Role of Lipid Phase Separations and Membrane Hydration in Phospholipid Vesicle Fusion†

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ABSTRACT: The relationship between lipid phase separation and fusion of small unilamellar phosphatidylserine-containing vesicles was investigated. The kinetics of phase separation were monitored by following the increase of self-quenching of the fluorescent phospholipid analogue *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine, which occurs when the local concentration of the probe increases upon Ca^{2+}-induced phase separation in phosphatidylserine (PS) bilayers [Hoekstra, D. (1982) *Biochemistry 21*, 1055–1061]. Fusion was determined by using the resonance energy transfer fusion assay [Struck, D. K., Hoekstra, D., & Pagano, R. E. (1981) *Biochemistry 20*, 4093–4099], which monitors the mixing of fluorescent lipid donor and acceptor molecules, resulting in an increase in energy transfer efficiency. The results show that in the presence of Ca^{2+}, fusion proceeds much more rapidly ($t_{1/2} < 5$ s) than the process of phase separation ($t_{1/2} \simeq 1$ min). Mg^{2+} also induced fusion, albeit at higher concentrations than Ca^{2+}. Mg^{2+}-induced phase separations were not detected, however. Subthreshold concentrations of Ca^{2+} (0.5 mM) or Mg^{2+} (2 mM) induced extensive fusion of PS-containing vesicles in poly(ethylene glycol)-containing media. This effect did not appear to be due to a poly(ethylene glycol)-facilitated enhancement of cation binding to the bilayer, and consequently Ca^{2+}-induced phase separation was not observed. The results suggest that *macroscopic* phase separation may facilitate but does not induce the fusion process and is, therefore, not directly involved in the actual fusion mechanism. The fusion experiments performed in the presence of poly(ethylene glycol) suggest that the degree of bilayer dehydration and the creation of "point defects" in the bilayer without rigorous structural rearrangements in the membrane are dominant factors in the initial fusion events.

The importance of membrane fusion in numerous biological processes and the potentially important role for divalent cations in some fusion events are well recognized [for a review, see Papahadjopoulos et al. (1979)]. Acidic phospholipid vesicles prepared by sonication have been used extensively as a model system for investigating molecular mechanisms underlying membrane fusion. Although Ca^{2+}-mediated fusion of vesicles has been demonstrated by monitoring intermixing of membrane lipids (Maeda & Ohnishi, 1974; Papahadjopoulos et al., 1975; Vanderwerf & Ullman, 1980; Struck et al., 1981) or the coalescence of aqueous compartments (Ingolia & Koshland, 1978; Hoekstra et al., 1979; Wilschut et al., 1980), the mechanism by which Ca^{2+} acts to promote fusion is still unclear. It has been suggested that Ca^{2+}-induced fusion of artificial vesicles is related to its ability to induce isothermal phase transitions and phase separations in model membranes, thus causing changes in molecular packing and the creation of phase boundaries. This, in turn, may result in a transient susceptibility of the membranes for fusion (Papahadjopoulos et al., 1977). More recently it has also been proposed that the formation of dehydrated Ca^{2+}/PS[1] complexes between apposed bilayers may be a key event in the initiation of fusion (Papahadjopoulos et al., 1978; Portis et al., 1979).

Although the interaction of Mg^{2+} with PS vesicles is less pronounced than that observed with Ca^{2+} (Newton et al., 1978; Portis et al., 1979), Mg^{2+} is still able to cause a slight shift in the phase-transition temperature (Jacobson & Papahadjopoulos, 1975; Newton et al., 1978) and induce fusion of small unilamellar PS vesicles, albeit to a limited extent (Papahadjopoulos et al., 1977; Wilschut et al., 1981). In contrast to the Ca^{2+}/PS complex, which involves a very close apposition between two adjacent bilayers and consequent exclusion of water, the Mg^{2+}/PS complex still contains considerable water between the bilayers with larger interbilayer separation (Hauser et al., 1977; Newton et al., 1978). Thus it could be suggested that the degree of bilayer dehydration may also be an important factor in the induction of membrane fusion (Portis et al., 1979), particularly since strong hydration forces have to be overcome in order to allow apposed membranes to come into intimate contact (Parsegian, 1977; Cowley et al., 1978).

These considerations have prompted us to investigate the effects of Ca^{2+}, Mg^{2+}, and a dehydrating agent, PEG, on PS vesicle fusion, using a recently described assay for vesicle fusion in which the energy transfer efficiency between a fluorescent donor (*N*-NBD-PE) and acceptor (*N*-Rh-PE) lipid molecule is continuously monitored (Struck et al., 1981). Recently, a method was also presented for monitoring lipid phase separations during the time course of vesicle–vesicle fusion, by using small amounts of fluorescent phospholipid analogues incorporated into the vesicle bilayer, which become self-quenched during the process of phase separation (Hoekstra, 1982). Together, these methods thus provided an opportunity to study the temporal relationship of phase separation to the fusion process.

Materials and Methods

Lipids and Lipid Vesicles. Bovine brain PS, *N*-NBD-PE, and DOPC were purchased from Avanti Biochemicals (Birmingham, AL). Cholesterol was obtained from Sigma Chemical Co. *N*-Rh-PE was synthesized and purified as

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[1] Abbreviations: PS, phosphatidylserine; DOPC, dioleoylphosphatidylcholine; *N*-NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-phosphatidylethanolamine; *N*-Rh-PE, *N*-(lissamine Rhodamine B sulfonyl)dioleoylphosphatidylethanolamine; NaCl/Hepes, 0.1 M sodium chloride/0.01 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4; PEG, poly(ethylene glycol); EDTA, ethylenediaminetetraacetic acid; NMR, nuclear magnetic resonance.

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previously described (Struck et al., 1981). All lipids were periodically monitored for purity by thin-layer chromatography and repurified as required. Small unilamellar vesicles were prepared in 0.1 M NaCl/0.01 M Hepes, pH 7.4, by ultrasonication at 15 °C under an atmosphere of argon gas. Vesicle lipid compositions were as indicated in the figure legends. Unless stated otherwise, the vesicles contained 5 mol % of the indicated fluorescent phospholipid derivative.

Fluorescence Measurements. Continuous monitoring of NBD fluorescence was carried out with an Aminco-Bowman spectrophotofluorometer (American Instrument Co.) equipped with a chart recorder. Samples were excited at 475 nm and monitored at 530 nm with narrow band-pass slits (1 mm) and crossed polarizers to minimize light scattering.

Vesicle–Vesicle Fusion. Vesicle fusion was monitored with the resonance energy transfer assay as described (Struck et al., 1981), except that the fluorescence donor *N*-NBD-PE and acceptor *N*-Rh-PE were incorporated in separate vesicle populations. Vesicles were mixed in a cuvette, and fusion was initiated by addition of Ca^{2+} or Mg^{2+}. Any fusion of the vesicles will result in intermixing of the membrane lipids so that the fluorescence energy donor and acceptor come into close proximity. This in turn results in fluorescence quenching of the *N*-NBD-PE by *N*-Rh-PE. The quenching process, a measure of the extent of fusion, was continuously monitored in the spectrophotofluorometer as described above.

An estimate for the initial number of fusion events can be made as follows: in an equimolar mixture of *N*-NBD-PE- and *N*-Rh-PE-containing vesicles the first round of fusion (i.e., when one vesicle fuses randomly with one other vesicle) should result in 50% of the maximal obtainable quenching of NBD fluorescence, since only half of the total number of fusion events leads to intermixing of *N*-NBD-PE and *N*-Rh-PE in the same bilayer. If all "dimers" thus formed would undergo one additional round of fusion, the remaining signal would be further decreased by 50%, resulting in a total quenching of 75%.

Lipid Phase Separation. Cation-induced phase separation was monitored continuously as described (Hoekstra, 1982) by using vesicles containing 5 mol % *N*-NBD-PE. The method is based on the self-quenching of the NBD fluorophore that occurs when the *local* concentration of NBD lipid in the bilayer increases during the segregation of the membrane lipids into discrete domains in the plane of the bilayer.

Other Methods. Light scattering was measured with excitation and emission wavelengths set at 400 nm. PEG 6000 was supplied by Fisher Scientific Co. A concentrated solution of PEG in NaCl/Hepes was diluted with NaCl/Hepes to the desired final concentration [percent (weight/weight)] and adjusted to pH 7.4 before addition of the vesicles.

Results

NBD Fluorescence Quenching Resulting from Fusion vs. Phase Separation. Addition of calcium to a mixture of two PS vesicle populations, one containing a small amount of *N*-NBD-PE and the other containing *N*-Rh-PE, resulted in efficient quenching of NBD fluorescence (Table I). As previously shown, this quenching results from lipid intermixing following the Ca^{2+}-induced fusion of the PS vesicles (Struck et al., 1981). Addition of Ca^{2+} to PS/*N*-NBD-PE vesicles alone also resulted in NBD fluorescence quenching. In this case, however, the quenching is due to phase separation, which causes an increase in local concentration of *N*-NBD-PE in the bilayer and hence self-quenching of fluorescence (Hoekstra, 1982). These two processes could be distinguished from one another by the addition of EDTA (Table I). Since fusion leads to intermixing of fluorescent donor and acceptor molecules in the *same* bilayer, NBD fluorescence quenching will persist following addition of EDTA. On the other hand, NBD quenching resulting from Ca^{2+}-induced phase separation is governed solely by the presence of the cation and should be reversible upon displacement of the cation from the membrane. These expectations were fulfilled as seen in Table I. From the results in Table I, we also concluded that exchange diffusion of *N*-Rh-PE molecules into *N*-NBD-PE-containing vesicles did not occur, since no decrease in fluorescence was observed in the absence of Ca^{2+}. As will be shown below, an exchange process is also not facilitated by Ca^{2+}.

Table I: Effect of EDTA on Fluorescence Quenching Resulting from Phase Separation or Fusion[a]

assay	vesicles	relative fluorescence: none	+5 mM Ca^{2+}	+10 mM EDTA
phase separation	PS/*N*-NBD-PE	1.000	0.453	0.997
fusion	PS/*N*-NBD-PE + PS/*N*-Rh-PE	1.000	0.097	0.097

[a] Approximately 40 nmol of PS/*N*-NBD-PE (95:5) or 20 nmol each of PS/*N*-NBD-PE (95:5) and PS/*N*-Rh-PE (95:5) was incubated in 2 mL of 0.1 M NaCl/0.01 M Hepes, pH 7.4, at 24 °C for 10 min, either in the presence or in the absence of Ca^{2+}, and the NBD fluorescence was recorded (λ_{ex} = 475 nm, λ_{em} = 530 nm). EDTA was then added and fluorescence was read again.

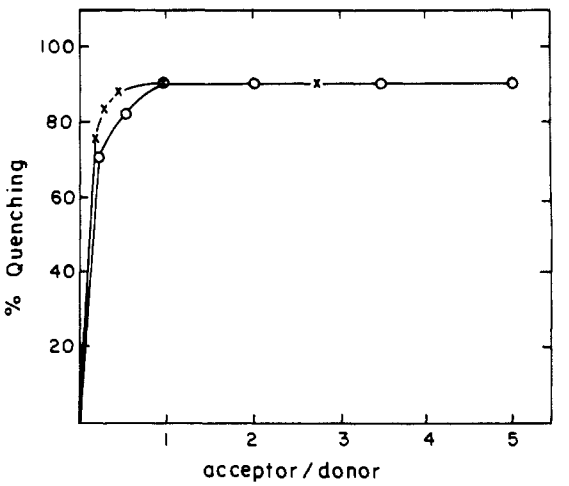

FIGURE 1: Efficiency of NBD fluorescence quenching by rhodamine as a result of resonance energy transfer. Various amounts of PS/*N*-NBD-PE (fluorescence donor) and PS/*N*-Rh-PE (fluorescence acceptor) vesicles were mixed in 2 mL of 0.1 M NaCl/0.01 M Hepes, pH 7.4. 5 mM Ca^{2+} was added and the extent of NBD quenching by *N*-Rh-PE was monitored as described under Materials and Methods. After 5 min, 10 mM EDTA was added and the percent NBD quenching was determined. (O) Variable amounts of acceptor vesicles with constant amount of donor vesicles (23.8 nmol); (×) various amounts of donor vesicles with constant amount of acceptor vesicles (23.8 nmol).

The efficiency of NBD quenching as a result of fusion with *N*-Rh-PE-containing vesicles was tested by changing the ratio of NBD to Rh vesicles (Figure 1). The maximal fractional quenching observed was 0.9. Furthermore, incorporation of equimolar concentrations of the probes in the same bilayer showed a residual (fractional) NBD fluorescence of approximately 0.1, which was consistent with the observed quenching of 0.9 when the probes, initially present in separate vesicles, became intermixed in the same bilayer as a result of fusion. The results in Figure 1 also indicate that upon mixing of equimolar concentrations of vesicles, multiple fusion events

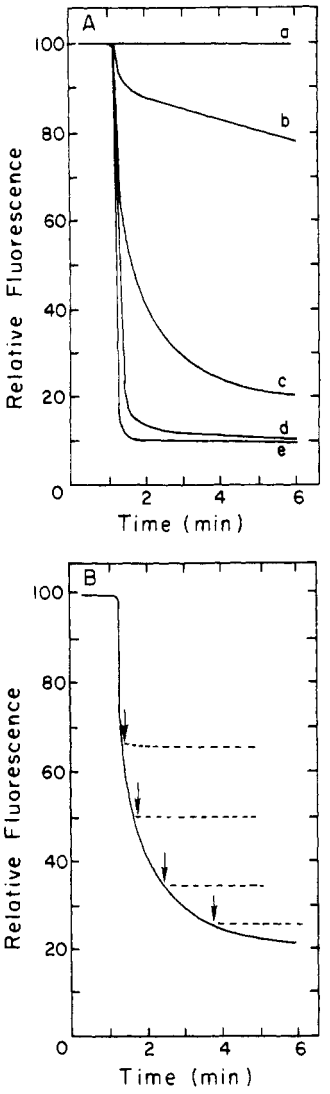

FIGURE 2: Dependence of PS vesicle fusion on Ca^{2+} concentration and termination of the fusion reaction by addition of EDTA. (A) Time course of NBD fluorescence quenching upon fusion of PS/*N*-NBD-PE and PS/*N*-Rh-PE vesicles as a function of Ca^{2+} concentration. Approximately 20 nmol of each vesicle type was mixed in 2 mL of NaCl/Hepes. Ca^{2+} was added to the final concentrations as indicated, and the NBD fluorescence quenching was monitored as described under Materials and Methods. Ca^{2+} concentrations were (a) 0.5, (b) 1, (c) 1.5, (d) 2, and (e) ≥3 mM. (B) Effect of EDTA on Ca^{2+}-induced fusion of PS vesicles. Experimental conditions were the same as in (A) except that fusion was initiated by addition of 1.5 mM Ca^{2+}. EDTA (3 mM) was added at several time intervals after initiation of the fusion (arrows). This figure is a composite of five separate experiments.

must have occurred between the two populations since one round of fusion (i.e., when one vesicle fuses randomly with one other vesicle) would have resulted in a fractional quenching of only 0.45.

Figure 2A shows the NBD fluorescence tracings that were obtained when different Ca^{2+} concentrations were added to equimolar mixtures of NBD- and Rh-containing PS vesicles. The rate of the quenching reaction was markedly dependent on the Ca^{2+} concentration, and while no significant fluorescence quenching was observed at Ca^{2+} concentrations ≤0.5 mM, effective immediate quenching occurred at concentrations ≥3 mM. Additions of excess EDTA at any time interval after addition of Ca^{2+} terminated the fusion reaction immediately, as indicated by an immediate fixation of the NBD fluorescence quenching level (Figure 2B). Interestingly, a (partial) recovery of fluorescence was not observed, thus excluding significant

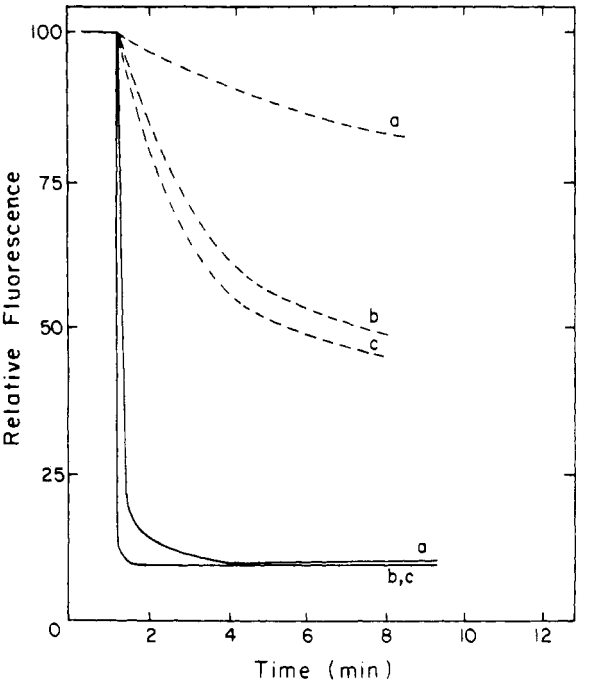

FIGURE 3: Kinetics of fusion and phase separation in PS vesicles as a function of Ca^{2+} concentration. 40 nmol of PS/*N*-NBD-PE vesicles or 20 nmol each of PS/*N*-NBD-PE and PS/*N*-Rh-PE vesicles was incubated in NaCl/Hepes. Ca^{2+} was added and *N*-NBD-PE fluorescence quenching was monitored as a function of time as indicated under Materials and Methods. Dashed lines, phase separation assay; solid lines, fusion assay. Ca^{2+} concentrations were (a) 2, (b) 4, and (c) 8 mM.

participation of phase separation induced NBD quenching in this process (see below). It therefore appeared that the decrease of NBD fluorescence intensity upon addition of Ca^{2+} to a mixture of NBD- and Rh-labeled PS vesicles (Figure 2A) represents the course of the fusion process alone.

Kinetics of Fusion vs. Kinetics of Lipid Phase Separation. The kinetics of phase separation and kinetics of fusion were compared by monitoring the course of NBD quenching in PS/*N*-NBD-PE and in mixtures of PS/*N*-NBD-PE and PS/*N*-Rh-PE vesicles, respectively. As shown in Figure 3, the rate of fusion at any Ca^{2+} concentration tested was much more rapid than the rate of phase separation (compare solid vs. dashed lines, respectively). Thus in the presence of 4 mM Ca^{2+}, for instance, the half-time of the fusion reaction was less than 5 s, whereas the half-time of the phase separation process approximated 1 min. This difference was even more pronounced when the kinetics of these processes were compared in the case of PS/PC (7:3) vesicles (Figure 4A). Upon addition of 5 mM Ca^{2+} a rapid initial rate of "fusion quenching" was observed, whereas the phase separation induced quenching was found to be virtually negligible during this incubation period.

In contrast to the observation in the PS and PS/PC system, fusion and phase separation appeared to occur at similar rates in the case of PS/cholesterol (6:4) vesicles (Figure 4B). Both reactions were virtually complete within seconds after addition of Ca^{2+}.

In the mixed lipid systems, addition of EDTA resulted in complete recovery of fluorescence in case of phase separation induced quenching (curve a) while no recovery was seen after fusion (curve b), which is consistent with the effects of EDTA in the PS system. Finally, from the observed NBD quenching due to fusion, we estimate that the extent of PS/PC vesicle fusion was limited to only two to three rounds of fusion, whereas multiple rounds of fusion apparently had taken place

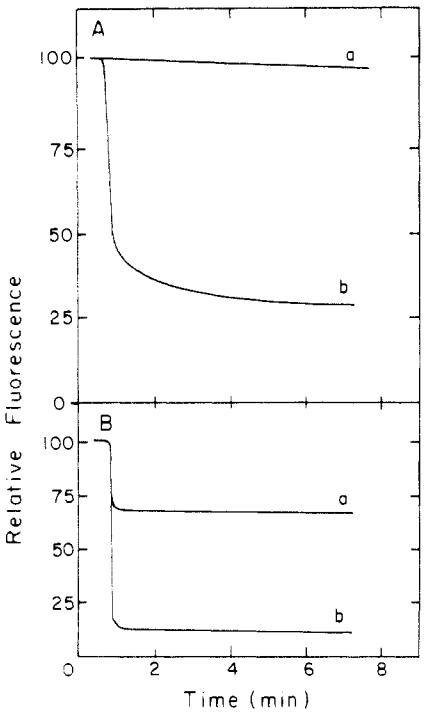

FIGURE 4: Kinetics of fusion and phase separation in PS/PC and PS/cholesterol vesicles. 22.5 nmol each of *N*-Rh-PE and *N*-NBD-PE labeled vesicles or 45 nmol of *N*-NBD-PE-containing vesicles was incubated in 2 mL of NaCl/Hepes. 5 mM Ca^{2+} was added and phase separation (a) or fusion (b) was monitored as a function of time. Vesicle compositions were (A) 7:3 PS/PC and (B) 6:4 PS/cholesterol.

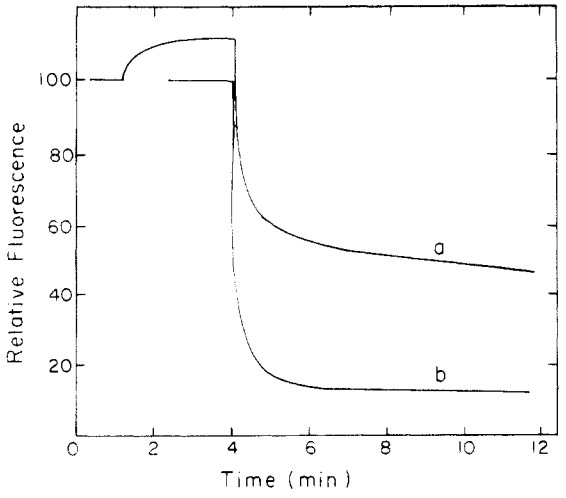

FIGURE 5: Effect of Mg^{2+} on PS vesicle fusion. (Curve a) 25 nmol of PS/*N*-NBD-PE vesicles was incubated in the presence of 10 mM Mg^{2+}. After 4 min, 25 nmol of PS/*N*-Rh-PE vesicles was added, and NBD fluorescence quenching was monitored as a function of time. (Curve b) 25 nmol of each vesicle type was premixed, followed by addition of 10 mM Mg^{2+}. Addition of EDTA (20 mM) at any time interval after initiation of the fusion process did not result in any relief of fluorescence quenching.

in the PS/cholesterol or PS systems.

Ability of Mg^{2+} To Induce Vesicle Fusion. The results shown in Figure 5 demonstrate that in the presence of 10 mM Mg^{2+} a considerable amount of fusion must have taken place since the fluorescence level dropped to approximately 10% of the initial value (curve b). In agreement with previous observations that Mg^{2+} does not induce phase separation in PS/*N*-NBD-PE vesicles (Hoekstra, 1982), this NBD quenching was not relieved upon addition of excess EDTA to the incubation medium, whereas the quenching process was completely prevented when EDTA was added prior to the

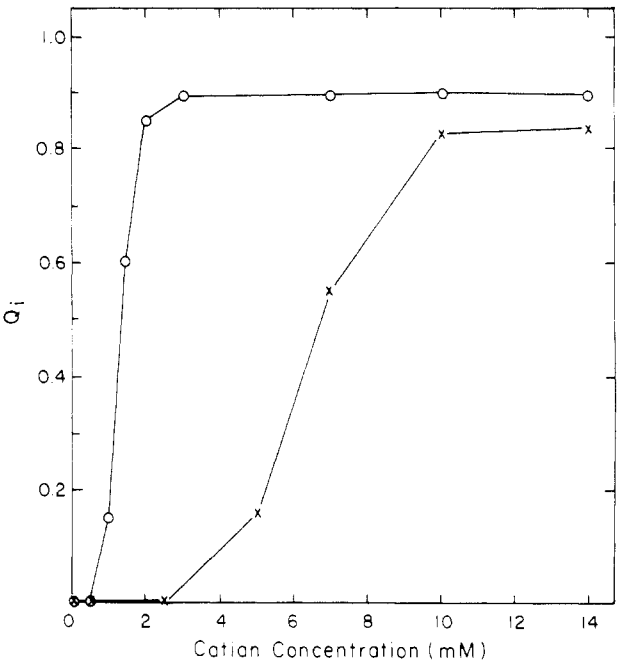

FIGURE 6: Effect of Ca^{2+} and Mg^{2+} on initial fusion rates of PS vesicles. Equimolar concentrations of PS/*N*-NBD-PE and PS/*N*-Rh-PE vesicles (approximately 45 nmol of total lipid) were mixed in 2 mL of NaCl/Hepes, and fusion was induced by addition of Ca^{2+} or Mg^{2+}. The extent of fusion after 30 s, as a measure for the initial fusion rate and expressed as the initial fractional quenching Q_i of *N*-NBD-PE fluorescence, was determined following the addition of a 2-fold molar excess of EDTA. (○) Ca^{2+}-induced fusion; (×) Mg^{2+}-induced fusion.

addition of Mg^{2+}. As an additional control, Mg^{2+} was added to an incubation mixture containing only *N*-NBD-PE-labeled PS vesicles (curve a). As expected, the fluorescence increased slightly, thus excluding the involvement of Ca^{2+} impurities in the Mg^{2+}-induced fusion process, since in the presence of trace amounts of Ca^{2+}, a Mg^{2+}-facilitated Ca^{2+}-induced phase separation would have been expected (see below).

Subsequent addition of PS/*N*-Rh-PE vesicles to Mg^{2+}-prefused PS/*N*-NBD-PE vesicles induced a decrease in fluorescence, which was not reversed upon addition of EDTA (Figure 5, curve a). However, the final fractional quenching in this case was ~50–60% of that observed for Mg^{2+}-induced fusion of premixed unilamellar vesicle populations (compare curve a vs. curve b). Presumably, the "prefused" vesicles had a reduced tendency to fuse with the nonfused vesicle population.

Finally, we determined the effectiveness of Mg^{2+} to induce vesicle fusion by measuring the extent of PS vesicle fusion as a function of the Mg^{2+} concentration. For convenience, the extent of quenching over the initial 30-s time interval was taken as an estimate for the initial rate of the fusion reaction. As can be seen in Figure 6, significant fusion occurred at cation concentrations ≥5 mM Mg^{2+}, although ~5-fold higher concentrations of Mg^{2+} were required in order to obtain similar initial fusion rates as those seen with Ca^{2+}.

Effect of Temperature on Mg^{2+}-Induced Vesicle Fusion. As demonstrated in Figure 7, we observed that the rate as well as the extent of Mg^{2+}-induced vesicle fusion increased with increasing temperature (10–37 °C). Addition of 10 mM Mg^{2+} at each temperature tested resulted in a virtually complete quenching of NBD fluorescence, which occurred immediately after addition of the cation. Since the main purpose of this work was to study the potential relationship between phase separation and fusion, we also examined the possible occurrence of Mg^{2+}-induced phase separation at the indicated

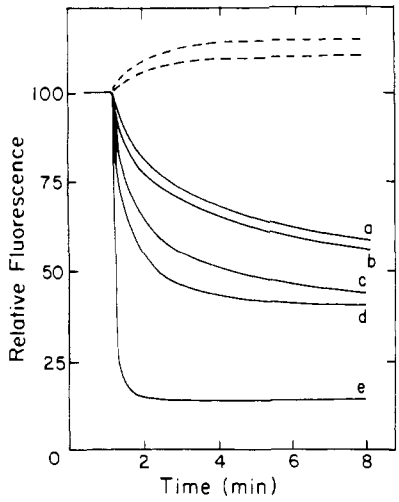

FIGURE 7: Effect of temperature on Mg^{2+}-induced fusion of PS vesicles. Approximately 25 nmol each of PS/*N*-NBD-PE and PS/*N*-Rh-PE vesicles was mixed at a given temperature. After equilibration of the mixture, 5 mM Mg^{2+} was added and the NBD fluorescence quenching was recorded as a function of time. Curves a, b, c, and d were recorded at 10, 20, 25, and 37 °C, respectively. Curve e was obtained over the temperature range of 10–37 °C when 10 mM Mg^{2+} was present. Dashed lines were obtained when 5 mM Mg^{2+} (lower curve) or 10 mM Mg^{2+} (upper curve) was added to PS/*N*-NBD-PE vesicles alone at 10 °C.

temperatures by adding Mg^{2+} to PS/*N*-NBD-PE vesicles and recording the fluorescence with time. The dashed curves in Figure 7 show the NBD fluorescence behavior at 10 °C [i.e., a temperature at which PS vesicles are fluid but become solid upon addition of Mg^{2+} (Portis et al., 1979)] after addition of either 5 mM (lower curve) or 10 mM Mg^{2+} (upper curve). The curves were similar for Mg^{2+}-induced dequenching at higher temperatures (not shown). Evidently no phase separation could be induced, in marked contrast to extensive Mg^{2+}-induced fusion.

Effect of Mg^{2+} at a Low Ca^{2+} Concentration on Vesicle Fusion. As described in previous work (Hoekstra, 1982), Mg^{2+} can facilitate Ca^{2+}-induced phase separation at low Ca^{2+} concentrations (where phase separation is not normally observed in the absence of Mg^{2+}). This synergistic effect of Mg^{2+} on Ca^{2+} is shown in Figure 8. In the presence of 0.5 mM Ca^{2+}, no significant vesicle fusion is observed (curve a). Addition of 0.5 mM Ca^{2+} plus 5 mM Mg^{2+} (curve c) to a mixture of PS/*N*-NBD-PE and PS/*N*-Rh-PE vesicles resulted in extensive fusion, which was virtually complete after 5 min (compare to curve d, 5 mM Ca^{2+} only). Curve b shows the fusion kinetics in the presence of 5 mM Mg^{2+}. Again, as has been shown above (Figure 2), the rate of lipid phase separation in PS/*N*-NBD-PE vesicles in the presence of 0.5 mM Ca^{2+} plus 5 mM Mg^{2+} appeared to be much slower than the rate of fusion under the same conditions (curve e vs. curve c, respectively), indicating that the time scales of the processes compared to one another are minutes vs. seconds, respectively.

Effect of PEG on Vesicle Fusion. When PS/*N*-NBD-PE and PS/*N*-Rh-PE vesicles were suspended in 4% (w/w) PEG, the initial NBD fluorescence level remained unchanged, indicating that intermixing of lipid had not occurred. At concentrations of 10–20% PEG, a slight decrease in NBD fluorescence was observed (~5–10% quenching during an 8-min incubation period). Thus, these results suggested that no substantial fusion of the vesicles took place. Vesicle fusion could be induced, however, when in addition, Ca^{2+} or Mg^{2+} were added at cation concentrations that, when added separately and in the absence of PEG, did not lead to significant fusion. As shown in Figure 9, this effect appeared to be

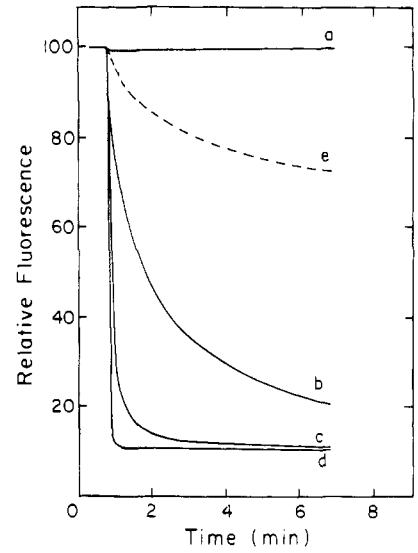

FIGURE 8: Enhancement of Ca^{2+}-induced fusion of PS vesicles by Mg^{2+} at a low Ca^{2+} concentration. Approximately 25 nmol each of PS/*N*-NBD-PE and PS/*N*-Rh-PE vesicles was mixed in 2 mL of NaCl/Hepes. Fusion of the vesicles was initiated by addition of (a) 0.5 mM Ca^{2+}, (b) 5 mM Mg^{2+}, (c) 0.5 mM Ca^{2+} + 5 mM Mg^{2+}, and (d) 5 mM Ca^{2+}. Curve e represents the phase separation in PS/*N*-NBD-PE vesicles in the presence of 0.5 mM Ca^{2+} + 5 mM Mg^{2+}.

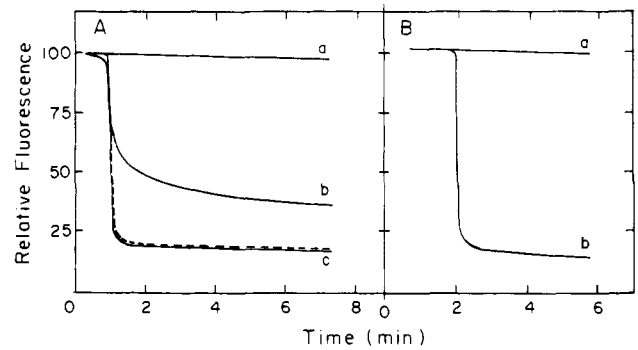

FIGURE 9: Effect of PEG on PS vesicle fusion at subthreshold cation concentrations. 25 nmol each of PS/*N*-NBD-PE and PS/*N*-Rh-PE vesicles was mixed in NaCl/Hepes containing various concentrations of PEG. (A) Fusion was initiated by addition of 0.5 mM Ca^{2+} to the medium containing (a) 4%, (b) 10%, and (c) 20% PEG. The dashed curve was obtained with 0% PEG and 5 mM Ca^{2+}. A curve identical with (a) was obtained by using PS/*N*-NBD-PE vesicles alone (phase separation assay) in the presence of 0.5 mM Ca^{2+} and 20% PEG. (B) Fusion was initiated by addition of 2 mM Mg^{2+} to the medium containing (a) 0% PEG and (b) 20% PEG.

dependent on the PEG concentration (at a fixed cation concentration). Thus, addition of 0.5 mM Ca^{2+} (final concentration) to a mixture of PS/*N*-NBD-PE and PS/*N*-Rh-PE vesicles suspended in buffer or buffer containing 4% PEG (w/w) did not result in any significant decrease of NBD fluorescence (curve a). However, when the final concentration of PEG was increased to 10%, addition of 0.5 mM Ca^{2+} caused a significant part of the vesicles to fuse within the experimental incubation period (curve b). A further enhancement of fusion was observed when the experiment was performed in a medium containing 20% PEG. Under these conditions, the extent of fusion appeared to be maximal and identical with that observed in the presence of 5 mM Ca^{2+} but in the absence of PEG (curve c).

This PEG-facilitated fusion at low Ca^{2+} concentration prompted us to examine also its effect on nonfusogenic concentrations of Mg^{2+}. As indicated in Figure 9B, 2 mM Mg^{2+} did not cause any fusion-induced quenching of NBD

fluorescence when the incubation was performed in the standard incubation medium (curve a). However, when the vesicles were suspended in 20% PEG, followed by addition of 2 mM Mg^{2+}, lipid intermixing occurred immediately as judged by a rapid quenching of NBD fluorescence by rhodamine. Addition of a 2-fold molar excess of EDTA at any time interval after addition of Ca^{2+} or Mg^{2+} did not result in a reversal of this quenching, indicating that actual fusion between the vesicles had occurred.

A possible explanation for the effect of PEG at low cation concentration could be that PEG might have facilitated an enhanced binding of the cations to the bilayer as observed for a Mg^{2+}-facilitated binding of Ca^{2+} at low Ca^{2+} concentrations (Portis et al., 1979). To investigate this possibility, we suspended PS/*N*-NBD-PE vesicles in 20% PEG medium, added Ca^{2+} or Mg^{2+}, and monitored the time course of NBD fluorescence. If enhanced binding of Mg^{2+} would have occurred, a partial dequenching of NBD would be expected (cf. Figure 7). However, in the presence of 2 mM Mg^{2+}, the initial fluorescence level remained unchanged, indicating that Mg^{2+} binding to the PS bilayer was not altered by PEG. Additional support for this conclusion was obtained when the fusion rates in standard medium (Figure 6) and PEG medium (Figure 9B) were compared. An effective Mg^{2+} concentration of approximately 10 mM is required in order to have a similar fusion rate as that observed in 20% PEG and only 2 mM Mg^{2+}. However, in contrast to 2 mM Mg^{2+} plus PEG, addition of 10 mM Mg^{2+} only to NBD-labeled vesicles leads to a significant dequenching (cf. Figure 5). We exclude therefore a PEG-facilitated enhancement in Mg^{2+} binding as the cause for the observed increase of Mg^{2+}-mediated fusion of the vesicles. Similarly, a PEG-facilitated enhancement of Ca^{2+} binding could not account for the observed increase in fusion at low Ca^{2+} concentration, since addition of 0.5 mM Ca^{2+} to the NBD-labeled vesicles in 20% PEG did not induce NBD quenching, which would be expected if Ca^{2+} binding per se would be enhanced (cf. Figure 8). These results thus indicate a specific effect of PEG on vesicle fusion per se, although Ca^{2+} or Mg^{2+} is clearly required to induce the fusion process.

Finally, the specificity of Ca^{2+} and *acidic* phospholipids in vesicle–vesicle fusion was emphasized by the inability of Ca^{2+} to fuse PC vesicles under similar conditions as described above. Thus, incubation of an equimolar mixture of *N*-NBD-PE-labeled DOPC vesicles and *N*-Rh-PE-labeled DOPC vesicles in NaCl/Hepes containing 20% PEG did not change the initial NBD fluorescence level. Subsequent addition of 20 mM Ca^{2+} (or 20 mM Cd^{2+}) similarly had no effect on the fluorescence level (incubation period 10 min). However, addition of either cation did cause extensive vesicle aggregation, as judged by the rapid increase in light scattering (not shown).

Discussion

In this paper the relationship between the kinetics of vesicle–vesicle fusion (as assessed by lipid intermixing) and the kinetics of lipid phase separation was examined. The former process was studied by using a recently described resonance energy transfer assay (Struck et al., 1981) in which small amounts of a fluorescence donor (*N*-NBD-PE) and fluorescence acceptor (*N*-Rh-PE) lipid were incorporated in separate populations of PS-containing vesicles. Addition of calcium to the vesicles resulted in intermixing of the fluorescent lipids and a resultant quenching of NBD fluorescence by rhodamine. This fluorescence quenching was not due to exchange of the fluorescent lipids between vesicles or to energy transfer between donor and acceptor molecules in colliding or closely aggregated bilayers (Struck et al., 1981; also, see below). It is emphasized that the fusion assay as presently used monitors lipid intermixing and that no study to date has compared the kinetics of intermixing of lipids vs. aqueous contents. In a separate assay, the calcium-induced phase separation in PS bilayers was also monitored by including small amounts of the fluorescent lipid *N*-NBD-PE in the membrane (Hoekstra, 1982). Phase separation resulted in segregation of the fluorescent lipid into domains in which fluorescence was self-quenched because of the high local concentration of the NBD lipid. Thus, the two processes, fusion vs. phase separation, could be followed with time in parallel experiments by using appropriately tagged vesicles. Furthermore, they could readily be distinguished from one another by addition of EDTA, which reverses phase separation (restoring NBD fluorescence) but had no effect in the fusion assay (Table I).

The results for Ca^{2+}-induced fusion of PS vesicles demonstrate that under the experimental conditions employed, the rate of phase separation was considerably slower than the rate of vesicle–vesicle fusion as assessed by lipid intermixing. Similar results were obtained with PS/PC vesicles. These results, along with the fact that Mg^{2+} induced substantial fusion of PS vesicles without the occurrence of phase separation, demonstrate that substantial phase separation was not required to *initiate* vesicle–vesicle fusion. For the PS/cholesterol system, phase separation and fusion appeared to occur at similar rates. The reason for this is unclear, but it may be related to preexisting phase separations in the membrane, an intrinsic property of cholesterol-containing vesicles (Shimshick & McConnell, 1973; Epstep et al., 1979; Snyder & Freire, 1980; Hoekstra, 1982).

Of further interest is the fact that both the rate and extent of vesicle–vesicle fusion in PS and PS/cholesterol vesicles are greater than those for PS/PC vesicles. It is then tempting to speculate that in order to initiate the fusion process, a certain number of Ca^{2+}/PS complexes (see below) are required. Such complexes can be readily formed in PS vesicles containing only 5 mol % *N*-NBD-PE and in PS/cholesterol vesicles where PS-enriched lipid phases may exist as an intrinsic property of such vesicles. Hence, rapid and extensive fusion of these types of vesicles may occur. However, for reasons of miscibility of PS and PC (Boggs, 1980), such Ca^{2+}/PS complexes will be less abundantly formed in PS/PC bilayers. Moreover, PS-enriched domains, which would facilitate Ca^{2+}/PS complex formation, do not readily form during the time course of PS/PC vesicle fusion, as inferred from the lack of Ca^{2+}-induced phase separation of membrane lipids in this system. Since fusion does, however, occur in PS/PC bilayers, it might be suggested that small PS domains ("point defects") rather than boundary phases in a demixed bilayer are involved in the initiation of vesicle fusion. This is further supported by the observations on the induction of fusion by Mg^{2+}/PEG and Ca^{2+}/PEG, where extensive vesicle fusion without phase separation was observed (Figure 9). The suggestion that only small PS domains (i.e., a limited number of Ca^{2+}/PS complexes) may be involved in the induction of fusion becomes particularly attractive when the relevant processes that participate in Ca^{2+}/PS complex formation are considered. The rate of fusion as well as phase separation will be determined by the rate of binding of Ca^{2+} to the PS bilayer. Subsequent aggregation of the Ca^{2+}/PS complexes by lateral diffusion along the plane of the bilayer will result in a separation of the lipid phases. Based on the binding rate of Ca^{2+} to PS molecules (cf. Ohki & Duzgunes, 1979) and the different events involved in both processes, it seems reasonable to expect a slower rate for the phase separation process than for the fusion

process, as was observed in Figure 3. Similar reasons may also explain why phase separation is more rapidly reversed (by addition of EDTA) than induced (upon addition of Ca^{2+}) (Ohnishi & Ito, 1974; Hoekstra, 1982). Apparently, Ca^{2+} displacement from the membrane is a much faster process (Figure 2B) than the rate of Ca^{2+} binding and subsequent aggregation of PS molecules by intermolecular Ca^{2+} chelation.

Finally, it cannot be excluded that some *microscopic* phase separation may occur at the sites where fusion nucleation sites are formed. If so, such changes were clearly not detected by the phase separation assay. Furthermore, even if local phase separation would occur at points of contact between two fusing membranes, it is unknown whether this process would be absolutely necessary to induce fusion. It is evident that, upon formation of fusion nucleation sites by binding of Ca^{2+} or Mg^{2+} to PS, locally induced changes in the physical properties of the bilayer will occur (Newton et al., 1978; Portis et al., 1979). However, this resulted in a slight *de*quenching and *not* quenching of NBD fluorescence in the case of Mg^{2+} binding (Figure 7). As discussed above, depending on the lipid composition of the vesicles, relatively enriched PS domains may preexist along the plane of the bilayer, obviating the necessity of lateral movement of Ca^{2+}/PS molecules in order to form the fusion nucleation sites.

Role of Bilayer Hydration on Vesicle Fusion. As has been pointed out by Parsegian and co-workers (Parsegian, 1977; Cowley et al., 1978), hydration forces become dominant with respect to other forces between vesicles (e.g., electrostatic and van der Waals interactions) when vesicles approach one another at distances $\leq$30 Å. These hydration forces must therefore be overcome if two vesicles are to fuse. In this context it is interesting to note that a trans Ca^{2+}/PS complex, which is essentially anhydrous, has been proposed on the basis of NMR and X-ray data with PS multilayers (Hauser et al., 1975, 1977; Newton et al., 1978), while a lesser degree of dehydration has been proposed for the Mg^{2+}/PS complex (Newton et al., 1978; Portis et al., 1979). This difference in hydration of Mg^{2+}/PS and Ca^{2+}/PS complexes may explain the observed differences in the fusion-inducing properties of these cations (Gresh, 1980; Wilschut et al., 1981) and is supported by the observations with PEG on Ca^{2+}- and Mg^{2+}-induced fusion (Figure 9).

The enhancement of Ca^{2+}- and Mg^{2+}-induced vesicle fusion by PEG also supports the idea that bilayer hydration plays an important role in the fusion process. PEG, in the absence of Ca^{2+} or Mg^{2+}, rapidly aggregated vesicles without resulting in fusion, while PEG in the presence of subthreshold concentrations of these cations facilitates fusion. This enhancement was not due to an effect of PEG on Ca^{2+}-induced phase separation (Figure 9), nor was it likely to be due to an effect of PEG on the phase-transition temperature of the vesicle lipids (Tilcock & Fisher, 1979; also, see below). Thus, the PEG effect might best be explained if the hydration layer at the lipid/water interface was destabilized by PEG (Tilcock & Fisher, 1979; Janiak et al., 1976), thereby facilitating the formation of (anhydrous) trans Ca^{2+}/PS or Mg^{2+}/PS complex(es) between adjacent vesicles. This conclusion is further supported by our observations with neutral PC vesicles, which, when suspended in PEG medium, were highly aggregated by Ca^{2+} (or Cd^{2+}) but did not fuse.

The results presented in this report on the temperature dependence of Mg^{2+}-induced fusion seem to be in contrast with observations reported by Papahadjopoulos et al. (1977). These authors proposed that the isothermal phase change induced by Mg^{2+} (or Ca^{2+}) is a key event leading to PS vesicle fusion. Their results suggested that a higher degree of fusion occurs at incubation temperatures between −3 and 19 °C, the temperature range over which these vesicles undergo a phase change upon addition of Mg^{2+}, whereas in the present study a decrease in the initial fusion rate was observed, as well as a slight decrease in the extent of fusion (after 8 min) with decreasing temperature (Figure 7). It is emphasized, however, that the present study focuses on initial events whereas in the study of Papahadjopoulos et al. (1977), observations were made after a 1-h incubation. Nevertheless, the results do not suggest that an isothermal phase transition (at least in the case of Mg^{2+}-induced fusion) is involved in *initiation* of the fusion process. Therefore, as suggested above, it appears that rather than a change in the physical state of the bilayer lipids, hydration of the bilayer is a major factor in the initiation of vesicle fusion.

Finally it is of interest to briefly discuss the observations on the inability of PC vesicles to fuse when suspended in PEG medium containing Ca^{2+} (or Cd^{2+}). Although both these cations can cause extensive aggregation of the PC vesicles, no quenching of *N*-NBD-PE by *N*-Rh-PE was seen. Thus, our assay for lipid intermixing (fusion) is not complicated by resonance energy transfer, which might occur between two closely apposed vesicles, or by spontaneous transfer (exchange) of the fluorescent lipids between vesicles.

In summary, it is concluded that *macroscopic* phase separation may *facilitate* but is not a prerequisite for the initiation of PS-containing vesicle fusion. The results emphasize the importance of vesicle surface properties, particularly bilayer hydration. It is proposed that dehydrated trans Ca^{2+}/PS or Mg^{2+}/PS complexes can be formed, in principle, without the induction of phase separations, and when sufficient in number, size, and state of dehydration, the fusion process will be initiated. Particularly in PC-containing PS bilayers, these parameters may determine the ability of such vesicles to fuse.

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Partial Molar Volumes of Some 1-Alkanols in Erythrocyte Ghosts and Lipid Bilayers†

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ABSTRACT: The partial molar volumes of 1-heptanol and 1-octanol in red cell ghosts, in egg phosphatidylcholine bilayers, and in water and phosphate buffer have been measured to a precision of better than 4% by using a density meter. In every case, the partial molar volume was independent of concentration in the range studied. In both membranes, the partial molar volume of each alcohol was close to its molar volume, whereas in aqueous solution it was considerably less. Comparison of the two membranes suggests that the major contribution to the partial molar volume arises from alcohol–lipid interactions in each case. Further comparison with partial molar volumes in bulk solvents suggests that on average the alcohols retain a hydrogen bond in the lipid bilayer. The magnitude of the volume change in ghosts is some 5 times less than the corresponding area changes previously reported by others [Roth, S. H., & Seeman, P. (1972) *Biochim. Biophys. Acta 255*, 190–198]. These two observations can only be resolved by assuming either that the bilayer expands anisotropically, experiencing a decrease in thickness with increasing volume, or that conformational changes in membrane-associated proteins can occur at constant volume to increase membrane area. Finally, these data are used to test the critical volume hypothesis of general anesthetic action. A volume change of 0.15% in red cell ghost membranes is found to be associated with anesthesia, which compares with a value of 0.2% predicted previously from pressure reversal of anesthesia studies. In egg phosphatidylcholine bilayers, a volume change of 0.36% is associated with anesthesia. The larger change in the lipid bilayer compared to the biomembrane originates solely in their different membrane/buffer partition coefficients.

Characterization of the thermodynamics of small hydrophobic molecules interacting with membranes may provide a basis for understanding the interactions between complex endogenous components of those membranes. Furthermore, small molecules may serve as structural perturbers, thus revealing information about membrane organization. In this paper, we have determined the volume changes which occur when alcohols interact with red cell ghost membranes.

Alcohols exert a wide variety of effects on membranes including effects on permeability (Kutchai et al., 1980a), transport (Sullivan et al., 1974; Kutchai et al., 1980b; Hara & Kasai, 1977), fluidity (Roth & Spero, 1976; Pringle & Miller, 1978; Zavoica & Kutchai, 1980), and excitability (Seeman, 1972; Swenson & Oxford, 1980). The anesthetic action of the alcohols has also been given considerable attention. Seeman and co-workers in particular have developed the idea that the action of alcohols may be mediated by membrane expansion, and this hypothesis is supported by the observation that anesthesia is reversed by pressure (Miller et al., 1973). Various attempts have been made to test this hypothesis by measuring the increase in surface area of erythrocytes exposed to alcohols (Roth & Seeman, 1972; Seeman, 1969; Seeman et al., 1969a,b) with the general conclusion that the area increase exceeds that calculated from the partition coefficient and molecular volume of the alcohols. One explanation for this would be that the alcohols were responsible, either directly or indirectly via lipid perturbations, for conformational changes in proteins which in turn result in a large area increase (Seeman, 1972, 1974). An alternative explanation suggested by several authors (Miller et al., 1973; Trudell, 1977) is that alcohols induce an anisotropic expansion of the membrane, such that its thickness decreases while its

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