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EFFECTS OF IONS ON VESICLES AND PHOSPHOLIPID DISPERSIONS STUDIED BY POLARIZATION OF FLUORESCENCE

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SUMMARY

1. The oxidation of egg lecithin leads to an increase in the degree of polarization of the fluorescent probe dansylphosphatidylethanolamine. This effect can be avoided by working under nitrogen.

2. The binding of cations on phospholipid dispersions or phospholipid vesicles significantly enhances the degree of polarization of the fluorescent probe dansylphosphatidylethanolamine. This enhancement is attributed to an increase in order of the phospholipid leaflet.

3. The observed effect is proportional to the negative charge of vesicles or to the positive charge of ions ($Na^+ < Ca^{2+} < La^{3+}$).

4. The temperature of the order–disorder transition of the aliphatic chains of the dimyristoylphosphatidylcholine caused by La^{3+} is 3 °C.

5. The kinetic study of the binding of La^{3+} on vesicles indicates that the cations do not diffuse through the bilayer of pure egg lecithin. However, in the case of vesicles charged with phosphatidic acid, the observed evolution can be explained by diffusion of the La^{3+} through the bilayer and binding on the internal layer of the vesicles, or by a rupture of the vesicles.

INTRODUCTION

The molecular structure and the mechanism of action of biological membranes pose challenging problems that require various and powerful experimental approaches in order to solve them. Electron paramagnetic resonance, nuclear magnetic resonance and fluorescence appear promising in this respect. Moreover, the use of well-defined model systems may facilitate the understanding at the submolecular level of the diversity of functions mediated by the organized molecular assemblies in membranes. Lipid dispersions and the closed vesicles which result from prolonged sonication of these dispersions have been extensively studied (see for example ref. 1–4).

The effect of ions on the fluorescence of liposomes or membrane extracts has been studied by Vanderkooi and Martonosi [5], Gomperts et al. [6] and recently

Abbreviation: ANS, 1-anilino-8-naphthalene sulfonate.

by Flanagan and Hesketh [7] using the 1-anilino-8-naphthalene sulfonate (ANS). In all cases, the presence of ions increases the fluorescence intensity of the ANS. The increased fluorescence has been interpreted as an increased ANS binding facilitated by the suppression of electrostatic repulsion between the anionic ANS and the negative phosphate or carboxylate groups of the lipid, as these are neutralised by the binding of cation [7].

Unfortunately, if this kind of study proves the binding of cation, it does not show the possible modification of the bilayer structure which can result from ion binding. It is possible to study such a possibility by using a label covalently bound to one of the membrane components. The dansylphosphatidylethanolamine consists of the dansyl chromophore attached to the phosphatidylethanolamine moiety. It probes the glycerol region of the bilayer, but it is also sensitive to the order–disorder transitions of the phospholipid aliphatic chains [8, 9]. We report here the effects of ions on the degree of polarization of the dansylphosphatidylethanolamine inserted in vesicles or in dispersions of a mixture of egg lecithin and phosphatidic acid.

MATERIALS AND METHODS

Materials

All reagents were A.R. grade and were used without further purification. The water was distilled and deionized. Aluminium oxide was obtained from Rhone Poulenc, Sepharose 4B from Fine Chemical Pharmacia; silicic acid from Mallinckrodt; dipalmitoylethanolamine from Fluka; dansylchloride from Baker. Egg phosphatidylcholine and dansylphosphatidylethanolamine were prepared as previously described [9].

Phosphatidic acid

The phosphatidic acid was prepared from the pure egg phosphatidylcholine. The choline moiety was removed by enzymatic hydrolysis using extracts from savoy cabbage. The preparation of cabbage extracts and the reaction mixture were essentially as described by Davidson and Long [10]. Approx. 1 g of phosphatidylcholine was dispersed in 100 ml of 0.1 M acetate buffer (pH 5.6) and mixed with 70 ml of crude enzyme solution, 20 ml of $CaCl_2$ 1 M and 80 ml of diethyl ether. The mixture was centrifuged at $10\,000 \times g$ for 1 h at 4 °C. A clear upper phase, a central white precipitate and a clear aqueous phase at the bottom were obtained. The white precipitate, consisting mostly of the Ca^{2+} salt of phosphatidic acid, was converted to the acid form by successive washings with 0.1 M HCl in the chloroform–methanol–water system of Folch et al. [11]. The lower phase then obtained was evaporated under reduced pressure. The residue was dissolved in absolute ethanol and evaporated to remove the last traces of water. The resulting compound was dissolved in chloroform and added dropwise to a magnetically stirred solution of 10 g of barium acetate and 20 ml of methanol water (1 : 1, v/v). The precipitate was collected, dried and washed with 0.1 M HCl in the chloroform–methanol Folch system. The lower phase was evaporated and the washing repeated. Then, the last lower phase was washed twice with chloroform–methanol–water and finally evaporated, dried, dissolved in benzene and lyophilized.

Preparation of the labeled vesicles

Fluorescence experiments were carried out on pure phosphatidylcholine vesicles or on negatively charged vesicles containing a variable amount of phosphatidic acid. In all cases, the convenient mixtures and the fluorescent probe (molar ratio 1 : 100) were dissolved together in chloroform which was then removed under reduced pressure. This process was repeated several times before the addition of the aqueous solution (buffer Tris–HCl, pH 7.4, $\mu = 0.05$).

The swelling solutions then obtained, containing about 40 mg of phospholipid per ml, were ultrasonically irradiated to clearness, with an Annemasse F_{50} sonifier, under nitrogen at 2 °C. The phospholipid dispersions were passed through a Sepharose 4B column and the elution diagrams automatically recorded at 280 nm. The elution profiles were typical, with peaks of both the large particles and of the vesicles (diameter 250–300 Å) [4]. The concentrations of solutions determined by phosphorus titration after $HClO_4$ hydrolysis were always about 1 mg of phospholipid per ml.

Fluorescence measurements

The degree of polarization $P = (I_{\parallel} - I_{\perp}/I_{\parallel} + I_{\perp})$ was determined with an apparatus built in the laboratory. The excitation wavelength was 366 nm and the fluorescence was observed above 450 nm. Exciting light was polarized vertically.

The study of turbid solutions such as phospholipid dispersions, is possible by decreasing the effect of the scattered light on the measured degree of polarization with horizontal slits, either in the incident or emitted beams (see ref. 9).

RESULTS

One of the main difficulties in experiments with egg phosphatidylcholine vesicles, is the oxidation effect of air during their preparation or subsequent manipulation. The oxidation of the aliphatic chains leads to a consequent variation in the ultraviolet spectra of the lecithin, new bands appear particularly at 233 and 270–280 nm. The measurement of the absorbance at 233 nm can be used to characterise the oxidized state of lecithin [12].

In Fig. 1, the degree of polarization P of the dansylphosphatidylethanolamine inserted in aqueous dispersions of lecithin is plotted as a function of the absorbance

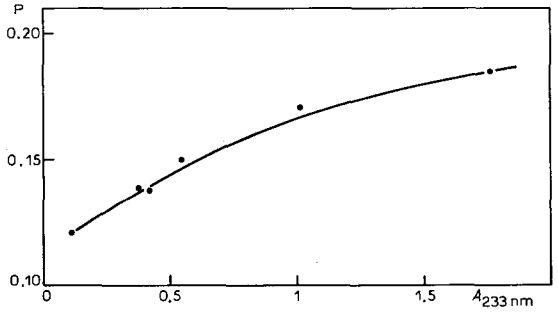

Fig. 1. Degree of polarization of the dansylphosphatidylethanolamine probe inserted in an egg lecithin dispersion as a function of the absorbance of lecithin at 233 nm. This absorbance is measured in methanol at a concentration of 0.5 mg/cm^3.

at 233 nm measured on a solution of lecithin in methanol at a concentration of 0.5 mg/cm^3. The variation of P from 0.120 to 0.190 seems to indicate an important modification of the probe environment.

These results show that the perturbation of the lipid bilayer induced by oxidation is very important. Consequently, it is necessary to avoid this effect in studies of lipid dispersions and also in studies of biological membranes. We have verified, in an egg lecithin sample prepared under nitrogen, that the effect disappears. Without nitrogen, the concentration of air in the sample is roughly constant and consequently the increase in the degree of polarization is proportional to the sample lipid concentration (Fig. 2). But, under nitrogen and with a degassed sample and high lipid concentration, the degree of polarization remains constant. It must be pointed out that solutions containing lipid vesicles can be conserved under nitrogen for 1 week at least without apparent degradation. Consequently, all the following experiments are carried out using this procedure.

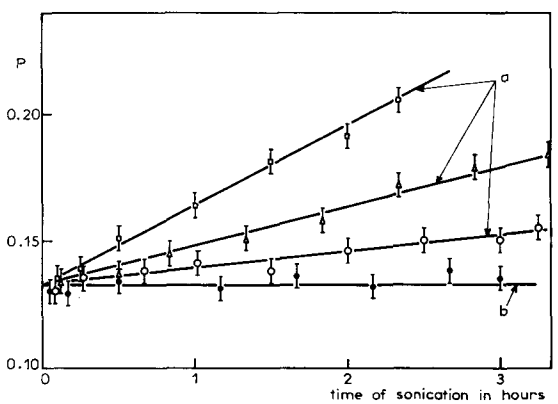

Fig. 2. Degree of polarization of the dansylphosphatidylethanolamine probe inserted in an egg lecithin dispersion as a function of time of sonication. (a) Sonication under air: lecithin concentration 0.3 mg/cm^3 (□–□); 3 mg/cm^3 (△–△); 30 mg/cm^3 (○–○). (b) Sonication under nitrogen: lecithin concentration 30 mg/cm^3 (●–●).

Effect of Na^+, Ca^{2+} and La^{3+} on egg lecithin and phosphatidic acid vesicles

The results reported here were obtained with samples prepared by adding the ions at the desired concentrations to the solution containing the vesicles. All the polarization measurements were done 24 h after the addition of ions. It can be seen on Fig. 3 that the presence of cations increases the degree of polarization of dansylphosphatidylethanolamine in some cases. The fixation of cations in the system leads to a decrease in the mobility of the probe, which certainly increases the packing of the lipid molecules. The results of Fig. 3 also show that two parameters play an important role: the charge of the vesicle and the valency of the cation. For each cation, the effect increases as the phosphatidic acid content of the vesicles increases; that is to say if their negative charge is larger. Similarly, the effect is directly correlated to the charge of the cation. For example, Na^+ has no detectable effect on vesicles charged with

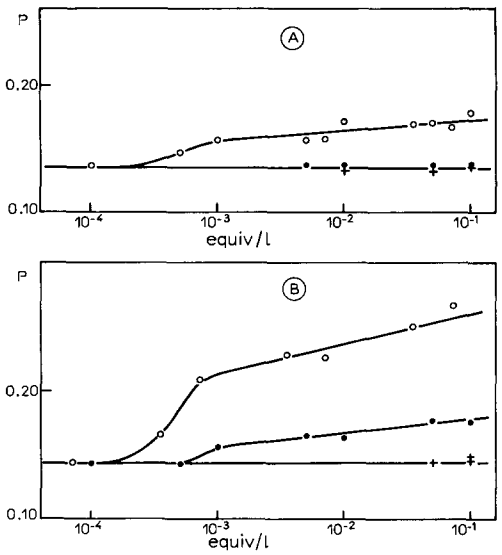

Fig. 3. Degree of polarization of the dansylphosphatidylethanolamine as a function of the cation concentration: (+–+) Na^+; (●–●) Ca^{2+}; (○–○) La^{3+}. (A) Egg lecithin vesicles, lecithin concentration 0.6 mg/cm^3; (B) egg lecithin vesicles charged with phosphatidic acid (1 mole of phosphatidic acid per 2 moles of lecithin) lecithin concentration 0.25 mg/cm^3. pH, 7.4; buffer, Tris–HCl.

33 mole% of phosphatidic acid. On the contrary, La^{3+} enhances the degree of polarization of the probe even on vesicles of neutral egg lecithin.

It is interesting to notice that the ions effects are reversible. At a basic pH, the constant of complexation (EDTA–cation), is very high so the addition of EDTA to the solution leads to a destruction of the phospholipid–cation complex, and consequently the degree of polarization decreases towards its initial value (Fig. 4). If the pH is then changed to an acid value, the fixation of the ions on the phospholipid system is again observed since at acid pH the stability of the EDTA–cation complex is then considerably reduced.

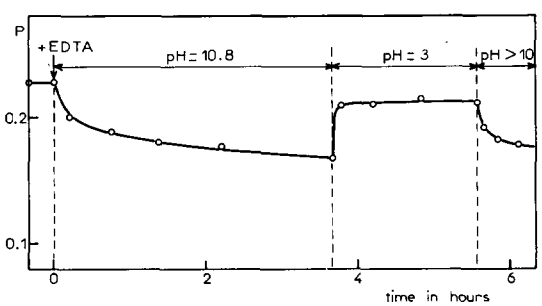

Fig. 4. Kinetic study of the effect of EDTA on the fixation of La^{3+} fixed on vesicles of egg lecithin charged with phosphatidic acid (1 mole of phosphatidic acid per 2 moles of lecithin). The pH is changed by the addition of NaOH or HCl to the solution. La^{3+} concentration, 11.5 mequiv/l; EDTA concentration, 17 mequiv/l.

The observed phenomena could also be explained primarily by a pH effect on the lipid cation interactions. At high pH, the increase of the total negative charge of the system must be followed by an increase of the lipid cation interactions which consequently must lead to an enhancement of the degree of polarization *P*. We observed the reverse effect (see Fig. 4).

Secondly, the pH could have an effect either on the lifetime of the excited state of the probe, or on the mobility of the probe in the system, leading to a variation of *P*. We have verified that *P* varies very little in charged vesicles, when the pH changes from 3 to 11.

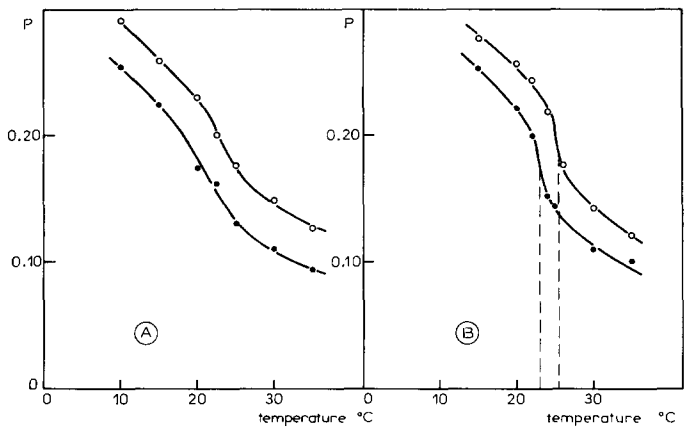

Fig. 5. Effect of La^{3+} on the transition temperature of the dimyristoyllecithin: without La^{3+} (●–●); with La^{3+} (○–○). La^{3+} concentration 16 mequiv/l. (A) vesicles; concentration 0.5 mg/cm^3, (B) lipid aqueous dispersion; concentration 0.1 mg/cm^3.

La^{3+} effect on the transition temperature of dimyristoyllecithin

In Fig. 5, the degree of polarization of the dansylphosphatidylethanolamine inserted in dimyristoyllecithin vesicles or dispersions is plotted as a function of temperature. The melting point of the aliphatic chains is about 23 °C in both systems, but the transition is better defined in a lipid dispersion, as we have pointed out elsewhere [9]. With La^{3+}, the values of the degree of polarization are greater in the whole temperature range and the transition temperature is slightly increased by about 3 °C. This result clearly shows that the interactions of phospholipid cations lead to an increase in order of the lipid leaflet.

The same phenomenon has been observed by NMR with Eu^{3+}, Nd^{3+} and UO_2^{2+} on vesicles of dipalmitoyllecithin [13].

Kinetic study of ion fixation on phospholipid vesicles

a. *Vesicles without net charge.* When La^{3+} is added to a solution containing vesicles of pure egg lecithin, the degree of polarization *P* reaches its maximum value (about 0.168 for $La^{3+} = 5 \cdot 10^{-2}$ equiv/l) in a few minutes and then remains constant even after several days (Fig. 6). When, however, the vesicles are prepared by sonication of a solution containing La^{3+}, the value of *P* is slightly larger (about 0.180) than the preceding one. In this latter case, the cations are fixed either on the

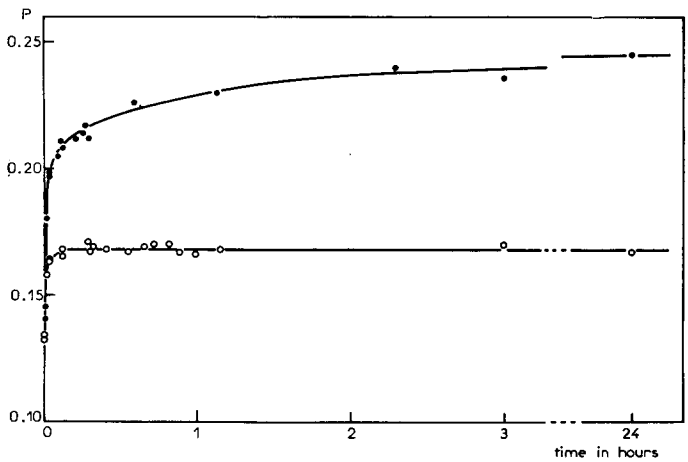

Fig. 6. Kinetic study of the fixation of La^3 on vesicles of egg lecithin (○-○) and vesicles of egg lecithin charged with phosphatidic acid (1 mole of phosphatidic acid per 2 moles of lecithin) (●-●). La^{3+} concentration, 35 mequiv/l.

external or internal layer of the vesicles. This experiment shows that the vesicles of pure egg lecithin are impermeable to La^{3+}. This effect agrees with the result of Levine et al. [13] using NMR spectroscopy.

b. *Vesicles charged with phosphatidic acid.* The addition of La^{3+} to a solution of vesicles negatively charged with phosphatidic acid, leads to a fast increase of P from 0.140 to 0.215 (Fig. 6). Then, P rises slowly for several hours. The maximum value reached is close to that of vesicles prepared by sonication of a solution containing La^{3+} ($P = 0.260$). The same phenomenon is observed with Ca^{2+}, but to a lesser extent because the effect of Ca^{2+} on the degree of polarization is smaller. The slow variation observed is probably due to a progressive fixation of La^{3+} on the inner layer of the vesicles. This fixation can be due to the diffusion of ions through the bilayer, or to a rupture of the vesicles followed by an aggregation.

CONCLUSION

The enhancement, in the presence of cations, of the degree of polarization of the dansylphosphatidylethanolamine inserted in phospholipid dispersion or phospholipid vesicles, can be explained by an increase in the microviscosity of the probe environment, or by a decrease in its excited state lifetime, since $P = f(\eta/\tau)$. In fact, we have always found that the fluorescence intensity, which can be considered as being proportional to the chromophore-excited-state lifetime, increases slightly when the degree of polarization increases. Also, the results obtained in this paper cannot be explained by the variation of the lifetime of the excited state. So, there is an enhancement of the microviscosity of the probe environment which can arise either from a strengthening of the lipid bilayer, or from a change in the normal situation of the bulky headgroup of the probe. It is not possible at this stage to decide between these two hypotheses, and this is one of the main difficulties encountered when using probes. Nevertheless, it is reasonable to think that it is a strengthening

of the lipid bilayer which occurs in the observed phenomena, as two recent and independent studies reach the same conclusion. These are the work of Butler et al. [14] using a spin-labelled probe and the results of Levine et al. [13] obtained by NMR spectroscopy.

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