Determination of the size of the packing defects in dimyristoylphosphatidylcholine bilayers, present at the phase transition temperature

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Multilamellar liposomes of dimyristoylphosphatidylcholine, containing 4 mol% egg phosphatidic acid show at the phase transition temperature an increased permeability for non-electrolytes of M_r values up to 900. This indicates that the packing defects occurring at the liquid crystalline/gel state phase boundary have a similar pore diameter (15–18 A) as the packing defects present in glycophorin–dioleoylphosphatidylcholine vesicles. This suggests that packing defects at the protein–lipid interphase are the major permeation pathway of the glycophorin–dioleoylphosphatidylcholine vesicles.

Dimyristoylphosphatidylcholine *Vesicle* *Bilayer permeability* *Phase transition temperature* *Pore size*

1. INTRODUCTION

At the transition temperature at which phospholipids undergo transformation from the liquid crystalline phase to the gel phase, the coexistence of phospholipids in two physical states disturbs the molecular arrangement of the bilayer. In model systems, consisting of pure phosphatidylcholine, such disturbances at the phase transition temperature result in an increased permeability for molecules like TEMPO [1], carboxyfluorescein [2] and cations like Na^+ and K^+ [3], an increased transbilayer movement of the phospholipids [4] and a maximal activity of pancreatic phospholipase A_2 [5].

Recently it has been shown that incorporation of integral membrane proteins of the human erythrocyte membrane, glycophorin and band 3, into a phosphatidylcholine bilayer also causes an increased bilayer permeability, at temperatures above [6–9] and below [10] the phase transition temperature of the phosphatidylcholine. In addition, it has been shown that in the protein containing vesicles, just like in the phosphatidylcholine vesicles at the phase transition temperature, the transbilayer movement of phospholipids is increased [11–13] and that the phospholipids have an increased susceptibility for phospholipase A_2 [14]. Because of these similarities, it has been hypothesized that the increased permeability in the protein–lipid vesicles is caused by packing defects at the protein–lipid interface. In addition, an alternative permeation pathway may be channels, present in protein aggregates [7,9].

Size determination of the packing defects (pores) present in glycophorin–DOPC vesicles, using permeability measurements with non-electrolytes of different size, showed that the pore diameter is 15–18 Å [9], allowing molecules up to M_r 900 to pass the bilayer. In [9], it was considered that the magnitude of the packing defects at the protein–lipid interface, may be comparable with the size of packing defects occurring between lipids

Abbreviations: DOPC, dioleoylphosphatidylcholine; TEMPO, 2,2,6,6-tetramethylpiperidine *N*-oxyl

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at the liquid crystalline/gel state phase boundary.

From osmotic experiments [5] it has been shown that these bilayer packing defects occurring at the phase transition temperature of DMPC results in a large permeability increase for Na^+ and K^+, but in a much smaller increase for Li^+ [5]. Assuming that the Li^+ ion is smaller than a non-electrolyte with an M_r of 900, it was concluded that the packing defects at the protein–lipid interface only contribute to a minor extent to the protein induced, increased bilayer permeability [9]. Therefore, it seemed that the channels present in protein aggregates constitute the major permeation pathway.

However, it should be realized that comparison of the permeability for an electrolyte and non-electrolyte, with respect to their M_r, is complicated by the presence of the charge of the electrolyte, which causes a watermantle with unknown hydrodynamic radius. For this reason, in order to allow a good comparison, the size of the packing defects occurring at the phase transition of dimyristoylphosphatidylcholine was determined, using the same permeability measurements with non-electrolytes of different size, as for the glycophorin–DOPC vesicles.

2. EXPERIMENTAL

1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and egg phosphatidic acid (egg PA) were prepared as in [15]. Chemicals were purchased from the following commercial sources: [*carboxyl*-^{14}C]dextran (M_r 70000, 0.99 mCi/g); [G-^{3}H]-inulin (175.5 mCi/g); [1,2-^{3}H]polyethyleneglycol 4000 (PEG 4000, 1.6 mCi/g); [1,2-^{3}H]polyethyleneglycol 900 (PEG 900, 4.5 mCi/g); L-[1(n)-^{3}H]-glucose (10.7 Ci/nmol) and [G-^{3}H]raffinose (7.8 Ci/nmol) from New England Nuclear; [6,6′(n)-^{3}H]sucrose (9.8 Ci/nmol) from Amersham; Sephadex G-75 and G-150 from Pharmacia.

2.1. *Efflux assay*

Efflux of ^{3}H-labelled solutes of different M_r relative to ^{14}C-labelled impermeable dextran, at the indicated temperature, was determined as in [7], as described in detail in [9].

Multilayered vesicles were prepared by hydration at 30°C of a mixture of 4.8 μmol DMPC and 0.2 μmol egg PA, previously dried under vacuum from chloroform, with 0.5 ml of 100 mM NaCl, 0.2 mM EDTA, 0.2 mM NaN_3, 1 mM solute ($M_r < 900$) or 0.1% (g/v) solute ($M_r > 900$), 0.1% (g/v) dextran, 10 mM Tris–HCl buffer (pH 7.4) which additionally contains 2 μCi ^{3}H-labelled solute/ml and 0.25 μCi [^{14}C]dextran/ml. Centrifugation (20 min, 37000 × *g*) was performed at the assay temperature.

The efflux assay gives an estimate of the amount of solute, still present in the vesicles, after a wash procedure of the vesicles for 4 h at the indicated temperature, relative to the trap of the large non-permeable solute dextran [7,9].

2.2. *Influx assay*

The influx of radiolabelled solutes under equilibrium conditions was measured using a modification of the method in [16], as described in detail in [9] and [17].

In this method the vesicles are prepared at 30°C in the same buffer as used in the efflux assay, but without radiolabelled dextran and permeant molecules. After equilibration at 30°C, labelled PEG 900 or PEG 4000 (60×10^6 dpm/ml) is added and the influx is monitored by taking samples at appropriate time intervals followed by rapid separation of vesicles and extra vesicular medium by gel filtration at 0°C. The influx curve is plotted as the amount of labelled solute, expressed in μl/μmol phospholipid *vs* time. The amount of labelled solute, present at time *t* in the vesicles is derived from the amount of radioactivity and the amount of phospholipid present in the eluent of the gel filtration column, related to the amount of radiolabelled permeant per volume unit of the vesicle suspension.

3. RESULTS AND DISCUSSION

Studying the permeability behaviour of DMPC liposomes, containing 4 mol% egg PA, the trap efficiencies for non-electrolytes of various molecular sizes, relative to the trap efficiency for large impermeable dextran molecules, have been determined with the efflux assay. Table 1 shows the results of such measurements at temperatures below (0°C), above (37°C), and at the phase transition temperature (23°C) [18]. At the phase transition temperature, the relative solute trap for molecules with an M_r up to 900 is zero. This indicates a loss

Table 1

Permeant	0°C	23°C	37°C
Glucose	54	0	30
Sucrose	87	0	53
Raffinose	110	0	83
PEG 900	75	0	12
PEG 4000	100	85	85
Inulin	238	78	237
Dextran	100	100	100

Relative solute/dextran traps (% values) of DMPC multilamellar liposomes, as determined with the efflux assay (as outlined in section 2) at the indicated temperature

of all the originally enclosed labelled permeant during the washing procedure of the efflux assay, and means that the DMPC liposomes at the phase transition temperature are extremely leaky towards non-electrolytes with an M_r up to 900. Upon further increase of the M_r, up to 4000 (PEG 4000) and 5200 (inulin), a decrease in permeability occurs, since the loss of the enclosed solites is now only, respectively, 15 and 22%, relative to the dextran trap.

Table 1 shows clearly that, at the phase transition temperature of 23°C, an increased permeability is observed for all the solutes, as compared to permeabilities at 0 and at 37°C. This is in agreement with previous studies, which showed an increased permeability at the phase transition temperature for Na^+ and K^+ [3], carboxyfluorescein [2] and TEMPO [1].

The vesicular volume as measured with [^{14}C]dextran, was found to be 1.5 l/mol phospholipid in all cases. This indicates that the temperature and presence of the various permeants tested has no influence on the average vesicular structure (see section 2.1).

The permeant, with the highest M_r, inulin, shows a trap value at 0 and at 37°C, which is 2.4 times higher than the dextran trap (table 1). This might be the consequence of a lack of space between the lamellae of the multilamellar liposomes, which results in a low trap efficiency of the very large dextran molecule as compared to the smaller inulin molecule. Specific adsorption of inulin to the vesicles is unlikely, since this would result in a relative inulin trap value at 23°C, comparable to that observed at 0 and at 37°C (see table 1). If the relative solute traps are related to the inulin trap at 0°C or at 37°C, instead of being related to the dextran trap, the trap values will decrease. However, since this decrease for every solute is the same, no change in the observed tendency of solute radius *vs* permeability degree will be observed.

To substantiate further the efflux assay results kinetic influx measurements were performed at the phase transition temperature. In fig.1 the influx of PEG 900 and PEG 4000 at 23°C is shown. In agreement with the efflux measurements (see table 1, middle column), PEG 900 enters the vesicles very fast reaching a level within 3 min of about 1 μl/μmol phospholipid. In addition, PEG 4000 shows a much slower influx rate. After 300 min, the level of 1 μl/μmol phospholipid is still not reached. Since these influxes of solutes are the average of permeation through more than one bilayer, it is hard to describe the kinetic parameters of the influx processes. After 18 h at 37°C both influx curves have reached the value of 1.4 μl/μmol phospholipid, which corresponds to the maximal relative solute traps at 0°C (see table 1, left column) which have the same value as the dextran trap (see section 2).

The comparison of the relative solute/dextran traps of the DMPC multilamellar liposomes as

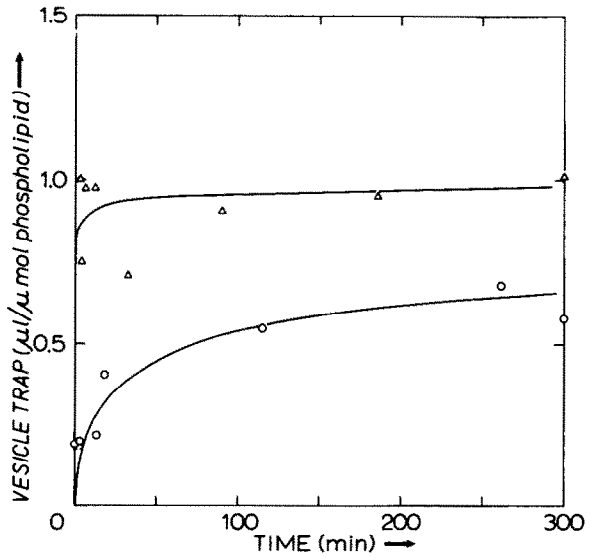

Fig.1. Influx assays of PEG 900 (Δ—Δ) and PEG 4000 (○—○) at 23°C. Vesicles were made and assays were performed as outlined in section 2.

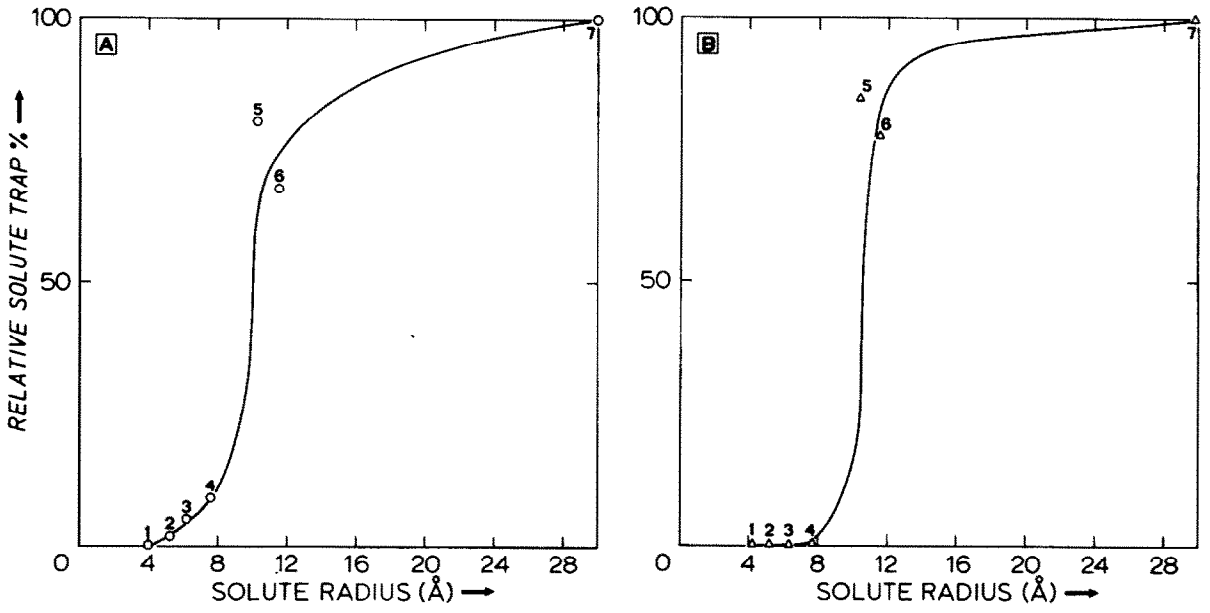

Fig.2. Relative solute/dextran traps of glycophorin–DOPC vesicles (A) at 0°C (taken from [9]) and DMPC liposomes at 23°C (B) as a function of solute radius. Solute: (1) glucose, (2) sucrose, (3) raffinose, (4) PEG 900, (5) PEG 4000, (6) inulin, (7) dextran. The molecular radius of the solutes are derived from [9] and references therein.

function of the molecular radius, with the relative solute/dextran trap of the unilamellar glycophorin–DOPC vesicles is depicted in fig.2. It is clear from this figure that the size of the packing defects in the glycophorin–DOPC bilayer (fig.2A) (pores), with a diameter of 15–18 Å [9] is comparable with the size of the packing defects (pores) occurring in the DMPC bilayer at the phase transition (fig.2B).

Since the magnitude of the packing defects occurring at the liquid crystalline/gel state phase boundary is unexpectedly high, having the same size as the packing defects in the glycophorin–phosphatidylcholine bilayer, the molecular origin of these defects has to be reevaluated. Previously it was stated that these packing defects are most likely channels present in the protein aggregates, while a possible participation of packing defects at the protein–lipid phase boundary could not be excluded [9]. However, taking into account the present results it is likely that packing defects at the protein–lipid interface are a permeation pathway of major importance as compared to the channels, probably present in protein aggregates.

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