Biochimica et Biophysica Acta, 1147 (1993) 237–244
© 1993 Elsevier Science Publishers B.V. All rights reserved 0005-2736/93/$06.00

BBAMEM 75940

The effect of positive and negative pH-gradients on the stability of small unilamellar vesicles of negatively charged phospholipids

B.Z. Lin, C.C. Yin and H. Hauser

Laboratorium für Biochemie, Eidgenössische Technische Hochschule Zürich, ETH-Zentrum, Zürich (Switzerland)

(Received 1 December 1992)

Key words: Phosphatidic acid; Unilamellar vesicle; Thermodynamic stability; pH-gradient

The stability of small unilamellar vesicles (SUV) made from negatively-charged phosphatidate by ultrasonication or pH-jump has been investigated. As criteria for the vesicle stability are used: (I) the bilayer integrity as judged from the permeability of the fluorescent probe carboxyfluorescein (CF) and (II) the susceptibility of the phospholipid vesicles to fusion as judged by gel filtration and freeze-fracture electron microscopy. Egg phosphatidate SUV (PA-SUV) whose internal cavity is in equilibrium with the dispersion medium are strictly speaking thermodynamically unstable by these criteria. They may, however, be regarded as stable from a practical point of view. CF-release is observed with a half-time of 14 days and also some vesicle fusion, particularly at low temperature (4°C). The small effects observed, e.g., the small tendency of the vesicles to undergo fusion is probably due to the high surface charge density of PA bilayers. A main finding of this work is that the same positive pH-gradient which is used in the pH-jump method to drive the formation of SUV from large phosphatidic acid bilayer sheets has a stabilizing effect on the resulting PA-SUV. Stabilization is achieved by positive pH-gradients of about two pH-units or more with the pH of the external medium exceeding the pH of the vesicle cavity. Under these conditions, up to about 8 weeks no significant loss of entrapped CF and no fusion of SUV was observed both at 4°C and room temperature. In contrast, a reverse or negative pH-gradient of several pH units applied to PA-SUV (with the external pH being lower than that of the vesicle cavity) destabilizes PA-SUV. Such a gradient can be shown to lead to a dramatic perturbation of the lipid bilayer packing as evident from a significant increase in CF permeability. The local perturbation of the phospholipid bilayer is accompanied by massive vesicle fusion which is prominent at low temperature (4°C).

Introduction

Spontaneous vesiculation is defined as the formation of vesicles or liposomes upon dispersing a dry lipid film in an aqueous medium. The dry lipid film consists of a lipid or a lipid mixture with a propensity for forming smectic (lamellar) phases. The work presented here is confined to such lipids. The term spontaneous implies that vesicle (liposome) formation occurs without external energy being supplied to the lipid dispersion. It also implies that the vesicles formed are thermodynamically stable.

It is known that lipids differ in their swelling behaviour: neutral and isoelectric lipids show limited swelling with water at temperatures above their crystal(gel)-to-liquid crystal transition temperature; their lamellar repeat distance *d* determined by X-ray diffraction increases with water content to a limiting value at a given water content above which a two phase system is formed [1–3]. In contrast, negatively-charged lipids exhibit continuous swelling with water; in this case the lamellar repeat distance *d* increases continuously with water content until at water contents > 70% the X-ray diffraction pattern becomes broad and diffuse. This is due to stacking disorder of the highly swollen bilayers, and under these conditions meaningful *d*-values can no longer be derived from X-ray diffraction. The former class of lipids comprises neutral lipids such as monoacylglycerols and glycolipids, e.g., mono- and diglycosyldiacylglycerols and cerebrosides, as well as isoelectric phospholipids such as phosphatidylcholine, phosphatidylethanolamine and sph-

---

Correspondence to: H. Hauser, Laboratorium für Biochemie, Eidgenössische Technische Hochschule Zürich, ETH-Zentrum, CH-8092 Zürich, Switzerland.

Abbreviations: ATP, adenosine 5′-triphosphate; CF, 4(5)-carboxyfluorescein; DPPC, 1,2-dipalmitoyl-*sn*-phosphatidylcholine; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; EPA, egg phosphatidic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N′*-2-ethanesulfonic acid; LUV, large unilamellar vesicle(s); MLV, multilamellar vesicle(s); PA, Phosphatidic acid; RT, room temperature; SUV, small unilamellar vesicle(s).

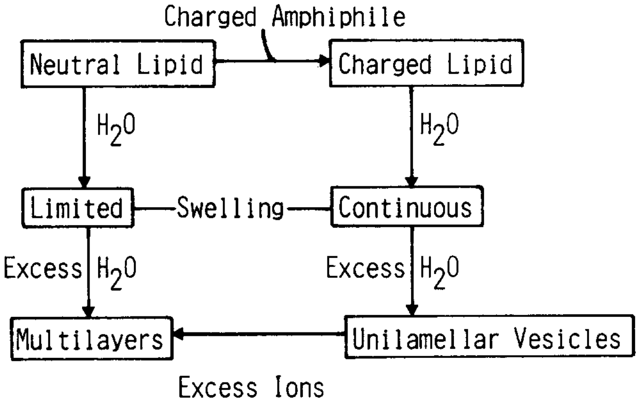

Fig. 1. Scheme summarizing the different swelling properties in water of neutral and charged lipids (from Hauser (1985) [28]).

ingomyelin. The latter class of lipids consists not only of negatively charged lipids, but more importantly of lipid mixtures of neutral (isoelectric) lipids and a minor charged component [3,4]. It was shown that doping phosphatidylcholine bilayers with a small quantity of a charged amphiphile confers continuous swelling to these bilayers provided the surface charge density is above 1–2 $\mu C/cm^2$ [4]. The swelling behavior of the two classes of lipids is summarized in Fig. 1. The thermodynamically stable structures that form when lipids are dispersed in excess water are obviously related to the swelling behavior. Neutral and isoelectric lipids form spontaneously large multilamellar vesicles (MLV) when dispersed in excess water while negatively charged lipids or lipid mixtures form spontaneously large unilamellar vesicles (LUV). The resulting populations of vesicles are inhomogeneous with respect to size: MLV vary in size ranging from 0.1 μm to several tens of micrometers, LUV are usually less inhomogeneous ranging in size from 0.1 to several micrometers [3,5]. For instance, negatively charged ox brain phosphatidylserine vesiculates spontaneously with 95% of the vesicles having diameters between 0.1 and 2 μm [3,5]. The major drawback of MLV and LUV is their heterogeneity in size. Small unilamellar vesicles (SUV) with diameters < 100 nm are usually formed by homogenization of MLV and LUV. The method most widely used in the past is ultrasonication, others include French press extrusion at generally very high pressures [6–8], the repeated extrusion at low or medium pressures through polycarbonate membrane filters of defined pore size [9] and more recently the pH-gradient method [10–12]. All these methods have in common that external energy is supplied to the lipid dispersion in one way or another. The resulting average vesicle size and size distribution depend on the input power and on the time the lipid dispersion is energized except for the extrusion through membrane filters. With this method the pore size is the main parameter determining the average vesicle size [13,14].

The pH-gradient method developed in our laboratory [11] involves a quick transient increase in pH of smectic phosphatidic acid dispersions to pH 10–12 followed by neutralization of the dispersion. This method originally referred to as pH-jump or pH-adjustment method produces SUV with a diameter of 20–60 nm. Before the mechanism of this method was disclosed, the vesiculation was considered as spontaneous implying that SUV are formed without input of external energy. However, ^{31}P-NMR [10] and infrared spectroscopy provide evidence that the driving force responsible for the formation of SUV is a pH-gradient of 3–4 pH units. Its orientation is such that the external (dispersion) medium is more alkaline than the pH of the vesicle cavity [10,12]. Like with other methods of homogenization the lipid dispersion is energized: the source of energy is the pH-gradient imposed on the PA bilayer. The term spontaneous is therefore misleading and inappropriate. Furthermore, it was shown that the method is not restricted to PA dispersions, but is of general applicability. A pH-gradient may be generated in any bilayer containing an amphiphile with one or several ionizable groups [12].

Here we study the thermodynamic stability of SUV made of PA by either sonication or the pH-gradient method. These vesicles are formed from LUV or MLV by homogenization, i.e., input of external energy. The bilayers of the resulting SUV are regarded as a distortion of the planar phospholipid bilayer and the SUV are therefore considered as thermodynamically unstable. PA vesicles are particularly attractive in addressing the question of thermodynamic stability. PA-SUV can be manipulated in a controlled way so that their stability can be determined under equilibrium conditions or in the presence of a pH-gradient. In the former case the dispersion medium is in equilibrium with the solvent entrapped in the internal cavity of the SUV. The presence of a pH-gradient can be readily monitored by ^{31}P high-resolution NMR [10].

Materials and Methods

Materials

The sodium salt of egg phosphatidic acid and 1,2-dioleoyl-*sn*-phosphatidic acid were purchased from Avanti Polar Lipids (Pelham, AL), 1,2-dilauroyl-*sn*-phosphatidic acid from Mr. R. Berchtold (Biochemisches Laboratorium, Bern, Switzerland), 1,2-dipalmitoyl-*sn*-phosphatidyl[*N-methyl*-^{3}H]choline (^{3}H-DPPC, specific activity 76 Ci/mmol) from Amersham (Amersham, UK), 4(5)-carboxyfluorescein from Fluka (Buchs, Switzerland), Sepharose CL-4B from Pharmacia (Dübendorf, Switzerland), EGTA and Hepes from Sigma (St. Louis, MO). All other chemicals used were of analytical grade. The phospholipids were pure by thin-layer chromatography standards.

Methods

A dry phospholipid film radiolabeled with ^{3}H-DPPC and deposited on the glass wall of a round bottom flask was dispersed in an appropriate volume of 0.01 M Hepes buffer (pH 7.2) containing 0.1 M KCl, 0.2mM EGTA and 0.02% NaN_3 and about 2 ml of the resulting dispersion were sonicated with a microtip sonicator (Branson B-30) under standard conditions [15]. If CF was incorporated in the vesicle cavity, the dispersion buffer contained CF at self-quenching concentrations, usually at 50 mM. External CF was separated from CF-loaded vesicles by gel filtration on Sephadex G-50 (column size, 40 × 1.1 cm), the column was equilibrated and run with the Hepes buffer at RT. The column effluent was analyzed for phospholipid content by determining the radioactivity in each fraction using a Beckman LS 7500 liquid scintillation counter. Fractions containing phospholipid vesicles were pooled and diluted with Hepes buffer to a final concentration of 0.1 or 0.2 mg phospholipid/ml (0.15 or 0.29 mM). Alternatively, EPA-SUV were prepared by the pH-gradient method [10,11].

pH-gradient

A pH-gradient across the lipid bilayer of EPA-SUV was produced by adding either KOH or HCl to the dispersion medium, and the final pH was measured with a pH electrode. If the pH in the external (dispersion) medium of the phospholipid vesicles is higher than the pH of the internal vesicle cavity, the pH-gradient is designated as positive, if the external pH is lower than the internal one the pH-gradient is negative: $pH_{ex} > pH_{in}$, then $\Delta pH > 0$; $pH_{ex} < pH_{in}$, $\Delta pH < 0$.

Gel filtration on Sepharose CL-4B

Gel filtration on calibrated Sepharose CL-4B was carried out as described before [16]. The column (49 × 0.9 cm) was equilibrated at RT with Hepes buffer, 0.2 ml of the sonicated EPA dispersions in the same buffer prepared as described above were injected in the top of the gel and the phospholipid eluted with the same buffer at a flow-rate of about 7 ml/h. The effluent was collected in an LKB-fraction collector and each fraction was analyzed for radioactivity by counting an aliquot in a Beckman LS 7500 liquid scintillation counter. The recovery of phospholipid was 90 ± 2%. The column parameters were V_o (void volume) = 11.4 ml and V_t (total volume) = 33.1 ml.

Fluorescence spectroscopy

CF was entrapped at self-quenching concentrations, and leakage due to diffusion of CF into the external medium was indicated by an increase in fluorescence intensity. The 100% value of the fluorescence intensity was determined by solubilizing EPA vesicles in 2% sodium cholate which released all CF into the external medium. Fluorescence intensities were measured with a SPEX DM1B fluorescence spectrophotometer (from Edison, NJ) with the excitation being at 470 nm, bandpass 4.5 nm, the emission at 520 nm, bandpass = 18 nm.

Results

CF (50 mM) was entrapped in the internal cavity of EPA-SUV at self-quenching concentrations, and the release of CF from these vesicles was determined as a function of time by monitoring the fluorescence inten-

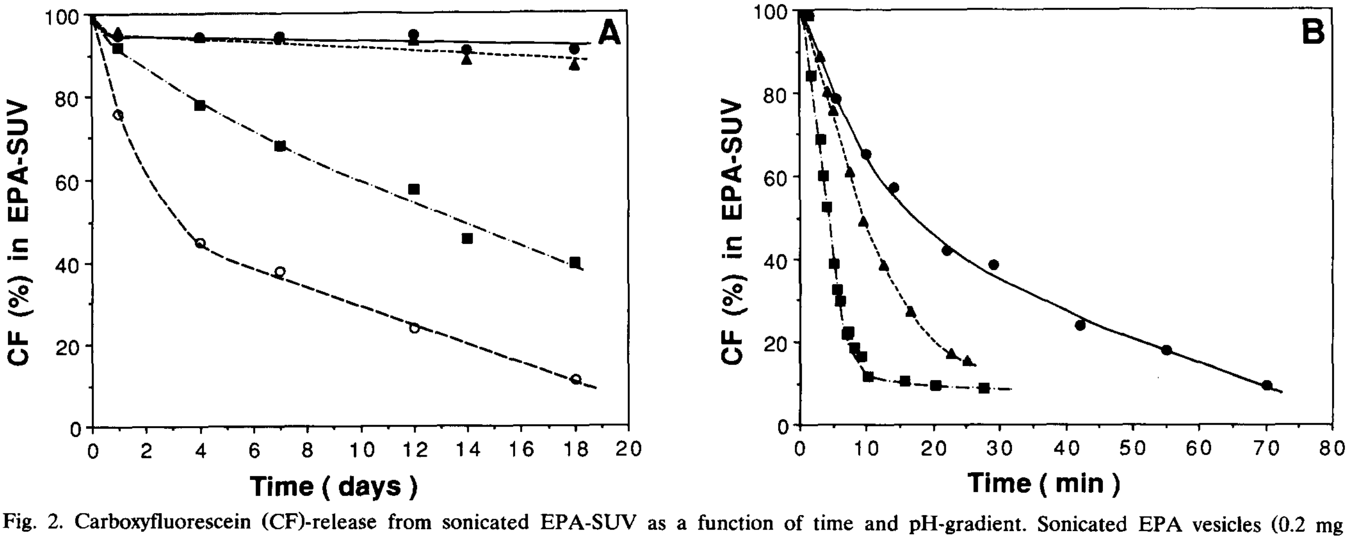

Fig. 2. Carboxyfluorescein (CF)-release from sonicated EPA-SUV as a function of time and pH-gradient. Sonicated EPA vesicles (0.2 mg lipid/ml) dispersed in 0.01 M Hepes buffer (pH 7.2), 0.1 M KCl, 0.2 mM EGTA and 0.02% NaN_3 containing 50 mM CF entrapped in the internal vesicle cavity were prepared and a pH-gradient was imposed on the EPA bilayer as described in Methods. The CF-release from EPA-SUV differing in the pH-gradient was measured at RT as a function of time. The pH-gradients were 2.3 (●——●), 1.7 (▲——▲), 0.36 (■–·–■) and −0.34 (○---○) in panel A and −3.2 (●——●), −3.76 (▲——▲) and −4.19 (■–·–■) in panel B.

sity increase. The effect of positive and negative pH-gradients on the CF-release from EPA-SUV is shown in Fig. 2. In the presence of a positive pH-gradient of two and more pH units the integrity of the bilayer of EPA vesicles appears to be maintained for weeks. Measurements were continued for up to 8 weeks and similar to the data shown in Fig. 2A no release of CF was detected over this period of time (data not shown). However, if the pH of the external medium exceeded pH 11 and the pH-gradient $\Delta pH \geq 4$ leakage of CF was observed. ^{31}P-NMR measurements indicate that degradation of PA occurred at high pH values, and the observed leakage of CF is very likely to be due to the presence of degradation products in the EPA bilayer. In the absence of a pH-gradient release of CF occurred on the time scale of days, and as shown in Fig. 2B CF-release from EPA-SUV was accelerated when the pH-gradient was reversed, i.e., the external medium was acidified relative to the pH of the vesicle cavity. In the presence of negative pH-gradients with the external pH being several pH units lower than the pH of the EPA vesicle cavity, the half-time of the CF-release was on the order of minutes; note the change in the time scale of Fig. 2 from days to minutes (cf. x-axis of Fig. 2A and Fig. 2B). In the absence of a pH-gradient and in the presence of a negative pH-gradient the CF-content of EPA-SUV decreased exponentially with time though with different time constants k_1. The kinetic data of Fig. 2 were linearized assuming that the CF-release from EPA-SUV is a pseudo-first-order reaction (Fig. 3). The pseudo-first-order rate constants k_1 derived from the linear relationships are summarized in Table I. As evident from this table the k_1 values increased with increasing negative pH-gradient. Quantitatively the k_1 values were found to be an exponential function of the negative pH-gradient (Fig. 4) indicating that the rate constant k_1 is directly proportional to the ratio $[H^+]_{ex}/[H^+]_{in}$, i.e., the ratio of proton concentration in the external medium and the internal vesicle cavity, respectively. The k_1 values were independent of the CF-concentration up to about 100 mM initially entrapped in the cavity of the EPA vesicles. Furthermore, the k_1 values were independent of phospholipid concentration between 0.01 and 0.2 mg lipid/ml indicating that the reaction is a true-first-order reaction. As regards the CF-release SUV made by sonication of dispersions of 1,2-dioleoylphosphatidic acid and 1,2-dilauroylphosphatidic acid behaved similarly to sonicated EPA dispersions.

Fig. 5 shows that the CF-release is temperature dependent. In the presence of a positive pH-gradient of several pH units, the CF-release was negligible both at 4°C and RT (Fig. 5A). In the absence of a pH-gradient or in the presence of pH-gradients of less than one pH unit very little CF was released from EPA-SUV at 4°C while some CF was released at RT. The half-time of the CF-release is about 14 days under these conditions. Consistent with the data in Fig. 2, negative pH-gradients accelerated the CF-release from EPA-SUV. This is demonstrated in Fig. 5B which also indicates that under these conditions the release of CF at 4°C is significantly reduced compared to that measured at RT.

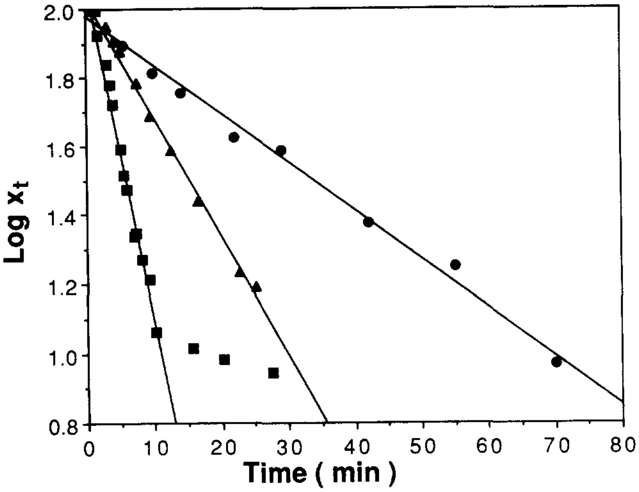

Fig. 3. The kinetic data of Fig. 2 were linearized according to log$(x_t/x_0) = -0.4343\ k_1 t$ where $x_0 = 100\%$ at $t = 0$, x_t is % CF entrapped in the cavity of EPA-vesicles at time t and k_1 is the pseudo-first-order rate constant.

Further data pertaining to the question of vesicle stability are presented in Fig. 6. This figure shows that practically superimposable gel filtration patterns on Sepharose CL-4B were obtained with EPA-SUV that were exposed to a positive pH-gradient of 2.6 pH units and immediately applied to Sepharose CL-4B and EPA-SUV that were stored under these conditions for 1 month at RT. EPA-SUV exposed to the same gradient and stored at 4°C for 1 month gave a gel filtration pattern similar to those presented in Fig. 6. The results of Fig. 6 indicate that EPA-SUV exposed to a pH-gradient ≥ 2 pH units are stabilized and do not undergo fusion to any significant extent. The average hydrodynamic radius derived from the elution volume

TABLE I

Pseudo-first-order rate constants k_1 and half-times $t_{1/2}$ for CF-release from the internal cavity of EPA-SUV

ΔpH	k_1 (min^{-1})	$t_{1/2}$ (min)
−4.65	0.430	1.61
−4.40	0.236	2.94
−4.20	0.214	3.24
−3.76	0.0883	7.85
−3.40	0.0435	15.9
−3.20	0.0401	17.3
−2.60	0.0172	40.3
−2.05	0.00535	129
−1.67	0.00218	318

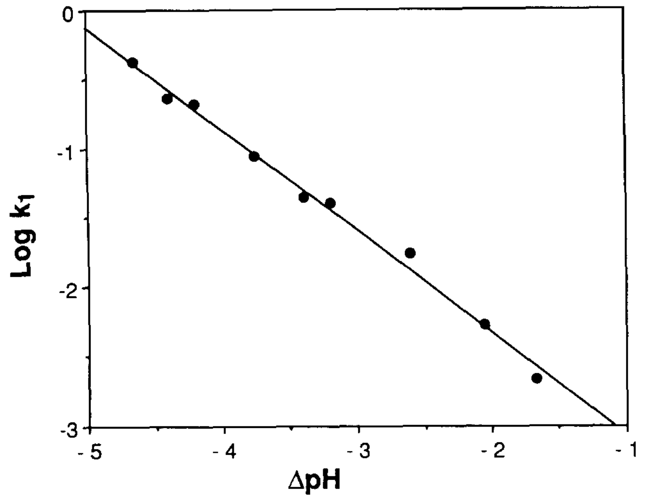

Fig. 4. Log k_1 as a function of the negative pH-gradient. The solid line was obtained by linear regression analysis yielding $y = -3.79-0.73\,x$ ($r^2 = 0.992$).

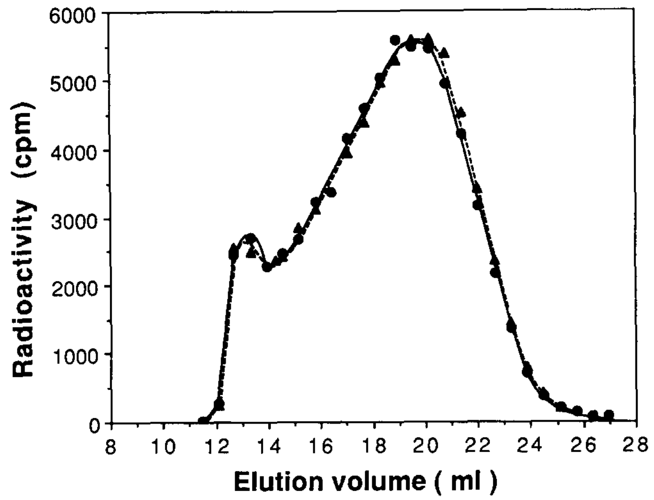

Fig. 6. Gel filtration patterns on Sepharose CL-4B. EPA radiolabeled with ^{3}H-DPPC (5 mg lipid/ml) was dispersed in 0.01 M Hepes buffer (pH 7.2) containing 0.1 M KCl, 0.2 mM EGTA and 0.02% NaN$_3$ and sonicated as described in Methods. After the preparation the pH of the medium was raised such that the external pH was 2.6 units higher than the internal pH of EPA-SUV. The dispersion (0.2 ml) thus obtained was immediately applied to the column (●——●) or after storage at RT for 31 days (▲——▲). The column (49×0.9 cm) was equilibrated and operated with the same buffer at RT with a flow-rate of about 7 ml/h.

of the main peak representing EPA-SUV was R_H = 10.9 ± 0.5 nm. The results obtained with gel filtration were corroborated by freeze-fracture electron microscopy. Within experimental error the average vesicle dimension derived from electron micrographs was consistent with that derived from gel filtration, and the vesicles appeared to be stable when incubated at 4°C or RT for 6 weeks (data not shown).

EPA vesicles produced by the pH-gradient method behaved similarly. Gel filtration on Sepharose CL-4B and freeze-fracture electron microscopy gave consistent results revealing the presence of SUV with a diameter ranging between 20 and 60 nm. When these SUV were exposed to a pH-gradient of more than two units and incubated under these conditions at RT for 8 weeks the average size and size distribution did hardly change as determined by a combination of gel filtration and freeze-fracture electron microscopy.

The effect of negative pH-gradients on the stability of sonicated EPA-SUV is depicted in Fig. 7. As a control sonicated EPA-SUV were stored at 4°C and RT (Figs. 7A and B). In sonicated dispersions the pH

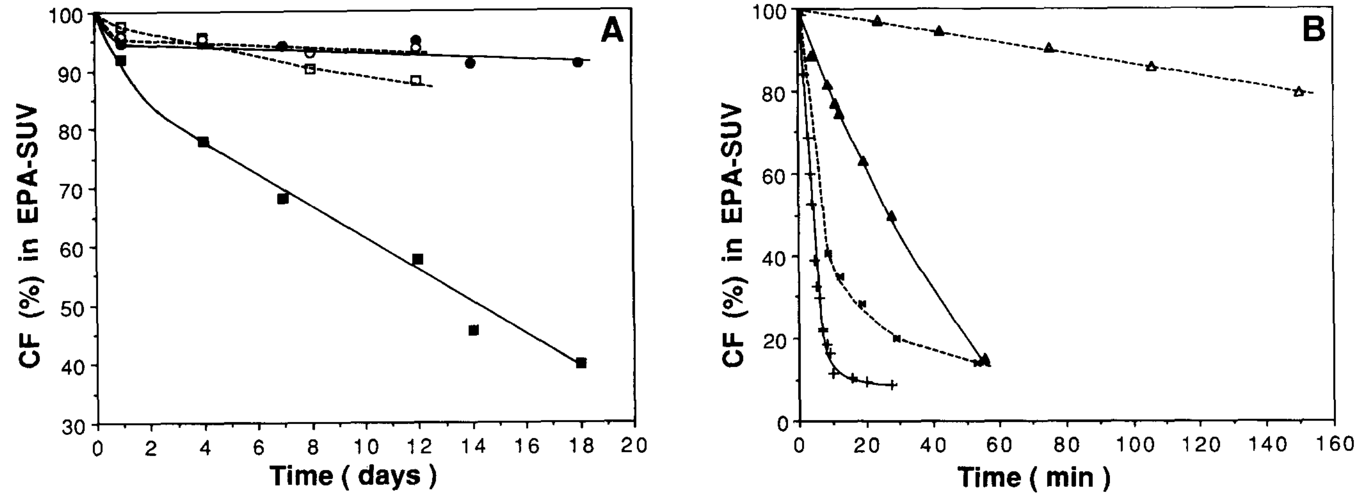

Fig. 5. Carboxyfluorescein (CF)-release from sonicated EPA-SUV at 4°C and RT. Sonicated EPA-SUV (0.2 mg lipid/ml) containing 50 mM CF in their internal cavity were prepared and subjected to a pH-gradient as described in the legends to Fig. 2 (cf. Methods). (A) EPA-SUV with a positive pH-gradient of 2.3 pH units were stored at 4°C (○——○) and at RT (●——●); EPA-SUV with a small positive gradient of 0.36 pH units were stored at 4°C (□——□) and at RT (■——■). (B) EPA-SUV with a negative pH-gradient of −2.6 units were stored at 4°C (△——△) and at RT (▲——▲); EPA-SUV with a negative pH-gradient of −4.19 units were stored at 4°C (★——★) and at RT (+——+).

of the internal cavity of SUV is at equilibrium with the external dispersion medium. Storage of these EPA-SUV at 4°C for 21 days led to vesicle fusion as indicated by an increase in the intensity of the peak at V_0 and the simultaneous decrease of the intensity of the main vesicle peak at $V_e = 20.5$ ml (Fig. 7A). This elution volume corresponds to an average hydrodynamic radius of $R_H = 10.3$ nm. After storage the elution volume of the main peak was reduced to 19.4 ml corresponding to an average hydrodynamic radius $R_H = 11.5$ nm. At RT, however, little fusion occurred during storage of equilibrated EPA-SUV for 22 days as indicated by a slight decrease of the elution volume V_e of the main peak and a very small increase in the intensity at V_0 (Fig. 7B). The decrease in elution volume V_e of the main vesicle peak corresponds to an increase in the average hydrodynamic radius from $R_H = 10.3$ nm to $R_H = 11.0$ nm. Vesicle fusion, however, was prominent in the presence of a negative pH-gradient. EPA-SUV exposed to a negative pH-gradient of 2.7 pH units underwent massive fusion then stored at 4°C for 1 day: under these conditions the main peak representing EPA-SUV disappeared almost completely and was replaced by a single peak at V_0 indicating that practically all SUV had aggregated and fused to larger particles (Fig. 7C). This interpretation is corroborated by evidence from freeze-fracture electron microscopy. Electron micrographs taken from these EPA-SUV samples ($\Delta pH = -2.7$, stored at 4°C for 1 day) revealed the presence of mainly large unilamellar phospholipid vesicles with a hydrodynamic radius of 80–200 nm (data not shown). EPA-SUV exposed to a negative

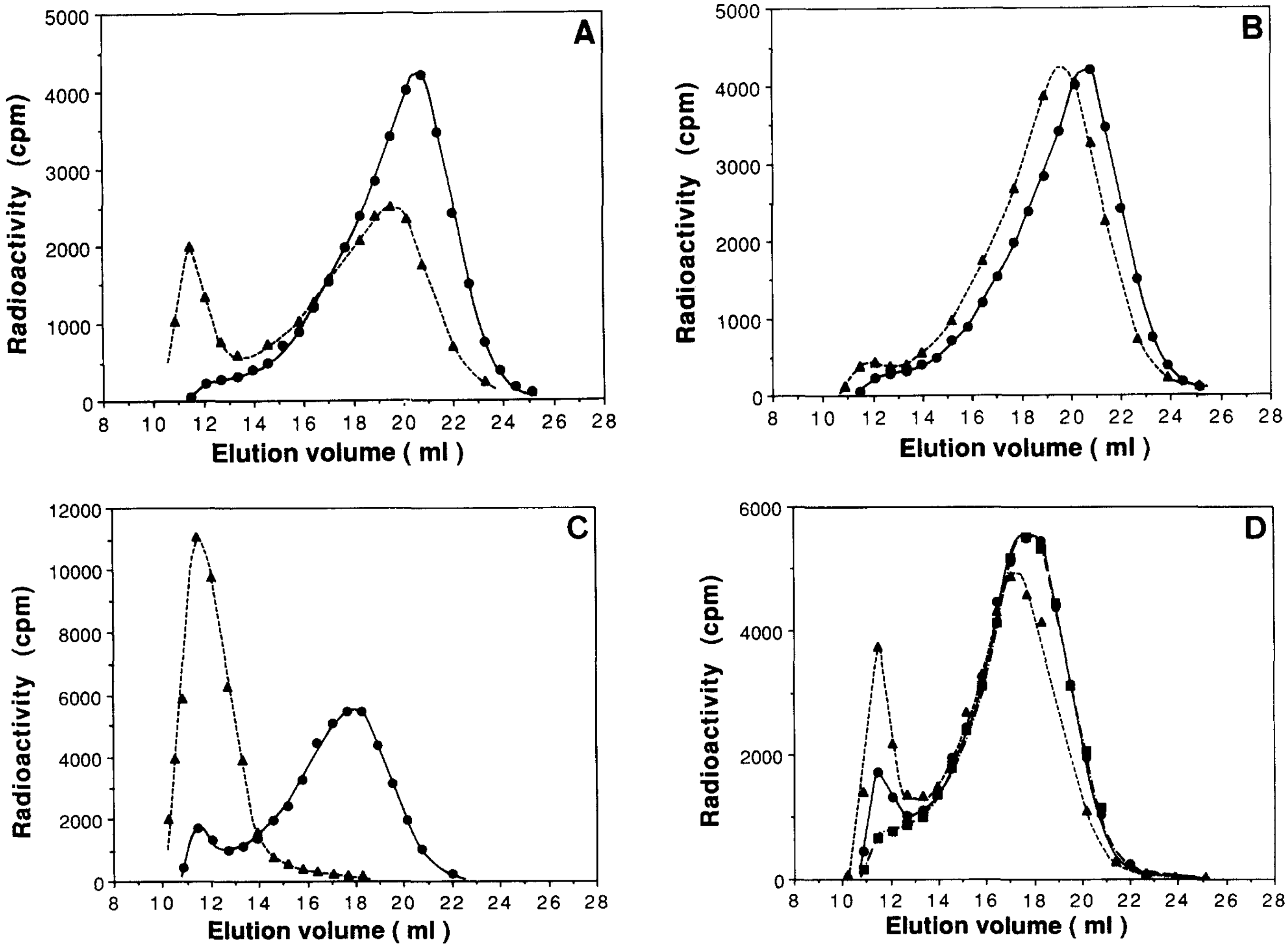

Fig. 7. Gel filtration patterns on Sepharose CL-4B. EPA radiolabeled with ^{3}H-DPPC (5 mg lipid/ml) was dispersed in 0.01M Hepes buffer (pH 7.2) containing 0.1 M KCl, 0.2 mM EGTA and 0.02% NaN_3 and sonicated as described in Methods. The resulting EPA-SUV were in equilibrium, i.e., there was no salt or pH-gradient across the EPA bilayer. In panel A and B 0.2 ml of the equilibrated EPA-SUV were applied to the column (49×0.9 cm) immediately after preparation (●——●) (A and B) and after storage at 4°C for 21 days (▲——▲) (A) and after storage at RT for 22 days (▲——▲) (B). In another sonicated EPA dispersion of the same composition as described for (A) and (B), the external pH was lowered so that a negative pH-gradient of 2.7 pH units was generated across the EPA bilayer. EPA-SUV (0.2 ml) with such a gradient were applied immediately after preparation (●——●) (C and D) and after storage at 4°C for 24 h (▲——▲) (C) and after storage at RT for 17 h (▲——▲). For comparsion the gel filtration pattern of equilibrated EPA-SUV is included (■——■) (D).

pH-gradient of 2.7 pH units, and stored at RT for 17 h exhibited the same trend although fusion was less marked than at 4°C (cf. Fig. 7C and D). Fig. 7D also shows that very short exposure of EPA-SUV to a negative pH-gradient of 2.7 pH units produced detectable vesicle fusion.

Discussion

SUV of EPA are produced by energizing the phospholipid dispersion. This is accomplished here by supplying external energy as ultrasonic irradiation or in form of a pH-gradient [10,11]. In the latter case the formation of SUV is driven by a pH-gradient of several pH units imposed on the EPA bilayer. Such pH-gradients may be regarded as a physical form of energy. They are known to participate in energy conversion in photosynthetic membranes and in mitochondria. For instance, in oxidative phosphorylation the energy gained from the oxidation of various metabolites is converted temporarily to a pH-gradient which in turn is used to produce chemical energy in form of ATP involving the enzyme ATP synthetase [17–20].

The main result of this work is the finding that the thermodynamic stability of EPA-SUV depends on experimental conditions. Under equilibrium conditions with the ionic composition of the vesicle cavity being equal to that of the external medium, EPA-SUV are strictly speaking unstable as judged from the bilayer permeability and the aggregation/fusion properties of EPA-SUV. However, both the diffusion of the fluorescent probe CF across the EPA bilayer and the aggregation/fusion of EPA-SUV are slow processes under these conditions. The CF release is characterized by a half-time of about two weeks (Figs. 2A and 5A) and the aggregation/fusion of EPA-SUV are minor events on this time scale, particularly at RT (cf. Figs. 7A and B).

The presence of a positive pH-gradient of about 2 pH-units with the external pH exceeding the internal pH ($pH_{ex} > pH_{in}$, $\Delta pH > 0$) stabilizes EPA-SUV as demonstrated in Figs. 2, 5 and 6. Under the conditions of positive pH-gradients the integrity of the bilayer is preserved (Figs. 2 and 5) and the aggregation and fusion of EPA-SUV are negligible (Fig. 6).

It should be noticed that positive pH-gradients that stabilize PA bilayers of SUV have the same direction as the pH-gradient used in the pH-gradient method to induce the formation of SUV. The mechanism of the pH-gradient method has been revealed by ^{31}P-NMR [10] and infrared spectroscopy [12] and discussed in some detail. These two spectroscopic methods provide unambiguous evidence that the pH-gradient method produces PA-SUV with the inner monolayer of the bilayer being at a lower pH (up to 4 pH units) than the external monolayer. It was suggested that the pH-gradient across the PA-bilayer gives rise to a molecular packing gradient [12]. If the pH of the dispersion medium is 10 and that of the vesicle cavity about 7, PA molecules in the outer monolayer will be fully ionized while PA molecules in the inner monolayer are partially ionized. There is greater electrostatic repulsion between doubly negatively-charged PA molecules in the outer monolayer than between partially ionized PA molecules in the inner one. Based on these electrostatic considerations a packing gradient is envisaged to exist along the bilayer normal with the inner PA monolayer being more tightly packed than the outer one. Imposing a positive pH-gradient on PA bilayers of SUV has a stabilizing effect by enforcing this kind of packing gradient. It may be regarded as equivalent to leaving the driving force on which is responsible for the formation of SUV. By comparison, with other methods of homogenization, e.g., ultrasonication, the equivalent situation would be to leave the sonicator on [21].

The time-course of the stabilization of the PA bilayer in the presence of a pH-gradient should be related to the time-constant of the dissipation of the proton gradient. The dissipation of the pH-gradient across PA bilayers can be followed conveniently by ^{31}P high-resolution NMR and preliminary NMR measurements indicate that pH-gradients decay exponentially with time constants of the order of days.

From the above discussion it should be clear that the stabilization of PA-SUV (and possibly other SUV consisting of lipids with ionizable groups) in the presence of a positive pH-gradient is achieved by maintaining a transverse packing gradient across the lipid bilayer that favours a small radius of curvature. Reversing this pH-gradient such that $pH_{ex} < pH_{in}$ would reverse the packing gradient and is therefore expected to have a destabilizing effect. This expectation is borne out by experiment. Under the conditions of a reversed pH-gradient, particularly if $(pH_{in} - pH_{ex}) \geq 2$, the bilayer integrity is significantly perturbed. This is evident from a dramatic increase in the permeability of CF (cf. Figs. 2B and 5B and Table I). Furthermore the local bilayer perturbation induced by reversing the pH-gradient leads to significant aggregation/fusion of EPA-vesicles as indicated by Figs. 7C and D. The gel filtration patterns alone indicate a significant increase in the average size of the lipid particles, they can, however not differentiate between aggregation and fusion. Electron micrographs of freeze-fractured preparation clearly show that mainly large unilamellar vesicles are formed (data not shown). This indicates that EPA-SUV undergo multiple fusion steps with the formation of large unilamellar vesicles with a diameter greater than 80 nm.

Under the conditions of negative pH-gradients with $\Delta pH < -2$ massive fusion occurs. It should be noted that under physiological conditions (pH = 7) each PA molecule bears at least one negative charge [22–25] while in the presence of negative pH-gradients $\Delta pH < -2$ the surface charge density and hence the electrostatic repulsion between apposing PA-bilayers is reduced which will also contribute to the fusion process. Figs. 7C and D give an approximate indication of the extent of fusion in 17 and 24 h, respectively, but since kinetic data of the fusion process were not collected, rate constants are not available. However, a comparison of the data in Figs. 2 and 5 and Table I with the data in Fig. 7D indicates that the rates of CF-release are faster than the fusion rates and that bilayer perturbation and an increase in bilayer permeability probably precede vesicle fusion. The release of CF from the vesicle cavity is a first-order reaction. Sometimes deviations from the first-order law were observed (cf. squares of Fig. 3). Such deviations could be due to the pH-gradient dissipating faster than the CF-gradient so that after pH equilibration and annealing of the PA-bilayer some CF remained entrapped. The slope in the semilogarithmic plot following this point would in this case represent the time constant for CF-diffusion across the intact EPA-bilayer.

Presently the dissipation of positive pH-gradients across PA-bilayers is measured using ^{31}P high-resolution NMR with the aim of deriving reliable values for the H^+ and OH^- permeability of bilayers. In the presence of positive pH-gradients of 3–4 units we observe some flip-flop motion of possibly protonated PA from the internal monolayer to the external one. Such transverse or flip-flop motion of PA molecules induced by positive pH-gradients was reported before [27]. The kinetics of the dissipation of pH-gradients across phospholipid bilayers and the permeability measurements will be the subject of a separate publication.

References

1 Chapman, D., Williams, R.M. and Ladbrooke, B.D. (1967) Chem. Phys. Lipids 1, 445–475.
2 Shipley, G.G. (1973) in Biological Membranes (Chapman, D. and Wallach, D.F.H., eds.), Vol. 2, pp. 1–89, Academic Press, London and New York.
3 Hauser, H. (1984) Biochim. Biophys. Acta 772, 37–50.
4 Gulik-Krzywicki, T., Tardieu, A. and Luzzati, V. (1969) Mol. Cryst. Liq. Cryst. 8, 285–291.
5 Atkinson, D., Hauser, H., Shipley, G.G. and Stubbs, J.M. (1974) Biochim. Biophys. Acta 339, 10–29.
6 Barenholz, Y., Amselem, S. and Lichtenberg, D. (1979) FEBS Lett. 99, 210–214.
7 Hamilton, R.L., Goerke, J., Guo, L.S.S., Williams, M.C. and Havel, R.J. (1980) J. Lipid Res. 21, 981–992.
8 New, R.R.C. (1990) in Liposomes A Practical Approach (New, R.R.C., ed.), Oxford University Press, New York.
9 Olson, F., Hunt, T., Szoka, F.C., Vail, W.J. and Papahadjopoulos, D. (1979) Biochim. Biophys. Acta 557, 9–23.
10 Hauser, H. (1989) Proc. Natl. Acad. Sci. USA 86, 5351–5355.
11 Hauser, H. and Gains, N. (1982) Proc. Natl. Acad. Sci. USA 79, 1683–1687.
12 Hauser, H., Mantsch, H.H. and Casal, H.L. (1990) Biochemistry 29, 2321–2329.
13 Hope, M.J., Bally, M.B., Mayer, L.D., Janoff, A.S. and Cullis, P.R. (1986) Chem. Phys. Lipids 40, 89–107.
14 Schurtenberger, P. and Hauser, H. (1992) in Liposome Technology (Gregoriadis, G., ed.), 2nd Edn., Vol. I, CRC Press, Boca Raton, FL.
15 Hauser, H.O. (1971) Biochem. Biophys. Res. Commun. 45, 1049–1055.
16 Schurtenberger, P. and Hauser, H. (1984) Biochim. Biophys. Acta 778, 470–480.
17 Mitchell, P. (1966) in Chemiosmotic coupling in oxidation and photosynthetic phosphorylation, Glynn Research, Bodmin, Cornwall, UK.
18 Mitchell, P. (1976) J. Theor. Biol. 62, 327–367.
19 Hatefi, Y. (1985) Annu. Rev. Biochem. 54, 1015–1069.
20 Qiu, Z., Yu, L. and Yu, C. (1992) Biochemistry 31, 3297–3302.
21 Finer, E.G., Flook, A.G. and Hauser, H. (1972) Biochim. Biophys. Acta 260, 49–58.
22 Papahadjopoulos, D. (1968) Biochim. Biophys. Acta 163, 240–254.
23 Träuble, H. and Eibl, H. (1974) Proc. Natl. Acad. Sci. USA 71, 214–219.
24 Galla, H.-J. and Sackmann, E. (1975) Biochim. Biophys. Acta 401, 509–529.
25 Van Dijck, P.W.M., De Kruijff, B., Verkleij, A.J., Van Deenen, L.L.M. and De Gier, J. (1978) Biochim. Biophys. Acta 512, 84–96.
26 Demel, R.A., Yin, C.C., Lin, B.Z. and Hauser, H. (1992) Chem. Phys. Lipids 60, 209–223.
27 Eastman, S.J., Hope, M.J. and Cullis, P.R. (1991) Biochemistry 30, 1740–1745.
28 Hauser, H. (1985) Chimia 39, 252–264.