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EFFECTS OF POLY(ETHYLENE GLYCOL) ON LIPOSOMES AND ERYTHROCYTES

PERMEABILITY CHANGES AND MEMBRANE FUSION *

TIMOTHY J. ALDWINCKLE [a], QUET F. AHKONG [a], ALEC D. BANGHAM [b], DEREK FISHER [a] and JACK A. LUCY [a]

[a] *Department of Biochemistry and Chemistry, Royal Free Hospital School of Medicine, University of London, 8 Hunter Street, London WC1N 1BP and* [b] *ARC Institute of Animal Physiology, Babraham, Cambridge (U.K.)*

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Poly(ethylene glycol) 6000 induced a concentration-dependent, time-dependent decrease in the latency of the reaction between Arsenazo III sequestered in liposomes and extraliposomal Ca^{2+}. This was mediated by a gross change in liposomal permeability, i.e. by a release of Arsenazo III from liposomes rather than simply by an entry of Ca^{2+}. The loss of latency was strongly temperature-dependent, and it was markedly diminished on increasing the cholesterol content of the liposomes. It was apparently not due to an osmotic stress of the polymer. The high activation energy found (63 $kJ \cdot mol^{-1}$) is thought to indicate that the loss of latency resulted from local discontinuities in the lipid bilayers, caused by dehydration, rather than from partial or total lysis. Related microscopy experiments indicated that the polymer also caused the liposomes to fuse, and it is suggested that membrane fusion may have occurred at the sites of dehydration-induced discontinuities in adjacent bilayers. in addition, the polymer was found to enhance the permeability of hen erythrocytes to Ca^{2+} in a manner that was comparable to its effect on liposomal latency, and it is proposed that cell fusion induced by poly(ethylene glycol) may occur at the sites of similarly induced discontinuities in the phospholipid bilayers of two closely adjacent cells.

Introduction

In recent years concentrated solutions of poly(ethylene glycol), (PEG), have been successfully used as a laboratory tool to promote the fusion of cells for various experimental purposes, e.g. in somatic cell genetics [1] and in the production of monoclonal antibodies [2]. This water-soluble fusogen induces many different types of cell to fuse with a high degree of efficiency[3–6]; it is relatively non-toxic by comparison with fusogenic lipids, and it possesses certain advantages over cell fusion induced by viruses [7]. In attempts to elucidate the mode of action of the polymer in promoting cell fusion, studies have been undertaken previously on several model membrane systems. Thus, poly(ethylene glycol), like other water-soluble fusogens, was found to decrease the surface potential of phospholipid monolayers [8], while erythrocytes exposed to fusogenic concentrations of poly(ethylene glycol) became permeable to $^{45}Ca^{2+}$ and other ions [9]. The latter finding was consistent both with the observation that hen erythrocytes fuse when treated with Ca^{2+} in the presence of the divalent-cation ionophore A23187 [10], and with the well-established importance of

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Ca^{2+} in the fusion of biological membrane systems. Differential scanning calorimetry studies also revaled that concentrated, fusogenic solutions of poly(ethylene glycol) bind appreciable quantities of water [11] and raise the transition temperature of phospholipids [12].

To further characterise and define the mechanism(s) by which poly(ethylene glycol) modifies membrane permeability, the interaction of the polymer with phospholipid liposomes containing the Ca^{2+}-sensitive dye, Arsenazo III [13], has been investigated. This metallochromic indicator was studied because of its high affinity for Ca^{2+} [14], because of the ease of detecting the formation of the dye-Ca^{2+} complex [15], and because Arsenazo III (mol. wt. 776) is not precipitated in solutions of the polymer [16,17]. Studies have also been undertaken to see if poly(ethylene glycol) induces liposomes to fuse, and whether or not the kinetics of the increase in permeability of hen erythrocyte membranes to Ca^{2+} that is induced by poly(ethylene glycol) [9] is similar to the kinetics of the action of the polymer on the permeability properties of liposomes.

Aspects of this work have been described previously [18].

Materials and Methods

Materials. Arsenazo III (A III) (practical grade containing 30–40% w/w A III), cholesterol and Triton X-100 were supplied by Sigma Chemical Company (Poole, Dorset): egg phosphatidylcholine and egg phosphatidic acid were obtained from Lipid Products (Nutfield Ridge, Surray). Ionophore A23187 was a gift from Eli Lilly (Indianapolis, IN, U.S.A.) and X537A was from Roche Products (Welwyn Garden City, Herts). Poly(ethylene glycol) 6000 was purchased from Koch-Light Ltd. (Colnbrook, Bucks) and BDH (Poole, Dorset). Solutions of poly(ethylene glycol), 10–50% w/w in the extraliposomal buffer (see below) were stored at 25°C. $^{45}CaCl_2$, [^{14}C]PEG 4000 and ^3H_2O were from Amersham International, Amersham. Silicone oils DC550 and DC710 were from Hopkin and Williams (Romford, Essex); Soluene-350 and Instagel were from Packard Instruments (Caversham, Berks).

Purification and spectrometry of Arsenazo III. Commerical Arsenazo III was purified [19], and the quantity of Arsenazo III in the final preparation was determined by the analytical method of Nemodruk [20]. After purification, the dye contained 82% of Arsenazo III and 12% of Arsenazo I. The bathochromic shift, from the absorption at 530–550 nm of uncomplexed Arsenazo III to that at 660 nm (for one of the two maxima of the A III-Ca^{2+} complex), was monitored with an SP1800 spectrophotometer equipped with an AR25 linear recorder and an SP871 temperature-controlled secondary cell housing (Pye Unicam Ltd., Cambridge). The absorption values measured for the A III-Ca^{2+} complex agreed with data previously published [17]. In experiments with liposomes containing Arsenazo III, the cell housing was maintained at 25°C.

Trapping of Arsenazo III in multilamellar vesicles. A mixture of egg phosphatidylcholine, egg phosphatidic acid and cholesterol (7:2:1, molar ratio) in chloroform/methanol (2:1, v/v), which was used in all experiments unless otherwise stated, was pipetted into glass-stoppered tubes and the solvent removed by rotary evaporation. Swelling solution (2 ml) was added to the dried lipid film (60 μmol), and multimellar vesicles were formed by alternate vortex-mixing and incubation at 45°C until all the lipid dispersed; the liposome preparation was then diluted with extraliposomal buffer and centrifuged at $150000 \times g$ for 80 min at 25°C. The swelling solution used contained 30 mM Arsenazo III and 0.32 M glucose in 50 mM Hepes buffer at pH 7.4, and it was iso-osmotic with the extraliposomal buffer (0.0725 M NaCl and 0.0725 M KCl in 50 mM Tris buffer, pH 7.4) as determined by freezing-point depression osmometry. Following centrifugation, untrapped Arsenazo III was removed by aspiration of the purple supernatant, and the pelleted multilamellar vesicles were resuspended in a small volume of extraliposomal buffer.

Aliquots of the multilamellar vesicle preparation were loaded onto Sephadex columns, and eluted with extraliposomal buffer. (Columns of pre-cycled Sephadex G-50 were equilibrated with extraliposomal buffer and washed with 5 mM Arsenazo III to remove contaminating Ca^{2+} before use.) Turbid, purple fractions that eluted in the void volume were pooled and centrifuged as

above to give purple pellets of Arsenazo III associated with lipid which were resuspended in a small volume of extraliposomal buffer to make the stock suspension of multilamellar vesicles. (In a typical experiment, 7.5 ml of swelling solution was added initially to dried lipid, and the final purple pellet was resuspended in 5 ml of extraliposomal buffer.) When this suspension was stored at 6°C the entrapped Arsenazo III remained latent for more than 40 h as demonstrated by the fact that no Arsenazo III was present in the extraliposomal buffer when the multilamellar vesicles were subsequently removed on an Amicon CF 25 membrane filter (Amicon Ltd., Woking, Surrey).

The percentage trapping of Arsenazo III in the liposomes (mean ± S.D.) was 5.58 ± 0.99% $n = 6$), in agreement with previous studies [21]; phospholipid analyses [22] showed that 83.6 ± 5.4% ($n = 4$) (mean ± S.D.) of the original phospholipid was present in the stock suspension of the multilamellar vesicles.

Interaction of poly(ethylene glycol) with multilamellar versicles containing Arsenazo III. Aliquots (3 ml) of the appropriate solution of poly(ethylene glycol) were pipetted into cuvettes. 10 mM $CaCl_2$ (10 μl) and 125 mM EGTA (100 μl) were added to the test and reference cuvettes, respectively, and aliquots (100 μl) of the stock suspension of vesicles containing entrapped Arsenazo III were added to both cuvettes. After stirring for 1 min, the difference spectrum was recorded from 630 to 670 nm. The cuvettes were then repeatedly stirred and difference spectra recorded at 2, 3.5, 5, 10 min and then at 5 min intervals until 60 min. After 1 h, ionophore A23187 (5 μl of 1 mg/ml in ethanol) was added to the test cuvette, to bring the formation of the A III-Ca^{2+} complex to completion; the difference spectrum was then monitored until it became stable. (The quantity of A III-Ca^{2+} complex formed on the addition of ionophore A23187 was equal to that produced on adding Triton X-100.) Standard curves were constructed to relate increments in absorption of the difference spectrum at 660 nm to the quantity of A III-Ca^{2+} complex present.

Using a PET microcomputer (Commodore model 3032) to test the fit of the data on the formation of the A III-Ca^{2+} complex in the presence of poly(ethylene glycol) to various kinetic curves, it was found that the experimental values could be described by a second order rate equation. A computer programme * was therefore prepared that enabled the experimental data on the formation of the A III-Ca^{2+} complex under different conditions to be used for calculating the rate constants of the reactions on the basis of second-order kinetica. This programme was also used to apply statistical smoothing to the measured values of differential absorption, thus eliminating small fluctuations arising from the aggregation of multilamellar vesicles in solutions of poly(ethylene glycol). A comparison of the experimental values obtained with the corresponding, computed smooth curve is shown in Fig. 1 for the formation of the A III-Ca^{2+} complex in the presence of 25% w/w poly(ethylene glycol). Experimental measurements from duplicate kinetic studies on each liposome preparation were averaged and entered into the computer programme; duplicate readings were usually reproducible to within ±3%.

To determine the quantity of Arsenazo III released from multilamellar vesicles by the treatment with poly(ethylene glycol), vesicles were subsequently separated from the concentrated solutions of polymer by density gradient centrifugation. The contents of the cuvettes were transferred to centrifuge tubes (6.5 ml), and a discontinuous gradient of poly(ethylene glycol) was layered on top (usually 4 × 0.5 ml layers of polymer decreasing in concentration by 5–10% w/w) terminating in a top layer (0.5 ml) of extraliposomal buffer. Lipid vesicles floated to the top of the tubes and were removed after centrifugation for 25 min (80000 × g at 25°C) in a swing out rotor. The coloured fractions containing extraliposomal A III-Ca^{2+} complex remaining in the dense solutions of polymer were then transferred to cuvettes. Excess Ca^{2+} (10μl of a 1 M solution) and excess EGTA (100 μl of 125 mM solution) were added to the test and reference preparations, respectively, and difference spectra were recorded as before.

Light and electron microscopy of liposomes. Extraliposomal buffer was added to a dried film of phosphatidylcholine, phosphatidic acid and cholesterol (7:2:1, molar ratio), prepared as described above; the lipid was dispersed by vortex

* Deposited in the Biochimica Biophysica Acta Data Bank.

mixing (30 μmol of lipid per ml of solution) and then left for 16 h at room temperature. Lipid dispersions were centrifuged for 1 min at approx. $10000 \times g$ in a Beckman 'microfuge' to sediment large lipid vesicles. Aliquots (75 μl) of the supernatant were mixed with an equal volume of a 60% w/w solution of poly(ethylene glycol), prepared in the extraliposomal buffer, and incubated at 37°C. Samples were removed at intervals for light and electron microscopy. For the latter, the samples were frozen from room temperature and then fractured using a Balzers freeze-etch apparatus.

Entry of $^{45}Ca^{2+}$ into hen erythrocytes. Hen erythrocytes were obtained and washed as previously [9]. Poly(ethylene glycol) 6000 was dissolved in extracellular buffer (116 mM NaCl, 5.55 mM glucose and 10 mM $CaCl_2$) to give a range of concentrations (10–40% w/w PEG). To study the effect of poly(ethylene glycol) on the permeability of hen erythrocytes to Ca^{2+}, packed cells (0.8 ml) were resuspended in 4 ml of the required solution of poly(ethylene glycol) at 37°C, containing approx. 40 μl of a mixture of $^{45}CaCl_2$ and ^3H_2O [9] that was sufficient to give 0.4 μCi of $^{45}Ca^{2+}$ and 4.0 μCi of $^3H^+$. The treated cells were incubated at 37°C and aliquots (250 μl) of the cell suspension were removed at intervals of 1 min for centrifugation in a Beckman 'microfuge', using poly(ethylene) tubes (400 μl capacity) containing approx. 100 μl of silicone fluid overlayed with 25 μl of 5.5 mM $LaCl_3$. Silicone fluids DC550 and DC710 were used with 10% w/w and with 20–40% w/w solutions of poly(ethylene glycol), respectively. After 8 min, the ionophore A23187 (5 μl of a 1 mg/ml solution in ethanol) was added to the cell suspension, which was incubated for a further 2 min before subjecting a final aliquot to the separation procedure. Centrifugation for 25 s was sufficient to separate cells from medium; cell fractions were prepared for scintillation counting as described previously [9]; solubilization of the erythrocytes was accelerated by incubating the preparations in Soluene at 60°C. Entry ratios for Ca^{2+} [9] were calculated from radioactivity (cpm) in treated cells compared with control cells, and expressed as a percentage of the maximum entry ratio (obtained with poly(ethylene glycol) followed by ionophore A23187 as described above). Experiments were performed in duplicate on each sample of erythrocytes; three samples were studied. Observations from each time course (usually seven averaged data points) were subjected to a geometric regression analysis using a PET model 3032 microcomputer. Data were interpolated from the fitted equation ($y = ax^{-b}$) to give smoothed time-courses. The average correlation coefficient for fitting the experimental data to this equation was 0.958 ± 0.03 ($n = 13$) (mean ± S.D.).

Results

Effects of poly(ethylene glycol) on the latency of liposomes containing Arsenazo III

Aliquots of the stock suspension of multilamellar vesicles containing entrapped Arsenazo III, which were incubated in 3 ml of extraliposomal buffer containing Ca^{2+} (10 μl of a 10 mM solution of $CaCl_2$, were latent for at least 1 h under the experimental conditions used in this work (constant stirring at 25°C). No A III-Ca^{2+} complex was detected by difference spectrophotometry until either Triton X-100 or a cation ionophore were added. Triton X-100 promoted a loss of liposomal latency caused by the release of Arsenazo III from the liposome. By contrast, the A III-Ca^{2+} complex formed in the presence of ionophores was intraliposomal and it remained so, as shown by Amicon filtration. Addition of the ionophores A23187 and X537A induced identical losses in the latency of the reaction between external Ca^{2+} and Arsenazo III sequestered in the vesicles.

For our experiments on the effects of poly(ethylene glycol), the quantity of A III-Ca^{2+} complex formed in solutions of the polymer is expressed as a percentage of the maximum quantity of complex produced on the subsequent addition of ionophore A23187 to the liposomes. Treatment of vesicles containing entrapped Arsenazo III with 20–50% w/w solutions of poly(ethylene glycol) 6000 in the presence of extraliposomal Ca^{2+}, induced a time-dependent, concentration-dependent loss of liposomal latency as monitored by the formation of the A III-Ca^{2+} complex (Fig. 1). Using the procedures described in Materials and Methods, the kinetics of the reaction between Arsenazo III and Ca^{2+} were found to be governed by a second-order rate equation (Fig. 2).

On completion of the kinetic experiments on

the formation of the A III-Ca^{2+} complex in the presence of poly(ethylene glycol), the contents of the cuvettes were subjected to density gradient centrifugation in order to determine whether the change in the permeability of the vesicles was the result of the entry of Ca^{2+} into the liposomes or of the release of dye from these vesicles. The data shown in Fig. 3 indicate that concentrated solutions of the polymer promoted a quite gross change in liposomal permeability, since increasing quantities of Arsenazo III were released on incubation of the vesicles for 80 min at 25°C with increasing concentrations of poly(ethylene glycol) 6000 rather than the poly(ethylene glycol) simply enhancing the permeability of the vesicles to Ca^{2+}.

Effect of cholesterol on the loss of latency by poly(ethylene glycol)

The incorporation of increasing quantities of cholesterol into the liposomes markedly reduced the loss of liposomal latency promoted by solutions of 25% w/w PEG 6000, as is shown in Fig. 4. This behaviour was paralleled in comparable experiments by a decrease in the rate constants for the formation of the Arsenazo III-Ca^{2+} complex on incubation of cholesterol-containing vesicles with various concentrations of poly(ethylene glycol) 6000 at 25°C, and also by a decrease in the quantities of extraliposomal Arsenazo III observed [18]. The molar ratio of cholesterol to total phospholipid was generally modified by altering the ratio of cholesterol to phosphatidylcholine, while the phosphatidic acid content was kept constant in order to maintain a maximum volume for the

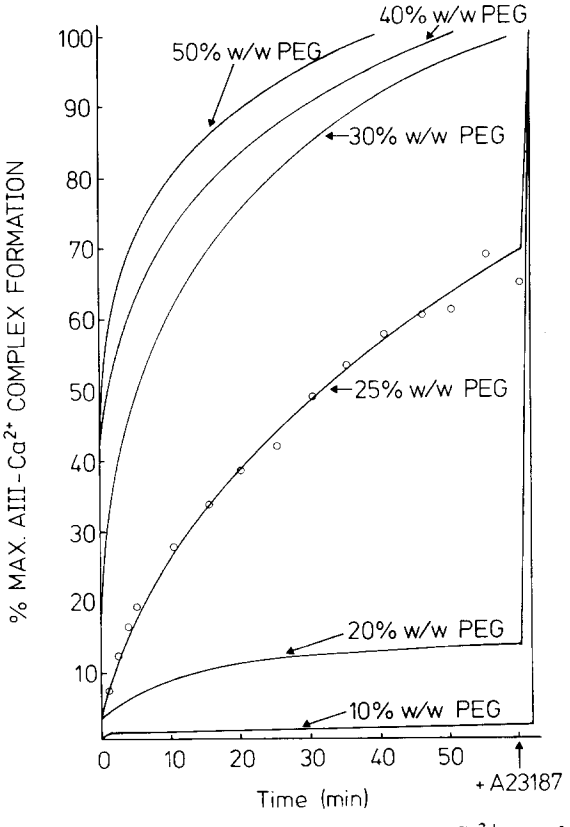


Fig. 1. Formation with time of the Arsenazo III-Ca^{2+} complex when multilamellar vesicles (phosphatidylcholine/phosphatidic acid/cholesterol, 7:2:1) were incubated at 25°C in solutions of poly(ethylene glycol) 6000 containing Ca^{2+} as described in Materials and Methods.

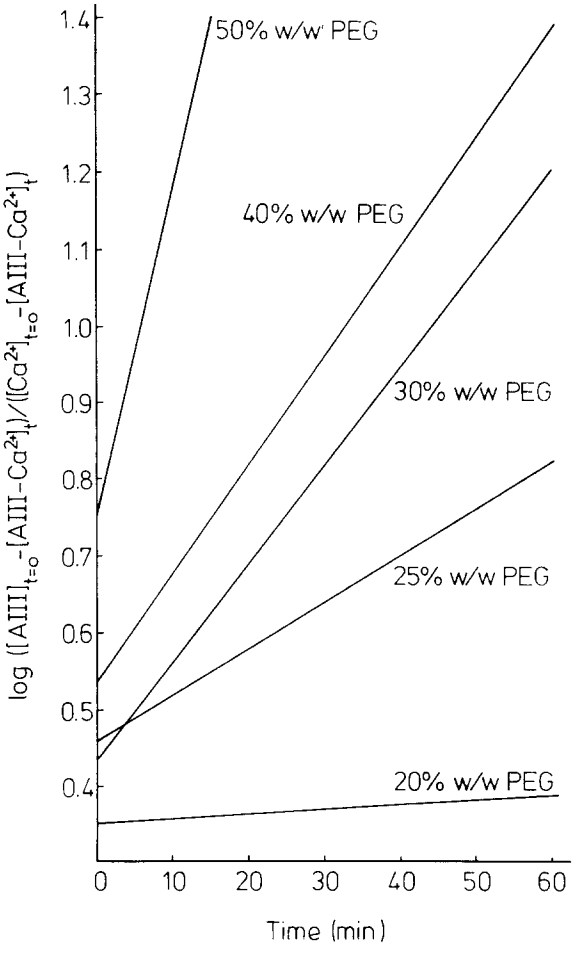


Fig. 2. Second-order reaction rate plots for the formation of the Arsenazo III-Ca^{2+} complex in experiments with poly(ethylene glycol) 6000 undertaken as described in Fig. 1.

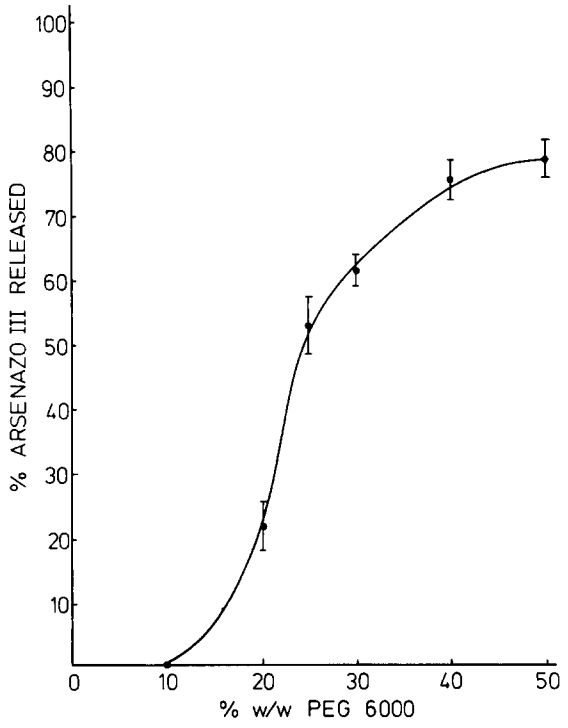


Fig. 3. The extraliposomal release of Arsenazo III from multilamellar vesicles on incubation for 80 min with poly(ethylene glycol) 6000 as described in Fig. 1.

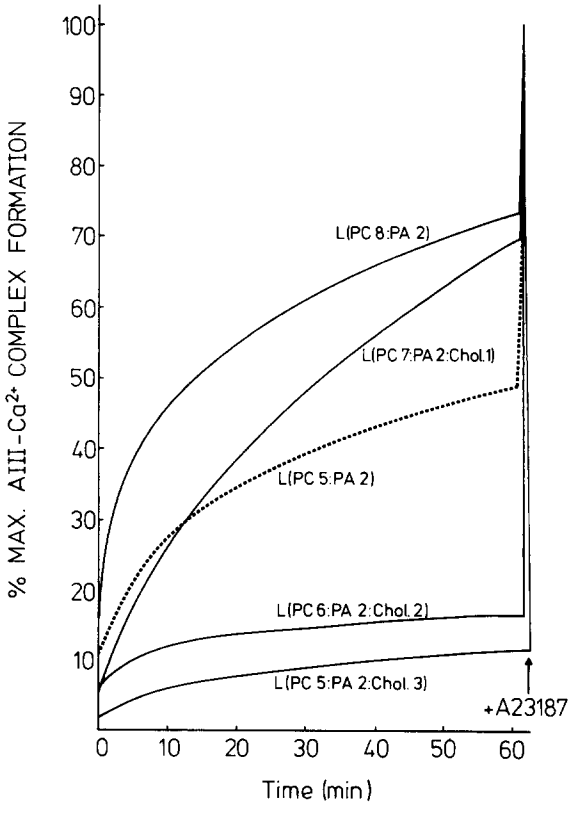


Fig. 4. The effect of varying the lipid composition of multilamellar vesicles on the rate of formation of the Arsenazo III-Ca^{2+} complex when the vesicles were incubated in 25% w/w poly(ethylene glycol) 6000 at 25°C as described in Materials and Methods.

aqueous compartment of the vesicles as in the investigations of Bangham et al. [23]. Further studies demonstrated, however, that the action of cholesterol in reducing the change in permeability of vesicles by poly(ethylene glycol) could be attributed to the presence of the sterol, and not to the altered ratio of phosphatidylcholine to phosphatidic acid (compare the data for liposomes composed of phosphatidylcholine/phosphatidic acid (5:2, molar ratio) with that for phosphatidylcholine/phosphatidic acid cholesterol (5:2:3, molar ratio) shown in Fig. 4).

Effect of temperature

Multilamellar vesicles composed of phosphatidylcholine/phosphatidic acid/cholesterol (7:2:1, molar ratio), containing sequestered Arsenazo III, that were incubated with 25% PEG 6000 in the presence of Ca^{2+} exhibited a loss of latency that was markedly dependent on temperature (Fig. 5). That the changes in permeability observed were due to the presence of poly(ethylene glycol) was demonstrated by the fact that vesicles containing Arsenazo III that were incubated in extraliposomal buffer with Ca^{2+} alone at the two extremes of temperatures studied retained their latency over the time scale of the experiments. Arrhenius plots were constructed from the computed values of rate constants (obtained in triplicate) at four different temperatures (17.5, 24.9, 31.0, and 37.7°C). These yielded activation energies of 58.7 and 66.5 kJ · mol^{-1} with two independent preparations of liposomes. Fig. 6 shows the Arrhenius plot obtained from the averaged values of these observations this gave a mean value for the activation energy of 62.6 kJ · mol^{-1}.

Further characterization of the loss of liposomal latency

To investigate whether or not molecules of larger

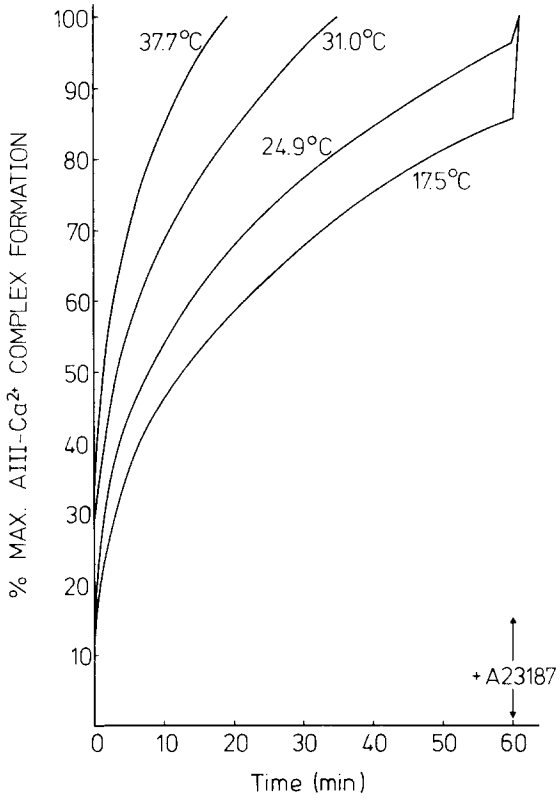


Fig. 5. The influence of temperature on the rate of formation of the Arsenazo III-Ca^{2+} complex in experiments with multilamellar vesicles incubated in 25% w/w poly(ethylene glycol) 6000 as described in Fig. 1.

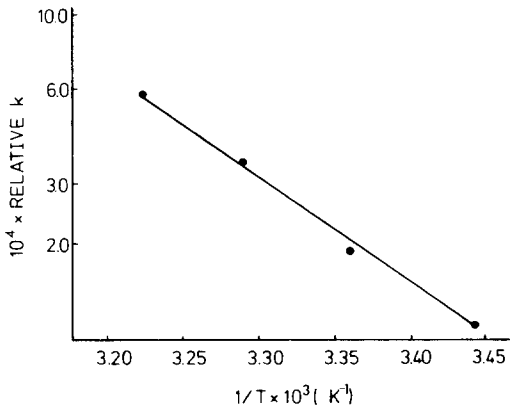


Fig. 6. A plot of the second-order rate constant (k) against the reciprocal of the temperature for the formation of the Arsenazo III-Ca^{2+} complex in experiments with multilamellar vesicles incubated with 25% w/w poly(ethylene glycol) 6000 as described in Fig. 5.

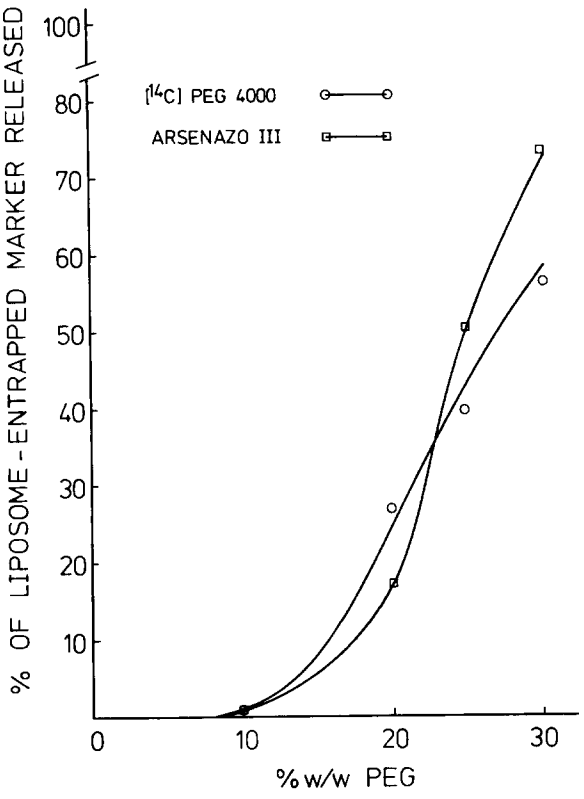
molecular weight than Arsenazo III are also released in the treatment of liposomes with poly(ethylene glycol) 6000, vesicles were formed in a swelling solution that contained Arsenazo III and approx. $4 \cdot 10^{-3}$% w/w [^{14}C]-labelled poly(ethylene glycol) of molecular weight 4000. It was found that both markers were released in a comparable manner from the vesicles in the presence of increasing quantities of poly(ethylene glycol) 6000 on incubation for 1 h at 25°C (Fig. 7). In the absence of poly(ethylene glycol), however, neither marker was released under the experimental conditions used.

To see if a simple cation, Ca^{2+}, might also be released by poly(ethylene glycol), the action of poly(ethylene glycol) on liposomes (phosphatidylcholine/cholesterol, 7:3, molar ratio) containing entrapped $^{45}Ca^{2+}$ was investigated (cf. Ref. 24). Solutions of the polymer were observed to mediate the release of the sequestered isotope in a time-dependent, concentration-dependent manner, which resembled the loss of latency observed for vesicles containing Arsenazo III on exposure to poly(ethylene glycol) (data not shown: cf. Ref. 18).

Taupin et al. [25] have reported that phospholipid vesicles subjected to an applied osmotic gradient undergo a process of transient pore-formation that results in the leakage of entrapped hydrophilic markers. To find whether the action of poly(ethylene glycol) on liposomal latency was likely to be determined by the osmotic properties of the polymer [26], vesicles containing entrapped Arsenazo III were incubated in solutions of sucrose that were either equal to or double the osmolarity of 30% w/w PEG 6000. In each experiment, the lipid vesicles remained latent during the experiment (1 h at 25°C), and no A III-Ca^{2+} complex was formed until ionophore A23187 was added subsequently. By contrast, as may be seen from the data of Fig. 1, treatment of the vesicles with 30% PEG 6000 allowed maximum formation of the A III-Ca^{2+} complex on incubation for 1 h at this temperature.

Morphology of liposomes treated with poly(ethylene glycol)

Using light and freeze-fracture microscopy, experiments were undertaken to see if poly(ethyl-



ene glycol) is capable of fusing liposomes. Despite the well-known fusogenic behaviour of poly(ethylene glycol) towards numerous different types of cell, no observations had been reported on the fusion of liposomes by poly(ethylene glycol) when this work was initiated. Fig. 8 shows that aggregated liposomes and large vesicles, which probably resulted from fusion of aggregated liposomes, were observed even with the light microscope within 30 min of treating the liposomes with 30% w/w PEG 6000 at 37°C, and it is relevant to note that 80–90% of the maximum loss of liposomal latency occurred in 30 min at 25°C in the presence of 30% PEG 6000 (Fig. 1). After 3 h with poly(ethylene glycol), very large vesicles were present (compare Fig. 9 with Fig. 10, and Fig. 12 with Fig. 11). During the course of this work, comparable observations were reported by Boni et al. [27]. Interestingly, both in the present studies and in the studies of Boni et al. the fusion of liposomes

Fig. 7. A comparison of the simultaneous extraliposomal release of isotopically-labelled poly(ethylene glycol) 4000 and of Arsenazo III from multilamellar vesicles that were incubated for 1 h with poly(ethylene glycol) 6000 as described in Fig. 1.

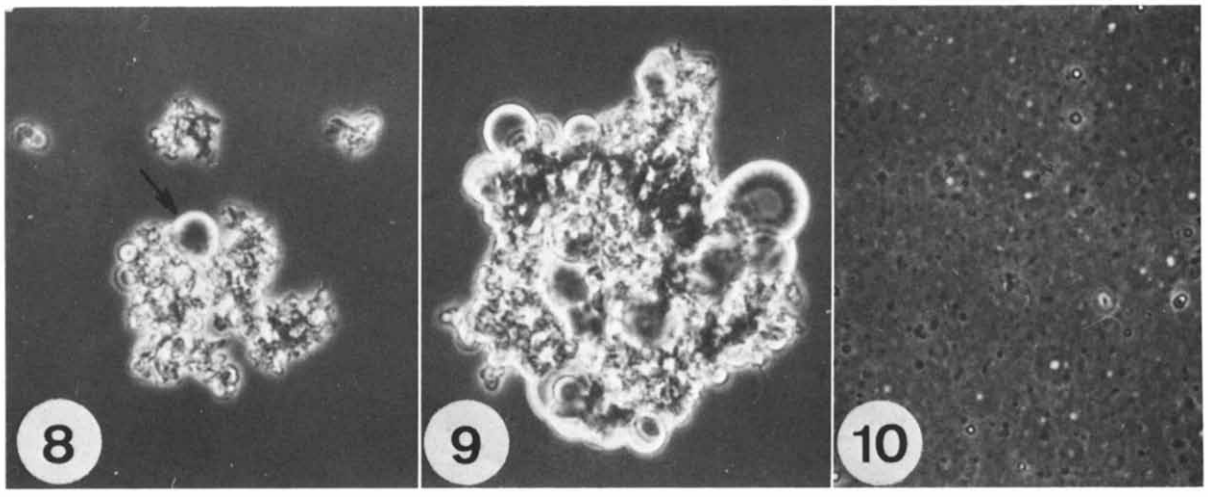


Fig. 8. A light micrograph of multilamellar vesicles (phosphatidylcholine/phosphatidic acid/cholesterol, 7:2:1) incubated at 37°C for 30 min with 30% w/w PEG 6000, showing a large vesicle (arrow) associated with aggregated liposomes. Magnification ×500.

Fig. 9. A light micrograph of the multilamellar vesicles incubated at 37°C for 3 h with 30% w/w PEG 6000, showing a larger aggregate of liposomes associated with several large vesicles. Magnification ×500.

Fig. 10. A light micrograph of the multilamellar vesicles incubated at 37°C for 3 h in the absence of poly(ethylene glycol) 6000, showing little aggregation and no large vesicles. Magnification ×500.

occurred in the poly(ethylene glycol) solution, without any need to remove the poly(ethylene glycol) by dilution as for cell fusion. However in their work, and in the related experiments of Sáez et al. [28], sonicated phospholipid vesicles were used which are inherently more unstable than the large multilamellar vesicles employed in the present investigation.

Hen erythrocytes treated with poly(ethylene glycol)

Hen erythrocytes are fused into multinucleated cells on treatment with poly(ethylene glycol) [8],

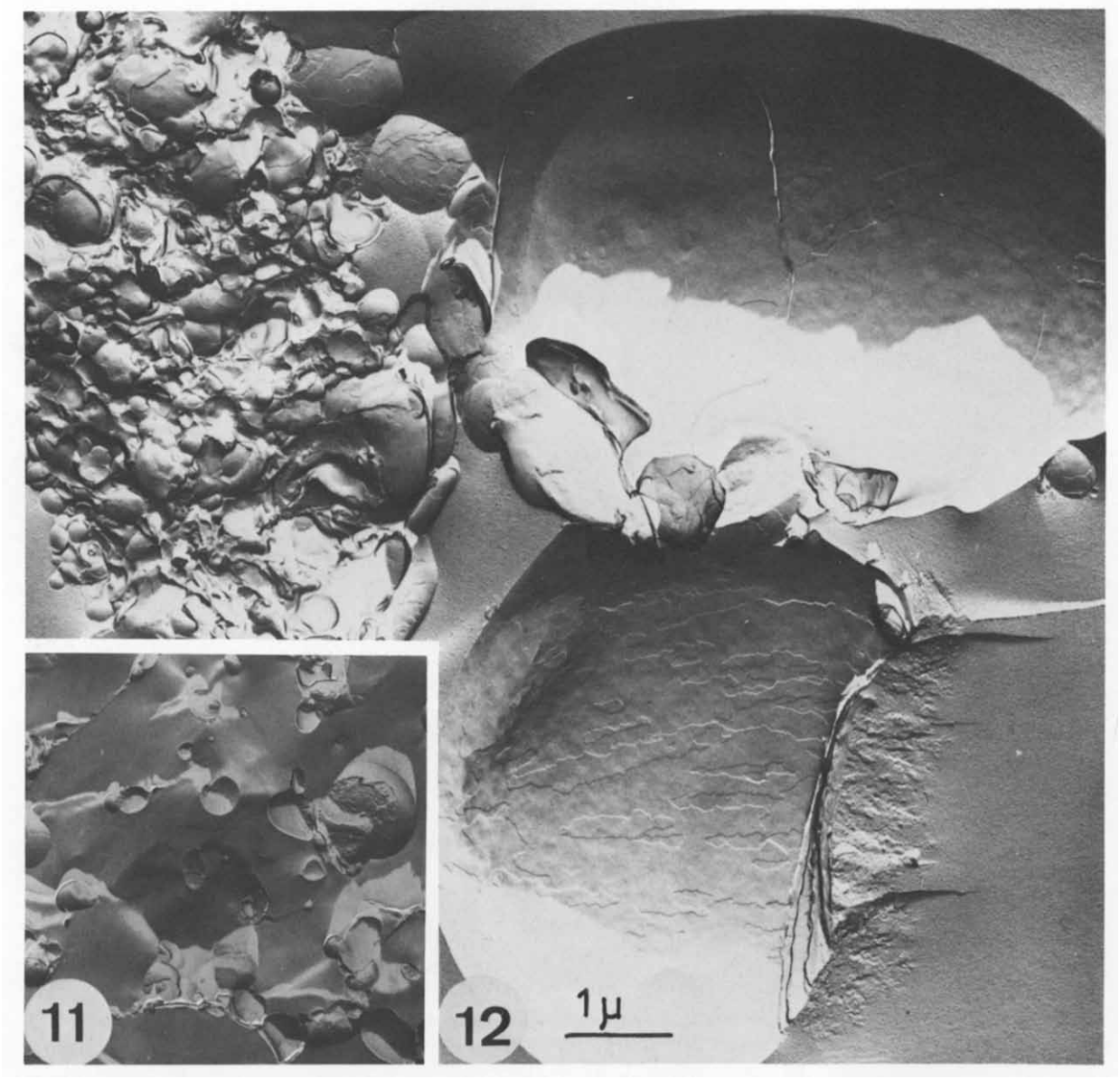


Fig. 11. An electron micrograph of a freeze-fractured preparation of the multilamellar vesicles incubated at 37°C for 3 h in the absence of poly(ethylene glycol) 6000 (cf. Fig. 10). (Magnification as for Fig. 12).

Fig. 12. An electron micrograph of a freeze-fractured preparation of the multilamellar vesicles incubated at 37°C for 3 h with 30% w/w PEG 6000 (cf. Fig. 9), showing aggregated liposomes and two large vesicles.

and it has been shown previously that incubation of these cells with fusogenic concentrations of poly(ethylene glycol) makes their plasma membranes abnormally permeable to $^{45}Ca^{2+}$ [9]. In the present work, the kinetics of this entry of $^{45}Ca^{2+}$ into the treated erythrocytes has been investigated and the cells were found to show a concentration-dependent, time-dependent increase in the entry ratio for this cation (Fig. 13). (Under the experimental conditions used, the increased $^{45}Ca^{2+}$ associated with the cells can be attributed, as previously [9], to $^{45}Ca^{2+}$ entering the erythrocytes rather than adhering to the cell surfaces.) Interestingly, the kinetic profiles of Fig. 13 for the poly(ethylene glycol)-mediated entry of Ca^{2+} into the hen erythrocytes closely resemble those for the effects of comparable concentrations of poly(ethylene glycol) on liposomal latency (Fig. 1). This indicates the existence of a basic similarity between the actions of poly(ethylene glycol) on the two systems, which is presumably a reflection in each case of the effect of the polymer on a lipid bilayer.

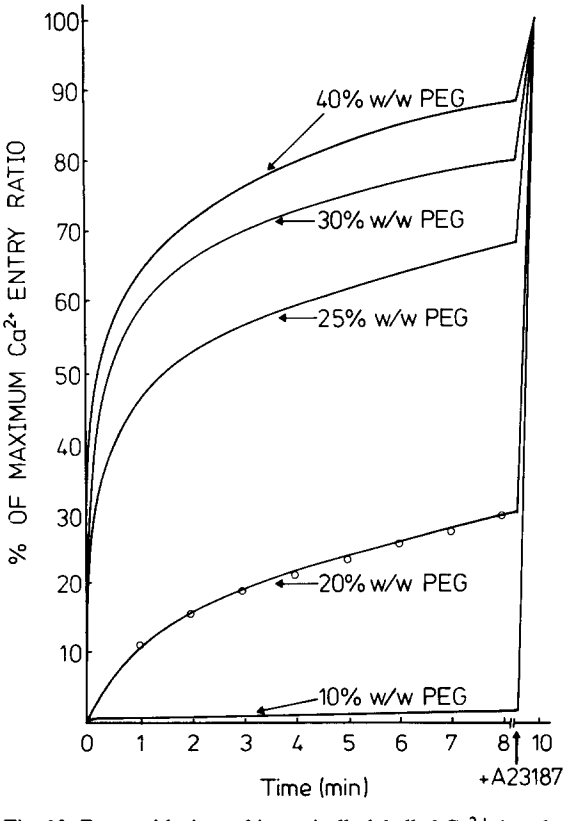


Fig. 13. Entry with time of isotopically-labelled Ca^{2+} into hen erythrocytes incubated in solutions of poly(ethylene glycol) 6000 at 37.4°C as described in Materials and Methods.

Discussion

It is interesting that the rate of loss of latency observed on treating multilamellar liposomes with 20% w/w PEG was quite slow after about 10 min (Fig. 1). With this concentration of poly(ethylene glycol) only some 12% of the maximum quantity of the A III-Ca^{2+} complex was formed in 1 h, corresponding approximately to the quantity of Arsenazo III present in the outermost aqueous compartment of the vesicles [29]. It thus seems conceivable that 20% w/w PEG affected only the outermost membrane of the multilamellar liposomes, and that increasing concentrations of polymer pertubed a greater number of concentric membranes thereby releasing progressively increasing quantities of dye.

With regard to possible explanations for the release of dye from liposomes treated with poly(ethylene glycol), it is clear from the light and electron microscopy work that total lysis and disruption of the liposomes did not occur. Localised lysis of liposomal membranes [30] also seems unlikely because the apparent activation energy found for the loss of liposomal latency mediated by 25% w/w PEG (63 $kJ \cdot mol^{-1}$) would appear to be too high for a lytic effect [31]. The observed value for the activation energy is more comparable to that for the release of glucose from liposomes (46 $kJ \cdot mol^{-1}$) via membrane defects induced by bovine serum albumin [31]; it may also be compared with the value of 84 $kJ \cdot mol^{-1}$ found both for the fusion of hen erythrocytes induced by lipid soluble fusogens [32] and for the spontaneous fusion of myoblasts in culture [33]. By contrast the activation energy observed here seems to be too low for the polymer to act as an ionophore for Arsenazo III or for the A III-Ca^{2+} complex (when an activation energy of about 116 $kJ \cdot mol^{-1}$ [34] would be anticipated), despite the fact that linear polyethers can complex ions [35]. Indeed, the low solubility of the polymer in hydrocarbons [36], and its high solubility in water [37], would appear to preclude an ionophore-type action for poly(ethylene glycol).

An alternative explanation for the action of

poly(ethylene glycol) observed in this work is that the polymer promotes the formation of transient discontinuities, pores, or other membrane defects in the liposomes through which Arsenazo III, or the A III-Ca^{2+} complex, is able to permeate [18]. As solutions of poly(ethlene glycol) 6000 released comparable quantities of entrapped [^{14}C]PEG 4000 and Arsenazo III in an identical manner, such defects in the phospholipid bilayer would be quite large. The leakage of labelled poly(ethylene glycol) 4000 may nevertheless be interpreted in terms of a 'pore-formation' process, in view of the efflux of entrapped inulin and other large markers from liposomes incubated at their phase-transition temperature [38]. It is also relevant that the plasma-induced release of solutes from small unilamellar liposomes of egg phosphatidylcholine has been reported to be associated with pore formation in the bilayers [39], which allows a relatively free passage of sucrose and of inulin (mol. wt. $\simeq$ 5000) but restricts the exit of polyvinylpyrrolidone (average mol. wt. 30000–40000). In this work, the presence of cholesterol appeared to decrease the size of the pores. In the present experiments, however, it was not feasible to investigate possible pore sizes by sequestering a range of molecular-weight markers in the vesicles, and then measuring their release in the presence of poly(ethylene glycol), owing to the precipitating action of concentrated solutions of this polymer [16,17].

During the preparation of the present work for publication, Boni et al. [40] have published data on the formation in the presence of poly(ethylene glycol) of structural defects in egg phosphatidylcholine multilamellar vesicles, as shown by freeze-fracture electron microscopy. They also used ^{31}P-NMR which indicated that the disruptions involve non-bilayer configurations of the phospholipid molecules which they consider may represent an intermediate stage in membrane fusion.

The relatively high concentration of poly(ethylene glycol) required to modify the permeability of liposomes and of erythrocytes, and also to induce cell fusion, indicates that these actions of the polymer may be mediated by its influence on water structure [11,41]. Ultrasonic relaxation experiments have indicated that poly(ethylene glycol) modifies water structure [42,43], and a number of investigations have been made on the water-binding properties of the polymer [44–47]. It has also been suggested that poly(ethylene glycol) may alter the properties of more water molecules than those to which it binds by exerting a 'structuring action' on several layers of molecular water surrounding the polymer, involving up to 16 water molecules per monomer unit [48]. This indicates that all the water would be structured in solutions more concentrated than 13% w/w PEG, and it may be relevant to our findings that multilamellar vesicles were latent in 10% w/w PEG but released Arsenazo III in solutions of 20% w/w PEG (Fig. 1). Structuring by poly(ethylene glycol) of water that would normally be associated with the polar headgroup of the phospholipids of membranes may induce structural perturbations in phospholipid bilayers that make them abnormally permeable to ions and molecules. Bilayers of phosphatidylcholine might be expected to be particularly susceptible to the presence of high concentrations of poly(ethylene glycol) since, with this phospholipid water molecules of hydration are incorporated into the headgroup lattice where they link phosphate groups into ribbons and shield them from the positively-charged choline groups [49].

The initial aim of the investigations reported in this paper was to see if a model phospholipid membrane system, free from protein, behaves similarly to erythrocytes [9] with regard to Ca^{2+} permeability on treatment with poly(ethylene glycol). Our findings indicate that liposomes are not lysed or destroyed by poly(ethylene glycol) 6000 but develop structural defects on treatment with this polymer and, as a result, can release trapped Arsenazo III and isotopically-labelled poly(ethylene glycol) 4000. In addition we have observed that not only does poly(ethylene glycol) induce the aggregation of liposomes but that very much larger vesicles are also produced, apparently by membrane fusion. It is therefore suggested that, when liposomes are treated with high concentrations of poly(ethylene glycol), membrane fusion occurs at the site of dehydration-induced discontinuities in adjacent lipid bilayers. Incidentally, it is interesting to note that both in the work reported here and in earlier unpublished studies undertaken by Tilcock and Fisher described in Ref. 50, and in the investigations of Boni et al. [27], the fusion of liposomal vesicles apparently induced by poly(eth-

ylene glycol) occurred in the absence of added Ca^{2+}. (In Ref. 27, the lipid was dispersed in the presence of 0.2 mM EDTA.) Furthermore, it has previously been reported that alkyl bromide-induced fusion of both egg phosphatidylcholine and phosphatidylcholine/phosphatidic acid vesicles shows no dependence on the concentration of Ca^{2+}, over a range of 0.5 to 10 mM, assayed either by light-scattering or by direct microscopical observation [51]. These several observations indicate that the need for Ca^{2+} seen in the fusion of fibroblasts [11] and hen erythrocytes [8] by poly(ethylene glycol) 6000, like the requirement for subsequent dilution of the poly(ethylene glycol) mentioned above, are features of cell fusion rather than of lipid bilayer fusion as such.

It is not, in fact, known if erythrocytes and other cells also become permeable to molecules of comparable molecular weight before they fuse in response to treatment with poly(ethylene glycol). It would appear to be significant, however, that the kinetic profiles for the loss of liposomal latency (Fig. 1) are directly comparable with changes in the Ca^{2+} permeability observed with hen erythrocytes incubated in solutions of the polymer (Fig. 13). This indicates that poly(ethylene glycol) may well act on the phospholipids of plasma membranes in a similar manner to that for liposomes although membrane proteins and, in the light of the present studies, membrane cholesterol will reduce instability in biological membranes treated with poly(ethylene glycol). It is concluded from our observations that the fusogenic action of PEG on liposomes and on cells probably involves an effect of the polymer on their common structural entity, the phospholipid bilayer. In both systems, membrane fusion may occur at the sites of discontinuities induced in the bilayer by the dehydrating action of poly(ethylene glycol).

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