Membrane Fusion due to Dehydration by Polyethylene Glycol, Dextran, or Sucrose†

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ABSTRACT: To determine whether polyethylene glycol (PEG) causes growth of liposomes by affecting them directly or indirectly, vesicles composed of phosphatidylcholine were exposed to increasing concentrations of M_r 15 000–20 000 PEG or M_r 40 000 dextran either by direct mixing or across a dialysis membrane. After incubation at room temperature and dilution below at least 5% (w/w) polymer, the vesicles were monitored for fluorescence energy transfer and for absorbance at 400 nm. PEG induced the same levels of dequenching or lipid mixing and increased turbidity, regardless of whether the vesicles had been mixed directly with or dialyzed against PEG. These changes occurred within 5–15 min of polymer application. It is concluded that the increased lipid mixing and/or increased turbidity, indicating vesicle growth, resulted from an indirect effect of PEG on the vesicles—most likely dehydration. Dextran, in contrast to PEG, induced less dequenching and/or less turbidity increase when vesicles were directly mixed with, as opposed to dialyzed against, dextran. Although dextran not in contact with vesicles and with osmotic activity comparable to PEG was able to cause a degree of membrane fusion similar to that of PEG, therefore, the dehydrating effect of dextran could be mitigated if it were allowed to interact with vesicles. In further support of membrane dehydration as a precursor to membrane fusion, lipid mixing among sonicated and sonicated, frozen–thawed vesicles dialyzed against sucrose increased as a function of sucrose concentration. Vesicle morphology generally determined the maximal degree of membrane fusion inducible by the polymers. Sonicated vesicles gave maximal responses to 20–30% (w/w) polymer, which were generally greater than maximal responses to 30–40% (w/w) polymer given by sonicated vesicles frozen–thawed 3 times (i.e., medium sized and uni- to paucilamellar). Large, multilamellar vesicles, in turn, gave the lowest maximal responses and also to 30–40% (w/w) polymer.

The cell-fusing activity of polyethylene glycol (PEG)[1] has been related to the ability of PEG to alter properties of purely lipid membranes, i.e., to reduce their surface potential (Maggio et al., 1976), to raise their transition temperature (Tilcock & Fisher, 1979), to solubilize bilayers in a detergent-like fashion (Saez et al., 1982), to increase bilayer permeability (Aldwinckle et al., 1982), and to bind to liposomes (Boni et al., 1984). Not only has it been unclear which of these perturbations leads to PEG-dependent membrane fusion, but also it has not been demonstrated whether these responses of lipid membranes signify direct or indirect effects of PEG. For example, it has been suggested, on the one hand, that PEG causes fusion by dehydrating lipid vesicles (Blow et al., 1978) or by lowering the polarizability of the aqueous medium (Arnold et al., 1983) but, on the other hand, that PEG itself interacts directly with phospholipid (Tilcock & Fisher, 1979; Ohno et al., 1981; Boni et al., 1984).

To answer the important, as yet unsettled, question as to whether PEG fuses membranes by acting on them directly or indirectly, two kinds of tests have been performed and the results reported here. First, liposome growth has been measured by monitoring vesicle turbidity and lipid mixing between vesicles by fluorescence energy transfer, before and after the vesicles were either mixed with or dialyzed against M_r 15 000–20 000 PEG. If liposome growth necessitated a direct interaction between polymer and vesicle, the separation of vesicles from polymer by a dialysis membrane should result in less liposome growth than direct mixing of vesicles and polymer. It is shown here that PEG effected the same degree of liposome growth whether or not a dialysis membrane separated PEG from the vesicles. LeNeveu et al. (1977) performed similar experiments to demonstrate that bilayer repeat distances can be reduced indirectly by osmotic forces alone.

Second, to test the inference from the foregoing experiments that any agent capable of dehydration should cause vesicle growth, experiments have been performed with two other agents capable of dehydration—one, like PEG, with a molecular structure associated with unusually high osmotic activity, i.e., dextran, and another at a molar concentration high enough to afford comparably high osmotic activity, i.e., sucrose. Unlike PEG, dextran was more effective when applied indirectly (by dialysis) than when applied directly (by direct mixing). Avid dextran binding to vesicles (Minetti et al., 1979; Evans & Metcalfe, 1984) may be able to mitigate its ability to cause liposome growth. Sucrose also caused membrane fusion, provided the vesicles were dialyzed against the sugar solution across a small pore dialysis membrane. Thus, dehydrating agents should induce bilayer fusion, as long as the bilayers are able to interact with each other and are not stabilized against fusion, e.g., as they are when in the form of multilayered vesicles.

MATERIALS AND METHODS

Preparation of Lipid Vesicles and Polymer Solutions. Diisostearoylphosphatidylcholine, synthesized in this laboratory (Johnson et al., 1973), was dried with N_2 either alone or with

[1] Abbreviations: PEG, polyethylene glycol; DiSPC, diisostearoylphosphatidylcholine; DnsPE, dansyldipalmitoylphosphatidylethanolamine; DiI-C_{18}, dioctadecylindocarbocyanine; RhPE, (lissamine rhodamine B sulfonyl)dioleoylphosphatidylethanolamine; MOPS, 4-morpholinepropanesulfonic acid.

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dansylphosphatidylethanolamine [made from dipalmitoylphosphatidylethanolamine (Sigma Chemical Co., St. Louis, MO) and dansyl chloride (Pierce Chemical Co., Rockford, IL), according to the method of Waggoner & Stryer (1970), or purchased from Molecular Probes, Junction City, OR] with or without dioctadecylindocarbocyanine (Molecular Probes, Junction City, OR) or (lissamine rhodamine B sulfonyl)-dioleoylphosphatidylethanolamine (Avanti Polar-Lipids, Birmingham, AL). Egg phosphatidylcholine could be substituted for DiSPC with similar results. All lipids gave one spot when periodically applied in excess and chromatographed on thin-layer silica gel plates. Lipids that had been under oil pump vacuum for at least 30 min were hydrated at 50 °C for 30 min with 10 mM MOPS,[1] pH 7, plus 0.02% NaN_3 at a concentration of 10 mg/mL (12.4 mM) DiSPC with or without probes, i.e., 1.8 mol % DnsPE, with or without 0.49 mol % DiI-C_{18} or 0.3 mol % RhPE. Multilayered vesicles were produced by vortexing the hydrated lipid; sonicated vesicles were produced by bath sonicating the multilayered vesicles for 12–36 min at room temperature, and uni- to paucilamellar vesicles were produced by freezing the sonicated vesicles 3 times in a dry ice–ethanol mixture, each freezing followed by thawing at room temperature. Samples were checked occasionally for phospholipid content by phosphate determinations according to a modification of the Bartlett procedure (1959) and were within 10% of the stated phospholipid content.

Polymers were from the following sources: M_r 6000–7500 PEG and M_r 15 000–20 000 PEG from J. T. Baker Chemical Co., Phillipsburg, NJ, and from Calbiochem, La Jolla, CA; M_r 10 000 dextran T10 and M_r 40 000 dextran T40 from Pharmacia Fine Chemicals AB, Uppsala, Sweden, and from Sigma Chemical Co., St. Louis, MO; M_r 184 000 clinical-grade dextran from Sigma Chemical Co., St. Louis, MO. They were dissolved in water; the solutions varied in pH from 6 to 7 and were generally not used if more than a few days old and usually used on the same day as made. The PEG preparations were used without further purification, after it was found that ether-extracted and dried PEG gave the same results as untreated PEG, in agreement with Smith et al. (1982).

Osmometry of Polymer Solutions. Freezing point depression was measured with an Osmette S automatic osmometer (Precision Systems, Inc., Sudbury, MA). The osmometer was calibrated with standards at 100 and 500 mOsm prior to measurement of the freezing points of 0.24-mL aliquots of each polymer solution.

Treatment of Vesicles with Polymer. Vesicles were exposed to polymer solutions in either of two ways: (1) The first method was direct mixing. Vesicles (100 μg of phospholipid in 0.01 mL) were injected into the appropriate polymer (0.04 mL). The pipet for accurately delivering solutions of high molecular weight polymers was a positive displacement type (Scientific Glass Apparatus, Bloomfield, NJ). Preliminary experiments indicated that lipid mixing reached plateau values within 10 min of polymer–vesicle contact (Figure 7), but for convenience, samples were left at room temperature for 1 h, after which time they had generally separated from the polymer phase and accumulated near the meniscus of the sample. After 1 h, 0.5 mL of 10 mM MOPS, pH 7, was added to each tube and vortexed several times during a 15–30-min period to ensure homogeneous dilution of each sample prior to fluorometry and spectrophotometry.

(2) The second method was dialysis. A 2-in. length of dry dialysis tubing (M_r 12 000–14 000 cutoff; Spectra/Por, Los Angeles, CA) was folded, closed at one end with a clamp, and left open at the other end to form a bag. The same results were obtained, whether or not the bag had been stirred overnight in a large volume of water before use. For sucrose experiments it was necessary to assemble a "double bag", consisting of a length of M_r 12 000–14 000 cutoff tubing within a length of M_r 1000 cutoff tubing (Spectra/Por), which were closed together at one end with a clamp. The outer, M_r 1000 pore-size bag prevented the influx of sucrose and so enhanced the dehydrating effect of the sugar. When placed *directly* in M_r 1000 pore-size bags, vesicles crept to unsubmerged regions of the bag, dried out, and underwent membrane fusion, even when the bags were placed in water. This creeping of the vesicles may be due to the chemical modification necessary to produce M_r 1000 sized pores, according to the manufacturer, and was avoided by putting the vesicles in bags of unmodified M_r 12 000–14 000 cutoff dialysis tubing, in which vesicle creeping did not occur, and drawing the small pore bags over the vesicle-containing large pore bags.

A total of 100–200 μg of phospholipid suspension was injected into each bag, which was then lowered into about 2 mL of the appropriate polymer or sucrose solution. Preliminary experiments indicated that the samples reached plateau levels within 15 min of vesicle addition (Figure 7) but were left at room temperature for 1–2 h for convenience. After incubation, the bags were flushed with three aliquots totaling 0.5 or 1 mL of 10 mM MOPS, pH 7, to resuspend all of the lipid therein which was transferred to a clean test tube. Permeability of the dialysis bags (M_r 12 000–14 000 cutoff) to M_r 15 000–20 000 PEG and to M_r 40 000 dextran, neither of which are claimed by the manufacturers to be monodisperse, was checked by substituting aliquots of vesicles with 10-μL aliquots of water. After 2 h at room temperature, each bag was flushed with three aliquots of water totaling 0.5 mL, the refractive index of which was measured at 25 °C. Essentially no PEG or dextran but measurable amounts of sucrose were found in the bags after 2 h.

Measurement of Lipid Mixing by Fluorescence Energy Transfer. Determination of membrane mixing by measurement of fluorescence energy transfer was based on the method of Fung & Stryer (1978) as previously used (MacDonald & MacDonald, 1984). A total of 25–50 μg of donor–acceptor-containing vesicles or only donor-containing vesicles were combined with 75–150 μg of unlabeled vesicles, which had been exposed to polymer prior to dilution with 0.5–1 mL of 10 mM MOPS as described in the previous paragraph. Each sample was excited at 355 nm (excitation maximum for DnsPE) while the fluorescence was scanned from 500 to 600 nm (the emission maximum for DnsPE being 515 nm, for DiI-C_{18} being 570 nm, and for RhPE being 590 nm). The value for quenched donor fluorescence in the absence of acceptor was obtained either by monitoring the fluorescence of a donor containing sample treated like the donor–acceptor-containing sample or by adding Triton X-100 to a concentration of 0.1% to the donor–acceptor-containing sample. An equivalent amount of unlabeled vesicles was also scanned to correct for nonfluorescent background.

The percent transfer was calculated according to the equation $E = 1 - F/F_0$ (Fung & Stryer, 1978), where F is the corrected donor fluorescence of vesicles containing both donor and acceptor and F_0 is the corrected donor fluorescence of vesicles containing donor only or vesicles containing both donor and acceptor and treated with Triton X-100. The degree of energy transfer in each sample was correlated with a percent lipid mixing given by a standard curve relating the energy transfer in vesicles with probe compositions made to simulate various degrees of mixing. Lipid mixing was measured by

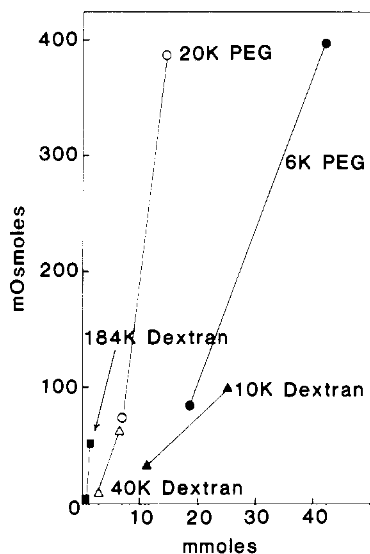

FIGURE 1: Osmotic activities (milliosmoles) of 10 and 20% (w/w) solutions of M_r 6000–7500 PEG (●), M_r 15 000–20 000 PEG (○), M_r 10 000 dextran (▲), M_r 40 000 dextran (△), and M_r 184 000 dextran (■) vs. millimoles in 1000 mL of water, as measured by freezing point depression osmometry.

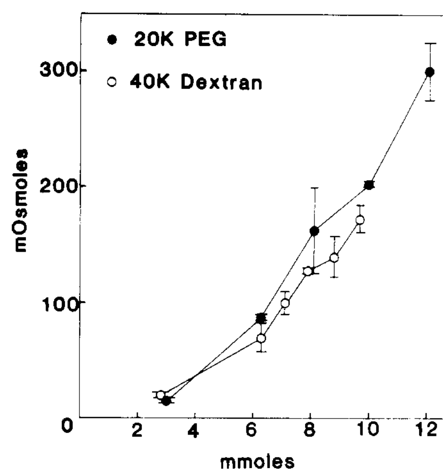

FIGURE 2: Osmotic activities (milliosmoles ± SD) of M_r 15 000–20 000 PEG (●) and M_r 40 000 dextran (○) vs. millimoles in 1000 mL of water, as measured by freezing point depression osmometry. Each point represents three measurements performed on two different samples.

monitoring dequenching of donor–acceptor-labeled vesicles.

Turbidity Measurements. Vesicles used in energy-transfer measurements were also checked by spectrophotometry for absorbance at 400 nm. Each value represents the mean ± SD of two or more triplicates, i.e., vesicles containing no probe, vesicles containing donor mixed with unlabeled vesicles, and vesicles containing both donor and acceptor mixed with unlabeled vesicles. Neither PEG nor dextran alone absorbed at 400 nm at concentrations equal to those in the dilute samples. The refractive indexes of solutions of polymer alone, as measured with an Abbe refractometer, were low enough that corrections pertaining to this parameter (Yi & MacDonald, 1973) would have reduced the reported turbidities by less than a few percent.

Electron Microscopy of Negatively Stained Vesicles. According to techniques described by Zingsheim & Plattner (1976), a drop of suspended vesicles containing 2 mg/mL phospholipid or less in 10 mM MOPS, pH 7, was placed on a carbon-coated, collodion-surfaced copper grid. After 20–30 s, excess fluid was drained with filter paper. Control vesicles or vesicles that had been directly exposed to polymer solutions were washed with a drop of 10 mM MOPS, pH 7, followed by a drop of 2 mg/mL dextranase (Sigma Chemical Co.) for 10 min at room temperature and drained. Both washed and unwashed vesicles were stained with a drop of 2% ammonium molybdate, pH 7, which was drained after 30 s, air-dried, and viewed at 48 000-fold magnification in a JEOL electron microscope.

Results

Dehydrating Activities of Various PEG and Dextran Preparations. Since preliminary results indicated that PEG and dextran cause vesicle growth by affecting vesicles indirectly, the osmotic or dehydrating activities of various PEG and dextran preparations were compared. Osmotic pressures of aqueous solutions of the polymers were measured by freezing point depression osmometry. The osmotic pressures in milliosmoles vs. millimoles per 1000 g of water in 10% (w/w) and 20% (w/w) solutions of M_r 15 000–20 000 and 6000–7500 PEGs and M_r 184 000, 40 000, and 10 000 dextrans are shown in Figure 1. Two points are important. First, as expected and reported by others [e.g., see Applegate (1960), Michel & Kaufmann (1973), and LeNeveu et al. (1977)], the polymers exert 3–39 times as much osmotic activity as can be accounted for by the numbers of molecules present. Furthermore, as indicated by the slopes of the lines joining the 10% (w/w) and 20% (w/w) points, this noncorrespondence between osmotic activity and numbers of molecules increases with an increase in polymer concentrations—least in the case of M_r 10 000 dextran and greatest in the case of M_r 184 000 dextran. Second, M_r 40 000 dextran and M_r 15 000–20 000 PEG may be the most suitable dextran and PEG pair among those tested for the purposes of this study, since they may afford comparable osmotic pressures while also being impermeant to dialysis membranes with an M_r 12 000–14 000 cutoff (see method 2 under Treatment of Vesicles with Polymer).

Figure 2 provides a direct comparison of the osmotic pressures (milliosmoles) of M_r 15 000–20 000 PEG and M_r 40 000 dextran from about 3 to 10 mmol [i.e., 5–17% (w/w) PEG and 10–28% (w/w) dextran]. Each measurement was repeated 2 or 3 times on two samples of the same concentration made at different times. It was not possible to measure the freezing point depression of more concentrated solutions [with the exception of 20% (w/w) PEG] because of their erratic freezing behavior. Nevertheless, the data in Figure 2 indicate that at the lower concentrations used to induce liposome growth in the following experiments, the dehydrating activity of M_r 15 000–20 000 PEG is nearly the same as that of M_r 40 000 dextran on a mole basis and, therefore, about twice that of the dextran on a weight per weight basis.

Lipid Mixing and Turbidity of Vesicles Exposed to Various Concentrations of M_r 15 000–20 000 PEG. To find out whether liposome growth caused by PEG (Tilcock & Fisher, 1979; Boni et al., 1981; Saez et al., 1982; Aldwinckle et al., 1982; Morgan et al., 1983) requires direct interaction of PEG with liposomes, probe-containing and unlabeled DiSPC vesicles were either directly mixed with (solid circles) or dialyzed against (open circles) various concentrations of PEG. The effects of PEG on three kinds of liposome preparations were examined. Figure 3A shows the lipid mixing (solid lines) and absorbance changes (broken lines) characteristic of sonicated vesicles treated with increasing concentrations of PEG. Note that maximal lipid mixing and turbidity begin to occur at about 20% (w/w) PEG for both directly mixed and dialyzed samples. The lipid mixing data include some values with particularly large standard deviations. This variability may be due to (1)

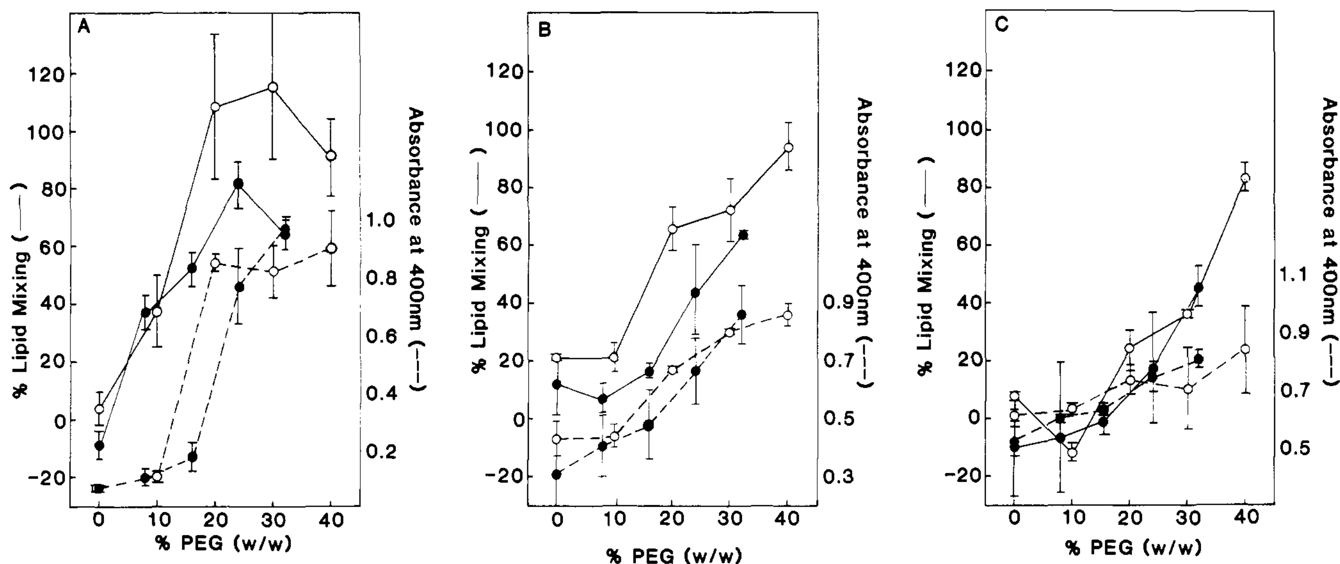

FIGURE 3: (A) Percent lipid mixing ± SD in (—) and absorbance at 400 nm ± SD of (- - -) sonicated, DiSPC vesicles either directly mixed with (●) or dialyzed against (○) solutions of M_r 15 000–20 000 PEG of increasing concentrations. (B) Percent lipid mixing ± SD in (—) and absorbance at 400 nm ± SD of (- - -) sonicated, 3 times frozen–thawed, DiSPC vesicles either directly mixed with (●) or dialyzed against (○) solutions of M_r 15 000–20 000 PEG of increasing concentrations. (C) Percent lipid mixing ± SD in (—) and absorbance at 400 nm ± SD of (- - -) large, multilamellar, DiSPC vesicles either directly mixed with (●) or dialyzed against (○) solutions of M_r 15 000–20 000 PEG of increasing concentrations. Concentrations of PEG given in the figure are those immediately prior to dilution of the PEG-treated vesicles with 10 mM MOPS, pH 7. Donor and acceptor probes used were DnsPE and DiI-C_{18}, respectively.

the use of the same calibration curve for most preparations of vesicles (the apparent variability would have been less had the data been expressed as percent differences between base line and experimental values in each experiment, rather than being pooled from several experiments), (2) some heterogeneity in vesicle size and/or morphology among different vesicle preparations, which were not fractionated prior to use, (3) the absence of stirring during polymer application, and (4) the use of viscous solutions. Nevertheless, these data allow valid conclusions to be drawn as to the dependence of lipid mixing on polymer concentrations, type, manner of application, and vesicle morphology of a quantitative nature not affordable by techniques such as electron microscopy.

The lipid mixing (solid lines) and absorbance (broken lines) of PEG-treated vesicles, which had been frozen and thawed 3 times after sonication, are presented in Figure 3B. Freeze–thawing of limit-sonicated PC vesicles in low-salt buffer is known to yield larger (140–180 nm; Gibson & Strauss, 1984), uni- to paucilamellar (Oku & MacDonald, 1983; Gibson & Strauss, 1984) vesicles, as well as some unaffected, limit-sonicated vesicles. After three cycles of freeze–thawing, however, no limit-sonicated vesicles remained, as determined by gel filtration chromatography (not shown). Since they have a moderate radius of curvature and consist of one or a few lamellae, the sonicated, 3 times frozen-and-thawed vesicles are the most, although admittedly not very, "cell-like" liposomes used in this study. As seen in Figure 3B, these vesicles, which are under significantly less strain than the sonicated vesicles of Figure 3A, underwent less growth than the sonicated vesicles exposed to the same concentration of PEG. As with the sonicated vesicles in Figure 3A, however, nearly the same amounts of lipid mixing and turbidity increase of these more cell-like vesicles were obtained whether exposure to PEG occurred during direct mixing or during dialysis (Figure 3B). Moreover, the degree of lipid mixing varied from 60 to 95% in the sample directly mixed with 32% (w/w) PEG (solid circles) or dialyzed against 40% (w/w) PEG (open circles).

Figure 3C shows the lipid mixing (solid lines) and absorbance changes (broken lines) characteristic of multilayered, DiSPC liposomes exposed to various concentrations of PEG. The effects of PEG on multilayered vesicles were of interest since their numerous lamellae in addition to their low curvature, should make this third type of vesicle least susceptible to PEG. Accordingly, the growth of multilamellar vesicles treated with PEG was less than that of unstressed, uni- to paucilamellar vesicles, which, it will be recalled, was, in turn, less than that of stressed vesicles. Like maximally stressed (Figure 3A) and unstressed, uni- to paucilamellar vesicles (Figure 3B), however, unstressed, multilamellar vesicles (Figure 3C) underwent liposome growth to the same extent, irrespective of whether they were dialyzed against (open circles) or directly mixed with (solid circles) PEG. Nevertheless, reduced as it was, the extent of growth of the multilamellar vesicles exceeded that expected had only the lipids in the outer monolayer participated in a biological type of fusion, i.e., 10% of the total lipid or 2 × 5%, which Schwartz & McConnell (1978) determined was the percentage of the total lipid in the outer monolayer of most vortexed, multilamellar preparations.

Lipid Mixing and Turbidity of Vesicles Exposed to Various Concentrations of M_r 40 000 Dextran. Experiments similar to those described with M_r 15 000–20 000 PEG were performed with M_r 40 000 dextran. These experiments were of particular interest because dextran by itself does not fuse cells (Ahkong et al., 1975; R. I. MacDonald, unpublished results), although it, like PEG, displays a high osmotic activity (Figures 1 and 2). These facts appear neither to support the hypothesis that high osmotic activity per se can effect fusion of membranes close enough to fuse nor to parallel the observations that as much lipid mixing and increased turbidity occurred in liposomes directly mixed with PEG as in liposomes dialyzed against PEG (Figure 3A–C). These apparent contradictions are resolved by the finding that dextran caused less membrane fusion when directly mixed with all types of vesicles than when dialyzed against those types of vesicles. In other words, dextran tends to inhibit fusion when in contact with liposomes and thus counteracts its own dehydrating activity.

Figure 4A shows the lipid mixing (solid lines) and turbidity changes (broken lines) characteristic of sonicated DiSPC

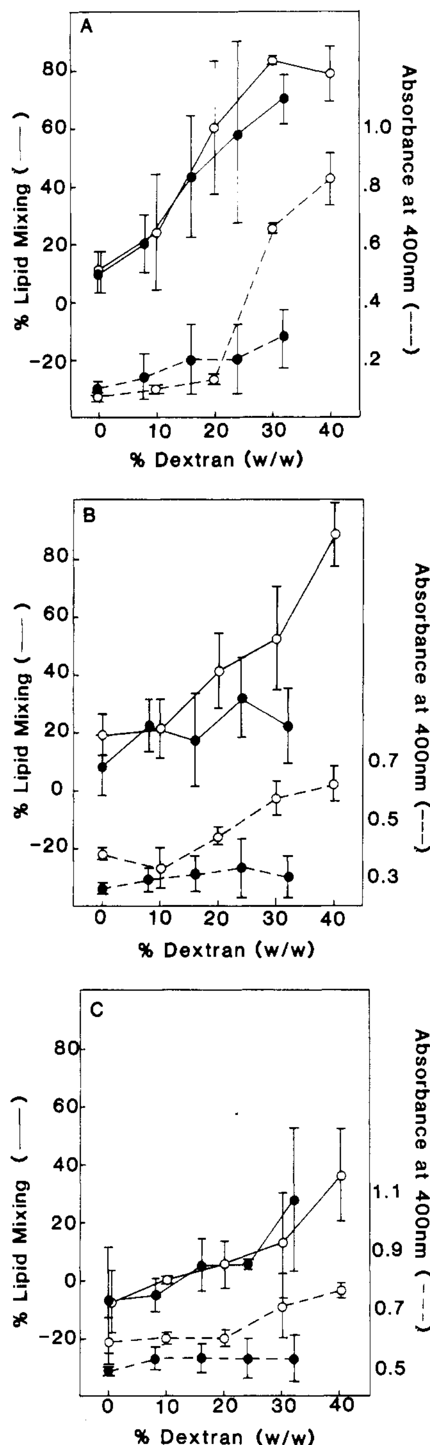

FIGURE 4: (A) Percent lipid mixing ± SD in (—) and absorbance at 400 nm ± SD of (---) sonicated, DiSPC vesicles either directly mixed with (●) or dialyzed against (○) solutions of M_r 40000 dextran of increasing concentrations. (B) Percent lipid mixing ± SD in (—) and absorbance of 400 nm ± SD of (---) sonicated, 3 times frozen–thawed, DiSPC vesicles either directly mixed with (●) or dialyzed against (○) solutions of M_r 40000 dextran of increasing concentrations. (C) Percent lipid mixing ± SD in (—) and absorbance at 400 nm ± SD of (---) large, multilamellar, DiSPC vesicles either directly mixed with (●) or dialyzed against (○) solutions of M_r 40000 dextran of increasing concentrations. Concentrations of dextran given in the figure are those immediately prior to dilution of the dextran-treated vesicles with 10 mM MOPS, pH 7. Donor and acceptor probes were DnsPE and DiI-C_{18}, respectively.

vesicles either mixed directly with (solid circles) or dialyzed against (open circles) increasing concentrations of dextran. As with PEG-treated vesicles, the degree of lipid mixing increased irrespective of whether polymer and vesicles were in contact and to a maximal 80% in the presence of 30% (w/w) dextran. In contrast with PEG-treated vesicles, however, turbidity increases of the dextran-dialyzed samples were much greater than those of the samples directly mixed with dextran and were of the same magnitude as found for samples either dialyzed against or directly mixed with 20% (w/w) PEG (Figure 3A). The sizable lipid mixing but small turbidity change in sonicated vesicles directly mixed with dextran in Figure 4A signifies that nearly all sonicated, unilamellar vesicles have participated in a small number of whatever molecular interactions result in lipid mixing. In the presence of PEG or dextran not mixed directly with vesicles, on the other hand, nearly all vesicles appear to have participated in a much larger number of those molecular interactions so that a large turbidity increase accompanied near maximal or maximal lipid mixing. These data suggest that dextran, given direct access to membrane surfaces, can interact with the vesicles so as to inhibit or mitigate the liposome growth, which would otherwise ensue on partial dehydration by dextran.

Figure 4B gives the lipid mixing (solid lines) and turbidity changes (broken lines) which occurred in unstressed, uni- to paucilamellar vesicles prepared by freeze-thawing sonicated vesicles 3 times prior to dialysis against (open circles) or direct mixing with (solid circles) increasing concentrations of M_r 40000 dextran. Like sonicated, freeze–thawed vesicles exposed to PEG (Figure 3B), similar vesicles exposed to dextran (Figure 4B) grew less than the sonicated vesicles (Figures 3A and 4A)—particularly those vesicles directly mixed with dextran, which evidenced almost no lipid mixing or increased turbidity. If these unstressed, uni- to paucilamellar vesicles can be likened to cells, the data in Figures 3B and 4B lead one to expect that cells mixed with PEG, but not dextran, would undergo fusion.

Completing the study of liposome responses to dextran are data on lipid mixing among and turbidities of multilamellar vesicles exposed to increasing dextran concentrations. As shown in Figure 4C, multilamellar vesicles mixed directly with dextran evidenced the same low level of lipid mixing but significantly less turbidity increase than vesicles dialyzed against dextran. Direct mixing with and dialysis against PEG, in contrast, gave the same results with multilamellar vesicles. Like vesicles treated with PEG, however, multilamellar vesicles underwent less lipid mixing and less turbidity change on exposure to dextran compared with sonicated, frozen–thawed vesicles exposed to dextran (Figure 4B).

Morphological Evidence for Polymer-Mediated Growth of Sonicated Liposomes. Vesicles which had been directly mixed with 40% (w/w) M_r 10000 dextran prior to their dilution with 10 mM MOPS could be seen with the electron microscope only after grids bearing the vesicles had been exposed to dextranase. Washing the liposome-bearing grids with 10 mM MOPS removed enough 40% (w/w) M_r 6000–7500 PEG to make PEG-treated liposomes visible, but the addition of dextranase was required to make dextran-treated liposomes visible. For that reason, control samples in direct-mixing experiments were also exposed to dextranase. Negatively stained control vesicles of a few hundred angstroms in diameter (Figure 5A) remained, whether the vesicles had been treated with dextranase (Figure 5B) or dialyzed against 10 mM MOPS (Figure 5E). The average liposome, directly mixed with polymer—either 40% (w/w) M_r 6000–7500 PEG (Figure 5C) or 40% (w/w) M_r 10000 dextran (Figure 5D)—diluted with 10 mM MOPS and treated with dextranase, was larger than the average control liposome, but (1) their numbers were very few as a result of the dextranase treatment, (2) they were much smaller than

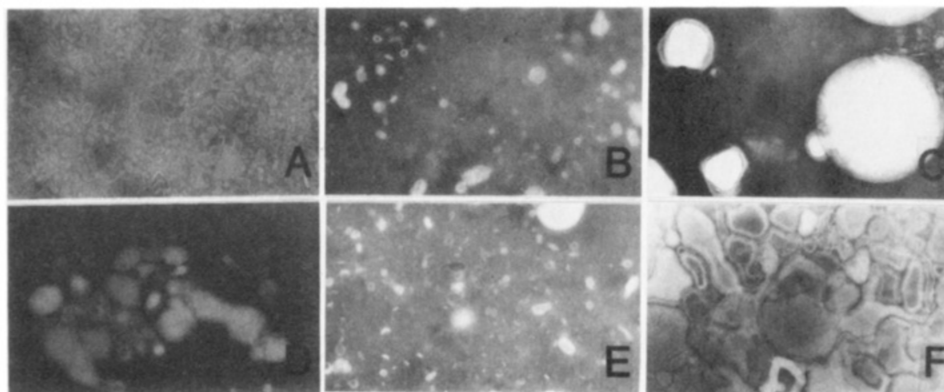

FIGURE 5: (A) Sonicated, DiSPC vesicles at 2 mg/mL phospholipid in 10 mM MOPS, pH 7. (B) Vesicles as in (A) on a grid were treated with 2 mg/mL dextranase. (C) Vesicles as in (A) were directly mixed with 40% (w/w) M_r 6000–7500 PEG and then diluted, prior to transfer to a grid and treatment with 2 mg/mL dextranase before negative staining. (D) Vesicles as in (A) were directly mixed with 40% (w/w) M_r 10 000 dextran and then diluted, prior to transfer to a grid and treatment with 2 mg/mL dextranase before negative staining. (E) Vesicles as in (A) were dialyzed against 10 mM MOPS, pH 7. (F) Vesicles as in (A) were dialyzed against 30% (w/w) M_r 184 000 dextran and then resuspended in 10 mM MOPS. All samples were stained with 2% ammonium molybdate and are shown at 19200× magnification.

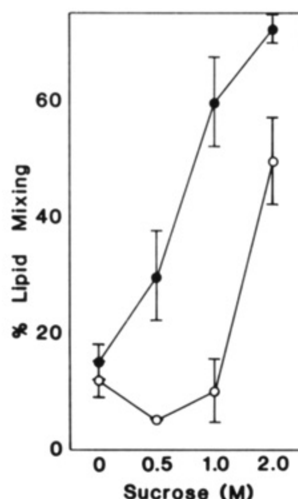

FIGURE 6: Percent lipid mixing ± SD among sonicated vesicles (●) and percent lipid mixing ± SD among sonicated, frozen–thawed vesicles (○), both dialyzed against various concentrations of sucrose for 1 h. Donor and acceptor probes were DnsPE and RhPE, respectively.

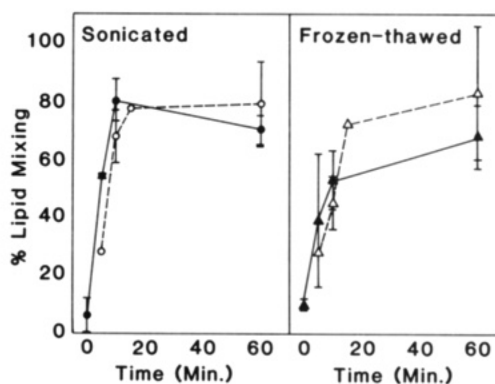

FIGURE 7: (Left panel) Lipid mixing ± SD among sonicated vesicles either mixed with (●) or dialyzed against (○) 50% (w/w) M_r 15 000–20 000 PEG for various lengths of time prior to dilution with 10 mM MOPS, pH 7. (Right panel) Lipid mixing ± SD among sonicated, frozen–thawed vesicles either mixed with (▲) or dialyzed against (△) 50% (w/w) M_r 15 000–20 000 PEG for various lengths of time prior to dilution with 10 mM MOPS, pH 7. Donor and acceptor probes were DnsPE and RhPE, respectively.

liposomes dialyzed against the polymer, and (3) their morphology was atypical, perhaps due to the electron density of residual polymer. In contrast, dialysis against 30% (w/w) M_r 184 000 dextran and resuspension in 10 mM MOPS transformed sonicated vesicles into morphologically typical, very large, multilamellar vesicles (Figure 5F).

Effects of Sucrose. In the preceding experiments, treatment of vesicles with PEG or with dextran resulted in lipid mixing and turbidity increases to a degree that cannot be explained solely as the transfer of lipid molecules between the outer monolayers of the treated vesicles. These observations suggest that any agent capable of dehydrating vesicles should produce a similar effect—even an agent of low molecular weight like sucrose—as long as the vesicles are amenable to fusion and are able to interact with each other. To test the ability of even a low molecular weight solute to induce fusion, sonicated and sonicated, frozen–thawed vesicles were dialyzed against 2, 1, or 0.5 M sucrose or water for 1 h prior to their dilution in 10 mM MOPS. Figure 6 shows that lipid mixing among both sonicated and sonicated, frozen–thawed vesicles increased as a function of sucrose concentration and, at least in the case of sonicated vesicles dialyzed against 2 M sucrose, was the same as that observed with the highest concentrations of PEG or dextran. However, 20% PEG which gave maximal membrane fusion has an osmolarity of about 0.2, whereas approximately 5–10 times that osmolarity of sucrose was required for maximal membrane fusion. Since maximal membrane fusion required the use of dialysis tubing of the smallest molecular weight cutoff available commercially—i.e., M_r 1000, which is still about 3 times the molecular weight of sucrose—on the other hand, the osmotic activity of sucrose was effectively reduced in the experiment of Figure 6 by the permeability of the dialysis tubing to sucrose. Direct mixing of sucrose with vesicles resulted in little membrane fusion (not shown).

Time Course of PEG Effects. Changes in fluorescence energy transfer (Morgan et al., 1983), dynamic light scattering (Boni et al., 1984), and NMR spectra (Boni et al., 1984) have been reported to occur within minutes of adding the polymer. To determine whether the lipid mixing reported here was similarly rapid, the time course of PEG-induced lipid mixing among sonicated and sonicated, frozen-and-thawed vesicles was monitored. A 10-μL sample of vesicles was mixed with 50 μL of 50% (w/w) PEG or dialyzed against 2 mL of 50% (w/w) PEG for various lengths of time before their dilution with 0.5 mL of 10 mM MOPS, pH 7. Figure 7 shows that most of the lipid mixing occurred within 5–10 min of exposure of the vesicles to the polymer, except for frozen–thawed vesicles dialyzed against PEG, which required another 5 min to reach

plateau levels. These times are similar to those required for PEG-induced fusion of liposomes (Morgan et al., 1983; Boni et al., 1984) and of biological membranes (Wojcieszyn et al., 1983).

Discussion

The major finding of this study is that PEG clearly causes growth or fusion of artificial membranes by an *indirect*, *dehydrating* action on the membrane lipid, since vesicles either mixed with or dialyzed against M_r 15 000–20 000 PEG exhibited equal amounts of growth. This indirect effect of PEG may depend on its high osmotic activity, since dextran with a comparably high osmotic activity induced a comparable degree of growth if a dialysis membrane prevented physical contact of dextran and the liposomes. Sucrose was much less effective than either PEG or dextran on the basis of osmotic activity but probably could not exert its osmotic activity maximally owing to its permeation of M_r 1000 cutoff dialysis tubing, the smallest pore size commercially available. Unlike PEG, sucrose must be separated from vesicles by a sucrose impermeable membrane in order to cause membrane fusion; if in contact with sucrose, vesicles will become dispersed and be unable to interact with each other. These observations that dehydration by osmoticants results in fusion of liposomes support the idea that hydration forces between bilayers must be overcome for membrane fusion to ensue (Parsegian & Rand, 1983).

It should be noted that the osmotic activities of different concentrations of PEG obtained by freezing point depression osmometry are approximations, since osmotic activities obtained by that method (Applegate, 1960; Figures 1 and 2) were 20–40% higher than those obtained by the thermocouple psychrometer method (Lagerwerff et al., 1961). On the other hand, the osmotic activity determined by freezing point depression osmometry for 20% (w/w) M_r 184 000 dextran (Figure 1) was also about 40% higher than that determined by vapor pressure osmometry for 20% (w/w) M_r 200 000–274 000 dextran (LeNeveu et al., 1977). Thus, although the absolute osmotic activities obtained by freezing point depression osmometry and by vapor pressure osmometry differ, the relative osmotic activities of PEG and dextran do not differ, and the data obtained by freezing point depressing osmometry are useful for the present purpose.

Some comment should be made on the legitimacy of measuring liposome growth by monitoring fluorescence energy transfer and turbidity. Changes in fluorescence energy transfer are now standard indicators of lipid mixing, although the distinction between mixing due to lipid exchange and mixing due to membrane fusion—e.g., by a kinetic method (Nichols & Pagano, 1981) not applicable in this instance—is not always made. The possibility that lipid exchange, rather than membrane fusion, occurs in the presence of PEG has been suggested by the report that DiI and NBD-PE undergo transfer between cells in the presence of 40% (w/w) PEG but under nonfusing conditions, as detected by fluorescence microscopy (Wojcieszyn et al., 1983). Lipid probes in protein-containing membranes, however, are more likely to undergo exchange than lipid probes in membranes composed of a single phosphatidylcholine species. Thus, when glycolipid-containing liposomes labeled with DiI-C_{16} or DiI-C_{18} and loaded with fluorescent albumin were mixed with cells in the presence of the appropriate lectin and PEG and washed, the numbers of cells labeled with DiI were nearly the same as the numbers of cells labeled with fluorescent albumin (Szoka et al., 1981).

Moreover, had lipid mixing resulted only from lipid exchange and not from membrane fusion, the degree of mixing observed in this study should *not have involved more than 50% of the vesicles* in the case of those which had been sonicated or sonicated and 3 times frozen–thawed or more than 5% of the multilamellar vesicles, since only the lipid in the outer monolayer is exchangeable (Pagano et al., 1981). Thus, a substantial amount of liposome growth by membrane fusion was indicated by the increase of lipid mixing to 80–100% and 70–80% in sonicated vesicles, to 60–94% and 30–90% in sonicated, 3 times frozen–thawed vesicles, and to 45–80% and 28–36% in multilayered vesicles, when each type of vesicle exposed to 32–40% (w/w) PEG and dextran, respectively. Another point against the possibility that the lipid mixing reflected exchange and not fusion is that lipid mixing peaked within 5–15 min of exposure of the vesicles to PEG (Figure 7).

Turbidity measurements provided more quantitative estimates of membrane fusion at the highest levels of lipid mixing, at which point turbidity continued to rise although lipid mixing had attained maximal values. It should be noted that turbidity measurements are also potentially ambiguous in the sense that increased absorbance could reflect an increase in particle size due to fusion of vesicles or to aggregation of vesicles. It has been demonstrated, however, that sufficient dilution, as performed here, reverses aggregation but not fusion (Boni et al., 1981; Tilcock & Fisher, 1982; Morgan et al., 1983). The combination of lipid mixing and turbidity data complemented by electron microscopy, therefore, ensures the reliability of the estimates of membrane fusion presented here. In this context, membrane fusion is not synonymous with vesicle fusion, which implies vesicle integrity not necessarily characteristic of membrane fusion. This issue has been addressed by Kendall & MacDonald (1982).

Whereas it is clear that PEG, dextran, and sucrose acted indirectly or by dehydrating vesicles to cause membrane fusion and liposome growth, the nature of this indirect effect remains to be characterized. In anticipation of a more quantitative treatment—e.g., following the approach of Evans, Parsegian, Rand, and co-workers (Parsegian & Rand, 1983; Evans & Parsegian, 1983)—it seems reasonable that two events must occur: (1) Membranes must be brought into contact. The characterization of systems composed of biological materials and dextran and/or PEG—although largely empirical—has increased their usefulness for separating membranes (Albertsson et al., 1982) and cells (Walter, 1977) and as impermeant osmoticants (Lagerwerff et al., 1961; LeNeveu et al., 1977). These effects may be explained by the rigorous demonstration that certain proteins can be precipitated in the presence of PEG due to their exclusion from the polymer phase and without any direct interaction between PEG and protein (Atha & Ingham, 1981). Correspondingly, therefore, the exclusion of membranes from the PEG phase may bring them into contact. In fact, aggregates of vesicles were seen within minutes after the vesicles had been mixed with high concentrations of polymers. This behavior contrasts sharply with that of vesicles (and presumably cells) which remain dispersed in high concentrations of sucrose and do not fuse, because they cannot interact although they have been dehydrated by the sucrose.

(2) Membranes in contact must be perturbed enough to break and reseal with each other. Given a typical trapped volume of 0.5 μL/μmol of phospholipid for sonicated liposomes (Oku & MacDonald, 1983) and the present experimental design, it can be calculated that only 3% of the originally trapped bulk water could have remained trapped on exposure of the vesicles to 20% (w/w) M_r 15 000–20 000 PEG, which

induced maximal growth of sonicated vesicles. Three kinds of consequences of partial dehydration are conceivable: (i) Withdrawal of nearly all the trapped bulk water from a vesicle could cause it to flatten like a pancake. The circumference of the pancake would have a high curvature under so much stress that it would collapse and expose hydrophobic acyl chains which would align with other exposed, hydrophobic acyl chains. Thus, two liposomes might fuse to relieve the stress on their highly curved boundaries. (ii) Alternatively, partial dehydration might result in the formation of fusion-prone, nonbilayer regions—somewhat like but not as dehydrated as the anhydrous complex of phosphatidylserine and Ca^{2+} (Portis et al., 1979). Nonbilayer arrangements have not been detected in PEG-treated, multilayered, lecithin vesicles by ^{31}P NMR spectroscopy, however (Tilcock & Fisher, 1982; Arnold et al., 1983). (iii) Fusion could also have occurred during rehydration of the dehydrated vesicles. For example, Miller et al. (1976) demonstrated that osmotic inflation appears to cause vesicles to burst into each other and fuse. Experiments are in progress to test this possibility.

In spite of dextran having enough osmotic activity to decrease interbilayer distances in multilayered vesicles either directly mixed with or dialyzed against dextran (LeNeveu et al., 1977), membrane fusion between uni- and paucilamellar vesicles, as determined here by fluorescence energy transfer and light scattering, was greater when the vesicles were dialyzed against than when directly mixed with dextran, prior to their dilution with hypotonic buffer. Three significant differences in experimental conditions between LeNeveu et al. (1977) and the present work can be cited: (a) the effect of dextran on two different parameters, i.e., interbilayer distance in the case of LeNeveu et al. (1977) but membrane fusion in the present case; (b) under two different conditions, in the presence of dextran in the case of LeNeveu et al. (1977) but following dilution of the dextran–vesicle mixture in the present case; (c) with two different kinds of vesicles in most experiments, i.e., multilayered vesicles in the case of LeNeveu et al. (1977) but uni- or paucilamellar vesicles in the present case. It is concluded that contact between vesicles and dextran can mitigate the membrane-fusing effect of dextran but not the reduction of interbilayer distance by dextran. The results reported here with dextran may explain the previous report (Ahkong et al., 1975) and unpublished results (R. I. MacDonald) that dextran, unlike PEG, does not fuse cells. First, the osmometry measurements show that dextran per weight has about half the osmotic activity of PEG. Thus, dextran's low cell-fusing activity may be attributable in some instances to the use of low dextran concentrations having insufficient osmotic activity. Even at concentrations of sufficiently high osmotic activity, however, directly mixing vesicles with dextran is not as effective as dialysis of vesicles against dextran. Hence, dextran's low cell-fusing activity may also be due to its directly interacting with bilayers so as to inhibit fusion. Apparently, the direct interaction of dextran and membranes (Minetti et al., 1979) can be very strong (Evans & Metcalf, 1984).

The dependence of the magnitude of the polymer effect on the curvature and number of lamellae of treated vesicles is also of interest with respect to the use of model lipid membranes to probe the mechanism of action of PEG on cells. In this regard, sonicated vesicles were too responsive to PEG and dextran to serve as reasonable models of cell membranes, since they responded maximally to 20% and higher concentrations of M_r 15 000–20 000 PEG and to 20–40% (w/w) dextran of M_r 40 000. Hoekstra (1981) also obtained substantial fusion, detected as lipid mixing, of phosphatidylserine-containing vesicles treated with 20% M_r 6000 PEG and subfusogenic concentrations of Mg^{2+} and Ca^{2+}. Moreover, sonicated vesicles directly mixed with dextran evidenced significant lipid mixing, whereas sonicated, 3 times frozen–thawed vesicles (Figure 3B) or cells (Ahkong et al., 1975; R. I. MacDonald, unpublished results) directly mixed with dextran evidenced significantly less or no fusion, respectively. Unlike sonicated vesicles, therefore, sonicated, 3 times frozen–thawed vesicles and large, multilamellar vesicles responded maximally at 40% (w/w) PEG and dextran, which is close to the 40–50% (w/w) PEG necessary for optimal fibroblast fusion (Blow et al., 1978; Wojcieszyn et al., 1983). Although the fusion of larger, unstressed vesicles did require concentrations of PEG similar to those required for cell fusion, however, the larger, stressed vesicles still did not behave entirely like cells. For example, lipid mixing and turbidity vs. PEG concentration curves characteristic of polymer-treated vesicles with low curvature increased gradually prior to attaining maximal values, whereas cell fusion vs. PEG concentration curves typically remain close to zero until a maximally effective PEG concentration is applied [e.g., see Blow et al. (1978)]. As expected, therefore, differences between cell membranes and model lipid membranes must condition the extent to which one can use model membranes to explain how PEG fuses cells. Useful information can be obtained, however, by maximizing the resemblance between the cell response and the model membrane response to PEG.

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Registry No. PEG, 25322-68-3; DiSPC, 58045-79-7; dextran, 9004-54-0; sucrose, 57-50-1.

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Reconstitution of Membrane Proteins. Spontaneous Association of Integral Membrane Proteins with Preformed Unilamellar Lipid Bilayers†

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ABSTRACT: We have developed a simple method for reconstituting pure, integral membrane proteins into phospholipid–protein vesicles. The method does not depend on use of detergents or sonication. It has been used successfully with three different types of integral membrane proteins: UDPglucuronosyltransferase (EC 2.4.1.17) from pig liver microsomes, cytochrome oxidase (EC 1.9.3.1) from pig heart, and bacteriorhodopsin from *Halobacterium halobium*. The method depends on preparing unilamellar vesicles of dimyristoylphosphatidylcholine (DMPC) that contain a small amount of myristate as fusogen. Under conditions that the vesicles of DMPC have the property of fusing, all of the above proteins incorporated into the bilayers. Two events appear to be involved in forming the phospholipid–protein complexes. The first is a rapid insertion of all proteins into a small percentage of total vesicles. The second is slower but continued fusion of the remaining phospholipid–protein vesicles, or proteoliposomes, with small unilamellar vesicles of DMPC. This latter process was inhibited by conditions under which vesicles of DMPC themselves would not fuse. On the basis of proton pumping by bacteriorhodopsin and negative staining, the vesicles were unilamellar and large. The data suggest that insertion of the above integral membrane proteins into vesicles occurred independently of fusion between vesicles.

Careful studies of the properties of proteins that are integral components of membranes depend on dissolution of the membrane with detergents, on separation of the protein of interest from other membrane components, and finally on reintroduction of the protein into a matrix of phospholipid. The latter may have a variety of physical structures, depending on the complex of protein and phospholipid being studied. The complex of most interest, because of its resemblance to naturally occurring biological membranes, is reconstitution of integral membrane proteins into unilamellar bilayers of

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