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Loading of amphipathic weak acids into liposomes in response to transmembrane calcium acetate gradients

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Abstract

We describe a novel procedure to load amphipathic weak acid molecules into preformed liposomes. Differences in calcium acetate concentrations across the liposomal membrane induce an increase of the internal pH. This pH imbalance serves as an efficient driving force to load and accumulate weak acids (5(6)-carboxyfluorescein and nalidixic acid) inside the lipid vesicles. The mechanism of loading and the relevance of the method in drug delivery systems are discussed.

Keywords: Liposome; Remote loading; pH gradient; Calcium acetate gradient; 5(6)-Carboxyfluorescein; Nalidixic acid

1. Introduction

The utilization of liposomes as drug delivery systems has stimulated the development of efficient encapsulation procedures. Imposing a difference in proton concentration across the membrane of liposomes can drive the loading of amphipathic molecules. For example, weak bases accumulate in the aqueous phase of lipid vesicles in response to a difference in pH between the inside and the outside of the liposomes ($pH_{in} < pH_{out}$) [1,2]. Usually this pH imbalance is generated by a two-step process: first the vesicles are prepared in a low pH solution, then the external medium is exchanged by gel-exclusion chromatography with a neutral solution.

Recently, this approach has been developed further by using transmembrane differences in ammonium sulfate concentrations [3]. This technique takes advantage of the large difference in the permeability coefficients across lipid bilayers of the sulfate anion ($P < 10^{-12}$ cm/s) and of the ammonia molecule ($P = 0.13$ cm/s), generated by the dissociation of the ammonium cation, to produce a decrease of the liposome internal pH. Then this transmembrane pH difference was used as a driving force for the remote loading of an amphipathic weak base, doxorubicin. In addition, the sulfate salt of this molecule has a very low solubility and aggregates inside the liposomes, resulting in even larger encapsulation efficiencies and the stabilization of the loading [3,4].

The capacities of this procedure prompted us to adapt it for the loading of amphipathic weak acids. To achieve this goal we had to find a weak acid salt with the following characteristics: (1) the salt is water soluble at high concentration; (2) the permeability coefficient of the weak acid is larger than 10^{-4} cm/s to ensure rapid transmembrane movements (on the order of 10 milliseconds); (3) the cation is practically impermeable ($P < 10^{-10}$ cm/s); (4) the cation is multivalent in order to facilitate the formation of intraliposomal insoluble salt complexes.

In this study we present results showing that calcium acetate fulfills these requirements. Differences in calcium acetate concentrations across the liposomal membrane indeed induce an increase of the internal pH. Then this pH imbalance serves as an efficient driving force to load and accumulate weak acids (5(6)-carboxyfluorescein and nalidixic acid) inside the lipid vesicles.

2. Materials and methods

2.1. Materials

Hydrogenated soybean phosphatidylcholine (HPC) was obtained from Lipoid KG (Ludwigshafen, Germany). 8-

Abbreviations: DPX, *p*-xylene-bis-pyridinium bromide; HPC, hydrogenated soybean phosphatidylcholine.

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Hydroxypyrene-1,3,6-trisulfonic acid (pyranine), *p*-xylene-bis-pyridinium bromide (DPX) and 5(6)-carboxyfluorescein were purchased from Molecular Probes (Eugene, OR). *tert*-Butanol was obtained from BDH Laboratory Supplies (Poole, UK). Sephadex G-50 was from Pharmacia (Uppsala, Sweden). HPLC-grade solvents were obtained from Labscan (Dublin, Ireland). All other chemicals used were purchased from Sigma (St. Louis, MO). Purity of the lipids was better than 98% as assessed by thin layer chromatography [5].

2.2. Methods

2.2.1. Preparation of the lipid vesicles

HPC/cholesterol (60:40, mol/mol) were dissolved in *tert*-butanol and lyophilized overnight. The lipid mixture was hydrated at 70°C (i.e., above the gel-to-liquid-crystalline phase transition temperature of HPC) to form multilamellar vesicles. The volume of hydration medium was adjusted to obtain a 10% (w/v) lipid concentration. The suspension was frozen in liquid N_2 and thawed in a water bath maintained at 70°C; the freeze-thaw cycle was repeated 5 times. The vesicles were downsized by extrusion through two stacked 0.1-μm-pore-diameter polycarbonate filters obtained from Poretics (Livermore, CA), using the LiposoFast™ device built by Avestin (Ottawa, Canada) [6]. Extrusion was performed 15 times at 70°C, incubating the extrusion apparatus in an oven set at that temperature. The vesicle size distributions were routinely determined by quasielastic light scattering with a Coulter submicron particle analyzer (model N4 SD, Coulter Electronics, Luton, UK). The phospholipid concentration was assayed using a modified Bartlett procedure [5].

2.2.2. Measurement of the inner pH of the liposomes

The pH of the internal compartment of the lipid vesicles was measured with the membrane-impermeant, pH-sensitive fluorescent molecule, pyranine [7,8], using a two-wavelength isosbestic-point method [9,10]. Since pyranine contains three sulfonate groups which are completely ionized over a large range of pH, it does not cross phospholipid bilayers. The degree of ionization of the 8-hydroxyl group ($pK_a = 7.2$) affects its fluorescence intensity at 507 nm when excited at 460 nm, while at 415 nm the emission is a function of the pyranine concentration only. The liposomes were prepared as described above in a solution containing 0.5 mM pyranine and either 150 mM sodium acetate or 120 mM calcium acetate, pH 6.0. The untrapped pyranine was removed by gel-exclusion chromatography on Sephadex G-50 minicolumns preequilibrated with a solution of the acetate salt used to prepare the liposomes (i.e., 150 mM sodium acetate or 120 mM calcium acetate, pH 6.0). The fluorescence emission intensity at 507 nm was measured for the excitation wavelengths of 460 and 415 nm with a Perkin-Elmer LS 50B luminescence spectrometer. The lipid concentration in the cuvette was 0.03% (w/v). DPX was added to the suspension at 2 mM final concentration to quench the fluorescence of any untrapped pyranine [9]. The internal pH was calculated from the ratio of the fluorescence intensities, $R = F_{460}/F_{415}$, as [11]:

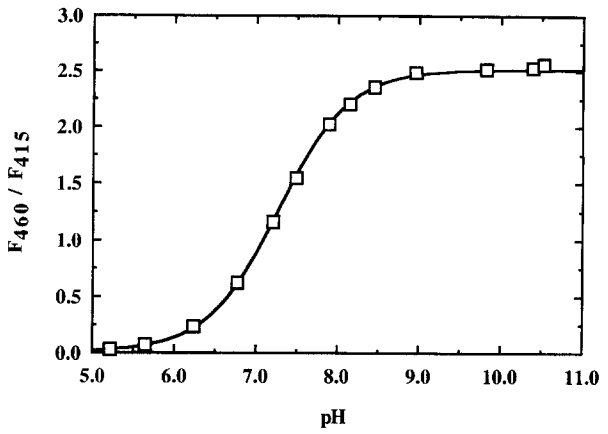

Fig. 1. pH dependence of the fluorescence intensity ratio, F_{460}/F_{415}, for pyranine (1 μM, excitation 460 and 415 nm, respectively, emission 507 nm) dissolved in 120 mM calcium acetate solutions with pH ranging from 5.2 to 10.5. The solid line represents the best fit to Eq. (2) described in Section 2 with $pK_a = 7.268 \pm 0.003$, $R_a = 0.0122 \pm 0.0007$, $R_b = 2.515 \pm 0.006$.

$$\mathrm{pH} = pK_a + \log\left(\frac{R - R_a}{R_b - R}\right) \quad (1)$$

where pK_a is the apparent pK_a of the fluorescent probe, R_a and R_b are the fluorescence intensity ratios of the protonated form and of the unprotonated form of the probe, respectively. A calibration curve was made with solutions of 1 μM pyranine in 120 mM calcium acetate at different pH values (Fig. 1). The values of pK_a, R_a and R_b were determined by fitting the experimental calibration curve to the reciprocal relationship:

$$R = R_a + \frac{R_b - R_a}{1 + \exp[(pK_a - \mathrm{pH}) \cdot \ln 10]} \quad (2)$$

The fitted parameters are: $pK_a = 7.268 \pm 0.003$, $R_a = 0.0122 \pm 0.0007$ and $R_b = 2.515 \pm 0.006$.

2.2.3. Formation of the transmembrane difference in acetate concentrations

The concentration of the acetate salt in the external compartment was controlled either by dilution of the liposome suspension in 120 mM Na_2SO_4, or by gel-exclusion chromatography on Sephadex G-50 minicolumns preequilibrated with 120 mM Na_2SO_4. Na_2SO_4 was chosen because of the low permeability coefficients of both the sodium and the sulfate ions (on the order of, or less than, 10^{-11} cm/s). The salt concentration was 120 mM to maintain the same osmolality on both sides of the lipid bilayers. The osmolalities of the solutions used in this study were measured with a Wescor 5500 vapor pressure osmometer (Wescor, Logan, UT).

2.2.4. Nalidixic acid assay

The amount of nalidixic acid present in the vesicle suspensions was quantified by HPLC [12]. Briefly, the samples were extracted with 9:1 (v/v) methylene chloride/2-propanol, and run on an Alltech C18 reversed-phase column (Alltech Associates, Deerfield, IL) (150 mm, 4.6 mm ID). The mobile phase was composed of 65:35 (v/v) methanol/55 mM K_2HPO_4, 18 mM NaH_2PO_4 and 5.5 mM hexadecyltrimethylammonium bromide, pH 7.4. The flow rate was 1.0 ml/min. Detection was achieved by UV absorption at 328 nm. Under these conditions, the retention time of nalidixic acid was typically 5.1 min.

3. Theoretical considerations

Let us consider the distribution of an amphipathic weak acid between the inside and the outside compartments of a liposome suspension. It is assumed that the dissociated ionized acid molecules are unable to cross the lipid bilayer, while the undissociated neutral molecules can permeate rapidly ($P > 10^{-4}$ cm/s). For spherical vesicles of radius r, the half-time of permeation is given by:

$$t_{1/2} = \frac{\ln 2 \cdot r}{3P} \tag{3}$$

Thus, molecules with permeability coefficients larger than 10^{-4} cm/s are released from 100-nm-diameter unilamellar vesicles with half-times less than 10 ms. At equilibrium the concentration of the undissociated form is uniform within the whole suspension, i.e., the same in both internal and external compartments. Thus, according to the acid-base equilibrium, the following relationships hold true:

$$\frac{[A^-]_{out}}{[A^-]_{in}} = \frac{[H^+]_{in}}{[H^+]_{out}} \tag{4}$$

$$\frac{C_{out}}{C_{in}} = \frac{[H^+]_{in}(K_a + [H^+]_{out})}{[H^+]_{out}(K_a + [H^+]_{in})} \tag{5}$$

where the subscripts in and out refer to the internal and external compartments, respectively. K_a is the dissociation constant of the acid, C is the total concentration of the acid, $[A^-]$ is the concentration of the dissociated form of the acid and $[H^+]$ is the proton concentration. These two expressions can be simplified when the pH values are much higher than the pK_a. Under these conditions, K_a is much larger than the proton concentrations, and the undissociated acid concentration is negligible. This simplifies Eqs. (4) and (5) to:

$$\frac{C_{out}}{C_{in}} \approx \frac{[H^+]_{in}}{[H^+]_{out}} \tag{6}$$

Therefore, a transmembrane concentration gradient of the weak acid generates a difference in pH between the inside and the outside compartments:

$$pH_{in} - pH_{out} = \log\left(\frac{C_{in}}{C_{out}}\right) \tag{7}$$

In the experiments described in this paper, the volume of the external compartment is at least three orders of magnitude larger than that of the internal compartment. Under these conditions, the number of transferred protons is not large enough to change significantly the external pH. In addition, if the initial pH values of both compartments are equal, i.e., $pH^0_{in} = pH^0_{out}$, then Eq. (7) becomes:

$$pH_{in} = pH^0_{in} + \log\left(\frac{C_{in}}{C_{out}}\right) \tag{8}$$

Let us suppose that a second amphipathic weak acid is added to the liposome suspension. Since its neutral form can cross the lipid bilayer, it distributes between the liposome internal compartment and the external compartment. The equilibrium distribution is determined by Eq. (6). Thus, the transmembrane gradient of the first weak acid can drive the loading of the second weak acid in the liposome internal compartment. This experiment was performed with 5(6)-carboxyfluorescein and with an antibacterial quinolone, nalidixic acid.

4. Results

4.1. Increases in the liposome inner pH in response to transmembrane differences in acetate concentrations

The measurements using the membrane-impermeant, pH-sensitive probe pyranine clearly show that the internal pH of the liposomes was linearly dependent on the logarithm of the acetate concentration ratios, C_{in}/C_{out} (Fig. 2). The presence of DPX, a very efficient quencher of the pyranine fluorescence [9], in the external compartment prevented possible traces of pyranine in that compartment from interfering with the measurements. In this experiment the internal concentration of the acetate salt was equal to that used for the preparation of the liposomes (either 150 mM sodium acetate or 120 mM calcium acetate, pH 6.0), while the external medium contained a lower concentration of acetate in Na_2SO_4, and 2 mM DPX at pH 6.0. The internal pH was calculated from the fluorescence intensity ratio of the trapped pyranine (Fig. 1). The pH values measured under equal concentration conditions ($C_{in} = C_{out}$) were, within experimental error, equal to the pH of the acetate solutions used for the preparation of the liposomes. This observation validated this experimental method for measuring the pH of the aqueous space trapped inside the lipid vesicles. The rise of the internal pH was very fast (less than 10 s). Our experimental settings did not permit measuring the actual rate. The pH increase depended only on the ratio of the internal to external concentration of acetate, and showed no dependence on the nature of the counterion, in our case sodium or calcium. This observation is expected since the permeability coefficients of both ions are of the same order of magnitude ($P^+_{Na} = 1.5 \cdot 10^{-11}$

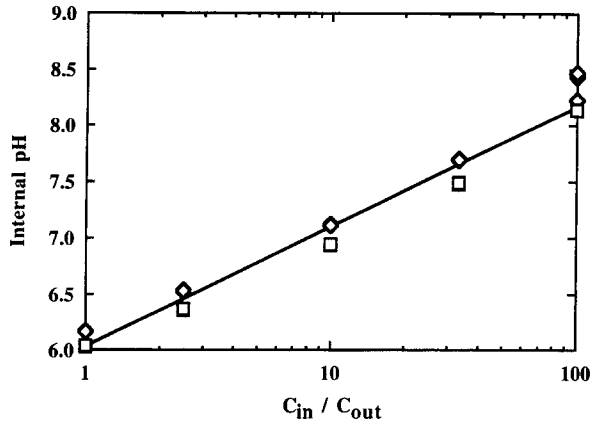

Fig. 2. Changes of the liposome internal pH as a function of the ratio of internal to external concentration of sodium acetate (□) or calcium acetate (◇). The liposomes were prepared in either 150 mM sodium acetate, pH 6.0, or 120 mM sodium acetate, pH 6.0. The acetate concentrations in the external medium were adjusted by dilution into mixtures made with the acetate salt solution used to prepare the liposomes and 120 mM Na_2SO_4, pH 6.0. The lipid concentration in the cuvette was 0.03% (w/v); DPX was added to the suspension at 2 mM final concentration. The internal pH's calculated from the entrapped pyranine fluorescence (see Section 2) are linearly dependent on the logarithm of the acetate concentration ratios: $pH = 6.03 + 1.07 \times \log(C_{in}/C_{out})$.

cm/s [13], $P_{Ca}^{2+} = 2.5 \cdot 10^{-11}$ cm/s [14]). The linear relationship between the inner pH and the logarithm of the concentration ratio is in good agreement with Eq. (8), derived from the acid-base equilibrium and the difference in the permeability coefficients as described in Section 3.

4.2. Transmembrane differences in acetate concentrations drive the loading of weak acids

Weak acids added to the external medium were loaded into liposomes with the driving force being the pH imbalance generated by the transmembrane difference in acetate concentrations. To demonstrate this remote loading technique, we used two fluorescent weak acids, 5(6)-carboxyfluorescein and nalidixic acid. First, 1 μM 5(6)-carboxyfluorescein was incubated in the presence of lipid vesicles loaded with calcium acetate at pH 7.0, and the fluorescence intensity of the suspension was monitored as a function of time (Fig. 3). When the external medium contained acetate at a concentration equal to that of the liposome inner space, the fluorescence remained practically constant. However, the fluorescence intensity decreased exponentially with a half-time of 54 min when no (or trace amounts of) acetate was in the external medium. It is known that the fluorescence of 5(6)-carboxyfluorescein is self-quenched at concentrations exceeding 1 mM [15]. The mechanism of quenching involves the formation of non-fluorescent dimers and the energy transfer from monomer to monomer and from monomer to dimer [16]. The observed decrease in fluorescence intensity is consistent with the movement of the weak acid from the external medium into the liposome interior, and its subsequent accumulation in this compartment. In separate experiments, we measured the amount of 5(6)-carboxyfluorescein associated with the liposomes by isolating the trapped material from the free material by gel-exclusion chromatography. To detect the fluorescence in the eluted fractions we used a 1 mM initial external concentration of the weak acid. In the absence of calcium acetate gradient, 5(6)-carboxyfluorescein eluted in the void volume of the column. After a 17 h-incubation in the presence of calcium-acetate-loaded vesicles at 70°C, 60% of the total 5(6)-carboxyfluorescein eluted with the liposomes. The quenching of the fluorescence in these fractions was 47% (±2%), as calculated by measuring the fluorescence intensity in the absence or in the presence of 3 mM Triton X-100; such a value is observed for trapped 5(6)-carboxyfluorescein of concentrations on the order of 10 mM [15]. In a second series of experiments we studied the loading of nalidixic acid. When this molecule was incubated at room temperature in the presence of calcium-acetate-loaded liposomes its fluorescence was a function of the internal-to-external ratio of calcium acetate concentration (Fig. 4). The interpretation of these experimental results is not straightforward since, at the wavelength used for excitation, the light scattered by the vesicles cannot be neglected (dashed line on both panels of Fig. 4), and above 10 μM concentration the fluorescence of nalidixic acid is affected by the inner filter effect ($\varepsilon_{328} = 10\,600$). At 10 μM initial external concentration (Fig. 4, bottom panel), under no-gradient conditions, i.e., 120 mM calcium acetate present inside and outside the liposomes, the addition of calcium-acetate-loaded vesicles produced a sudden increase in the signal

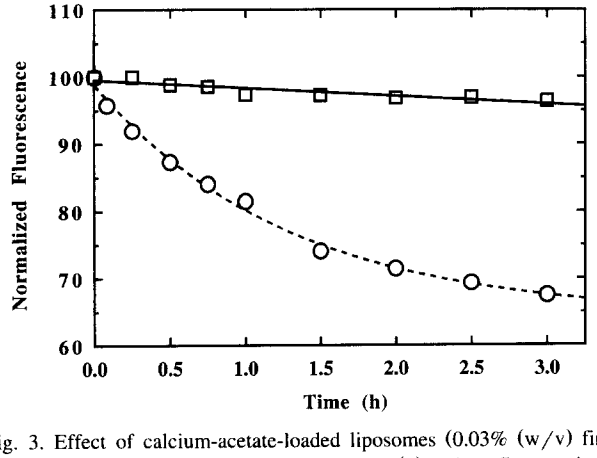

Fig. 3. Effect of calcium-acetate-loaded liposomes (0.03% (w/v) final lipid concentration) on the fluorescence of 5(6)-carboxyfluorescein (1 μM) dissolved in either 120 mM calcium acetate, pH 7.0 (□) or 1.2 mM calcium acetate and 118.8 mM Na_2SO_4, pH 7.0 (○). Experimental temperature: 65°C. The fluorescence intensities (excitation 470 nm, emission 520 nm) were normalized to the values measured at time 0. In the absence of any transmembrane difference in acetate concentrations the fluorescence remains practically constant (solid line: $F/F_0 = 0.996 - 0.012t$), while the fluorescence decreases exponentially when the acetate concentration in the external medium was lower than that inside the liposomes (dashed line: $F/F_0 = 0.35 \times \exp(-0.77t) + 0.65$).

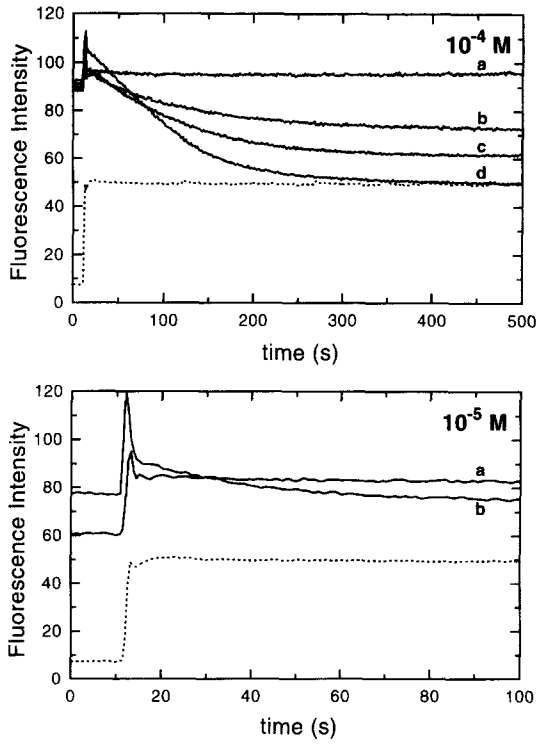

Fig. 4. Effect of calcium-acetate-loaded liposomes (0.033% (w/v) final lipid concentration) on the fluorescence of nalidixic acid (excitation 328 nm, emission 364 nm) at 25°C. The liposomes were added at $t = 12$ s. The light-scattering caused by the vesicles was determined under the same conditions with no nalidixic acid in solution (dashed line). (Top panel) Initial external concentration of nalidixic acid: 100 μM. The suspension medium was composed of (a) 120 mM calcium acetate, pH 7.0, (b) 2.4 mM calcium acetate, 117.6 mM Na_2SO_4, pH 7.0, (c) 1.2 mM calcium acetate, 118.8 mM Na_2SO_4, pH 7.0 or (d) 0.4 mM calcium acetate, 119.6 mM Na_2SO_4, pH 7.0. The resulting internal-to-external concentration ratios of calcium acetate: (a) 1, (b) 50, (c) 100 or (d) 300. (Bottom panel) initial external concentration of nalidixic acid: 10 μM. The suspension medium was composed of either (a) 120 mM calcium acetate, pH 7.0 or (b) 0.4 mM calcium acetate, 119.6 mM Na_2SO_4, pH 7.0. The resulting inside-to-outside concentration ratios of calcium acetate: (a) 1 or (b) 300.

intensity due to the scattering of light, then the signal remained stable (trace a). This experiment was repeated under the same conditions but the internal-to-external ratio of calcium acetate concentration was set to 300 by controlled dilution (trace b). The initial jump in signal produced by the light-scattering of the lipid vesicles was observed, but then the fluorescence intensity decreased and reached a stable value lower than that obtained under no-gradient conditions. In this experiment, the initial intensities reflected the difference in fluorescence of the calcium salt (trace a) and of the sodium salt (trace b) of nalidixic acid. At 100 μM concentration (Fig. 4, top panel), the optical density of the nalidixic acid solution was 1.06, and the fluorescence signal was lower due to the inner filter effect. However, the addition of calcium-acetate-loaded liposomes produced the same effects as those observed at 10 μM concentration; namely, the fluorescence remained constant under no-gradient conditions (trace a), and decreased when there was a gradient of calcium acetate (traces b–d). The reduction in fluorescence intensity was dependent on the gradient: the larger the gradient, the lower the final fluorescence. The limited water-solubility of nalidixic acid did not allow us to measure its fluorescence above 1 mM concentration, and we are not aware of any reports of a concentration-dependent mechanism of fluorescence self-quenching for this molecule. Nevertheless, the observed decrease of the fluorescence intensity points to a process similar to that proposed for the loading of 5(6)-carboxyfluorescein. The amount of trapped nalidixic acid was also measured directly by gel-exclusion chromatography and HPLC. At 100 μM initial external concentration, and after a 15 min incubation at room temperature in the presence of calcium-acetate-loaded lipid vesicles, 72% of the nalidixic acid was associated with the liposomes. The molar ratio of nalidixic acid to phospholipid was equal to $(3.8 \pm 0.3) \cdot 10^{-3}$, and from this value the intra-liposomal concentration of the acid was estimated to be about 1 mM. Thus, for both weak acids tested in this study, we found that the transmembrane differences in acetate concentrations were able to drive their loading inside the liposomes.

4.3. Release of the trapped nalidixic acid

Once the loading of nalidixic acid was completed, we used two approaches to induce its release into the external medium. The loading and the release were monitored by the changes in the fluorescence intensity of a 0.1 mM solution of nalidixic acid. First, a detergent (Triton X-100 at 5 mM final concentration) was added to a suspension of nalidixic-acid-loaded vesicles (Fig. 5). The addition of the detergent produced an increase in the fluorescence inten-

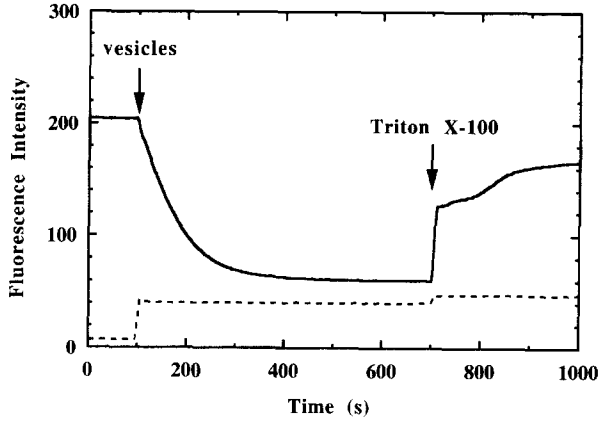

Fig. 5. The disruption of the lipid bilayers causes the release of trapped nalidixic acid. The fluorescence intensity (excitation 328 nm, emission 364 nm) of 10^{-4} M nalidixic acid was monitored at 25°C as a function of time (solid line). At $t = 100$ s, calcium-acetate-loaded vesicles (0.033% (w/v) final lipid concentration) were added, and at $t = 700$ s, the lipid vesicles were permeabilized by addition of Triton X-100 (5 mM final concentration). The scattering caused by the vesicles was measured under the same conditions with no nalidixic acid in solution (dashed line).

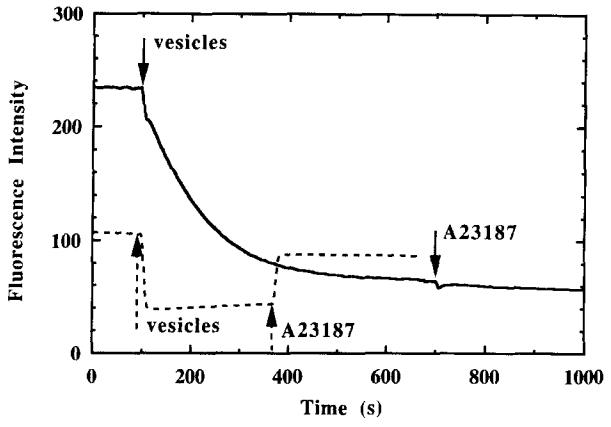

Fig. 6. Equilibrating the calcium concentrations induces the release of the trapped nalidixic acid. The fluorescence intensity (excitation 328 nm, emission 364 nm) of a 10^{-4} M nalidixic acid was monitored as a function of time at 25°C (solid line) and at 60°C (broken line). At $t = 100$ s, calcium-acetate-loaded vesicles (0.033% (w/v) final lipid concentration) were added, and the calcium ionophore A23187 (10 μM final concentration) was added at either $t = 700$ s (25°C experiment) or 350 s (60°C experiment).

sity (solid line). It is likely that the sudden change in fluorescence reflects the partitioning of untrapped nalidixic acid molecules into the surfactant micelles. In this experiment, the loading of nalidixic acid was not complete: if one assumes total quenching of the fluorescence of the trapped molecules, the amount of the untrapped molecules represented 15% of the total nalidixic acid. After addition of Triton X-100, the untrapped nalidixic acid can distribute between the aqueous phase and the surfactant micelles. Since the dielectric constant in the core of the micelle is lower than in water, one expects an increase in the fluorescence quantum yield. Actually, we found in separate experiments that the addition of Triton X-100 at 5 mM final concentration increased by 25% the fluorescence of a 0.1 mM solution of nalidixic acid (data not shown). The absence of effect of the detergent on the light scattered by the liposomes alone (broken line) suggests that the permeabilization process involves the formation of holes in the membrane rather than the total solubilization of the bilayer by formation of lipid-detergent micelles. Therefore, in the experiment presented in Fig. 5, two independent processes with different kinetics are likely to occur: first, the partitioning of the untrapped nalidixic acid between the aqueous phase and the surfactant micelles; second, the formation of pores in the bilayer and the release of the trapped molecules. In a second experiment we used the calcium ionophore A23187 at 10 μM concentration (Fig. 6). At 60°C the ionophore induced a rapid increase in fluorescence (broken line). The dequenching of the nalidixic acid fluorescence can be explained by the release of the acid. Hence, bringing to equilibrium the proton and the calcium concentrations across the liposome membrane induces the collapse of the driving force for the loading and the release of the trapped acid. This experiment also illustrated the difference in the fluidity of the lipid bilayers between the gel state and the fluid state, and how these changes affect the penetration of molecules into the bilayer. When the suspension was incubated at 25°C, i.e., a temperature below the phase transition temperature of HPC, the ionophore had no effect on the fluorescence (solid line). Setting the experimental temperature at 60°C had a dramatic effect on both the loading and the action of the ionophore (broken line). The rate of loading was faster by one order of magnitude ($t_{1/2} = 84$ s at 25°C, and 7 s at 60°C).

4.4. Stability of 5(6)-carboxyfluorescein-loaded liposomes

5(6)-carboxyfluorescein was loaded into 60:40 HPC/cholesterol extruded vesicles using the calcium acetate gradient method. After removal of the untrapped 5(6)-carboxyfluorescein by gel-exclusion chromatography, the 5(6)-carboxyfluorescein-loaded liposomes (10 mM HPC concentration) were stored at 4°C. After 10 months, we measured the liposome size and the amount of entrapped 5(6)-carboxyfluorescein. The vesicle size distribution showed a single population similar to that of the starting vesicles (initial size: 112 ± 28 nm, final size: 96 ± 24 nm). An aliquot of the suspension was loaded on a Sephadex G-50 column, and the fluorescence intensity was measured in the eluted fractions after addition of Triton X-100 (3 mM final concentration). 96% of the total fluorescence was recovered in the liposomal fraction. This experiment showed that the size and the entrapment capacity of the 5(6)-carboxyfluorescein-loaded liposomes did not change appreciably over a 10 month storage period at 4°C.

5. Discussion

The data presented in this paper indicate that transmembrane gradients of calcium acetate generate an increase of the liposome internal pH, and can be used as the driving force to load amphipathic weak acid compounds into liposomes. In some ways, this system is similar to one of the cellular organelles, the mitochondrion. The pH of the mitochondrial matrix is about 8.8; the other cellular compartments are either close to neutrality (e.g., the cytoplasm) or acidic (e.g., the lysosomes, the chloroplasts and the chromaffin granules).

It is important to realize that the permeability coefficients of acetic acid ($6.6 \cdot 10^{-4}$ cm/s) and of calcium ion ($2.5 \cdot 10^{-11}$ cm/s [14]) differ by seven orders of magnitude. While calcium ions remain trapped inside the liposomes, acetic acid molecules behave as proton shuttles. In response to calcium acetate concentration differences across the membrane (inner concentration higher than outer concentration), a net transfer of protons occurs from the inside of the liposomes to the external medium, resulting in

changes of pH in both compartments. The increase of the liposome internal pH was observed experimentally (Fig. 2) and its extent was equal to that predicted from theoretical considerations. However, under the conditions used in this study, it was not possible to measure changes in the external pH. In a typical experiment the internal pH increased from 7 to 10 and the internal-to-external volume ratio was less than 10^{-3}; thus, the change in proton concentration in the external medium was less than $(10^{-7}-10^{-10})\cdot 10^{-3} \approx 10^{-10}$ M, i.e., below the sensitivity limit of the pH measurement method.

Amphipathic weak acid molecules added in the external medium can cross the lipid bilayer. The kinetics of this process depends on the weak acid permeability coefficient; this coefficient can be estimated from the partition coefficient of the weak acid between *n*-octanol and water (Table 1). In the absence of a pH gradient, the concentration of the compound inside the liposomes equals that outside. However, the high internal pH generated by transmembrane calcium acetate concentration gradients affects the equilibrium of the charged and uncharged forms of the weak acid in favor of the charged, non-permeable form. The overall result is the loading of the weak acid inside the liposomes. Since the calcium acetate gradient provides the driving force for this process, we can talk of 'active' loading, while loading in the absence of gradient is 'passive'. This new method is based on the same principles as the ammonium sulfate gradient method [3]. In both methods, a salt exists in equilibrium with two species whose permeability coefficients differ by several orders of magnitude. However, the natures of the fast-permeating species and of the non-permeating species are opposite, allowing, in the one case, the loading of amphipathic weak acids and, in the other, the loading of amphipathic weak bases.

The gel-exclusion chromatography experiments indicate that the final concentration of the loaded weak acids (either 5(6)-carboxyfluorescein or nalidixic acid) was 10-times larger than the initial external concentrations. The increase in concentrations of the weak acids inside the liposomes, relative to the external medium, demonstrates the active loading. However, even though the concentration of calcium acetate present in the external medium was not measured directly, it is estimated to be less than 10^{-4} M. Consequently the internal-to-external concentration ratios of calcium acetate used in these experiments were larger than 1000, and such ratios should generate higher entrapment than those observed (Eq. (6)). This difference can be explained by a partial release of the loaded molecules that may occur during the loading procedure. The experiment with nalidixic acid carried out at 60°C (Fig. 6, broken line) shows that once the loading is completed, the weak acid molecules can cross the fluid lipid bilayer back into the external medium. The permeation half-time of 5(6)-carboxyfluorescein is about 1 h at 65°C (Fig. 3), thus 99% of the weak acid is encapsulated in 6 h; longer incubation time at high temperature can only induce release of the weak acid in the incubation medium.

It can be argued that such a loading can be achieved simply by creating a pH difference across the liposome membrane, e.g., by preparing the liposomes in a medium at pH 10 and replacing the external medium with a medium at pH 7 by gel exclusion chromatography. However, the difference in concentrations of the hydroxide ion is only on the order of 1 mM; the gradient can be canceled totally by leaks of protons or hydroxide ions. Even in the presence of buffers inside and outside the liposomes, their limited buffer capacities may not be sufficient for ensuring the stability of the loading. On the contrary, in the method described here, the difference in calcium ion concentrations across the lipid bilayer is about 0.1 M; this excess of

Table 1
Dissociation constants (pK_a), permeability coefficients (P) and *n*-octanol/water partition coefficients (K_p) of the weak acids used in this study

Molecules	pK_a	P (cm/s)	K_p
Acetic acid	4.74 [a]	$(6.6 \pm 1.3)\cdot 10^{-4}$ [b]	0.49 [c]
Pyranine	7.22 [d]	N.D. [e]	$< 3\cdot 10^{-5}$ at pH 9 [f]
5(6)-Carboxyfluorescein	6.3 [g]	$8.1\cdot 10^{-11}$ at pH 7 [h]	$2.8\cdot 10^{-3}$ at pH 6.9 [i]
		$7.2\cdot 10^{-12}$ at pH 8.2 [h]	$6.0\cdot 10^{-5}$ at pH 8.0 [i]
Nalidixic acid	6.13 [j]	$1.4\cdot 10^{-8}$ at 25°C [k]	3.34 ± 0.08 at pH 7 [l]
		$1.7\cdot 10^{-7}$ at 60°C [k]	

[a] Ref. [19].
[b] In egg phosphatidylcholine planar bilayer, at 22°C; Ref. [20].
[c] At 25°C; Ref. [21].
[d] Ref. [7].
[e] N.D.: not determined, below the experimental sensitivity limit; Ref. [7].
[f] At 25°C; Clerc and Barenholz, unpublished results.
[g] Ref. [15].
[h] Sodium salt of 5(6)-carboxyfluorescein in sonicated dioleoylphosphatidylcholine vesicles, at 37°C; Ref. [15].
[i] Ref. [22].
[j] Ref. [23].
[k] In 60:40 (mol/mol) HPC/cholesterol extruded vesicles, from our results.
[l] Ref. [24].

calcium ions acts as a reservoir for sustaining the pH difference across the membrane for longer periods of time. The experiment with the calcium ionophore A23187 (Fig. 6) stresses the central role played by calcium ions in the stability of the loaded liposomes. As soon as the calcium ions trapped inside the liposomes are replaced by protons, i.e., when the proton and calcium gradients are collapsed, there is no more driving force for keeping the nalidixic acid inside the liposomes, and the loaded weak acid molecules leak out.

In the experimental design of the loading procedure, we have chosen a bivalent cation, calcium, to favor the formation of a complex between calcium and a weak acid inside the liposomes. Since molecules distribute between the two compartments according to their concentrations, i.e., the amounts of the soluble species, intraliposomal precipitation of the weak acid in fact removes these molecules from the equilibrium mechanisms controlling the loading, and the liposomes act as 'sinks' in which the weak acid molecules accumulate. We did not investigate the solubility of the calcium salts of 5(6)-carboxyfluorescein and nalidixic acid. However, in order to select rationally the trapped cation, it will be necessary to include in future developments the study of the solubility products and the kinetics of dissolution of the complexes composed of different cations and of the molecule to be loaded.

We used another procedure to increase the stability of 5(6)-carboxyfluorescein-loaded liposomes, namely, loading the liposomes and storing them at different temperatures. Phospholipid bilayers containing cholesterol at concentrations above 30 mol% do not exhibit gel-to-liquid-crystalline phase transitions, but their permeability is still dependent on the temperature, the higher the temperature the more permeable the membrane. Thus, the loading is performed under conditions of high permeability of the liposome membrane, e.g., at 70°C. When the loading is completed, the experimental temperature is lowered to room temperature or below; the liposome membrane is then in a low permeability state, and the suspension can be further purified and stored while retaining the loaded weak acid molecules. Our results show that 5(6)-carboxyfluorescein-loaded liposomes remained stable and retained their contents for 10 months at 4°C.

5(6)-Carboxyfluorescein-loaded vesicles offer convenient tools for studying cation permeation across lipid bilayers. 5(6)-Carboxyfluorescein is negatively charged at alkaline and neutral pH; thus, in order to maintain electroneutrality in both the intraliposomal and the external compartments, 5(6)-carboxyfluorescein efflux is coupled either to the efflux of the counter-cations present inside the liposomes, or to the influx of anions from the external medium [17,18]. This principle can be extended to other fluorescent amphipathic weak acids. Our experimental procedure allows the proper choices of the cation to be studied, by using its acetate salt to prepare the liposomes, and of the fluorescent probe to monitor the efflux of the cation. In addition to the study of cation permeation itself, these liposomes can be used to characterize factors or molecules inducing or affecting the cation efflux. For examples, the release experiments of nalidixic acid illustrate the action of Triton X-100 on lipid bilayers (Fig. 5) and the effect of the physical state of the lipid bilayer on the efficiency of the calcium ionophore A23187 (Fig. 6). This method combines two advantages over the conventional 5(6)-carboxyfluorescein-loaded liposomes. First, the amount of the fluorescent weak acid needed for the experiment is very small; second, different time regimes can be explored by the proper choice of the fluorescent probe.

The method presented in this paper describes a novel procedure to load amphipathic weak acid molecules into preformed liposomes. The release experiments (Figs. 5 and 6) indicate also that the loaded molecules are available, i.e., they are not irreversibly modified during the loading process and they can be released either by disrupting the liposome membrane or by collapsing the transmembrane gradient. The transmembrane calcium acetate gradient method combines a simple experimental protocol with high entrapment capacity and stability of the loaded liposomes. These features are very important for future applications of the method for delivery of drugs which are weak acids; possible candidates include non-steroidal anti-inflammatory drugs and anti-microbial agents.

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