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Phase behaviour of mixtures of lipid X with phosphatidylcholine and phosphatidylethanolamine

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The effect of increasing concentrations of lipid X (2,3-bis(3-hydroxymyristoyl)-α-D-glucosamine 1-phosphate) on the phase behaviour of EPC (egg phosphatidylcholine) and EPE (egg phosphatidylethanolamine) is studied at a pH ⩾ 7 where lipid X carries one to two negative charges. Small amounts of lipid X (molar ratio ≈ 0.01) induce continuous swelling of EPC and EPE bilayers and consequently the formation of large unilamellar vesicles in excess water. In many respects, the effect of lipid X on EPC and EPE bilayers is similar to that of phosphatidic acid. However, lipid X/EPC mixtures form micelles in excess lipid X whereas mixtures of phosphatidic acid/EPC vesiculate at all ratios. The same is true for lipid X/EPE mixtures. Small unilamellar vesicles of an average diameter of 40 nm form spontaneously upon dispersion of a dry lipid X/EPE film (molar ratio = 10). Unsonicated dispersions of lipid X/EPC (molar ratio = 1) are subjected to pH-jump treatment which involves raising of the pH to 11–12 and subsequent lowering of the pH to between 7.5 and 8.5. Such a treatment has little effect on the vesicle size and size distribution as compared to a control dispersion at pH 8.2. The mean size is determined to be 92 ± 60 nm. Electron micrographs of freeze-fractured samples of lipid X/EPC (molar ratio = 1) reveal the presence of mainly micelles at pH 12. Upon lowering the pH to neutrality these micelles become unstable and aggregate/fuse rapidly to unilamellar vesicles (average diameter 95 ± 40 nm). Sonication of equimolar mixtures of lipid X and EPC at pH 7 yields small unilamellar vesicles of a diameter of 20–25 nm as well as mixed micelles of a size between 15 and 17 nm. This behaviour is again different from that of mixed EPC/phosphatidic acid dispersions which form small unilamellar vesicles. The presence of lipid X in such mixtures does not prevent the aggregation/fusion to larger vesicles during freezing of the dispersion. As with pure EPC bilayers, stabilization is, however, achieved in the presence of 10% sucrose. This indicates that the covalently bonded glucosamine group of lipid X cannot substitute water of hydration in neighbouring EPC molecules.

Introduction

Lipid X (2,3-bis(3-hydroxymyristoyl)-α-D-glucosamine 1-phosphate) is a precursor of lipopolysaccharides [1], which are a major component of the outer cell wall of Gram-negative bacteria. Lipopolysaccharides (endotoxins) are known to have a number of biological activities, for instance stimulatory and inhibitory effects on the immune system of mammals [2,3]. Since lipid X shows some of the activities of the lipopolysaccharides but little toxicity – it might even protect from the harmful effects of endotoxins [4,5] – there is considerable interest in its potential medical and clinical application.

The spontaneous formation of unilamellar lipid vesicles has been described in a series of recent publications [6–10]. It was shown that vesicles form spontaneously when a dry mixed film consisting of a bilayer-forming neutral or isoelectric phospholipid such as EPC and an appropriate detergent is dispersed in H_2O. It was also shown that the neutral lipid/detergent molar ratio is a critical parameter. An extension of this method is the production of small unilamellar vesicles of phosphatidic acid (diameter < 50 nm) by the pH-jump method [6]. This method involves the transient increase in the pH of the phosphatidic acid dispersion to 10–12. At this pH the phosphatidic acid molecule is fully ionized bearing two negative charges, i.e., one negative charge per hydrocarbon chain. In this state it resembles structurally a negatively charged detergent. Lipid X exhibits detergent-like properties at pH 7 when it carries one to two negative charges [11]. Under these

Abbreviations: EPC, egg phosphatidylcholine; EPE, egg phosphatidylethanolamine; EPA, egg phosphatidic acid.

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conditions it may be regarded as a structural analogue of phosphatidic acid. In the light of such an analogy the question arises as to whether lipid X has similar effects on bilayers of isoelectric EPC and EPE as phosphatidic acid.

In this work we study therefore the effect of increasing amounts of lipid X on the phase behaviour of EPC and EPE and related to this the effect of the pH jump method on mixed lipid X/EPC (EPE) dispersions. Furthermore, the stabilizing effect of mono- and disaccharides on small unilamellar EPC vesicles subjected to either freeze-drying and rehydration or freeze-thawing has been reported recently [12–14]. Lipid X is a glycophospholipid with a glucosamine ring as the central part of the molecule. The question arises whether this covalently bonded sugar moiety has a stabilizing effect on EPC bilayers, in a way mimicking the effects of externally added mono- or disaccharides. This question is important because the thermodynamic stability of small unilamellar vesicles is of main concern if these vesicles are to be used as drug carriers. Therefore, the question of the stability of small unilamellar lipid X/EPC vesicles (molar ratio = 1) during freeze-thawing is also addressed here.

Materials and Methods

Lipid X was synthesized and crystallized as described before [11]. All experiments reported here were carried out with the bis-Tris salt unless otherwise stated. Lipid X was pure by TLC, C, H, N, P microanalysis and mass spectrometry. The molecular weight of the bis-Tris salt used to calculate concentrations is 954. Egg phosphatidylcholine (EPC) and egg phosphatidylethanolamine (EPE) were purchased from Lipid Products (South Nutfield, U.K.) and were pure by TLC standards.

Preparation of protonated lipid X. The bis-Tris salt of lipid X was converted to the protonated form by ion-exchange chromatography using the cation exchange resin AG 50W-X2 (Bio-Rad, Glattbrugg, Switzerland). 5 g of the resin was suspended in water and poured into a small column. The resin was rinsed with 1 M HCl until the pH of the eluate was strongly acid. Excess HCl was removed by washing with distilled water until the effluent was neutral. About 40 mg of the bis-Tris salt marked with [^{3}H]dipalmitoylphosphatidylcholine was dispersed in water, applied to the column and eluted with distilled water. The radioactive fractions of the eluate were pooled and dried down in a rotary evaporator. The yield was 42%. The acid form of lipid X could be dispersed in water by heating to above 70 °C for about 1 min and subsequent vortexing. This procedure was repeated once or twice until a turbid, homogeneous dispersion was formed with an apparent pH of 1.8. Its ^{1}H-NMR spectrum showed no signal characteristic of the Tris cation.

Preparation of mixed lipid dispersions. Stock solutions of lipid X in CH_3OH and of phospholipids in $CHCl_3/CH_3OH$ (2:1, v/v) were prepared. Aliquots of each solution together with a trace of [^{3}H]dipalmitoylphosphatidylcholine as a marker were mixed to give the desired final lipid X/phospholipid molar ratio. The solvent was removed by rotary evaporation at room temperature and the lipid film formed was kept at a pressure of 12–16 hPa for about 15 min as indicated by a pressure gauge attached to the evaporator (PVK 600, Büchi, Switzerland). For experiments where the permeability of the vesicles was important, the film was dried under high vacuum at a pressure of 0.01 hPa or better for at least 1 h. The lipid mixture was dispersed in water or one of the following buffers (10 mM Tris (pH 7.0)/0.05% NaN_3; 10 mM Tris (pH 7.0)/0.05% NaN_3/10% sucrose; 10 mM Tris (pH 7.0)/0.05% NaN_3/50 mM NaCl; 10 mM sodium phosphate (pH 7.0); 5 mM Hepes (pH 8.2)/0.02% NaN_3) by vortexing for about 5 min.

Sonication of vesicles. A dispersion of a mixture of lipid X and EPC was made as described above. 3 ml of this dispersion were sonicated for about 45 min using a Branson E-30 sonicator with a titanium microtip. During sonication, samples were cooled with an ice-water mixture under a steady N_2 stream to minimize lipid degradation.

pH-jump treatment. Equimolar mixtures of lipid X and EPC were dispersed in H_2O/0.05% NaN_3 or in 5 mM Hepes buffer (pH 8.2)/0.02% NaN_3. The apparent pH of the lipid dispersion was raised to pH 10–12 by adding a small amount of 1 M NaOH. Solutions were kept at the high pH for 5 min; this treatment did not cause any significant lipid degradation as shown by TLC. The pH was then reduced with 1 M HCl to a pH between 7.5 and 8.5.

Freeze-thawing of lipid dispersions. Dispersions of lipid mixtures labeled with [^{3}H]dipalmitoylphosphatidylcholine in water or buffer were frozen at −30 °C for about 1 h and subsequently thawed at room temperature. The amount of multilamellar vesicles formed during freeze-thaw cycles was determined as described in the following paragraph.

Separation of multilamellar lipid vesicles. Dispersions were centrifuged at 12000 × *g* for 10 min. Large multilamellar vesicles sedimented under these conditions. The total lipid and the lipid remaining in the supernatant after centrifugation were determined by radiocounting aliquots in a Beckman LS 7500 scintillation counter.

Gel filtration. For particle size analysis lipid dispersions were chromatographed on calibrated Sepharose CL-4B (42–46 cm × 1 cm). Different columns were used in the course of this study varying in bed height. Lipid X-containing samples varied in viscosity. More viscous samples produced some contraction of the gel bed,

leading to a decrease in the void volume V_0 and the total column volume V_t. The column was equilibrated and eluted with the same buffer used to disperse the lipid. The flow rate was 6.3 ml/h. Fractions of 0.3 ml were collected and analyzed for their lipid content. The recovery of lipid was 91 ± 14% (mean and S.D. of the mean of nine experiments). For the determination of the lipid X/EPC ratios in the eluent two consecutive fractions were combined and dried under N_2. The lipid was taken up in 100 μl of $CHCl_3/CH_3OH/H_2O$ (130:60:10, v/v) and 30 μl were applied to a TLC plate (Merck, Silica gel 60). The same $CHCl_3/CH_3OH/H_2O$ mixture was used as the solvent. The plates were exposed to I_2 vapour for a short period of time and lipids were detected by I_2-staining. Lipid bands were extracted with 1 ml of the same solvent mixture at 50°C and subsequent vortexing for 30 s. The silica gel was removed by centrifugation at 2500 rpm for 10 min. The extraction was repeated twice, the extracted lipids were pooled, and phosphate was determined [10]. For the determination of the internal volume of vesicles the lipid was dispersed in buffer containing 50 mM NaCl and a trace of $^{22}NaCl$ (2–10 μCi per experiment). The $^{22}Na^+$-labeled lipid dispersions were chromatographed and the $^{22}Na^+$ present in each fraction was determined by radiocounting using a Kontron γ-ray counter (GAMMAmatic I). 97 ± 5% (13 measurements) of the total amount of $^{22}NaCl$ was recovered.

^{31}P-NMR. Proton-decoupled ^{31}P powder NMR spectra were recorded at room temperature on a Bruker CXP-300 Fourier transform spectrometer operating at a ^{31}P frequency of 121.47 MHz. Chemical shifts were referenced to external 85% orthophosphoric acid.

Electron microscopy. Lipid X/phospholipid mixtures were prepared as described above. Electron microscopy of freeze-fractured samples was carried out as published previously [7,15].

Results

Phase behaviour of unsonicated mixed lipid X/EPC and lipid X/EPE dispersions

Fig. 1 shows the effect of increasing proportions of lipid X incorporated into bilayers of EPC and EPE. The absorbance at 340 nm and the amount of phospholipid recovered in the supernatant after centrifugation of the mixed phospholipid dispersions at 12000 × *g* for 10 min are plotted as a function of the lipid X/phospholipid molar ratio. For both mixed lipid X/EPC (Fig. 1A) and lipid X/EPE dispersions (Fig. 1B) there are three regions where the absorbance and the amount of lipid in the supernatant are constant. At molar ratios under 0.005, most of the lipid is sedimented: 65% and 80% for lipid X/EPC and lipid X/EPE, respectively. At molar ratios between 0.01 and 0.5 most of the lipid is recovered in the supernatant: 77% for lipid X/EPC and

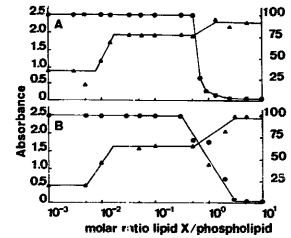

Fig. 1. Absorbance (●) and percentage of lipid recovered in the supernatant (▲) of unsonicated lipid X/EPC (A) and lipid X/EPE (B) as a function of the lipid/phospholipid molar ratio. The phospholipid mixtures were dispersed in H_2O at 10 mg/ml. Absorbance at 340 nm was used as a measure of turbidity. The amount of lipid in the supernatant was determined by radiocounting after centrifugation of the lipid dispersion at 12000 × *g* for 10 min.

65% for lipid X/EPE. Up to a molar ratio of 0.5 both dispersions are turbid and their absorbance remains at the original value of 2.5. At molar ratios of at least 3, dispersions of both phospholipids become optically clear and all lipid is present in the supernatant after centrifugation at 12000 × *g* for 10 min (cf. Fig. 1A and B).

In order to characterize the lipid aggregates prevalent at different lipid X/phospholipid molar ratios (cf. Fig. 1), freeze-fractured preparations were investigated by electron microscopy. Electron micrographs of lipid X/EPC and lipid X/EPE dispersions are shown in Fig. 2A–D and Fig. 2E–H, respectively. At a lipid X/phospholipid molar ratio of 0.001, large, multilayered, particles prevail in both lipid dispersions (Fig. 2A and E). At molar ratios of 0.01, large, highly swollen multilamellar (Fig. 2 B and F, insets), oligolamellar as well as unilamellar vesicles are present. Frequently the smooth fracture face characteristic of EPC is replaced by faces exhibiting patching or beading (cf. Fig. 2E and 2F). Mixtures of approximately equimolar composition (Fig. 2C and G) consist of single-shelled vesicles which are considerably smaller in size than the lipid particles in the preparations discussed above. The size of the vesicles is 50–200 nm with a mean size of 100 nm; sometimes larger vesicles are seen that contain several smaller vesicles within their cavity (Fig. 2G). These latter, oligomeric vesicles are, however, clearly different from the multilayered (multilamellar) vesicles seen in Figs. 2A, B, E and F. The mixtures with the highest lipid X/phospholipid molar ratio (= 10) contain the smallest particles. They are significantly smaller than unilamellar vesicles and presumably represent mixed micelles (see arrows. Fig. 2D and H). Many of these micellar structures appear to be rod-shaped, sometimes bent at least for lipid X/EPC (Fig. 2D). Besides these very small

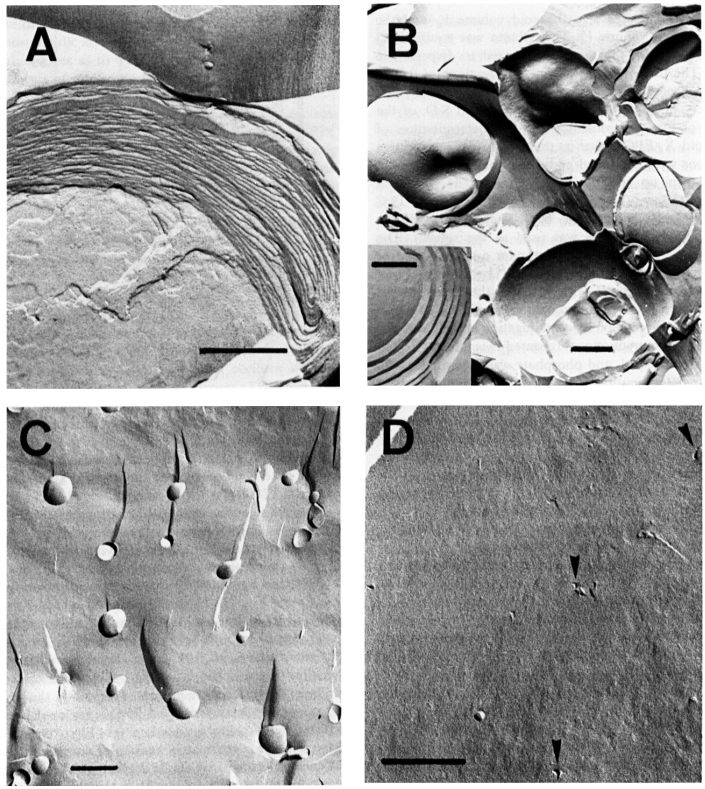

Fig. 2. Electron micrograph of freeze-fractured samples of unsonicated mixed lipid X/EPC (A–D) and lipid X/EPE dispersions (E–H). The lipid/phospholipid molar ratios were 0.001 (A and E); 0.01 (B, F; the insets show highly swollen multilamellar vesicles which are observed at this molar ratio); 0.7 (C); 0.5 (G); 10 (D, H). The phospholipid mixtures were dispersed in H_2O at 10 mg/ml. The bars represent 300 nm.

particles, small unilamellar vesicles of a diameter of 20–60 nm with a mean diameter of 40 nm are also present in excess lipid X; in lipid X/EPE dispersions (molar ratio = 10) these vesicles are even the predominant particles (Fig. 2H).

The conclusions drawn from electron microscopy are supported by ^{31}P-NMR. Mixed lipid X/EPC dispersions (molar ratio = 0.001) give a ^{31}P powder spectrum which is identical to that of a pure EPC dispersion (Fig. 3A and B). The line shape is characteristic of an axially symmetric chemical shielding tensor. This and the chemical shielding anisotropy, $|\Delta\sigma| = 53$ ppm, indicate that the phospholipid is present in a liquid-crystalline bilayer undergoing rapid anisotropic motion about the bilayer normal. At a lipid X/EPC molar ratio of 0.1 (Fig. 3C) the chemical shielding anisotropy is reduced

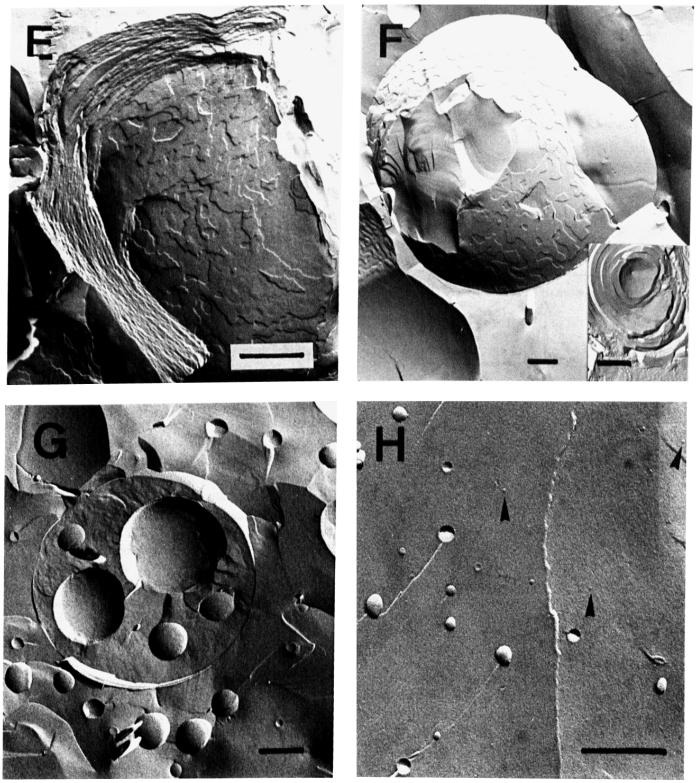

Fig. 2 (continued)

to 45 ppm and a small isotropic signal appears. As the lipid X/EPC molar ratio increases further, the intensity of the isotropic signal grows at the expense of the powder pattern (Fig. 3D). In excess lipid X the powder pattern collapses and is replaced by a high-resolution spectrum (Fig. 3E). It consists of two resonances: the major downfield one is assigned to lipid X, the minor upfield signal to EPC based on the intensity ratio of 9; the value of this ratio calculated from the chemical composition is 10. Similar effects are observed when increasing amounts of lipid X are incorporated into EPE bilayers. Pure EPE gives a ^{31}P powder spectrum characteristic of an axially symmetric shielding tensor with $|\Delta\sigma| = 42$ ppm (Fig. 3F). There is a very small isotropic component at 0 ppm superimposed on the powder spectrum. With increasing quantities of lipid X the intensity of the isotropic signal grows at the expense of the intensity of the powder spectrum (Fig. 3G and H). Excess lipid X (lipid X/EPE molar ratio > 1) produces a ^{31}P high-resolution NMR spectrum consist-

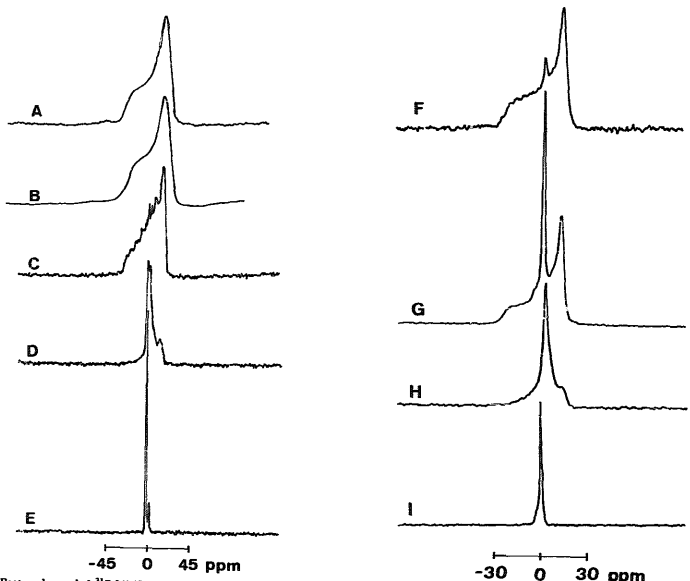

Fig. 3. Proton-decoupled ^{31}P-NMR spectra of unsonicated lipid X/EPC(EPE) dispersions recorded at room temperature (22° C) on a CXP 300 Bruker Fourier transform spectrometer operating at 121.47 MHz. Phospholipids were dispersed in H_2O pH 8.2 at a concentration of about 100 mg/ml. (A) Pure EPC; (B) to (E) lipid X/EPC dispersions of molar ratio of 0.001 (B), 0.1 (C), 0.7 (D) and 10 (E); (F) pure EPE; (G) to (I) lipid X/EPE dispersions of molar ratio of 0.001 (G), 0.7 (H) and 10 (I).

ing of a main signal at 1 ppm and a high-field shoulder (Fig. 3I). In analogy to the lipid X/EPC dispersion (Fig. 3E) and based on the chemical composition, the main signal is assigned to lipid X and the upfield shoulder to EPE. The slight shoulder at low field could be due to some lysoEPE formed during the NMR experiment.

Mixed dispersions of lipid X and EPC (molar ratio = 1) were originally adjusted to a pH between 1.8 and 8.3; the pH was then raised to pH 11 ± 0.5 and immediately decreased to values of 8.0 ± 0.5. This treatment is referred to as pH jump [6–10]. After this treatment the dispersions were centrifuged at 12000 × *g* for 10 min to remove large multilamellar particles. Results are summarized in Table I. If the original pH measured after dispersing the lipids is lower than 4.5, about 30% of the phospholipid is spun down; if the original pH is higher than 4.5, the proportion of large particles that sedimented is about 5%. The average size and the size distribution of the phospholipid particles present in the supernatant after the pH jump were analyzed by gel filtration on Sepharose CL-4B. Unsonicated lipid X/EPC dispersions (molar ratio = 1) at pH 8.2 served as control. The elution patterns of such dispersions are shown in Fig. 4A and B. In this control experiment all the phospholipid is eluted as a broad asymmetric peak at or close to the void volume. Subjecting the unsonicated lipid X/EPC dispersion to the pH-jump treatment had little effect on the elution pattern: the main peak is still broad and eluted at about V_0; however, the peak is clearly asymmetric, with a shoulder towards larger V_e, indicating that some smaller particles are produced by this procedure. Fig. 4B (+) shows the elution pattern of lipid X/EPC dispersion (molar ratio = 1) whereby the protonated form of lipid X was used to make up the mixture. The pH jump was started at pH 1.8. The pattern is representative of mixtures dispersed at an original pH < 4.5. The phospholipid particles

are eluted as a single asymmetric peak at V_0 with a shoulder towards higher V_e values. The shoulder is more pronounced the higher the final pH of the dispersion. The higher the final pH, the greater is apparently the fraction of smaller particles. The phospholipid particles present in mixed lipid X/EPC dispersions are fairly stable when stored at 4°C for 2 days. This is shown in Fig. 4C. The elution curve (Fig. 4C) is characteristic of unsonicated lipid/EPC dispersions (molar ratio = 1) which were subjected to the pH-jump treatment similarly to the dispersion described in Fig. 4A. The elution pattern after incubating this dispersion for 2 days at 4°C is similar but not identical to the original one (Fig. 4C). The phospholipid vesicles are eluted as a single peak which is narrower and shifted towards V_0, indicating that some aggregation has taken place during incubation. The gel filtration results indicate that unsonicated mixed dispersions of lipid X/EPC (molar ratio = 1) at about neutral pH both before and after the pH-jump treatment consist mainly of vesicles larger than 60 nm. This is concluded from the fact that most of the lipid is eluted in the void volume of the Sepharose CL-4B column. The presence of some smaller vesicles with a diameter less than 60 nm is indicated by the asymmetry and the shoulder of the elution peak.

The conclusions derived from gel filtration are confirmed by freeze-fracture electron microscopy (Fig. 5A and B). Mixed lipid X/EPC dispersions (molar ratio = 1, 5 mM Hepes (pH 8.2)) were subjected to the pH-jump method. Their pH was raised to 11.6 and then returned to 8.2. The electron micrograph (Fig. 5A) shows that such dispersions consist of unilamellar vesicles with an average diameter of 95 ± 40 nm. Electron micrographs

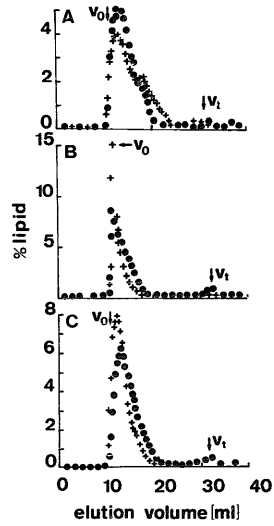

Fig. 4. Gel filtration of lipid X/EPC dispersions (molar ratio = 1) on Sepharose CL-4B. Effect of pH jump and storage on average vesicle size and size distribution. Phospholipid mixtures were dispersed in 5 mM Hepes buffer (pH 8.2)/0.02% NaN_3 at 16 mg/ml except for the dispersion under (B). The Sepharose CL-4B column was equilibrated and eluted with the same solvent in which the phospholipid mixture was dispersed. Amounts of lipid eluted at each elution volume, V_e, are given as percentage of the total lipid applied to the column. (A) Phospholipid dispersion in 5 mM Hepes (pH 8.5)/0.02% NaN_3 (●); the same dispersion was brought to pH 11.5 by adding NaOH and then returned to pH 8.5 by adding HCl (+). (B) Phospholipid dispersion in H_2O (pH 8.2)/0.02% NaN_3 (●); this sample was compared with a phospholipid dispersion in HCl (pH 1.8)/0.02% NaN_3 subjected to a pH jump by raising the pH to 11 by adding NaOH and bringing the pH back to 7.6 by adding HCl (+). (C) A mixed lipid X/EPC dispersion (molar ratio = 1) in Hepes buffer (pH 8.2) was adjusted to pH 11.5 and then titrated back to pH 8.2 and chromatographed immediately (●). An aliquot of the same dispersion was stored at 4°C for 2 days and then chromatographed (+). All phospholipid mixtures contained a trace amount of [^{3}H]dipalmitoylphosphatidylcholine for lipid analysis.

TABLE I

The effect of pH-jump treatment on unsonicated mixed lipid X/EPC dispersion

For the phospholipid dispersions, the pH jump is defined by three pH values, the first one is the original pH of the dispersion, the second is the maximum pH to which the pH of the dispersion is raised, and the third value given is the final pH. The amount (percent) phospholipid present in the supernatant of unsonicated lipid X/EPC mixed dispersions (molar ratio = 1) was determined after centrifugation at 12000 × *g* for 10 min. Results are presented as the mean ± S.D. of the mean (n = 5–10 experiments).

Phospholipid dispersion	% phospholipid in supernatant	Dispersion medium
Lipid X/EPC (control experiment)	94 ± 4	5 mM Hepes (pH 8.2)/ 0.02% NaN_3
Lipid X/EPC subjected to pH jump:		
pH ≤ 4.5 → 11 → 8.0	70 ± 10	dilute HCl
4.5 < pH < 8.2 → 11 → 8.0	95 ± 3	dilute HCl
pH 8.2 → 11 → 8.2	100 ± 2	5 mM Hepes (pH 8.2)/ 0.02% NaN_3

of the control experiment which is a lipid X/EPC mixture of the same composition dispersed in buffer pH 8.2 show also mainly unilamellar vesicles with an average diameter of 92 ± 60 nm. This indicates that the pH-jump treatment has practically no effect on the size and size distribution of lipid X/EPC dispersions (molar ratio = 1). Fig. 5C is an electron micrograph of an equimolar lipid X/EPC dispersion which was taken to

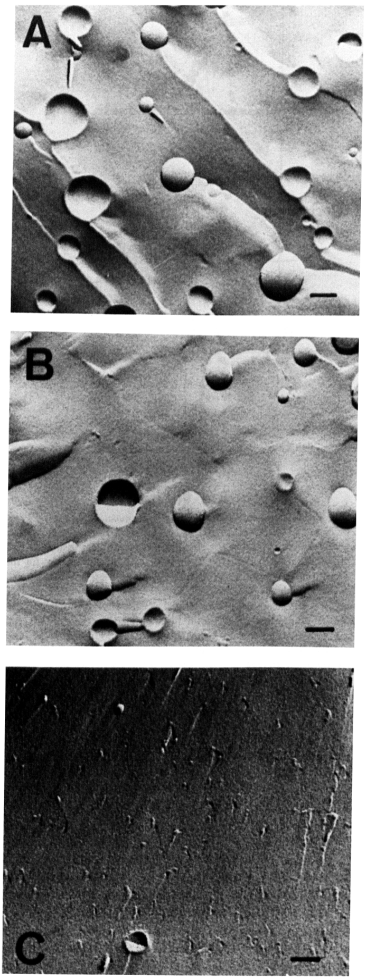

Fig. 5. Electron micrographs of freeze-fractured preparations of unsonicated mixed lipid X/EPC dispersions (10 mg/ml, molar ratio = 1) in 5 mM Hepes (pH 8.2)/0.02% NaN_3. (A) The dispersion was subjected to pH-jump treatment: the pH was raised to 11.6 and then returned to 8.2. (B) Dispersion in 5 mM Hepes (pH 8.2)/0.02% NaN_3 (control experiment). (C) The pH of the dispersion was raised to 12 and the sample was immediately freeze-fractured. The bars represent 100 nm.

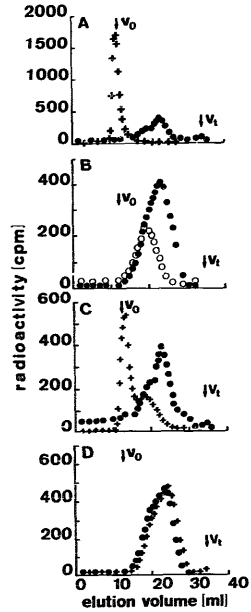

Fig. 6. Gel-filtration on Sepharose CL-4B of sonicated mixed lipid X/EPC dispersions (10 mg/ml, molar ratio = 1) in 10 mM Tris (pH 7.0)/0.05% NaN_3. Effect of freeze-thawing. (A) Sonicated dispersion (●); for comparison, the elution profile of an unsonicated lipid X/PC dispersion (molar ratio = 1) is included (+); (B) sonicated dispersion in the same Tris buffer, containing 50 mM NaCl with a trace of $^{22}NaCl$, lipid analysis (●), $^{22}Na^+$ (○); (C) sonicated dispersion in Tris buffer before (●) and after (+) one freeze-thaw cycle; (D) sonicated dispersion in Tris buffer containing 10% sucrose before (●) and after (+) one freeze-thaw cycle. For the lipid analysis a trace amount of [^{3}H]dipalmitoylphosphatidylcholine was added to the lipid dispersion. The two chromatograms in A, C and D were normalized to constant areas.

pH 12 and immediately freeze-fractured. At this pH, lipid X is fully ionized and acts as a detergent solubilizing the host EPC bilayer to small mixed micelles. The prevailing particles present in this electron micrograph (Fig. 5C) are mixed micelles. Their size is significantly smaller than the average size of small unilamellar phospholipid vesicles of 20–30 nm. Some residual small unilamellar vesicles with a diameter less than 100 nm are still present under these conditions (Fig. 5C).

Effect of sonication on mixed lipid X/EPC dispersions

The effect of sonication on mixed dispersions of lipid X/EPC (molar ratio = 1) in 10 mM Tris buffer (pH 7.0)/0.05% NaN_3 is shown in Fig. 6A. While an unsonicated lipid mixture of the same composition is eluted as a single sharp peak at the column void volume, the elution profile of a sonicated dispersion consists essentially of a peak at $V_e = 23.3$ ml and a shoulder at $V_e = 19.2$ ml. The origin of the shoulder becomes evident when a trace amount of $^{22}NaCl$ is added to the buffer. After sonication $^{22}Na^+$ will be entrapped in the cavity of vesicles surrounded by an impermeable bilayer. As shown in Fig. 6B, ^{22}Na is eluted at $V_e = 19.2$ ml and not with the main peak at $V_e = 23.3$ ml. The radioactivity returns to background level before the elution of the main peak is completed. It is well known that $^{22}Na^+$ is entrapped in unilamellar phospholipid vesicles; since it was shown that $^{22}Na^+$ does not bind to lipid X micelles [11] the shoulder at $V_e = 19.2$ ml in Fig. 6B is assigned to vesicles of lipid X/EPC and the main peak at $V_e = 23.3$ ml to mixed micelles. The elution pattern of Fig. 6B can be used to decompose the composite elution pattern shown in Fig. 6A (●). The data derived from this analysis are summarized in Table II. In sonicated lipid X/EPC dispersions about 40% of the phospholipid is present as unilamellar vesicles with an average hydrodynamic radius of 11.5 ± 0.5 nm and about 60% as micelles with an average hydrodynamic radius of 8.5 ± 0.5 nm. Within the error of the measurement the molar ratio lipid X/EPC was unity over the total chromatogram, i.e., the molar ratio lipid X/EPC of both unilamellar vesicles and micelles was identical with the original composition of the dispersion. From the $^{22}Na^+$ entrapment the average internal volume of the unilamellar vesicle is determined as $3.4 \cdot 10^{-3}$ cm^3/mol of lipid, whereas the value calculated from the average radius of 11.5 nm is $0.24 \cdot 10^{-3}$ cm^3/mol of lipid. This discrepancy is probably due to a counterion effect. ^{22}Na will be enriched at the bilayer/water interface due to the negative surface potential, ψ_0, of the lipid X/EPC bilayer. As a result of the higher $^{22}Na^+$ concentration at the inner monolayer of the bilayer more $^{22}Na^+$ is encapsulated when the bilayers seal and unilamellar vesicles are formed.

Stability of small unilamellar vesicles made of lipid X/EPC

The question of whether or not small unilamellar vesicles of lipid X/EPC (molar ratio = 1) are stable against freezing and drying is of considerable interest. For this purpose, small vesicles were produced by sonication of aqueous dispersions of lipid X/EPC (molar ratio = 1) in the presence and absence of 10% sucrose. These dispersions were then subjected to freeze-thaw cycles as described under Materials and Methods. When sonicated mixed lipid X/EPC dispersions in H_2O (pH 8.2) or in 10 mM phosphate buffer (pH 7.0) were freeze-thawed once and then centrifuged at $1.000 \times g$ for 10 min, 75% and 65% of the phospholipid, respectively, were spun down to a pellet. This indicates that in the absence of sucrose one freeze-thaw cycle is sufficient to induce marked aggregation/fusion. In contrast,

TABLE II

Vesicle size analysis of sonicated and unsonicated lipid X/EPC dispersions (molar ratio = 1) by gel filtration on Sepharose CL-4B. The effect of freeze-thawing on the vesicle size and vesicle size distribution

Results are presented as the mean ± S.D. of the mean. Averages were calculated from 2–4 experiments. Peak intensities are expressed as % of the eluted lipid.

Phospholipid dispersion		Particle size analysis							
Preparation	solvent [a]	V_e (ml)	peak intensity	V_e (ml)	hydrodynamic radius (nm)	peak intensity	V_e (ml)	hydrodynamic radius (nm)	peak intensity
Unsonicated	buffer, no sucrose	V_0	88 ± 5						
Sonicated	buffer, no sucrose	–	–	19.2	12.1 ± 0.8	45 ± 10	23.3	8.5 ± 0.6	46 ± 12
Sonicated	buffer with 10% sucrose	–	–	18.9	10.5 ± 0.2	42 ± 5	23.8	7.7 ± 0.4	60 ± 10
Sonicated	buffer with 50 mM NaCl, no sucrose	–	–	18.8	11.0 ± 0.2	48 ± 10	22.8	8.4 ± 0.3	65 ± 10
Sonicated, after first freeze-thaw cycle	buffer, no sucrose	V_0	50 ± 10	18.1	12.0 ± 0.4	45 ± 1	–	–	–
Sonicated, after first freeze-thaw cycle	buffer, with 10% sucrose	–	–	21.4	11.4 ± 0.1	62 ± 15	25	8.2 ± 0.2	34 ± 5

[a] Unless otherwise stated the dispersion buffer was 10 mM Tris (pH 7.0)/0.05% NaN_3.

if dispersions were made in either Tris buffer (pH 7.0)/40 mM NaCl or in 10% sucrose solutions with or without buffer (10 mM Tris (pH 7.0) or 10 mM phosphate (pH 7.0)) and subjected to freeze-thaw cycles as described above, most if not all of the lipid (95 and 100%, respectively) was recovered in the supernatant.

The effect of freeze-thawing on the vesicle size of sonicated mixed lipid X/EPC dispersions was also studied by gel filtration on Sepharose CL-4B. In Fig. 6C elution profiles of sonicated lipid X/EPC dispersions in 10 mM Tris buffer (pH 7.0)/0.05% NaN_3 are presented before and after freeze-thawing. Comparison of the two elution profiles reveals (Fig. 6C) that freeze-thawing leads to aggregation/fusion in the absence of sucrose; about 40–60% of the phospholipid is eluted in the void volume, indicating that large particles with a diameter above 60 nm are present. At the same time, the peak at $V_e = 23.3$ ml corresponding to mixed micelles is greatly reduced, while the fractional population of unilamellar vesicles eluted at $V_e \simeq 18.1$ ml and corresponding to a hydrodynamic radius of 11 ± 1 nm remains approximately the same (Table II). In the presence of 10% sucrose the aggregation/fusion process described in Fig. 6C is suppressed: there is no peak in the void volume (Fig. 6D, Table II) and almost identical elution profiles were obtained if the sample was applied before and after freeze-thawing.

Discussion

The two pK values of lipid X are 1.3 and 8.2 [11]; at pH $\geq$ 7 lipid X carries therefore one to two negative charges. Lipid X spontaneously forms micelles when dispersed in H_2O [11]. As discussed previously [11], it may be regarded as a structural analogue of phosphatidic acid, with the glucosamine ring replacing the glycerol group of phosphatidic acid. Both lipid X and phosphatidic acid bear two negative charges when fully ionized, i.e., one negative charge per hydrocarbon chain. Under these conditions both lipids can be expected to behave like detergents. This is indeed the case (cf. Refs. 6–11). However, there is a significant difference in the behaviour of the two fully charged molecules. While lipid X forms small micelles when dispersed in H_2O at a pH > 7, phosphatidic acid has been shown to undergo spontaneous vesiculation at pH > 7 [6–10]. Phosphatidic acid apparently remains in the smectic (lamellar) phase throughout the pH range 2–12 [6,7]. Subjecting phosphatidic acid to the pH-jump treatment by transiently raising the pH to 10–12 leads to the formation of small unilamellar vesicles with a diameter of 20–50 nm [6,7,10].

It was shown first by Luzzati and his co-workers [16,17] and is now well accepted that EPC doped with only a few percent of a positively or negatively charged lipid exhibits continuous swelling with increasing water content. As a result, the mixed dispersion forms large unilamellar vesicles in excess water [18–21]. This type of behaviour has been verified for mixed dispersions of EPC/phosphatidic acid [6,7,10,19,20,22].

Here we study the effect of lipid X on EPC and EPE bilayers over a range of lipid X/phospholipid molar ratios of about four orders of magnitude, from 0.001 to 10. The results derived from different physical methods including freeze-fracture electron microscopy, ^{31}P-NMR and gel filtration on calibrated Sepharose CL-4B are in good agreement. At very low contents of lipid X (molar ratios $\leq$ 0.001) multilamellar vesicles are formed which are indistinguishable from those of pure EPC and EPE (Fig. 2A and E). Incorporating lipid X in EPC or EPE bilayers at molar ratios $\geq$ 0.01 has the effect that the limiting swelling properties of EPC and EPE [23–25] are changed to continuous swelling characteristic of charged lipids. This conclusion is based on the observation that at lipid X/phospholipid molar ratios of at least 0.01, multilamellar vesicles are increasingly replaced by large unilamellar vesicles. At neutral pH lipid X bears one negative charge and behaves therefore similarly to phosphatidic acid or any other negatively charged diacylphospholipid of approximately cylindrical shape. Mixed lipid X/EPC and lipid X/EPE dispersions become optically clear in excess lipid X (lipid X/phospholipid molar ratio $\geq$ 3). A centrifugation routine that removes large multilamellar particles fails to produce any significant pellet (Fig. 1), indicating the presence of small lipid particles under these conditions. Their nature is revealed by ^{31}P-NMR (Fig. 3D and I) and freeze-fracture electron microscopy (Fig. 2D and H). These methods provide unambiguous evidence that mixed micelles are the dominant lipid particles in lipid X/EPC dispersions (molar ratio = 10, Fig. 2D). In contrast, in lipid X/EPE dispersions at molar ratios in excess of 1, small unilamellar vesicles are predominant and mixed micelles represent a minor fraction (Fig. 2H). Both EPE [26] and lipid X [11] have a tendency to form highly curved aggregates, at least in the liquid-crystalline state [26]. The fact that in mixtures of these two molecules the bilayer structure is stabilized (cf. Fig. 2H) may be due to a complementary shape effect. The area/molecule for EPE in the liquid-crystalline state is determined by the space requirement of the two hydrocarbon chains which exceed the space occupied by the headgroup significantly [26]. The projection of the two hydrocarbon chains onto the bilayer plane is 50 Å^2, the projection of the headgroup is 42 Å^2. Thus the shape of the EPE molecule is conical expanding towards the hydrocarbon chain end. This is opposite to the shape of lipid X as discussed previously [11]. Therefore, relatively small amounts of EPE might counteract the tendency of lipid X for forming highly curved micelles and hence stabilize mixed bilayers.

Sonicated mixed dispersions of lipid X/EPC (molar

ratio = 1) are also different compared to EPA/EPC dispersions. More than half of the lipid of such lipid X/EPC dispersions is present in small mixed micelles with a diameter of 17 nm (Table II). These mixed micelles are larger than the micelles present in pure lipid X dispersions which have a diameter of 12.6 nm [11]. The larger size of these mixed micelles may be attributed to the presence of EPC, the proportion of which probably determines the micellar size. In contrast, sonicated EPA/EPC dispersions (molar ratio = 1) consist of small unilamellar vesicles similar to those vesicles present in pure sonicated EPA or EPC dispersions [6,27].

Lipid X also behaves differently from phosphatidic acid as to the pH jump. As shown by gel filtration (Fig. 4) and electron microscopy (Fig. 5), the pH-jump treatment of lipid X/EPC dispersions (molar ratio = 1) has relatively little effect on the vesicle size and size distribution compared to control dispersions. The average vesicles size and size distribution resulting after the pH-jump treatment depends somewhat on the starting and final pH. This is also different from the pH-jump treatment of EPA/EPC dispersions. In the latter case the resulting average vesicle size is rather insensitive to the final pH; size and size distribution depend mainly on the maximum pH to which the dispersion is raised transiently and the kinetics of the pH increase [6,7]. Whatever the mechanism of the pH-jump method of mixed EPA/EPC dispersions, the difference in the behaviour of the two lipid dispersions during the pH jump is a mechanistic one. When lipid X/EPC mixed dispersions are exposed to high pH (pH 10–12) the bilayers disintegrate and small mixed micelles are formed (Fig. 5C). Apparently, upon reducing the pH to about 8 these micelles become unstable and aggregate/fuse rapidly to unilamellar vesicles (cf. Fig. 5A and B). The vesicles formed by this treatment are significantly larger than small unilamellar vesicles produced by sonication or pH-jump treatment of unsonicated phosphatidic acid dispersions.

The experiments on the stability of small unilamellar lipid X/EPC vesicles (molar ratio = 1) clearly show that lipid X incorporated into the EPC bilayer has no stabilizing effect, at least not up to equimolar concentrations. Addition of 10% sucrose is required to suppress the fusion of vesicles during freeze-thaw cycles. Hence, lipid X containing vesicles are not different in their stability from pure EPC vesicles [12–14]. This result is consistent with the water replacement hypothesis, which stipulates that stabilization of phospholipid bilayers during dehydration or freezing in the presence of externally added mono- or disaccharides is brought about by sugar molecules replacing water of hydration [28–30]. Evidently, the covalently bonded glucosamine ring of lipid X cannot replace water of hydration in neighbouring EPC molecules and thus stabilize the bilayer. Like the glycerol group in glycerophospholipids, the sugar ring is the central part or backbone of the lipid X molecule. As such, it can be envisaged to be motionally restricted and therefore to have a preferred orientation at the lipid/water interface. This preferred orientation and the lack of flexibility probably do not allow the glucosamine ring of lipid X to interact effectively with the hydration sites of neighbouring phospholipid molecules.

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