) of the vesicle suspension increased from 150 ± 54 to 509 ± 170 nm within 5 min. It should be

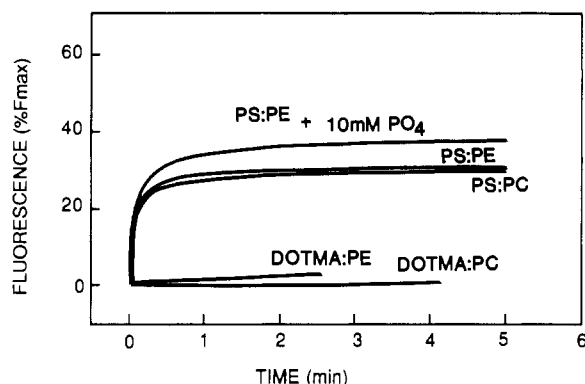


FIGURE 6: Time course of lipid mixing during fusion of labeled DOTMA/PE (1:1) liposomes with unlabeled PS/PE (1:1) liposomes (in the presence or absence of 10 mM phosphate), PS/PC (1:1) liposomes, DOTMA/PE (1:1) liposomes, or DOTMA/PC liposomes. Unlabeled vesicles (final concentration 40 nmol lipid/mL) were injected into a suspension of labeled vesicles (10 nmol of lipid/mL).

noted, however, that light scattering measurements do not distinguish between fusion products and vesicle aggregates.

Addition of 10 mM phosphate together with the PS/PE vesicles caused a slight increase in the extent of fusion (Figure 6). Control additions of DOTMA/PE or DOTMA/PC vesicles to the DOTMA/PE suspension in NaCl/TES buffer did not affect the fluorescence intensity. Addition of PS/PC vesicles to the DOTMA/PE vesicle suspension also resulted in membrane fusion to an extent similar to that obtained with PS/PE liposomes.

It was also of interest to establish whether PC-containing DOTMA liposomes, which did not fuse among themselves in the presence of multivalent anions, would undergo fusion with negatively charged liposomes and whether the zwitterionic lipid component of the latter would influence the fusion reaction. The addition of unlabeled PS-containing liposomes to a suspension of labeled DOTMA/PC (1:1) vesicles again resulted in fusion, as measured by the probe dilution assay, but the extent of fusion was considerably less in the case of PS/PC (1:1) vesicles ($11.5 \pm 1.6\% F_{\max}$) compared to that of PS/PE (1:1) vesicles ($26.8 \pm 2.9\% F_{\max}$).

DISCUSSION

Our results demonstrate that liposomes containing the cationic lipid DOTMA and PE can fuse with each other in the presence of multivalent anions, and with other liposomes containing negatively charged phospholipids. The effectiveness of the anions tested decrease in the sequence citrate > EDTA > phosphate >> sulfate. It is possible that monovalent anions such as acetate or Cl^- may induce aggregation at very high concentrations, analogous to the aggregating effect of Na^+ on PS vesicles at concentrations above 0.5 M (Day et al., 1980; Düzgüneş et al., 1987b); this point remains to be investigated. Carmona-Ribeiro et al. (1985) have reported that sonicated vesicles composed of the surfactant dioctadecyldimethylammonium chloride (DODAC), and prepared in glucose solutions, aggregate at a threshold concentration of 132 mM NaCl or 89 mM CaCl_2 and suggested that aggregation is controlled by Cl^- .

We have utilized a fluorescence assay for lipid mixing which we have shown previously not to be affected by the mere aggregation (without fusion) of phospholipid vesicles (Düzgüneş et al., 1987b). The change in resonance energy transfer between NBD and rhodamine in this assay is thus a reliable indicator of lipid mixing. Mixing of outer monolayers in the absence of intermixing of aqueous contents is also a

possibility which must be considered in experiments utilizing lipid mixing assays (Rosenberg et al., 1983; Bondeson & Sundler, 1985; Ellens et al., 1985; Düzgüneş & Bentz, 1988). In the case of DOTMA-containing vesicles, it was not possible to encapsulate any of the components of commonly used, and reliable, aqueous contents mixing assays, because of the negative charge they carry. It was also not possible to encapsulate carboxyfluorescein to monitor the membrane permeability during fusion. We therefore examined DOTMA/PE vesicles by electron microscopy under conditions where lipid mixing was observed with the fluorescence assay. Extensive fusion of vesicles was observed both in the presence of multivalent anions and upon addition, of negatively charged phospholipid vesicles, confirming the results of the lipid mixing assay.

Rupert et al. (1985, 1986) have shown that large (~ 300 -nm diameter) vesicles composed purely of the cationic surfactant didodecyltrimethylammonium bromide (DDAB) fuse in the presence of dipicolinic acid, sulfate, or *p*-toluenesulfonic acid at pH 6 in a solution of HEPES buffer only (without NaCl). DOTMA/PE vesicles, however, do not fuse in the presence of dipicolinate or sulfate. This difference may be accounted for by the different headgroup and acyl chain structures of the two cationic lipids, the presence of a zwitterionic lipid in the vesicles used in our studies, and the presence of 100 mM Cl^- in the medium. It will be of interest to compare the fusogenic properties of the two lipids under identical conditions. A remarkable observation made on the DDAB system is the vesicle size dependence of membrane fusion, vesicles of 200 nm or smaller diameter being unable to fuse despite aggregation in the presence of dipicolinic acid (Rupert et al., 1985, 1986). Clearly, DOTMA-containing vesicles of a smaller mean diameter do not have a comparable restriction. Nevertheless, the fusion susceptibility of vesicles composed of pure DOTMA needs to be investigated before a proper comparison can be made. Small vesicles composed of DODAC, however, appear to undergo slow fusion in the presence of NaCl (Carmona-Ribeiro et al., 1985).

In addition to partially neutralizing the surface charge of DOTMA/PE vesicles, multivalent anions are expected to form a complex between positively charged trimethylammonium groups on apposed bilayers. The formation of the complex is likely to be accompanied by the dehydration of the membrane surface due to replacement of the bound water by the anion-cation complex, as suggested by fluorescence studies on the DDAB/dipicolinic acid complex (Rupert et al., 1986). The interbilayer DOTMA-anion-DOTMA complex would be analogous to the dehydrated trans PS-Ca^{2+} -PS complex proposed earlier (Portis et al., 1979; Düzgüneş & Papahadjopoulos, 1983). The difference between the fusogenic activities of sulfate and phosphate ions is surprising, since both anions are expected to be divalent at neutral pH. However, the size of the anion and its binding constant to DOTMA would also be expected to influence its fusogenic activity (Bentz et al., 1983; Nir et al., 1983b). Further studies are necessary to quantitate the binding affinity of various anions for DOTMA and to examine the possibility of dehydration in the presence of the anions.

Substitution of PC for PE as the zwitterionic lipid component mixed with DOTMA renders the vesicles completely resistant to fusion with anions. This observation is analogous to the effect of PC on divalent cation induced fusion, when it is mixed with negatively charged phospholipids (Düzgüneş et al., 1981a,b; Sundler et al., 1981; Nir et al., 1983a; Düzgüneş, 1985), and indicates that the zwitterionic lipid plays a crucial role in DOTMA-mediated fusion. PE can partially

overcome the inhibitory effect of PC in the case of asymmetric fusion between DOTMA/PE and DOTMA/PC vesicles in the presence of citrate. However, here the citrate concentration necessary for fusion is considerably higher than that in the case of DOTMA/PE vesicles fusing among themselves. The inclusion of a high mole fraction of PE in negatively charged membranes was found to be essential for the induction of fusion by the polyvalent cations spermine or spermidine (Schuber et al., 1983).

One instance where PC does not appear to be inhibitory is the fusion of DOTMA/PE liposomes with PS-containing liposomes. Fusion with PS/PC or PS/PE proceeds with very similar kinetics (Figure 6). This result suggests that it is the negatively charged lipid and not the zwitterionic lipid that dominates the lipid mixing process in this asymmetric fusion system. Whether this effect of the negatively charged lipid is primarily at the aggregation or fusion step of the overall fusion process (Nir et al., 1983a) remains to be investigated. Fusion of cationic lipid vesicles with liposomes containing anionic lipids and PC has also been observed by Stamatas et al. (1988), who used an analogue of DOTMA as the positively charged lipid species. Our results also indicate that the interaction between the trimethylammonium on DOTMA and the carboxyl and phosphate groups on PS is sufficient to bring the two membranes into close proximity and to mediate the destabilization step leading to intermixing of lipids. Interaction between the positive charge and the zwitterionic headgroup on PC or PE is less likely to be involved in the process, since stable vesicles composed of mixtures of DOTMA with PC or PE can be prepared and do not fuse spontaneously. The PE component of the DOTMA/PE vesicles appears also to be involved in mediating fusion with negatively charged vesicles. When PE is replaced by PC in DOTMA-containing vesicles, the extent of fusion with negatively charged vesicles containing PC as the zwitterionic component is diminished substantially compared to fusion with PS/PE vesicles. Thus, when both of the vesicle types contain PC, fusion is inhibited. The observation that DOTMA/PC vesicles fuse with PS-containing vesicles is striking, since the former vesicles are completely resistant to fusion with each other in the presence of multivalent anions. It is likely that the surface of a PS-containing vesicle appears as a polyvalent anion to DOTMA/PC or DOTMA/PE vesicles.

The mechanism of interaction of DOTMA/PE vesicles with the plasma membrane of cultured cells is not known. It is possible that binding of DOTMA to gangliosides with negatively charged sialic acid residues near the surface of the lipid bilayer may lead to the destabilization of the PE component due to its low level of surface hydration on its own (Jendrasiak & Hasty, 1974; Lis et al., 1982; Düzgüneş et al., 1981a) and to subsequent destabilization of the plasma membrane. Diffuse Rh-PE fluorescence is observed on the surface of cultured cells after incubation with DOTMA/PE/Rh-PE vesicles (Felgner et al., 1987; J. Goldstein and N. Düzgüneş, unpublished data). The cytoplasmic delivery of highly charged fluorescent probes and macromolecules encapsulated in pH-sensitive liposomes with high PE content, via endocytosis in coated vesicles and subsequent destabilization of endosome membranes, has been proposed to involve a similar mechanism (Straubinger et al., 1985; Connor & Huang, 1985; Papahadjopoulos et al., 1987). We are currently investigating the intracellular fate of membrane markers and of fluorescent macromolecules encapsulated in DOTMA-containing vesicles interacting with various cultured cell lines and erythrocyte ghosts. We have observed substantial lipid mixing between the membranes of DOT-

MA/PE liposomes and erythrocyte ghosts and cultured cells. DOTMA-containing liposomes also appear to deliver their aqueous contents into the cytoplasm of cultured cells (J. A. Goldstein, D. Alford, and N. Düzgüneş, unpublished data). Recent studies have shown that, in addition to mediating transfection, DOTMA-containing liposomes facilitate the delivery of transcriptional regulatory proteins into cultured cells and that the liposome membrane undergoes fusion with the plasma membrane (R. Debs, L. Freedman, S. Edmunds, K. Gaensler, D. S. Friend, N. Düzgüneş, and K. Yamamoto, unpublished results).

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Proteolytic Fragments of the Nicotinic Acetylcholine Receptor Identified by Mass Spectrometry: Implications for Receptor Topography[†]

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ABSTRACT: A triple-state quadrupole or a tandem quadrupole Fourier-transform mass spectrometer was used to detect and sequence the peptides released by proteolytic cleavage of the acetylcholine receptor (AChR) from *Torpedo californica* electroplax. Fragments in mass range up to 3479 daltons were characterized on the above instrumentation and used to determine proteolytically accessible sites on the receptor. These data were consistent with the cleavage points determined for membrane-bound fragments of the same AChR samples using gas-phase microsequencing. Each subunit of the receptor is readily cleaved near the C-terminus in the region between the proposed transmembrane hydrophobic α -helices MIII and MIV. This region includes the putative regulatory phosphorylation sites and the amphipathic α -helix. Cleavage is also observed in the N-terminal domain, but occurs much more slowly than in the C-terminal region. No cleavage was detected in the middle third of the receptor, which includes the proposed transmembrane α -helices MI and MII. An evaluation of these data in terms of the transmembrane topography of the AChR peptides is consistent with a synaptic or extracellular disposition for the region between MIII and MIV.

Membrane ion channels are an extremely important class of membrane proteins that have an essential role in many biological processes. Unfortunately, the structure of ion channels and the events leading to their gating and voltage dependence are not yet understood at a molecular level. The nicotinic acetylcholine receptor (AChR)¹ is a ligand-gated ion channel that can be purified from the electric organ of *Torpedo californica* [for a review, see Changeux et al. (1984)]. In size, it is similar to other intrinsic ion channels, and it is

an ideal system for physical and chemical studies because of the relative ease with which nanomolar quantities of the protein can be purified. The linear structure has been obtained from cDNA sequences for each of the four subunits of *T. californica*

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¹ Abbreviations: AChR, acetylcholine receptor; BTX, bungarotoxin; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; MOPS, 3-(N-morpholino)propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid; PVDF, poly(vinylidene difluoride); egg PC, egg yolk phosphatidylcholine; DOPC, dioleoylphosphatidylcholine; POPA, palmitoylphosphatidic acid; DTT, dithiothreitol; CHS, cholesteryl hemisuccinate; FTMS, Fourier-transform mass spectrometry; TX-100, Triton X-100; DEAE, diethylaminoethyl; BSA, bovine serum albumin.