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Lipid-Polyethylene Glycol Interactions: I. Induction of Fusion between Liposomes

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Summary. Fusion between unilamellar vesicles of both egg phosphatidylcholine and bovine phosphatidylserine was induced by polyethylene glycol. Aggregation and fusion events were monitored by electron microscopy and turbidity measurements. The threshold concentration of polyethylene glycol for aggregation and fusion is found to be independent of lipid concentration. Typically, aggregation of phosphatidylcholine vesicles starts at 2.5% (wt/wt) polyethylene glycol, but fusion is not significant until the polyethylene glycol concentration reaches 35%. Multilamellar vesicles were formed as a result of fusion.

Key words: Fusion, polyethylene glycol, unilamellar vesicles, aggregation, freeze fracture, turbidity

Abbreviations

PEG – Polyethylene glycol
IMP – Intramembranous particle
PC – Phosphatidylcholine
PS – Phosphatidylserine
SUV – Small unilamellar vesicles
MLV – Multilamellar vesicles
DPPC – Dipalmitoyl phosphatidylcholine
DSC – Differential scanning calorimetry

High molecular weight polymers of polyethylene glycol (PEG) have been widely employed in mediating cell-cell fusion in cell hybridization studies (Davidson, O'Malley & Wheeler, 1976; Davidson & Gerald, 1977). The advantages of PEG-induced fusion are its low cytotoxicity, high fusion rate, ease of handling, reproducibility, and the ability to fuse a wide variety of cell types (Knutton, 1979; Robinson, Roos, Davidson & Karnovsky, 1979). The protocol usually employed calls for a 1-min exposure of the cells to PEG followed by a wash (removal of PEG) and incubation at 37 °C (Davidson et al., 1976; Davidson & Gerald, 1977; Knutton, 1979; Robinson et al., 1979). Aggregation is achieved during PEG exposure, with fusion taking place during the incubation period.

An important known property of PEG is its ability to bind and structure water (Baran, Solomentseva, Mank & Kurilenko, 1972; Blow et al., 1978; Tilcock & Fisher, 1979). This property appears to be related to a critical PEG concentration (45–50%, wt/wt) for fusion to occur (Davidson & Gerald, 1977). During the process of PEG-induced fusion between cells, intramembrane particle (IMP) free regions in the plasma membranes of cells have been observed by freeze fracture electron microscopy (Knutton, 1979; Robinson et al., 1979). These IMP-free regions, possibly exposed lipid bilayers, are thought to be the sites of cell fusion (Lawson et al., 1977; Zakai, Kulka & Loyter, 1977; Knutton, 1979). What is known of PEG-induced fusion seems to indicate that PEG acts primarily on the lipid bilayer of membranes. However, the details of the molecular mechanism is far from being understood. The methods of PEG-induced fusion thus remains mainly empirical.

In order to elucidate the mechanism of PEG-induced fusion, we have studied its effect on a pure lipid system – small unilamellar vesicles (SUV). This will permit us to examine exclusively the role of lipid components in the process of membrane fusion.

Methods and Materials

Lipid Vesicles

Hen egg phosphatidylcholine (PC) and bovine phosphatidylserine (PS), both stored in chloroform, were obtained from Avanti Polar-Lipids, Inc. (Birmingham, Ala.). Both lipids were shown to be pure by thin-layer chromatography. For specimen preparation, the chloroform was evaporated from known aliquots of lipid from a glass tube under vacuum at 25 °C for 1 hr. Aqueous buffer (7 mM Tris-HCl, 0.2 mM EDTA, pH 7.4) was added to the dry lipid and

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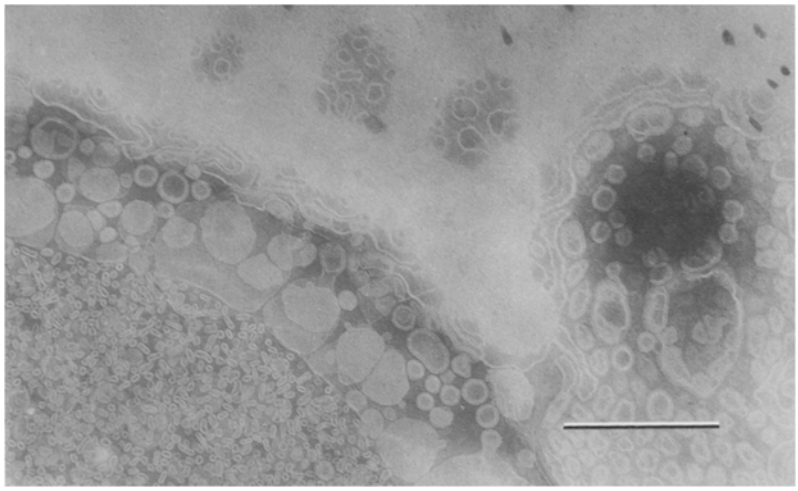

Fig. 1. Negative stain electron micrograph of egg phosphatidylcholine in 2.5% PEG. Large aggregates of SUV with some MLV fusion products can be discerned. Bar = 0.3 μm

dispersed by vortexing for 5 min at 25 °C. All transfers were done under nitrogen. Lipid concentrations in the final aqueous suspensions were approximately 12.5 mM. Small unilamellar vesicles were prepared by sonication in a water bath ultrasonicator (Laboratory Supplies Co., Hicksville, N.Y.) for 3 hr under an atmosphere of nitrogen followed by centrifugation at 6,000 × *g* for 15 min to remove any large vesicles. Vesicle size was measured by freeze-fracture electron microscopy to be less than 300 Å in diameter, with no noticeable larger vesicles.

Polyethylene Glycol (PEG)

Polyethylene glycol (Carbowax) of average mol wt 6,000 was obtained from Fisher Scientific. The buffer used was the same as that used in vesicle preparation.

Freeze-Fracture Electron Microscopy

Aliquots of samples containing lipid suspensions in 50% (wt/wt) PEG were placed in Balzers specimen holders (center recessed gold discs) and frozen in Freon 22 from room temperature. Samples containing < 50% PEG were mixed with glycerol to a final concentration of 30%. In some instances samples were frozen without cryoprotectants, employing a quick freeze apparatus (Costello & Corless, 1978) built in this laboratory. In this method, 0.1 μl of sample was placed between a 75-μm thick copper foil sandwich and rapidly quenched in liquid propane from room temperature. All samples were stored in liquid nitrogen. Samples were fractured and replicated at either −115 or −120 °C in a Polaron E 7 500 freeze-fracture module evacuated by a Perkin-Elmer Ultek TNB-X ion pump at a vacuum of 5×10^{-7} Torr or better. Replicas were cast by resistance evaporation, floated off in distilled water, cleaned in full strength Clorox, and picked up on uncoated 460 Hex mesh copper grids. Representative micrographs were taken on a Siemens 101 electron microscope.

Negative Staining Electron Microscopy

A drop of the specimen was placed on a Formvar-carbon coated grid (treated with 0.1% Bacitracin). The bulk of the sample was removed with filter paper while applying a 2% ammonium molybdate stain for 10 sec. The grids were air dried and examined in a Siemens 101 electron microscope at a typical magnification of 55,000.

Turbidity Measurements

Turbidity measurements were performed on a Cary 219 spectrometer with the absorbance measured at 660 nm. The appropriate percent PEG was placed in a precision cuvette into which concentrated egg PC SUV suspensions were added to give the desired final concentrations. The suspensions were immediately mixed by vigorous shaking. Following dilutions, readings were taken when the suspensions had equilibrated. Dilutions were achieved with aqueous buffer, which was used as a reference.

X-Ray Diffraction

Concentrated lipid suspensions were pelleted by centrifugation at 4 °C for up to 1 hr at 20,000 × *g*. Pellets were placed in Teflon-lined aluminum cells with mica windows. Diffraction lines were recorded using a Frank camera with slit focusing. The X-ray source was a Jarrell-Ash microfocusing unit with copper target and nickel filter. The temperature of the cell was controlled by a thermoelectric module (MELCO). Each exposure required approximately 15 hr.

Results

A. Determination of PEG Threshold Concentration for Fusion

Clear suspensions of egg PC SUV of concentrations as low as 0.8 mM revealed a cloudy appearance immediately upon the addition of PEG at as low as 2.5%. This is indicative of the aggregation or fusion of SUV into larger entities. Figure 1 shows, at this PEG concentration, large aggregates of SUV with large vesicles already formed, some of which are MLV (selected field). Some SUV seem to take on a cylindrical appearance in negative staining micrographs and, depending on their orientation, may appear < 250 Å in cross-section. Bovine PS SUV, which are negatively charged, did not show any noticeable increase in turbidity until a final concentration of approximately 40% PEG was obtained.

In order to determine whether an increase in tur-

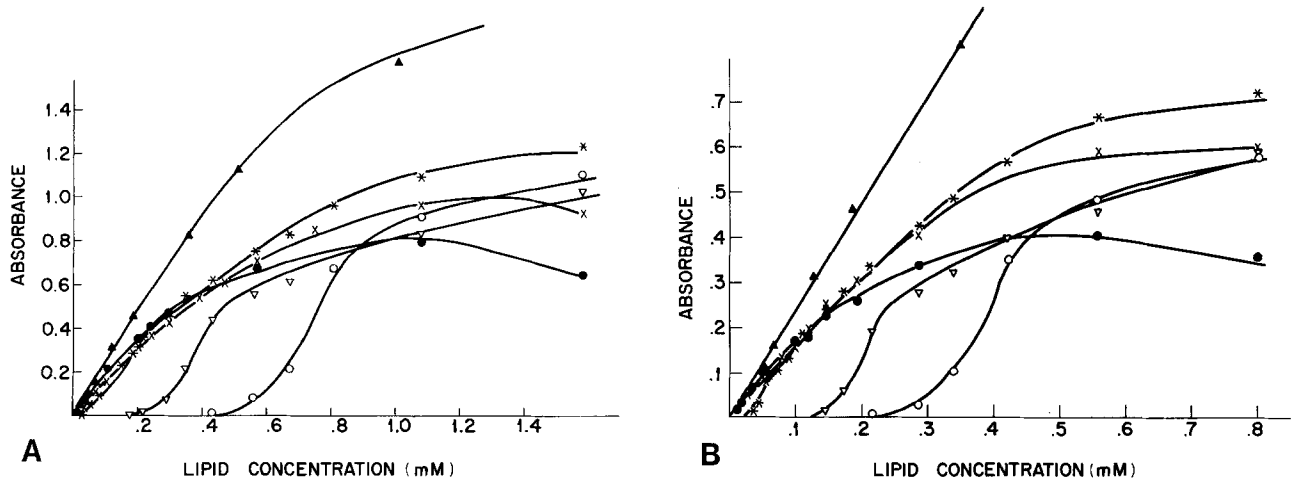

Fig. 2. Variation in turbidity upon dilution of mixtures of egg phosphatidylcholine SUV and PEG at initial lipid concentrations of (*A*) 1.6 mM and (*B*) 0.8 mM. Initial PEG concentrations are denoted as ○=9%, ▽=19%, *=28%, ×=38%, and ●=47%. The turbidity of egg PC MLV at various concentrations in the absence of PEG is given by ▲

bidity pertained to vesicle aggregation or fusion, we measured the reversibility of turbidity upon dilution for two different lipid concentrations in various PEG percentages. Assuming that fusion is irreversible, diluting a fusion product would yield a linear decrease in turbidity, as shown by the MLV dilution curve (▲) in Fig. 2*b*, whereas dilution of aggregates would show a sharp decrease in turbidity at a point corresponding to deaggregation. The resulting sigmoidal curves in Fig. 2 below a starting concentration of 20% implies that considerable deaggregation of SUV occurred. The turbidity measurements also show a maximum in the deaggregation (a maximum decrease in turbidity or inflection point in Fig. 2) consistently in the range of 4.2–4.4% PEG. Above an initial concentration of 28% PEG, the dilution curves approach linearity. The curves approximate dilutions in which a substantial amount of vesicles have irreversibly fused, and deaggregation is not significant. At 38% PEG or greater, fusion appears to be complete, and the dilution curve approaches that found for the dilution of large vesicles (MLV).

Turbidity measurements were also performed at constant lipid concentration by diluting the initial lipid-PEG mixture with SUV suspensions of the mixture's initial lipid concentration (results not shown). The addition of SUV to the high initial PEG concentration results in the formation of a large amount of new aggregates or MLV, making the interpretation of the deaggregation effect difficult.

It was noted that in the presence of 7% PEG or greater, PEG lipid suspensions are less turbid than expected (an optical masking effect at high PEG concentrations). This was exemplified by the fact that pure MLV in 50% PEG is markedly less turbid than equal concentrations of MLV in aqueous buffer without PEG. This masking effect is believed to be mainly responsible for the nonlinearity of the dilution curves at high PEG concentrations.

B. Morphology of the Fusion Product

The above results indicate that at 50% PEG, fusion between vesicles is extremely common. We have monitored the fusion process through the dilution step following the procedure of Knutton (1979) for the fusion of erythrocytes. Egg PC SUV were added to 50% PEG and then diluted after 1 min. Aliquots were taken at each step for freeze-fracture electron microscopic observation. Figure 3*a* shows the initial SUV were all small, approximately 300 Å. Fusion to large MLV occurs without the need for the removal of the PEG, a necessary step in cell fusion (Davidson & Gerald, 1977; Knutton, 1979; Robinson et al., 1979). A worm-like texture is apparent in all fracture faces of egg PC lipids in 50% PEG (Fig. 3*b*). X-ray diffraction patterns of this sample shows distinct lamellar structures. After the dilution step, multilamellar structures still predominante, but the fracture surfaces are devoid of texture (Fig. 3*c*). Similar results were obtained for bovine PS SUV.

In a quick freeze experiment a drop of both egg PC SUV and PEG were adjacently placed and sandwiched in a specimen holder to allow for partial mixturing and were then quickly quenched. MLV were found to have formed immediately. Figure 4 reveals smaller vesicles attached to larger ones.

X-ray diffraction of the lipid pellet of egg PC and bovine PS after the PEG dilution step gave repeat spacings of 61 and 77 Å, respectively, at 20 °C. These are the expected values for hydrated MLV of these particular lipids (Shipley, 1973). Before dilution, egg PC SUV in 50% PEG has a lamellar repeat spacing

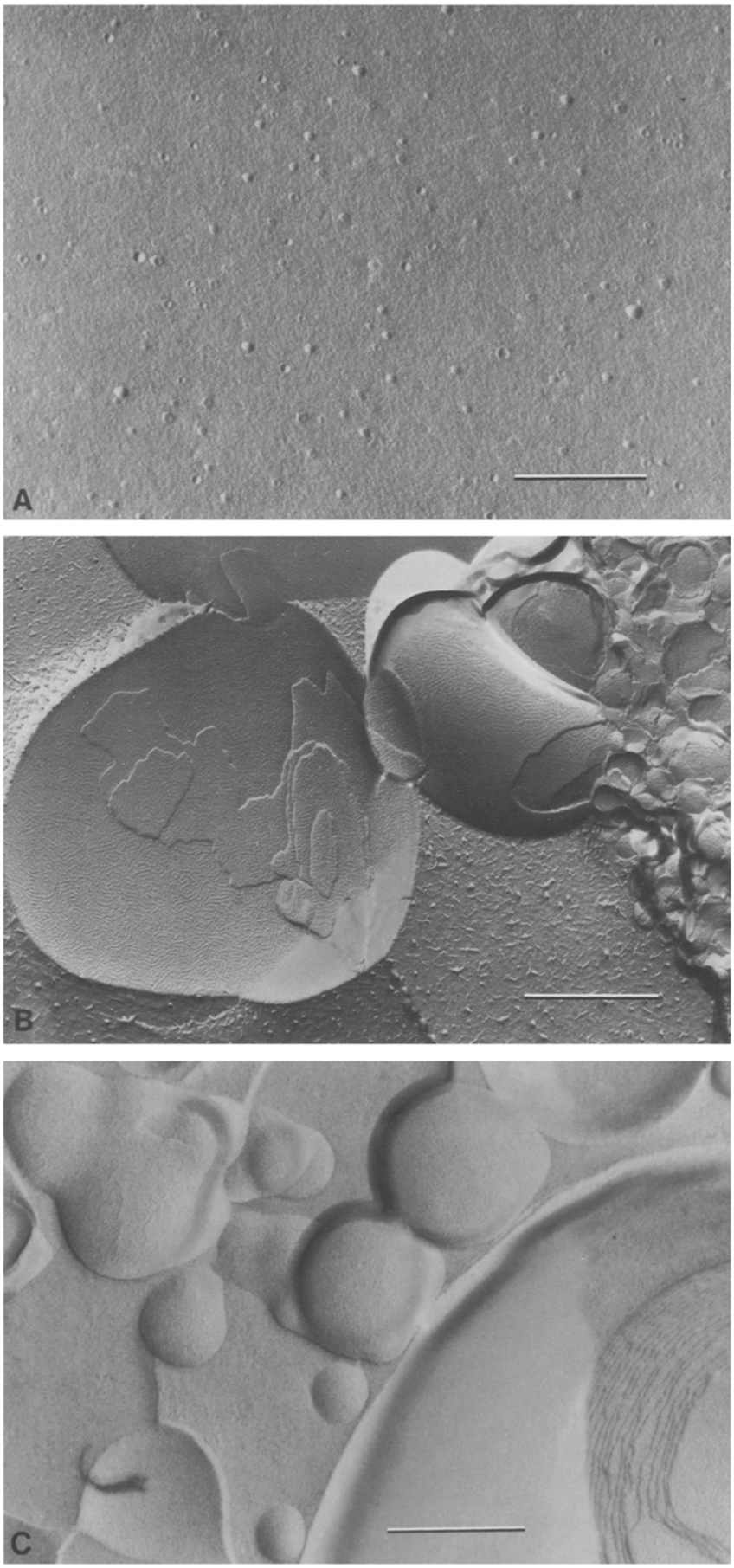

Fig. 3. Freeze-fracture electron micrographs of egg phosphatidylcholine at various stages of PEG-induced fusion: (*A*) SUV before the addition of PEG; (*B*) after mixing in 50% PEG for 1 min; and (*C*) after dilution and removal of PEG. Bar = 0.3 μm

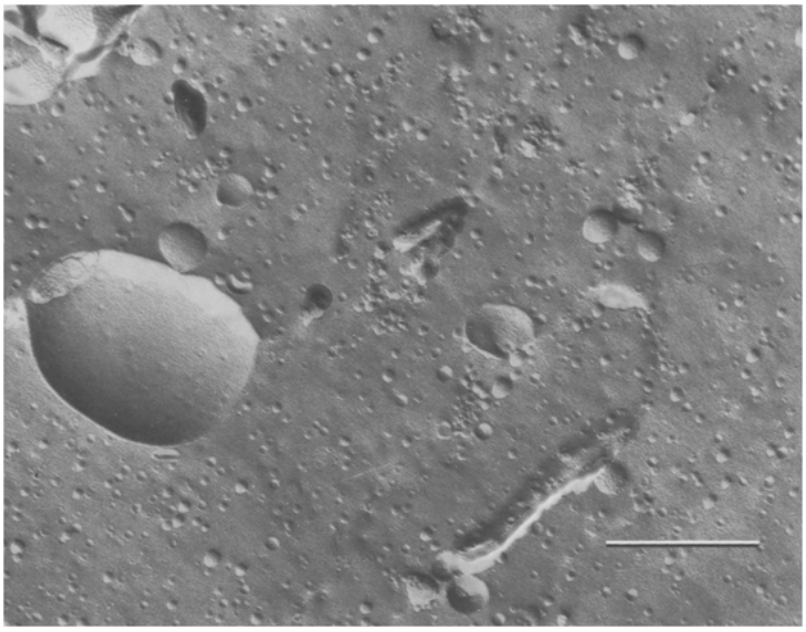

Fig. 4. Freeze fracture electron micrograph of rapidly quenched egg phosphatidylcholine (12.5 mM) immediately following contact with 50% PEG. Note the smaller vesicles on the surface of the larger one, suggesting the fusion of SUV. Bar = 0.3 μm

of 54.5 Å, which is the expected value for dehydrated MLV (LeNeveu, Rand, Parsegian & Gingell, 1977; Ranck et al., 1974).

Discussion

We have found that aggregation of vesicles occurs at lower percentages of PEG, whereas fusion occurs at higher percentages. The competition of PEG with the lipid bilayer for water is believed to be an important step in overcoming the primary barrier to membrane fusion – the stabilizing hydration layer. PEG has a high capacity to bind water. By measuring the proton broadening via high resolution proton NMR, it was determined that 3–4 water molecules were bound per monomer unit (CH_2-CH_2-O) of PEG, along with a gradual decrease in linewidth in going from 4–16 waters per monomer (Baran et al., 1972). This corresponds to the complete binding of water in 38–45% PEG, with the structuring of up to 16 waters per monomer at 13% PEG. These values also agree with observations of the disappearance of a differential scanning calorimetry (DSC) melting exortherm of frozen free water at 45% PEG-6,000. (Blow et al., 1978). Our finding that complete fusion occurs at 38% PEG and above thus correlates with the percentages of PEG required to completely bind water. It should be noted that dehydration alone is not sufficient to induce membrane fusion, since dextran is not fusogenic (Ahkong, Fisher, Tampion & Lucy, 1975). A combination of dehydration and structural destabilization is required for fusion (Papahadjopoulos, Portis & Pangborn, 1978). Thus, PEG is more specific in its mode of lipid interaction.

For vesicles or cells to approach and then make subsequent contact, the electrostatic repulsive forces must also be overcome. We have shown that PEG induces both contact (aggregation) and extensive fusion of PC and PS vesicles to MLV. The fusion of PS vesicles, which are negatively charged, occurs at a higher PEG concentration but without the need of any divalent cation to facilitate close approach and contact. By diminishing the electrostatic field perpendicular to the surface of the lipid molecules, as found in monolayer studies using DPPC, extensive aggregation would be expected between opposed membranes (Maggio & Lucy, 1978). A considerable decrease in the surface potential for PEG was found in the range of 0.5–5% PEG (Maggio & Lucy, 1978). Our results also indicate the occurrence of extensive aggregation at PEG concentrations as low as 2.5%. Maximal deaggregation occurs at 4.2–4.4% PEG in all experimental conditions. These factors are consistent with the more generalized model of Knutton and Pasternak (1979), in which aggregation precedes fusion.

We have found that fusion of vesicles occurs in PEG, without the need of the final step of removing the PEG by dilution, as required for cell fusion. We attribute this to the fact that vesicles are metastable due to their high curvature (Lawaczeck, Kainosho & Chan, 1976) and can readily undergo fusion upon introduction of a perturbing force, in this case PEG. In addition, vesicles, being devoid of more rigid structural components (such as the cytoskeleton and inte-

gral membrane proteins), are more flexible than cells. The dilution step in cell fusion experiments, which causes cell swelling, thereby expanding the contact points (Knutton & Bachi, 1980), may not be required for vesicle fusion.

Cell fusion is a complex phenomenon in which the mixing of membrane lipids may not be the only factor. However, IMP-free areas, presumably exposed lipid bilayers, have been observed in oleic acid effected erythrocyte ghost fusion (Cullis & Hope, 1978), calcium phosphate-induced erythrocyte ghost fusion (Zakai et al., 1977), fusion of erythrocytes by bivalent-cation ionophore (Vos et al., 1976), uranyl acetate-induced erythrocyte fusion (Majumdar, Baker & Kalra, 1980), and the natural occurrence of membrane fusion during exocytosis in rat peritoneal mast cells (Lawson et al., 1977). We have not at this point addressed the importance of membrane protein in the PEG-induced fusion process. However, the fact that pure lipid, as in lipid denuded areas, can be induced to fuse by PEG has been proven by this study.

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