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INFLUENCE OF DICARBOXYLIC PHOSPHATIDYLCHOLINES ON THE STABILITY AND PHASE TRANSITION OF PHOSPHATIDYLCHOLINE LIPOSOMES

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Summary

The effect of dicarboxylic phosphatidylcholines (glutaryl phosphatidylcholine) on the stability and phase transition of phosphatidylcholine liposomes is examined by using liposomes prepared with egg phosphatidylcholine or dipalmitoyl phosphatidylcholine and by varying the surface charge by addition of dicetyl phosphate.

Light-scattering and osmotic behaviour studies showed that the stability of liposomes containing dicarboxylic phosphatidylcholine is influenced by the charge and the fatty acid saturation of the liposomes. Increasing the glutaryl phosphatidylcholine-to-phosphatidylcholine molar ratio in liposomes caused the formation of mixed glutaryl phosphatidylcholine/phosphatidylcholine micelles. The sensitivity of the lipid bilayers towards glutaryl phosphatidylcholine action increases with the fatty acid saturation of liposomes. Dipalmitoyl phosphatidylcholine liposomes are most sensitive to the dicarboxylic phosphatidylcholine effect. Dicetyl phosphate addition enhances the solubilization of liposomes prepared from saturated phospholipids.

The effect of increasing concentrations of glutaryl phosphatidylcholine on the gel-to-liquid crystal thermal transition of dipalmitoyl phosphatidylcholine was observed. Glutaryl phosphatidylcholine modifies the thermal phase transition of the constituents of the liposome. The presence of dicetyl phosphate in liposomes affects the phase transition temperature of these liposomes. It is suggested that the formation of the mixed micelles is responsible for the phase transition modifications.

These data show that the solubilization of liposomes by dicarboxylic phosphatidylcholines depends on the fatty acid composition of phosphatidylcholine and on the presence of dicetyl phosphate.

Introduction

It is well known that the fatty acid composition of phosphatidylcholines affects considerably the stability of artificial and natural membranes [1,2]. Several authors studied the effects of detergents on the integrity of liposomal membranes. Inoue and Kitagawa [3] have demonstrated that 'solid' liposomes are more susceptible to the effects of Triton X-100 than 'fluid' liposomes. Hertz and Barenholz [4] also reported a correlation between the microviscosity of bilayers and their sensitivity towards Triton X-100. One of the important characteristics of a phospholipid/water lamellar phase is the ordered-disordered phase transition [5]. The lipid phase transition has been reported to have a significant effect upon the mixing of phospholipid bilayer vesicles [6] and the permeability of the bilayer [2,7,8]. Furthermore, the phase transition of a bilayer membrane can be influenced by altering the surface charge [9—11].

Irradiation of plasma high density lipoproteins determines the formation of dicarboxylic phosphatidylcholines [12]. These molecules are formed by the shortening of an unsaturated fatty acid in position 2 with the formation of malonaldehyde. These phosphatidylcholines are hemolytic [13], behave as detergents and enhance liposome permeability to glucose [14].

In the present paper, we describe the effects of dicarboxylic phosphatidylcholines on the stability and phase transition of non-sonicated phospholipid liposomes prepared from two different phosphatidylcholines (egg phosphatidylcholine and dipalmitoyl phosphatidylcholine), under conditions which alter the surface charge.

Materials and Methods

Phospholipid preparation. 1-Acyl-2-glutaryl-*sn*-glycero-3-phosphorylcholine was synthesized as described previously [15]. The purity of the glutaryl phosphatidylcholine preparation was checked by thin-layer chromatography on silica gel plates with $CHCl_3/CH_3OH/7$ M NH_4OH (76 : 30 : 5, v/v) as solvent system. Dipalmitoyl phosphatidylcholine and dicetyl phosphate were purchased from Sigma. Egg phosphatidylcholine was prepared by chromatography on Al_2O_3 and silicic acid. Lysophosphatidylcholine was obtained from egg phosphatidylcholine by treatment with phospholipase A_2 from snake venom (*Naja naja*). The fatty acid composition of egg phosphatidylcholine and dipalmitoyl phosphatidylcholine was determined by gas chromatography.

Preparation of liposomes. Non-sonicated liposomes were prepared from mixtures of egg phosphatidylcholine or dipalmitoyl phosphatidylcholine, glutaryl phosphatidylcholine or lysophosphatidylcholine in different proportions, dicetyl phosphate and cholesterol (when present). The lipid combinations are given in the figures. The dry lipid mixtures were prepared by dispersion above the transition temperature of the phosphatidylcholine on a vortex mixer in an aqueous solution (phase transition measurements) or in a 50 mM KCl solution, following the technique of Bangham et al. [16].

Measurement of lipid transition temperature. Phase transition temperatures of the lipid dispersions were determined by measuring apparent light absorb-

ances of liposomal suspensions at 400 nm as a function of temperature [17]. This study was performed with a Beckman Acta CIII spectrophotometer equipped with a temperature programmer.

The pH of the lipid mixed dispersions was adjusted to the desired values by addition of HCl or NaOH in water. The pH of the dispersions at 20°C was controlled.

Osmotic behaviour. The water permeability of liposomes was studied according to the methods of Bangham et al. [16] and de Gier et al. [1]. They have shown that the initial rate of volume change, upon exposure to osmotic pressure, is proportional to $1/A$, where absorbance, A, is entirely due to light scattering. For these studies, lipid dispersions were prepared in 50 mM KCl, transferred into different KCl concentrations and turbidity changes measured.

For the osmotic shrinkage experiments, the liposomes (lipid concentration 1 mM) were prepared in 20 mM glucose/10 mM Tris-HCl (pH 7.5) and transferred into a thermostatically controlled cuvette and stirred. After temperature equilibration, an osmotic shock was given by rapidly injecting 0.2 ml of 1.0 M glucose/20 mM Tris-HCl (pH 7.5), preincubated at the same temperature [8]. Changes in the turbidity were determined with a spectrophotometer (Beckman Acta CIII) at 450 nm.

Measurement of turbidity. The turbidities of mixed dispersions of phosphatidylcholine and glutaryl phosphatidylcholine in water were measured by recording the absorbance at 400 nm at 20°C in cuvettes of 1 cm light path.

Results

Effect of lipid composition on osmotic behaviour of liposomes

As shown by Bangham et al. [16] and de Gier et al. [1], a linear relationship is obtained between $1/A$ (450 nm) and 1/osmolarity with liposomes displaying an ideal osmotic behaviour. To determine the stability of liposomes prepared from various lipid mixtures containing 2.85 mol dicetyl phosphate per 10 mol phosphatidylcholine, we measured their turbidity changes over a range of KCl concentrations. As shown in Fig. 1A, egg phosphatidylcholine and glutaryl

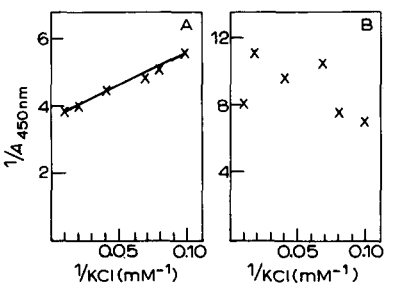

Fig. 1. Relationship between the reciprocal of the absorbance at 450 nm of liposome suspensions and the reciprocal of the KCl concentration in which they are osmotically treated until equilibrium. Liposomes contain 2.85 mol dicetyl phosphate per 10 mol phospholipid. Studies with dipalmitoyl phosphatidylcholine liposomes were performed over the transition temperature at 50°C for 1 h. (A) Egg phosphatidylcholine/glutaryl phosphatidylcholine (9 : 1, mol/mol). (B) Dipalmitoyl phosphatidylcholine/glutaryl phosphatidylcholine (9 : 1, mol/mol).

TABLE I

OSMOTIC BEHAVIOUR OF LIPOSOMES CONTAINING VARIOUS AMOUNTS OF PHOSPHOLIPIDS AND CHOLESTEROL

Liposomes contained 2.85 mol dicetyl phosphate per 10 mol phospholipid. The presence of osmotically active liposomes was determined by following $1/A$ variation as a function of 1/KCl concentration (see Fig. 1). Obtainment of a straight line was taken as indicating the presence of osmotically active liposomes (see Fig. 1). Egg PC, egg phosphatidylcholine; DPPC, dipalmitoyl phosphatidylcholine; GL, glutaryl phosphatidylcholine; LPC, lysophosphatidylcholine; CL, cholesterol.

Liposome composition (mol/mol)					Osmotically active liposomes
Egg PC	DPPC	GL	LPC	CL	
8	0	2	0	0	+
0	9	1	0	0	—
0	8	0	2	0	+
0	5	5	0	10	+
0	5	0	5	10	+

phosphatidylcholine (molar ratio 9 : 1) form multilayer liposomes which are osmotically active. On the other hand, Fig. 1B gives an example of non-osmotically active liposomes (dipalmitoyl phosphatidylcholine/glutaryl phosphatidylcholine, molar ratio 9 : 1), where linearity between $1/A$ and 1/[KCl] was no longer observed.

Table I summarizes the essential results obtained by such a method. One can notice that replacement of 20% egg phosphatidylcholine by glutaryl phosphatidylcholine leaves the liposomes intact, whereas only 10% of glutaryl phosphatidylcholine is sufficient to destabilize liposomes containing dipalmitoyl phosphatidylcholine. Lysophosphatidylcholine was less perturbing than glutaryl phosphatidylcholine and liposomes containing 8 mol dipalmitoyl phosphatidylcholine per 2 mol lysophosphatidylcholine were still osmotically active. The stabilizing effect of cholesterol is also evident from the data of Table I, since addition of the sterol in equimolar amounts to phospholipid allowed dipalmitoyl phosphatidylcholine liposomes to support as much as 50%

TABLE II

OSMOTIC SHRINKAGE OF LIPOSOMES CONTAINING VARIOUS AMOUNTS OF PHOSPHOLIPIDS

Egg PC, egg phosphatidylcholine; DPPC, dipalmitoyl phosphatidylcholine; GL, glutaryl phosphatidylcholine; DCP, dicetyl phosphate.

Liposome composition (mol/mol)				Volume change
Egg PC	DPPC	GL	DCP	
0	10	0	0	0
0	10	0	0.57	decrease
0	9	1	0	0
0	9	1	0.57	0
10	0	0	0	0
9	0	1	0	decrease
8	0	2	0	0
8	0	2	0.57	decrease

glutaryl phosphatidylcholine or lysophosphatidylcholine.

Osmotic properties of liposomes containing glutaryl phosphatidylcholine are the same above and below the phase transition temperature (T_c). In contrast, we observed that in the absence of dicetyl phosphate, the dispersion of pure phosphatidylcholine showed no variation in osmotic swelling and shrinkage.

Another way to investigate the osmotic behaviour of liposomes was to follow their volume decrease, as detected from the increase in A (450 nm), upon addition of a hyperosmolar glucose solution. As previously reported [16], a volume change occurred only with liposomes carrying a net ionic charge. This is illustrated in Table II when comparing dipalmitoyl phosphatidylcholine without or with dicetyl phosphate. Addition of glutaryl phosphatidylcholine (10% molar ratio) to these liposomes suppressed the volume change, whether or not dicetyl phosphate was present. Conversely, addition of 10% glutaryl phosphatidylcholine (which confers a net ionic charge on the liposomes) to egg phosphatidylcholine restored the osmotic behaviour of the liposomes, which was suppressed with 20% glutaryl phosphatidylcholine. However, inclusion of dicetyl phosphate in the latter liposomes allowed the recovery of the volume change. The osmotic shrinkage behaviour of liposomes confirmed the instability of dipalmitoyl phosphatidylcholine/glutaryl phosphatidylcholine dispersions.

Turbidity measurements

The turbidity studies at 400 nm are summarized by Fig. 2. For liposomes containing dipalmitoyl phosphatidylcholine, the decrease in turbidity caused by the presence of glutaryl phosphatidylcholine was pronounced when the liposomes also contained dicetyl phosphate (2.85 mol per 100 mol total phosphatidylcholine). In contrast, in the absence of dicetyl phosphate, mixed liposomes (dipalmitoyl phosphatidylcholine/glutaryl phosphatidylcholine) were obtained up to a concentration of about 40 mol% glutaryl phosphatidylcholines. At a

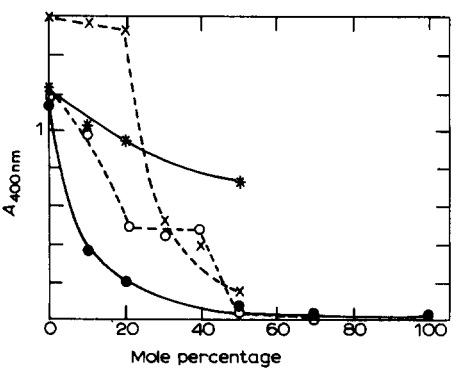

Fig. 2. Turbidity (400 nm) of phosphatidylcholine/glutaryl phosphatidylcholine mixtures dispersed in an aqueous phase. The total amount in each tube was 2.5 μmol phospholipid/2.5 ml H_2O. Dipalmitoyl phosphatidylcholine/glutaryl phosphatidylcholine, o------o; dipalmitoyl phosphatidylcholine/glutaryl phosphatidylcholine with dicetyl phosphate (2.8%), •———•; egg phosphatidylcholine/glutaryl phosphatidylcholine, X------X; egg phosphatidylcholine/glutaryl phosphatidylcholine with dicetyl phosphate (2.8%), ★———★.

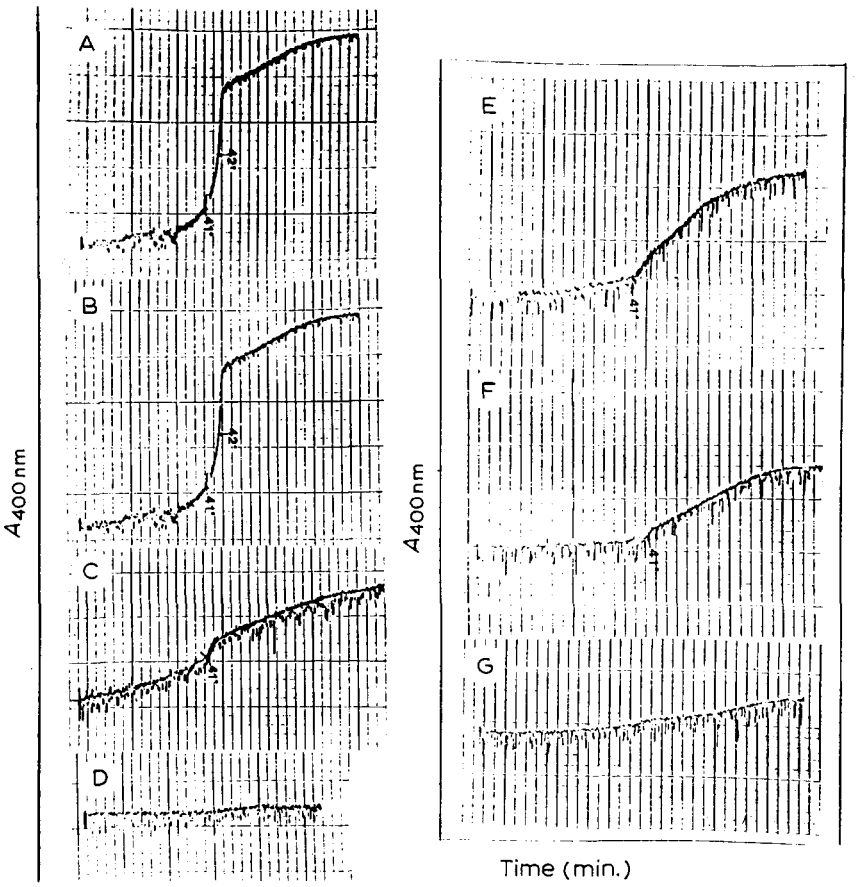

Fig. 3. Influence of glutaryl phosphatidylcholine on the apparent light absorbance at 400 nm of suspensions of dipalmitoyl phosphatidylcholine as a function of temperature. (A) dipalmitoyl phosphatidylcholine; (B) dipalmitoyl phosphatidylcholine/glutaryl phosphatidylcholine (9 : 1, mol/mol); (C) dipalmitoyl phosphatidylcholine/glutaryl phosphatidylcholine (7 : 3, mol/mol); (D) dipalmitoyl phosphatidylcholine (5 : 5, mol/mol); (E) dipalmitoyl phosphatidylcholine/dicetyl phosphate (10 : 0.28, mol/mol); (F) dipalmitoyl phosphatidylcholine/glutaryl phosphatidylcholine/dicetyl phosphate (9 : 1 : 0.28, mol/mol); (G) dipalmitoyl phosphatidylcholine/glutaryl phosphatidylcholine/dicetyl phosphate (8 : 2 : 0.28, mol/mol). Lipid concentration 2.5 mM.

glutaryl phosphatidylcholine/dipalmitoyl phosphatidylcholine molar ratio of 50 : 50, a breakdown of the liposomes occurs, followed by a decrease in absorbance. The breakdown is probably caused by the formation of mixed micelles of lipid and detergent [18]. In this case, mixed micelles of phosphatidylcholine and glutaryl phosphatidylcholine are formed [14]. Electron microscopy studies support these data (unpublished results). For liposomes containing egg phosphatidylcholine, the influence of dicetyl phosphate was different. Mixed liposomes (egg phosphatidylcholine/glutaryl phosphatidylcholine) were obtained in the presence of dicetyl phosphate (2.85 mol per 100 mol total phosphatidylcholine) up to a concentration of about 50 mol% glutaryl phosphatidylcholine. In contrast, in the absence of dicetyl phosphate, the turbidity of the system is drastically reduced. At a concentration of 50

mol% glutaryl phosphatidylcholine, glutaryl phosphatidylcholine solubilizes the liposomes to form mixed egg phosphatidylcholine/glutaryl phosphatidylcholine micelles.

Fig. 2 also shows that, for natural egg phosphatidylcholine liposomes, more glutaryl phosphatidylcholine is required for the solubilization of the membranes.

Effect of glutaryl phosphatidylcholine on the phase transition temperature of dipalmitoyl phosphatidylcholine

We studied the effect of glutaryl phosphatidylcholine on the gel-to-liquid thermal transition of dipalmitoyl phosphatidylcholine liposomes. For liposomes without dicetyl phosphate, at an equimolar ratio (dipalmitoyl phosphatidylcholine to glutaryl phosphatidylcholine, thermal phase transition measurements showed an absence of the usual lipid phase transition (Fig. 3, curve D). Fig. 3 also demonstrates the effect of glutaryl phosphatidylcholine on the thermal transition of dipalmitoyl phosphatidylcholine liposomes containing 2.8 mol% dicetyl phosphate. No transition was observed in the range of 20—60°C for a dipalmitoyl phosphatidylcholine/glutaryl phosphatidylcholine ratio of 8 : 2 (Fig. 3, curve G).

It is clear that dicetyl phosphate affects considerably the liposomes. In the presence of dicetyl phosphate (2.8 mol%), pH values of lipid dispersions are lower than those of dispersions without this compound (6.5 and 6.9, respectively, for mixed lipid dispersions containing dipalmitoyl phosphatidylcholine and glutaryl phosphatidylcholine at a molar ratio of 8 : 2). A pH of 12 or

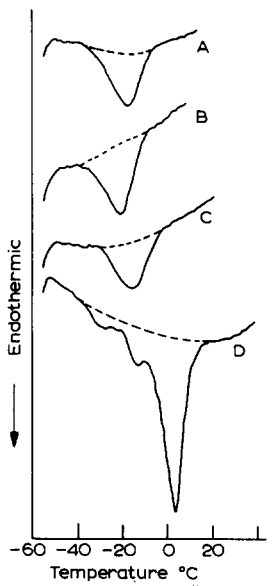

Fig. 4. Phase transition of glutaryl phosphatidylcholine and egg lysophosphatidylcholine. (A) Glutaryl phosphatidylcholine dispersed in 25 mM Tris, 100 mM NaCl/ethylene glycol (1 : 1, v/v), pH 7.8. (B) Glutaryl phosphatidylcholine dispersed in 75 mM KCl, 75 mM NaCl, 100 mM Tris/ethylene glycol (1 : 1, v/v), pH 8.0. (C) Glutaryl phosphatidylcholine dispersed in 75 mM KCl, 75 mM NaCl, 100 mM Tris/ethylene glycol (1 : 1, v/v), pH 2.5. (D) Egg lysophosphatidylcholine dispersed as B.

TABLE III

EFFECT OF DICETYL PHOSPHATE ON THE TRANSITION TEMPERATURE OF DIPALMITOYL PHOSPHATIDYLCHOLINE/GLUTARYL PHOSPHATIDYLCHOLINE DISPERSIONS

Transition temperature of mixed lipid dispersions containing glutaryl phosphatidylcholine and dipalmitoyl phosphatidylcholine at different molar ratios. DPPC, dipalmitoyl phosphatidylcholine; GL, glutaryl phosphatidylcholine; DCP, dicetyl phosphate.

DPPC/GL (molar ratio)	Transition temperature (°C)	
	without DCP	with DCP (2.8%)
1	41.0	41.0
9 : 1	41.0	41.0
8 : 2	41.0	no transition *
7 : 3	41.0	no transition *
6 : 4	41.0	no transition *
5 : 5	no transition *	no transition *
0	no transition *	no transition *

* No transition in the range of 20—60°C.

0.5 resulted in the main transition centered at 38°C. The dispersions at pH values larger than 10 or smaller than 4 were unstable and were only used for one temperature cycle. At low pH (0.5), dipalmitoyl phosphatidylcholine was found to have been decomposed into lysophosphatidylcholine and fatty acid chains. Fig. 4 shows that glutaryl phosphatidylcholine in a medium of 75 mM KCl, 75 mM NaCl, 100 mM Tris at pH 8.0 undergoes a thermotropic phase transition centered at −20°C. This transition temperature did not seem to be significantly altered when the pH was lowered to pH 2.5.

The results of transition temperatures obtained for liposomes with or without dicetyl phosphate (2.8 mol% total phosphatidylcholine) are summarized in Table III.

Discussion

In this study, the effect of dicarboxylic phosphatidylcholines on phosphatidylcholine liposomes was followed by using multilamellar liposomes made of various glutaryl phosphatidylcholine-to-monocarboxylic phosphatidylcholine (egg phosphatidylcholine or dipalmitoyl phosphatidylcholine) molar ratios and by studying the osmotic behaviour, the turbidity measurements and the phase transition temperature of these dispersions.

Incorporation of cholesterol into liposomes made of dipalmitoyl phosphatidylcholine and glutaryl phosphatidylcholine enhances the stability of mixed dispersions. In fact, we have observed that cholesterol incorporation in dipalmitoyl phosphatidylcholine bilayers, at a concentration of 5 mol cholesterol/10 mol phospholipid, in the absence or presence of glutaryl phosphatidylcholine, abolishes the transition. These results may be compared to those of de Kruijff et al. [19] and Ladbrooke et al. [20]. Cholesterol increases the chain mobility and has a 'liquefying' effect [21]. On the other hand, our results confirm that liposomal membranes must have a net ionic charge in order to vary with salt concentration [16].

Our data also demonstrate that glutaryl phosphatidylcholine micelles are able to solubilize phospholipids and thus to perturb the stability of liposomes. The structures obtained depend essentially on the relative percentage of these two phospholipids. The process of micelle formation by glutaryl phosphatidylcholine depends on the saturation of fatty acid in phosphatidylcholine dispersions and on the presence of dicetyl phosphate. Dicetyl phosphate added in dipalmitoyl phosphatidylcholine liposomes enhances the transformation of liposomes into micelles by glutaryl phosphatidylcholine. Dicetyl phosphate causes a separation of adjacent layers of phospholipid but dicetyl phosphate also may induce the formation of micelles [22]. Much of the added dicetyl phosphate in liposome dispersions may exist as a population of anionic micelles which are not necessarily part of the liposome population [23]. Like lysophosphatidylcholine, a glutaryl phosphatidylcholine dispersion has a single hydrophobic chain, behaves as an anionic detergent and forms micelles [14]. Mixed micelles of glutaryl phosphatidylcholine and dicetyl phosphate might be formed (unpublished results). In dipalmitoyl phosphatidylcholine bilayers, the rigidity prevents the glutaryl phosphatidylcholine from being distributed evenly throughout the bilayer. Regions enriched in glutaryl phosphatidylcholine induce the formation of mixed glutaryl phosphatidylcholine/dipalmitoyl phosphatidylcholine micelles. This phenomenon is enhanced by the presence of anionic dicetyl phosphate in bilayers. In fact, surface charge differences affect the fluidity of the polar region of liposomes [24]. In dipalmitoyl phosphatidylcholine/dicetyl phosphate/glutaryl phosphatidylcholine liposomes, there is probably an electrostatic repulsion between the negatively charged lipid molecules in the liposome and there is formation of mixed glutaryl phosphatidylcholine/dipalmitoyl phosphatidylcholine micelles.

Concerning egg phosphatidylcholine, due to the less rigid and less dense structure, glutaryl phosphatidylcholine is evenly distributed throughout the liposomes. In the presence of dicetyl phosphate, mixed liposomes of egg phosphatidylcholine/glutaryl phosphatidylcholine are formed. It is to be noticed that dicetyl phosphate is itself capable of forming liposomes [25]. In the absence of dicetyl phosphate, up to a molar percentage of glutaryl phosphatidylcholine of 30 mol%, the liposomes remain mostly intact. At intermediate values of the molar percentage of glutaryl phosphatidylcholine (30—50 mol%), mixed liposomes and mixed micelles are formed. Above a molar percentage of glutaryl phosphatidylcholine of 50 mol%, the formation of mixed micelles is observed. The interrelationship between the bilayer solubilization and its lipid composition is supported by the results of Hertz and Barenholz [4]. These authors showed that the sensitivity of the lipid bilayer towards Triton X-100 increased in parallel with increasing the mole fraction of sphingomyelin in the membrane and that higher mole fractions of sphingomyelin resulted in more viscous membranes.

The present study shows that glutaryl phosphatidylcholine and dicetyl phosphate affect considerably the transition temperature of mixed dispersions. A pH of 0.5 results in a minor modification of T_c. A decrease of T_c of ditetradecylphosphatidic acid was reported associated with acidic pH [26]. We notice that the transition temperature of glutaryl phosphatidylcholines does not seem to be significantly altered when the pH is lowered to pH 2.5. Van Dijck et al.

[27] reported a similar result for dimyristoyl phosphatidylglycerol. But for dilauroyl phosphatidylglycerol, a considerable increase in T_c was observed at acidic pH as a consequence of a decrease in surface charge [9]. The disappearance of T_c for a molar ratio of glutaryl phosphatidylcholine to dipalmitoyl phosphatidylcholine of 2 : 8 in the presence of dicetyl phosphate does not seem to be due to a pH difference of lipid dispersions. The reduction of turbidity, attributed to liposome solubilization, suggests that the formation of micelles is responsible for these modifications.

Similar findings were recently reported by Brendzel and Miller [28] for dicetyl phosphate which increases membrane fluidity of dipalmitoyl phosphatidylcholine liposomes, but not with egg lecithin.

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