Shyamala, G., & Yeh, Y. F. (1975) *Biochem. Biophys. Res. Commun. 64*, 408-415.
Simons, S. S., Martinez, H. M., Garcea, R. L., Baxter, J. D., & Tomkins, G. M. (1976) *J. Biol. Chem. 251*, 334-343.
Spelsberg, T. C., Steggles, A. W., & O'Malley, B. W. (1971) *J. Biol. Chem. 246*, 4188-4197.
Spelsberg, T. C., Steggles, A. W., Chytil, F., & O'Malley, B. W. (1972) *J. Biol. Chem. 247*, 1368-1374.
van Eekelen, C. A. G., & van Venrooij, W. J. (1981) *J. Cell Biol. 88*, 554-563.
Wilson, E. M., & French, F. S. (1976) *J. Biol. Chem. 251*, 5620-5629.
Wilson, E. M., & French, F. S. (1979) *J. Biol. Chem. 254*, 6310-6319.
Wilson, E. M., & French, F. S. (1980) *J. Biol. Chem. 255*, 10946-10953.

Modulation of Membrane Fusion by Ionotropic and Thermotropic Phase Transitions†

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ABSTRACT: We have studied the relationship of ionotropic and thermotropic phase transitions to divalent cation induced fusion of large unilamellar phospholipid vesicles. Fusion was monitored by the Tb/dipicolinic acid fluorescence assay for the intermixing of internal aqueous contents of vesicles. The phase behavior of the membranes was followed by differential scanning calorimetry. (1) Sr^{2+} and Ba^{2+} shifted the phase transition temperature (T_c) of bovine brain phosphatidylserine vesicles from 6 to 27 and 31.5 °C, respectively. These cations induced vesicle fusion at temperatures above or below the T_c of that cation/phospholipid complex, indicating that an isothermal phase change from the liquid-crystalline to the gel phase is not a requirement for membrane fusion. (2) The temperature dependence of the initial rate of fusion of phosphatidylserine/dipalmitoylphosphatidylcholine (1:1) vesicles in the presence of Ca^{2+} exhibited a pronounced maximum at 17 °C, at the lower part of the broad phase transition endotherm whose T_c was about 25 °C; fusion was inhibited completely at 30 °C when the membrane was in the liquid-crystalline state. These observations suggest that molecular clusters rich in phosphatidylserine, formed when the membrane is in the phase transition region, allow the vesicles to fuse. (3) The fusion of phosphatidylserine/dimyristoylphosphatidylethanolamine (1:1) vesicles, whose T_c was also around 25 °C, had a different temperature dependence in that the initial rate increased sharply above the T_c, with a local maximum within the transition region. Phase separation of dimyristoylphosphatidylethanolamine was induced by Ca^{2+} but not by Mg^{2+}, although both ions induced fusion. The observation that phosphatidylserine/egg phosphatidylethanolamine (1:1) vesicles fused in the presence of Ca^{2+} or Mg^{2+} at temperatures below or above the lamellar to hexagonal (H_{II}) transition temperature of the phosphatidylethanolamine and that Mg^{2+} could induce fusion without causing a transition into the H_{II} phase suggests that this transition is not essential for membrane fusion. On the basis of all three systems, it is proposed that fusion occurs via defects in molecular packing and dehydration of the polar groups of phospholipids at the region of interbilayer contact.

The molecular mechanism by which divalent cations, in particular Ca^{2+}, induce the fusion of liposomes made of acidic phospholipids or their mixtures with zwitterionic lipids is not understood clearly. It has been proposed that the isothermal phase change induced by Ca^{2+} or Mg^{2+} in the bilayer membrane is the key event leading to the fusion of the liposomes and that this phase change causes the transient destabilization of the membranes which then fuse at the domain boundaries between fluid and solid phases in the same membrane (Papahadjopoulos et al., 1977). To test this proposal, it is necessary to correlate the isothermal phase change with the early kinetics of membrane fusion. Although Ca^{2+} causes extensive fusion of both small unilamellar vesicles (SUV;[1] approximately 30 nm in diameter) and large unilamellar vesicles (LUV; approximately 100 nm in diameter) (Papahadjopoulos et al., 1974, 1977; Wilschut et al., 1980), it shifts the transition temperature of PS bilayers to such high temperatures (greater than 100 °C; Portis et al., 1979; Newton, et al., 1978; Jacobson & Papahadjopoulos, 1975) that it is impossible to compare the kinetics of fusion above and below this transition temperature. The fusogenic effect of Mg^{2+} on PS membranes is strongly dependent on the curvature of the bilayer: SUV undergo a few rounds of fusion when the Mg^{2+} concentration exceeds a threshold value but stop fusing when they reach a

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[1] Abbreviations: PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; DPPC, dipalmitoylphosphatidylcholine; DMPE, dimyristoylphosphatidylethanolamine; DPA, dipicolinic acid; NTA, nitrilotriacetic acid; SUV, small unilamellar vesicles; LUV, large unilamellar vesicles; NBD, 7-nitro-2,1,3-benzoxadiazole; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

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limiting size (Wilschut et al., 1981). The same study also showed that when Mg^{2+} is added to LUV the vesicles aggregate but do not fuse. Mg^{2+} appears to have an intrinsic inability to fuse PS vesicles of large radii, and thus neither Ca^{2+} nor Mg^{2+} can be used conveniently to examine the role of isothermal phase changes in membrane fusion.

It has been reported recently that the other alkaline earth metals, Ba^{2+} and Sr^{2+}, also induce fusion of (SUV) PS (Düzgüneş et al., 1981a; Bentz et al., 1983a). The interaction of these ions with PS bilayers causes an upward shift (by about 20 °C) of the gel–liquid-crystalline phase transition temperature (T_c) of the Na^+ complex of PS, accompanied by an exothermic reaction (Düzgüneş et al., 1981a). These cations can thus be used to assess the effect of ionotropic phase changes on fusion since the T_c of their complexes with PS is within an experimentally accessible range. We have found that the rates of fusion increase exponentially above the phase transition temperature of the divalent cation/PS complex, suggesting that an isothermal phase change from the liquid-crystalline to the gel phase is not a prerequisite for fusion and that the increased fluidity of the bilayer enhances the rate of fusion.

The importance of a fluid bilayer in membrane fusion has been shown previously for the Ca^{2+}-induced fusion of dipalmitoylphosphatidylglycerol vesicles (Papahadjopoulos et al., 1974). It has also been suggested that the lateral segregation of the molecular components induced by Ca^{2+} in mixed phospholipid membranes could be involved in the fusion of these membranes by allowing the negatively charged components in apposed vesicles to interact with each other (Papahadjopoulos et al., 1974; Papahadjopoulos, 1978). Here we have studied the role of lateral phase separations in the fusion of membranes composed of mixtures of PS with dipalmitoylphosphatidylcholine (DPPC) or dimyristoylphosphatidylethanolamine (DMPE). Our results show a striking modulation of membrane fusion by temperature, especially in the phase transition region of PS/DPPC membranes. The initial rate and extent of fusion are greatly enhanced at temperatures below the midpoint of the transition endotherm. However, our studies also indicate that macroscopic phase separations, as observed by differential scanning calorimetry, are not strictly correlated with the induction of fusion. Some of these observations have been reported in preliminary form (Düzgüneş et al., 1981b, 1983a). Studies in which phase separation was monitored by the fluorescent quenching of NBD attached to the head group of phosphatidylethanolamine (PE) have also indicated that the rate of phase separation is slower than that of fusion (Hoekstra, 1982).

The phase transition of certain PE species from the lamellar to the hexagonal (H_{II}) phase has been suggested to be a crucial step in membrane fusion (Cullis & Verkleij, 1979; Hope & Cullis, 1979; Tilcock & Cullis, 1981). We have investigated this possibility by following the fusion of PS/egg PE vesicles in the presence of Ca^{2+} or Mg^{2+}, at temperatures above and below the temperature of this transition. We have found that fusion can be induced at temperatures well below this transition temperature and that Mg^{2+} also induces fusion although it does not cause transformation of the membrane into the H_{II} phase.

Experimental Procedures

Bovine brain PS and egg phosphatidylcholine (PC) were prepared as described by Papahadjopoulos & Miller (1967). DPPC was obtained either from Sigma (St. Louis, MO) and repurified by high-performance liquid chromatography or from Avanti Polar Lipids (Birmingham, AL). DMPE was from Fluka (Hauppauge, NY) and Avanti. Egg PE was purchased from Avanti. Lipids were stored under argon in sealed ampules at −40 °C. $TbCl_3$ was obtained from Ventron (Danvers, MA). Nitrilotriacetic acid (NTA) and dipicolinic acid (DPA) were purchased from Sigma. Cholate (Calbiochem) was recrystallized twice. $CaCl_2$, $MgCl_2$, $BaCl_2$, and $SrCl_2$ were from Fisher, and NaCl was from Mallinckrodt. Water was twice distilled, the second time in an all-glass apparatus.

Large unilamellar vesicles (0.1 μm in diameter) were prepared by reverse-phase evaporation (Szoka & Papahadjopoulos, 1978) with some modifications (Wilschut et al., 1980; Düzgüneş et al., 1983b). The PS/DMPE and PS/DPPC vesicles were prepared at 45 °C by the use of diisopropyl ether; the other vesicles were prepared at 30 °C with diethyl ether. The two populations of vesicles used for the fusion assay were prepared essentially as described by Wilschut et al. (1980). One population contained 2.5 mM $TbCl_3$, 40 mM NTA (Na salt), and 5 mM TES, pH 7.4 in the internal aqueous space. The other population contained 50 mM DPA (Na salt), 20 mM NaCl, and 5 mM TES, pH 7.4.

The fusion assay was performed as described previously (Wilschut et al., 1980). Maximal fluorescence (100%) was determined by lysing the appropriate concentration of Tb-containing vesicles with 0.5% sodium cholate (pH 7.4) in the presence of 20 μM DPA and sonicating for 5 min under argon in a bath-type sonicator to ensure the complete reaction of Tb and DPA. The assay was carried out in a quartz fluorometer cell in a total volume of 1 mL of 100 mM NaCl, 5 mM TES, and 0.1 mM EDTA, pH 7.4, and containing equimolar amounts of Tb- and DPA-containing vesicles, at a total lipid concentration of 50 μM (determined by lipid phosphorus; Bartlett, 1959). The fusion reaction was initiated by injecting a small aliquot of a concentrated solution of divalent cations into the cuvette, stirred continuously by a magnetic stirrer under the chamber. Complete mixing was achieved in 1 s. Fluorescence was measured at wavelengths above 530 nm by using a Corning 3-68 cutoff filter and an excitation wavelength of 276 nm in an SLM 4000 fluorometer; 90° light scattering at 276 nm was measured simultaneously in the second channel of the fluorometer. The release of internal aqueous contents was followed by the enhancement of carboxyfluorescein fluorescence (Wilschut et al., 1980; Düzgüneş et al., 1981c) and by monitoring the dissociation of preencapsulated Tb/DPA complex (1.25 mM $TbCl_3$, 20 mM NTA, 25 mM DPA, 10 mM NaCl, and 5 mM TES, pH 7.4). The dissociation is also a measure of the entry of divalent cations and EDTA into the vesicle interior and hence can be used to correct the fluorescence arising from the fusion of Tb vesicles with DPA vesicles (Bentz et al., 1983a). Temperature was maintained within 0.2 °C by circulating water through the metal jacket around the fluorometer cell. The pH of the solution was corrected for the effects of temperature.

For differential scanning calorimetry, LUV were prepared in 100 mM NaCl, 5 mM TES, and 0.1 mM EDTA. Ba^{2+} or Sr^{2+} was added to PS vesicles (1 μmol of lipid/mL) at 35 °C and incubated for 30 min. Ca^{2+} or Mg^{2+} was added to PS/DPPC or PS/DMPE vesicles at the temperatures indicated in the figure legends and incubated for 30 min. The vesicles were collected by centrifugation (aggregated vesicles at 10000g for 20 min; dispersed vesicles at 150000g for 3 h at 25 °C) and transferred to aluminum calorimeter pans which were then sealed hermetically. Thermograms were obtained in a Perkin-Elmer DSC-2 calorimeter at a scan rate of 5 °C/min and a sensitivity setting of 1 mcal/s.

Results

Fusion of Phosphatidylserine Vesicles Induced by Ba^{2+} and

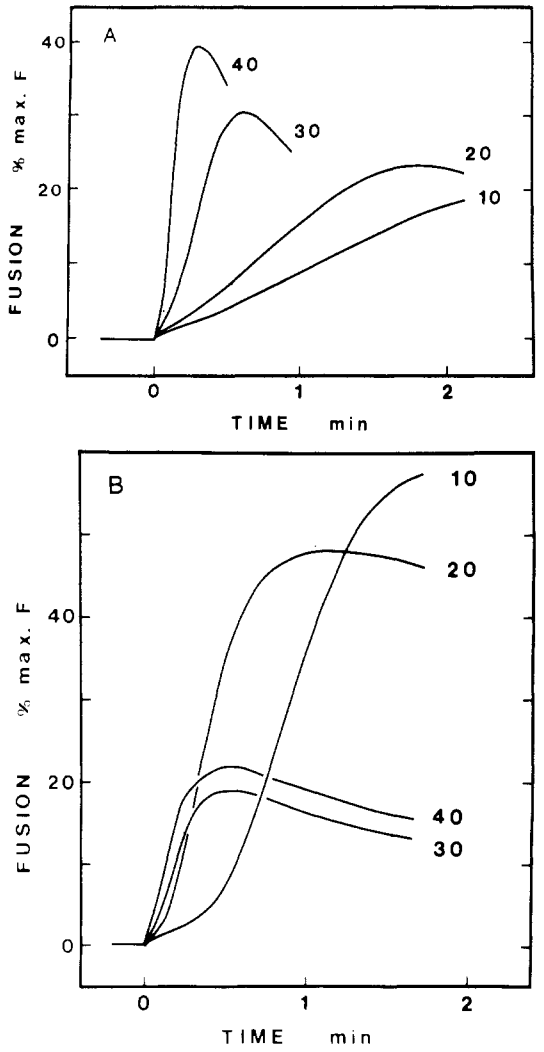

FIGURE 1: Fusion of phosphatidylserine vesicles induced by (A) 10 mM Sr^{2+} and (B) 3 mM Ba^{2+} at the indicated temperatures (in degrees centigrade). Tb vesicles and DPA vesicles were mixed in a 1:1 ratio at a total lipid concentration of 50 μM, in 100 mM NaCl, 5 mM TES, and 0.1 mM EDTA, pH 7.4. Fusion is given as the percent of maximal fluorescence obtained if all the encapsulated Tb were chelated by DPA. Divalent cations were injected at $t = 0$.

Sr^{2+}. The Tb/DPA fusion assay measures the enhancement in Tb fluorescence upon its interaction with DPA, each encapsulated in different populations of vesicles. The leakage of contents which may occur during fusion does not contribute to this fluorescence because the Tb/DPA reaction is inhibited outside the vesicles. During Ca^{2+}-induced fusion of vesicles, this inhibition is provided by the presence of EDTA and Ca^{2+} in the medium (Wilschut et al., 1980). For studies of fusion induced by Ba^{2+} or Sr^{2+}, we have confirmed that the Tb/DPA complex dissociated rapidly in the aqueous medium used in the fusion experiments (100 mM NaCl, 5 mM TES, pH 7.4, and 0.1 mM EDTA with either 3 mM Ba^{2+} or 10 mM Sr^{2+}) (data not shown).

The time course of the fusion of LUV (PS) induced by 10 mM Sr^{2+} at various temperatures is shown in Figure 1A. Fusion was observed at all temperatures between 10 and 40 °C, and the rate and extent of fusion increased with temperature. Figure 1B displays a similar set of results for 3 mM Ba^{2+}. The initial rate of fusion again increased with temperature; however, the apparent extent of fusion was considerably higher at the lower temperatures. The slower dissociation of the preencapsulated Tb/DPA complex at the lower temperatures (results not shown) is consistent with this observation. The initial rate of dissociation is 0% maximum fluorescence per minute and thus does not affect the measurement of the fusion rate in the fusion assay.

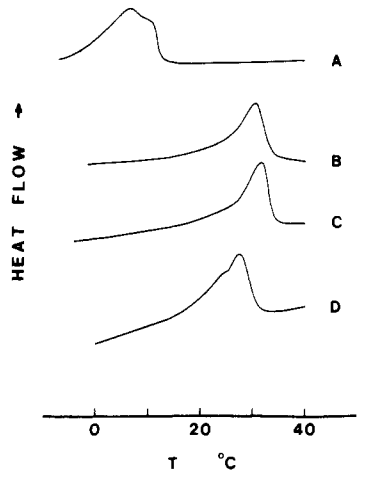

FIGURE 2: Differential scanning calorimetry scans of phosphatidylserine vesicles in the presence of divalent cations. (A) Untreated vesicles in the presence of Na^+ only; (B) 2 mM Ba^{2+}; (C) 10 mM Ba^{2+}; (D) 10 mM Sr^{2+}. The arrow shows the direction of heat flow in an endothermic transition.

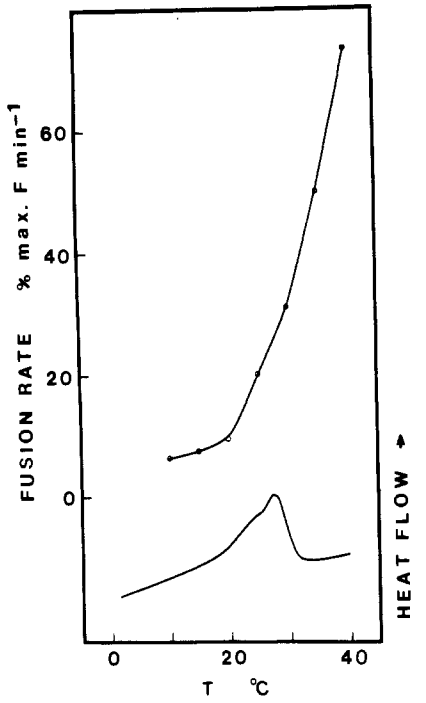

FIGURE 3: Initial rate of fusion of phosphatidylserine vesicles induced by 10 mM Sr^{2+} as a function of temperature (upper curve) and the differential scanning thermogram of the vesicles in the presence of 10 mM Sr^{2+} (lower curve). The initial rate is given as the percent maximum fluorescence per minute.

To examine the relationship of the temperature dependence of fusion to the phase transition of these membranes, we have studied the phase behavior of PS vesicles in the presence and absence of divalent cations by differential scanning calorimetry (Figure 2). In 100 mM NaCl, pH 7.4, PS vesicles displayed a broad, endothermic transition between −5 and 12 °C with a peak temperature, T_c, of 6 °C (scan A). In the presence of 10 mM Sr^{2+}, the transition temperature shifted to 27 °C (scan D). With 10 mM Ba^{2+}, the T_c occurred at 31.5 °C (scan C). The relationship of the gel–liquid-crystalline transition of the metal ion/PS complex to membrane fusion is shown in Figure 3 for the case of 10 mM Sr^{2+}. Here the initial rate of fusion of PS vesicles (upper curve) is plotted as a function

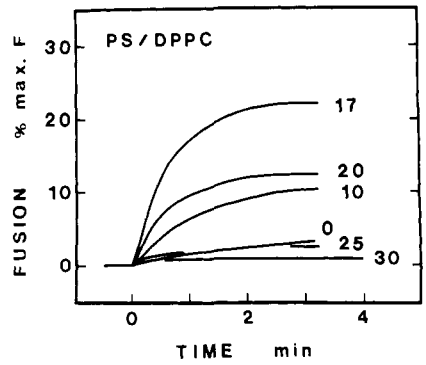

FIGURE 4: Fusion of vesicles composed of phosphatidylserine/dipalmitoylphosphatidylcholine (1:1) induced by 10 mM Ca^{2+} at different temperatures (in degrees centigrade).

of temperature, together with the differential scanning thermogram (lower curve) of the Sr^{2+}/PS complex. Clearly, the rate of fusion increased exponentially as the membrane became more fluid. A slow rate of fusion was observed at temperatures in which the PS vesicles had undergone an isothermal phase transition (from T_c = 6 °C in Na^+ to T_c = 27 °C in Na^+ and Sr^{2+}), for example, at 15 °C. However, the rate of fusion was much faster at temperatures where the Sr^{2+}/PS complex was in the liquid-crystalline state. A similar observation was made for the Ba^{2+}-induced fusion. Hence, for these cations, the induction of an isothermal phase transition is not a necessary requirement for fusion.

Fusion of Phosphatidylserine/Dipalmitoylphosphatidylcholine Vesicles in the Presence of Ca^{2+}. We have demonstrated previously that LUV composed of egg PC mixed with PS at an equimolar ratio do not fuse in the presence of Ca^{2+}, although the vesicles aggregate (Düzgüneş etal., 1981c). When the egg PC component was replaced by DPPC, however, the fusion behavior of these vesicles was altered drastically. Figure 4 shows the time course of the fusion of PS/DPPC (1:1) vesicles induced by 10 mM Ca^{2+} at various temperatures. Fusion was inhibited completely at 30 °C in spite of extensive aggregation, but at lower temperatures, considerable fusion occurred. When the initial rate of fusion induced by 5 and 10 mM Ca^{2+} was plotted as a function of temperature (Figure 5), there was a pronounced maximum at 17 °C. Although the rate of fusion again increased above 40 °C, its extent was less than 5% maximum fluorescence (data not shown). No fusion was detectable between 0 and 50 °C with PS/egg PC (1:1) vesicles (data not shown). Differential scanning calorimetry of the PS/DPPC (1:1) vesicles showed a broad gel–liquid-crystalline phase transition endotherm with a T_c of about 25 °C. At 30 °C, the bilayer was in the liquid-crystalline state. The peak fusion temperature (17 °C) corresponded to the lower end of the endotherm. In the presence of 10 mM Ca^{2+}, the endotherm shifted upward by about 3 °C (Figure 5, dashed line of upper curve). Identical calorimetry scans were obtained with vesicles incubated with Ca^{2+} at 0, 16, or 40 °C (data not shown).

The absence of Tb/DPA fluorescence at 30 °C could be ascribed to the possible loss of internal contents into the medium without any intermixing. We therefore investigated the release of carboxyfluorescein (Portis et al., 1979; Wilschut et al., 1980, 1983) and the dissociation of the Tb/DPA complex (Bentz et al., 1983a; Nir al., 1983a) preencapsulated in these liposomes. No appreciable release or dissociation occurred over a period of 2 min following Ca^{2+} addition (data not shown). After this period, a slow dissociation of the Tb/DPA complex occurred, probably as a result of the entry of Ca^{2+} and EDTA into the vesicle interior. No release of contents

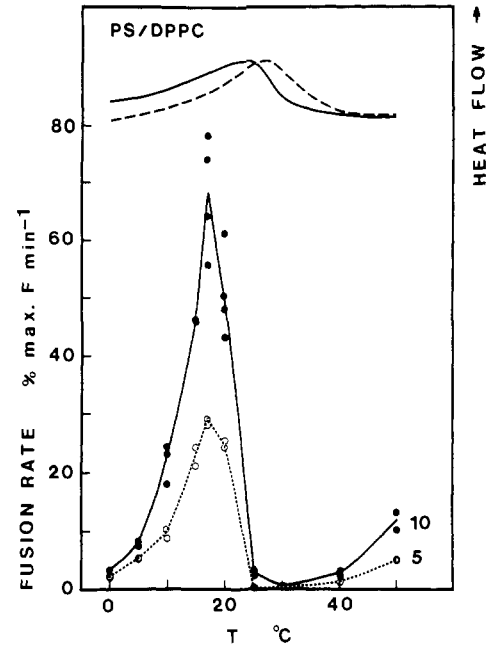

FIGURE 5: Initial rate of fusion of phosphatidylserine/dipalmitoylphosphatidylcholine (1:1) vesicles in the presence of 5 or 10 mM Ca^{2+} as a function of temperature (lower curves) and their differential scanning calorimetry thermograms in the absence (solid line) and presence (dashed line) of 10 mM Ca^{2+} (upper curves).

was observed during this time, indicating that the vesicles were stable.

Fusion of Phosphatidylserine/Dimyristoylphosphatidylethanolamine Vesicles in the Presence of Ca^{2+} or Mg^{2+}. Vesicles composed of a 1:1 mixture of PS with phosphatidylethanolamine (PE) transphosphatidylated from egg PC exhibit a different fusion susceptibility compared to PS/PC (1:1) vesicles in that they are readily induced to fuse by the presence of Ca^{2+} (Düzgüneş et al., 1981c). Furthermore, although Mg^{2+} is unable to induce fusion of LUV composed of pure PS (Wilschut et al., 1981), it does cause fusion in PS/PE vesicles (Düzgüneş et al., 1981c). We therefore investigated the temperature dependence of the Ca^{2+}- and Mg^{2+}-induced fusion of PS/DMPE (1:1) membranes which have a phase transition at a temperature similar to that of PS/DPPC membranes. The kinetics of the fusion of LUV (PS/DMPE) in the presence of 3 mM Ca^{2+} are shown in Figure 6. Although the initial rate of fusion at 0 °C was very low, the extent of fusion was close to that obtained at 40 °C. The extent of fusion was considerably greater at 10 and 20 °C. At these temperatures, the dissociation of the preencapsulated Tb/DPA complex was considerably slower than at the higher temperatures. However, the initial rates of dissociation at all temperatures were 0% of maximum fluorescence per minute and hence did not affect the measurement of the fusion rate.

The initial rates of fusion plotted as a function of temperature (Figure 7) showed a local maximum just below the T_c of the mixture and a minimum just above it; however, this was not obvious in the case of 5 mM Ca^{2+} where the fusion rates were considerably higher. The initial rates of fusion in the presence of 10 mM Mg^{2+} exhibited a behavior similar to 3 mM Ca^{2+}. When the membrane was completely in the liquid-crystalline state, the fusion rates in all three cases increased sharply with temperature (Figure 7).

The thermotropic behavior of PS/DMPE vesicles in the presence of Ca^{2+} or Mg^{2+} is shown in Figure 8. Scan A was obtained without Ca^{2+} or Mg^{2+}. If Ca^{2+} was added to the vesicles at 0 °C and all subsequent operations such as centrifugation and loading of the calorimeter pans were carried

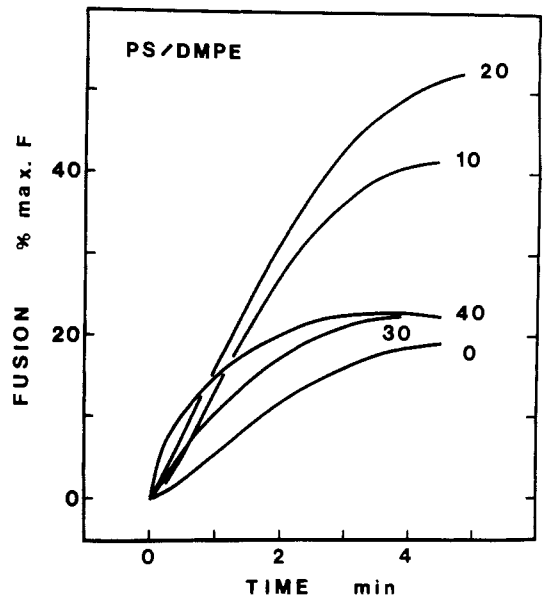

FIGURE 6: Fusion of phosphatidylserine/dimyristoylphosphatidylethanolamine (1:1) vesicles in the presence of 3 mM Ca^{2+} at the indicated temperatures (in degrees centigrade).

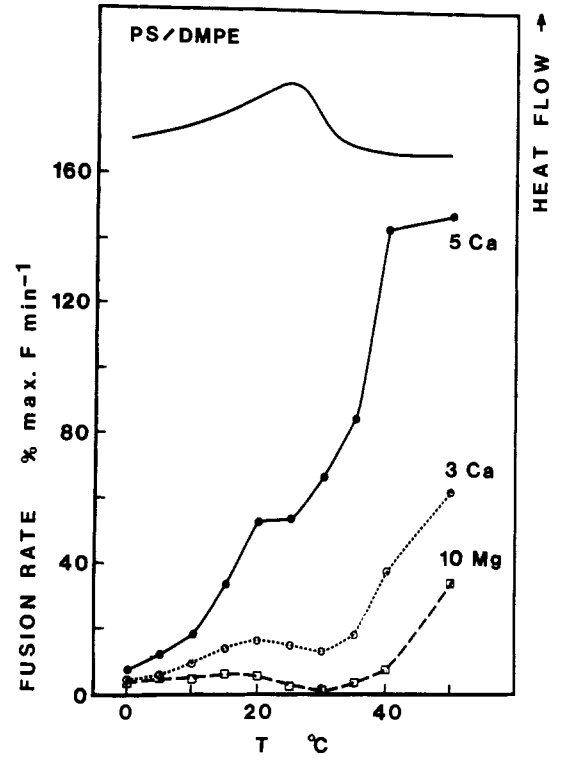

FIGURE 7: Initial rates of fusion of phosphatidylserine/dimyristoylphosphatidylethanolamine (1:1) vesicles in the presence of 3 (open circles) or 5 mM (filled circles) Ca^{2+} or 10 mM Mg^{2+} (open squares) as a function of temperature (lower curves). The upper curve is the differential scanning calorimetry scan of these vesicles in the absence of divalent ions.

out at 0–4 °C, and the sample was heated for the first time during the calorimeter scan, an endothermic peak was observed at 32 °C followed by an exothermic peak around 36 °C (scan B). The peak at 48 °C corresponding to DMPE became more pronounced following cooling and reheating, but the other peaks did not reappear (scan C). We tentatively ascribe the endotherm at 32 °C to the melting of the Ca^{2+}/phospholipid complex and the exotherm to the transformation of the acyl chains from a hexagonal close-packed configuration to the orthorhombic perpendicular packing observed in the case of

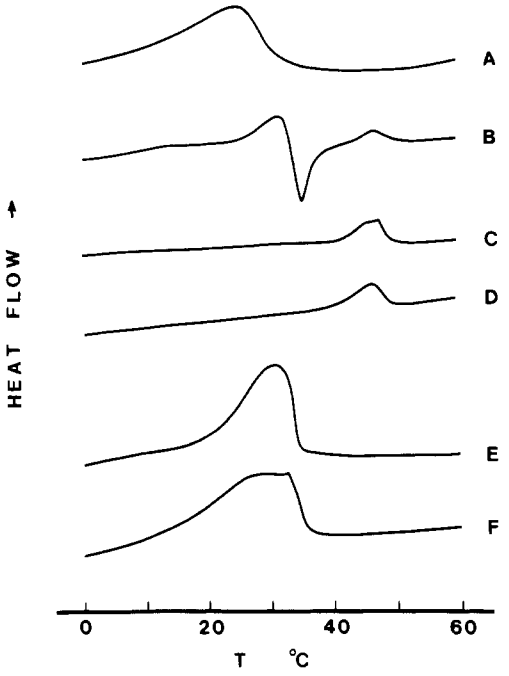

FIGURE 8: Differential scanning calorimetry of phosphatidylserine/dimyristoylphosphatidylethanolamine (1:1) vesicles incubated with 10 mM Ca^{2+} or Mg^{2+} at 0 or 40 °C. (A) Vesicles in Na^{+} only; (B) vesicles incubated with 10 mM Ca^{2+} at 0 °C and heated for the first time in the calorimeter; (C) the sample in (B) heated for the second time; (D) vesicles incubated with 10 mM Ca^{2+} at 40 °C; (E) vesicles incubated with 10 mM Mg^{2+} at 0 °C and heated for the first time in the calorimeter; (F) vesicles incubated with 10 mM Mg^{2+} at 40 °C.

the "trans" Ca^{2+}/PS complex (Portis et al., 1979). The extensive phase separation of the membrane may be associated with the formation of such a complex. If Ca^{2+} was added at 40 °C, only a single endothermic peak at 48 °C was observed (scan D). The addition of Mg^{2+} at 0 or 40 °C did not cause any observable phase separation (scans E and F) but only a shift in the T_c to a slightly higher temperature (from 25 to 31 °C).

Fusion of Phosphatidylserine/Egg Phosphatidylethanolamine Vesicles Induced by Ca^{2+} or Mg^{2+}. Above the gel–liquid-crystalline transition temperature of certain PE species, a second transition from the bilayer into the hexagonal (H_{II}) phase takes place (Reiss-Husson, 1967; Cullis & de Kruijff, 1978). DMPE, which was used in the experiments described in the previous section, does not transform into the H_{II} phase in the physiological temperature range (Harlos & Eibl, 1981; Wilkinson & Nagle, 1981). Since egg PE shows a bilayer–hexagonal phase transition around 28 °C (Cullis & de Kruijff, 1978), it was of interest to investigate whether this transition influences the rate of divalent cation induced fusion. Although PE would not be expected to undergo this transition when mixed with PS, the phase separation caused by Ca^{2+} has been shown to transform the PE part of the membrane into the hexagonal phase (Cullis & Verkleij, 1979). Mg^{2+} does not induce a phase separation of the two phospholipid components, and it does not induce a bilayer–hexagonal transformation (Cullis & Verkleij, 1979; Tilcock & Cullis, 1981).

The time course of the fusion of PS/egg PE (1:1) vesicles in the presence of 5 mM Ca^{2+} or 8 mM Mg^{2+} at different temperatures is shown in panels A and B, respectively, of Figure 9. It is noteworthy that 5 mM Ca^{2+} could induce fusion even at 1 °C (Figure 9A), well below the bilayer–hexagonal H_{II} transition temperature, and that this rate was faster than the fusion of vesicles composed of pure PS at this

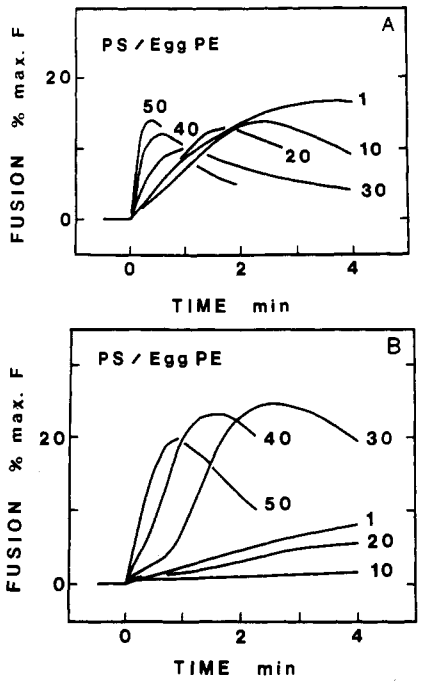

FIGURE 9: Fusion of phosphatidylserine/egg phosphatidylethanolamine (1:1) vesicles in the presence of (A) 5 mM Ca^{2+} or (B) 8 mM Mg^{2+} at the indicated temperatures (in degrees centigrade).

temperature (J. Wilschut, N. Düzgüneş, D. Hoekstra, and D. Papahadjopoulos, unpublished results). The extent of fluorescence obtained in this system was considerably less than that in PS/DMPE vesicles, especially at the lower temperatures. It was shown in parallel experiments with carboxyfluorescein-containing PS/egg PE vesicles that no aqueous contents were released into the medium at the early stages of fusion. Thus, the initial rate of fluorescence increase reflects accurately the rate of intermixing of aqueous contents and, hence, of fusion. The time of onset of release decreased as the temperature was increased; for example, 20 s after the addition of 5 mM Ca^{2+} at 5 °C and 5 s at 40 °C. Once the release started, it proceeded rapidly. The release was considerably slower in the case of Mg^{2+} (data not shown). This observation could explain the apparent higher extent of fusion in the presence of Mg^{2+} (Figure 9).

The initial rates of fusion are shown in Figure 10 as a function of temperature. It should be noted that since leakage is negligible at early times, the initial rate of fusion need not be corrected for the leakage of the reactants of the fusion assay. The temperature dependence of the Ca^{2+}-induced fusion was very similar to that of Mg^{2+}, except that the rates were higher. In the case of Mg^{2+} (Figure 9B), fusion did occur at 1 °C, was inhibited at 10 °C, and increased again above this temperature. A similar decrease in the initial rate of fusion at 10 °C was also observed for Ca^{2+} (Figure 10). At 30 °C, a pronounced biphasic behavior was observed in the kinetics of Mg^{2+}-induced fusion (Figure 9B).

Discussion

In this paper, we have examined the questions of whether the isothermal phase change induced by divalent cations in acidic phospholipid membranes and the bilayer to hexagonal H_{II} phase transition in PE-containing vesicles are requirements for the initiation of membrane fusion. Our results indicate that fusion can be induced by Ba^{2+} or Sr^{2+} at temperatures where the membrane is in the liquid-crysalline state before and after the addition of the divalent cations, for example, at 35 °C. At this temperature, the membrane has not undergone

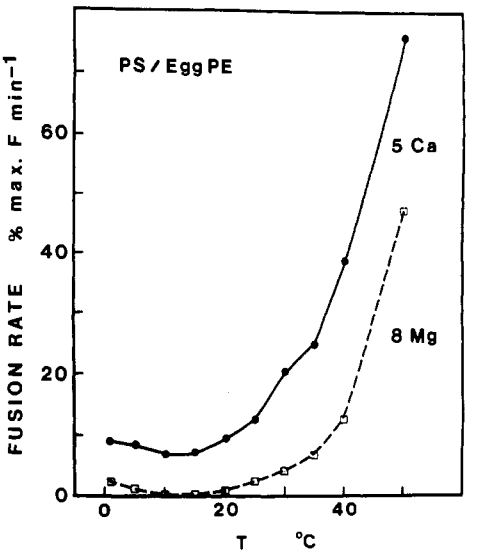

FIGURE 10: Initial rate of fusion of phosphatidylserine/egg phosphatidylethanolamine (1:1) vesicles induced by 5 mM Ca^{2+} or 8 mM Mg^{2+} as a function of temperature.

a transition from the liquid-crystalline to the gel state, and therefore, fusion has occurred without the necessity of an isothermal phase transition. X-ray diffraction studies have indicated that there are indeed no detectable domains of crystalline (gel state) Ba^{2+}/PS complex above the T_c (G. W. Feigenson, personal communication). At 15 °C, the membrane has indeed undergone such a transition, but the fusion rate is considerably slower than at temperatures above the transition temperature of the divalent cation/PS complex.

Previous studies have suggested that the formation of a trans Ca^{2+}/PS complex between two apposed membranes leads to the crystallization of the acyl chains and that the domain boundaries between the solid and fluid domains could be the points of membrane fusion (Portis et al., 1979; Papahadjopoulos et al., 1978). Our observation that Ba^{2+} and Sr^{2+} can induce fusion when the metal ion/PS complex is in the liquid-crystalline state suggests that if a trans metal ion/PS complex is necessary for fusion, it may still form without concomitant crystallization of the acyl chains. Recent Raman spectroscopic evidence indicates that the Ba^{2+} and Sr^{2+} complexes of dimyristoyl-PS have an intermediate head-group hydration between the Na^+ and Ca^{2+} complexes (Sheridan & Düzgüneş, 1983), which suggests that the nature of the trans complexes in these cases may be different from the anhydrous Ca^{2+}/PS complex.

The isothermal phase changes induced by divalent cations have been detected in this study by differential scanning calorimetry. The calorimetry scans reflect phase transitions in phospholipid membrane/divalent cation systems which have reached equilibrium after undergoing extensive aggregation and fusion. However, fusion has been monitored as a kinetic process. It is possible that there are differences in phase state between vesicles which are dispersed, vesicles which are in the process of aggregating and fusing, and membranes which have reached equilibrium after interaction with the divalent cations. The rate of ionotropic phase transitions is likely to be fast; Strehlow & Jähnig (1981) have found a relaxation time on the order of 10 ms for the Ca^{2+}-induced phase transition of dimyristoylmethylphosphatidic acid. However, intermembrane contact may affect the extent of the upward shift in T_c and the mode of ion binding to the membrane (Portis et al., 1979; Ekerdt & Papahadjopoulos, 1982; Düzgüneş & Papahadjopoulos, 1983).

The initial rates of fusion reflect the rates of both the aggregation step preceding fusion and the fusion reaction per se (Nir et al., 1982, 1983a,b; Bentz et al., 1983a,b). The rates given in the present study pertain to the overall process. It would be important to resolve the rates of the fusion reaction at temperatures below and above the phase transition region, with respect to the aggregation and fusion steps separately.

Our results, taken together with previous studies on the effect of Mg^{2+} and Ca^{2+} on the phase behavior of PS membranes (Jacobson & Papahadjopoulos, 1975; Newton et al., 1978; Portis et al., 1979), establish the sequence of effectiveness of divalent cations in increasing the phase transition temperature of PS membranes as $Ca^{2+} > Ba^{2+} > Sr^{2+} > Mg^{2+}$. This sequence differs from the effectiveness sequence of these ions in inducing the fusion of PS/PC vesicles (SUV) with PS monolayers (Ohki & Düzgüneş, 1979) or of SUV (PS) with each other (Düzgüneş et al., 1981a; Ohki, 1982; Bentz et al., 1983a), $Ba^{2+} > Ca^{2+} > Sr^{2+} > Mg^{2+}$, assessed by the bulk concentration of the divalent cations which induce fusion on a time scale of seconds to minutes. Recent studies have shown, however, that when the amount of divalent cation bound per PS molecule in a nonaggregated membrane is considered, Ca^{2+} is more effective than Ba^{2+}; that is, less Ca^{2+} is bound than Ba^{2+} at the threshold of fusion of SUV (PS) (Bentz et al., 1983a). It will be of interest to determine whether the binding of Ba^{2+} or Sr^{2+} to PS is enhanced upon intermembrane contact, as is the binding of Ca^{2+} (Portis et al., 1979; Rehfeld et al., 1981; Ekerdt & Papahadjopoulos, 1982).

An additional aim of this study has been to elucidate the role of the membrane phase state in divalent cation induced membrane fusion. The experiments with PS/DPPC vesicles show that when the membrane is in a fluid state, fusion is completely inhibited. The PS and DPPC molecules would be expected to be well dispersed in the plane of the membrane at this temperature, so that the fusion susceptibility of the membrane would be similar to that of PS/egg PC vesicles, which are fluid at the temperature range studied and do not fuse (Düzgüneş et al., 1981c). However, when the temperature is within the phase transition region of the PS/DPPC membrane, these vesicles fuse in the presence of Ca^{2+} (Figure 5). A tentative explanation for this observation is that the membrane is laterally phase separated into PS-rich and DPPC-rich domains, particularly within the broad phase transition (Shimshick & McConnell, 1973; Luna & McConnell, 1977; Stewart et al., 1979; Hui, 1981). Indeed, freeze-fracture electron micrographs of PS/DPPC (1:1) liposomes quenched from 18 °C reveal coexisting terraced, banded, and smooth fracture faces (Stewart et al., 1979) which probably arise from lipid clusters of varying composition. Furthermore, the observation that at low temperatures (0–10 °C) the rate of fusion of PS/DPPC vesicles is similar to that of pure PS vesicles (J. Wilschut et al., unpublished results) lends support to this explanation. Thus, the domains rich in PS would undergo fusion if they were to come in contact with other such domains in apposed membranes. This observation may have important implications for the control of membrane fusion by biological membranes in which lipid domains may exist (Bearer & Friend, 1980; Karnovsky et al., 1982; Severs & Robenek, 1983). Molecular factors such as proteins and divalent cations, which may regulate the formation of these domains, could also influence the fusogenicity of the membranes (Papahadjopoulos, 1978; Düzgüneş et al., 1980, 1984).

In contrast to PS/DPPC membranes, the fusion of PS/DMPE membranes is enhanced monotonically above the T_c of the mixture. Fusion is observable starting at temperatures close to the lower end of the phase transition endotherm where most of the membrane is in the solid (gel) state. The difference in the temperature dependence of fusion between the PS/DPPC and PS/DMPE vesicle systems may be attributed to the differences in hydrogen bonding, water of hydration, relative configuration of the head group and hydrocarbon chains, and interbilayer repulsive forces (Hauser et al., 1981; Jendrasiak & Hasty, 1974; Lis et al., 1982); these differences in the physicochemical properties between PC and PE in relation to membrane fusion have been discussed previously (Düzgüneş et al., 1981c; Nir et al., 1983b).

Concerning the possible role of the bilayer to hexagonal phase transition of PE in membrane fusion, it is important to note that PS/egg PE vesicles fuse at temperatures where the PE would be in the lamellar phase even if phase separated from the PS. The initial rates of fusion in the presence of Ca^{2+} or Mg^{2+} do not exhibit a discontinuity at the temperature of this transition. The fact that Mg^{2+} can induce the fusion of PS/egg PE vesicles (Figure 9B) without causing a phase transition into the hexagonal phase (Cullis & Verkleij, 1979; Tilcock & Cullis, 1981) indicates that this transition is not strictly necessary for membrane fusion. In addition, DMPE appears to facilitate membrane fusion without undergoing a bilayer to hexagonal phase transition within the physiological temperature range (Harlos & Eibl, 1981; Wilkinson & Nagle, 1981; Sundler et al., 1981).

Extensive and complete lateral phase separation in phospholipid mixtures can be observed by differential scanning calorimetry as the appearance of endotherms characteristic of the individual phospholipids (Papahadjopoulos et al., 1974; Jacobson & Papahadjopoulos, 1975; van Dijck et al., 1978). Phase separations occurring transiently or on a smaller scale, such as the formation of local domains enriched with one of the phospholipid species, may be difficult to detect with calorimetry. Nevertheless, phase diagrams may be utilized to estimate the proportion of the components which are in the liquid-crystalline phase and the gel phase, when the system is at a temperature within the phase transition region (Lee, 1977). Recently Hui et al. (1983) have produced evidence for microdomains in PS/DPPC membranes in the presence of Ca^{2+} by means of microprobe x-ray analysis and ^{31}P NMR. Sensitive and rapid spectroscopic techniques may be necessary to detect local and transient phase separations or phase transitions which may be involved in membrane fusion.

The relationship of the macroscopic (complete) lateral phase separations of phospholipid mixtures and the induction of membrane fusion is complex. Ca^{2+} induces both fusion and phase separation in PS/DMPE vesicles, whereas Mg^{2+} induces fusion but no phase separation (Figure 8). Similar to the latter case, Ca^{2+} can induce fusion of PS/DPPC vesicles without causing complete phase separation observable in calorimetry scans. Previous studies had utilized small unilamellar vesicles composed of PS/DPPC mixtures in which Ca^{2+} (but not Mg^{2+}) does induce a complete lateral phase separation and fusion (Papahadjopoulos et al., 1974). Massive or complete phase separation of PS from DPPC or DMPE thus does not appear to be necessary for membrane fusion.

Conclusion

Membrane fusion can occur locally at points of contact between vesicles, such that the number of phospholipids involved in the reaction may be a very small fraction of the total phospholipid content of the vesicles. The observation of divalent cation induced phase transitions at equilibrium, such as the crystallization of PS membranes (Papahadjopoulos et al., 1977; Portis et al., 1979) or the formation of lipidic par-

ticles or the hexagonal H_{II} phase in cardiolipin membranes (Rand & Sengupta, 1972; Vail & Stollery, 1979; Verkleij et al., 1979), does not necessarily imply that such transitions occur on a time scale of the fusion reaction. Indeed, quick freezing, freeze–fracture electron microscopy of cardiolipin/PC vesicles fusing in the presence of Ca^{2+} has indicated that lipidic particles are not necessary intermediates in membrane fusion although they are observed after long-term incubation with Ca^{2+} (Bearer et al., 1982). Intermediate structures at the site of membrane fusion may be too transitory or too small to be visualized in morphological studies. In mixed phospholipid membranes, the different molecular packing arrangements of the acidic and zwitterionic phospholipids in the presence of divalent cations could create local defects, exposing the hydrophobic interior of the bilayers at the points of contact between two membranes (Düzgüneş et al., 1984). The dehydration of the polar groups of specific phospholipids at the region of adhesion may also be involved in allowing molecular contact and producing discontinuities in molecular packing (Portis et al., 1979; Wilschut et al., 1981; Hoekstra, 1982). The lipids of the two membranes would then intermix at these defect points and form a continuous bilayer. If the local perturbations are dependent on intermembrane contact, once the two vesicles fuse to form one vesicle, the conditions for fusion would be eliminated momentarily until another vesicle established contact with the fusion product.

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Registry No. DPPC, 2644-64-6; DMPE, 20255-95-2; Ba, 7440-39-3; Sr, 7440-24-6; Ca, 7440-70-2; Mg, 7439-95-4.

References

Bartlett, G. R. (1959) *J. Biol. Chem. 234*, 466–468.

Bearer, E. L., & Friend, D. S. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 6601–6605.

Bearer, E. L., Düzgüneş, N., Friend, D. S., & Papahadjopoulos, D. (1982) *Biochim. Biophys. Acta 693*, 93–98.

Bentz, J., Düzgüneş, N., & Nir, S. (1983a) *Biochemistry 22*, 3320–3330.

Bentz, J., Nir, S., & Wilschut, J. (1983b) *Colloids Surf. 6*, 333–363.

Cullis, P. R., & de Kruijff, B. (1978) *Biochim. Biophys. Acta 513*, 31–42.

Cullis, P. R., & Verkleij, A. J. (1979) *Biochim. Biophys. Acta 552*, 546–551.

Düzgüneş, N., & Papahadjopoulos, D. (1983) in *Membrane Fluidity in Biology* (Aloia, R. C., Ed.) Vol. 2, pp 187–216, Academic Press, New York.

Düzgüneş, N., Hong, K., & Papahadjopoulos, D. (1980) in *Calcium Binding Proteins and Calcium Function* (Siegel, F. L., Carafoli, E., Kretsiner, R. H., MacLennan, D. H., & Wasserman, R. H., Eds.) pp 17–22, Elsevier/North-Holland, New York.

Düzgüneş, N., Rehfeld, S. J., Freeman, K. B., Newton, C., Eatough, D. J., & Papahadjopoulos, D. (1981a) Abstracts of the VIIth International Biophysics Congress, p 226, Mexico City, Mexico.

Düzgüneş, N., Hong, K., Wilschut, J., Lopez, N., & Papahadjopoulos, D. (1981b) Abstracts of the VIIth International Biophysics Congress, p 105, Mexico City, Mexico.

Düzgüneş, N., Wilschut, J., Fraley, R., & Papahadjopoulos, D. (1981c) *Biochim. Biophys. Acta 642*, 182–195.

Düzgüneş, N., Paiement, J., Freeman, K., Lopez, N., Wilschut, J., & Papahadjopoulos, D. (1983a) *Biophys. J. 41*, 30a.

Düzgüneş, N., Wilschut, J., Hong, K., Fraley, R., Perry, C., Friend, D. S., James, T. L., & Papahadjopoulos, D. (1983b) *Biochim. Biophys. Acta 732*, 289–299.

Düzgüneş, N., Wilschut, J., & Papahadjopoulos, D. (1984) in *Physical Methods on Biological Membranes and Their Model Systems* (Conti, F., Ed.) Plenum Press, New York (in press).

Ekerdt, R., & Papahadjopoulos, D. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2273–2277.

Harlos, K., & Eibl, H. (1981) *Biochemistry 20*, 2888–2892.

Hauser, H., Pascher, I., Pearson, R. H., & Sundell, S. (1981) *Biochim. Biophys. Acta 650*, 21–51.

Hoekstra, D. (1982) *Biochemistry 21*, 2833–2840.

Hope, M. J., & Cullis, P. R. (1979) *FEBS Lett. 107*, 323–326.

Hui, S. W. (1981) *Biophys. J. 34*, 383–395.

Hui, S. W., Boni, L. T., Stewart, T. P., & Isac, T. (1983) *Biochemistry 22*, 3511–3516.

Jacobson, K., & Papahadjopoulos, D. (1975) *Biochemistry 14*, 152–161.

Jendrasiak, G. L., & Hasty, J. H. (1974) *Biochim. Biophys. Acta 337*, 79–91.

Karnovsky, M. J., Kleinfeld, A. M., Hoover, R. L., & Klausner, R. D. (1982) *J. Cell Biol. 94*, 1–6.

Lee, A. G. (1977) *Biochim. Biophys. Acta 472*, 285–344.

Lis, L. J., McAlister, M., Fuller, N., Rand, R. P., & Parsegian, V. A. (1982) *Biophys. J. 37*, 657–666.

Luna, E. J., & McConnell, H. M. (1977) *Biochim. Biophys. Acta 470*, 303–316.

Newton, C., Pangborn, W., Nir, S., & Papahadjopoulos, D. (1978) *Biochim. Biophys. Acta 506*, 281–287.

Nir, S., Wilschut, J., & Bentz, J. (1982) *Biochim. Biophys. Acta 688*, 275–278.

Nir, S., Düzgüneş, N., & Bentz, J. (1983a) *Biochim. Biophys. Acta 735*, 160–172.

Nir, S., Bentz, J., Wilschut, J., & Düzgüneş, N. (1983b) *Prog. Surf. Sci. 13*, 1–124.

Ohki, S. (1982) *Biochim. Biophys. Acta 689*, 1–11.

Ohki, S., & Düzgüneş, N. (1979) *Biochim. Biophys. Acta 552*, 438–449.

Papahadjopoulos, D. (1978) *Cell Surf. Rev. 5*, 765–790.

Papahadjopoulos, D., Poste, G., Schaeffer, B. E., & Vail, W. J. (1974) *Biochim. Biophys. Acta 352*, 10–28.

Papahadjopoulos, D., Vail, W. J., Newton, C., Nir, S., Jacobson, K., Poste, G., & Lazo, R. (1977) *Biochim. Biophys. Acta 465*, 579–598.

Papahadjopoulos, D., Portis, A., Pangborn, W., & Newton, C. (1978) in *Transport of Macromolecules in Cellular Systems* (Silverstein, S. C., Ed.) pp 413–430, Dahlem Konferenzen, West Berlin.

Portis, A., Newton, C., Pangborn, W., & Papahadjopoulos, D. (1979) *Biochemistry 18*, 780–790.

Rand, R. P., & Sengupta, S. (1972) *Biochim. Biophys. Acta 255*, 484–492.

Rehfeld, S. J., Düzgüneş, N., Newton, C., Papahadjopoulos, D., & Eatough, D. J. (1981) *FEBS Lett. 123*, 249–251.

Reiss-Husson, F. (1967) *J. Mol. Biol. 25*, 363–382.

Severs, N. J., & Robenek, H. (1983) *Biochim. Biophys. Acta 737*, 373–408.

Sheridan, J. P., & Düzgüneş, N. (1983) *Biophys. J. 41*, 344a.

Shimshick, E. J., & McConnell, H. M. (1973) *Biochemistry 12*, 2351–2360.

Stewart, T. P., Hui, S. W., Portis, A. R., Jr., & Papahadjopoulos, D. (1979) *Biochim. Biophys. Acta 556*, 1-16.
Strehlow, V., & Jähnig, F. (1981) *Biochim. Biophys. Acta 641*, 301-310.
Sundler, R., Düzgüneş, N., & Papahadjopoulos, D. (1981) *Biochim. Biophys. Acta 649*, 751-758.
Tilcock, C. P. S., & Cullis, P. R. (1981) *Biochim. Biophys. Acta 641*, 189-201.
Vail, W. J., & Stollery, J. G. (1979) *Biochim. Biophys. Acta 551*, 74-84.
van Dijck, P. W. M., de Kruijff, B., Verkleij, A. J., van Deenen, L. L. M., & de Gier, J. (1978) *Biochim. Biophys. Acta 512*, 84-96.
Verkleij, A. J., Mombers, C., Leunissen-Bijvelt, J., & Ververgaert, P. H. J. Th. (1979) *Nature (London) 271*, 162-163.
Wilkinson, D. A., & Nagle, J. F. (1981) *Biochemistry 20*, 187-192.
Wilschut, J., Düzgüneş, N., Fraley, R., & Papahadjopoulos, D. (1980) *Biochemistry 19*, 6011-6021.
Wilschut, J., Düzgüneş, N., & Papahadjopoulos, D. (1981) *Biochemistry 20*, 3126-3133.
Wilschut, J., Düzgüneş, N., Hong, K., Hoekstra, D., & Papahadjopoulos, D. (1983) *Biochim. Biophys. Acta 734*, 309-318.

Photoaffinity Labeling of the Solubilized, Partially Purified Muscarinic Acetylcholine Receptor from Porcine Atria by *p*-Azidoatropine Methyl Iodide†

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ABSTRACT: The synthesis of a tritiated photoaffinity analogue of the muscarinic antagonist atropine, [^{3}H]-*p*-azidoatropine methyl iodide, is described. The compound appeared to bind to a single class of sites in membrane-bound, solubilized, and partially purified preparations of muscarinic receptor from porcine atria with a dissociation constant (determined by competition vs. [^{3}H]-L-quinuclidinyl benzilate) of about 1.0×10^{-7} M. This value was in agreement with the apparent dissociation constant (8.5×10^{-8} M) determined by measuring the concentration dependence of covalent incorporation into a partially purified receptor preparation. Competition experiments indicated that the specific covalent labeling could be blocked by the muscarinic agonist carbamylcholine and the antagonists L-quinuclidinyl benzilate and atropine. An apparent molecular weight of 75 000 ± 5000 was found for specifically labeled peptide(s) in a solubilized, partially purified receptor preparation by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Although ligand interactions with mAcChR[1]-rich preparations from many tissues have been well characterized (Ehlert et al., 1981), structural studies have been limited due to difficulties in solubilization and, thus, purification. The techniques of affinity and photoaffinity labeling have been extremely useful, both in structural studies and as probes to characterize receptors at various stages of purification (Fewtrell, 1976; Heilbronn & Bartfai, 1978). Only two covalent probes for the mAcChR have been reported (Gill & Rang, 1973; Burgen et al., 1974; Amitai et al., 1982). Photoaffinity labeling of soluble mAcChR preparations from any tissue has not been reported to date.

The purpose of this investigation is to report the synthesis and characterization of a photoaffinity label, [^{3}H]-*p*-azidoatropine methyl iodide, specific for the mAcChR L-QNB binding site(s). Photolabeled digitonin/cholate-solubilized partially purified mAcChRs from porcine atria were characterized by gel electrophoresis to determine an apparent subunit molecular weight of the ligand binding peptide(s). The specificity of the probe for the mAcChR was verified, upon the basis of the agreement between reversible and covalent interaction with muscarinic antagonist and agonist binding sites. A preliminary report of this work has been previously published (Cremo & Schimerlik, 1983).

Experimental Procedures

Materials. Digitonin (lot no. 92F-0661) was purchased from Sigma Chemical Co., 2.4-cm DEAE (DE-81) filters were from Whatman, the preparative and analytical TLC plates were from Merck (silica gel 60 F254). (*p*-Aminophenyl)acetic acid and tropine were obtained from Aldrich, and [^{3}H]methyl iodide was from Amersham. [^{3}H]L-QNB (33.1 Ci/mmol), purchased from New England Nuclear, cochromatographed with a nonradiolabeled standard (the generous gift of Dr. W. E. Scott, Hoffman-La Roche Inc.) on TLC plates in solvent systems A (R_f 0.58) and D (R_f 0.38) with 95% of the radioactivity in the QNB spot. All other reagents were the highest purity commercially available. TLC plates were visualized by UV light at 254 nm and an iodoplatinate spray solution (Krebs et al., 1969).

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[1] Abbreviations: mAcChR, muscarinic acetylcholine receptor; nAcChR, nicotinic acetylcholine receptor; WGA, wheat germ agglutinin; PMSF, phenylmethanesulfonyl fluoride; TLC, thin-layer chromatography; L-QNB, L isomer of quinuclidinyl benzilate; [^{3}H]L-QNB, tritiated L isomer of quinuclidinyl benzilate; EDTA, ethylenediaminetetraacetic acid; [^{3}H]PrBCM, tritiated propylbenzilylcholine mustard; SDS, sodium dodecyl sulfate; THF, tetrahydrofuran. TLC solvent systems were (A) chloroform–methanol–acetic acid–water (65:25:5:5), (B) chloroform–methanol (4:1), (C) 1-butanol–acetic acid–water (66:17:17), and (D) acetone–methanol–diethanolamine (10:10:0.3).

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