Preparation and Characterization of Vesicles from Mono-*n*-alkyl Phosphates and Phosphonates

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The aggregation properties of different linear, single-chain alkyl phosphates and phosphonates in water were investigated at concentrations of up to 50 mM as a function of pH, focusing in particular on spontaneous vesicle formation. Under conditions where about half the molecules are monoionic and half the molecules are completely protonated (pH ≈ 2), *n*-dodecylphosphoric acid, *n*-decylphosphonic acid, and *n*-dodecylphosphonic acid spontaneously form vesicles at room temperature. For *n*-hexadecylphosphoric acid, stable vesicles only form above ∼40 °C. The presence of vesicles was evidenced by light and electron microscopy and in the case of *n*-dodecylphosphoric acid by entrapment experiments using as water soluble probes glucose, dextran, and pepsin. The phase-transition temperature of vesicles of *n*-dodecylphosphoric acid was 2.3 °C, as determined by differential scanning calorimetry. For *n*-hexadecylphosphoryladenosine evidence for micelle formation has been obtained with a cmc of 20−50 μM at 25 °C. In an experimental extension of the vesicle self-reproduction principles to phosphoamphiphiles, results are also presented on the alkaline hydrolysis of the water-insoluble di-*n*-decyl-4-nitrophenyl phosphate, which led to the formation of 4-nitrophenol and di-*n*-decyl phosphate, the latter being a known vesicle-forming amphiphile.

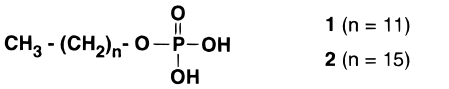
Introduction

So far, most of the studies on lipid bilayers (liposomes, black lipid membranes) have been carried out with glycerophospholipids[1]—phospholipids that contain phosphate in the polar head group and that have a glycerol backbone with two esterified fatty acids. This is because glycerophospholipids, such as phosphatidylcholine, are easily available from biological samples (e.g., hen egg yolk, soybeans), since these are the most abundant lipids present in the bilayered biological membranes of contemporary cells.[2]

From a prebiotic point of view, glycerophospholipids are rather complex molecules. More "primitive" amphiphiles able to form closed bilayers are single-chain fatty acids when dispersed in water under appropriate conditions (about half the molecules have to be ionized).[3] More recently, terpenoid-based polyprenyl phosphates have been considered by Wächtershäuser[4] and Ourisson et al.[5] as possibly relevant prebiotic membrane-forming lipid. This hypothesis is mainly based on the fact that no terpenoid-free cellular membrane is known today and that many terpenoid-based molecular fossils have been isolated. Furthermore, it has been argued that phosphate could have been of prebiotic importance, since phosphate may have arisen from volcanoes in prebiotic times.[6] With respect to this latter point, alkyl phosphonates have been found in the carbonaceous Murchison meteorite,[7] and recently, they have been synthesized under plausibly prebiotic conditions,[8] supporting the possible role of phosphorus-containing amphiphiles on the primitive Earth.

In view of the biological relevance of alkyl phosphate and phosphonate vesicles, it is important to better understand their aggregation behavior and to physically characterize the corresponding supramolecular aggregates. There are actually only few data in the literature.[9] Particularly important is the spontaneous vesiculation of these amphiphiles, namely, the process whereby they form stable vesicles by dispersing them in aqueous solution without any further supply of mechanical energy such as sonification or extrusion.

In this paper, we are describing the spontaneous association of linear monoalkyl phosphates and phoshonates. The vesicles will be occasionally prepared by extrusion, and actually, the comparison between the two preparations will permit us to learn something about the chemical equilibrium in these systems. We will focus here in particular on micelle and vesicle formation of *n*-dodecylphosphoric acid (**1**), *n*-hexadecylphosphoric acid (**2**), *n*-decylphosphonic acid (**3**), and *n*-dodecylphosphonic acid (**4**).



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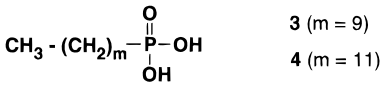

3 (m = 9)
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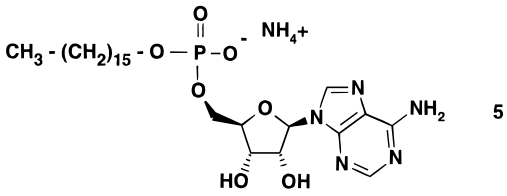

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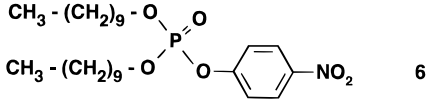

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Furthermore, preliminary aggregation studies of *n*-hexadecylphosphoryladenosine (**5**) will be reported, and experiments on the hydrolysis of di-*n*-decyl-4-nitrophenyl phosphate (**6**) will be described. In this latter case a spontaneous vesicle formation occurs as a consequence of a simple chemical reaction.

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Materials and Methods

Reagents. *n*-Dodecylphosphoric acid (**1**) was obtained from Tokyo Kasei Kogyo Co., Ltd. (Japan) as a mixture of the mono- and disodium salts, also containing di-*n*-dodecyl and tri-*n*-dodecyl phosphate as impurities. The free acid **1** was purified by first treating 5 g of the commercial product with 10 g Dowex 50WX8 mesh 50−100 in 300 mL methanol for 1 h. After removal of the ion exchanger by filtration, **1** was crystallized from isooctane. Product purity was confirmed by thin-layer chromatography, elemental analysis, and ^{31}P and ^{1}H NMR spectroscopy ($CDCl_3$ solution). *n*-Hexadecylphosphoric acid (**2**, commercial name "amphisol A") was a gift of Givaudan, Switzerland, and used without further purification. *n*-Decylphosphonic acid (**3**) was from Lancaster Synthesis, Ltd., France, and *n*-dodecylphosphonic acid (**4**) was from Oryza (U.S.A.) and used as received.

n-Hexadecylphosphoryladenosine (**5**) was synthesized enzymatically in a two-phase system, using the phospholipase D-catalyzed trans-alkylphosphorylation reaction with *n*-hexadecylphosphorylcholine[10] and the corresponding nucleoside (adenosine), basically following the procedure described by Shuto et al.[11] A quantity of 900 units of phospholipase D from *Streptomyces* sp. AA 486 (from Genzyme Diagnostics, U.K.) was added to 20 mL of a 200 mM sodium acetate buffer solution containing 2.1 g (8.0 mmol) adenosine and 250 mM $CaCl_2$, pH 6.0. The solution was kept at 45 °C, and 20 mL of a $CHCl_3$ solution containing 0.8 g (1.9 mmol) *n*-hexadecylphosphorylcholine was added under stirring. After 5 h the reaction was stopped by adding 5 mL of 1 M HCl followed by addition of 150 mL $CH_3OH/CHCl_3$ (1:3, v/v). The organic phase was separated and concentrated about two times by rotatory evaporation. The ammonium salt of **5** was obtained in 43% yield after adding NH_4OH at −10 °C, and the purity of the product was confirmed by TLC, ^{1}H, ^{13}C, and ^{31}P NMR, and MS-FAB.

Di-*n*-decyl-4-nitrophenyl phosphate (**6**) was synthesized from decanol and 4-nitrophenylphosphorodichloridate in THF/pyridine following a procedure described previously.[12] After purification by silica gel chromatography, the product was obtained in 68% yield, pure according to TLC and ^{1}H, ^{13}C, and ^{31}P NMR.

CAPS (3-[cyclohexylamino]-1-propanesulfonic acid), ammonium molybdate (>99%), K_3PO_4, $CuSO_4$, orcinol, sodium potassium tartrate, Triton x-100, adenosine, 4-nitrophenyl phosphorodichloridate, dextran (from *Leuconostoc* ssp., M_r 15 000−20 000), porcine pepsin (crystallized twice), Dowex 50WX8 mesh 50−100, decanol, and phosphorus oxychloride (the latter two liquids were distilled before use) were obtained from Fluka, Switzerland. Pinacyanol chloride, *n*-hexadecylphosphorylcholine, and bicinchoninic acid reagent were from Sigma (U.S.A.). Hide powder azure was obtained from Calbiochem (U.S.A.). Citric acid was a product of Hoffmann-LaRoche, Switzerland.

Instrumentation. UV−vis absorption spectra were recorded either on a Cary 1E UV−visible spectrophotometer from Varian (Australia), on a HP8452 diode array spectrophotometer from Hewlett-Packard (U.S.A.), or on a DU-68 spectrophotometer from Beckman (U.S.A.).

Bath sonications were performed in a Brandlin Sonorex RK100 H instrument at room temperature.

Thermal phase transitions were determined by differential scanning calorimetry (DSC) using a DSC 7 differential scanning calorimeter from Perkin-Elmer (U.S.A.).

Electron micrographs were taken by the freeze−fracture method,[13] and light micrographs were obtained by using a Axioplan microscope from Zeiss (Germany) as described previously.[14]

Titration Curves. For the acid/base titration curves, a certain amount of appropriate phosphoamphiphile was dispersed in water and equivalents of HCl or NaOH (or KOH or LiOH) were added and stirred with a magnetic stirrer. The concentration of the amphiphile was kept constant within the same titration curve. After equilibration, the pH was measured and the samples were analyzed by light microscopy and electron microscopy.

Preparation of Vesicles. Vesicles of **1** were prepared by dispersing **1** under mild stirring at the desired concentration in 50 mM citric acid. The resulting pH was between 3.0 (5 mM **1**) and 2.2 (50 mM **1**). For the extrusions of the vesicle suspensions, "The Extruder" from Lipex Biomembranes (Canada) and polycarbonate membranes from Nucleopore (U.S.A.) were used.[15] In the entrapment experiments, the vesicles were prepared in the presence of the molecules to be entrapped. For these experiments 30 mM **1** was used with 40 or 56 mM D-glucose, 7.1 mg dextran/mL, or 1.4 mg pepsin/mL.

Gel Permeation Chromatography. Vesicles containing entrapped solutes were separated at room temperature from nonentrapped material by gel permeation chromatography, using sepharose 4B from Pharmacia, Sweden (column diameter, 1.4 cm; length, 40−50 cm). Fractions of 2.0−2.5 mL were collected by using a 2112 Redirac fraction collector from LKB-Pharmacia (Sweden). Elutions were made with 50 mM citric acid (pH 2.0). Entrapment yields are given as a percentage of solute coeluting with the vesicles relative to the amount of solute used during vesicle preparation.

Quantification of the Phosphoamphiphiles, Glucose, Dextran, and Pepsin. All phosphoamphiphiles were quantified as described before,[16] using ascorbic acid and ammonium molybdate to determine inorganic phosphate after hydrolyzing the amphiphiles with $H_2SO_4/HClO_4$ (1:1). The absorbance was measured at 820 nm and K_3PO_4 was used as a standard (20−100 nmol).

Glucose was quantified in two different ways.[17] (a) The first is with the orcinol/H_2SO_4 test, using glucose standards in the range 0.1−0.5 mg. To 1 mL of the glucose-containing samples 8.5 mL of 1.32 mM orcinol in 53% (v/v) H_2SO_4 was added, vortexed, and then heated for 30 min at 80 °C. After the samples were cooled to room temperature, the absorbance was read at 422 nm. (b) The second way is with alkaline $CuSO_4$. To 1 mL of the glucose-containing samples 0.3 mL of 217 mM $CuSO_4$ in a 17% (w/v) solution of sodium potassium tartrate containing 5% (w/v) NaOH was added and heated for 14 min at 80 °C under magnetic stirring. After cooling to room temperature, the samples were centrifuged at 8000 rpm for 45 s using an ALC microcentricugette 4214. Glucose standards were used in the range 0.1−1.0 mg, and absorbance readings were performed at 672 nm.

Dextran was quantified with the phenol/H_2SO_4 assay.[17,18] To 2 mL of dextran-containing samples 5 mL of concentrated H_2SO_4 and 110 μL of 90% (v/v) phenol were added and left at 25 °C for 30 min. The optical density was measured at 488 nm. Calibrations were made with known amounts of dextran, ranging from 10 to 70 μg.

Pepsin was quantified in two different ways. (a) The protein content was determined with alkaline bicinchoninic acid and $CuSO_4$,[19] using pepsin as a standard in the range 10−100 μg. Absorbance readings were at 562 nm. If necessary, vesicle fractions containing pepsin were first lyophilized to concentrate before measuring. (b) The enzymatic activity was measured with the water-insoluble hide powder azure as substrate.[20] To 8 mg of hide powder azure, 2 mL of a pepsin-containing sample in 50 mM citric acid (pH 2.0) at 37 °C was added. After

incubation at 37 °C for 1 h, the reaction mixture was cooled in an ice bath and the remaining insoluble hide powder azure was separated from the solution by centrifugation. The absorbance of the supernatant was then measured at 595 nm. For the calibration 10−70 μg pepsin was used. In the case of the vesicle fractions containing entrapped pepsin, all the assays were carried out in the presence of 13% (w/v) Triton x-100 and in the presence of the appropriate concentration of **1** previously determined by the molybdate assay (see above).

cmc Determinations. The cmc determinations were carried out by using the colorimetric method with pinacyanol chloride.[21] A quantity of 8 μL of a 1.3 mM methanolic solution of pinacyanol chloride was added to 1 mL of an appropriate aqueous solution of the phosphate-containing surfactant. The absorbance at 605 nm was then plotted against surfactant concentration, and the cmc value was taken as that surfactant concentration at which the absorbance at 605 nm with increasing concentration started to increase. Unless otherwise specified, the final concentration of pinacyanol chloride was always 10 μM.

Hydrolysis of Di-*n*-decyl-4-nitrophenyl Phosphate (6). The hydrolysis of **6** was carried out in a closed 3 mL flat-bottom flask (diameter 1.3 cm) in a Reacti-Therm heating/stirring module 18971X from Pierce (U.S.A.) under slight magnetic stirring (position 1.5) at 68 °C. The aqueous phase initially was either 0.25 M KOH or 0.2 M CAPS buffer (pH 10.5). From time to time 10 μL of the aqueous phase (1 mL) was removed and diluted (1:1 v/v) by adding 10 μL of 0.4 M CAPS buffer (pH 10.5) followed by a further dilution with 1 mL of CH_3OH. The *p*-nitrophenolate was quantified spectrophotometrically at 391 nm ($\epsilon_{391\ nm} = 17\ 600\ M^{-1}\ cm^{-1}$). All reactions were carried out in triplicates, and product concentrations are given as mean values with standard deviations.

Results and Discussion

Although much is known on the formation of (large)[22] vesicles of di-*n*-alkyl phosphates, such as di-*n*-decyl phosphate,[23] di-*n*-dodecyl phosphate,[23−25] di-*n*-tetradecyl phosphate,[23,25] di-*n*-hexadecyl phosphate,[23,25−27] and di-*n*-octadecyl phosphate,[23,25,28,29] there are only a few reports describing the capability of single-chain phosphates to form closed bilayer structures when dispersed under appropriate conditions in water. Without detailed investigations, Hargreaves and Deamer[3b] noted that *n*-octadecyl phosphate (1−5 mM), upon being briefly heated above 70 °C, can form small vesicles (diameters below 50 nm) as well as large vesicles (diameters up to 5 μm). Ravoo and Engberts[28] studied the vesicle-forming properties of a series of phosphates with branched alkyl chains at a concentration of 15−30 mM, and they have observed that the pH critically influences the morphology of the vesicles. Ourisson et al.[5c] investigated the pH dependence of the vesicle formation of a variety of monopolyprenyl phosphates and pyrophosphates, mentioning—without further detail—that *n*-dodecyl phosphate was used as a control.

As we will show below, the aggregation properties of linear alkylphosphoric or -phosphonic acids in water not only depend on the concentration, temperature, or the presence of additional salt but also on the degree of protonation of the head group. This is analogous to the behavior of fatty acids when dispersed in water.[3,14,30]

In the following, we will first report on the pH-dependence of the aggregation of **1**, **2**, and **3**, in particular focusing on the conditions under which vesicles are formed.

Effect of pH on the Supramolecular Aggregation of *n*-Dodecylphosphoric Acid (1) in Water. Initially, we found

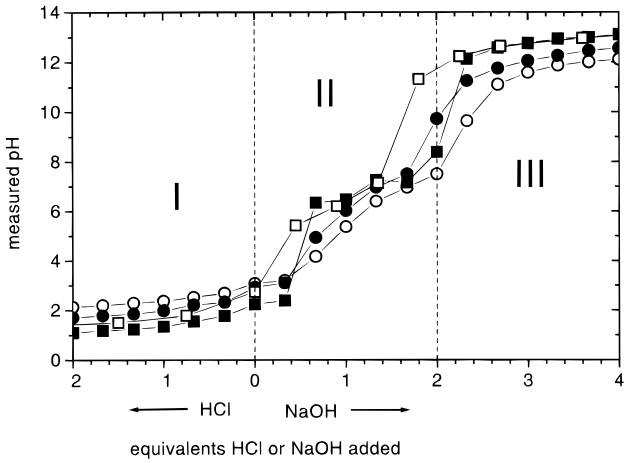

Figure 1. Titration curves for **1** at room temperature. The concentrations of *n*-dodecylphosphoric acid **(1)** were 4.4 mM (○), 10.0 mM (●), 17.6 mM (□), and 43.0 mM (■). For details see text.

that upon dispersing **1** in pure water at a concentration of 5 mM, vesicles spontaneously form, as evidenced by light microscopy and freeze−fracture electron microscopy. These vesicles vary in size and lamellarity; multilamellar vesicles are seen as well as unilamellar, oligolamellar, or multivesicular vesicles (vesicles containing in their interior two or more nonconcentrically localized smaller vesicles). The presence of vesicles in aqueous dispersions of **1** was detected down to a concentration of at least 0.6 mM, again using light and electron microscopy.

Based on these initial observations, full titration curves of **1** were determined using 4.4, 10.0, 17.6, or 43.0 mM **1** and adding either HCl or NaOH. In Figure 1, the measured pH is reported as a function of added HCl or NaOH. All four curves appear very similar and can be divided in basically three different regions: I, II, and III (Figure 1).[31]

In region I (pH below ∼3), the samples are turbid, and light microscopy (parts a−c of Figure 2) and electron microscopy (Figure 3) evidenced the presence of vesicles. These vesicles were stable for several weeks at room temperature without precipitation. In samples where more than 2.5 equiv of HCl was added, formation of crystals occasionally occurred. In region II (pH between ∼3 and 7−9) crystallization of **1** occurred (Figure 2d), while in the alkaline region III (more than 2 equiv of NaOH was added) the solution was transparent. In some of the samples formation of precipitates was also observed. Generally, the extent of precipitation decreases with increasing pH.

From all these titration curves it is evident that the charge of the head group of **1** very much influences the aggregation behavior. If the phosphate is bearing two negative charges, electrostatic head group repulsions will prevent the formation of bilayers (vesicles), the apparent head group size will increase, and highly curved micellar structures are formed, which are much smaller than vesicles and which therefore do not scatter light.

Indeed, from literature it is known that **1** micellizes in the alkaline pH region.[32] To determine the concentration at which micelles are formed, we determined the cmc of **1** at high pH and obtained 42 mM (at pH 11.4 with NaOH) and 26 mM (at pH 11.7 with KOH) at 25 °C. These values are in the range of the literature values reported before.[32]

From all the titration curve measurements it was clear that stable vesicles were only present in region I (Figure 1), where the measured pH did not markedly change with the addition of HCl. Stable vesicles are therefore only formed around the pK_{a1}

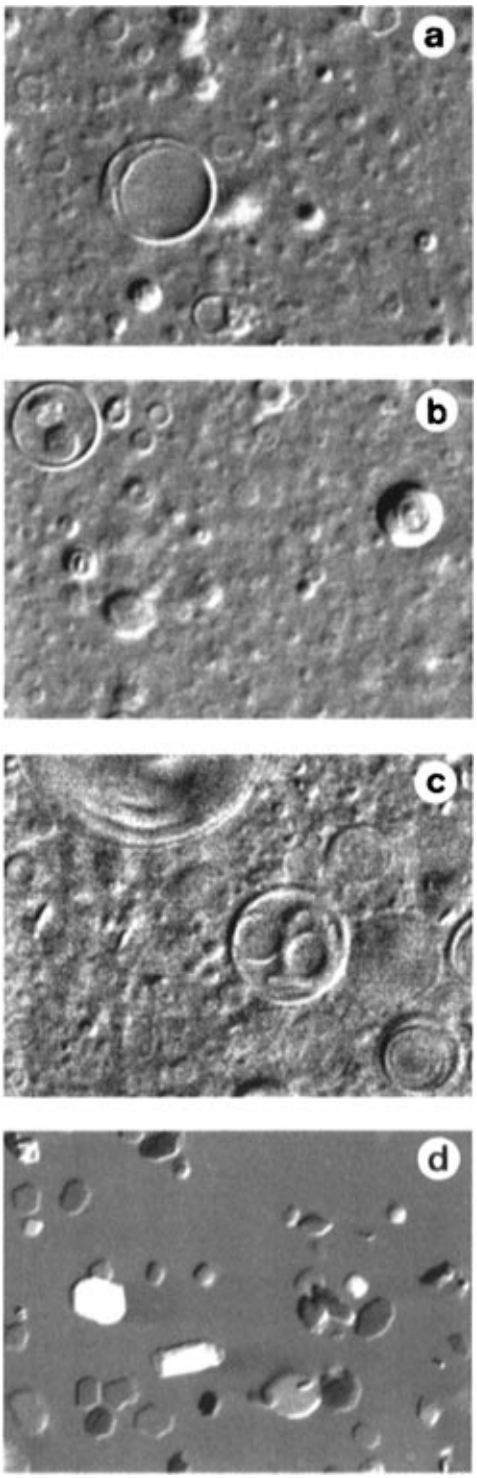

Figure 2. Light micrographs of 17.6 mM **1** dispersed in water in the presence of different amounts of HCl or NaOH: (a) pH 1.4 (2.25 equiv of HCl added); (b) pH 1.7 (0.75 equiv of HCl added); (c) pH 2.6 (no HCl or NaOH added); (d) pH 6.4 (0.90 equiv NaOH added). The micrographs were taken at room temperature. The width of the micrographs in parts a−c is 50 μm and that of part d is 100 μm.

of **1**. As described in the following, vesicles of **1** have been characterized to some extent by microscopic methods and by entrapment experiments.

A general consideration about the relationship between spontaneous vesiculation and chemical equilibrium must be addressed at this point. The observation that formation of vesicles is a spontaneous process, together with the observation that dilution brings about an immediate decrease in the number of aggregate particles, is the first indication that we are in the

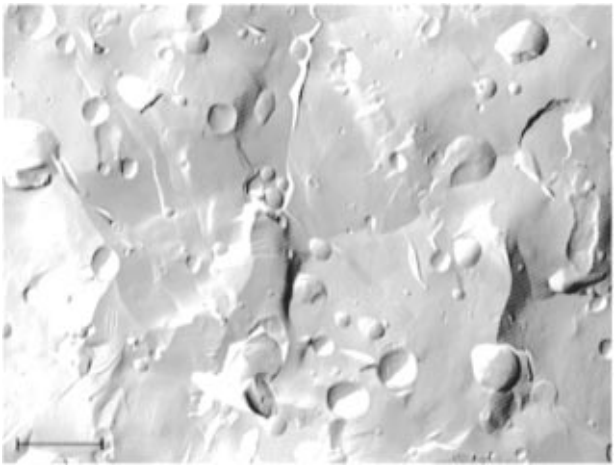

Figure 3. Freeze−fracture electron micrographs of 43 mM **1** dispersed in water without addition of HCl or NaOH (pH 2.2). The samples were frozen from room temperature, and the length of the bar is 1 μm.

presence of a chemical equilibrium—a system in which monomers and aggregates are in rapid equilibrium as in the case of micelles in water. However, in the case of vesicles, the situation is in most cases not as simple as for micelles. In fact, the spontaneity of a process does not ensure automatically the formation of a chemical equilibrium, a spontaneous process, which may also represent a chemically irreversible process, may produce kinetically trapped ("irreversible") forms that do not reequilibrate again. This is indeed the case for several of these spontaneous vesiculation reactions in which the size and the lamellarity degree are not well reproduced in different experiments. As a consequence of this mixed situation, once the vesicular system is prepared by, say, extrusion, it is not expected to relax to the most stable statistical size and lamellarity distribution simply because the mechanical stress of the extrusion has produced forms that are kinetic traps, removed from the normal chemical equilibrium. In conclusion, there is only a partial chemical equilibrium.

Characterization of Vesicles from *n*-Dodecylphosphoric Acid (1). As shown above, the formation of vesicles of **1** occurs under conditions where about half the phosphate head groups is completely protonated and half the molecules is in the monoanionic form. The estimated pK_{a1} of **1** in the system at 10 mM **1** is 1.8 (for 10 mM **1**), similar to the value reported for *n*-propylphosphoric acid (pK_{a1} = 1.88).[33] This situation leads to attractive intermolecular head group interactions, probably through a hydrogen-bonding network as shown in Figure 4, analogous to the case of vesicles from fatty acids,[3,14,30] where dimers stabilized by hydrogen bonds are probably formed, and analogous to the case of di-*n*-alkyl phosphate vesicles.[34] If all the head groups are completely ionized, the solubility of the molecule increases, intermolecular repulsions destabilize the bilayer of the vesicles, and micelles are formed—if the concentration is high enough (see above and region III in Figure 1).

Owing to this pH sensitivity of vesicles of **1**, all additional investigations of the vesicles were carried out under controlled pH conditions, namely, in the presence of 50 mM citric acid. Since cations may interact with the negatively charged phosphate head groups in the vesicles,[35] possibly causing a vesicle disaggregation, the use of additional salt, such as NaCl, KCl, or LiCl, was avoided. Indeed, in the presence of cations, generally, no stable vesicles could be prepared (as checked by turbidity changes).

Vesicles of **1** (30 mM) formed in 50 mM citric acid are rather polydisperse and mainly multilamellar. Some vesicles were so large that they could be observed by light microscopy (in

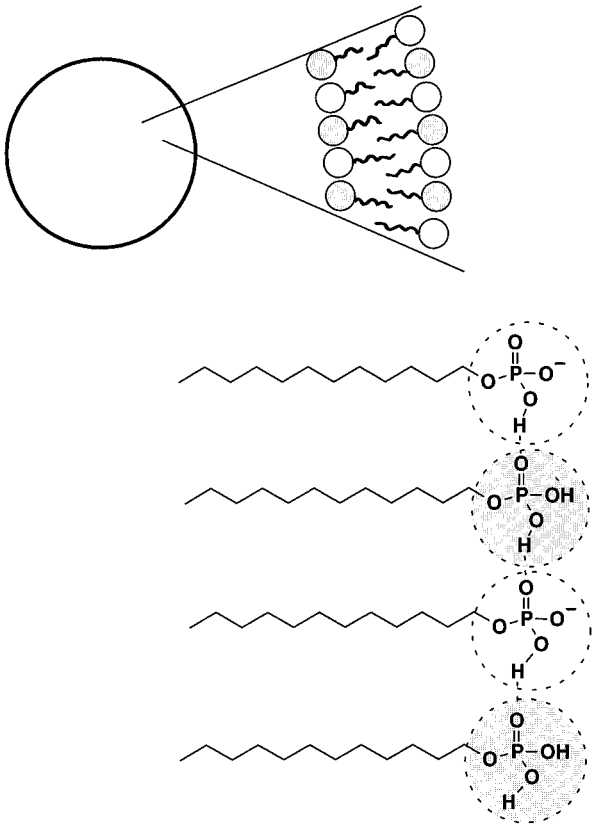

Figure 4. Schematic illustration of the cross section of an unilamellar vesicle of **1** and of the possible intermolecular hydrogen bonding in the head group area of **1** under conditions where about half of the molecules have a head group that is monoanionic and where the other half of the molecules is completely protonated. See also the discussion of the hydrogen-bonding possibilities in phosphatidic acid aggregates.[34]

analogy to the spontaneous formation of vesicles at the same pH but without citric acid). This vesicle suspension could be passed through polycarbonate membranes containing pores with defined diameters of 400, 200, or 100 nm, just as in the case of conventional phosphatidylcholine vesicles.[15] A freeze−fracture electron microscopy analysis indicated that the extruded vesicles had a considerably smaller size than the unextruded samples. The maximal vesicle size, however, does not correspond to the size of the filter pores; there were always some vesicles present that were larger than the diameter of the pores of the polycarbonate membranes (Figure 5). The reason for this follows from the observations made initially on the spontaneity and chemical equilibrium of the system. After forced filtration through the pores, the system relaxes only partly into the thermodynamically most stable distribution. Some aggregates remain, however, trapped in a fixed size.

With respect to the equilibrium between **1** in the vesicles and the relatively high amount of monomeric **1**, there must be a rather rapid exchange of molecules, since experimentally, it is observed that the total number and size of vesicles rapidly changes upon dilution, just as in the case of fatty acid vesicles.[14,30] This is also the reason why no unambiguous dynamic light-scattering measurements can be performed; sample dilutions always lead to a change in the whole system, just as in the case of micelles, where generally the concentration of nonassociated single-chain amphiphile is also rather high.

To know whether vesicles of **1** show a phase transition from the solid-analogue to the liquid-analogue state at a temperature above the freezing point of water, DSC measurements were performed (Figure 6a). These measurements indicated that such a phase transition occurs around 2.3 °C if a vesicle sample is

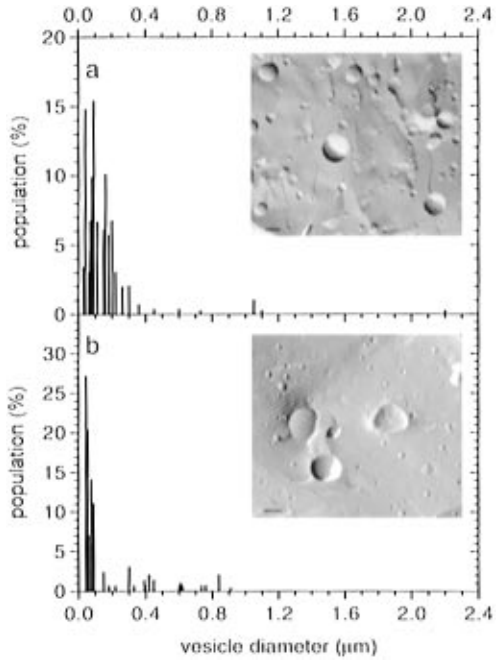

Figure 5. Size distribution of vesicles of **1** that had been passed through polycarbonate membranes with pores of 400 nm (a) and 100 nm (b). [**1**] = 30 mM in 50 mM citric acid. The inserts show freeze−fracture electron micrographs taken for the analysis. The length of the bar corresponds to 200 nm.

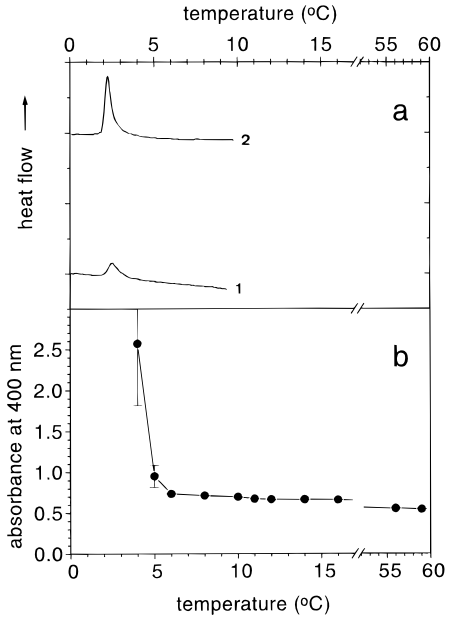

Figure 6. (a) DSC traces of vesicles of **1** in 50 mM citric acid (heating curves). [**1**] = 30 mM (1) and 100 mM (2). (b) Turbidity of vesicles of **1** in 50 mM citric acid measured at 400 nm as a function of temperature. [**1**] = 30 mM.

first cooled to −10 °C and then heated to 30 °C (Figure 6a). Around 2−4 °C, the vesicles precipitate as shown by turbidity measurements (Figure 6b). This behavior is again analogous to the case of vesicles from fatty acids.[30] From a practical point of view, it is important to note that vesicles of **1** are stable above ∼5 °C, therefore allowing us to perform all additional measurements at room temperature without any stability problems.

To test whether vesicles of **1** can entrap and retain water soluble molecules, a series of entrapment experiments were performed using D-glucose, dextran, and pepsin. These molecules were present in the aqueous solution (50 mM citric acid) in which the vesicles were prepared. The vesicles (containing

TABLE 1: Entrapment of Glucose, Dextran, and Pepsin in Vesicles of 1 ([1] = 30 mM)

entrapped solute	solute concentration during vesicle preparation (mg/mL)	final extrusions (mean pore diameter) (nm)	entrapment yield[a] (%)
glucose	7.9 (=40 mM)	not extruded	17, 19
	7.9 (=40 mM)	400	8.8
	11.1 (=56 mM)	400	7.5
	11.1 (=56 mM)	100	3.1
dextran	7.1	100	16
pepsin	1.4	200	29

[a] The theoretically calculated entrapped volume for monodisperse, unilamellar vesicles of **1** ([**1**] = 30 mM) with a diameter of 100 nm is 5.8%. With a diameter of 200 nm it is 12.4%, and with a dimater of 400 nm it is 25.6%. The bilayer thickness was taken as 3.3 nm,[38] and for the mean head group area 44 Å^2 was considered.[39]

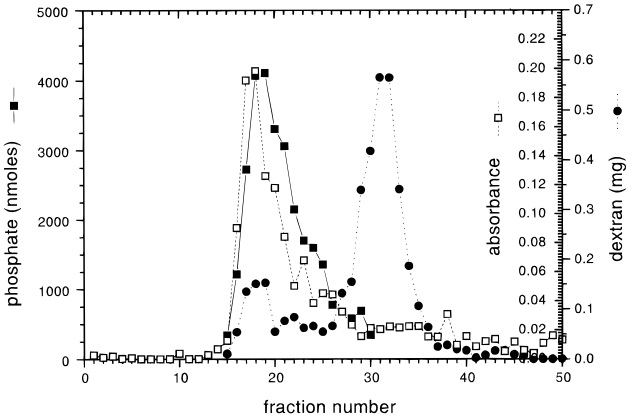

Figure 7. Gel permeation chromatographic separation of dextran-containing vesicles of **1** from free dextran on sepharose 4B. A quantity of 1 mL of a vesicle suspension containing 30 mM **1** and 7.1 mg of dextran/mL was first extruded (final extrusions through pores of 100 nm diameter) and then applied on a sepharose 4B column (length, 45 cm; diameter, 1.8 mL; flow rate, 0.85 mL/min; 2.3 mL/fraction; elution with 50 mM citric acid, pH 2.0). The amounts of dextran and of **1** in each fraction are plotted against the fraction number.

entrapped solute molecules) were then separated from nonentrapped molecules by gel-permeation chromatography using sepharose 4B, and it was shown that all three molecules tested can be entrapped inside vesicles of **1** (Table 1). Figure 7 shows a chromatogram for the separation of dextran-containing vesicles of **1** (first peak) from free dextran (second peak). As seen from Table 1, the entrapment yield varies with the conditions of the experiments and the type of solute entrapped. Two experimental observations are worthwhile to mention. First of all, addition of solute molecules to preformed vesicles did not lead to solute uptake. This documents that vesicles of **1** showed negligible permeability for all solutes tested. For glucose, the measured entrapment yields correspond roughly to the theoretically expected values (see Table 1), indicating that glucose does not significantly interact with the vesicles. In contrast, for the two macromolecules, dextran and pepsin, the high entrapment yields indicate interactions with the vesicles. In the case of pepsin, it was observed by light microscopy that this enzyme interacts indeed with the vesicles, leading to vesicle aggregation (micrographs not shown). Furthermore, extrusions of the vesicle samples containing pepsin were difficult to perform. In particular, polycarbonate filters with pore diameters smaller than 200 nm could not be used, since the vesicles could not be pressed through the pores.

Case of *n*-Hexadecylphosphoric Acid (2). The titration of **2** (5 mM) with NaOH and HCl was carried out at 40 °C (to avoid extensive precipitation). A titration curve was obtained that was very similar to the curves shown in Figure 1 for **1**.

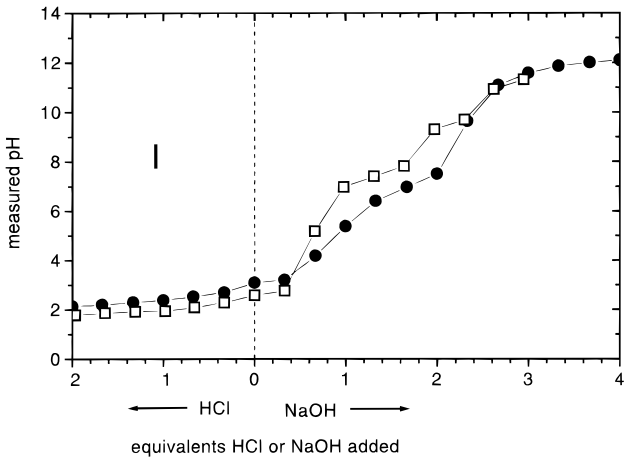

Figure 8. Titration curves of 19 mM **3** and 9 mM **4** at 30 °C. In region I, vesicles could clearly be seen by light microscopy.

Again, vesicle formation could be observed in the low pH region (pH < 4), but only after the samples were first heated for about 1 min to ~70 °C. If a preheated sample was cooled to about 40 °C and immediately observed under the light microscope, spherical uni- and oligolamellar vesicles could easily be detected, and the presence of vesicles was also confirmed by electron microscopy. Prolonged storage at room temperature led to a reversible transformation of the spherical vesicles to partly crystallized structures with a lot of edges, a kind of polyhedral vesicle. Further storage at room temperature resulted in vesicle precipitation. Owing to this temperature instability, we did not further investigate this particular system.

Case of *n*-Decylphosphonic Acid (3) and *n*-Dodecylphosphonic Acid (4). The titration curves of **3** and **4** are rather similar to the corresponding curves for **1** and **2**, and vesicles formed again in the low pH region (Figure 8). By use of 10−20 mM **3** or **4**, unilamellar and in particular also many multilamellar vesicles were present below pH ≈ 3.7, as shown by light and electron microscopy. As in the case of **2**, the temperature played a critical role, the vesicles not being very stable at room temperature. To avoid precipitation and crystal formation, the samples were kept at 30 °C. By use of pinacyanol chloride, it was shown that **3** and **4** form micelles at high pH, the cmc being 40−70 mM (50 °C).

***n*-Hexadecylphosphoryladenosine (5).** In contrast to **1**−**3**, the amphiphile **5** has a complex chiral head group that is relatively bulky (phosphoryladenosine). The study of this compound is part of an extensive investigation, now in progress in our group, on the aggregation behavior of lipids containing nucleobases. We have, for example, characterized already liposomes obtained from phosphatidyl nucleosides,[36] and we have also studied morphological transformations of these supramolecular aggregates.[37] Clearly, monoalkylphosphoryl nucleosides offer the advantage of a larger molecular simplicity with respect to the phosphatidyl analogues. They are, however, more water soluble and have only one alkyl chain, and as such, they form stable micelles, as will be reported in detail elsewhere. **5** forms micelles, and as evidenced by pinacyanol chloride, the cmc is 20−50 μM. Preliminary experiments show that monoalkylphosphoryl nucleosides form vesicles at low pH (Heiz C. et al., unpublished data). Detailed characterizations are in progress.

Chemically Induced Vesicle Formation. In an extension of our studies on the autocatalytic self-reproduction of fatty acid vesicles (hydrolysis of water-insoluble fatty anhydride catalyzed by fatty acid vesicles),[14a,30] we have asked the question whether a similar effect could also be observed with phosphate-based amphiphiles. For this, we investigated the alkaline hydrolysis

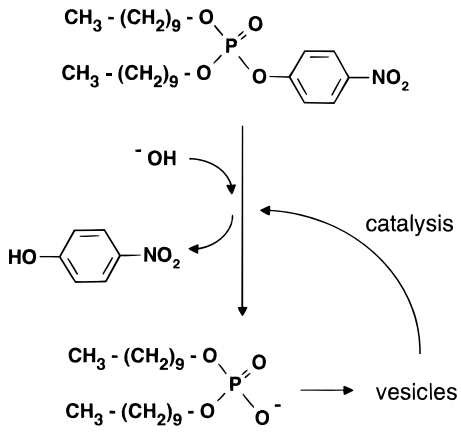

Figure 9. Chemical reaction scheme representing the hydrolysis of **6** leading to di-*n*-decyl phosphate, which forms vesicles that in turn may catalyze the hydrolysis of **6**.

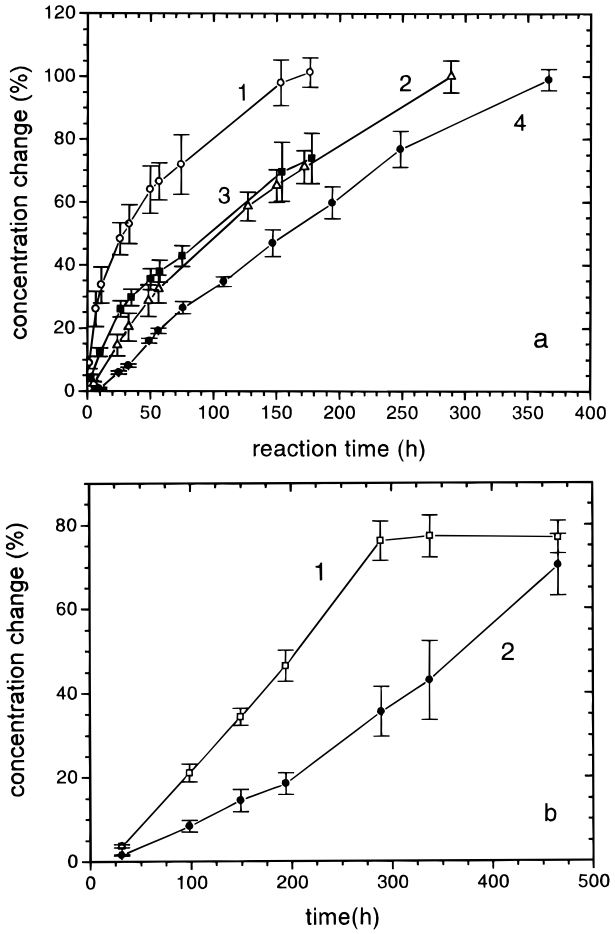

Figure 10. (a) Effect of ultrasound treatment on the rate of hydrolysis of **5** (10 μmol) overlaid to 1 mL of an aqueous 0.25 M KOH phase (pH 13.4). (2) The aqueous phase initially contained vesicles of di-*n*-decyl phosphate (10 mM) that were sonicated for 10 min. (1) The aqueous phase initially contained vesicles of di-*n*-decyl phosphate (10 mM) that were cosonicated with **5** for 10 min. (3) The aqueous phase initially contained no vesicles, but the aqueous phase and **5** were sonicated for 10 min. (4) The aqueous phase initially contained no vesicles, and there was no sonication at all. (b) Effect of vesicles of di-*n*-decyl phosphate on the rate of hydrolysis of **5** (10 μmol) overlaid to 1 mL of an aqueous CAPS-buffered phase (0.2 M CAPS, pH 10.5), no sonication. (1) Initially, no vesicles of di-*n*-decyl phosphate were present. (2) Initially, vesicles of di-*n*-decyl phosphate were present (10 mM).

of the water-insoluble di-*n*-decyl-4-nitrophenyl phosphate (**6**), leading to di-*n*-decyl phosphate, which forms vesicles,[23] and 4-nitrophenolate (Figure 9). In this simple experiment vesicles are formed (as evidenced by light and electron microscopy) during a chemical reaction, leading to an increase in the number of vesicles as time progresses, analogous to the case of the fatty anhydride/fatty acid system.[14a,30] We were particularly interested in knowing whether the hydrolysis of **5** is autocatalytic, namely, catalyzed by the vesicles formed possibly through an uptake mechanism, which would involve the spontaneous incorporation of **6** into the vesicles. It may be that electrostatic repulsions between the negatively charged vesicles and the hydroxyl ions (0.25 M KOH) hinder the triester attack by OH^-. To test these two possibilities, experiments were carried out where **5** (10 mM overall) was initially cosonicated with vesicles of di-*n*-decyl phosphate (10 mM) and the rate of hydrolysis of **5** was compared with the rate of hydrolysis of **5** in the absence of vesicles (see Figure 10a). Only this mechanical triester/vesicle dispersion showed an increased hydrolysis rate in comparison with the control measurements. This finding indicates that the spontaneous triester uptake by the vesicles (without ultrasound treatment) is unlikely to occur under the conditions used. With respect to the possible electrostatic repulsion effects, the triester hydrolysis was also performed at lower OH^- concentration in the presence of CAPS buffer (0.2 M, pH 10.5, Figure 10b). To our surprise, in this case the vesicles showed indeed a catalytic effect. Detailed studies of the general role of buffer ions during vesicle self-reproduction are now under investigation.

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