The influence of poly(ethylene glycol) 6000 on spermine-induced aggregation of liposomes

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Poly(ethylene glycol) 6000 affected the aggregation of mixed liposomes induced by spermine. It lowered the concentration of spermine causing 50% maximal aggregation, accelerated the rate and increased the extent of aggregation. The effect was inversely proportional to the density of the acidic phospholipid in the vesicles. These effects were not due either to poly(ethylene glycol) 6000-induced permanent structural modification of the liposome or increased binding of spermine to the vesicles. These findings are discussed in relation to a decreased hydration force caused by the ability of poly(ethylene glycol) 6000 to alter the water of hydration of the phospholipid polar groups in the liposome.

INTRODUCTION

The naturally occurring polyamines (putrescine, spermidine and spermine) are ubiquitous components of living material (Bachrach, 1973). At physiological pH, polyamines are polycations, and it is generally accepted that they are bound to anionic cell constituents among which are acidic phospholipids in biomembranes. The phospholipid–polyamine complex appears to carry out several functions: regulation of enzymic activities (Jellinck & Perry, 1967; Solaini & Tadolini, 1984), membrane stabilization against peroxidation (Igarashi et al., 1981; Tadolini et al., 1984, 1985b), mediation of the transduction of hormonal signals (Koenig et al., 1983a,b) and mediation in membrane fusion (Hong et al., 1983; Schuber et al., 1983).

To elucidate the molecular mechanism by which polyamines affect membrane fusion, aggregation studies were utilized. Aggregation of phospholipid vesicles by cations has been frequently used as a model system, since the close contact of two membranes is considered to be an initial step and a necessary condition for two membranes to fuse.

Spermidine and spermine induced the aggregation of large unilamellar vesicles containing acidic phospholipids. The kinetics of aggregation were markedly dependent on the polyamine concentration and exhibited a threshold (Schuber et al., 1983).

A systematic study of the mechanism of binding spermine to phospholipid vesicles of different composition was conducted and it showed that the threshold concentration was dependent on the type and density of the acidic phospholipid component of the vesicles (Tadolini et al., 1985a). It was proposed that polyamines, as polycations and thus as counterions to acidic phospholipids, greatly decrease the surface charge density and surface potential. The consequent reduction of the mutual electrostatic repulsion between vesicles could induce the vesicles to aggregate as a result of the increase in the contribution of the van der Waal's attractive forces to the total free energy of interaction (Schuber et al., 1983). A direct relationship between the

binding of spermine and the aggregation of the vesicles would then be expected. However, the complex formed at the acidic phospholipid/spermine ratio causing aggregation is characterized by a formation constant independent on the type and density of the acidic phospholipid on the vesicles (Tadolini et al., 1985a). Precipitation of a complex is dependent on its solubility as well as on its formation.

In order for two particles to adhere to one another, it is necessary that the driving force acting on a particle be equal to, or greater than, the repulsive force corresponding to the steepest part of the repulsive interaction energy curve. The net repulsive force, P, is equated to the balance of hydration (P_H) and electrostatic repulsion (P_{ES}) and van der Waal's attraction (P_A):

$$P = P_H + P_{ES} - P_A \text{ (Rand, 1981).}$$

Hydration force is greater than electrostatic repulsion at a separation of less than about 2 nm (20 Å). This force is related to the work required to remove water from hydrophilic surfaces as they approach. In order to arrive at an understanding of the various influences on spermine-induced aggregation of liposomes, hydration force must be considered. In view of this we have investigated the effect of PEG 6000, a compound that is known to bind water and to alter the water of hydration of the phospholipid polar groups in cell membranes (Blow et al., 1978). The results are described in the present paper.

MATERIALS AND METHODS

Egg-yolk phosphatidylcholine (PC) and phosphatidic acid (PA) were obtained from Lipid Products, Redhill, Surrey, U.K. Spermine tetrahydrochloride, spermidine trihydrochloride, putrescine dihydrochloride and Mops were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.); [14C]spermine was from The Radiochemical Centre, Amersham, Bucks., U.K.; heparin–Sepharose CL-6B was from Pharmacia Fine Chemicals, Uppsala, Sweden; PEG 6000 and all reagents of
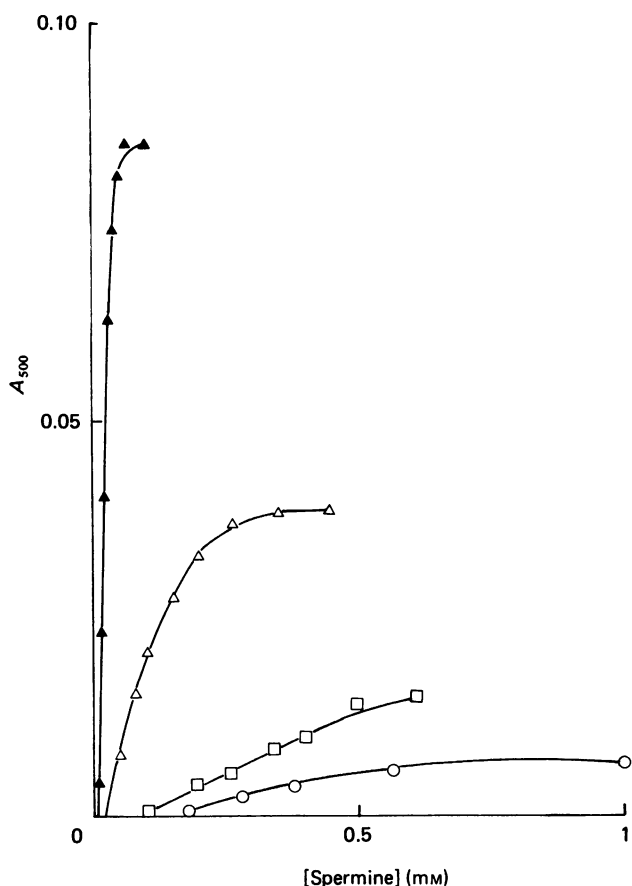

Abbreviations used: PEG 6000, poly(ethylene glycol) 6000; PC, phosphatidylcholine; PA, phosphatidic acid; PS, phosphatidylserine; PE, phosphatidylethanolamine.

Fig. 1. Aggregation of vesicles composed of different ratios of PC and PA versus spermine concentration

Aggregation was evaluated by measuring turbidity changes (A_{500}) after spermine addition. ▲, PC/PA (1:1); △, PC/PA (8:2); □, PC/PA (9:1); ○, PC/PA (49:1).

analytical grade were purchased from Merck (Darmstadt, Germany). Unilamellar phospholipid vesicles were prepared by a published method (Huang, 1969) in 5 mM-Mops buffer, pH 7. Phosphorus determination was by the method of Marinetti (1962). The binding of [^{14}C]spermine to phospholipid vesicles was determined by a cation-exchange-resin method previously described (Tadolini et al., 1985a) and similar to the anion-exchange-resin method developed by Walaas (1958). Equations and derivations described by Nakai & Glinsmann (1977) were used to interpret our experimental data.

Aggregation was monitored by the turbidity changes (at 500 nm) in the vesicle suspension. The concentration of phospholipid was about 0.3 mg/ml in 5 mM-Mops buffer, pH 7, at room temperature. The change of absorbance, after addition of spermine, was recorded. The absorbance change obtained 5 min after spermine addition was plotted as a function of the polyamine concentration.

RESULTS

We studied the aggregation of vesicles of different PC/PA molar ratios in the presence of increasing concentrations of spermine. At a constant PC/PA ratio the extent of maximal aggregation depends on the

amount of vesicles in the reaction mixture. When vesicles composed of a high PC/PA ratio (1:1; 4:1) were used, the titration curves shifted with vesicle concentration, and the spermine concentrations causing half-maximal aggregation ($AC_{0.5}$) differ, as previously shown (Tadolini et al., 1985a). To compare the aggregation curves of vesicles of different PC/PA ratio we incubated amounts of vesicles containing the same concentration of acidic phospholipid (10 μg of PA). As shown in Fig. 1, the aggregation curves greatly differ in $AC_{0.5}$. Also the extents of maximal aggregation differ: at the maximum spermine concentration tested (7 mM) the maximal A_{500} of 9:1 (PC/PA) vesicles was 0.014 compared with 0.086 of 1:1 (PC/PA) vesicles, although the latter sample contained one-fifth of the total phospholipid of the former (results not shown).

When 49:1 (PC/PA) vesicles were aggregated by spermine in the presence of increasing concentrations of PEG 6000, both the $AC_{0.5}$ and the extent of maximal aggregation vary (Fig. 2).

The time-dependence of the phenomenon was studied. Incubation of the vesicles for various times with PEG 6000 (from 1 to 15 min) did not show any difference and, moreover, the addition of vesicles to a cuvette containing PEG 6000 and spermine resulted in an almost instantaneous aggregation.

To obtain further information on spermine aggregation of charged phospholipid vesicles we studied the effect of PEG 6000 on vesicles containing different densities of PA. Vesicles containing 1:1; 8:2, 9:1 and 49:1 molar ratios of PC to PA were utilized.

Fig. 3 shows plots of the logarithm of the spermine concentration causing half-maximal aggregation of these vesicles against PEG 6000 concentration. The ability of PEG 6000 to decrease $AC_{0.5}$ is inversely proportional to the density of the acidic phospholipid in the vesicles.

The curves intersect at a point defined by a spermine concentration that depends on the amount of acidic phospholipid present in the reaction mixture and by a PEG 6000 concentration of about 17.5 mM. PEG 6000 is known to promote fusion of liposomes (Aldwinckle et al.,
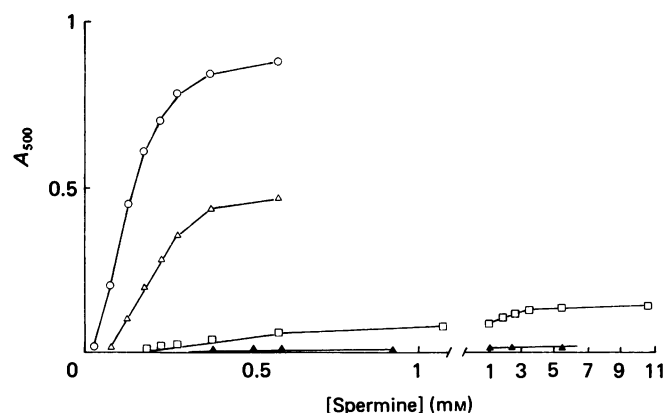

Fig. 2. Effect of PEG 6000 concentration on spermine-induced aggregation of PC/PA (49:1) vesicles

Aggregation was evaluated by measuring turbidity changes (A_{500}) after spermine addition in the absence of PEG 6000 (▲) or presence (open symbols) of 2.5 mM-(□), 5 mM-(△) and 10 mM-(○) PEG 6000.
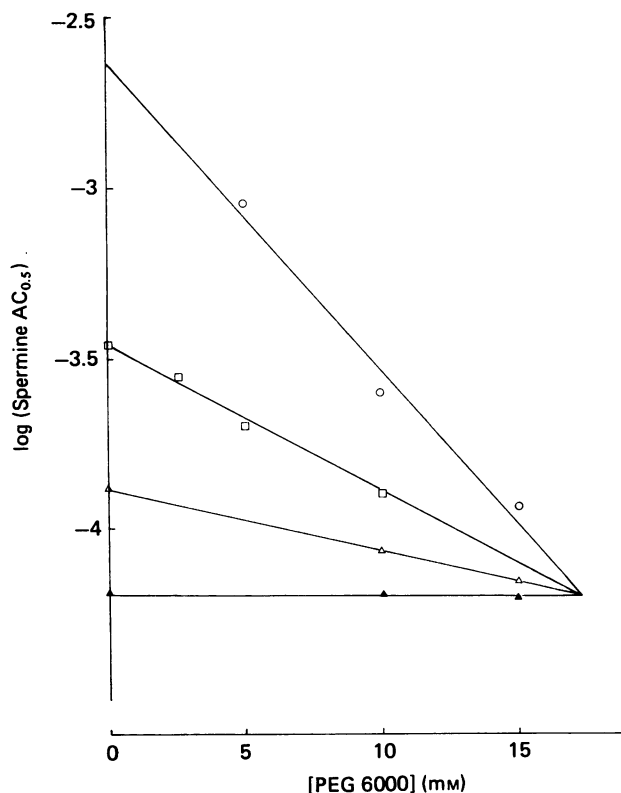
Fig. 3. Plot of spermine $AC_{0.5}$ of vesicles made of different ratios of PC and PA versus PEG 6000 concentration

$AC_{0.5}$ was determined from aggregation curves obtained as indicated in the Materials and methods section. ▲, PC/PA (1:1); △, PC/PA (8:2); □, PC/PA (9:1); ○, PC/PA (49:1).

presence of 15 mM-PEG 6000, a higher concentration of vesicles is required to remove spermine.

The secondary plot of the data produced straight lines with a positive slope both in the absence and presence of PEG 6000. The intersections of the plots with the ordinate are similar and represent a measurement of the formation constant (K_1) of the acidic phospholipid–spermine complex formed at low ratio of these reactants in the reaction mixture (Tadolini et al., 1985b). The slope of the curves, by contrast, differs. As the slope represents a measurement of $K_1 \cdot K_2$, the formation constant, K_2, appears to be decreased by the presence of PEG 6000. K_2 is the formation constant of the complex formed at a high phospholipid/spermine ratio (Tadolini et al., 1985a). PEG 6000 therefore decreases spermine binding to the vesicles under this experimental condition.

PEG 6000 may interact with vesicle components different from those interacting with spermine. We have evaluated the effect of PEG on 100% PC vesicles that were previously shown not to bind this amine (Tadolini et al., 1985a). When a 100% PC vesicle suspension was added with increasing concentrations of PEG, aggregation occurred and the presence of 4 mM spermine (ineffective in itself in causing aggregation) did not interfere (Table 1).

DISCUSSION

The results clearly indicate that the aggregation of negatively charged liposomes by spermine is greatly affected by the presence of PEG. PEG 6000 decreases the
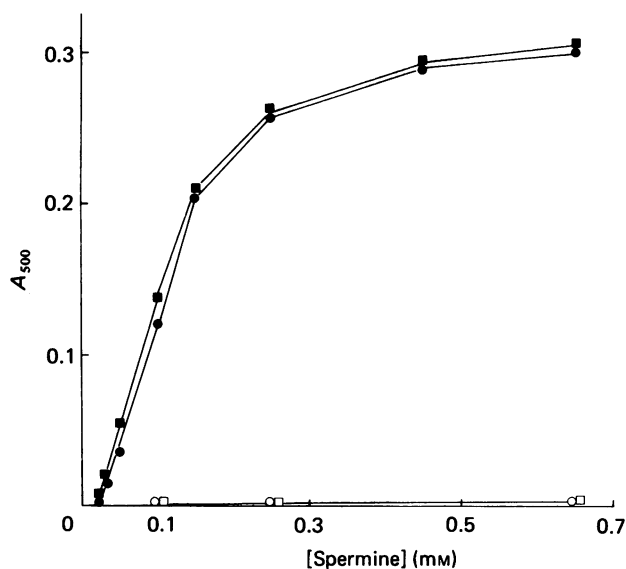
1982). During this event, leakage of a dye sequestered into liposomes has been described (Aldwinckle et al., 1982), suggesting the occurrence of a transient continuity between the two layers of the bilayer. During this event, a redistribution of the charged phospholipid may occur. The effect on aggregation exerted by PEG 6000 may be due to such a structural modification of the vesicles that remains after removal of PEG 6000. To assess this possibility, 49:1 (PC/PA) vesicles were incubated with 30 mM-PEG 6000 for 15 min and then the reaction mixture was submitted to chromatography on a Sephadex G-50 column to remove PEG 6000. Untreated vesicles and vesicles eluted from the column were aggregated by addition of increasing concentrations of spermine in the absence or presence of PEG 6000. The results are reported in Fig. 4. Chromatographed vesicles do not differ from untreated vesicles and are not aggregated by spermine up to 0.7 mM, whereas both types of vesicles when incubated in the presence of 15 mM-PEG 6000 have an $AC_{0.5}$ of about 0.11 mM-spermine. The hypothesis that PEG 6000 may facilitate the binding of spermine to the vesicles was verified.

Increasing concentrations of 9:1 (PC/PA) vesicles were incubated in the presence of the anionic resin heparin–Sepharose and [^{14}C]spermine. The vesicles compete with the resin for spermine and decrease the formation of the heparin–Sepharose–spermine complex (Fig. 5). When the experiment is conducted in the

Fig. 4. Effect of PEG 6000 removal on spermine-induced aggregation of mixed vesicles

Aggregation of PC/PA (49:1) vesicles was evaluated by measuring turbidity changes (A_{500}) after spermine addition in the absence (○) or presence of 15 mM-PEG 6000 (●). A portion of vesicles was incubated in the presence of 30 mM-PEG 6000 for 15 min. PEG was then removed by gel filtration on a Sephadex G-50 column. Fractions containing the vesicles were pooled and aggregated by spermine in the absence (□) or presence of 15 mM-PEG 6000 (■).
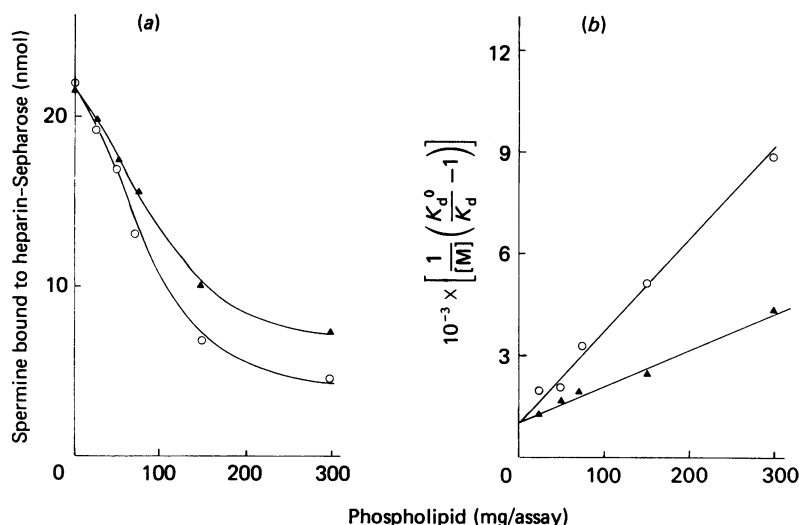
Fig. 5. Effect of PEG 6000 on the interaction of spermine with mixed vesicles

(a) Inhibition of spermine binding to heparin–Sepharose by increasing concentration of PC/PA (9:1) vesicles in the absence (○) or presence of 15 mM-PEG 6000 (▲). (b) Secondary plots of the data in (a). Values of $1/K_d$ were determined by the cation-exchange-resin method described in the Materials and methods section. [M] represents the concentration of phospholipid.

Table 1. Aggregation of 100% PC vesicles induced by PEG 6000 in the absence or presence of 4 mM-spermine

Aggregation was monitored by the turbidity changes at 500 nm in the vesicle suspension, 5 min after PEG 6000 addition.

Treatment [Concn. (mM) of PEG 6000 added]	A_{500}	
	No spermine	4 mM-Spermine
0	0.005	0.006
2.3	0.005	0.006
4.4	0.009	0.012
6.3	0.040	0.034
8.1	0.092	0.103
9.7	0.164	0.184
11	0.248	0.278
13.7	0.387	0.419

$AC_{0.5}$ of spermine, increases the maximal rate and also the final extent of aggregation. The effect on aggregation is dependent on PEG 6000 concentration and it is maximal at about 17.5 mM-PEG 6000. At this PEG 6000 concentration the spermine $AC_{0.5}$ is similar for all the vesicles tested.

The different aggregation of these vesicles observed in the absence of PEG 6000 is therefore most unlikely to be due to a difference in the binding of spermine to the vesicles. This is in agreement with our previous results showing that K_1 (formation constant of the complex formed at a low phospholipid/spermine ratio) is fairly constant for all vesicles tested (Tadolini et al., 1985a).

The aggregation of 100% PC vesicles by PEG 6000 in the absence of spermine suggests that PEG affects aggregation by a mechanism different from enhancing spermine binding to the vesicles. In fact measurements of spermine binding to liposomes have shown that whereas PEG 6000 has no effect on the formation of the complex

occurring at a low acidic phospholipid/spermine ratio (K_1), it exerts a small inhibition of the formation of the complex formed at a high acidic phospholipid/spermine ratio (K_2).

The influence of PEG 6000 on the aggregation of liposomes induced by spermine is not due to irreversible modification of the liposomes themselves. The effect of PEG 6000 on aggregation appears to be mediated by its influence on water structure (Blow et al., 1978; Bangham, 1981). Structuring by PEG of water that would normally be associated with the polar head group of the phospholipids of membranes may induce structural perturbations in phospholipid bilayers. Bilayers of PC might be expected to be particularly susceptible to the presence of high concentrations of PEG, since, with this phospholipid, water molecules of hydration are incorporated into the head-group lattice where they link phosphate groups into ribbons and shield them from the positively charged choline groups (Hauser et al., 1981).

The ability of PEG to bind water affects the hydration force (P_H), which is related to the work required to remove water from hydrophilic surfaces as they approach (Rand, 1981). The structural changes in the water of hydration introduced by PEG apparently also induce alterations in the orientation of the phospholipid head groups, decreasing the surface potential of monolayers of phospholipid (Maggio et al., 1976) and then the electrostatic repulsion (P_{ES}). The correlation between the increased PC content of the vesicles and the increased effect of PEG on aggregation is consistent with the hypothesis of a PEG 6000 effect on the hydration water. The lack of effect of PEG on the aggregation of vesicles containing a high proportion of PA may be due to the direct effect of the large amount of spermine bound to the membrane surface on the hydrated PC head groups, since it is known that the hydrocarbon segments of bound polyamines perturb and release structured water (Loftfield et al., 1981). Hydration force thus appears to have a major influence in determining the spermine-induced aggregation of liposomes. Aggregation

of negatively charged vesicles by spermine is not simply due to the formation of an acidic phospholipid/spermine complex and to the consequent reduction of the electrostatic repulsion between vesicles. Spermine binding is a necessary condition, but the solubility of the complex depends also on its hydration force. It was also shown that the difference observed between the aggregation properties of PS/PE and PS/PC vesicles in the presence of calcium were not due to differences in their ability to bind Ca^{2+} (McLanghlin et al., 1981). The ease of PE-vesicle aggregation compared with the improbability of PC-vesicle aggregation also correlates well with hydration force (Kolber & Haynes, 1979). However, other effects of PEG 6000, due to its interaction with water, may be involved. It is known that the strong interaction of this polymer with water molecules (Molyneux, 1975) decreases the exclusion volumes (Nichol et al., 1981; Tellam et al., 1983), increasing the thermodynamic activity of the species in solution, which in turn influences chemical equilibria involving that species (Minton, 1981).

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