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# Rate and Extent of Poly(ethylene glycol)-Induced Large Vesicle Fusion Monitored by Bilayer and Internal Contents Mixing†

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ABSTRACT: Poly(ethylene glycol) (PEG) of average molecular weight 8000 was used to mediate the fusion of large unilamellar vesicles composed of dipalmitoylphosphatidylcholine. Fusion was monitored by fluorescence assays of lipid mixing and aqueous contents mixing. The extent of lipid mixing, as monitored by DPHpPC fluorescence lifetime, indicated that large unilamellar vesicles underwent a single fusion cycle when incubated with PEG and subsequently diluted into buffer. The ANTS/DPX assays for contents mixing and leakage indicated that, while addition and dilution of PEG were accompanied by extensive contents leakage, this occurred on a much different time scale as compared to contents mixing. Both the lipid-mixing and contents-mixing assays gave comparable estimates for the number of rounds of fusion that occurred in a given time following PEG addition, although the contents-mixing assay always yielded an estimate 10–15% larger than the lipid-mixing assay. These assays were used to evaluate several factors purported to influence PEG-induced fusion. First, the initial rate of fusion was found to be dependent on PEG concentration in the range of 0–35 wt %, while the extent of fusion was not. In addition, a substantial rate enhancement occurred when vesicles were incubated with greater than 26% PEG. Second, the creation of an osmotic gradient upon dilution of vesicle–PEG mixtures was shown to have no effect on either the extent or the initial rate of fusion. Consistent with this observation, both contents and lipid mixing were found to occur prior to and independent of the dilution of the PEG–vesicle suspension. Third, impurities, either present in our commercially available PEG or added to vesicle–PEG mixtures, also had no effect on the rate or extent of fusion. Fourth, another dehydrating polymer, dextran (average mol wt 9000), was capable of promoting fusion, though at a much lower rate than PEG. These results suggest that even partial bilayer dehydration accompanied by vesicle collapse and close interbilayer contact may be sufficient to induce vesicle fusion.

The juxtaposition of cellular or vesicular membranes is widely accepted to be necessary for fusion. However, this close association may be insufficient to kindle the mixing of bilayer lipids and the interaction of trapped internal components, both of which occur during a fusion event. The ability of several agents to induce reversible vesicle or cell aggregation (i.e., not leading to fusion) supports this idea (Honda et al., 1981; Boni et al., 1981; Wilschut et al., 1981). These findings imply that the continuity of bilayer structure must be disrupted or altered by some agent or event before fusion can occur. Lateral-phase separations (Papahadjopoulos et al., 1976), fusogenic im-

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purities (Honda et al., 1981), osmotic swelling (Wojcieszyn et al., 1983; Cohen et al., 1982), and increased surface tension (Ohki, 1984) have been proposed to cause bilayer destabilization leading to fusion. By inducement of bilayer juxtaposition independent of bilayer destabilization, it should be possible to determine how the bilayer must be disrupted to complete the fusion process.

Poly(ethylene glycol) (PEG)[1] was chosen as a fusogen for our initial attempts to address this issue because Honda et al. (1981) had reported that purified PEG induced the close association of erythrocyte membranes but not their fusion. We set out to test this report in a model vesicle system by using techniques that could provide information about the molecular events involved in the fusion process. Fluorescence fusion assays which monitor either the mixing of lipids or the mixing of trapped internal contents were chosen as methods that can provide insight into molecular events not ascertainable by freeze–fracture electron microscopy, light scattering, or turbidity measurements. In addition, we chose to use both lipid-mixing and contents-mixing assays to ensure that vesicle leakage and intervesicular lipid exchange are not mistaken for the fusion process.

This study has focused on several issues related to PEG-induced membrane fusion. First, we asked whether PEG-induced vesicle fusion was a "true" fusion event as opposed to a process involving vesicle rupture and reformation, as suggested by Sâez et al. (1982). Second, we questioned whether a critical PEG concentration has to be attained before fusion of large unilamellar vesicles would occur, as has been suggested for the fusion of small unilamellar vesicles (Boni et al., 1981). Third, using dextran as an alternative dehydrating agent, we investigated the importance of bilayer dehydration as a requirement for fusion. Fourth, we considered whether osmotic swelling resulting from dilution of PEG was necessary for vesicles to fuse or if fusion occurred before vesicle–PEG mixtures were diluted; this question was raised by the work of Wojcieszyn et al. (1983). Finally, we determined whether impurities were a key requirement for PEG-induced fusion, as has been proposed by Honda et al. (1981).

Three features distinguish the present study from previous reports on PEG-induced vesicle fusion. First, we have focused on the fusion of a reasonably well-defined population of large unilamellar vesicles (LUV), which are stable in the absence of an added fusogen such as PEG. Most previous reports (Boni et al., 1981a, 1984a; MacDonald, 1985) have emphasized the effects of PEG on small unilamellar vesicles (SUV) or ill-defined phospholipid dispersions. SUV are prone to fusion and spontaneously form larger vesicles both below (Suurkuusk et al., 1976; Wong et al., 1982) and even above (Lentz, Carpenter, & Alford, unpublished data) their phospholipid-phase transition. Second, this is the first report of PEG-induced fusion to combine measurements of both mixing and leakage of vesicle contents with precise quantitation of bilayer lipid mixing. Finally, this study considers not only the extent of vesicle fusion induced by PEG but also the rate of fusion. Rate determinations are expected to be essential to understanding the molecular events central to the fusion process.

[1] Abbreviations: DPPC, 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine; DPHpPC, 1-palmitoyl-2-[[[2-[4-(6-phenyl-*trans*-1,3,5-hexatrienyl)-phenyl]ethyl]oxy]carbonyl]-3-*sn*-phosphatidylcholine; ANTS, disodium 8-aminonaphthalene-1,3,6-trisulfonate; DPX, *N,N'-p*-xylylenebis(pyridinium bromide); PEG, poly(ethylene glycol); GMO, glycerol monooleate; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; $C_{12}E_8$, dodecyloctaethylene glycol monoether; REV, reversed-phase evaporation vesicle(s); LUV, large unilamellar vesicle(s); SUV, small unilamellar vesicle(s).

## Experimental Procedures

### *Materials*

Chloroform stock solutions of 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine (DPPC) and 1-palmitoyl-2-[[[2-[4-(6-phenyl-*trans*-1,3,5-hexatrienyl)phenyl]ethyl]oxy]carbonyl]-3-*sn*-phosphatidylcholine (DPHpPC) were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). DPPC stocks were filtered over Norit A neutral activated charcoal to remove trace fluorescent contaminants. Lipids were found to be greater than 98% pure by thin-layer chromatography on Analtech GHL plates containing 0.01 M dipotassium oxalate. Plates were developed in a 65:25:4 (v/v) $CHCl_3$:$CH_3OH$:$H_2O$ mixture and were stained with iodine vapors. DPHpPC was also viewed under near-UV light. The disodium salt of 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) and *N,N'-p*-xylylenebis(pyridinium bromide) (DPX) were purchased from Molecular Probes (Junction City, OR). Poly(ethylene glycol) (average mol wt 8000) was obtained from Fisher Scientific (lot 721900). Dextran (average mol wt 9000, lot 53F-0240) and 1-monooleoyl-*rac*-glycerol (GMO) were purchased from Sigma (St. Louis, MO). Dodecyloctaethylene glycol monoether ($C_{12}E_8$) was purchased from Nikko Chemical Co. (Tokyo, Japan). Ultrapure KCl was obtained from Heico, Inc. (Delaware Water Gap, PA; lot 2179) and *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES) was from Research Organics, Inc. (Cleveland, OH; lot 1344). Buffer solutions were filtered through 0.22-$\mu$m GS filters prior to use (Millipore Corp., Bedford, MA). All other chemicals were of the highest quality available.

### *Methods*

*Vesicle Preparation.* Reversed-phase evaporation vesicles (REV) (Szoka et al., 1980) were prepared from DPPC or mixtures of DPPC–DPHpPC at a 25:1 mole ratio. Isopropyl ether for vesicle preparation was distilled over $LiAlH_4$, then passed through an alumina column immediately before use to remove impurities (Parente & Lentz, 1984). Vesicles were filtered through 0.4- and 0.2-$\mu$m polycarbonate filters (Nuclepore Corp., Pleasanton, CA). Phosphate analysis to determine the phospholipid concentration of vesicle samples was performed by a modification of the procedure of Chen et al. (1956). In most cases, vesicles were prepared in 50 mM KCl, 50 mM TES, and 0.1 mM EDTA, pH 7. For the contents-mixing experiments, vesicles were prepared in buffers containing 25 mM ANTS (or 90 mM DPX), 40 mM NaCl, and 10 mM TES, pH 7.5. For leakage experiments, the buffer contained 12.5 mM ANTS, 45 mM DPX, 20 mM NaCl, and 10 mM TES, pH 7.5. Vesicles were eluted from a Sephadex G-75 column (0.7 × 10 cm) with 100 mM NaCl and 10 mM TES, pH 7.4, to remove untrapped ANTS or DPX immediately before use.

*PEG Purification and Storage.* Commercial PEG 8000 was purified by a modification of the procedure outlined by Honda et al. (1981). PEG was dissolved in the minimum amount of water and slurried with Chelex 100 (Bio-Rad, Richmond, CA) for 1 h. Chelex was removed by filtration, and the PEG solution was lyophilized to dryness. PEG was then dissolved in chloroform and recrystallized twice from diethyl ether according to Honda et al. The resulting powder was stored at room temperature in a desiccator. PEG solutions were prepared in doubly distilled, autoclaved water and were always used within 48 h.

*Osmolality Measurements.* The osmolality of buffers and polymer solutions were determined by a freezing-point depression osmometer ($\mu$Osmette, Precision Systems, Inc., Na-

tick, MA). Reported osmolality values represent the average of three measurements.

*PEG Trapping.* DPPC REV (0.55 μmol of lipid) were incubated in 35 or 8 wt % [$^{14}$C]PEG 6000 (Amersham Corp., Arlington Heights, IL) for 20 and 60 min, respectively. The mixture was either diluted with 1 mL of water or applied directly to a 0.45-μm filter (type HA, Millipore Corp., Bedford, MA). Filters were prepared for use by washing sequentially with 1 mL of water, 1 mL of 1 M EDTA, and 0.195 mL of unlabeled PEG, then 5 mL of water. This procedure was necessary to reduce background counts from the nonspecific binding of PEG to the filter. Following application of vesicle–PEG samples, filters were rinsed 8 times with 1-mL aliquots of water, after which both the filter and filtrates were counted in a LKB RackBeta liquid scintillation counter. Separate control experiments showed that greater than 85% of the vesicular phospholipid was trapped by the filter while greater than 99% of the PEG passed through the filter. Counts associated with the filter indicated some form of PEG–vesicle interaction.

*Fluorescence.* All fluorescence measurements were made on an SLM 4800 spectrofluorometer (Urbana, IL) equipped with a modified, three-position, multitemperature cuvette holder (Barrow & Lentz, 1985) and a 200-W mercury–xenon light source (Canrad-Hanovia, Newark, NJ). The temperature of two sample positions was kept at 48 °C. The 366-nm mercury line was used to excite DPHpPC for lifetime measurements, while emission was monitored through a 3-mm high-pass KV-450 filter (50% transmittance at 450 nm; Schott Optical Glass, Duryea, PA). A polarizer set at 35° from vertical was used in the emission path. Phase-angle shifts and modulation ratios were recorded at 30-MHz modulation frequency as described by Barrow and Lentz (1983) using a reference standard of DPH in heptane measured at 23 °C in the third cuvette position ($\tau$ = 6.78 ns; 2 × $10^{-7}$ M). The derived lifetimes were calculated by the method of Spencer and Weber (1969). Corrections for background intensities of nonfluorescent vesicle samples were made when they were greater than 0.5% of the intensity of the fluorescent samples (Barrow & Lentz, 1985).

Fluorescence intensity measurements of ANTS were made at an excitation wavelength of 366 nm, and emission was observed through a 2-mm OG-515 filter (50% transmittance at 515 nm; Schott Optical Glass, Duryea, PA).

*Fusion Assays.* For both the contents-mixing and lipid-mixing assays, vesicles were incubated at 48 °C with defined concentrations of PEG for specific times. These mixtures were then diluted 10-fold with glass-distilled water and transferred to a cuvette for the fluorescence measurements. In all cases, dilution dramatically slowed the fusion process. When high concentrations of PEG were being used, this step was essential for measurement, due to the high viscosity of the medium. It should be noted that stock PEG solutions could not be prepared at concentrations greater than 45% (w/w).

*DPHpPC Lipid Mixing.* This assay works on the principle that the DPHpPC fluorescence lifetime is dependent on the probe concentration in the bilayer (Parente & Lentz, 1986). Vesicles containing 4 mol % of DPHpPC (0.05 μmol of total lipid) were mixed with a 10-fold excess of probe-free vesicles (0.5 μmol of lipid) in the presence of polymer. After dilution to a final volume of 3 mL, about 10 lifetime measurements were recorded over a period of about 20 min. Each lifetime value was an average of 10 individual measurements and carried an error of 0.05–0.1 ns. Lipid mixing between probe-free and probe-containing vesicles was indicated by an increase in fluorescence lifetime. Plots of the lifetime variation with time following dilution were made, and a linear regression was performed to quantitate the drift in lifetime following dilution. The $y$ intercept of such a plot was taken as the fluorescence lifetime at the time of vesicle dilution. These intercept values were the lifetimes used to make plots of lifetime vs. time of PEG incubation, as in Figures 1, 4, and 5. A standard curve of lifetime vs. lipid:probe ratio (Parente & Lentz, 1986) was used to estimate the number of fusion events that had occurred, as described in detail in the Appendix.

*ANTS/DPX Leakage and Contents Mixing.* Both of these assays (Ellens et al., 1984; Ellens et al., 1985) work on the principle that ANTS fluorescence is efficiently quenched by DPX via collisional transfer (Smolarsky et al., 1977). In the leakage experiment, ANTS and DPX were coencapsulated in one vesicle population such that DPX quenched over 90% of ANTS fluorescence. When leakage occurred, fluorescence intensity increased due to dilution of ANTS and DPX in solution, resulting in lower quenching efficiency. In the contents-mixing experiments, two vesicle populations were used in a 1:1 ratio. The first contained trapped ANTS molecules and the second, trapped DPX molecules. Mixing of aqueous contents resulted in a decrease in the fluorescence intensity of ANTS due to collisional quenching by DPX. Because of the substantial dilution involved, ANTS and DPX leakage was not observable in and did not interfere directly with the contents-mixing assay (see Appendix). For our purposes, vesicles were mixed with PEG as described above and diluted to a final volume of 3 mL. After dilution, fluorescence intensity and light scattering (at 366 nm) were monitored continuously for 16 min, although only intensity was recorded.

For each type of experiment, the fluorescence intensity scale was normalized as follows: To monitor leakage, the background fluorescence of vesicles in which ANTS and DPX were coencapsulated was set at 0% fluorescence, while the fluorescence resulting from vesicle lysis (using the detergent, $C_{12}E_8$) was taken as 100%. This detergent was chosen because it did not contribute any fluorescence at 366 nm. To monitor contents mixing, 0% fluorescence was again set by the residual fluorescence of coencapsulated ANTS and DPX. The 100% fluorescence level was taken as the fluorescence intensity obtained immediately after mixing equal amounts of ANTS- and DPX-containing vesicles with PEG at concentrations obtained after final dilution of the assay mixture. With the fluorescence scale defined in this manner, one complete fusion cycle would be expected to lower the fluorescence from 100 to 50%.

## Results

*PEG-Induced Lipid Mixing.* The DPHpPC lifetime dependent lipid-mixing assay (Parente & Lentz, 1986) was chosen for use in this study because PEG was found to interfere with the fluorescence of probes employed in the more widely used fluorescence energy transfer assay of Struck et al. (1981).

Using the DPHpPC lipid-mixing assay, we have measured the initial rate and extent of PEG-induced vesicle fusion as a function of polymer concentration. In Figure 1, the phase-derived lifetime is plotted vs. the time of vesicle incubation in 8, 15, 23, and 35% (w/w) PEG. Vesicle–PEG incubation mixtures were diluted prior to recording lifetime measurements. Each time point in Figure 1 corresponds to the DPHpPC fluorescence lifetime at the time of dilution (see Methods and Appendix). The lifetime at time zero, corresponding to vesicles containing an initial 25:1 lipid-to-DPHpPC ratio, was unaltered by the presence of probe-free vesicles in the absence of PEG. However, lipid mixing, as indicated by

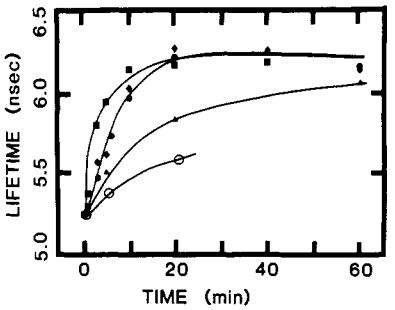


FIGURE 1: DPHpPC fluorescence lifetime as a function of vesicle–polymer incubation time. REV containing DPPC–DPHpPC at a 25:1 molar ratio (0.05 μmol of total lipid) were incubated with a 10-fold excess of unlabeled DPPC REV (0.5 μmol of lipid) in the presence of 8 (closed trianges), 15 (closed diamonds), 23 (closed circles), and 35 (closed squares) wt % PEG and 23 (open circles) wt % dextran for varying times. Samples were diluted 10-fold to a final volume of 3 mL before the fluorescence lifetime was measured. Each time point corresponds to the lifetime at the time of dilution (see Methods and Appendix).

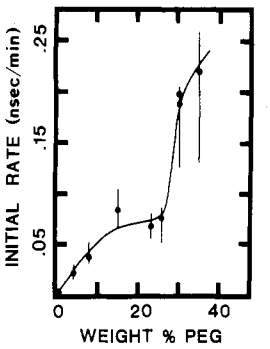


FIGURE 2: Initial rates of lipid mixing as a function of wt % PEG. Initial rates in terms of the change in DPHpPC fluorescence lifetime with time (ns/min) were estimated from tangents to curves such as shown in Figure 1. Vertical error bars indicate the estimated standard deviation in the rates determined from the known standard deviations in lifetime measurements and the uncertainty in the times at which lifetimes were measured. The value at 3.8% PEG was determined from an experiment where the vesicle–PEG mixture was not diluted before lifetime measurements were made (see Figure 7).

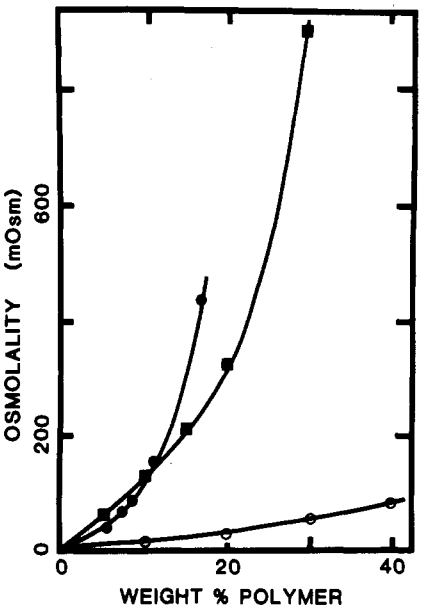


FIGURE 3: Osmolality, measured by freezing point depression osmometry, of PEG (8000 mol wt; filled circles) and dextran (9000 mol wt; filled squares) solutions as a function of polymer concentrations. The osmolality calculated for a hypothetical 8000 mol wt globular (i.e., nonpolymeric) molecule is plotted (open circles) for comparison.

an increase in lifetime, was observed at all concentrations of PEG studied. From the standard curve of lifetime vs. lipid:probe ratio (Parente & Lentz, 1986), the final lifetime value attained after a 60-min incubation in the presence of 15, 23, or 35% (w/w) PEG (approximately 6.2 ns) corresponded to an average lipid:probe ratio of about 50:1. Thus, the probe was diluted approximately twofold indicating that, on the average, each probe-containing vesicle fused only once with a probe-free vesicle. After a 60-min incubation with a PEG concentration of 8%, the DPHpPC fluorescence lifetime was still increasing but, by 2 h (not shown), approached the same limiting value evident in Figure 1 for the higher PEG concentrations.

The use of the DPHpPC lifetime assay for fusion allowed us to make an estimate of the size heterogeneity of the vesicle population resulting from the fusion process. The DPHpPC fluorescence lifetime values observed after completion of the PEG-induced fusion process (Figure 1) could result from a nearly uniform population of fused vesicles, each containing DPPC and DPHpPC at a 50:1 molar ratio. Alternatively, the observed lifetime could represent the average of several lifetimes occurring in different sized vesicles resulting from multiple fusion events. From the difference between phase and modulation lifetime values, it was evident that more than one fluorescence decay component contributed to the observed lifetimes. Unfortunately, with data collected at only one frequency, we could not perform a quantitative analysis for lifetime heterogeneity. However, the data were adequate to establish the limits of lifetime heterogeneity that could exist in our samples. Expected apparent lifetimes were simulated, assuming lifetimes for three components: (1) unfused vesicles, (2) vesicles having undergone one fusion cycle, (3) vesicles having undergone unlimited fusion. The observed lifetime could be modeled only if greater than 50% of the vesicles had undergone one fusion event, with equal contributions from the other two lifetime components. Larger contributions from an extensively fused population would have resulted in average lifetimes greater than those observed. It would appear that widespread heterogeneity, which would be detectable by our measurements, did not occur in this system. However, more subtle sample heterogeneity would have been undetectable under the conditions of our measurements.

The data in Figure 1 demonstrate that the ultimate extent of fusion was independent of PEG concentration, while the initial rate of fusion increased with PEG concentration. Initial rates of lipid mixing were estimated from the slope of the tangent lines drawn through the zero time points of several experimental time courses, such as those shown in Figure 1. These initial rates have been plotted as a function of PEG concentration in Figure 2. Although a rigorous kinetic analysis would require data collected at earlier times in the fusion process, our crude initial rate estimates were quite reproducible (see Figure 2) and sufficient to illustrate clear trends in fusion rate with PEG concentration. This concentration dependence serves to make two important points. First, the rate of large vesicle fusion was a continuous function of PEG concentration, although a dramatic enhancement of fusion efficiency occurred between 26 and 30 wt % PEG. Second, fusion occurred even at low concentrations of PEG, albeit at a slow rate.

*Effect of Dextran on the Fusion of DPPC REV.* Dextran is a naturally occurring polymer with colligative properties similar to those of PEG, suggesting that it should also be capable of causing bilayer dehydration and result in the close association of vesicles. We have investigated whether this polymer would be similar to PEG in its ability to promote

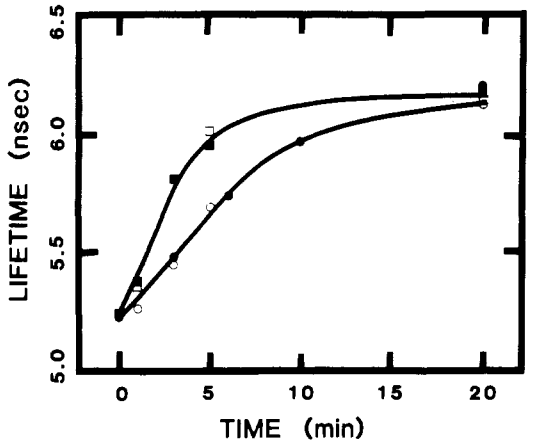


FIGURE 4: Effect of impurities on PEG-induced lipid mixing. Vesicles were incubated with 23 wt % (circles) and 35 wt % (squares) purified (filled symbols) or unpurified (open symbols) PEG. No effects of impurity on the rate or extent of lipid mixing were observed under these conditions.

fusion. In Figure 3, the concentration dependences of the osmolality of PEG 8000 and dextran 9000 solutions are plotted along with the osmolality calculated for an 8000 molecular weight nonpolymer particle in an ideal solution. It is evident that the observed osmotic behaviors of these polymers were similar, although the ability of PEG to lower the activity of water increased more dramatically with concentration than did that of dextran.

We investigated the extent and initial rate of fusion induced by incubation of DPPC REV in a 23 wt % solution of dextran. As seen in Figure 1, dextran did promote lipid mixing associated with fusion, but at a much slower rate than PEG. Although the osmolality of 8 wt % PEG 8000 was less than the osmolality of 23 wt % dextran (see Figure 3), 8% PEG was substantially more effective in promoting fusion. This suggests that some properties of either dextran or PEG other than their osmotic behavior must enter into their fusogenic abilities. It may be that binding of dextran to lipid vesicles protects them from fusion, as suggested by MacDonald (1985).

*Effect of Impurities and Additional Fusogens on the Fusion of DPPC REV.* To check a previous report that impurities (i.e., possibly antioxidants or polymerizing agents) present in commercially available PEG were responsible for its high-fusion efficiency (Honda et al., 1981), vesicles were incubated with both purified PEG (see Methods) and unpurified PEG at 35 and 23% (w/w). When lipid mixing was monitored as a function of time, the two PEG preparations behaved identically at both 35 and 23% (w/w), as shown by the curves in Figure 4. The effects of glycerol monooleate (GMO) on vesicle fusion were also investigated as a further test of the influence of fusogenic impurities. GMO has previously been shown to enhance cell fusion efficiency (Ahkong et al. 1973). When up to 4 mol % GMO was added to either the purified or the unpurified PEG–vesicle incubation mixtures or dextran–vesicle mixtures, no increase in either the rate or the extent of fusion was observed (data not shown).

*Effect of an Osmotic Gradient on the Fusion of DPPC REV.* Osmotic swelling was reported to be a driving force for vesicle–planar bilayer fusion (Miller et al., 1976; Cohen et al., 1982) and necessary for cell fusion after incubation with PEG (Wojcieszyn et al., 1983). We have used the DPHpPC lipid-mixing assay to determine if the initial rate or extent of fusion induced by PEG was altered in response to the creation of an osmotic gradient at the time of sample dilution. Vesicles were prepared in a 162 mosmolal buffer. Both positive and

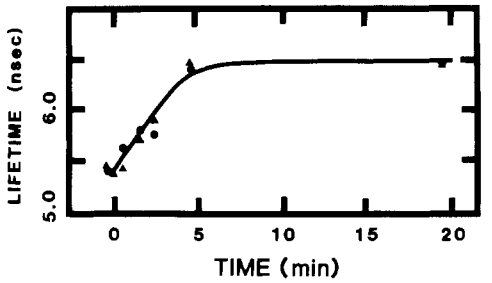


FIGURE 5: Effect of negative (circles) or positive (triangles) transbilayer osmotic gradients on PEG-induced lipid mixing, as detected by DPHpPC fluorescence lifetime (ordinate) changes with time (abscissa). Vesicles prepared in 162 mosmolal buffer and mixed with 35% (w/w) PEG were diluted in to different osmolality buffers (circles, 241 mosmolal; triangles, 100 mosmolal) to attain a negative or positive osmotic gradient (defined as osmolality inside vesicles minus the osmolality of the final dilution medium, assuming no leakage of trapped solutes or entry of PEG or other external solutes into the vesicles).

negative osmotic gradients were created by diluting vesicles into buffers of lower or higher osmolality, respectively. Buffer osmolality was altered by varying the KCl concentration, and expected osmolalities were confirmed by direct measurements. For our purposes, the osmolality gradient was calculated by subtracting the osmolality of the final diluted medium (containing vesicles plus PEG) from the osmolality of the buffer initially inside the vesicles, assuming leakage had not occurred. When this definition was used, vesicles would be expected to swell and experience increased bilayer surface tension when a positive osmotic gradient was created. A positive osmotic gradient might be responsible for the fusion event itself (Ohki, 1984) as well as for substantial leakage of vesicle contents associated with fusion and dilution (see below).

Figure 5 shows the changes in DPHpPC lifetime occurring with time after dilution of DPPC REV incubated in 35% PEG. The circles correspond to values obtained when vesicles were diluted into a low-osmolality buffer (creating a positive osmolality gradient), and the squares represent the lifetime recorded after vesicles were diluted into a high-osmolality buffer (creating a negative osmolality gradient). These results demonstrate that attempts to induce an osmotic gradient by dilution of concentrated PEG–vesicle solutions had no effect on either the initial rate or the extent of vesicle fusion. Smaller positive or negative gradients as well as a zero gradient produced the same result, as did a larger positive gradient of 191 mosmolal. This result indicated that dilution-induced osmotic swelling was not a driving force in PEG-induced vesicle fusion. This does not preclude the possibility that vesicles might be collapsed due to the substantial negative osmotic gradient created when vesicles are first incubated with PEG, as will be addressed below.

*PEG-Induced Contents Mixing and Leakage.* Fusion was monitored by contents mixing as a function of time at two concentrations of PEG, 8 and 35 wt %, under the same conditions used to demonstrate lipid mixing. Leakage experiments were also performed to ensure that fluorescence changes indicating contents mixing were not misinterpreted and to determine to what extent PEG-induced vesicle fusion is a leaky process. Both leakage and fusion were monitored with the ANTS/DPX assay system (Ellens et al., 1984, 1985), since PEG interfered with the fluorescence of other assay systems tested ($Tb^{3+}$/dipicolinic acid: Wilschut et al., 1980; $Co^{2+}$/calcein: Kendall & MacDonald, 1982).

Figure 6A shows typical data obtained from a *leakage experiment* in which ANTS and DPX were coencapsulated in DPPC REV. For this experiment, vesicles were incubated in 35% PEG for 10 min prior to dilution. Due to the high

Table I: Summary of Leakage- and Contents-Mixing Data with Comparison to Lipid-Mixing Data

| PEG (wt %) | incubn time (min) | % of vesicles involved in one fusion event | | % leakage[a] | |
|---|---|---|---|---|---|
| | | lipid mixing | contents mixing | before dilution | after dilution |
| 8 | 20 | 56 | 73 | 62 | 85 |
| 8 | 30 | 64 | 95 | 76 | 88 |
| 8 | 60 | 75 | 100 | 88 | 92 |
| 35 | 3 | 52 | 88 | 30 | 74 |
| 35 | 5 | 67 | 83 | 34 | 75 |
| 35 | 10 | 88 | 100 | 40 | 78 |
| 35 | 40 | 88 | 100 | 60 | 85 |

[a] "Before dilution" encompasses the first 30 s after dilution, since this was the time required to make the first measurement. "After dilution" refers to the value calculated as described in the text by extrapolation to zero time (see Figure 6).

viscosity of these PEG mixtures, fluorescence could not be measured continuously until roughly 30 s after dilution (the time necessary to mix and transfer the sample to a cuvette). Since DPX effectively quenched ANTS when present in the same vesicle, but not when free in solution, leakage of contents was observed as an increase in ANTS fluorescence. From the data in Figure 6A, it is clear that substantial leakage (roughly 30–40% of contents) had occurred by the time fluorescence intensity was first recorded. These results could mean that leakage had occurred in the concentrated PEG solution. It is also apparent that leakage occurred rapidly for a short period (~2 min) immediately following (and possibly due to) dilution. Thereafter, leakage occurred very slowly and may have been due to the low level of PEG remaining in the diluted solution. We fit this region of slow change to a line and used the intercept of this line with the ordinate as the total fraction of vesicle contents that leak due to exposure to PEG and to the dilution event. In the case illustrated in Figure 6A, 78% of the contents leaked in response to these treatments. This meant that we could expect only 22% of the contents originally trapped inside vesicles to be available to monitor the mixing of vesicle contents.

In Figure 6B, the fluorescence intensity data from a *contents-mixing experiment*, under conditions equivalent to the leakage experiment in Figure 6A, are plotted vs. time after dilution. In this experiment, ANTS-containing vesicles were mixed in a 1:1 ratio with DPX-containing vesicles in the presence of PEG. Contents mixing was observed as a decrease in ANTS fluorescence resulting from collisional quenching by DPX molecules. In control experiments, ANTS vesicles and DPX vesicles were mixed in the presence of buffer containing 3% PEG and lysed with the detergent, $C_{12}E_8$. No increase in fluorescence was observed, which confirmed that, under the conditions of this assay, leakage of internal contents from vesicles before the occurrence of contents mixing was undetectable due to the large dilution of trapped material during leakage. Reference to Figure 6B reveals that a rapid drop in intensity occurred within 4 min after dilution, presumably due to the mixing of trapped contents. This delay in observing contents mixing may reflect the time required for diffusion of contents in the dehydrated interior of fused vesicles or may indicate that fusion did not occur until dilution. Comparison of frames A and B demonstrates that both contents leakage (frame A) and contents mixing (frame B) occurred in the same time frame following dilution of the samples. The continued slow drop in fluorescence following the rapid initial drop could represent further mixing of contents due to slow fusion in the presence of diluted PEG. Presumably, leakage also occurred during this time period but at a rate slightly slower than that for contents mixing, leading to the net drop in intensity seen in frame B. In analogy to our treatment of leakage, we fit the region of slow fluorescence decline to a line and used the

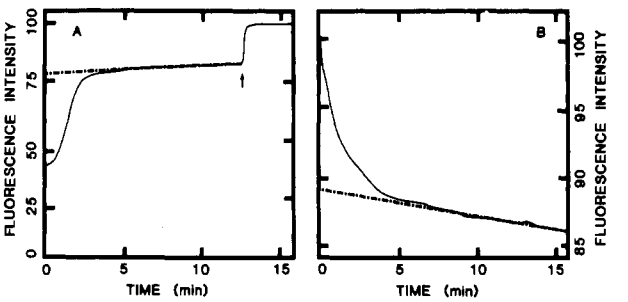


FIGURE 6: Relative leakage and contents mixing of ANTS and DPX upon dilution of DPPC REV incubated in 35% (w/w) PEG for 10 min. (A) Leakage: Fluorescence intensity (see Methods for determination of the scale) was monitored continuously upon dilution of a mixture of PEG and vesicles containing coencapsulated ANTS and DPX. Detergent ($C_{12}E_8$) was added after 12 min (arrow) to set the 100% fluorescence level. This plot is typical of leakage experiments performed at different PEG concentrations and times of incubation, the results of which are summarized in Table I. The dashed line is a linear fit to the data in the region where maximal leakage had occurred. The intercept was used to calculate the fraction of contents remaining inside the vesicles after fusion and dilution had occurred. (B) Contents mixing: Fluorescence intensity (see Methods for determination of the scale) was monitored continuously upon dilution of ANTS-containing vesicles and DPX-containing vesicles mixed in the presence of PEG. The observed decrease in fluorescence intensity of ANTS due to quenching by DPX is indicative of contents mixing. The dashed line is a linear fit to the data over the region where maximal mixing of contents had occurred. The zero-time intercept was taken to be the percent of contents mixing occurring immediately after fusion and dilution had occurred.

intercept to determine the percent of contents mixed at the point of dilution in response to fusion or dilution. In the case illustrated in Figure 6B, an 11% drop in fluorescence was observed. By the referencing of all measurements to the moment of dilution, it was most convenient to correct contents-mixing results for the effects of leakage that occurred either before or after dilution, as descibed in the Appendix.

*Comparison of Fluorescence Fusion Assays.* Both the lipid-mixing and contents-mixing assays that we have used are most sensitive to the observation of initial fusion events. In Table I, we have compared the results obtained with these two assays in terms of the percent of vesicles involved in at least one fusion event after incubation in 8 or 35% (w/w) PEG for the times indicated. The calculations used to obtain these numbers are described in the Appendix. Reference to the table indicates that the contents-mixing assay gave somewhat larger estimates of the extent of fusion than were obtained by the lipid-mixing assay. This could reflect uncertainties associated with the contents-mixing results due to the need to correct for leakage or the possibility that contents mix more rapidly than lipids in the presence of PEG. Within a reasonable level of uncertainty, however, there was good correlation between the two assays, indicating that the observed lipid and contents

Table II: Vesicle–PEG Interaction

| experiment | [$^{14}$C]PEG retained (dpm) | % of effective trapped vol[a] |
|---|---|---|
| 35% PEG, 20-min vesicle incubation, 1-mL dilution | 4000 ± 400 | 31 ± 3 |
| 8% PEG, 60-min vesicle incubation, no dilution | 8500 ± 2300 | 30 ± 8 |
| vesicles applied and rinsed in 8% PEG | 12300 ± 3400 | 50 ± 14 |
| background | 2700 ± 800 | |

[a] Trapped volume was assumed to be 3 $\mu$L/$\mu$mol of $P_i$ calculated by carboxyfluorescein trapping (Parente & Lentz, 1984). The % of effective trapped volume = [([$^{14}$C]PEG retained on filter − background)/([$^{14}$C]PEG concn × [$^{14}$C]PEG sp act. × trapped volume × lipid concn × lipid aliquot)] × 100.

mixings were indicative of fusion.

*PEG Trapping.* We have monitored the ability of PEG to enter into and become trapped in vesicles either prior to dilution of vesicle–PEG mixtures or after dilution. If PEG were incorporated into vesicles before or during dilution, an osmotic gradient could not have been created as a result of the fusion process. Vesicles were incubated with [$^{14}$C]PEG at concentrations used in fluorescence experiments, and mixtures were treated as described in Methods and summarized in Table II. From the radioactively labeled PEG remaining associated with the filter, it appeared that roughly 30% of the total vesicular space contained trapped PEG before dilution (line 2, Table II). This represented less than 1% of the total radioactive PEG in the incubation mixture, which contained 8% PEG (w/w). There was no difference in this result after dilution of a 35% (w/w) PEG sample (line 2, Table II). These results could reflect trapping or could equally be interpreted as indicating binding of PEG to the outside of the vesicles. To distinguish between these possibilities, vesicles were added to the filter and washed with 5 mL of water. Eight percent PEG was then rapidly passed through the filter, which was then rinsed with a total volume of 8 mL of water. The results (line 3, Table II) implied that 50% of the vesicle internal volume was occupied by PEG. Vesicles and PEG were only in contact for a short time (less then 5 min) on the filter, and, after this short an incubation time, only minimal lipid mixing would have occurred (Figure 1). Therefore, vesicle rupture and resealing needed for PEG trapping could not have been appreciable on the time scale of this experiment. Thus, the radioactivity observed associated with the filter in these trapping experiments likely resulted totally from external association of PEG with the vesicles or with the filter in the presence of vesicles. This possibility is supported by reports that both PEG (Boni et al., 1984a) and dextran (Evans & Metcalfe, 1984) associate with synthetic phospholipid vesicles. We can conclude that PEG trapping by vesicles was minimal under the conditions of our experiments.

*Fusion before Dilution*? As outlined, our fluorescence fusion assays have required dilution of viscous PEG-rich samples before measurements could be obtained. Thus, we do not know whether fusion occurred in undiluted PEG or only during or after the dilution process. In order to address this issue, we measured lipid mixing, contents mixing, and leakage in 3.8% PEG without a dilution step. This approach is valid since we have shown that fusion occurred at different initial rates but to the same final extent at all concentrations of PEG (Figures 1 and 2). In Figure 7, the percentages of lipid and contents mixing and of total possible leakage are presented as a function of time after vesicles were added to a 3.8 wt % solution of PEG. The lipid-mixing assay indicated that greater than 50% of the

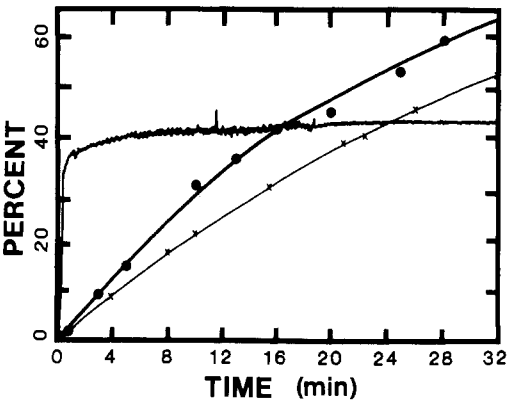


FIGURE 7: Percent of leakage (solid line), contents mixing (circles), and lipid mixing (×) as a function of time after addition of vesicles to 3.8% (w/w) PEG. Maximum leakage (100%) was determined by addition of $C_{12}E_8$ to ANTS/DPX-containing vesicles. 100% mixing for both lipids and contents was taken to be the value (fluorescence lifetime or intensity) expected for one complete cycle of fusion.

vesicles had fused within a half hour, and a lifetime value (6.2 ns, not shown in the figure) consistent with one fusion cycle was obtained by 2 h. The initial rate of fusion obtained from this undiluted experiment fit exactly on the curve of initial rates constructed from the incubation/dilution experiments (Figure 2).

Leakage of contents occurred within 1 min of vesicle addition and thereafter remained fairly constant at 41% for the entire time that it was followed. This immediate leakage upon addition of vesicles to a PEG solution can be explained by the creation of a negative transbilayer osmotic gradient, leading to partial collapse of the vesicles. From the previous dilution experiments (Figure 6A and Table I), some leakage had been expected during vesicle–PEG incubation, or within 30 s after dilution, since for several experiments, the first recorded value was always in the range of 30–60% leakage.

Contents mixing was also observed when vesicles were added to 3.8% PEG, as shown by the circles in Figure 7. When the contents-mixing experiment was performed under conditions of no dilution, the sample contained a 10 times greater concentration of lipid than was present in diluted samples. These more concentrated conditions necessitated correction for ANTS quenching by DPX which had leaked into the solution (see Appendix). As a result of difficulties involved in obtaining this correction factor, results from three separate experiments (one of which is presented in Figure 7) were inconsistent as to the exact extent of contents mixing (range 45–60% fusion at 30 min). However, all three experiments demonstrated unambiguously that contents mixing did occur prior to dilution of PEG–vesicle mixtures and that this mixing of contents occurred on a much different time scale than leakage (Figure 7).

## Discussion

Membrane fusion involves the intermixing of internal aqueous contents and bilayer components of two individual vesicles to form a single trapped compartment surrounded by one bilayer. To rigorously state that vesicle fusion has occurred, both mixing processes must be demonstrated. We have shown, for the first time, that PEG-induced vesicle fusion fulfills both of these requirements. Results obtained with the lipid-mixing assay, which monitors changes in the fluorescence lifetime of the probe DPHpPC (Parente & Lentz, 1986), were in good agreement with those obtained with the contents-mixing assay, which measures the quenching of ANTS fluorescence by DPX (Ellens et al., 1985). Since both of these

assays are most sensitive to the detection of early fusion events, data could be interpreted in terms of the fraction of vesicles involved in a single fusion event. On the average, large unilamellar vesicles experienced only one fusion event, irrespective of the amount of time that they were in contact with PEG (Figure 1, Table I). By comparison with observed lifetime values, simulation of sample heterogeneity indicated that greater than 50% of the vesicles would have undergone only one fusion event with no more than 25% of the signal arising from vesicles involved in multiple fusion events. Our evidence lends support to results from freeze–fracture electron microscopy which showed that the median diameters of egg PC large vesicles, incubated for 1 h in 45% PEG ($M_r$ 6000) and subsequently washed, shifted from 140 to 220 nm (Boni et al., 1984a). The calculated surface area of a sphere with a diameter of 140 nm is $6.2 \times 10^4$ nm$^2$ while that for a sphere with a diameter of 220 nm is $1.5 \times 10^5$ nm$^2$. The latter is close to the value expected for the surface area of vesicles formed from the fusion of two 140-nm vesicles ($1.2 \times 10^5$ nm$^2$).

It is not clear at this point why PEG-mediated fusion of LUV stopped after only one round of fusion. By contrast, PEG-induced fusion of phosphatidylcholine SUV led to formation of large multilamellar structures, as visualized by freeze–fracture electron microscopy (Boni et al., 1981a, 1984a). It could be that the inherent instability of SUV causes fusion of these vesicles to follow an entirely different pathway than is followed for LUV fusion (e.g., a rupture-type mechanism as opposed to a true fusion mechanism). The different responses of SUV and LUV to dehydration and osmotic stress have recently been discussed (Evans & Parsegian, 1983). Alternatively, it could be that unfused SUV can fuse with large fusion products (Düzgünes & Ohki, 1981), making the formation of larger products possible. This would be a likely explanation only if SUV–LUV fusion is much more probable than SUV–SUV fusion. Further studies will be necessary to resolve this issue.

Vesicle fusion is often accompanied by leakage of contents in a manner not directly associated with the fusion process, as we have shown for PEG-induced fusion. Two stages of vesicle leakage were apparent from our experiments. The first, releasing 30–60% of vesicle contents, regardless of PEG concentration, occurred before dilution (Figure 6 and Table I). This first stage of leakage was essentially complete within 1 min after vesicles were added to solutions containing even low concentrations of PEG (Figure 7). This initial leakage occurred well before any substantial fusion (Figure 7) and, thus, appeared to be caused by the osmotic compression of vesicles associated with mixing with PEG, rather than by the fusion process itself. It is not clear to what extent this osmotic compression was necessary for the subsequent fusion events to occur. The second stage of vesicle leakage was not complete until a few minutes after vesicle–PEG mixtures were diluted, regardless of either the time of vesicle incubation with PEG or the PEG concentration. $Ca^{2+}$-induced fusion of LUV has also been shown to be accompanied by substantial contents leakage, although, as in our studies of PEG fusion, the time course of leakage was different from the time course of fusion (Wilschut et al., 1981). Calcium-initiated leakage from phosphatidylserine LUV probably reflects vesicle collapse (Bentz et al., 1985), in analogy with the first stage of leakage observed in our studies.

Early work on PEG-induced cell fusion showed that incubation in high concentrations of PEG [greater than 40%, but below 50% (w/w) to avoid cell rupture] was necessary for fusion to occur (Blow et al., 1978). Boni et al. (1981a) have also reported that a threshold concentration of PEG (greater than 28%) was required to obtain fusion of SUV. Although interpretation of their light scattering data was not straightforward, these workers suggested that the rapid reversibility of vesicle aggregation at low PEG concentrations prevented vesicles from being held in contact long enough for fusion to occur. MacDonald (1985) also reported a maximal response in lipid mixing and turbidity at greater than 20–30% (w/w) PEG for SUV and 30–40% (w/w) PEG for larger vesicles; however, the extent of fusion was not zero when vesicles were incubated with PEG below these threshold concentrations.

No distinction between the rate and extent of fusion was attempted in most earlier studies, making it difficult to draw a complete parallel between these results and ours. Both MacDonald (1985) and Boni et al. (1981a) varied the PEG concentration, while the time of vesicle incubation with polymer was held fixed (at about 1 h). We have varied not only the PEG concentration but also the time of vesicle incubation on the premise that the key bilayer destabilizing event of the fusion process would be most precisely defined in terms of the rates of the molecular processes involved in fusion. In contrast to the results of MacDonald, roughly the same extent of fusion was obtained after 1 h of incubation for all PEG concentrations above 15% (w/w) (Figure 1). Our results did not indicate that a threshold concentration of PEG was necessary for fusion activity, although initial rate analysis did indicate a substantial enhancement in rate between 26 and 30% (w/w) PEG (Figure 2). This enhancement in fusion rate occurred over the same range of PEG concentration that Tilcock and Fisher (1979) found necessary to cause a significant increase in the phase-transition temperature of DPPC multilamellar vesicles. It is well-known that limiting the bilayer hydration layer significantly increases the phospholipid phase-transition temperature (Ranck et al., 1974). It may be that PEG alters bilayer structure at these concentrations through disruption of the water layer intimately associated with and involved in the lamellar bilayer structure.

In order to gain insight into the properties of PEG that lead to fusion, another dehydrating agent, dextran, was used to induce vesicle fusion. This polymer has been reported to have no effect on cell fusion (Ahkong et al., 1973) or on small vesicle fusion monitored by dynamic light scattering (Boni et al., 1984a). We found that dextran can induce limited large-vesicle fusion, but at a much slower rate than PEG. On the basis of osmolality measurements alone (Figure 3), we would have expected 23 wt % dextran to behave in a manner similar to that of 15 wt % PEG. MacDonald (1985) also reported observing only limited fusion when dextran was mixed directly with vesicles, but she reported fusion levels comparable to PEG when a vesicle suspension was dialyzed against a dextran solution. MacDonald interpreted results as indicating that direct interaction of vesicles with dextran acts to inhibit fusion (possibly by binding to the vesicle surface).

Bilayer destabilization may be important for enhancing the fusion efficiency of PEG, and for this reason, two factors influencing this were investigated. Honda et al. (1981) suggested that "pure" PEG acted to aggregate cells, while impurities present in commercial preparation were responsible for promoting fusion. In our hands, purified PEG was as capable of promoting fusion as the unpurified material (obtained from Fisher Scientific). This confirmed other reports (Smith et al., 1982; Boni et al. 1984a; MacDonald, 1985) that both purified and unpurified PEG from a variety of suppliers induced both cell and vesicle fusion. Certain preparations, however, might contain such high levels of impurity that the

rate of fusion might be enhanced (Smith et al., 1982).

It has been proposed that osmotic swelling is responsible for destabilizing the bilayer during PEG fusion. Miller et al. (1976) and Cohen et al. (1982) observed that osmotic swelling was necessary to obtain lipid vesicle and planar bilayer fusion. Knutton (1979) and Wojcieszyn et al. (1983) reported that PEG induced the close association of cells but that it was osmotic swelling, when PEG was removed or diluted, that induced fusion. In this regard, Ohki (1984) has postulated that the increase in the surface free energy of a membrane associated with the dilution step of PEG-induced fusion might account for the bilayer destabilization thought to be necessary for fusion to occur. However, our attempts to create either a positive or a negative osmotic gradient upon dilution of vesicle–PEG mixtures had no effect on fusion monitored by lipid mixing (Figure 5), indicating that dilution-induced osmotic swelling was not a driving force in PEG-induced vesicle fusion. This could mean that an increase in membrane surface tension associated with a positive osmotic gradient was not a contributor to the fusion process. Alternatively, leakage of internal contents out of the vesicles and of PEG into the vesicles prior to dilution could have prevented formation of an osmotic gradient during dilution. Finally, our results could be explained by the occurrence of fusion in the concentrated PEG solution, i.e., prior to dilution. These issues will be addressed below.

First, as to leakage, a fraction of the internal contents was observed to leak from vesicles almost immediately after contact with even low percentages of PEG. PEG could possibly enter vesicles by complete rupture of vesicles or through pores created when vesicles were forced into close contact. Experiments to determine the likelihood of PEG trapping were performed, and the results (Table II) indicated that PEG trapping by vesicles was minimal under the conditions of our experiments. In addition, a mechanism of complete vesicle rupture and resealing could have accounted for the observed lack of osmotic gradient effects but would not have explained results from the ANTS/DPX assays for leakage and contents mixing. If vesicles ruptured completely, all contents would have been lost. In fact, we observed only a limited and constant amount of leakage before dilution (Table I and Figure 7). Most significantly, some fraction of trapped contents remained trapped and capable of mixing in a nonleaky fashion with the trapped contents of other vesicles (see Figure 6B and Table I). The most plausible explanation for these seemingly contradictory results is that the vesicles experienced at least some osmotic stress at the point of sample dilution, but, while this stress may have accounted for additional contents leakage immediately following the dilution process, it was not a key factor in bilayer destabilization to induce fusion.

Second, as to the exact moment of fusion, lipid-mixing and contents-mixing experiments performed in 3.8% (w/w) PEG solutions without a dilution step provided direct evidence for fusion occurring before dilution (Figure 7). Freeze–fracture electron micrographs of large vesicles (Boni et al., 1984a), taken before, during, and after incubation with PEG, clearly demonstrated aggregation during incubation with PEG but were difficult to interpret in terms of fusion because of the distortion of vesicle shape in the aggregated state. Fusion of small unilamellar vesicles did appear to occur before dilution (Boni et al., 1984a), but this could have reflected the inherent instability of these small vesicles. *All our results, however, firmly support the conclusion that dilution and the osmotic swelling that might accompany dilution are not required for PEG-induced large vesicle fusion.* The conflict between this conclusion and the cell fusion results of Knutton (1979) and Wojcieszyn et al. (1983) may reflect differences in the mechanism of cell and vesicle fusion.

Several proposals have been made to explain the ability of PEG to induce membrane fusion. Most of these recognize the dehydrating ability of PEG, but stress different structural consequences of this property. The importance of nonbilayer structures has been stressed by Boni et al. (1981b; 1984a,b), although Tilcock and Fisher (1982) and Arnold et al. (1983), also using $^{31}P$ NMR techniques, found no evidence for nonbilayer structures induced by PEG. Arnold et al. (1983) suggested that impurities in the PEG used by Boni et al. could have explained their detection of nonbilayer structures. From electron microscopy and turbidity measurements, Sâez et al. (1982) have suggested that PEG locally solubilized the membrane bilayer, causing fusion by a process of rupture and resealing of the membrane. However, while our results demonstrate substantial leakage of vesicle contents associated with PEG treatment (Figure 6A, Table I, Figure 7), they also indicate that this leakage is not an integral part of the fusion process (compare the time courses of leakage and fusion in Figure 7). Our demonstration that fusion precedes sample dilution very much weakens the proposal that increases in bilayer surface tension which can be induced by osmotic swelling play a key element in the fusion process (Ohki, 1984). Finally, as to the importance of membrane dehydration in PEG-induced fusion (Blow et al., 1978; Arnold et al., 1983), our results show a small but appreciable rate of fusion even at concentrations of PEG (Figures 1 and 2) much lower than those necessary to structure all free water (Tilcock & Fisher, 1979). This suggests that, if dehydration of intimately juxtaposed bilayers is responsible for bilayer destabilization and fusion, then even partial dehydration and loose association of vesicle membranes caused by low concentrations of PEG can still substantially increase the probability of a fusion event. It will be of interest to determine what effect PEG has on bilayer structure at these low concentrations and how variations in vesicle composition might influence the rate of fusion under these conditions. In addition to slow fusion at low concentrations of PEG, we observed a significant increase in rate at concentrations that might be expected to disrupt the hydration shell of the phospholipid bilayer. If we accept that closely associated water molecules are an inherent and essential structural component of the lipid bilayer, it would seem plausible that perturbation or elimination of these oredered water molecules might constitute the long sought-after bilayer destabilizing event that would increase the probability of local fluctuations to nonlamellar structures leading to membrane fusion.

Aside from bilayer dehydration, there is another effect of PEG that could account for the bilayer destabilization necessary for fusion. Thus, it may be that the highly curved edges of osmotically collapsed vesicles provide the points of structural defect that are needed for fusion. Additional study will be required to distinguish between these two possible mechanisms.

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APPENDIX

*Data Analysis for Fluorescence Fusion Assays. (A) Lipid Mixing.* The lifetime values obtained and plotted in Figures 1, 4, and 5 were compared to our standard curve of DPHpPC

lifetime vs. lipid-to-probe ratio (Parente & Lentz, 1986) to determine the extent of probe dilution. When starting with vesicles at a 25:1 lipid-to-probe ratio, a fusion event between one probe-containing and one probe-free vesicle would be expected to result in a 50:1 lipid-to-probe ratio in the fused vesicle. The experiment was designed such that there was a 10-fold excess of unlabeled vesicles at the start of the experiment. Therefore, on average, only nine of 10 interactions would occur between a labeled and an unlabeled vesicle. For this reason, the lifetime change expected for vesicles starting at a 25:1 lipid-to-probe ratio and ending at 50:1 (obtained from the standard curve) was multiplied by 0.90. This expected lifetime change was equated with 100% of all vesicles experiencing one round of vesicle fusion. A lifetime value corresponding to greater than 100% fusion was taken to indicate that some vesicles experienced more than one fusion event. The lifetime observed for vesicles containing a 25:1 lipid:DPHpPC ratio corresponded to 0% fusion. By this method, a calibration curve of percent fusion vs. expected lifetime change was constructed. This analysis makes the assumption that the proportion of vesicles undergoing multiple fusion events during the initial phases of the fusion process was small, that is, fusion was uniform and random. From a simulation of lifetime heterogeneity, no widespread heterogeneity was detectable in our experiments, indicating that this assumption was reasonable (see Results).

(*B*) *ANTS/DPX Contents Mixing and Leakage.* To determine the leakage in response both to addition of PEG and to dilution of vesicle–PEG mixtures, the regions of curves such as Figure 6A showing slow drift in fluorescence intensity were fit to a line (e.g., see the dashed line in Figure 6A). The zero-time intercept, on an intensity scale normalized from 0 to 100% as described under Methods, was taken as the percent of maximal possible leakage ($L_{max}$). From this, we obtained the maximum percentage of vesicle contents that could mix under identical experimental conditions ($C_{max} = 100 - L_{max}$). Since ANTS vesicles and DPX vesicles were mixed in a 1:1 ratio for the contents mixing assay, statistically, only half of the initial vesicle interactions should result in a change in fluorescence (i.e., ANTS–DPX or DPX–ANTS vs. ANTS–ANTS or DPX–DPX; Nir et al., 1980). Therefore, $C_{max}/2$ was taken as the maximum percentage of contents mixing that could be observed by this assay, given the measured extent of contents leakage. The observed percentage of mixed contents resulting from fusion or dilution ($C_{obsd}$) was obtained from data such as presented in Figure 6B by subtracting from 100% (fluorescence was normalized on a scale from 0 to 100% as described in Methods) the zero-time intercept of a line drawn through the region of slow fluorescence change. Finally, the percentage of vesicles that would be involved in one fusion event ($C$, i.e., the value reported in Table I) was obtained as $C = [C_{obsd}/(C_{max}/2)] \times 100$.

The contents-mixing assay necessarily involved interference from leakage. In the case of experiments involving dilution of concentrated PEG samples (e.g., Figure 6), the contribution of leakage was determined by performing parallel control leakage experiments. All measurements of leakage and contents mixing were extrapolated to the time of dilution as described above and illustrated in Figure 6. When this extrapolated value was used, our analysis was not influenced by the time course of leakage induced as a result of dilution. Instead, we obtained measures of total leakage (pre- and postdilution) and contents mixing at the point of dilution, after incubation in concentrated PEG for a given period of time. This procedure was used to obtain the data presented in Table I. By contrast, for the experiment performed with dilute PEG, in which the rate of contents mixing was measured directly (Figure 7), leakage of contents was essentially completed before contents mixing occurred and could be treated as a time-dependent correction. Each contents-mixing time point in Figure 7, then, was corrected for a constant amount of leakage by the same procedure described above for the dilution experiments.

**Registry No.** PEG, 25322-68-3; DPPC, 63-89-8; dextran, 9004-54-0.

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# Possible Basis for the Apparent Surface Selectivity of the Contact Activation of Human Blood Coagulation Factor XII†

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ABSTRACT: The activation of factor XII by the proteases factor XIIa and kallikrein is known to be greatly enhanced by certain negatively charged surfaces. Studies that compared factor XII surface binding to factor XII activation found that binding alone was insufficient to account for surface enhancement of the activation rate. The temperature dependence of the reaction showed unusual behavior that may be related to the conformational change of factor XII following binding; the rate of factor XII activation had a relatively low temperature optimum (0-47 °C) that was sensitive to choice of surface and salt concentration. In temperature studies, below 47 °C, the decrease in the activation rate was not related to the thermal denaturation of enzyme or substrate, nor to the choice of activator enzyme (factor XIIa or kallikrein), nor to the species of factor XII (human or bovine) but to a behavior, designated a thermal transition, associated with the surface or the protein–surface interaction. The previously reported surface selectivity of contact activation is possibly due to the temperature characteristics and other properties of the thermal transition; a surface that has a low-temperature thermal transition and that is highly sensitive to salt will be a "poor" contact surface under the usual choice of reaction conditions (~150 mM ionic strength and 37 °C). However, solution conditions were identified that allowed the following negatively charged surfaces to function, in nearly equal potency, in the activation of factor XII: phosphatidylserine, phosphatidylglycerol, phosphatidic acid, phosphatidylinositol 4-phosphate, heparin, and 5-kDa dextran sulfate, as well as the previously characterized sulfatide and 500-kDa dextran sulfate. The thermal transition may also explain the phenomenon of cold-promoted activation of plasma; plasma, or its storage containers, may contain "poor" contact surfaces that become active at low temperature. The surface property that is responsible for the thermal transition has not been identified but appears to account for several properties of contact activation.

Glass tubes, when compared to silicon-coated glass tubes, shorten the clotting time of normal platelet-poor plasma (Conley et al., 1949). This effect has been termed contact activation because a component of plasma becomes activated upon contact with glass. This observation led to the hypothesis that a plasma deficient in the contact protein could be identified by its long clotting time in either type of tube. Four contact proteins, factor XI (Rosenthal et al., 1953), factor XII[1] (Ratnoff & Colopy, 1955), prekallikrein (Wuepper, 1973), and high molecular weight kininogen (Colman et al., 1975; Saito et al., 1975; Wuepper et al., 1975), have since been identified. With the purified proteins and several different surfaces, the complex interactivations have been studied and a model for the in vitro contact activation scheme has been

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; factor XII, 78-kDa single-chain factor XII (596 residues); factor XIIa, 78-kDa two-chain disulfide-linked factor XII that is enzymatically active (596 residues with a heavy chain from $Ile^{1}$ to $Arg^{353}$ and a light chain from $Val^{354}$ to $Ser^{596}$); factor XIIf, 28-kDa two-chain disulfide-bonded fragment of factor XII that is enzymatically active (251 residues with a heavy chain from $Val^{354}$ to $Ser^{596}$ and a light chain from $Asn^{335}$ to $Arg^{343}$); HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; kDa, kilodalton(s); PA, egg phosphatidic acid; PG, egg phosphatidylglycerol; PIP, bovine brain phosphatidylinositol 4-phosphate; PS, bovine brain phosphatidylserine; Tris, tris(hydroxymethyl)aminomethane.

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