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INFLUENCE OF DICARBOXYLIC PHOSPHATIDYLCHOLINES ON PHOSPHATIDYLCHOLINE LIPOSOMES AS REVEALED BY GEL CHROMATOGRAPHY AND ELECTRON MICROSCOPY

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The effect of dicarboxylic phosphatidylcholines (glutarylphosphatidylcholine) on the structural changes of phosphatidylcholine liposomes is examined by using multilamellar liposomes prepared with egg phosphatidylcholine or dipalmitoylphosphatidylcholine and by varying the surface charge by addition of dicetyl phosphate. Investigations are performed by gel chromatography and electron microscopy. Glutarylphosphatidylcholine is in micellar form (rod-like micelles or globular micelles). The structures obtained depend on the fatty acid saturation of liposomes and on the charge of liposome (addition or not of dicetyl phosphate). With egg phosphatidylcholine/glutarylphosphatidylcholine dispersions, an aspect more similar to myelinic figures than liposomes is observed, while in the presence of dicetyl phosphate, liposomes similar to control egg phosphatidylcholine liposomes are obtained. Gel chromatography on Sepharose 4B and turbidity measurements prove that dicetyl phosphate increases the stability of egg phosphatidylcholine/glutarylphosphatidylcholine mixtures. On the other hand, in dipalmitoylphosphatidylcholine/glutarylphosphatidylcholine dispersions, incorporation of dicetyl phosphate destabilizes bilayer structure and the formation of mixed micelles occurs. Viscosity measurement shows, in the presence of dicetyl phosphate, an increased fluidity for dipalmitoylphosphatidylcholine/glutarylphosphatidylcholine dispersions, in agreement with the micellar organization. These data confirm that the disorganization of liposomal membranes by dicarboxylic phosphatidylcholine depends on the fatty acid composition of phosphatidylcholine and on the presence of dicetyl phosphate.

Introduction

The structure and permeability of biological and model membranes are modified by lysophosphatidylcholines [1,2] or short-chain phosphatidylcholines [3] or synthetic phosphatidylcholines [4]. On the other hand, lipid peroxidation also increases the permeability of liposomes [5,6]. We have reported that irradiation of plasma high-density lipoproteins determines the formation of dicarboxylic phosphatidylcholines (glutarylphosphatidylcholines) [7]. These phosphatidylcholines, formed by peroxidative attack of unsaturated fatty acid in the position 2 of phosphatidylcholines, display lytic properties against both red blood cells [8] and multilamellar liposomes [9].

Furthermore, increasing the glutarylphosphatidylcholine to phosphatidylcholine molar ratio in liposomes caused the formation of mixed glutarylphosphatidylcholine/phosphatidylcholine micelles [10]. The sensitivity of the lipid bilayers towards glutarylphosphatidylcholine action increases with the fatty acid saturation of liposomes. On the other hand, inclusion of anionic dicetyl phosphate in dipalmitoylphosphatidylcholine/glutarylphosphatidylcholine dispersions affects the

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phase transition temperature of these liposomes [10].

In the present study, the nature of the structural changes has been investigated using multilamellar liposomes prepared with glutarylphosphatidylcholine and monocarboxylic phosphatidylcholine under conditions which alter the surface charge. The structures have been studied by gel filtration, turbidity measurement and microscopic studies. The results are interpreted in the light of the viscosity measurement data.

Materials and Methods

Materials

The 1-acyl-2-glutaryl-*sn*-glycero-3-phosphorylcholine (glutarylphosphatidylcholine) was synthesized as previously described [11]. The purity of the glutarylphosphatidylcholine preparation (free form) was checked by thin-layer chromatography on silica gel plates with chloroform/methanol/7 N ammonia (70:30:5, v/v) as solvent system. The lipid sample showed no TLC-detectable impurity and gave a single spot. Dipalmitoylphosphatidylcholine, dipalmitoyl-DL-α-phosphatidic acid (free acid), L-α-phosphatidyl-L-serine from bovine brain and dicetyl phosphate (free form) were purchased from Sigma, St Louis, MO, U.S.A. Pure egg phosphatidylcholine was prepared according to Hanahan et al. [12]. Lysophosphatidylcholine was obtained from egg lecithin by treatment with phospholipase A_2 from snake venom (*Naja naja*). The fatty acid composition of egg phosphatidylcholine and dipalmitoylphosphatidylcholine was determined by gas chromatography.

Preparation of liposomes

Non-sonicated liposomes were prepared from mixtures in chloroform/methanol (1:1) solutions of egg phosphatidylcholine or dipalmitoylphosphatidylcholine, of glutarylphosphatidylcholine or lysophosphatidylcholine in different proportions, of dicetyl phosphate, phosphatidic acid or phosphatidylserine (when present). The dry lipid mixtures were prepared by mechanical shaking above the transition temperature of the phosphatidylcholines (6.25 mM) on a vortex mixer in an aqueous solution (microscopic studies), following the technique of Bangham et al. [13]. Egg phosphatidylcholine liposomes were prepared at room temperature and dipalmitoylphosphatidylcholine liposomes at 55°C.

Analytical gel chromatography on Sepharose 4B

The method was essentially derived from that of Huang [14]. A column of Sepharose 4B (1.6 × 20 cm) was equilibrated at room temperature with the following elution solution: 0.1 M NaCl/0.01 N H_3BO_4/0.02% NaN_3 (pH 7.5). 0.5 ml of the phospholipid dispersion (10 mM) was applied to the column. The flow-rate was 1 ml/min and fractions of 1 ml were collected. The molar percentage of glutarylphosphatidylcholine, (100 × glutarylphosphatidylcholine)/(phosphatidylcholine + glutarylphosphatidylcholine), in the eluates was determined by thin-layer chromatography and by phosphorus determination of the scraped spots.

Turbidity measurements

The turbidities of dispersions in water were determined by measuring the apparent absorbance at 20°C at 400 nm in cuvettes of 1 cm light path.

Electron microscopy

All the samples were prepared in water, at the same pH (6.5) and at the same temperature (25°C).

Negative staining. A drop of lipid suspension in water was placed on 200-mesh copper grids previously coated with carbon film and rendered hydrophilic by glow discharge before use. After excess samples had been drained off with filter paper, 2% sodium phosphotungstate (pH 7.2) was applied to the grids. The excess of staining solution was removed with filter paper after 10–15 s and the grid was examined immediately under an electron microscope; magnifications were from 15000 to 40000.

Thin sections. A method described by Hamilton et al. [15] was slightly improved. Samples were fixed by adding 2% osmium tetroxide in sodium veronal buffer, pH 7.4. The lipid turned from light yellow to brown, depending on the number of double bonds. After 48 h at 4°C, the fixed lipid was mixed with 2% gelatin solution, and then filtered on Millipore filter (22 nm average pore diameter) at low pressure. After 3 h, the sample formed a uniform film on the Millipore membrane

which was transferred to 2% aqueous uranyl acetate and left at 37°C for 48 h. The sample was dehydrated by the usual grade of ethanol from 70 to 100% and by propylene oxide. The filter could be removed and fragments of lipid film were embedded in Epon. Sections were performed on Reichert ultramicrotome, stained by 5% uranyl acetate followed by lead citrate. Micrographs were taken with a JEMC or AEI Corinth 275 at 80 kV and magnifications were from 10000 to 20000.

Viscosity determination

Viscosity measurements were performed at 37°C with a Rotovisco RV 100 (Haake).

Results

Physicochemical characterization of mixtures of egg phosphatidylcholine and glutarylphosphatidylcholine

Electron microscopic images of negatively stained and thin-sectioned preparations show that there were difference in the products observed when dicetyl phosphate was added or not. Egg phosphatidylcholine/glutarylphosphatidylcholine (molar ratio 5:5) dispersions in the presence of dicetyl phosphate showed a mixture of multilamellar and unilamellar liposomes (Fig. 1A) similar to that observed with egg phosphatidylcholine control liposomes (Fig. 1B). On the other hand, in the absence of dicetyl phosphate, the micrograph showed aspects more similar to myelinic figures than liposomes (Fig. 1C). Glutarylphosphatidylcholine showed, in negative staining, large amounts of micelles: rod-like micelles and globular micelles (Fig. 2A and B). However, the same glutarylphosphatidylcholine preparation gave no visible particles in ultrathin sections.

Turbidity experiments support these data (Table I). Mixed liposomes (egg phosphatidylcholine/glutarylphosphatidylcholine) were obtained in the presence of dicetyl phosphate up to a concentration of about 50 mol% glutarylphosphatidylcholine. On the other hand, in the absence of dicetyl phosphate, the turbidity of the system at high molar percentage of glutarylphosphatidylcholine was drastically reduced. At a concentration of 50 mol% glutarylphosphatidylcholine, liposomes were solubilized to form mixed egg phosphatidylcholine/glutarylphosphatidylcholine micelles. These results were confirmed by the phospholipid elution profiles obtained from dispersions on Sepharose 4B.

An equimolecular dispersion of glutarylphosphatidylcholine and egg phosphatidylcholine without dicetyl phosphate gave a unique peak corresponding to an elution volume $V_e = 2V_0$. V_e was found close to the elution volume of glutarylphosphatidylcholines which were in spherical micelle states. This suggested that this dispersion might also be in a micellar state. The molar percentage of glutarylphosphatidylcholine in the eluates showed that there were mixtures of phosphatidylcholine and glutarylphosphatidylcholine and that the glutarylphosphatidylcholine was completely incorporated into micelles.

Under the same conditions, an equimolecular dispersion of glutarylphosphatidylcholine and egg phosphatidylcholine in the presence of dicetyl phosphate gave a single peak of multilamellar liposomes, eluted at the void volume V_0. Thin-layer chromatography confirmed the presence of a homogeneous mixture of egg phosphatidylcholine and glutarylphosphatidylcholine.

Physicochemical characterization of mixtures of dipalmitoylphosphatidylcholine and glutarylphosphatidylcholine

Fig. 3A shows that dipalmitoylphosphatidylcholine/glutarylphosphatidylcholine (molar ratio 5:5) dispersions existed as multilayered liposomes similar to dipalmitoylphosphatidylcholine control liposomes (Fig. 3B). When dicetyl phosphate was present in dipalmitoylphosphatidylcholine/glutarylphosphatidylcholine mixture, no structure was visualized in thin-sectioned preparations. But by negative staining, the same equimolar mixture of dipalmitoylphosphatidylcholine/glutarylphosphatidylcholine produced discoid particles which aggregated in rouleaux (Fig. 4). However, when liposomes were formed from only dipalmitoylphosphatidylcholine, we did not observe any stacking of liposomes.

Influence of glutarylphosphatidylcholine and/or dicetyl phosphate on membrane fluidity

The results in Table II show that dicetyl phosphate increased viscosity of dipalmitoylphosphatidylcholine dispersions. Similarly, glutarylphospha-

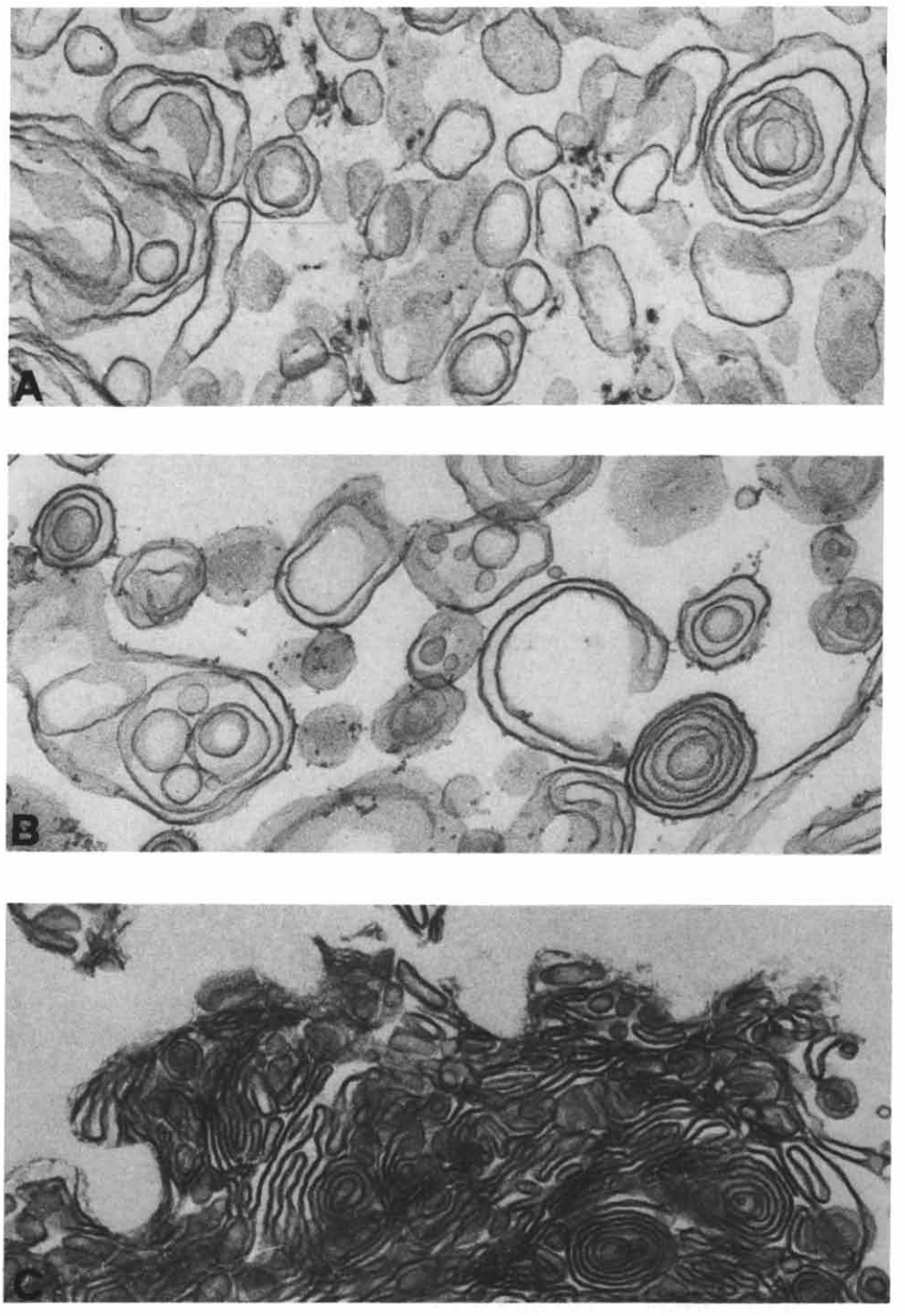

Fig. 1. Thin-sections of osmium-fixed lipids (×60000). (A) egg phosphatidylcholine/glutarylphosphatidylcholine/dicetyl phosphate (5 : 5 : 2.8); (B) egg phosphatidylcholine; (C) egg phosphatidylcholine/glutarylphosphatidylcholine (5 : 5).

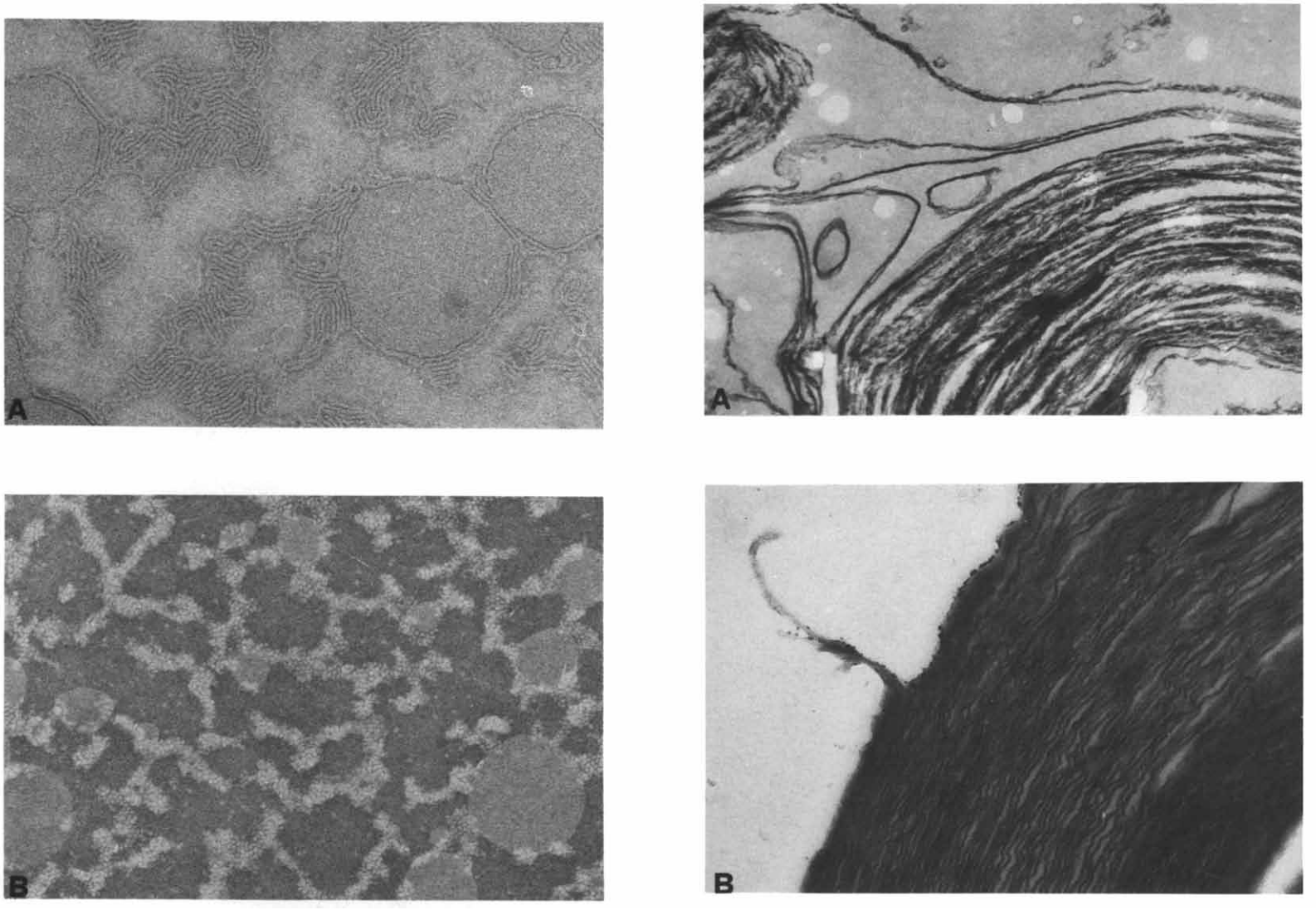

Fig. 2. Negative staining of glutaryl phosphatidylcholine micelles prepared with dicetyl phosphate (28%) (×140400). (A) Rod-like micelles; (B) Globular micelles.

Fig. 3. Thin sections of osmium-fixed lipids (×31200). (A) Dipalmitoylphosphatidylcholine/glutarylphosphatidylcholine (5:5); (B) Dipalmitoylphosphatidylcholine.

TABLE I

TURBIDITIES (400 NM) OF EGG PHOSPHATIDYLCHOLINE/GLUTARYLPHOSPHATIDYLCHOLINE MIXTURES DISPERSED IN A WATER PHASE

The total amount in each tube was 2.5 μmol phospholipid/2.5 ml water. egg PC, egg phosphatidylcholine; GL, glutarylphosphatidylcholine; DCP, dicetyl phosphate.

	Without DCP	With DCP (28.5%)
egg PC	1.6	1.2
egg PC/GL		
9:1	1.6	1.17
8:2	1.55	0.96
7:3	0.50	0.82
6:4	0.40	0.80
5:5	0.13	0.71

tidylcholine increased also viscosity and the inclusion of dicetyl phosphate in dipalmitoylphosphatidylcholine/glutarylphosphatidylcholine (molar ratio 5:5) mixture was followed by the restitution of the initial viscosity, i.e, a viscosity similar to that of control dipalmitoylphosphatidylcholine liposome.

TABLE II

INFLUENCE OF GLUTARYLPHOSPHATIDYLCHOLINE AND/OR DICETYL PHOSPHATE ON VISCOSITY OF DIPALMITOYLPHOSPHATIDYLCHOLINE

DPPC, dipalmitoylphosphatidylcholine; GL, glutarylphosphatidylcholine; TP, total phospholipid.

	Viscosities at 37°C			
	Without dicetyl phosphate		With dicetyl phosphate (28.5%)	
	TP, 6 mM [a]	TP, 10 mM	TP, 6 mM [a]	TP, 10 mM
DPPC	1	1.4	3	8
DPPC/GL (5:5)	1.5	14	1	3

[a] This phospholipid concentration is the same as that used for microscopic investigation.

These effects of dicetyl phosphate and/or glutarylphosphatidylcholine on viscosity of dispersions were more accentuated at a concentration of 10 mM phospholipid (Table II).

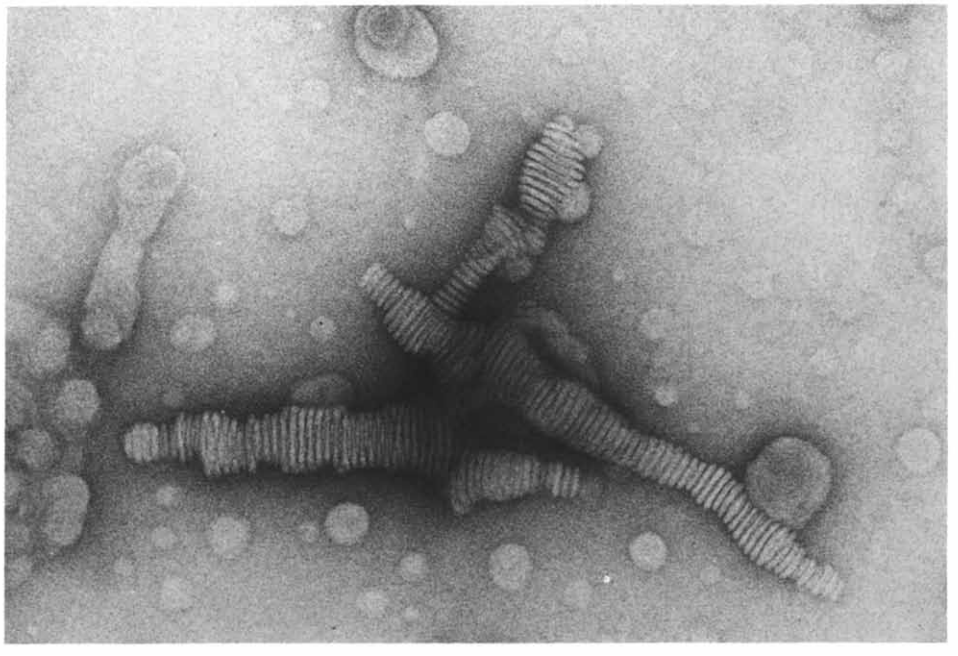

Fig. 4. Negative staining of dipalmitoylphosphatidylcholine/glutarylphosphatidylcholine/dicetyl phosphate (5:5:2.8) (×180000).

Discussion

In this study, the effect of glutarylphosphatidylcholine on phosphatidylcholine liposome morphology was examined by using multilamellar liposomes made of various glutarylphosphatidylcholine to monocarboxylic phosphatidylcholine (egg phosphatidylcholine or dipalmitoylphosphatidylcholine) molar ratios under conditions which altered the surface charge. In addition, the influence of glutarylphosphatidylcholine and/or dicetyl phosphate on membrane fluidity was measured.

Our data show that structural modifications in liposomes induced by glutarylphosphatidylcholine depend on the saturation of fatty acids of the phospholipids and on the surface charge.

With mixed egg phosphatidylcholine/glutarylphosphatidylcholine dispersions, at a glutarylphosphatidylcholine molar percentage of 50%, almost all the multilamellar structures are absent, with formation of myelinic-like figures (Fig. 1C). Addition of dicetyl phosphate is followed by the formation of mixed liposomes. At 50% of glutarylphosphatidylcholine, multilayered liposomes predominate with some unilamellar liposomes (Fig. 1A). Our turbidity observations and gel chromatography confirm these data on the effect of dicetyl phosphate.

The morphology of mixed dipalmitoylphosphatidylcholine/glutarylphosphatidylcholine suspensions is differently modified by dicetyl phosphate addition. We have performed microscopic observations only on its qualitative aspect as control of physicochemical investigations. On thin-sectioned preparations, in the absence of dicetyl phosphate, multilayered liposomes exist, similar to control dipalmitoylphosphatidylcholine liposomes, whereas with dicetyl phosphate the preparation gives no visible particle. Glutarylphosphatidylcholine also gives no visible particle. A similar observation is reported for a succinylphosphatidylcholine preparation [16]. For dipalmitoylphosphatidylcholine/glutarylphosphatidylcholine/dicetyl phosphate dispersions, the turbidity measurements reported previously [10] and the viscosity data are in favour of micelles. In fact, glutarylphosphatidylcholine, in the absence of dicetyl phosphate, increases the viscosity of dipalmitoylphosphatidylcholine liposomes. On the other hand, in the presence of dicetyl phosphate, the viscosity is similar to that of control liposomes (Table II). A lower microviscosity observed with dipalmitoylphosphatidylcholine/glutarylphosphatidylcholine in the presence of dicetyl phosphate compared to the microviscosity observed in the absence of dicetyl phosphate is likely to reflect the difference in the structure of these both dispersions. In fact, a higher viscosity is associated with a lamellar organization and a micellar organization with a reduced viscosity. In dipalmitoylphosphatidylcholine/glutarylphosphatidylcholine/dicetyl phosphate dispersions, there is probably an electrostatic repulsion between the negatively charged lipid molecules, followed by a highly fluid structure and there is formation of mixed dipalmitoylphosphatidylcholine/glutarylphosphatidylcholine/dicetyl phosphate micelles. This effect is not observed when phosphatidylserine or phosphatidic acid are included in dipalmitoylphosphatidylcholine/glutarylphosphatidylcholine dispersions, instead of dicetyl phosphate, because these anionic phospholipids provoke an increasing bilayer organization of the lipid structure.

With negative staining, mixed dispersions of dipalmitoylphosphatidylcholine/glutarylphosphatidylcholine/dicetyl phosphate exist as small bilayered structures which aggregate to form the rouleau structures observed (Fig. 4). Some disc-shaped structures were also observed by Inoue et al. [17]. This stacking is similar to that observed in mixtures of lecithin, cholesterol and bile salts [18]. We have observed structural differences between samples stained and dried at 25°C and 50°C. At 25°C, many large unilamellar liposomes are seen with a few disc-shaped structures. At 50°C, liposomes are smaller and a large number of disc-shaped structures is seen. In agreement with our results, Hui et al. [19], studying temperature-dependent morphology of natural sphingomyelin, have reported a change in structure observed in and above the transition temperature. At 55°C, sphingomyelin exhibits small disc-shaped structures. Negative staining is by far the simplest and the fastest technique available for structural studies of liposomes. However, this technique is prone to produce some artifacts by stacking of single vesicles and multilamellar structures [20] and the

extent of stacking is somewhat variable and depends on incubation time. Thus, negative staining of unfixed vesicles can result in a variety of structures that may be artifacts [21]. Nevertheless, negative staining has shown modifications confirmed by other techniques.

In conclusion, the present study shows clearly that the action of glutarylphosphatidylcholine on the organization of lipid mixtures depends on the nature of the lipids constituting the bilayer and on the presence of dicetyl phosphate. Previous results [10] have indicated that the presence of dicetyl phosphate in liposomes containing glutarylphosphatidylcholine affects the phase transition temperature of these liposomes. Viscosity measurements confirm the hypothesis that the formation of mixed micelles occurs in dipalmitoylphosphatidylcholine/glutarylphosphatidylcholine/dicetyl phosphate lipid dispersions. On the other hand, for egg phosphatidylcholine/glutarylphosphatidylcholine/dicetyl phosphate mixtures, an increase of the liposome stability is observed in the presence of dicetyl phosphate.

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