Biochimica et Biophysica Acta, 433 (1976) 1–12

BBA 77257

EFFECT OF THE GEL TO LIQUID CRYSTALLINE PHASE TRANSITION ON THE OSMOTIC BEHAVIOUR OF PHOSPHATIDYLCHOLINE LIPOSOMES

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(Received October 8th, 1975)

SUMMARY

Aspects of osmotic properties of liposomes, prepared from synthetic lecithin, above, at and below the gel to liquid crystalline phase transition temperature are described. The experiments show that liposomal membranes with their lipids in the gel state are still permeable to water. The rate of water permeation changes drastically on passing the transition temperature. The water permeation has activation energies of 9.5 ± 1.28 and 26.4 ± 0.9 kcal/mol above and below the transition temperature, respectively, indicating that the diffusion processes take place by different mechanisms.

With respect to the barrier properties of the liposomes in the vicinity of the transition temperature, the following conclusions can be made.

(1) Studying the osmotic shrinkage of liposomes at a fixed temperature near the transition point, the experiments indicate that dimyristoyl phosphatidylcholine membranes are highly permeable to glucose under these conditions, where liquid and solid domains co-exist. Under the same conditions the osmotic experiments did not indicate a strong increase in glucose permeability of dipalmitoyl phosphatidylcholine membranes as compared to the situation above and below the transition temperature.

(2) On the other hand, perturbations of the phase equilibrium by temperature varations resulted in a marked increase of the glucose permeation through dipalmitoyl phosphatidylcholine bilayers. Once a new phase equilibrium of liquid and solid regions is established the permeation rate of glucose is much less.

INTRODUCTION

In studies on the effect of lipid phase transitions on the barrier properties of liposomes most attention was drawn to the permeability behaviour in the vicinity of the gel to liquid crystalline phase transition temperature. Near this temperature, usually abbreviated as T_c, the barrier characteristics of lipid membranes are profoundly changed, resulting in an enhanced release of trapped solutes from the liposomes [1–5]. Recently we demonstrated that the enhanced permeability near T_c exhibits strong selectivity with respect to the molecular size of the permeating compound [5]. Furthermore, it appeared that the extent of this permeability increase is

strongly dependent on the length of the paraffin chain of the saturated lecithins [5].

Information concerning the permeability properties of liposomal membranes with their lipids in the gel state is limited. Studying the osmotic swelling of liposomes in isotonic solutions of permeable non-electrolytes, striking changes have been noticed in the swelling rate on lowering the temperature below T_c [6, 7]. For the explanation of such data it is very important to know more about the general osmotic behaviour of the liposomes with their lipids in different physical conditions. In this paper experiments are described dealing with the osmotic shrinkage behaviour of liposomes prepared from synthetic lecithins.

MATERIALS AND METHODS

The phosphatidylcholines with defined fatty acid composition were synthesized as described before [8]. Phosphatidylglycerols were prepared from lecithins as described elsewhere [9]. Purity of the phospholipids was checked by thin-layer chromatography. Fatty acid impurities were less than 1 % as shown by gas chromatography. Egg phosphatidic acid was prepared from egg lecithin by phospholipase D degradation [10]. Sodium dicetylphosphate (Sigma) was recrystallized from ethoxyethanol. All other reagents were commercial and of analytical reagent grade.

Multilayered liposomes, normally containing 4 mol % of egg phosphatidic acid, were prepared at temperatures above the T_c of the lecithins in 20 mM glucose/10 mM Tris · HCl (pH 7.5) as previously described [6]. For the osmotic shrinkage measurements the liposomes were first diluted in the medium in which they were prepared and then transferred into a thermostatted cuvette, which was vigorously stirred. After temperature equilibration an osmotic shock was given by rapidly injecting a small volume of a concentrated solute preincubated at the same temperature. Changes in the turbidity were determined with a spectrophotometer (Vitatron, MPS type) at 662 or 450 nm. These data were used to calculate the following parameters (Fig. 1): (1) A_{t_0}, used as a parameter for the temperature dependence of the

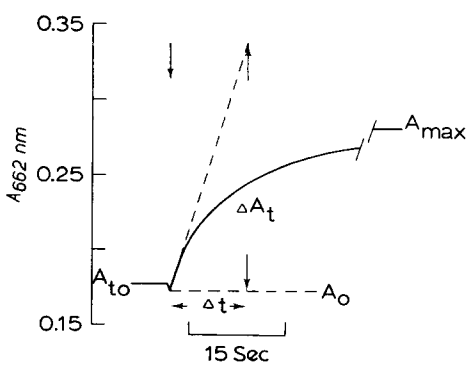

Fig. 1. Recorder tracing showing the increase in turbidity of a liposome dispersion following the addition of concentrated glucose. At the moment indicated by the first arrow 0.4 ml 1.0 M glucose/20 mM Tris · HCl (pH 7.5) was rapidly injected into 9.6 ml of a liposome suspension (0.3 μmol lipid/ml in 20 mM glucose/10 mM Tris · HCl, pH 7.5). For further explanation see Materials and Methods.

turbidity of the suspension; (2) $d(1/A)/dt$ %, the initial shrinkage velocity, given by $(\Delta At/\Delta t) \cdot (100/A_0)$; and (3) $\Delta(1/A)\% = (1/A_0 - 1/A_{max})\ A_0 \cdot 100$, being a measure for the extent of shrinkage [6].

Measurement of ^3H_2O permeation through liposomal membranes below the transition temperature: liposomes of dipalmitoyl phosphatidylcholine with 4 mol % of egg phosphatidic acid (50 μmol lipid) were prepared at 55 °C in 1.0 ml 100 mM glucose, containing 1.0 μCi ^3H_2O and 0.2 μCi [^{14}C]glucose (both obtained from The Radiochemical Centre, Amersham, England). After cooling the suspension to room temperature, 0.75 ml was transferred into a dialysis bag, mounted on a small funnel at its open side and dialyzed against about 1000 ml of nonradioactive 100 mM glucose. During the dialysis procedure, which was carried out at room temperature, samples of 25 μl of suspension were taken via the funnel for counting radioactivity. In a control experiment the ^3H_2O and [^{14}C]glucose were added after preparing the liposomes at 55 °C and cooling them to room temperature. Radioactivity (^{3}H and ^{14}C) was measured in a Packard Tri-Carb liquid scintillation spectrophotometer and was corrected to 100 % relative efficiency using an external standard.

The release of glucose from liposomes was measured continuously with a double beam spectrophotometer (Varian Techtron, model 635), equipped with thermostatted cuvette holders, according to the procedure originally developed by Kinsky et al. [11]. After equilibrating the complete test system at the desired temperature the experiment was started by adding a small volume of the dialyzed liposome suspension, which was stored at 0 °C, to both the test cell and the reference cuvette. Hexokinase, glucose-6-phosphate dehydrogenase, ATP and $NADP^+$ were omitted from the reference cell. The temperature of the medium in the cuvettes was changed rapidly by using two waterbaths, each at a desired temperature, that could be separately connected to the cuvette holders. Hexokinase (EC 2.7.1.1), glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and $NADP^+$ were from Boehringer, Mannheim (Germany); ATP was from Sigma Chemical Co., St. Louis (U.S.A.).

RESULTS AND DISCUSSION

Studying the osmotic properties of egg lecithin liposomes, Bangham et al. [12] showed that liposomes behave as perfect osmometers, obeying the Boyle-van 't Hoff law. They showed that the reciprocal of absorbance at 450 nm of osmotically treated liposome dispersions, at equilibrium, is proportional to the pellet volumes and the reciprocal of the concentration of the impermeable solute in which the liposomes are suspended. Assuming that these relationships are generally applicable to liposome dispersions, the existence of a proportionality between $1/A_{450\ nm}$ and 1/osmolarity can be taken as an indication for an ideal osmotic behaviour of liposomes. Fig. 2 shows the results of typical experiments with dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine liposomes. Straight lines were obtained by plotting $1/A_{450\ nm}$ against 1/[glucose] for both liposome preparations above and below T_c, and with dipalmitoyl phosphatidylcholine liposomes also in the vicinity of T_c.

For osmometers with membranes completely impermeable to the solute (e.g. glucose) a proportionality exists between the volume change and the difference in osmotic pressure of the outer and inner solution according to the following equation [13]:

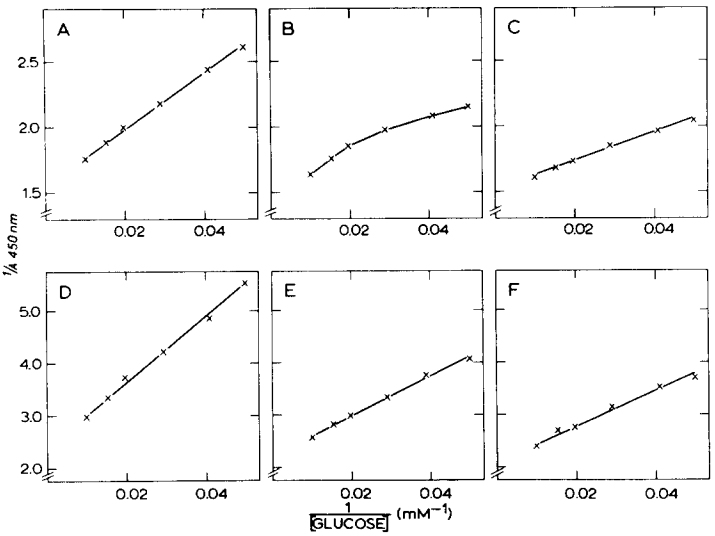

Fig. 2. Relationship between the reciprocal of the absorbance at 450 nm of liposome suspensions and the reciprocal of the glucose concentration in which they are osmotically treated until equilibrium. Dimyristoyl phosphatidylcholine/egg phosphatidic acid = 96 : 4 liposomes, prepared in 20 mM glucose, were osmotically shrunken in hypertonic glucose at 39.6 °C for 0.5 h (A), at 23.1 °C for 1h (B) and 10.5 °C for 18 h (C). Dipalmitoyl phosphatidylcholine/egg phosphatidic acid = 96 : 4 liposomes were treated in an identical way at 49.8 °C for 0.5 h (D), at 39.6 °C for 1 h(E) and at 22.0 °C for 18 h (F). In all experiments the final lipid concentration was 0.30 μmol · ml^{-1}.

$$dV/dt = K_w SRT(c_i^0 - c_i^i) \quad (1)$$

in which dV/dt is the volume change with time, K_w is the permeability constant for water, S is the area occupied by the membrane, R is the gas constant, T is the absolute temperature and c_i^0 and c_i^i are the concentrations of the impermeable solutes outside and inside the liposomes, respectively. Since the relative initial change in the absorbance is directly proportional to the initial volume change, the following relation holds:

$$d(1/A)/dt\ \% = k\,dV/dt = kK_w SRT\,(c_i^0 - c_i^i) \quad (2)$$

As a second test of the osmotic behaviour of the liposomes we studied the dependence of the initial shrinkage velocity on the difference in glucose concentrations on both sides of the membrane. Fig. 3 shows that the initial shrinkage velocity of dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine liposomes was directly proportional to the glucose concentration difference, both above and below T_c. However, there were remarkable differences in the shrinkage velocities of liposomes in the fluid and in the solid state. For dipalmitoyl phosphatidylcholine liposomes this direct proportionality also existed in the vicinity of T_c (Fig. 3B). With dimyristoyl phosphatidylcholine liposomes the changes in $A_{450\ nm}$ were too small to determine the initial shrinkage velocity. The small changes in $A_{450\ nm}$ are due to a high glucose permeability at this temperature [3, 5].

Summarizing, from the findings that, on the time scale of the experiments, lipid bilayers are practically impermeable to glucose both above [14, 15] and below

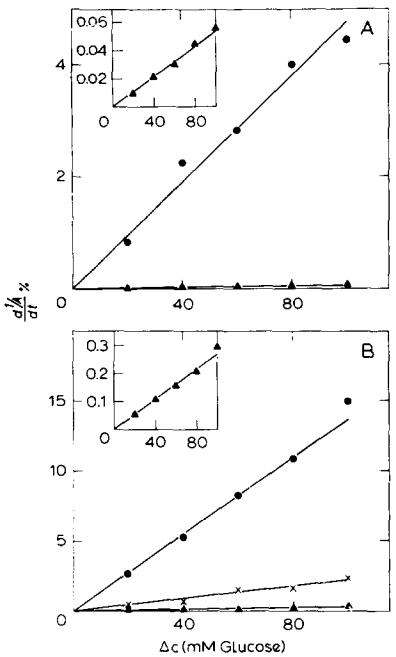

Fig. 3. The dependence of the initial shrinkage velocity on the difference in glucose concentration over the liposomal membrane. (A) dimyristoyl phosphatidylcholine/egg phosphatidic acid = 96 : 4 at 30.5 °C (●) and 14 °C (▲); (B) dipalmitoyl phosphatidylcholine/egg phosphatidic acid = 96 : 4 at 46.0 °C (●), 39.5 °C (×) and 35.0 °C (▲). Insets: enlargements of the data below T_c. For experimental details see Materials and Methods.

[3] the transition temperature and from the experiments of Figs. 2 and 3 it can be concluded that liposomes prepared from synthetic lecithins are perfect osmometers both above and below T_c.

This result contradicts the conclusion of Cohen [16]. Based on an experiment similar to that in Fig. 2, with the exception that glucose was replaced by KCl, Cohen concluded that liposomes are osmotically insensitive structures below their transition temperature [16]. To exclude the possibility that this difference should be ascribed to the different solutes, liposomes of dimyristoyl phosphatidylcholine with 4 mol % of egg phosphatidic acid were prepared in 20 mM KCl, and osmotically treated above T_c (32 °C) and below T_c (10 °C). At both temperatures the liposomes were osmotic sensitive structures, as could be concluded from the changes in the absorbance at 450 nm. With liposomes prepared both in glucose and in KCl it was found that the rate of shrinkage at these temperatures differed by about a factor 100 (Fig. 3). Since the rate of shrinkage of liposomes below T_c is very slow (Figs. 2 and 3) it may be that the time period Cohen used for the osmotic treatment, which unfortunately is not mentioned in the paper [16], was too short.

To prove directly that lipid membranes in the gel state are still permeable to water, the diffusion of ^3H_2O through dipalmitoyl phosphatidylcholine bilayers below T_c was measured (Fig. 4). From this figure it can be seen that a significant amount of [^{14}C]glucose, which was trapped inside the liposomes with the ^3H_2O, is

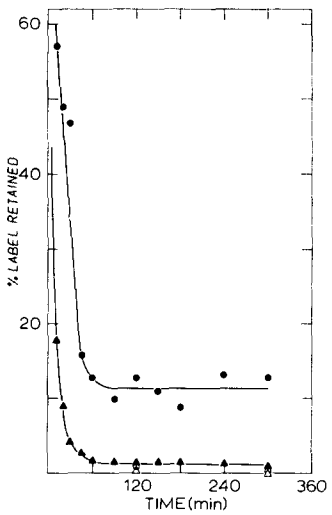

Fig. 4. The retention of ^3H_2O and [^{14}C]glucose by a dialysis bag containing dipalmitoyl phosphatidylcholine/egg phosphatidic acid = 96 : 4 liposomes. Retention of ^3H_2O (▲) and [^{14}C]glucose (●) by a liposome suspension, prepared in the presence of the labeled compounds. Retention of ^3H_2O (△) and [^{14}C]glucose (○) by a liposome suspension to which the labeled compounds were added after preparation of the liposomes and cooling them to room temperature. The dialysis procedure was carried out at room temperature (about 22 °C). For experimental details see Materials and Methods.

retained even after prolonged dialysis. This amount of glucose (about 11 %) is trapped by the liposomes during their preparation, since it was found in a control experiment, that less than 1 % of the [^{14}C]glucose added after the liposomes had been prepared was retained after 2h dialysis (Fig. 4). If liposomes below their T_c were impermeable to water it would be expected that roughly equal amounts of [^{14}C]glucose and ^3H_2O would be retained by the dialysis bag, both representing the amount of label trapped during liposome preparation. Fig. 4 shows that, whereas the glucose was retained, the retention of ^3H_2O went down to a negligible level, indicating that the bilayer was still permeable to water.

According to Eqn. 1 the rate of volume change is directly proportional to the permeability constant of water. However, in comparing the optical data some care is necessary as the optical parameters may change upon passing the transition temperature.

Fig. 5 shows the temperature dependence of the absorbance of dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine liposomes, equilibrated at the indicated temperatures. As has been shown already [17, 18], there is an abrupt change in the absorbance on passing T_c. Fig. 5 shows that the gel to liquid crystalline phase transition temperature of dipalmitoyl phosphatidylcholine liposomes was shifted to a somewhat lower temperature (Fig. 5B), owing to the incorporation of 4 mol % of egg phosphatidic acid. The T_c as found by measuring the temperature dependence of the turbidity of the liposome dispersions (Fig. 5) correlated well with the transition temperature found by calorimetry (Fig. 6). The T_c obtained by differential scanning calorimetry is determined from the point at which the predominant slope of the

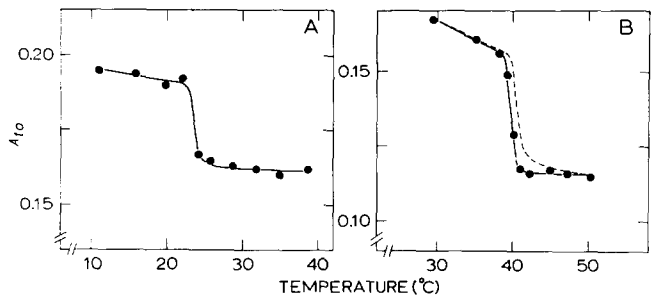

Fig. 5. Temperature dependence of the turbidity of liposome dispersions, prepared from synthetic lecithins. After equilibration of the liposomes at the temperature indicated, the turbidity was measured. (A) Dimyristoyl phosphatidylcholine liposomes and (B) dipalmitoyl phosphatidylcholine liposomes, both with 4 mol % of egg phosphatidic acid. The dotted line represents the temperature dependence of the turbidity of pure dipalmitoyl phosphatidylcholine liposomes. Experimental details are given in Materials and Methods.

descending arm of the major endothermic peak intercepts the base line [19]. Fig. 5 further demonstrates that above T_c there is virtually no temperature dependence, whereas below T_c there is a slight increase on lowering the temperature.

The osmotic experiments of Fig. 2 confirmed, with the possible exception of dimyristoyl phosphatidylcholine liposomes near T_c, a linear relationship between the change in the volume and the change in the reciprocal of the absorbance above, at and below T_c. This proportionality was used in Eqn. 2. The coefficient k in this formula, being defined as $k = \Delta(1/A\%)/\Delta V$, however, may have different values

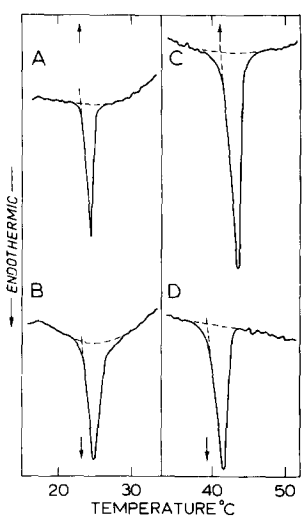

Fig. 6. The effect of egg phosphatidic acid on the thermotropic behaviour of synthetic lecithins. Dimyristoyl phosphatidylcholine in the absence (A) and presence (B) of 4 mol % of egg phosphatidic acid; dipalmitoyl phosphatidylcholine without (C) and with (D) 4 mol % of egg phosphatidic acid. The calorimetric experiments were performed with a Perkin Elmer DSC-2B apparatus as described elsewhere [9]. The lipids were dispersed in a 40 mM Tris-acetate/ethyleneglycol (1 : 1, v/v) solution (pH 7.0). The T_c of the lipid dispersions, determined as described in the text, is indicated by the arrow.

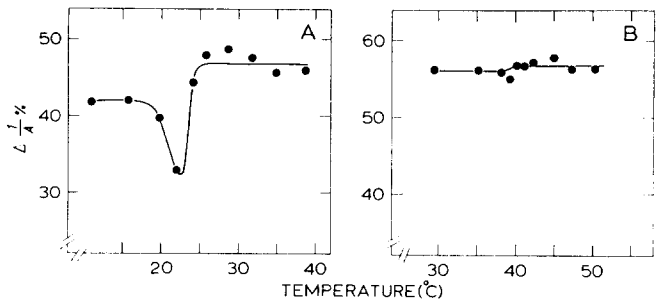

Fig. 7. Temperature dependence of the factor $\Delta(1/A)$ %. For an explanation of the factor $\Delta(1/A)$ % see Materials and Methods. Dimyristoyl phosphatidylcholine (A) and dipalmitoyl phosphatidylcholine (B) liposomes, both containing 4 mol % of egg phosphatidic acid. The liposome dispersions used in this experiment were the same as in Fig. 5.

above and below T_c. Assuming that differences in the geometric parameters of the liposomes above and below T_c can be neglected, ΔV will not change when passing T_c. Then k is directly proportional to $\Delta(1/A)$%. Fig. 7 shows that the variation of $\Delta(1/A)$ %, not considering the temperature region near T_c for dimyristoyl phosphatidylcholine liposomes, was limited.

If we now consider Fig. 8, showing the temperature dependence of the initial shrinkage velocity, it can be concluded that the drastic change in the shrinkage velocity on passing T_c should be largely ascribed to changes in the permeation rate of water. The drastic change in the permeation rate of water when going from the gel to the liquid crystalline state is also clearly visible from an Arrhenius plot, in which the logarithm of the initial shrinkage velocity is plotted against the reciprocal of the absolute temperature (Fig. 9). It is also evident from this plot that below T_c the activation energy of the shrinkage process was much higher than above T_c. Table I gives the activation energies for dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine liposomes above and below T_c. Although there is a rather large scattering for the value obtained above T_c, which should be ascribed largely to the rapidity of the process, the activation energy below T_c was always significantly higher. This suggests that there are different mechanisms of water permeation, which operate above and below the T_c. The activation energy for the permeation of water at temperatures

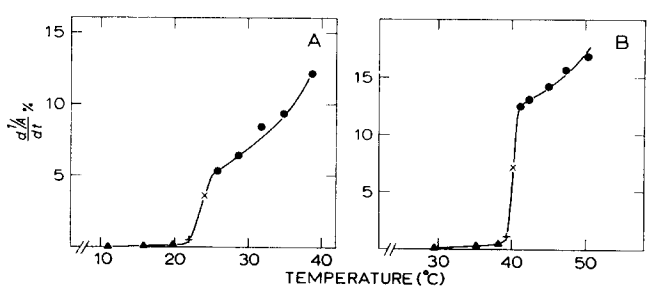

Fig. 8. Temperature dependence of the initial shrinkage velocity of liposomes after osmotic shock. Dimyristoyl phosphatidylcholine (A) and dipalmitoyl phosphatidylcholine (B) liposomes, both containing 4 mol % of egg phosphatidic acid. The liposome dispersions used in this experiment were the same as in Figs. 5 and 7.

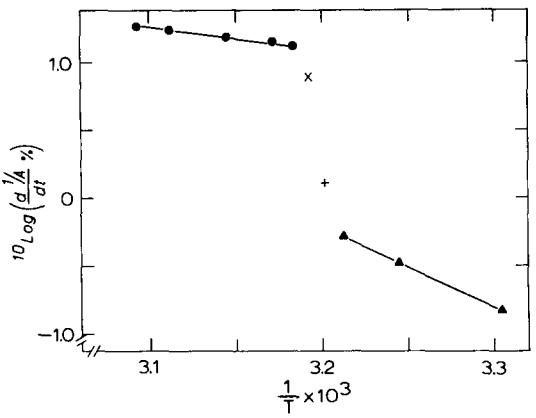

Fig. 9. Arrhenius plot of the osmotic shrinkage of dipalmitoyl phosphatidylcholine liposomes. The data of this plot are those of Fig. 7B.

above T_c correlated rather well with data published earlier (see Table I and refs. 16, 20 and 21).

The experiments discussed so far clearly demonstrate the pronounced effect of the gel to liquid crystalline phase transition on the osmotic shrinkage of liposomes, containing phosphatidylcholine as the main membrane component. Since liposomal membranes should have a net ionic charge in order to trap aqueous medium between the concentric bilayers of the multilayered structures [12], 4 mol % of egg phosphatidic acid is usually incorporated into the phosphatidylcholine bilayers. The question now

TABLE I

ACTIVATION ENERGIES FOR THE PERMEATION OF WATER THROUGH LIPID BILAYERS ABOVE AND BELOW THE GEL TO LIQUID CRYSTALLINE PHASE TRANSITION TEMPERATURE

Lipid	Activation energy (kcal/mol)	
	$T > T_c$	$T < T_c$
14 : 0/14 : 0 phosphatidylcholine/egg phosphatidic acid = 96 : 4	9.3±1.3*	–
	12.1±0.9	27.4±3.9*
16 : 0/16 : 0 phosphatidylcholine/egg phosphatidic acid = 96 : 4	11.3±3.4	20.5±1.9
	7.3±1.1	26.6±0.9
	7.1±0.7	27.6±2.3
16 : 0/16 : 0 phosphatidylcholine/egg phosphatidic acid = 92 : 8	7.0±1.5	26.0±2.1
16 : 0/16 : 0 phosphatidylcholine/dicetyl phosphate = 92 : 8	11.1±2.3	28.3±1.5
16 : 0/16 : 0 phosphatidylcholine/16 : 0/16 : 0 phosphatidylglycerol = 96 : 4	14.1±2.6	28.5±2.2
egg phosphatidylcholine/egg phosphatidic acid = 96 : 4	6.6±0.4	–
	9.2±1.1	–
	9.5±1.2**	26.4±0.9**

* Standard error.
** Standard deviation.

arises of whether the lipid membrane is completely in the gel state below the T_c of the phosphatidylcholine or whether small patches of egg phosphatidic acid in the liquid crystalline state are present in the plane of the membrane besides large areas of phosphatidylcholine in the gel state. Therefore the osmotic shrinkage of dipalmitoyl phosphatidylcholine liposomes with 4 mol % of dipalmitoyl phosphatidylglycerol or 8 mol % of dicetyl phosphate was studied. The membranes of these liposomes are assumed to be completely solid below approximately 38 °C for the following reasons. At pH 7.5 dipalmitoyl phosphatidylglycerol has its transition temperature at about 38 °C [22]. Calorimetric studies showed one single endothermic peak for dipalmitoyl phosphatidylcholine/dicetyl phosphate = 90 : 10 liposomes, whereas a comparable mixture of dimyristoyl phosphatidylcholine/dicetyl phosphate showed lateral phase separation, dicetyl phosphate being the highest-melting component (van Dijck, P. W. M., personal communication). This indicates that liposomal membranes of dipalmitoyl phosphatidylcholine with 8 mol % of dicetyl phosphate are completely solid below about 39 °C. It was found that dipalmitoyl phosphatidylcholine liposomes with 4 mol % of dipalmitoyl phosphatidylglycerol or 8 mol % of dicetyl phosphate still responded to an osmotic gradient by giving changes in the absorbance at 450 nm below 38 °C. This was also found with the liposomes that contained 4 mol % of egg phosphatidic acid. Table I also shows that the activation energies for the permeation of water, as determined by the optical method, were of comparable values both above and below T_c. Based on these results it can be concluded that there is permeation of water molecules through completely solid lipid bilayers.

With respect to the osmotic behaviour of liposomes in the vicinity of the transition temperature, it should be noted that it has been shown previously that the increased permeability near T_c [1–5] is very much dependent on the acyl chain length of the saturated phosphatidylcholines [5] and also strongly depends on the size of the permeating compound [5]. Fig. 7A shows that the factor $\Delta(1/A)\%$ has a minimum at the transition temperature for dimyristoyl phosphatidylcholine liposomes. This was due to a markedly reduced change in absorbance after giving an osmotic shock near T_c, as compared to the change in $A_{450\ nm}$ above and below T_c. For the same reason a straight line was not obtained in the plot of Fig. 2B. Both these experiments suggested a strongly increased glucose permeability near T_c for dimyristoyl phosphatidylcholine liposomes. On the other hand, dipalmitoyl phosphatidylcholine liposomes did not indicate a high permeability for glucose near T_c. This result appears to contradict the observation of Inoue [3], who demonstrated a rapid release of part of the glucose trapped inside dipalmitoyl phosphatidylcholine/dicetyl phosphate = 1 : 0.1 liposomes if the incubation was done at 38–40 °C. We have repeated the experiment and continuously measured the release of glucose by an enzymatic method. Qualitatively identical results were obtained with dipalmitoyl phosphatidylcholine/egg phosphatidic acid = 96 : 4 liposomes. It was found that the release of glucose near T_c is strongly biphasic, as was found by Inoue [3]. Most of the glucose was released during the first short incubation period. This prompted us to do the following experiment (Fig. 10). Dipalmitoyl phosphatidylcholine liposomes containing glucose were first incubated at 22 °C to determine the amount of untrapped glucose present in the dialyzed suspension. Then the temperature of the test cell was elevated to 39.5 °C, decreased to 22 °C and again raised to 39.5 °C. Fig. 10 shows that the release of glucose was increased only during the short periods of time when the temperature was changed in

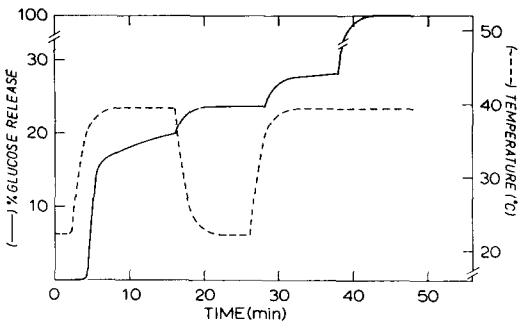

Fig. 10. The effect of perturbation of a lipid phase equilibrium on the release of glucose from dipalmitoyl phosphatidylcholine liposomes, containing 4 mol % of egg phosphatidic acid. The experiment was carried out as described in Materials and Methods. After an incubation period of 38 min, Triton X-100 was added to lyse the liposomes in order to determine the total amount of glucose trapped. It can be seen that there is an increased release of glucose only during the short periods of time when the temperature was changed near 39.5 °C.

the vicinity of T_c. This strongly indicates that a perturbation of a phase equilibrium results in an increased release of the trapped glucose. Once a new phase equilibrium is reached, the glucose permeability is much less. Thus it is now apparent that the lack of strongly increased glucose permeation in osmotic experiments on dipalmitoyl phosphatidylcholine liposomes in the vicinity of the T_c resulted from equilibration of the liposomes at the desired temperature before introduction of the osmotic shock.

The finding that the perturbation of a lipid phase equilibrium greatly enhances the release of trapped solutes needs further exploration. The results of the experiments presented here indicate that misleading results may be obtained if the effects of lipid phase transitions on transport and diffusion processes are studied using techniques in which the cells or liposomes undergo temperature changes. Finally, the finding that the perturbation of a lipid phase equilibrium results in an increased release of trapped solutes indirectly supports the concept that a dynamic equilibrium at the boundaries of two co-existing phases is important for the formation of statistical pores, through which the solutes would permeate [2, 5].

ACKNOWLEDGEMENTS

The present investigations have been carried out under the auspices of the Netherlands Foundation for Biophysics, with financial aid from the Netherlands Organization for the Advancement of Pure Research (ZWO). We are grateful to Mr. P. Standaart and Mrs. A. M. W. Hermkens for synthesizing the lecithins, to Mr. R. J. van Kooij for part of the experimental work, to Drs. P. W. M. van Dijck for doing the differential scanning calorimetric studies, and to Drs. E. J. J. van Zoelen for stimulating discussions.

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