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EFFECT OF ION CONCENTRATION ON PHOSPHATIDYLETHANOLAMINE DISTRIBUTION IN MIXED VESICLES

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The membrane of mixed phosphatidylcholine-phosphatidylethanolamine vesicles was found to be impermeable to the fluorescent label fluorescamine. Added to the vesicle solution, fluorescamine labels only the phosphatidylethanolamine molecules in the outer layer. The separation of labeled and free phospholipid by thin-layer chromatography permits the determination of the inner to outer phosphatidylethanolamine ratio. This ratio can be independently obtained by multiple sonication and the addition of fluorescamine, which results in a progressive increase of the labeled phosphatidylethanolamine. These two methods give identical results for the phospholipid distribution. The ratio of the outer to the total phosphatidylethanolamine decreases with the increase in the mole fraction of phosphatidylethanolamine, in agreement with the results published by Litman (Litman, B.J. (1973) Biochemistry 12, 2545–2554). It was also found that the lipid distribution is sensitive to changes of the electrolyte concentration in the aqueous phase. At high concentrations the distribution is close to the symmetrical one; a decrease in ionic strength results in preferential localisation of phosphatidylethanolamine molecules in the inner vesicle layer.

Introduction

It is now well established that the protein and phospholipid components of a variety of biomembranes exhibit nonrandom distribution between the two layers of the membrane [1–4]. However, the nature and the origin of the forces which govern this transmembrane asymmetry are not yet clearly understood. Useful information on this subject can be obtained from studies on mixed phospholipid vesicles. In these model systems the asymmetric distribution of the phospholipids is completely determined by the physical interactions of the lipid molecules among themselves and with the surrounding electrolyte solution. Specific factors, such as lipid metabolism, which may prove to be essential in native membranes are not involved at all. Recently, several papers have been published which show the existence of transmembrane asymmetry in mixed vesicles prepared from binary mixtures of the major phospholipid species, e.g., PC-PE, PC-phosphatidylserine, PC-phosphatidylglycerol, PC-phosphatidylinositol [5–9]. The distribution of charged phospholipids in vesicles has also been studied theoretically using the Gouy-Chapman theory of the electric double layer [10–13]. According to the conclusions in Refs 11 and 12, the asymmetric lipid arrangement between the two layers of a vesicle should be strongly dependent not only on the mole fraction of the charged molecules but also on the electrolyte concentration in the aqeous phase.

The influence of the lipid mole ratio on the trans-membrane distribution has been studied ex-

Abbreviations: PC, phosphatidylcholine, PE, phosphatidylethanolamine.

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perimentally [6,7,9], while the role of the electrolyte concentration has been overlooked in the works published up to now. In this paper, the considerable influence of the ionic concentration on the lipid distribution in PC-PE vesicles is demonstrated. For the measurements of the outer to inner PE ratio a new procedure has been developed using the fluorescent label fluorescamine. Added to the outer aqueous phase, fluorescamine binds only to the PE molecules in the outer layer. The subsequent separation by TLC of labeled and free PE permits the determination of the outer to total PE ratio.

Materials and Methods

Vesicle preparatoin. Egg L-α-phosphatidylcholine (Koch-Light) and DL-β,γ-dipalmitoyl-α-phosphatidylethanolamine (Fluka) were used. Both phospholipids were chromatographically pure. Vesicles were prepared in phosphate buffer (pH 8.0) by sonication for 45 min under nitrogen. During the sonication the sample was maintained at a temperature higher than the PE transition temperature of 63°C [14]. The phospholipid concentration was 0.1 mg/ml. The sample was then centrifuged at $100\,000 \times g$ for 30 min. The ionic strength of the buffer solution was varied in the range 0.065–0.0065.

Electron microscopy. The vesicles were negatively stained with 1% uranyl acetate solution. The grids were examined with a JEOL-100B electron microscope. The examination revealed that more than 95% of the vesicles were of diameter ranging from 25 to 35 nm. Occasionally, vesicles of diameter up to 75 nm were encountered. From the general size range and substructure we conclude that the vesicles are unilamellar.

Labeling of PE. The fluorescent label fluorescamine, which binds covalently to primary aminogroups was synthesized in the Special Reagents Laboratory, Center of Chemistry, Bulgarian Academy of Sciences, Sofia. It was added to the vesicle suspension from a 2% acetone solution. The excess fluorescamine rapidly hydrolyzes to nonreacting and nonfluorescent products [15]. The fluorescent measurements were carried out on a Jobin Yvon-D3 spectrofluorimeter. The excitation wavelength of fluorescamine was 390 nm and its emission wavelength was at 470 nm. The determination of labeled and free PE was carried out by separation by TLC and the subsequent determination of lipid amounts in the different spots by standard methods [16]. The spot of labeled PE was easily detected also by its fluorescence after illumination of the plate with an ultraviolet lamp. No labeling was detected by TLC when fluorescamine was added to PC vesicles.

Results and Discussion

The application of the present procedure for the investigation of the lipid distribution was possible due to the fact that labeled and free PE could be separated by TLC. Their R_F values in the phase chloroform/methanol/water (65 : 25 : 4, v/v) were 0.325 for the labeled PE and 0.385 for the free PE. In order to use this circumstance for the determination of the PE distribution between the inner and outer layer of the vesicles, it was necessary to clarify whether the following two conditions were fulfilled:

(1) The reaction between fluorescamine and PE amino groups reaches completion under these experimental conditions so that the whole amount of available PE is labeled.

(2) Fluorescamine does not permeate across the vesicle membrane and does not label PE molecules located in the inner monolayer.

Since fluorescamine reacts with nonprotonated amino groups only, it is important to know what is the state of PE amino groups at pH 8.0. The published data concerning the apparent pK of protonation of the PE amino group are rather inconsistent [17,18]. We shall use the data of Bangham [17] obtained by free microelectrophoresis. The electrophoretic measurements demonstrated the existence of negative electrokinetic potential in PE suspensions over a wide range of pH values above 3, so that at pH 8.0 the amino groups of PE should be to some extent nonprotonated. This is also confirmed by our qualitative observations showing that particles of crude PE suspensions placed in an electrophoretic chamber move toward the anode in the stationary planes of the chamber at pH 8.0. On the basis of these results, one may expect that the reaction of fluorescamine with PE amino groups would reach

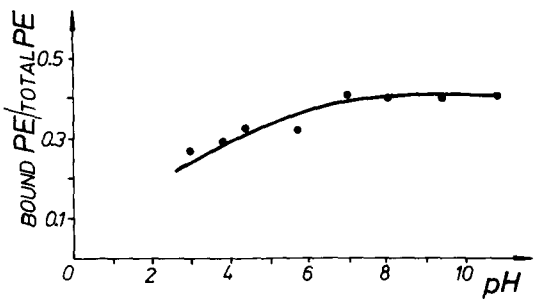

Fig. 1. The dependence of the ratio of labeled PE to total PE on pH of the solution (ionic strength 0.026).

completion. In order to check this point, we measured the pH-dependence of the binding of fluorescamine with PE (Fig. 1). The figure shows that the binding is constant in a wide range of pH and begins to decrease at pH below 6, where the reaction becomes controlled by pH. So one may think that at pH 8.0 fluorescamine completely labels the available PE. Another and more definite proof of this conclusion is given by the fact that after a multiple resonication of the sample and the addition of fluorescamine to the outer aqueous volume after each sonication, a complete labeling of PE is reached which is established chromatographically (Fig. 3).

The second very important question is whether fluorescamine permeates through the membrane of the vesicles under our experimental conditions. In order to clarify this question the following experimental results can be used. First, the dependence of the ratio of labeled to free PE on the added

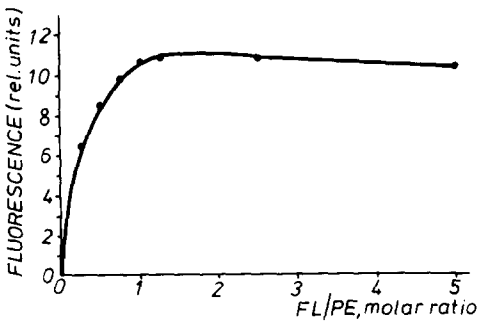

Fig. 2. The dependence of the fluorescence on the mole ratio of fluorescamine to the total vesicle PE (pH 8.0, ionic strength 0.026).

fluorescamine reaches saturation at a certain amount of fluorescamine (Fig. 2). Further addition of fluorescamine does not decrease the free PE, in accordance with the assumption that the free PE is the inner layer PE and is not accessible for fluorescamine. Second, if vesicles labeled by fluorescamine up to saturation are resonicated and then fluorescamine is added again, the fluorescence rises and TLC also shows an increase in the labeled PE. This, too, is easily explained by the assumption that the inner layer PE is inaccessible for fluorescamine but during the resonication a part of it turns out to the outer layer and later binds the additional portion of fluorescamine. In this way, a complete labeling of PE is reached by multiple resonication and addition of fluorescamine (Fig. 3). As should be expected, the amount of bound PE increases after each cycle in geometric progression. This is a natural result showing that the free PE amount becoming accessible to labeling by fluorescamine after given sonication is proportional to the amount of unlabeled inner layer PE left after the previous cycle of sonication and addition of fluorescamine. On the basis of these considerations, we conclude that the fluorescamine molecules do not penetrate through the vesicle membrane and label only the PE molecules in the outer layer, so that the separation of bound and free PE by TLC corresponds to a separation of outer and inner surface PE, respectively. Thus, the ratio of outer to total PE in the vesicles is determined.

The procedure of resonication of the suspension and addition of fluorescamine after each sonication is an independent method for the determination of the outer to total PE ratio. This procedure is repeated until the fluorescence of the solution reaches a plateau and TLC shows complete labeling of PE. As explained above, the number of labeled PE molecules, N^*, must increase in a geometric progression during the multiple sonicatoin:

$$N^*/N = 1 - (N_{in}/N)^n$$

Here N is the mean total number of PE molecules in a vesicle, N_{in} is the mean number of PE molecules in the inner layer, n is the serial number of sonication. The increase in bound PE registered by TLC and fluorescence increase during this proce-

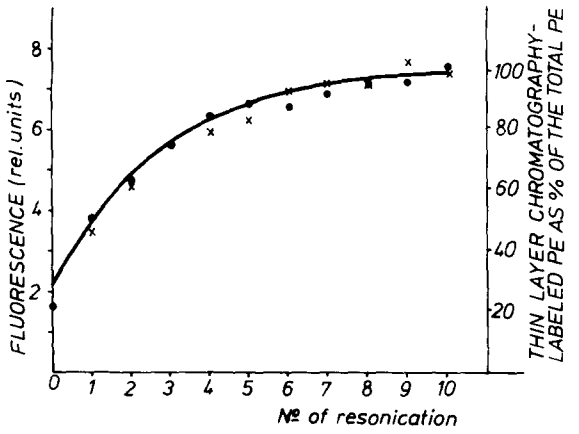

Fig. 3. The increase of the labeled PE in PE-PC (2:3) vesicles (ionic strength 0.026) during multiple sonication and subsequent addition of fluorescamine; ×, fluorescence intensity of the sample; ●, amount of labeled PE as a percentage of the total PE, determined by TLC; ———, the best fitting theoretical curve, calculated using the formula for geometric progression.

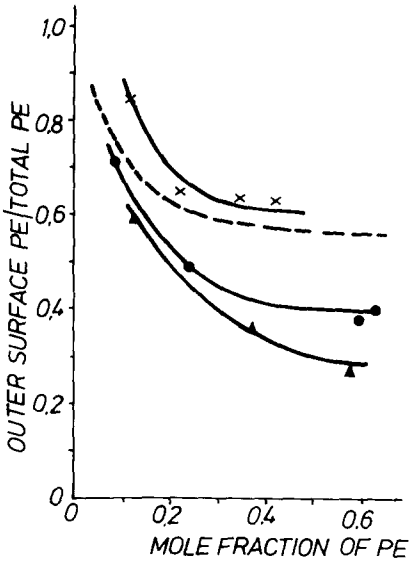

Fig. 4. The dependence of the ratio of outer to total PE on the mole fraction of PE in mixed PC-PE vesicles for different ionic strength of buffer solution: ▲, 0.0065; ●, 0.026; ×, 0.065; ------, Litman [6]; 100 mM KCl (pH 6.0).

dure are shown in Fig. 3. By suitable choice of N_{in}/N in the above equation, the experimental points can be very well fitted by a theoretical curve. In this way the ratio of the outer to total PE could be obtained independently. The values of the outer to total PE ratio (PE_{out}/PE_{tot}) obtained by this method practically coincide with the values obtained directly by TLC after a single sonication and addition of fluorescamine. For example, $PE_{out}/PE_{tot} = 0.355$ for the case shown in Fig. 3, which is quite close to the corresponding curve in Fig. 4 (mole fraction of PE, 0.4 and ionic strength, 0.026).

After the establishment of the procedure for measuring the outer to total PE ratio in mixed PC-PE vesicles, our next purpose was to investigate the effects of the PE/PC mole ratio and electrolyte concentration of the aqueous phase on the lipid distribution. The obtained results are summarized in Figs. 4 and 5. The dependence of the lipid distribution on the mole fraction of PE is similar for different ionic concentrations (Fig. 4). A substantial decrease in the PE_{out}/PE_{tot} ratio is observed as the mole fraction of PE relative to PC is increased. The same dependence for PC-PE vesicles has been obtained earlier by Litman by labeling the outer PE with trinitrobenzensulfonate [6]. The question whether this dependence reveals asymmetry of the PC-PE vesicles has been later discussed in [19]. It has been argued that if the increase in the vesicle size is taken into account, then, even for a symmetric distribution of both

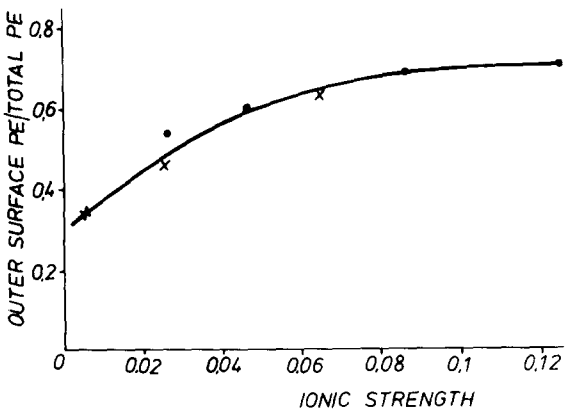

Fig. 5. The effect of the electrolyte concentration on the PE distribution in PE-PC (2:3) vesicles at pH 8.0; ×, ionic strength of buffer solutions; ●, ionic concentrations obtained by addition prior to sonication of KCl to a buffer solution of initial ionic strength 0.0065.

phospholipids, a decrease of PE_{out}/PE_{tot} is to be expected. The values of the outer to inner PE ratio have been found to be quite close to the calculated ratio of the outer to inner vesicle surfaces and therefore it has been concluded that any asymmetry, if it exists at all, is small. It is important to note, however, that this conclusion seems to be valid only in the range of high electrolyte concentrations – the data of Litman [6] and also the highest curve in Fig. 4. Quite different is the situation at lower electrolyte concentrations presented by the two lowest curves in Fig. 4. In this range the ratio PE_{out}/PE_{tot} becomes smaller than 0.5 for sufficiently great mole fractions of PE. As the inner vesicle surface cannot exceed the outer surface in any way, this means that a certain asymmetry exists characterized by a preferential localization of PE molecules in the inner layer. The increase of the electrolyte concentration either by the addition of KCl before sonication or by using more concentrated buffer solutions results in an increase of the outer PE with a marked tendency to saturation (Fig. 5) presumably reaching the symmetric state discussed above. The results of Litman do not contradict this electrolyte effect. Ideally, Litman's curve in Fig. 4 should coincide with or be placed slightly above the highest of our curves because of the higher electrolyte concentration in his samples (see legend to Fig. 4). It is obvious, however, that such an ideal agreement cannot be required and the slight discrepancy between the two sets of experimental points can be attributed to many factors – a different method of investigation, different phospholipid sources and so on.

An entirely different picture was obtained when the electrolyte (120 mM KCl) was added to the suspension after the preparation of the vesicles. The initial ionic strength of the suspension was 0.0065. Fluorescamine was added at several time intervals from 0 to 24 h after the addition of KCl. The sample was kept at room temperature during this time. In all the experiments the values of PE_{out}/PE_{tot} were found to be equal to the value corresponding to the initial ionic strength of 0.0065. This is an indication that for at least 24 h no structural rearrangements, including phospholipid flip-flop, occur after the addition of KCl to the outside of the vesicles. These experiments also show that the reaction between fluorescamine and PE at pH 8.0 is not affected by the electrolyte concentration.

At present we are not able to propose a satisfactory theoretical explanation of the observed electrolyte effect. If we take into account that the PE molecules are partially negatively charged, then electrical double layers should exist on both surfaces of the vesicles. According to the Gouy-Chapman model, their thickness is of the Debye screening length order. It may happen that at low electrolyte concentrations the Debye length becomes greater than the inner radius of the vesicle and the inner electrical double layer is only partially developed. In such a case, it has been theoretically predicted in Ref. 11 that the equilibrium distribution of the surface charges is asymmetric and depends on the electrolyte concentration. It is worth noting, however, that this situation was not realized in our experiments. The electrolyte concentrations used (0.0065–0.1265 M) correspond to Debye screening lengths in the range from 38 Å to 8 Å. These values are smaller than the inner radius of the vesicles, so that the electric double layer inside the vesicles should be fully developed. That is why we think that the observed effect could be explained by a detailed examination of the packing properties of the phospholipid polar groups. It is evident that the considerations about the packing properties of the phospholipids should take into account not only some constant geometric dimensions of the molecules, but also the electrostatic interactions of their polar head groups. It seems possible that the head-group conformation is also determined, to some extent, by internal and intermolecular electrostatic forces and it can be influenced by changes in the electrolyte concentration by means of a screening effect or ionic adsorption, so that an electrolyte effect on the packing properties of polar groups may exist.

Acknowledgements

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