Role of Membrane Hydration and Membrane Fluidity in the Mechanism of Anion-Induced Fusion of Didodecyldimethylammonium Bromide Vesicles

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Abstract: The mechanism by which dianions of dipicolinic acid (DPA^{2-}) induce fusion of didodecyldimethylammonium bromide (DDAB) vesicles has been investigated. Labeling of the DDAB bilayers with fluorescent probe molecules indicates that the alterations in the hydrophilic head group region are essential for fusion to occur. An isothermal phase transition in the vesicle bilayer is not a prerequisite for fusion. The effect of binding of DPA^{2-} to the vesicles causes a blue shift in the emission spectrum of one of the membrane-associated probes and a concomitant increase in the fluorescence quantum yield. These results together with the increase in fluorescence polarization are interpreted in terms of dehydration of the bilayer–water interface by DPA^{2-} and an increase in the lateral packing of the DDAB head groups. Since these effects already occur below the DPA^{2-} aggregation threshold concentration, a subsequent process appears to be required to initiate the actual fusion event. The possible formation of a specific "trans" (interbilayer) dianion–DDAB complex is discussed. Monitoring the kinetics of fusion as a function of temperature with a membrane fusion assay based on resonance energy transfer shows that the vesicles have a low tendency to fuse below the phase transition temperature (17 °C) although a transient increase in the fusion rate is observed around the pretransition temperature (7 °C). DDAB vesicles appear to be most susceptible to fusion when the bilayers are in an overall fluid state.

In many respects, vesicles prepared from simple synthetic surfactants mimic the properties of vesicles prepared from synthetic or natural phospholipids.[1] Thus, insight into the dynamic alterations in aggregate morphology and membrane structure of these surfactant vesicles can result in a better understanding of comparable processes occurring in natural membranes.[2,3] Membrane fusion is an essential and ubiquitous event in numerous physiologically relevant processes.[4] Hitherto, investigations aimed at clarifying the molecular mechanisms underlying membrane fusion have been mainly performed with (phospho)lipid vesicles as model membranes.[5] It has been proposed[5b,6] that lipid head group dehydration, as a result of cation (e.g., Ca^{2+}) binding, may play an essential role in the eventual induction of membrane fusion by lowering the repulsive hydration forces[7] between two apposed bilayers. Once dehydration and close bilayer apposition have been established, local fluctuations in lipid packing may create "point defects" at the site of vesicle interaction, thus leading to membrane merging.

Recently, we have demonstrated[3] that fusion of vesicles prepared from the synthetic, positively charged amphiphilic didodecyldimethylammonium bromide (DDAB) can be induced by the dianion of dipicolinic acid (DPA^{2-}). Remarkably, fusion of DDAB vesicles is restricted to vesicles with a diameter of at least 3000 Å, smaller vesicles only possessing the ability to aggregate. Most interestingly, however, the two distinct steps in fusion, i.e., (i) vesicle aggregation, resulting in close apposition of the membranes, and (ii) the actual fusion event, involving (local) bilayer destabilization, can be readily distinguished in this surfactant vesicle system, as the threshold DPA^{2-} concentration required to induce these processes is 15 and 30 μm, respectively. This observation provides a unique opportunity to investigate the fusion mechanism, since membrane alterations induced by DPA^{2-} during vesicle aggregation, which, therefore, could be relevant to the actual fusion mechanism, can be examined under carefully controlled experimental conditions. Furthermore, in contrast to phospholipids, the molecular structure of the synthetic amphiphiles is readily amenable to chemical modification, thus providing a tool to systematically alter the properties of membrane packing and the bilayer/water interface. Such alterations, when carried out in a controllable manner, will be of great advantage to improve the insight into the fundamental mechanism of processes such as membrane fusion.

In this work we have examined the mechanism of DPA^{2-}-induced fusion of DDAB vesicles by investigating the effect of anion binding to DDAB bilayers on the fluorescence properties of membrane-associated 8-anilinonaphthalene-1-sulfonate (ANS) and diphenylhexatriene (DPH) as a function of the DPA^{2-} concentration. Since the apolar probe DPH[8] is intercalated in the hydrophobic part of the bilayer, whereas ANS[9] binds to the membranes in the region of the polar head groups, a possibility was provided to monitor potential membrane alterations, induced by DPA^{2-}, both at the level of the bilayer/water interface and the bilayer interior. The results indicate that the DDAB head

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(1) (a) Fendler, J. H. *Membrane Mimetic Chemistry*; Wiley-Interscience: New York, 1982. (b) Kano, K.; Romero, A.; Djermaini, B.; Ache, H. J.; Fendler, J. H. *J. Am. Chem. Soc.* **1979**, *101*, 4030. (c) Stein, J. M.; Tourtellotte, M. E.; Reinert, J. C.; Mc Elhaney, R. N.; Rader, R. L. *Proc. Natl. Acad. Sci. U.S.A.* **1969**, *63*, 104. (d) Okahata, Y.; Ando, R.; Kunitake, T. *Ber. Bunsenges. Phys. Chem.* **1981**, *85*, 789. (e) Carmona Ribeiro, A. M.; Chaimovich, H. *Biochim. Biophys. Acta* **1983**, *733*, 172. (f) Blok, M. C.; Van Deenen, L. L. M.; De Gier, J. *Biochim. Biophys. Acta* **1976**, *433*, 1. (g) Sudhölter, E. J. R.; De Grip, W. J.; Engberts, J. B. F. N. *J. Am. Chem. Soc.* **1982**, *104*, 1069.

(2) Recent studies include: (a) Nakashima, N.; Asakuma, S.; Kim, J.-M.; Kunitake, T. *Chem. Lett.* **1984**, 1709. (b Yamada, K.; Ihara, H.; Ide, T. Fukomoto, T.; Hirayama, C. *Chem. Lett.* **1984**, 1713. (c) Ueuka, R.; Matsumoto, Y.; Ihara, Y. *Chem. Lett.* **1984**, 1807. (d) Brady, J. E.; Evans, D. F.; Kachar, B.; Ninham, B. W. *J. Am. Chem. Soc.* **1984**, *106*, 4279. (e) Nakashimara, N.; Asakuma, S.; Kunitake, T. *J. Am. Chem. Soc.* **1985**, *107*, 509. (f) Murakami, Y.; Kikuchi, J.-i.; Takahi, T.; Uchimura, K.; Nakano, A. *J. Am. Chem. Soc.* **1985**, *107*, 2161. (g) Murakami, Y.; Kikuchi, J.-i.; Takahi, T.; Uchimura, K. *J. Am. Chem. Soc.* **1985**, *107*, 3373.

(3) Rupert, L. A. M.; Hoekstra, D.; Engberts, J. B. F. N. *J. Am. Chem. Soc.* **1985**, *107*, 2628.

(4) Poste, G.; Nicolson, G. L. In *Cell Surf. Rev.* **1978**, *5*, 1.

(5) (a) Papahadjopoulos, D.; Poste, G.; Vail, W. J. *Methods Membr. Biol.* **1979**, *10*, 1. (b) Wilschut, J.; Hoekstra, D. *Trends Biochem. Sci.* **1984**, *9*, 479.

(6) (a) Portis, A.; Newton, C.; Pangborn, W.; Papahadjopoulos, D. *Biochemistry* **1979**, *18*, 780. (b) Hoekstra, D. *Biochemistry* **1982**, *21*, 2833.

(7) (a) Rand, R. P. *Annu. Rev. Biophys. Bioeng.* **1981**, *10*, 277. (b) Israelachvili, J. M. *Chem. Scripta* **1985**, *25*, 7. (c) Rand, R. P.; Das, S.; Parsegian, V. A. *Chem. Scripta* **1985**, *25*, 15.

(8) Grunberger, D.; Haimovitz, R.; Shinitzky, M. *Biochim. Biophys. Acta* **1982**, *688*, 764.

(9) Slavik, J. *Biochim. Biophys. Acta* **1982**, *694*, 1.

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groups become dehydrated upon binding of DPA^{2-}, which occurs prior to vesicle aggregation. It is shown that the actual fusion susceptibility is conferred to the vesicles during vesicle aggregation. Membrane fluidity is only marginally affected by anion binding and it appears that the vesicles are most prone to fusion when the bilayer is in a fluid or a perturbed crystalline state.

Experimental Section

Materials. Didodecyldimethylammonium bromide (DDAB) was purchased from Eastman Kodak and crystallized twice from acetone. *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (*N*-NBD-PE) and *N*-(lissamine Rhodamine B sulfonyl)phosphatidylethanolamine (*N*-Rh-PE) were obtained from Avanti Polar Lipids Inc. Diphenylhexatriene (DPH) and 8-anilinonaphthalene-1-sulfonate (ANS, Mg^{2+} salt) were bought from Aldrich and Pierce, respectively. Dipicolinic acid (DPA) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were from Sigma Chemicals. Picolinic acid was obtained from Fluka, sodium sulfate from Baker and *p*-toluenesulfonic acid from Janssen Chimica. All chemicals were of the highest grade available.

Vesicle Preparation. DDAB vesicles of various sizes were prepared by ultrasonication and the ethanol injection procedure, as described elsewhere.[3] Briefly, vesicles with an average diameter of ca. 3000 Å (defined hereafter as *large* vesicles) were prepared by injecting an ethanolic solution of DDAB (0.87 M) into 2 mL of aqua bidest under stirring. Vesicles with an average diameter of ca. 2000 Å (*small* vesicles) were prepared similarly, except that the final DDAB concentration in ethanol was 0.29 M, while, in addition, they were sized to the desired diameter by extrusion[10] through 0.2-μm Unipore polycarbonate membranes (Bio-Rad). Part of this vesicle suspension was sonicated, using a Branson cell disrupter (20 min, 40 W, temperature 20 °C) to generate *sonicated* vesicles with an average diameter of ca. 500 Å.

ANS- and DPH-labeled vesicles were prepared by adding small aliquots of an ANS (in ethanol) or DPH (in tetrahydrofuran) stock solution to the vesicle suspensions, to give a DPH:DDAB ratio of ca 1:11 000 and an ANS:DDAB ratio of ca 1:120. The effect of THF in the vesicle solution (0.05% (v/v)) can be neglected.[11] The vesicle solutions were allowed to equilibrate for 1 h at room temperature.

Fusion Measurements. Vesicle fusion was monitored continuously with the resonance energy-transfer assay.[3,6b,12] Vesicles containing 0.8 mol % each of *N*-NBD-PE and *N*-Rh-PE were prepared as described above, by solubilizing appropriate amounts of DDAB and fluorophores in ethanol. Fusion measurements were carried out in HEPES buffered solutions (pH 6.0) with equimolar amounts of labeled and nonlabeled DDAB vesicles as previously described.[3] After equilibration at the desired temperature (see legends) fusion was initiated by adding the appropriate (di)anion with a Hamilton syringe. Fusion was monitored continuously by following the relief of energy transfer between *N*-NBD-PE and *N*-Rh-PE as the two probes dilute upon merging of a labeled and an unlabeled vesicle bilayer. As a measure of fusion, the increase of *N*-NBD-PE fluorescence, which increases linearly upon dilution, is measured. The excitation and emission wavelength were set at 475 and 530 nm, respectively. The fluorescence scale was calibrated such that the zero level corresponded to the initial residual NBD fluorescence of the labeled vesicles and the 100% level (infinite dilution) to the fluorescence obtained after addition of Triton X-100 (1%(v/v), final concentration), corrected for sample dilution and the effect of the detergent on the NBD fluorescence quantum yield.[6b]

Fluorescence Measurements. The fluorescence measurements were performed on a Perkin-Elmer MPF-43 spectrofluorometer equipped with a thermostatically controlled cell holder, a magnetic stirring device, and a polarization accessory. ANS fluorescence was measured at an excitation wavelength of 380 nm, while DPH was excited at 360 nm. For fluorescence polarization measurements the emission wavelengths were 465 (ANS) and 428 nm (DPH). The degree of fluorescence polarization (P)[13] was calculated according to the equation

$$P = I_{\parallel} - I_{\perp}/I_{\parallel} + I_{\perp}$$

where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities detected with the polarizers oriented parallel and perpendicular, respectively, to the direction of polarization of the excitation light, with a correction factor[14] for $I_{\perp}$ to account for the intrinsic polarization of the instrument.

Electron Microscopy. Aliquots of DDAB vesicles, before or after induction of fusion, were stained with a 1% (w/v) solution of uranyl acetate, as previously described.[15] The samples were examined in a Philips EM 300 electron microscope, operating at 80 kV.

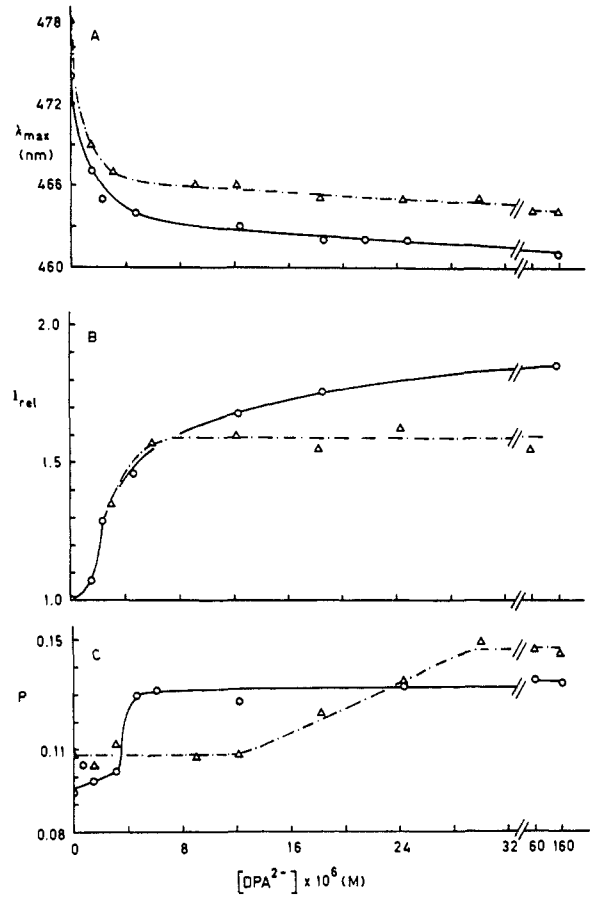

Figure 1. Modification of ANS fluorescence properties upon interaction of DPA^{2-} with small (Δ) and large (O) ANS-labeled DDAB vesicles as a function of the DPA^{2-} concentration: (A) wavelength of the ANS emission maximum, λ_{max}; (B) relative increase of the fluorescence intensity (I_{rel}) at the emission maximum; (C) ANS fluorescence polarization, P. [DDAB] = 55 μM, [ANS] = 0.47 μM, the pH of the incubation medium was 6.0, and the incubation temperature was 25 °C.

Results and Discussion

Properties of the DDAB Bilayer/Water Interface. As shown previously,[3] large DDAB vesicles readily fuse upon addition of DPA^{2-} dianions, while the smaller vesicles do not respond beyond the stage of bilayer aggregation. To investigate whether vesicle size dependent differences in the surface properties of the DDAB bilayers could explain this remarkable distinction, ANS-labeled vesicles were prepared which were subsequently incubated with various concentrations of DPA^{2-}. At each concentration the following parameters were determined for both the small and large DDAB vesicles: (i) the wavelength of the ANS-emission maximum (λ_{em}); (ii) the fluorescence intensity of this maximum, relative to the fluorescence intensity obtained at the emission maximum in the absence of DPA^{2-} (I_{rel}); (iii) the ANS fluorescence polarization value (P). The results of these measurements are summarized in Figure 1. In the absence of DPA^{2-}, the ANS-labeled DDAB bilayers possess a polarity comparable to that of methanol, which agrees well with a location of the fluorophore in the bilayer/water interface. A similar result was obtained for artificial membranes composed of phospholipids.[16]

(10) Hoekstra, D.; Düzgünes, N.; Wilschut, J. *Biochemistry* **1985**, *24*, 565.
(11) Lentz, B. R.; Barenholz, Y.; Thompson, T. E. *Biochemistry* **1976**, *15*, 4521.
(12) Struck, D. K.; Hoekstra, D.; Pagano, R. E. *Biochemistry* **1981**, *20*, 4093.
(13) Shinitzky, M.; Barenholz, Y. *J. Biol. Chem.* **1974**, *249*, 2652.
(14) Chen, R. F.; Bowman, R. L. *Science (Washington, D.C.)* **1965**, *147*, 729.
(15) Sudhölter, E. J. R.; Engberts, J. B. F. N.; Hoekstra, D. *J. Am. Chem. Soc.* **1980**, *102*, 2467.
(16) (a) Bashford, C. L.; Morgan, C. G.; Radda, G. K. *Biochim. Biophys. Acta* **1976**, *426*, 157. (b) Podo, F.; Blasie, J. K. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 1032.

Figure 1 shows that binding of DPA^{2-} results in a blue shift of λ_{em} from 478 to 464 nm and from 474 to 461 nm for small and large vesicles, respectively. This reflects in both cases a polarity change of the ANS microenvironment comparable to a change from a methanol to a *n*-propyl alcohol solution.[9] Concomitantly, the relative fluorescence intensities increased by a factor of 1.8–1.9 for the large vesicles and approximately 1.5–1.6 for the smaller ones.[17] The decrease in bilayer surface polarity with increasing DPA^{2-} concentration suggests that the environment of the polar DDAB head groups becomes more hydrophobic, most likely because of a DPA^{2-}-induced displacement of part of the bound water molecules. Furthermore, it appears that the efficiency of DPA^{2-}-induced dehydration is slightly more effective for the larger vesicles than for the smaller ones. This conclusion is based on the observation that the ANS emission maxima are shifted to lower wavelengths for the larger vesicles with a relative higher enhancement of the fluorescence intensity. A more substantial distinction between large and small DDAB vesicles became apparent when determining ANS fluorescence polarization as a function of the DPA^{2-} concentration. At low DPA^{2-} concentrations (3–5 μM) the bilayer/water interface of the large DDAB vesicles displayed a sudden increase in the fluorescence polarization, with a threshold anion concentration of 3.5 μM. Remarkably, at similar DPA^{2-} concentrations, and when the vesicle size dependent amphiphile concentration available for DPA^{2-} interaction is taken into account, small DDAB vesicles were essentially not affected. For the latter type of vesicles the fluorescence polarization increased at higher DPA^{2-} concentrations, showing a threshold concentration of 12 μM, while the polarization change displayed a much lower degree of cooperativity than that observed for the larger vesicles.

The above results indicate that a certain amount of water tightly bound to the amphiphilic head groups in the larger vesicles is readily removable. As this first hydration layer[18] follows the global movements of the head group,[19] the motions of the ANS molecules will be directly affected as reflected by an increase in molecular ordering near the amphiphilic polar head group region. Apparently, for the smaller vesicles, the first hydration layer is more tightly bound and consequently a higher DPA^{2-} concentration is required to perturb the primary hydration shell. Thus a perturbation of the less strongly bound hydration shell, adjacent to the primary hydration shell[20] may then explain why at lower DPA^{2-} concentrations the emission maximum and fluorescence intensity (Figure 1, parts A and B) are affected without a concomitant effect on the fluorescence polarization. These binding characteristics are similar to the interaction of ions such as Mg^{2+} and Na^{+} with large negatively charged phospholipid vesicles, which causes vesicle aggregation without disrupting the most tightly bound hydration shells surrounding the phospholipid head groups and the ions remain in the "trapped" water region of the lamellar phase.[7a,19a,21] It is possible that this distinction between the tightness of bound water of small vs. large vesicles arises from the lower degree of DDAB head group ionization for the large vesicles, which, as outlined previously,[3] may be expected to exist in order to accommodate the amphiphilic molecules in a bilayer structure with a relative small curvature.[22]

The observed abrupt increase in ANS fluorescence polarization in case of the large vesicles can be interpreted as an increase in head group packing density. This can be rationalized in terms of a reduction in the area per DDAB molecule in the head group region[7a,23] upon binding of DPA^{2-}. A reduced inter-head group electrostatic repulsion in the presence of the anion may be invoked to explain this result. In addition, subsequent dehydration as a result of anion binding will further decrease the repulsive inter-head group interactions.[7b]

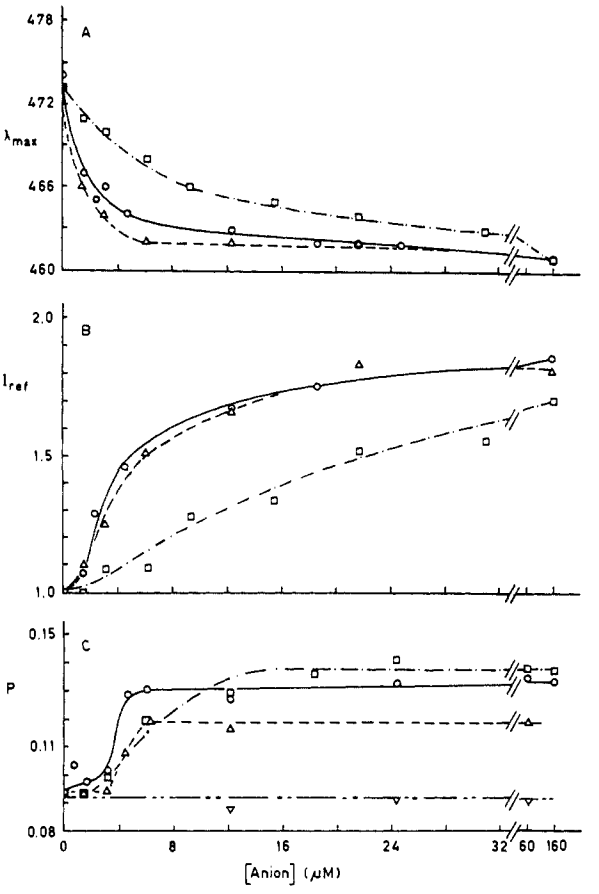

Figure 2. Effect of the fusogenic anions SO_4^{2-} (△) and *p*-toluenesulfonate (□) on the fluorescence properties of large ANS-labeled DDAB vesicles as a function of the anion concentration: (A) wavelength of the ANS emission maximum, λ_{max}; (B) relative increase of the fluorescence intensity (I_{rel}) at the emission maximum; (C) ANS fluorescence polarization, *P*. For comparison, the data obtained for DPA^{2-} (○) were included. In C, the effect of the nonfusogenic anion of picolinic acid (◇) on ANS fluorescence polarization is also shown. [DDAB] = 55 μM, [ANS] = 0.47 μM, pH 6.0, and the incubation temperature was 25 °C.

It is important to note that for both small and large vesicles the threshold DPA^{2-} concentration for the occurrence of aggregation is ca. 13 μM, while the threshold concentration for fusion, which is only observed for the large vesicles,[3] is ca. 30 μM. This implies that for the large vesicles all the DPA^{2-}-induced changes as shown in Figure 1 take place *before* the DPA^{2-} threshold concentration for aggregation has been reached. Hence, the main alterations at the bilayer/water interface are induced on individual, not-aggregated vesicles. It follows that these alterations are apparently not sufficient to induce instantaneous fusion of the bilayers, once they were brought into close proximity when the DPA^{2-} concentration is raised above the aggregation threshold concentration (i.e., ≥13 μM). Thus, additional structural changes in the bilayer are required before the actual fusion event is initiated.

As far as the small vesicles are concerned, ordering of the head group region was also indicated, but now the process occurred *during* aggregation rather than prior to this event. It is relevant to note that as bilayers approach one another, their structures can change,[7a] and, hence, the increased ordering may be accomplished in a manner different from that observed for the large vesicles.

(17) On the basis of the increase in quantum yield with polarity, however, an increase in fluorescence intensity with a factor of ca. 3 would be expected.[9] Presumably, ANS and DPA^{2-} compete for the same binding sites, which results in a reduced binding of ANS to the DDAB vesicles.

(18) For the C_{18} analogue of DDAB the first hydration layer consists of four water molecules: Kumano, A.; Kajiyama, T.; Takayanagi, M.; Kunitake, T.; Okahata, Y. *Ber. Bunsenges. Phys. Chem.* **1984**, *88*, 1216.

(19) (a) Pope, J. M.; Cornell, B. A. *Chem. Phys. Lipids* **1979**, *24*, 27. (b) Kreissler, M.; Lemaire, B.; Bothorel, P. *Biochim. Biophys. Acta.* **1983**, *735*, 23.

(20) Pullman, B.; Berthod, H.; Gresh, N. *FEBS Lett.* **1975**, *53*, 199.

(21) Finer, E. G. *J. Chem. Soc., Faraday Trans. 2* **1973**, *69*, 1590.

(22) Mc Neil, R.; Thomas, J. K. *J. Colloid Interface Sci.* **1980**, *73*, 522.

(23) Cullis, P. R.; De Kruijff, B.; Hope, M. J.; Verkleij, A. J; Nayar, R.; Farren, S. B.; Tilcock, C.; Madden, T. D.; Bally, M. B. In *Membrane Fluidity in Biology*; Aloia, R. C., Ed.; Academic: New York, 1983; Vol. I, p 39.

Furthermore, a much lower cooperativity was observed for the smaller vesicles, indicating that relatively few neighboring molecules underwent the head group packing alterations simultaneously. This could imply that if such areas would be involved as fusion intermediates, the local surfactant concentration might be too low to provide a surface area large enough for the putative fusion sites to form[24] (see below). It was of interest, therefore, to examine whether similar changes in ANS fluorescence properties could be induced by other fusogenic anions,[3] such as those of *p*-toluenesulfonic acid (pTSA) and Na_2SO_4. In addition, the interaction of the nonfusogenic[3] anion of picolinic acid (PA) with ANS-labeled DDAB bilayers was investigated. The results of these experiments are shown in Figure 2 and indicate that the fusogenic anions, in marked contrast to the nonfusogenic PA anion, exhibit similar characteristics to DPA^{2-} on the ANS fluorescence properties upon binding to the large DDAB vesicles. Compared to DPA^{2-}, interaction of SO_4^{2-} with the vesicle bilayer causes almost identical changes with respect to the blue shift of the emission maximum as well as the increase in the relative fluorescence intensity of the ANS-labeled vesicles. Although the monovalent $pTSA^-$ causes a more gradual change in these properties, its effect is similar as for DPA^{2-} and SO_4^{2-} and completed before vesicle aggregation is induced. Furthermore, both SO_4^{2-} and $pTSA^-$ induced an increase in head group packing density as reflected by the increase in ANS fluorescence polarization. In marked contrast to the effect of the fusogenic anions, the nonfusogenic PA^- did not induce any significant changes in the ANS fluorescence properties, indicating its inability to dehydrate the DDAB bilayer or influence the lateral head group packing. Thus, these observations provide further support that membrane dehydration and the concomitant increase in the lateral packing of the amphiphilic head groups represent key events in the anion-induced fusion of the DDAB vesicles. Based on the observation that charge neutralization of the vesicles does not lead to dehydration or packing alterations, as may be inferred from PA^-/DDAB bilayer interaction, the results also support the view that, prior to aggregation, a specific complex between the fusogenic anions and DDAB is formed. This complex presumably involves a so-called (intrabilayer) "cis" complex[6a] and is formed when a divalent ion binds two DDAB molecules within the same bilayer, very similar to that observed for the interaction of divalent cations with negatively charged phospholipid bilayers.[6a] In the case of $pTSA^-$, such a type of interaction presumably occurs[3] as a result of the dual functional properties of this molecule, because it contains a charged and a hydrophobic side group. In fact, it is quite conceivable that this cis complex formation would enhance a tightening of inter-head group packing, thereby further facilitating the expulsion of water from the head group region.[20] Obviously, the formation of this complex as such is not a sufficient condition for the occurrence of fusion (see above), which is in accordance with observations on phospholipid vesicle systems.[6a] In the latter systems, an additional complex needs to be formed which entails a so-called "trans" binding[6a,25] in which a divalent ion binds to two phospholipid molecules on different bilayers. We suggest that such a complex also needs to be formed in the DDAB system. It is reasonable to assume that the formation of such a complex will be greatly facilitated after prior perturbation of the water structure surrounding the head group region.[25]

Studies on the fusogenic properties of negatively charged phospholipid vesicles have revealed the requirement for an overall bilayer fluidity in order to be susceptible toward Ca^{2+}-induced fusion.[26] Eventually, however, collapsed cochleate structures are formed, consisting of tightly packed Ca^{2+}/phospholipid complexes. In earlier work this observation has led to the suggestion that the key event in vesicle fusion is the isothermal phase transition induced by the metal ion.[27] To find out whether the alterations induced by DPA^{2-} on the DDAB vesicle surface are propagated to the interior of the bilayer and whether the physical state of DDAB bilayers influences the fusogenic behavior of the vesicles, the effect of anion binding on membrane fluidity and the effect on fusion of membrane fluidity per se was examined.

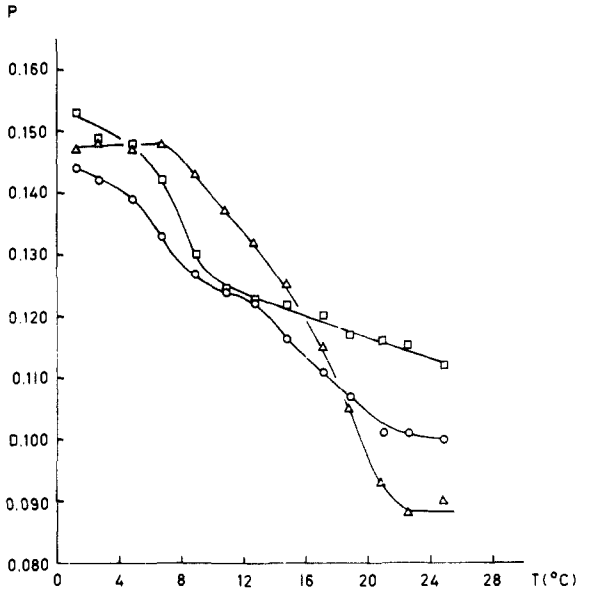

Figure 3. Membrane fluidity of DDAB vesicles, determined by DPH fluorescence polarization, as a function of vesicle size: (□) sonicated vesicles; (△) small vesicles; (○) large vesicles. [DDAB] = 0.52 mM; [DPH] = 47 nM.

Anion Binding and Membrane Fluidity. The occurrence of a liquid crystalline phase transition in diphenylhexatriene (DPH)-labeled DDAB bilayers was revealed by the DPH fluorescence polarization as a function of temperature. As shown in Figure 3, the temperature at which the phase change occurred, T_c, is dependent on the size of the vesicles, very similar to that in phospholipid vesicles.[28] Sonicated DDAB vesicles, with an average diameter of approx. 500 Å, display a transition between 5 and 11 °C, the midpoint being centered around 8 °C. The small vesicles (diameter ca. 2000 Å) show a rather broad transition that takes place between 6 and 22 °C (midpoint 14 °C). Interestingly, the large DDAB vesicles show two breaks in the fluorescence polarization curves, the first transition taking place between 4 and 9 °C (midpoint ca. 7 °C), while the second transition is seen between 13 and 21 °C (midpoint approximately 17 °C). Previously, Okahata et al.[1d] reported a phase transition temperature of 16 °C for large DDAB vesicles, determined with differential scanning calorimetry. This temperature correlates fairly well with the second transition we observed, indicating that this transition represents the main thermotropic transition. The most obvious explanation for the transition, occurring at 7 °C in the large vesicle population, would be that the vesicle fraction is heterogeneous in size, the transition being due to the presence of vesicles comparable in size to sonicated vesicles. Alternatively, it is possible that the transition reflects a so-called pretransition similar to that detected in various phospholipid and surfactant bilayers.[1d,28b] As will be described below, the temperature-dependent fusion experiments allowed us to discriminate between these two possibilities, favoring the latter one. Having established the liquid crystalline properties of DDAB vesicles, we next examined the extent to which these properties are affected upon binding of DPA^{2-}. To this end, large DPH-labeled vesicles were incubated with DPA^{2-} at various temperatures. The concentration of DPA^{2-} relative to that of the DDAB vesicles in the incubation mixture was taken such that it would result in an immediate and maximal

(24) Siegel, D. P. *J. Colloid Interface Sci.* **1984**, *99*, 201.
(25) Prévost, M.; Gallez, D. *J. Chem. Soc., Faraday Trans 2* **1984**, *80*, 517.
(26) Wilschut, J.; Düzgünes, N.; Hoekstra, D.; Papahadjopoulos, D. *Biochemistry* **1985**, *24*, 8.
(27) Papahadjopoulos, D. *Cell Surf. Rev.* **1978**, *5*, 765.
(28) (a) Van Dyck, P. W. M.; De Kruijff, B.; Aarts, P. A. M. M.; Verkleij, A. J.; De Gier, J. *Biochim. Biophys. Acta* **1978**, *506*, 183. (b) Jain, M. K. In *Membrane Fluidity in Biology*; Aloia, R. C., Ed.; Academic: New York, 1983; Vol. I, p 1.

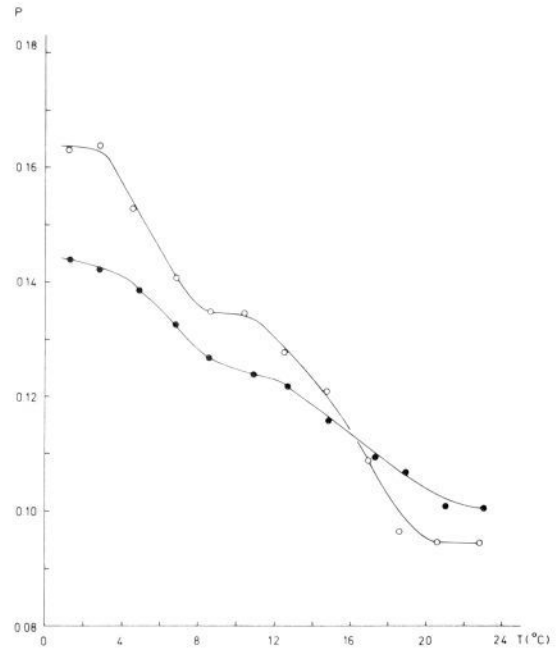

Figure 4. Effect of DPA^{2-} on the membrane fluidity of large DDAB vesicles. DPH fluorescence polarization was determined at the indicated temperatures in the absence (●) or presence (○) of DPA^{2-}. [DDAB] = 0.52 mM; [DPH] = 47 nM; [DPA^{2-}] = 40 μM.

increase in ANS fluorescence polarization (4.1 μM, cf. Figure 1) without causing aggregation or fusion ("subthreshold" DPA^{2-} concentration). As shown in Figure 4, the transition temperatures remain unaltered upon DPA^{2-} binding, indicating that anion/DDAB interaction does not culminate in extensive solidification of the the membranes. Such an effect does occur for Ca^{2+} binding to PS bilayers, in which systems a large upward shift of the phase transition temperature is generally observed. Furthermore, also at higher DPA^{2-} concentrations (up to 150 μM, i.e., well above the threshold concentration for fusion), no shift in the transition temperature for DDAB vesicles could be detected. The higher P values at temperatures below 15 °C, determined for vesicles incubated in the presence of DPA^{2-}, indicate that only in a gellike state DPA^{2-} further induced the structural ordering of aliphatic chains.

From the above results it is concluded that DPA^{2-}-induced fusion of large DDAB vesicles does not require the induction of an isothermal phase transition. Rather the data obtained for ANS-labeled vesicles indicate that the physical and structural alterations induced in DDAB bilayers upon binding of DPA^{2-} are restricted to changes at the membrane surface.

Membrane Fluidity vs. Fusion. To investigate the fusion susceptibility of the large DDAB vesicles as a function of the physical state of the bilayer (solid/fluid), both the initial rate and the extent of fusion were determined (see Experimental Section) and plotted as a function of temperature. As shown in Figure 5, fusion proceeds over the entire temperature range (4–24 °C) studied. An apparent maximum is seen around 7 °C. After decreasing and subsequently leveling off with increasing temperatures (9–16 °C), these parameters sharply raise again at $T > 17$ °C. The apparent fusion maximum seen at approximately 7 °C coincides with the lower transition temperature as determined by DPH fluorescence polarization (Figure 3). Furthermore the onset of the rapid increase in the fusion kinetics at ca. 17 °C clearly parallels the second phase transition which we attributed to the main gel/liquid crystalline transition of the large DDAB vesicles. To obtain further support for the remarkable fusion event seen around 7 °C and, hence, to exclude the possibility of transfer of individual fluorescent molecules between labeled and nonlabeled membranes, DDAB vesicles were incubated with DPA^{2-} at 6.5 °C and subsequently examined by electron microscopy. As shown in Figure 6, large fused vesicles were observed with an average

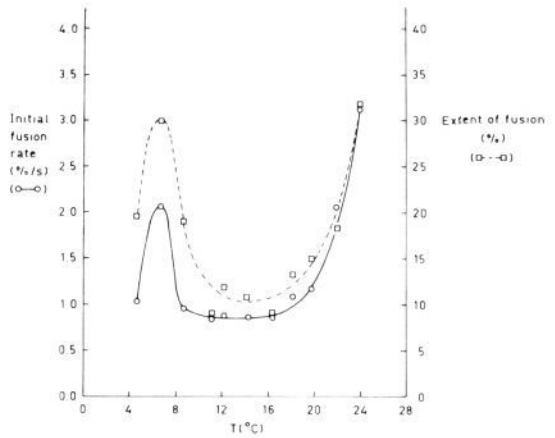

Figure 5. Effect of temperature on the initial fusion rate (○) and the extent of fusion (□) of large DDAB vesicles. Fusion was monitored with the resonance energy-transfer fusion assay. The ratio of vesicles labeled with N-NBD-PE and N-Rh-PE to nonlabeled vesicles was 1:1. Total [DDAB] = 55 μM; [DPA^{2-}] = 63 μM. The pH of the incubation medium was 6.0.

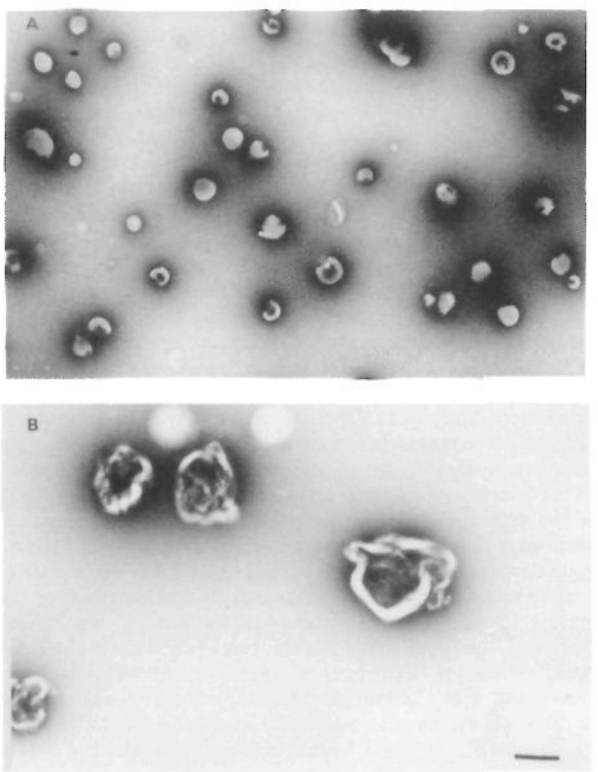

Figure 6. Electron micrographs of large DDAB vesicles before (A) and after (B) an incubation with DPA^{2-} at 6.5 °C for 45 min. Bar indicates 0.35 μm.

diameter of ca. 1 μm. The size of the fused structures observed under these conditions appears to be two- to three-fold smaller than that observed for vesicles formed upon fusion at $T > 17$ °C, showing[3] an average diameter of ca. 2–3 μm. Thus the lower extent of fusion observed around 7 °C, using the fusion assay, is consistent with the electron microscopic observations.

In the previous section, we indicated the possibility that the transition temperature around 7 °C for large vesicles might be due to small sonicated-type-like vesicles, present as contaminant in the large vesicle population. If so, sonicated DDAB vesicles should fuse in the presence of DPA^{2-} when incubated at 7 °C, as may be inferred from the results presented in Figure 5. However, we did not detect any fusion when sonicated N-NBD-PE/N-Rh-PE-labeled vesicles were incubated with nonlabeled vesicles. This result provides strong evidence for the occurrence

of a pretransition in the DDAB bilayer and argues against the presence of smaller vesicles as contaminant in this fraction.

Concluding Remarks

The results described in this study indicate that disturbance in the hydrophilic region of the bilayer causes anion-induced fusion of large DDAB vesicles. The inability of DPA^{2-} to perturb the head group packing in the small vesicles *prior to aggregation* and the resistance of such vesicles to fusion[3] further support the conclusion that the observed initial changes induced in the bilayer interface of the large vesicles are essential for fusion to occur. Since the actual fusion susceptibility was evidently conferred to vesicles within the aggregated state, prior dehydration and subsequent head group packing alterations would thus constitute a necessary though not sufficient prerequisite for the induction of fusion. Thus, an additional process must take place. As a likely possibility, we suggest a further tightening of adjacent vesicles via formation of a specific "trans" complex between anions and amphiphiles in apposed vesicles, very similar to that proposed for cation-induced fusion of negatively charged phospholipid vesicles.[6a] Prior dehydration of the vesicle surface will allow the apposed membranes to come into sufficiently close proximity for such a complex to be formed.[6b,25] It is interesting to note that such a sequence of events is fully consistent with observations previously reported on the effect of the dehydrating agent polyethylene glycol on cation-induced fusion of phosphatidylserine vesicles.[6b]

Several possibilities may explain the highly resistant properties of small vesicles toward fusion: (i) the strongly bound water surrounding the head groups was only partially removed, preventing the bilayers from coming into sufficiently close, i.e., fusion susceptible, contact; (ii) as a result, and analogous to phospholipid systems (e.g., the interaction between Mg^{2+} and phosphatidylserine[6a]), the ability of forming a trans-anion complex will be abolished; (iii) the low cooperativity in head group packing alterations, compared to that observed in the large vesicles, could be indicative for molecular areas formed too small for allowing stable fusion contact sites to be created.

Modification of either the head group structure or the aliphatic chain may allow further insight into the physical properties of the bilayer interface and the dynamic behavior of these surfactant vesicles to be obtained. Such experiments are currently in progress.

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Unusual Selectivities of Radical Reactions by Internal Suppression of Fast Modes

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Abstract: Radical reactions involving two or more intermediates and many mutual reaction channels may lead to the specific formation of (ideally) only one product if one species is more persistent than the others and if persistent and transient species are generated with equal rates. This is caused by an initial buildup of the persistent intermediate which thereafter steers the system to follow (ideally) a single path. A kinetic analysis explains recent data on the photochemistry of nitrosamines and cobalamine. The effect is a likely reason for the high selectivities observed in many photoreactions involving NO as intermediate, e.g., the Barton reaction or the photooxidation of alkanes by NOCl.

Reactions involving two and more free radical intermediates have been reported which exhibit unusually high selectivities of product formation though no chain processes are involved: Gas-phase photolysis of dimethylnitrosamine in the $n\pi^*$-transition region leads to cleavage with a quantum yield of unity.[1]

$$(CH_3)_2N\text{-}NO \rightarrow (CH_3)_2N\cdot + NO\cdot \quad (1)$$

During continuous photolysis in inert atmospheres the only reaction of the radicals appears to be cross-termination.

$$(CH_3)_2N\cdot + NO\cdot \rightarrow (CH_3)_2N\text{-}NO \quad (2)$$

Thus, the nitrosamine exhibits an apparent high photostability under inert conditions. Similarly, the photolability of methylcobalamine in aqueous solution is drastically reduced in the absence of free radical traps.[2] Photolysis in the presence of 0.03 M CO leads to acetylcobalamine with high yield (≅70%). The mechanism

$$CH_3\text{-cob} \xrightarrow{h\nu} \cdot CH_3 + \cdot cob \quad (3)$$

$$\cdot CH_3 + \cdot cob \rightarrow CH_3\text{-cob} \quad (4)$$

$$\cdot CH_3 + CO \rightarrow CH_3\dot{C}O \quad (5)$$

$$CH_3\dot{C}O + \cdot cob \rightarrow CH_3CO\text{-cob} \quad (6)$$

was suggested.[2] If the mechanisms are correct the selectivity for the cross-terminations 2, 4, and 6 is surprising. The reactions involve radicals ($(CH_3)_2N\cdot$, $\cdot CH_3$, $CH_3\dot{C}O$) which are known to self-terminate with high rate constants,[3] and one would intuitively

(1) Geiger, G.; Huber, J. R. *Helv. Chim. Acta* **1981**, *64*, 989.
(2) Kräutler, B. *Helv. Chim. Acta* **1984**, *67*, 1053.

(3) $(CH_3)_2N\cdot$ and $\cdot CH_3$ self-terminate in liquids with rates close to diffusion control.[4] The rate constant for self-termination of $CH_3\dot{C}O$ in liquids is nearly equal[5] to that of $(CH_3)_3\dot{C}$ which is diffusion controlled.[4] $2k$ for $(CH_3)_2N\cdot$ in the gas phase seems unknown, but the constant is high[6] for $H_2N\cdot$ at pressures similar to those used in ref 1.
(4) *Radical Reaction Rates in Liquids*; Fischer, H., Ed.; Springer: Berlin, 1983; Landolt-Börnstein, New Series, Group II, Vol. a and c.
(5) Vollenweider, J. K.; Fischer, H.; Hennig, J.; Leuschner, R. *Chem. Phys.* **1985**, *97*, 217.
(6) *Handbook of Bi- and Termolecular Gas Reactions*; Kerr, J. A., Moss, S. J., Eds.; CRC-Press: Boca Raton, 1981.

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