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A metastable state of high surface activity produced by sonication of phospholipids

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Abstract

Sonication of phosphatidylcholine dispersions generates a metastable high energy assembly of molecules, the existence of which is revealed by its conspicuous surface activity. Freshly sonicated liposome dispersions release molecules to the air/water interface at rates sufficient to produce a close-packed monolayer within minutes. In contrast, monolayers at the surface of multilamellar and extruded vesicles take hours to form. The highly surface active species appears within the first few minutes of sonication, long before a major reduction in turbidity occurs, and accumulates over the course of a few hours of sonication. It disappears upon exhaustive sonication, extrusion, addition of extruded vesicles, or, more slowly, simply on standing. Tests for extraneous substances in the lipids before as well as after sonication revealed amounts of degradation products too small to represent the observed surfactant. Direct evidence that the metastable aggregate releases intact phospholipids was provided by a novel procedure to characterize monolayer composition by comparing surface tension with surface potential, both as a function of surface density. Centrifugation and gel filtration chromatography indicate that the surface activity is associated with a particle of diameter larger than a lysophosphatidylcholine micelle but not larger than limit sonicated vesicles. The metastable material appears to be lipid molecules in other than the normal stable vesicular state, perhaps an incompletely closed vesicle, one in which the inner and outer monolayers have not equilibrated, or possibly a micellar form.

Key words: Surface activity; Phospholipid; Sonication; SUV; Vesicle

1. Introduction

Lipid bilayer vesicles serve as model membranes for basic research in many areas of science and technology [1]. Although lipid vesicles are often regarded as invariant structures, they are non-covalently linked aggregates of molecules, the actual state of which depends on the conditions of formation and environment of storage. Common preparations of vesicles consist of populations of non-equilibrium species. Thus, not only will the mean structure change with time but at a given time the extremes of the distribution may differ considerably from the mean. This variability is important, since the extremes can dictate the behavior of the entire sample.

In the process of measuring the tension of monolayers that form at the surface of liposome suspensions [2–7], we observed remarkable effects of sonication of the liposomes on the rate of monolayer formation. Hand-shaken or extruded dispersions equilibrated with their air/water interface quite slowly, as would generally be expected from the relatively slow exchange of constituent molecules in and out of these vesicles [8]. In contrast, when a suspension of multilamellar vesicles (MLVs) is sonicated and examined shortly thereafter, it is found that a monolayer forms at the air/water interface very rapidly. Further investigation showed that the surface tension-lowering activity is due to a population of metastable particles, the molecules of which readily migrate from the particle to the air/water interface. Although the surfactive particle has a transient existence and presumably represents a

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Abbreviations: MLVs, multilamellar vesicles; SUVs, small unilamellar vesicles; LUVETs, large unilamellar vesicles made by extrusion; DOPC, dioleoylphosphatidylcholine; POPC, palmitoyloleoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; LPC, monooleoyl phosphatidylcholine; OA, oleic acid.

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minor fraction of the total population, it constitutes by far the major source of either loosely associated molecules or exposed hydrophobic surface in the population. It could therefore have an inordinate influence on measurements of processes that involve free molecules, such as intervesicle molecular exchange, or on hydrophobically driven binding interactions such as interactions of intrinsic membrane proteins with vesicles.

It has long been recognized that small unilamellar vesicles (SUVs) are not the lowest energy form of lipid bilayers, since molecules migrate from the small to the large vesicles in such populations [9]; however, only recently has it been appreciated that other structures may be generated by high energy processes like sonication. Indeed, theoretical examinations of vesicle formation and stability have indicated that non-vesicle bilayer forms can have energies that are comparable to those of SUVs and hence are expected to be present in detectable quantities in dispersions subjected to the kinds of forces needed to generate SUVs [10–13]. Defects in gel phase lipids were noted years ago [14], but the present report seems to provide the first indication that liquid crystalline phase phospholipids can be organized in a form that, at least kinetically, if not also thermodynamically, is considerably more unstable than SUVs.

2. Materials and methods

Chemicals

Dioleoylphosphatidylcholine (DOPC), palmitoyloleoylphosphatidylcholine (POPC), dimyristoylphosphatidylcholine (DMPC) and monooleoylphosphatidylcholine (LPC) were purchased from Avanti Polar Lipids (Alabaster, AL). Their purity was verified by thin-layer chromatography. Oleic acid (OA) and sodium oleate were purchased from Sigma (St. Louis, MO). Building supply deionized water was charcoal-filtered, redeionized and successively passed through a Barnstead organic-free cartridge and a 0.22 micron filter.

Vesicle preparation

Multilamellar vesicles (MLVs). The lipids were stored in HPLC-grade chloroform. After most of the solvent was removed under a stream of argon, the samples were placed under oil pump vacuum for 2 h to assure removal of residual solvent. Subsequently, the lipid was hydrated to a concentration of 10 mg/ml water and vortexed for about 1 min to produce a uniform, milky suspension of MLVs.

Small unilamellar vesicles (SUVs). Unless otherwise indicated, 1 ml of MLV suspension at a concentration of 10 mg/ml was sonicated under argon in a screw cap, borosilicate glass tube with a bath sonicator (Laboratory Supplies, Hicksville, NY). Ice was added to the bath water to keep the temperature under 14°C. The sonicated suspension was then diluted to 1 mg/ml with deionized water. For some experiments, the suspension was centrifuged at $27000 \times g$ for 40 min at 10°C.

Large unilamellar vesicles made by extrusion (LUVETs). MLVs were extruded with a syringe extruder [15] containing a polycarbonate membrane (Costar) with 100 nm pores. After extrusion eleven times, the radii of vesicles are uniformly distributed around 100 nm as determined by analysis of dynamic light scattering of an 488 nm Argon ion laser beam with a Brookhaven Instruments BI2030 Digital correlator.

Surface tension measurement

Surface tension was determined by means of the detachment variation of the Wilhelmy method [5]. A 0.5 mm diameter platinum wire was used as probe. Teflon troughs of 6 mm radius were raised and lowered four times per min on an oscillating platform. The force exerted on the probe was continuously measured with a Cahn electrobalance. The latter was interfaced (Scientific Solutions LabMaster, Solon, OH) to a computer running under Asyst software, allowing for data acquisition and analysis. When the probe detached from the liquid surface, the maximum force exerted on it was determined and the program computed the surface tension from that force. Calibration was with clean, freshly aspirated water.

Since surface tension reflects the molecular density of the monolayer, the higher the rate at which surface tension falls, the faster the monolayer forms. To compare the rate at which lipid molecules are transported from vesicle suspensions to the monolayer at the air/water interface under various conditions of vesicle treatment, we measured the time-courses of surface tension change for vesicle suspensions and calculated the initial rate at which surface tension fell. This initial rate was averaged over the first 10–20 min following removal of residual monolayer by aspiration of the surface.

Surface potential measurement

Surface potential was measured according to the ionizing electrode method [16] with a polonium air electrode (Cartridge Model 1C200R, NRD, Grand Island, NY) and a commercial pH meter reference electrode connected to an electrometer voltmeter (Model 610B, Keithley Instruments, Cleveland, OH). The surface tension was monitored simultaneously.

Gel-filtration

DOPC vesicles sonicated in water or 1 mM NaCl. MLVs of DOPC at 10 or 25 mg/ml were sonicated for 90 min and a 0.2 ml aliquot taken for chromatography

on a 1 × 14 cm Sepharose 4B column. 40 0.3 ml fractions were collected at a flow rate of 0.15 ml/min. After the absorbance at 400 nm was measured to identify fractions containing vesicles, their surface tensions were measured at various times in troughs of 0.5 cm^2 surface area and 0.3 cm depth. Suspensions were aspirated individually to remove surface molecules and to generate a relatively clean surface of approximately 72 mN/m tension. The time of aspiration was taken as zero time and the surface tension of each solution was determined at recorded intervals. Some experiments were performed in 1 mM NaCl solution as well as in water, but no effect of this amount of electrolyte on the formation of SUVs or on gel filtration elution profiles was detected.

To generate a population of SUVs, 1 ml of 10 mg/ml DOPC in 1 mM NaCl solution was sonicated for 6 h, 0.2 ml of which was chromatographed on a Sepharose 4B column as described in the previous paragraph.

DOPC vesicles in the presence of sodium oleate or lysophosphatidylcholine. DOPC-OA: A mixture of 100 μl of 2 mg/ml DOPC MLVs and 10 μl of a 1 mg/ml sodium oleate solution (OA represents 11 mol% of the total lipid) were eluted from a Sepharose CL-4B column (1 × 14 cm). 40 0.3 ml fractions were measured for absorbance and surface tension.

DOPC-LPC: A mixture of 100 μl of 10 mg/ml DOPC MLVs and 30 μl of an aqueous solution of 1 mg/ml LPC (LPC represents 4 mol% of the total lipid) were eluted from the Sepharose CL-4B column (1 × 14 cm) with water. 0.3 ml fractions were measured for absorbance and surface activity.

DOPC vesicles suspended in 0.1 M NaCl. Some gel filtration experiments were done in 0.1 M NaCl to compare the elution volume of the surface activity with that of authentic SUVs; the latter vesicles form less readily in water than in the presence of this concentration of electrolyte. Chromatography was done on several different media with different exclusion limits.

A 0.5 ml portion of 20 mg/ml DOPC in 0.1 M NaCl was sonicated for 60 min in the bath sonicator. 100 μl of this suspension was applied to each of three gel filtration columns (1 × 14 cm) packed with Sephacryl S-1000, Sepharose 4B or Sepharose CL-4B. 0.3 ml fractions were measured for absorbance and surface activity.

Thin-layer chromatography

Lipids were analyzed by thin-layer chromatography on plates of Silica gel 60 F (MC/B) developed with chloroform/methanol/water (65:25:4, v/v). Lipids were stained in iodine vapor. Hydrolysis products in sonicated DOPC were quantified by the following procedure. Before and after sonication for 1 h, 0.05 ml of a 10 mg/ml DOPC vesicle solution was vacuum dried. 25 μl of chloroform was added to dissolve the lipid, which was chromatographed as described above. The limits of detection were 1 μg for LPC and 2 μg for OA.

3. Results

The rate of formation of a monolayer at the air / water interface of a vesicle suspension depends greatly on vesicle preparation

Previous investigations have revealed that the transfer of lipid from bulk phase to the air/water interface is influenced by vesicle size, specifically, that the tension of the monolayer is considerably higher at the surface of a suspension of small vesicles than at the surface of larger vesicles [17]. Although theoretical analyses have rationalized this experimental finding [2,18,19], it is not entirely understood. We therefore sought to investigate the phenomenon more thoroughly and began by comparing the tension at the interface of several different types of vesicles.

Fig. 1 shows the surface tension as a function of time at the air/water interface of DOPC MLVs (circles), LUVETs (filled circles) and sonicated vesicles (triangles). As may be seen, large differences in vesicle-to-monolayer transport rates are observed; however, differences in the tensions at equilibrium were difficult to establish for monolayers that formed slowly. One of

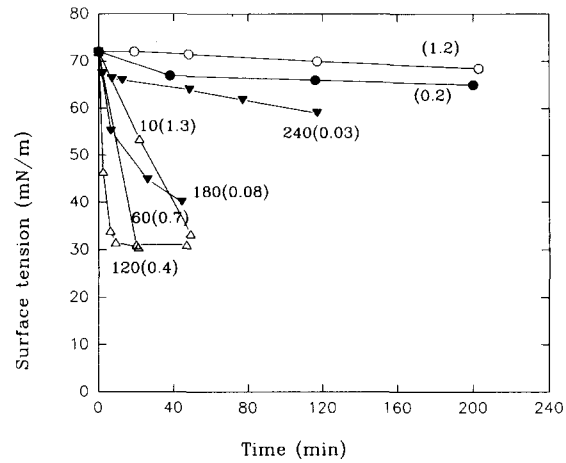

Fig. 1. Surface tension, as a function of time, of vesicle suspensions formed by different methods. The surface tension of sonicated vesicle suspensions (triangles) fell quickly, whereas that of MLV (circles) or LUVET suspensions (filled circles) remained above 60 mN/m for more than 3 h. The longer the sonication at temperatures lower than 14°C, the faster the surface tension fell (triangles). Experiments were done at several intermediate times but were omitted from the figure for clarity. When sonication is continued beyond about 2 h in the absence of cooling (temperatures between 25 and 40°C), the rate of surface tension decrease was reduced (filled triangles). Numbers outside parentheses are sonication times and numbers within parentheses are absorbance at 400 nm.

the sets of open circles shows that, in the case of multilamellar vesicle dispersions, the surface tension of the air/water interface scarcely changed from that of water for at least 3 h. The other set of open circles shows that, during the same time, the surface tension of a LUVET suspension fell to about 65 mN/m. This represents a significant and easily-measured rate of transfer, which nevertheless was small compared to a MLV suspension that had been briefly sonicated (triangles). A monolayer with a surface tension as low as 30 mN/m was formed in as few as 10 min at the surface of a vesicle suspension which had been subjected to sonication for 120 min. Corresponding phenomena were observed with sonicated vesicles of DMPC or POPC.

Also shown in Fig. 1 are several experiments in which sonication was continued for more than 2 h (filled triangles). The absorbance at 400 nm (numbers in parentheses) of vesicle suspension diminished with duration of sonication (numbers outside the parentheses), suggesting that the multilayered vesicles were indeed being broken down into smaller particles. Initially, surfactivity increases with decrease in absorbance. This pattern changes at about 2 h of sonication; when vesicles are subjected to longer periods of sonication, the surface active species is lost. Indeed, after 4 h of sonication, the rate of fall of tension has nearly returned to the low rate exhibited by LUVETs. As will be seen below, the surface activity produced by sonication is metastable and elevated temperatures accelerate its disappearance; however, as discussed below, it seems unlikely that the temperature effect alone is responsible for the loss of surface activity observed in the experiments of Fig. 1.

To rule out possible contaminants from the glass tube used in sonication, a Teflon container was substituted for the glass container. Sonication with a probe sonicator (Microson sonifier; 30% output power) of DOPC in this vessel resulted in suspensions with essentially the same surfactive properties as those prepared by bath sonication in glass containers.

The surface tension-lowering activity of sonicated dispersions is not removed by centrifugation

A MLV suspension of 1 mg/ml DOPC was sonicated until the absorbance at 400 nm decreased from 1.1 to 0.8 A_{400}, a much smaller change than that which accompanies preparation of true limit-sonicated SUVs (1.1 to < 0.1 A_{400}). Following sonication, the solution was centrifuged at $27\,000 \times g$ for 40 min and the supernatant decanted from the pellet. The latter was resuspended in water so that its turbidity was equal to that of the original dispersion. A portion of the supernatant was extruded through a polycarbonate membrane filter with 100 nm diameter pores. The results of surface tension measurements on these three preparations are shown in Fig. 2. The resuspended pellet behaved like MLVs, while the supernatant behaved like a sonicated vesicle suspension even though its concentration of 0.6 mg/ml, as determined by phosphate assay, was significantly lower than that prior to centrifugation. The behavior of the extruded sample with respect to rate of monolayer formation was intermediate between that of the pellet and that of the supernatant. Centrifugation was sufficient to pellet the largest MLVs, and subsequent extrusion of the supernatant visibly reduced its turbidity from 0.1 to 0.05.

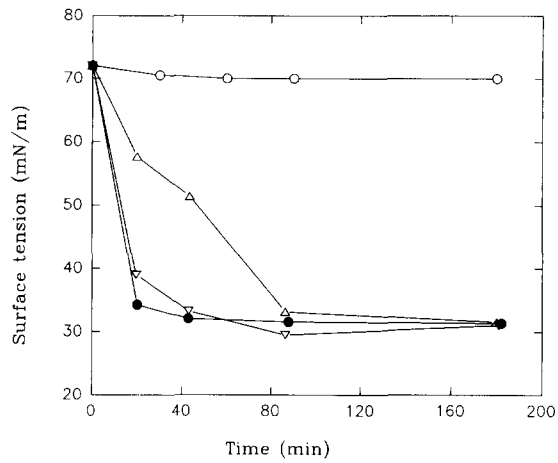

Fig. 2. Time-course of surface tension of briefly sonicated vesicles (filled circles), their supernatant after centrifuging (inverted triangles), resuspended pellet (circles) and supernatant subjected to a subsequent extrusion through a filter with 100 nm pores (triangles). The supernatant exhibited the same rate of monolayer formation as the sonicated vesicle suspension. A subsequent extrusion reduced the adsorption rate.

It is evident from the results shown in Fig. 2 that extrusion causes the surface activity produced during sonication to diminish. Since small (relative to the 100 nm pores of the filter) particles should not be affected by extrusion, it appears likely that the effect of extrusion results from fragmentation of residual large vesicles rather than passage of small vesicles through the filter. As will be seen below, the addition of a large amount of bilayer surface in the form of extruded vesicles also reduces the surface activity of sonicated suspensions. The concentration of lipid in the supernatant did not change after extrusion, at least to within experimental error of the phosphate assay, about $\pm 5\%$, so the reduction of surface activity cannot be attributed to a loss of lipid.

Monooleoylphosphatidylcholine and oleic acid mimic some of the effects of sonication on monolayer formation

To explore the effect of possible breakdown products, such as lyso lipid and fatty acid, generated by sonication [20], we prepared two kinds of vesicle suspensions containing these compounds. One prepara-

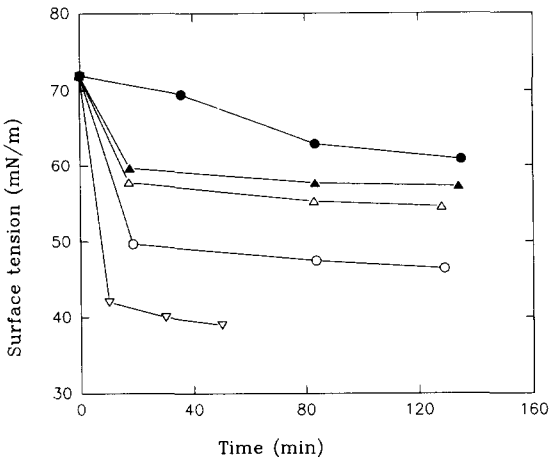

Fig. 3. Time-course of monolayer formation at the surface of vesicle suspensions containing DOPC mixed with LPC and OA prepared in two ways: (a) All lipids were mixed in chloroform followed by removal of solvent and then hydration to form MLVs (filled circles) or LUVETs (filled triangles); (b) A suspension of DOPC multilamellar vesicles was mixed with a solution of LPC and OA of 8 mol% (inverted triangles) or 4 mol% (circles). The suspension prepared by method (b) had higher surface activity than that prepared by method (a). A subsequent extrusion of solution prepared by method (b) containing 4 mol% reduced the surface activity (triangles).

tion consisted of MLVs generated from mixtures of DOPC, LPC and OA in chloroform. The other preparation consisted of preformed DOPC MLVs suspended in an aqueous phase in which LPC and OA were dissolved. In this case, the vesicle suspension was added to the solution and surface tension measurements were begun thereafter. These two preparation methods were followed since it was not clear whether breakdown products were more likely to be produced in the interior of the liposome and subsequently equilibrate with the aqueous phase, or whether they might be preferentially generated at the surface of vesicles from which equilibration with the aqueous phase and, potentially, mixed micelle formation, would be rapid. The two samples thus represent the two extremes of component distribution; however, after complete equilibration, both samples would be expected to give the same results (equilibration times were not determined). As shown in Fig. 3, the preparation in which the degradation products were dissolved in the aqueous phase reached a lower surface tension within a shorter time than that in which the single chain compounds were included in the liposome phase. This result clearly shows that greater surface activity is produced by the hydrolysis products when they are present in micelles or as monomers. As the concentrations of LPC and OA dissolved in the aqueous phase were increased from 4 mol% to 8 mol% (corresponding to 28 and 55 μg/ml of LPC and 15 and 31 μg/ml of OA), the time-course of surface tension decline came to resemble that of the sonicated vesicle suspensions.

Thin-layer chromatographic analysis shows the concentration of hydrolysis products in sonicated DOPC is too low to give rise to significant surface activity

By measuring the rate of surface tension decline of solutions of OA and LPC, it was determined that the lowest concentrations that exhibit a surface activity similar to that of the sonicated vesicle suspensions were 10 and 50 μg/ml, respectively. To determine whether such large amounts of OA and LPC were produced by sonication, a suspension of DOPC (10 mg/ml) sonicated for 1 h was vacuum dried and the residue taken up in chloroform for TLC analysis. By comparing this sample with standards containing the same amount of DOPC as the sonicated sample but varying amounts of OA and LPC, it was determined that the sonicated material contained less than 2 μg/ml or 0.6 mol% of LPC and less than 5 μg/ml or 2.7 mol% of OA. It is thus evident that even if all of the LPC and OA produced during sonication were in solution (as micelles and monomers), the solution could not exhibit as high a surface activity as the sonicated DOPC suspension. Vesicles were omitted from the surface tension tests but, as indicated in the previous section, the surface activity of such concentrations of hydrolysis products would be even lower in the presence of vesicles. Thus, decomposition of phosphatidylcholine during sonication cannot account for the surface activity generated during sonication of the lipid.

Surface potential measurements show that the monolayer that forms over sonicated DOPC vesicle suspensions consists overwhelmingly of DOPC

Although the surface activity of OA and LPC was inadequate to explain the surfactivity of sonicated vesicles, it was nevertheless desirable to be able to directly examine the composition of the monolayer itself to verify that it did indeed consist of DOPC and that we had not overlooked some mechanism that could lead to rapid transfer of a minority component to the air/water interface. Such a test was devised which involved the simultaneous measurement of surface tension and surface potential of the monolayer.

Although isotherms of the relationship between surface area and either surface potential or surface pressure are lipid dependent, information about monolayer composition cannot be acquired from the tension or the potential relationship alone. We found, however, that the relationship between surface tension and surface potential is quite characteristic of a given lipid. Therefore, such potential-tension plots can, in principle, be used to distinguish a monolayer composed of pure lipids from one composed of a mixture. To vali-

date this procedure, aliquots of lipid in chloroform were delivered onto the surface of water or suspension of MLV with a microliter syringe. After the chloroform had evaporated in about 2 min, the surface potential and the surface tension were measured. The tension and potential were thus changed stepwise between their extreme values corresponding to a clean surface and a collapsed monolayer, i.e., between 72 and approximately 30 mN/m. Fig. 4 shows the relation between surface tension and surface potential for monolayers of pure DOPC, LPC, OA and a mixture with molar ratios of 3 : 1 : 1 (DOPC/LPC/OA). At any given surface tension, pure DOPC had the highest surface potential and OA the lowest. The differences are large and easily measurable. The mixture had a potential-tension relationship that was intermediate between those of the pure materials.

It was evident from results such as those in Fig. 4 that this simple procedure could reveal whether or not the monolayer which formed over sonicated lipid suspensions was predominantly DOPC or not. Accordingly, we obtained a time-course of values of tension and potential on the surface of sonicated vesicles simultaneously. As the monolayer forms, the tension falls and the potential becomes more positive. As also shown in Fig. 4, the tension-potential relationship obtained from the monolayer generated by the sonicated

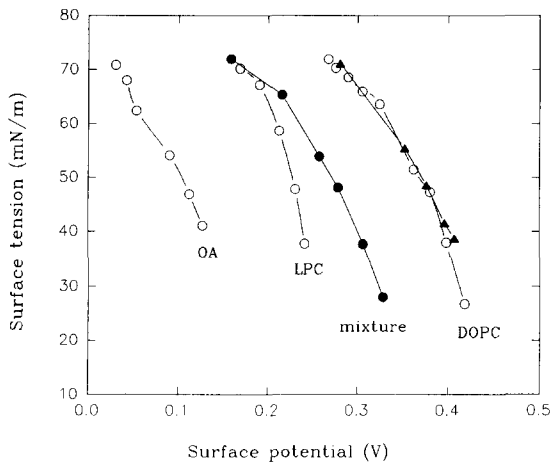

Fig. 4. Surface potential depends on the monolayer composition. Surface tension and surface potential were measured simultaneously and plotted against each another. All monolayers were formed by spreading from chloroform solution except that represented by the triangles. For a given tension, monolayers of DOPC had highest surface potential, LPC the next highest and OA the lowest. A mixture of lipids (filled circles, DOPC/OA/LPC in mole ratio 3:1:1) gives a surface potential that is intermediate between those of pure components. A monolayer formed from sonicated vesicles exhibited a surface tension-surface potential relationship (filled triangles) indistinguishable from that of a DOPC monolayer formed by spreading (circles).

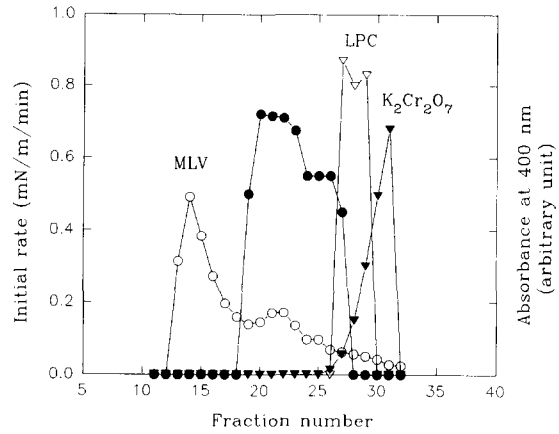

Fig. 5. Gel filtration chromatography of sonicated vesicle suspensions. 0.5 ml of a 20 mg/ml dispersion of DOPC was sonicated for 30 min. 200 μl of the suspension was applied to a Sepharose 4B column and eluted with 1 mM NaCl. The absorbance as well as the initial rate of change of the surface tension of the column fractions was measured immediately after their collection. Filled circles represent the initial rate of surface tension decline. Triangles and filled triangles represent the surface activity of an LPC solution and the absorbance of dichromate ion, respectively, which were used to calibrate the column in a separate experiment. In another experiment, a DOPC vesicle solution sonicated for 6 h was used to indicate the elution of small vesicles. The turbidity measurement (open circles) shows that small vesicles are eluted between fraction numbers 19 and 25. The peak of initial rate of surface tension change overlaps with the peak representing the small vesicles, well before the elution of either micelles or small molecules.

vesicle suspension (filled triangles) did not show significant differences from that of pure DOPC. Since a contamination of 20% of LPC or 10% of OA is easily seen, it is clear that the monolayer could not contain more than these amounts of impurities, at the very most. Furthermore, similar amounts of any other impurity, unless it has a tension-potential relationship very much like that of DOPC, is also ruled out for the same reasons.

Gel-filtration indicates that the surface active species generated by sonication resides in a particle nearly as large as SUVs

If the high adsorption rate of lipid molecules to the air/water surface from sonicated vesicle suspensions is due to single molecules in solution or to some particle of unusually small size, removal of those species would presumably reduce the rate of formation of a monolayer from the residual vesicles. To further discriminate among the components of the vesicle suspension, a sonicated vesicle suspension was fractionated on a Sepharose 4B column (Fig. 5). This column was calibrated with MLVs and potassium dichromate. The peak of MLVs appeared at the 15th fraction, while that of chromate appeared at the 32nd fraction. By adding 100 μl of 1 mg/ml LPC to the column and measuring

the time-course of the change of surface tension of the fractions subsequently eluted with 1 mM NaCl, we established that LPC was eluted at fractions 27–29. After a sonicated vesicle suspension (0.2 ml of 20 mg/ml of DOPC containing 1 mM NaCl) was also passed through the column, the time-courses of surface tension were measured for the fractions of interest. The initial rate at which the surface tension fell, along with the turbidity of each of those fractions, is plotted vs fraction number in Fig. 5. The initial rate reached a maximum in those fractions eluted before the position of elution of micelles of LPC and indicates that the surface activity is not associated with monomeric species such as lyso compounds or fatty acids, or micelles thereof. Indeed, when the chromatographic behavior of SUVs, prepared by sonicating DOPC for 6 h, was compared with that of the surfactivity generated by sonication for 1 h, surface activity emerged with small vesicles, but the peak was less skewed toward the leading edge than was the small vesicle peak. The trailing of the latter was not due to size differences; dynamic light scattering measurements showed that the small vesicles in all fractions were about 200 Å in diameter. The surface active particle is thus clearly larger than LPC micelles, and the same as or slightly smaller than DOPC SUVs.

Gel filtration chromatography of DOPC vesicles was also carried out in the presence of OA and LPC to further characterize the behavior of these phospholipid degradation products. It was found that when DOPC MLVs were mixed with an aqueous phase containing OA (12 mol% relative to DOPC) and chromatographed, the surface activity eluted at the position of the MLVs. Evidently, OA partitioned into the MLVs and although these may have fragmented, the resultant particles were too large to be retarded on Sepharose 4B. In contrast, when DOPC MLVs were mixed with an LPC solution (4 mol% LPC relative to DOPC) and chromatographed, no significant surface activity was detected at any elution volume, although there was a large light scattering peak at the position expected for MLVs. It thus appears that with relatively small concentrations used here, the LPC dissociates from micelles (and presumably also vesicles into which it had partitioned) while the sample is moving down the column, so that the LPC is too dilute in all the fractions to generate significant surface activity.

The surface active material is metastable and disappears more rapidly, the higher the temperature

If the high transfer rate of lipid to the air/water interface is a consequence of a metastable structure, one might expect that thermal energy would facilitate the conversion of the metastable state to a stable state. To test this hypothesis, we incubated sonicated vesicles

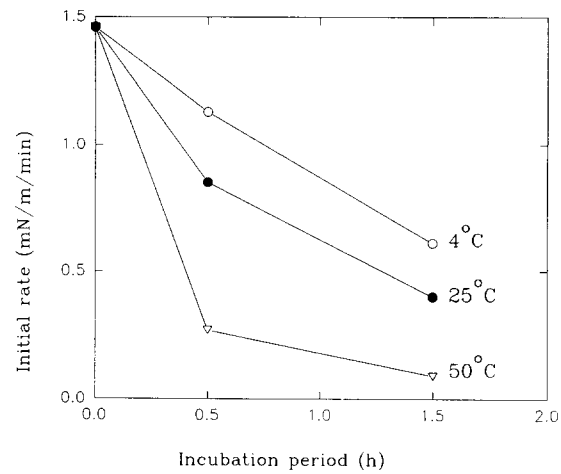

Fig. 6. Initial rate of change of surface tension of suspensions of sonicated vesicles that were incubated at elevated temperatures. DOPC was sonicated for 1 h at a temperature below 14°C. The sample was divided into three parts and each was incubated at a different temperature, as indicated in the figure. At the times shown on the abscissa, portions were removed and the rate of change of the tension of their air/water surface was measured. Surface activity generated by sonication was lost at a rate that increased with increasing temperature.

at different temperatures and observed the consequences of these manipulations on the rate of monolayer formation at their surfaces. A vesicle suspension of 10 mg/ml DOPC was sonicated for 60 min, diluted to 1 mg/ml and then incubated at room temperature for 30 min. The rate of decline of the surface tension of this sample was measured and it was immediately divided into three portions which were then incubated at 4, 25 and 50°C. Subsequently, the time dependence of the surface tension of these samples was measured twice, after 30 min and again after 90 min. The initial rates of surface tension decline vs. incubation time at these different temperatures are plotted in Fig. 6. At the beginning of the incubation, the initial rate was 1.5 mN/m per min for all samples. The loss of surface activity is seen at all temperatures, but its elimination is dramatically faster at 50°C than at 4°C. It is noteworthy that, in spite of the large decrease in surface activity in the sample held at 50°C for 1.5 h, its absorbance at 400 nm did not measurably change.

The surface active species is inactivated by interaction with MLVs or LUVETs

Since both MLVs and LUVETs release lipid at low rates to the surface of their suspensions, it was of interest to examine the surface activity of mixtures of these vesicles with solutions containing the surfactive species generated by sonication. We mixed MLVs and LUVETs with the supernatant obtained by centri-

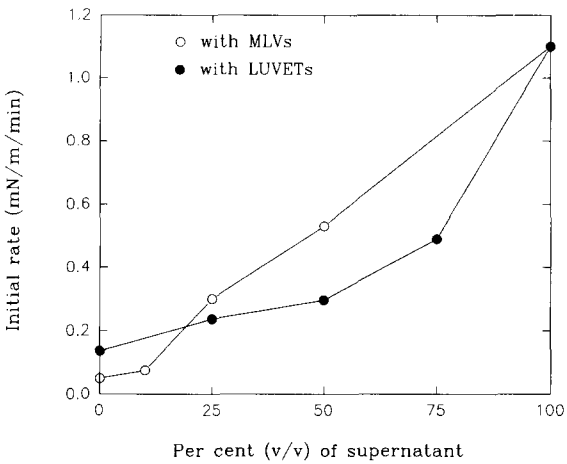

Fig. 7. Effect of hand-shaken and extruded vesicles on the surface activity generated by sonication of DOPC. DOPC was sonicated in water for 1 h. The suspensions were then mixed with DOPC vesicles prepared by extrusion (filled circles) or by simple shaking (circles) to give mixed suspensions of the composition shown on the abscissa. The initial rate of change of surface tension of these mixtures was then determined. The effect of combining sonicated vesicles with MLVs was linear, indicating simple dilution of the sonication-induced surfactivity. The curve in the case of LUVET mixtures is non-linear; it increased with the volume percentage of LUVETS slowly up to 75%, whereupon it rose much more rapidly. This dependence indicates that the LUVETS dictated the surface activity until they became the minority species, i.e., LUVETS are capable of partially neutralizing the surface activity of sonicated vesicles.

fuging sonicated vesicles and subsequently measured the initial rate of decline of surface tension. In Fig. 7, these initial rates are plotted vs. the volume percentage of supernatant contained in the sample. In the MLV mixtures, the initial rate varied linearly with the volume fraction of sonicated supernatant. In the LUVET mixtures, however, this relationship was highly non-linear; for mixtures of between 50 and 75% supernatant, surface activities are low and increase only slowly with an increasing proportion of supernatant. The lines between 0 and 50% supernatant are reasonably linear and the ratio of their slopes, about 10:1, indicates that the LUVETs are an order of magnitude more efficient than MLVs in suppressing the surface activity of sonicated lipid suspensions. It would not be surprising if 90% of the bilayers were internal in the case of MLVs, so the behavior represented by Fig. 7. may well reflect the amount of membrane surface facing the bulk aqueous phase.

4. Discussion

Contaminants or lipid degradation products are not alone responsible for high surface activity of sonicated vesicle solutions

Brief sonication of multilayer vesicle suspensions leads to a change of an order of magnitude in the rate of spreading of lipid molecules at the air/water interface. Moderately sonicated suspensions generate a monolayer at close packing, whereas MLVs and LUVETs have low lipid release rates at their air/water surfaces. Since probe sonication in a Teflon container gave rise to the same phenomenon as bath sonication in glass, it is unlikely that the active material was derived from the container. Other potential candidates for the surface active material were the hydrolysis products of DOPC, LPC and OA. Indeed, when an extruded or hand-shaken suspension of DOPC was treated with these two single-chain compounds, the result was a mixture whose surface tension also fell quite rapidly (compare Figs. 1 and 3). Nevertheless, for a number of reasons, it appears very unlikely that hydrolysis products can account for the surface activity of the sonicated vesicle suspension: (1) The amount of hydrolysis products required to mimic the surface activity of sonicated vesicles is far greater than the amounts detected by TLC analysis, which were much too low for a significant effect on the surface activity. (2) Although the concentrations of hydrolysis products were too small to account for the surface activity of the sonicated vesicle suspension, they would be adequate to cover the surface. Nevertheless, the surface potential-surface tension relationship of the monolayer formed from the sonicated vesicle supernatant was such that molecules other than DOPC could have represented no more than 10–20% of the total. (3) Sepharose 4B column chromatography behavior was inconsistent with the surface activity being associated with micelles or single molecules. (4) The observed metastability of the surface activity is inconsistent with a degradation product generated during sonication.

The observed surface activity of sonicated suspensions appears to be due to an intermediate state that has defects and / or structural instabilities

Since chemical change per se seems not to account for sonication-induced surface activity, a physical change must be considered. This cannot be a simple size reduction, since more extensive sonication reduces rather than produces the activity. The only other alternative seems to be an aggregate that is arranged such that the constituent molecules can transfer to the air/water interface unusually readily. Indeed, non-vesicular forms are likely to arise during sonication, according to recent analyses which suggest that conversion of MLVs to small vesicles may involve a two-stage process; fragmentation into open sheets of bilayer upon sonication and subsequent spontaneous closure of the sheets into closed shells [10–13,21–24]. The stability of the various possible intermediate forms depends upon the balance between edge energy and bending energy, so structures should be present ranging from small disks to almost-closed vesicles [12,25].

Any of the postulated intermediate states in SUV formation would be expected to be highly surface active, since all have exposed edges from which lipid could spread to the air/water interface. It appears, however, that small bilayer disks are unlikely to be the surface active agent, since, as shown by the calculation in the Appendix, their existence is expected to be ephemeral. In contrast to fragments of bilayer that are too small to be highly curved, spheres that are *almost* closed should be relatively stable to fusion with each other yet be quite surface active, since the energy to remove a molecule from an edge is only a fraction of that required to remove an internal molecule.

Although sonication-induced lipid degradation products appear not to represent the surfactant per se, it is possible that trace amounts of such species could stabilize various of the intermediates that are likely to be generated during sonication. Small bilayer disks can, for example, be stabilized by certain detergents, which have 'edge-activity' [26]. Partially open vesicles should be similarly stabilized. Our results indicate that lysolipids and fatty acids do not act in this fashion, however, it is possible that sonication generates other species that do.

The existence of *any* kind of defect would, in fact, be advantageous for lipid exchange, provided that the exchanging surfaces come nearly into contact so that a molecule could move from one to the other without becoming completely dissociated from either. Thus, it should be considered that newly-formed SUVs may have defects due to a transient disequilibrium between the two surfaces. This would come about if they seal faster than equilibium can be established between the inner and outer monolayers. This kind of defect is stable in gel phase vesicles [14], and even limit-sonicated liquid-crystalline vesicles have different densities in the inner and outer monolayers [26]. In our case, continued sonication and heating could bring the inner and outer monolayers into equilibrium, with concomitant loss of surface activity, as observed. Alternatively, it is conceivable that prolonged sonication generates enough degradation products to occupy voids in the external monolayer and thereby stabilize previously non-equilibrium structures.

A LUVET suspension generates a monolayer at its air interface at a low rate, suggesting that almost all of the vesicles are closed and do not possess defects or surface density imbalances that could lead to ready release of molecules. This is perhaps to be expected from vesicles formed under conditions involving relatively low energies. Nevertheless, the recent observation that a fraction of LUVET populations is unusually susceptible to penetration by cytochrome b_5 [27] suggests that LUVETS should also be regarded as having at least the potential to exist in non-equilibrium forms.

The rate of formation of the monolayer at the air / water interface provides a measure of the disintegration of metastable surfactive structures

By using a surface pressure-surface area isotherm to relate surface tension to number of molecules per unit surface area, the rate at which surface tension falls can be used to evaluate the rate of adsorption of lipid molecules onto the surface. For a typical initial rate, 1 mN/m per min, the surface pressure would change from 0 to 10 mN/m in a time interval of 10 min. The average area of one lipid molecule would then correspondingly vary from infinity to 0.85 nm^2 according to the π-a curve of DOPC we have obtained. During this time interval, the lipid adsorption rate would be on the order of $2 \cdot 10^{11}$ $cm^{-2} s^{-1}$. This rate is an order of magnitude greater than the maximum adsorption rate which would obtain if the transfer process were due to lipid molecule diffusion, i.e., approximately $5 \cdot 10^9$ $cm^{-2} s^{-1}$ (Appendix). This calculation is in agreement with the gel filtration results, namely that monomer diffusion through the aqueous phase cannot be responsible for the rapid adsorption observed. On the other hand, vesicles of 100 Å in radius can give rise to an adsorption rate as high as 10^{13} $cm^{-2} s^{-1}$ from a 1 mg/ml DOPC suspension (Appendix). As indicated above, however, such structures would have to have some kind of packing defect if their constituent molecules were to rapidly transfer to the air/water interface.

5. Conclusions

Surface tension measurements of the air/water surface over sonicated phospholipid vesicles reveal the existence of a metastable state with a high lipid transferability. Such a metastable state is consistent with models of SUV formation which predict intermediate states approaching, but not corresponding to a stable vesicular structure. These structures appear to possess an exposed hydrocarbon core in a form not yet elucidated.

We found sonication-induced metastability in all three phospholipids examined, so the phenomenon appears likely to be a general one. Potential effects of sonication-induced surface activity should therefore be considered for applications of SUVs where such activity could have an influence, particularly those involving molecular exchange. Incubation of SUVs for several hours before use, to allow decay of the surface activity, is suggested as a test of such potential influence. The observations presented here underscore the fact that bilayer vesicles are not invariant structures, but rather collections of non-covalently interacting molecules. In principle, perturbations of many kinds besides those

studied here, can generate minority populations of non-equilibrium structures which can bias the behavior of the entire sample.

Appendix

Rate of aggregation of bilayer disks into larger bilayer sheets

We assume that the amount of bilayer sheet has a total area equal to the total area of the vesicles. This is a very conservative assumption; presumably only a small proportion of bilayer is in the metastable form. We choose an intermediate disk radius r, 100 Å, i.e., too small to roll into a vesicle, but large enough that the rate of transfer to the surface would be close to maximal. According to Smoluchowski's theory [28] the diffusion controlled rate of disappearance of primary spherical particles due to collision is determined by

$$-\mathrm{d}n/\mathrm{d}t = 4\pi DRn^2 \quad (1)$$

where D is the diffusion coefficient of a sphere, n the total number of primary particles and R the collision radius of the particles. We use that equation for a conservative estimate of the rate of disappearance of small disks by choosing the thickness, l, of bilayer as R and $D = k_B T/6\pi\eta r$, where k_B is the Boltzmann constant, T is temperature and η is the viscosity of the solution. If the original number of disks is n_o, at time t, we have:

$$1/n(t) - 1/n_o = 2k_B Tlt/3\eta r \quad (2)$$

To reduce $n(t)$ to 1% of n_o, it takes

$$t_{1\%} = 297\eta r/2k_B Tln_o \simeq 10^{-1}\ \mathrm{s}$$

Since the monolayer area is typically about 0.5% of the total bilayer area, this means that the active material would be reduced to an amount less than that needed for surface (air/water) coverage from the suspension very shortly after the sonication were over. This would be true even if the aggregation rate were 100 × lower than we assume, and our rate assumption would appear to be conservative, given the likely probability that any collision that came close to involving an edge would be expected to cause merging of sheets. Only a few rounds of aggregation of sheets of the size chosen would lead to bilayer pieces large enough to form vesicles.

Diffusion of monomers to the air / water surface

Assuming that all monomers arriving at the surface are irreversibly adsorbed, we use Fick's first law to find the total number of molecules adsorbed to a unit area on the surface at time t after aspirating the surface [29]:

$$n(t) = 2CN(Dt/\pi)^{1/2} \quad (3)$$

where C is the molarity of the monomer, N is Avogadro's number and D is the diffusion coefficient of monomer, which depends on the shape of the molecule. The average rate of adsorption during this time period will be $n(t)/t$. Since a molecule which departs from a spherical shape will have a smaller diffusion coefficient than does a spherical molecule of the same volume, we can estimate the maximum adsorption by replacing the diffusion coefficient for an elongated rod with that for spherical molecules of the same volume. According to Stokes' equation, the diffusion coefficient of a sphere of radius r in a solution of viscosity η is

$$D_s = k_B T/6\pi\eta r \quad (4)$$

where k_B is the Boltzmann constant, T the temperature, η the coefficient of viscosity (0.01 mg/s per cm) and r the radius of the solute molecules, which relates to the volume of a DOPC molecule by

$$r = (3al/4\pi)^{1/3}$$

If we choose the head group area $a = 60$ Å^2, the maximum length of the molecule $l = 24$ Å and monomer concentration, using an impossibly high value for the critical micelle concentration of DOPC, $C = 10^{-7}$ M, the average adsorption rate during the first 10 min would be

$$n(t)/t \simeq 6 \cdot 10^9\ \mathrm{cm}^{-2}\,\mathrm{s}^{-1}$$

This value is lower than our experimental value by almost an order of magnitude.

Diffusion of vesicles to the air / water surface

If lipid adsorption is due to vesicle diffusion, following the preceding calculation and using the minimum radius of vesicles, 100 Å, and the concentration of 1 mg/ml for DOPC, during the same time period as in monomer diffusion, the average adsorption rate will be

$$n(t)/t \simeq 2 \cdot 10^{13}\ \mathrm{cm}^{-2}\,\mathrm{s}^{-1}$$

This is higher than the required value by two orders of magnitude. In other words, one percent of total DOPC in the metastable state is sufficient to endow the suspension with a high surface activity.

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