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Transmembrane ammonium sulfate gradients in liposomes produce efficient and stable entrapment of amphipathic weak bases

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Gradients of ammonium sulfate in liposomes $[(NH_4)_2SO_4]_{lip.} > [(NH_4)_2SO_4]_{med.}$ were used to obtain 'active' loading of amphipathic weak bases into the aqueous compartment of liposomes. The loading is a result of the base exchange with the ammonium ions. This approach was applied to encapsulate anthracyclines and acridine orange inside the liposomes at very high efficiency (> 90%). Doxorubicin was accumulated in the aqueous phase of the liposomes where it reached a level as high as 100-fold the doxorubicin concentration in the remote loading medium. Most of the intraliposomal doxorubicin was present in an aggregated state. The active entrapment and loading stability were dependent on liposome lipid composition, lipid quality, medium composition and temperature, as well as on the pK_a and hydrophobicity of the base. The ammonium sulfate gradient approach differs from most other chemical approaches used for remote loading of liposomes, since it neither requires preparation of the liposomes in acidic pH, nor to alkalinize the extraliposomal aqueous phase. The stability of the ammonium ion gradient is related to the low permeability of its counterion, the sulfate, which also stabilizes anthracycline accumulation for prolonged storage periods (> 6 months) due to the aggregation and gelation of anthracycline sulfate salt.

Introduction

The main medical and pharmaceutical application of liposomes is delivery of drugs and other bioactive agents for therapy or for in vivo diagnostics. Such application will require sufficient loading of the agent into the liposomes. A number of loading strategies are currently available. The selection of the optimal loading procedure should be based on a scientific rationale. The various agents can be classified into three groups based on their oil/water and octanol/water partition coefficient (K_p). The first group includes hydrophilic agents with very low oil/water and octanol/water K_p. The second group includes amphipathic agents of low oil/water K_p but variable, and in some cases even high, octanol/water K_p, which is controlled by the pH and, to a lesser extent, the ionic strength of the medium. Good examples of this group are the anthracyclines, the main subject of this paper. The third group includes hydrophobic agents having high oil/water and octanol/water K_p. Agents of group III are associated with the liposome bilayer(s) though their loading efficiency is rather low (for review see Ref. 1). Agents of group I do not interact with the liposome bilayer, and their encapsulation is dependent on, and limited mainly by, the vesicle trapped volume. The smaller the liposome size, the lower the trapped volume and, therefore, the encapsulation [2,3].

Recently, Pfeiffer and co-workers [4] found that for some solutes of group I (only at relatively low concentration) the intraliposomal concentration can exceed the medium concentration. However, the absolute amount of drug loading into small liposomes still may not be sufficient to obtain a therapeutic level.

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Abbreviations: DNR, daunorubicin; DXR, doxorubicin; DPPC, dipalmitoylphosphatidylcholine; HPC, hydrogenated soybean phosphatidylcholine; HPI, hydrogenated soybean phosphatidylinositol; FTMLV, frozen and thawed MLV; lip., liposomes; med., medium; MLV, multilamellar vesicles; OLV, oligolamellar vesicles; PC, phosphatidylcholine; PEG-DSPE, *N*-carbamyl-poly-(ethylene glycol methyl ether)-1,2-distearyl-*sn*-glycero-3-phosphoethanolamine triethylammonium salt; SPLV, stable plurilamellar vesicles; sup., supernatant; dial., last dialysis medium; SUV, small unilamellar vesicles; T_m, gel to liquid crystalline phase-transition temperature.

Drugs of group II present a better chance to obtain loading at a dose needed for therapy in humans, even for small liposomes. This is explained either by the high affinity of the drug for the liposomal membrane (reviewed in Refs. 1,5) or by its ability to be remote loaded, reaching a very high concentration in the intraliposomal aqueous phase. Some of the amphipathic weak bases of group II can diffuse through the liposome bilayer as unprotonated species which then get trapped in the intraliposomal aqueous phase due to a proton gradient which shifts it to group I. Therefore, it cannot cross the lipid bilayer and it is accumulated in the intraliposomal aqueous phase. Nichols and Deamer [6] were the first to demonstrate remote loading of liposomes by a pH gradient for amphipathic amines such as catecholamines. They used liposomes prepared in acidic medium, and created the pH gradient by elevating the pH of the extraliposomal acidic medium. This concept was extensively used by Cullis and coworkers (reviewed in Ref. 7) who demonstrated that liposomes loaded by such an approach when compared with the free drug have lowered toxicity and improved efficacy [8,9]. Liposomes containing doxorubicin (DXR) which was remote loaded through a pH gradient have already been used in clinical trials [10]. Recently, Deamer and Harang [11] demonstrated that a pH gradient can be created by a photochemical reaction. However, application of this approach for drug delivery is questionable, since it depends on liposomes containing ferrocyanide.

We used another remote loading approach in which an ammonium sulfate gradient $[(NH_4)_2SO_4]_{lip.} > [(NH_4)_2SO_4]_{med.}$ is used as the driving force for loading of amphipathic weak bases such as anthracyclines into liposomes [12,13].

Small liposomes loaded by the ammonium sulfate gradient with either DXR or epirubicin were used by us and our collaborators extensively in animal studies using rodents and dogs [14,15], and very recently also in clinical trials [16]. In all cases, liposomes loaded by this method show high stability of DXR during storage and during circulation in vivo, as well as better tumor localization, lower toxicity and higher efficacy than the free drug or conventional liposomes. This paper describes in detail the characteristics of the ammonium sulfate gradient as a means to obtain efficient and stable loading of amphipathic weak bases.

Materials and Methods

Materials

Lipids. Dipalmitoylphosphatidylcholine (DPPC) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Egg phosphatidylcholine (egg PC) and hydro-

TABLE I

Types of liposomes used in the study

Ammonium-ion gradient was created by gel-exclusion chromatography of liposomes prepared in 120 mM $(NH_4)_2SO_4$, except preparations IV and V which were hydrated in 110 mM ammonium sulfate.

Preparation	Composition (molar ratio)	Liposome characterization		Liposomal DXR/phospholipids (mol/mol)	Sizing method	Mean size (±S.D.) (nm)	% DXR leakage at 4°C in 100 h
		Type	Method of hydration				
I	Egg-PC/Chol 2:1	MLV	Thin lipid film	0.26	None	large and heterogenous	26.9
II	Egg-PC/Chol 2:1	FTMLV	Thin lipid film plus freezing and thawing	0.42	None	large and heterogenous	15.6
III	Egg-PC/Chol 2:1	SPLV	Organic solvent replacement	0.51	None	large and heterogenous	18.7
IV	Egg-PC/Chol 2:1	OLV	Thin lipid hydration	0.17	Extrusion (0.2 μm)	214±86.0	–
V	DPPC/Chol 2:1	OLV	Thin lipid hydration	0.32	Extrusion (0.2 μm)	323±154	<1%
VI	Egg-PC/Egg-PG/Chol 7:3:4	SUV	Thin lipid hydration	0.10	Extrusion (0.05 μm)	85±21	–
VII	HPC/HPI/Chol 9:1:4	SUV	Thin lipid hydration	0.12	Extrusion (0.05 μm)	85±20	<1%
VIII	HPC/PEG-DSPE/Chol 55:5:40	SUV	Thin lipid hydration	0.1	Extrusion (0.05 μm)	93±25	<1%
IX	HPC/PEG-DSPE/Chol 55:5:40	SUV	Thin lipid hydration	0.1	Homogenization	92±31	<1%
X	HPC/PEG-DSPE/Chol 55:5:40	SUV	Ethanol injection	0.1	Homogenization	91±32	<1%
XI	HPC/Cholesterol	SUV	Thin lipid hydration	0.1	Homogenization	84±19	<1%

genated soybean phosphatidylcholine (HPC) were obtained from Lipoid (Ludwigshafen, Germany). *N*-carbamyl-poly-(ethylene glycol methyl ether)-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine triethyl ammonium salt (PEG-DSPE) (the polyethylene moiety of this phospholipid having a molecular mass of 1900 Da) was obtained as a gift from Liposome Technology (Menlo Park, CA, USA). Cholesterol was obtained from Sigma (St. Louis, MO, USA).

Drugs. Doxorubicin-HCl (DXR) and epirubicin-HCl were obtained from Farmitalia Carlo Erba (Milan, Italy). Daunorubicin-HCl (DNR) was obtained from Sigma. The purity of all lipids and anthracyclines exceeded 98%, as assessed by TLC and HPLC, as described by Barenholz and co-workers [17,18].

pH indicators. Pyranine (8-hydroxy-pyrene-1,3,6-trisulfonate) was purchased from Molecular Probes (Junction City, OR, USA). Acridine orange was purchased from Aldrich (Milwaukee, WI, USA).

Other reagents. Nigericin, desipramine, D-α-tocopherol acid succinate, D,L-α-tocopherol, Hepes, Sephadex G-50, Sepharose 6B (Pharmacia) and Dowex 50 WX-4 400 mesh (Dow) were obtained from Sigma. Deferoxamine mesylate (Desferal^{R}) was obtained from Ciba-Geigy (Basel, Switzerland). [^{3}H]Inulin was obtained from Amersham (Amersham, England). Ammonium sulfate, 99.999% pure, was obtained from Aldrich.

Methods

Liposome preparation. Table I describes 11 types of liposomes used in this study which differ from each other in one or more of the following parameters: lipid composition, size, number of lamellae and method of liposome preparation. Table II describes the general scheme of liposome preparation. Lipid hydration for all preparations, except III and X, was performed by thin lipid hydration. For preparations I, II, IV, V, VI and VII, thin lipid films were formed by evaporation from chloroform solution followed by hydration using mechanical shaking with ammonium sulfate solution [17]. For preparations VIII, IX and X the lipids were freeze-dried from a *tert*-butanol solution and fast hydration was obtained by adding the ammonium sulfate solution to the lipid 'cake' [19]. Preparation II was prepared from multilamellar vesicles (MLV) (as in preparation I) followed by 10 repetitive freezing and thawing cycles between liquid air and 25°C, as described by Mayer et al. [20]. For preparation III, lipid hydration was performed by reverse evaporation at high lipid concentration after the removal of the diethyl ether under the conditions described by Gruner et al. [21] to produce MLV. The product of this hydration is referred to as stable plurilamellar vesicles SPLV [21]. For preparation XI, lipids were hydrated by injecting a concentrated solution of lipids in ethanol into the ammonium sulfate solution. Ethanol was removed by evaporation at 65°C under vacuum. All aqueous phases used for lipid hydration contained either 110 or 120 mM of ammonium sulfate. To minimize DXR degradation and lipid peroxidation (for unsaturated phospholipids and cholesterol) we included 0.1 mol% D-α-tocopherol and 1 mol% D,L-α-tocopherol succinate in all lipid mixtures; 0.5 mM Desferal was included in all ammonium sulfate solutions used for lipid hydration and in all final liposome preparations [5,17,19]. Preparations I, II and III were not downsized; preparation II went through 10 cycles of freezing and thawing between liquid air and 25°C [3,20]. Frozen and thawed MLV are referred to as FTMLV. In all cases the hydration step was carried out at temperatures above the gel to liquid crystalline phase transition (T_m) of the phospholipids (25°C for egg-PC, 49°C for DPPC and 60°C for HPC [2,22]. Preparations IV and V were downsized to form oligolamellar vesicles (OLV) by low-pressure extrusion of MLV. We used a two-step extrusion: first, three consecutive extrusions through a 0.4-μm pore diameter polycarbonate filter (Nuclepore, Pleasanton, CA, USA), followed by three consecutive extrusions through a 0.2-μm pore diameter polycarbonate filter under argon pressure of 60–150 psi using a medium-pressure thermostated extruder device (Lipex Biomembrane, Vancouver, British Columbia, Canada) [23] under conditions described by Amselem et al. [24].

For preparations VI, VII and VIII, MLV were downsized to small unilamellar vesicles (SUV) ($\leq$ 100 nm) by stepwise medium-pressure extrusion using the extruder device described above under the conditions described by Mayer et al. [25], at 60–65°C. For larger volumes of SUV we used the extruder system and conditions described by Amselem et al. [19]. Recently, we found that 10–30 s of ultrasonic irradiation in a bath sonicator (Transsonic 460/H 35 kHz, 285 W, from Elma (Bergwies, Austria)) facilitates and accelerates the extrusion without reducing the quality (trapped volume) or affecting the properties of the extruded OLV. More recently, the Rannie high-pressure lab homogenizer, Model Minlab type 8.30H (from APV Rannie (Albertslund, Denmark)), modified to work with batch sizes of 40 ml and larger, has been used. SUV prepared by extrusion (0.05-μm pore size filter)

TABLE II

Scheme of liposome preparation

Step 1	Lipid hydration in ammonium sulfate solution to form MLV
	Downsizing of MLV to form either OLV or SUV [a]
Step 2	Formation of ammonium sulfate gradient
Step 3	Liposome loading with doxorubicin
Step 4	Removal of nonentrapped doxorubicin

[a] Was not used for preparations I, II, III.

and high-pressure (8000–10 000 psi) homogenization were almost identical in their size distribution and encapsulation efficiencies.

In all cases the extrusion was carried out above the T_m of the PC in the liposome lipids.

Formation of ammonium sulfate gradient. The following methods were used to obtain ammonium sulfate gradients:

(a) Dilution of the liposomes in isoosmotic solutions composed either of sodium chloride, potassium chloride or of nonelectrolytes such as glucose or sucrose, in which ammonium sulfate is dissolved at concentrations to give the desired $[(NH_4)_2SO_4]_{med.}/[(NH_4)_2SO_4]_{lip.}$ gradient in the range of 1.0–0.001. This method gives a diluted liposome preparation.

(b) To obtain complete removal of the external ammonium sulfate (with only a small dilution of liposomes) we used gel-exclusion chromatography (Preparations I–V). To reduce loss of the large vesicles (MLV, SPLV and FTMLV) on the column due to liposome aggregation, the vesicle dispersions were sonicated for 10 s in a bath sonicator prior to their chromatography. The Sephadex G-50 column was preequlibriated with either NaCl or KCl (isoosmotic with the ammonium sulfate solution). The column was eluted with the same salt solution used for column equilibration but containing 0.5 mM Desferal. The void volume, containing the liposomes 'free of' external ammonium sulfate, was collected and used for loading experiments.

The ammonium sulfate gradient in the liposomes was determined by measuring the ammonia concentraton at pH 13–14 (using an ammonia electrode, see below) in the 100 000 × *g* supernatant of the Sephadex G-50 void volume $[(NH_4)_2SO_4]_{sup.}$ and the ammonia concentration at pH 14.0 for the whole liposome dispersion at the void volume $[(NH_4)_2SO_4]_{lip.}$.

For determination of the gradient we used the following equation:

$$\text{Gradient} = \frac{[(NH_4)_2SO_4]_{sup.}}{[(NH_4)_2SO_4]_{lip.} - [(NH_4)_2SO_4]_{sup.}} \quad (1)$$

In all cases $[(NH_4)_2SO_4]_{lip.}/[(NH_4)_2SO_4]_{sup.}$ was $\geq$ 1000, so the gradient $[(NH_4)_2SO_4]_{med.}/[(NH_4)_2SO_4]_{lip.}$ was ≤ 0.001. The gel-exclusion chromatography step introduced a 3–6-fold dilution of the liposome preparation.

(c) Four consecutive dialysis exchanges against 25 vols. of 5% glucose or 10% sucrose were used. For larger-scale preparation the dialysis was replaced by a three-step tangential flow dialysis using the Minitan ultra-filtration system (Millipore, Bedford, MA, USA) equipped with '300K' polysulfone membranes (having an exclusion limit of 300 kDa). For both processes of the dialysis the ammonium sulfate gradient was determined according to the following equation:

$$\text{Gradient} = \frac{[(NH_4)_2SO_4]_{dial.}}{[(NH_4)_2SO_4]_{total} - [(NH_4)_2SO_4]_{dial.}} \quad (2)$$

where $[(NH_4)_2SO_4]_{dial.}$ is the ammonium sulfate concentration post dialysis in the last dialysis medium and $[(NH_4)_2SO_4]_{total}$ is the total ammonium sulfate concentration in the retentate. In both cases ammonium sulfate was measured as ammonia concentration using an ammonia electrode at pH 13–14 (see below).

In all preparations in which the ammonium gradient was created by dialysis or tangential flow dialysis the $[(NH_4)_2SO_4]_{out}/[(NH_4)_2SO_4]_{in}$ was ≤ 0.001 and no dilution of liposomes occurred. The tangential flow dialysis permits concentrating the liposomes in a controlled way.

Conductivity measurements. Conductivity measurements were performed using a conductivity meter (Radiometer, Copenhagen, Denmark) type CDM3 equipped with a CDC 304 immersion electrode with manual temperature compensator type CDA 100. The instrument was calibrated as specified by the manufacturer. The determination of the $(NH_4)_2SO_4$ concentration from the conductivity measurements was done at constant temperature (4°C) using a calibration curve of 0.016–120 mM ammonium sulfate in glucose or sucrose (total osmolarity 285 mosmol). For this calibration curve the conductivity range was 7 μS to 22 S.

pH and ammonia measurements. pH and ammonia measurements were carried out using a Corning 250 pH/ion analyzer (Corning Science Products, Corning, NY, USA) equipped with an automatic temperature compensation (ATC) stainless steel probe. For the determination of ammonium ion concentration we used the Corning ammonia combination electrode (Corning 476130). A 5-ml sample volume was used in all measurements. The calibration curve was performed using NH_4Cl and $(NH_4)_2SO_4$ as described in the manufacturer's operating instructions. Calibration curves were obtained at pH 7.0, 8.5 and 13.5. The relationship between the NH_3-related elecrode potential in millivolts and the log of $(NH_4)_2SO_4$ conductivity (mS) is linear; the slope and intercept are pH dependent. This calibration curve enabled us to determine the ammonium ion concentration over a broad pH range.

The extraliposomal ammonium ion concentration $[(NH_4)^+_{med.}]$ was measured as ammonia with the ammonia electrode at pH 13.5. Under these conditions no leakage of intraliposomal ammonium occurred during the measurement. For measurements of total ammonia $[(NH_4)^+_{med.} + (NH_4)^+_{lip.}]$ the vesicles were sonicated in acidic conditions (pH 1.5–2.0) using the Transonic 460/H bath sonicator in sealed vials for 45 min. Then NaOH was added to bring the pH to 13.0–13.5 and the total ammonia concentration was measured. The total

ammonia concentration determined for the liposome dispersion after the complete replacement of the medium ammonium sulfate by nonelectrolyte, was identical to the ammonium sulfate determined by conductivity meter after complete disruption of the liposomes (see Conductivity measurements).

Liposome loading with DXR. A solution of DXR (0.5–20 mM) was added to the liposome dispersion (1–120 mM phospholipids) after the creation of an ammonium sulfate gradient. The loading was performed at temperatures above the T_m of the liposome PC [2,22]: 37°C, 45°C and 65°C for egg-PC, DPPC and HPC, respectively. The loading was terminated at the desired time by removal of unencapsulated DXR using Dowex 50WX-4 as described below.

Removal of non-entrapped DXR. The separation and removal of non-liposome-associated DXR (free DXR) from the liposome-entrapped DXR (liposomal DXR) was achieved through the complete binding of free DXR (but not of the liposomal DXR) to the cation exchange resin Dowex 50WX-4 [26], as modified by Amselem et al. [17,19]. Dowex also binds the extraliposomal Desferal, and therefore Desferal (0.5 mM) was added after the Dowex treatment.

Liposome quality control assays. (a) DXR: DXR concentration was determined spectrophotometrically based on the molar extinction coefficient of 12500 $A\,M^{-1}$ in a dual-beam spectrophotometer (either Perkin-Elmer Lambda 3B or Kontron Uvikon 860). The DXR quantification was confirmed by HPLC [17–19]. Purity of DXR and its degree of degradation during the processes of liposome preparation and liposome storage were determined by a combination of HPLC and TLC, as described by Barenholz and coworkers [18,19].

(b) Level of free DXR: Two approaches were used: (i) the selective adsorption of free anthracycline to Dowex 50 WX-4 either in tips of automatic pipets (range 0.1–1.0 ml) or in small columns; (ii) small gel-exclusion chromatography columns containing 2 ml preswollen beaded 12% cellulose, having an exclusion limit of 5000 Da (Excellulose GF-5, 40–100 μ, Pierce (Rockford, IL, USA)) [18,19].

(c) Lipids: Phospholipid concentration was determined using our modification [18] of Bartlett's procedure [27]. Cholesterol concentration and purity were determined by HPLC [18,19]. Purity of phospholipids as raw materials, and the extent of their hydrolysis during various steps of liposome preparation and liposome storage, were assessed by TLC [18,19]. Peroxidation of egg-PC acyl chains during liposome preparation and storage was assessed by gas liquid chromatography [17,18].

(d) DXR/phospholipid ratio: This ratio was determined from the phosphorus and liposomal DXR content (after removal of the free drug released from liposomes by Dowex 50 WX-4). It was used to assess efficiency of loading, as well as to indicate drug leakage during storage.

(e) Size distribution of liposomes: Liposome size distribution was determined by photon correlation spectroscopy (PCS) [2,18], using either the Malvern 4700 Automeasure laser light scattering spectrometer system (Malvern Instruments, UK) or a Coulter N_4SD submicron particle analyzer with size distribution processor analysis (Coulter Electronics, Luton, UK). Mean sizes are described in Table I. Size distribution analysis was performed using the CONTIN algorithm [18]. All size distributions described in Table I were unimodal.

(f) Trapped volume determination: The trapped volume of the vesicles was determined by entrapping [^{3}H]inulin in the vesicles during their preparation [28]. The non-entrapped inulin was removed from the liposomes using a Sepharose 6B column; we found Sephadex G-50 to be unsatisfactory, since traces of [^{3}H]inulin overlap with the OLV and SUV. The ratio of radioactivity (inulin content) to phospholipid content was determined before and after the gel-exclusion chromatography and was used to calculate the liposome-trapped aqueous volume [28].

(g) Determination of the inner pH of liposomes: (i) Using pyranine (8-hydroxy-1,3,6-pyrene trisulphonate): Liposomes were prepared as above with the exception that pyranine (0.5 μM) was included in the hydration solution. Removal of untrapped pyranine was achieved by gel filtration on a Sephadex G-50 column, preequilibrated with an ammonium sulfate solution also containing 10 mM Hepes buffer (pH 7.5). The ammonium sulfate gradient was obtained by replacing the extraliposomal ammonium sulfate with an isoosmotic solution of KCl in ammonium sulfate containing 10 mM Hepes adjusted to pH 7.5, over the molar ratio $KCl/(NH_4)_2SO_4$ of 0–100. The internal pH of the vesicles was determined from the fluorescence emission intensity of the pyranine [29]. A calibration curve was constructed by preparing liposomes in which the hydration was done using solutions of NaCl adjusted to different pH values by adding appropriate buffers. Gel-exclusion chromatography on a Sephadex column, as mentioned above, yielded a series of liposome preparations with a fixed external pH (pH 7.5) but different internal pH values, determined by the buffer used for lipid hydration. KI, which quenches the fluorescence of aqueous solutions of pyranine, did not have any effect on the fluorescence intensity of pyranine in the void volume after gel-exclusion chromatography, which indicates the complete removal of the pyranine from the extraliposomal medium.

The fluorescence intensity of the liposomes containing pyranine free of non-entrapped pyranine (F) was determined at 520 nm (excitation 460 nm). Then 5 μM nigericin was added to disrupt the pH gradient and the

fluorescence intensity (F_N) of the sample was remeasured. The ratio

$$\frac{F}{F_N} \times 100 \quad (3)$$

gave the pH-dependent % change in fluorescence.
(ii) Using acridine orange: 1 μM acridine orange was added to the desired solution containing various ratios of potassium chloride to ammonium sulfate, then an aliquot of the liposomes loaded with ammonium sulphate was added to the cuvette, and the decrease of fluorescence intensity at 525 nm (excitation 490 nm) due to quenching was monitored continuously following immediate mixing (Fig. 2, inset). After equilibration, the pH gradient was abolished by addition of nigericin to a final concentration of 5 μm, and the increase in fluorescence due to dequenching was monitored (Fig. 2, inset). The ratio $F/F_N \times 100$ was used to calculate the % of acridine orange quenched due to the pH gradient (Fig. 2). This ratio was used to plot the calibration curve relating fluorescence in tensity and pH. The main problem with using acridine orange is that it cannot distinguish between the contribution of a pH gradient and an ammonium ion gradient.

All fluorescence intensity measurements were performed using a Perkin-Elmer LS-5 luminescence spectrometer.

Results

Ammonium sulfate gradient in liposomes

When liposomes are formed by hydration in ammonium sulfate solution this salt is present in both the intra- and extraliposomal aqueous phases. Removal of the extraliposomal ammonium sulfate creates an ammonium sulfate gradient in which the concentration of the salt inside the liposome is greater than its concentration in the extraliposomal medium. We measured the ammonium sulfate gradient using two independent methods: (i) conductivity measurements in liposomes dialyzed against 5% glucose or 10% sucrose (both are isoosmotic with 120 mM ammonium sulfate; (ii) direct determination of the ammonium ion as ammonia by ammonia electrode. Identical ammonium sulfate concentrations were obtained for conductivity and direct ammonia determinations. For example, 5–6% of the ammonium sulfate was encapsulated in preparation IX (Table I) in which lipid concentration was 10%. The ammonium sulfate concentration of intraliposomal ammonium sulfate based on trapped volume as determined by [^{3}H]inulin gave a value of approx. 120 mM, which is identical to the concentration of ammonium sulfate used for the lipid hydration. After removal of extraliposomal ammonium sulfate by gel-exclusion chromatography or dialysis, its concentration in the extraliposomal medium is < 0.1 mM, thereby establish-

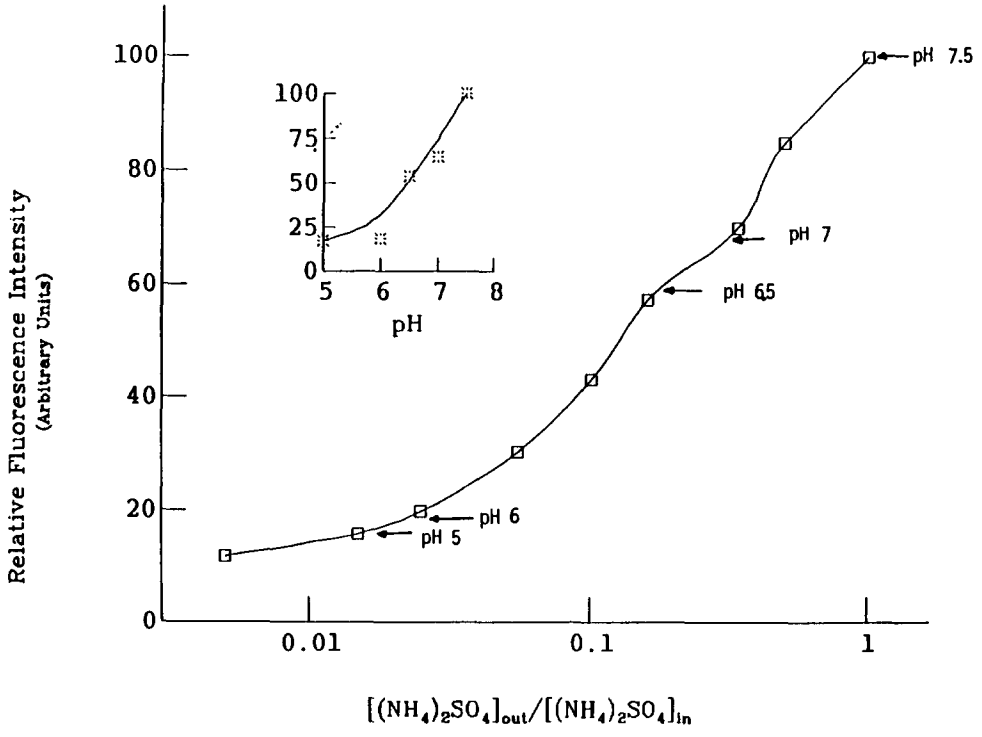

Fig. 1. Changes of the fluorescence intensity of pyranine entrapped within ammonium sulfate liposomes as a function of the ratio of external to internal concentration of ammonium sulfate. For more details, see Materials and Methods. The arrows in the curve describe the internal pH of ammonium-sulfate-loaded liposomes derived from the inset to this figure. Inset: Calibration curve that relates the fluorescence of liposome-entrapped pyranine and the liposome internal pH.

ing an ammonium ion gradient > 1000. This gradient is retained for over six months for preparations of liposomes stored below the T_m of their PC (i.e., HPC), though the gradient decays slowly when liposomes are stored above the T_m of their PC (i.e., egg-PC). For the latter, the gradient is reduced with time, the rate of decay being dependent on temperature. For example, at 4°C, 25–30% of the gradient is lost in two weeks.

Ammonium sulfate gradient in liposomes as a driving force to create trans-membrane pH gradient

The creation of a pH gradient in liposomes having an ammonium sulfate gradient was proved using two independent methods for measuring the internal pH of liposomes. First we trapped pyranine inside extruded OLV (preparations IV, V). The pyranine fluorescence emission intensity is pH dependent, since the degree of ionization of its 8-hydroxy group ($pK_a = 7.2$) affects its extinction coefficient appreciably [29]. We constructed a calibration curve by varying the pH inside the vesicles in the range 5.0–7.5 under isoionic strength conditions and keeping the extraliposomal medium pH constant at pH 7.5. This calibration curve was used to quantify the intraliposomal pH, and therefore the pH gradient induced by the creation of the ammonium gradient in the liposomes. This was done by dilution of the liposomes with solutions containing descending ratios of buffered (pH 7.5) ammonium sulfate to buffered (pH 7.5) KCl under isoosmotic conditions. It is shown that as the ratio $(NH_4)_2SO_4/KCl$ is lowered, the internal pH decreases. As expected from the determination of the ammonium sulfate gradient, the largest gradient was obtained after complete removal of extraliposomal ammonium sulfate. Under these conditions the pyranine fluorescence is indicative of a pH change of at least 3 units. The calibration curve cannot give an exact answer at the low end of the pH range (see Fig. 1) because it is much lower than the pyranine pK_a. The rate of the establishment of the pH gradient is very fast (< 10 s). Addition of nigericin, an ionophore which exchanges potassium ions and protons, brought the fluorescence intensity (F_N) of all samples described in Fig. 1 to the value obtained for 'no gradient' conditions. In order to further substantiate the existence of the gradient, we followed it using a second approach. Ammonium sulfate loaded liposomes (preparations IV, V, IX, X) were added to a solution containing 1 μm acridine orange. Being an amphipathic weak base (pK_a 9.25), acridine orange partitions between the external medium and the inner aqueous phase of the liposomes. Its partitioning into the liposomes can be determined from the degree of its fluorescence emission quenching [30,31]. This method is used routinely to determine the pH in various types of vesicles [30,31].

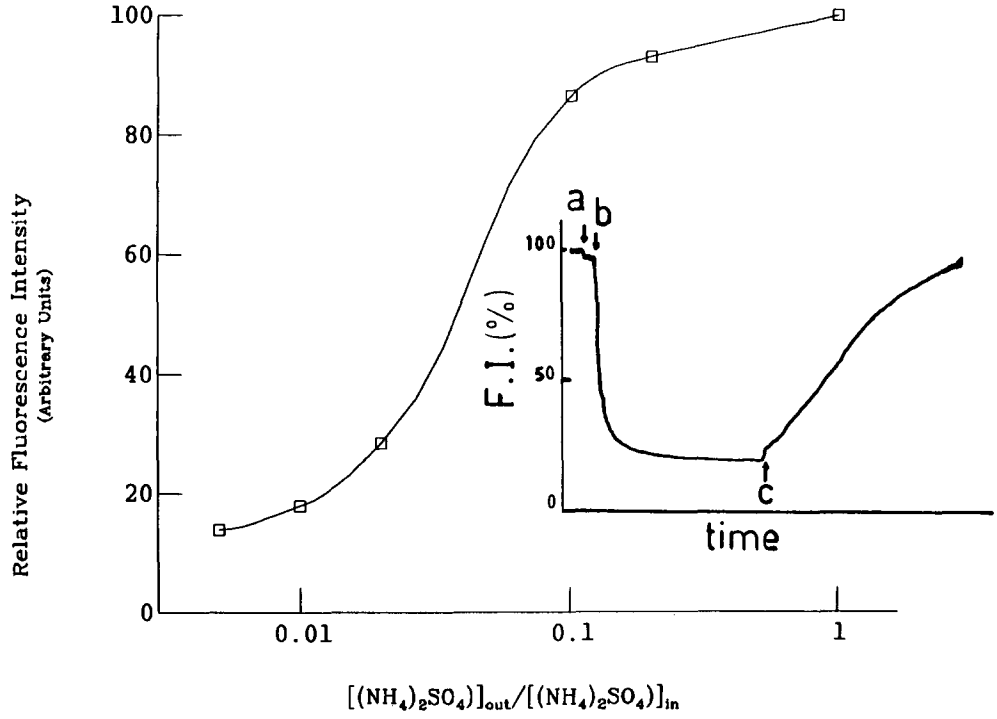

Fig. 2. The effect of the gradient of ammonium sulfate between the inside and the outside of the liposomes on the quenching of acridine orange fluorescence. Inset: An example of one of the experiments described in this figure. Fluorescence intensity of acridine orange was monitored continuously (a), and at the specified time (b) liposomes were added, and the quenching was followed. At steady state, nigericin (5 μM) was added (c), and the extent of the dequenching was used to calculate the ammonium-sulfate-gradient-dependent fluorescence intensity. For more details, see Materials and Methods. Full time-scale of the nigericin experiment was 5 min.

TABLE III

Liposome characterization

Trapped volume was determined using [^{3}H]inulin entrapment as described in Materials and Methods. Ratio of doxorubicin to phospholipid after loading to 'equilibrium' (mol/mol) and removal of free drug by Dowex. Calculated doxorubicin concentration inside liposomes was determined in liposomes prepared in 110 mM ammonium sulfate. DXR concentration in the medium was 5 mM. For more details, see text.

Preparation (Table I)	Liposome composition (mol/mol)	Mean diameter ±S.D. (nm)	Trapped volume (l/mol)	Doxorubicin/ phospholipid (mol/mol)	Doxorubicin concentration inside liposomes (mM)
IV	Egg-PC/chol (2:1)	214.5± 86.0	1.5	0.17	115
V	DPPC/chol (2:1)	2.1±15.9	2.7	0.32	119

Fig. 2 demonstrates that the degree of quenching is determined by the ammonium sulfate gradient. The sigmoidity of the curve is related to a high pK_a of the acridine orange, indicating that, as expected, it is not a suitable probe to quantify pH at the range of low pH. After addition of nigericin to samples having an ammonium ion gradient, a complete dequenching occurred (Fig. 2, inset), suggesting that the quenching is determined by the pH gradient. The acridine orange approach is problematic since the partitioning of acridine orange into the liposome may be related to driving forces other than the pH gradient such as direct exchange with NH_3, and the relative contribution of the ammonium ion gradient per se to the distribution of the acridine orange between the intra- and extraliposomal aqueous phases.

Loading of the liposomes with DXR: Since DXR, like the acridine orange discussed above, is a weak amphipathic base (pK_a of the DXR amino group = 8.15 [32], it was assumed that the ammonium ion gradient created between the inside and outside of the liposomes may act as a driving force for DXR influx and accumulation in the aqueous compartment of the liposomes.

Table I demonstrates the loading efficiency obtained for 11 different liposome preparations. Loading of all these preparations was done using extraliposomal medium at acidic pH (approx. 5.5) using either electrolytes (0.15 M NaCl or KCl) for preparations I–IX, or nonelectrolytes (5% glucose or 10% sucrose) for preparations IX–XI. (Under these loading conditions the pH gradient was ≤ 1 pH unit. The loading obtained was better than when the extraliposomal pH was basic (data not shown).

As expected, the higher the liposome trapped volume, the larger the DXR/lipid ratio. Table III, which gives complete characterization of two preparations)

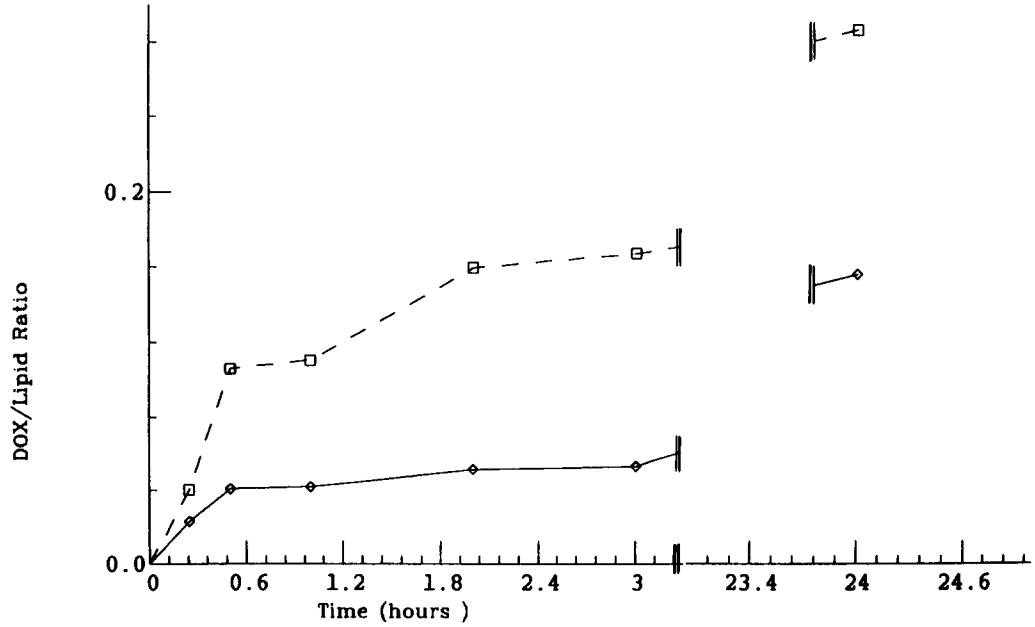

Fig. 3. Kinetics of the loading of doxorubicin into ammonium-sulfate-loaded liposomes. The loading of DXR was started after removal of external untrapped ammonium sulfate by Sephadex G-50 gel-exclusion chromatography. DXR was incubated for the indicated time with either egg-PC/cholesterol liposomes (preparation IV, Table I) at 25°C or dipalmitoyl phosphatidylcholine/cholesterol liposomes (preparation V, Table I) at 49°C. At the end of the incubation period, unincorporated DXR was removed by adsorption on a Dowex 50WX-4 column (see Materials and Methods), and the concentrations of the phospholipid and drug were determined and used to calculate the DXR/lipid molar ratio.

demonstrates that the intraliposomal DXR concentration exceeds at least 20-fold the DXR concentration of the liposome hydration medium. The calculated DXR concentration inside the liposomes is much higher than DXR solubility in the aqueous phase [5]. Finally, for all 11 preparations used in this study, close to 100% encapsulation efficiency of anthracyclines was obtained for a broad range of drug to lipid ratios. For identical ammonium gradients the range was determined by the liposome size (trapped volume).

Fig. 3 describes the kinetics of DXR influx as followed in preparations IV and V (Table I). It is clear that the loading process is biphasic, with a fast phase in the first hour, followed by a slower phase which continues for about 24 h. The amount of drug incorporated into DPPC/ cholesterol liposomes (preparation V) was twice the amount incorporated into the egg-PC/ cholesterol liposomes (preparation IV). This difference is related to the difference in the trapped volume (Table III). The intraliposomal DXR concentration for both preparations was almost identical in spite of the large differences in their physical properties (Fig. 3), and it was directly related to the magnitude of the ammonium ion gradient. Under conditions of no ammonium ion gradient, the influx was minimal. It was dependent only on lipid drug association and therefore on the exact lipid composition of the liposomes [5,34–36]. For egg-PC/cholesterol (preparation IV, Table I, 12.5 mM PC), the liposome DXR to phospholipid ratio was < 0.01 for 'no gradient' conditions compared with 0.17 (Tables I, III) obtained in the presence of an ammonium ion gradient. The ammonium-ion-gradient-related loading efficiency was also dependent on the lipid quality. For example, no ammonium-dependent loading of DXR was obtained when a peroxidized egg-PC preparation was used (data not shown).

Exchange between DXR and ammonium ions

10 ml of 30 mM preparation XI (Table I) was dialyzed against 1 liter of 5% glucose (pH 5.5). After 4 1-liter changes, ammonia concentration in the extraliposomal medium was below $1 \cdot 10^{-5}$ M. The intraliposomal ammonium concentration was higher than 0.1 M. The molar ratio of intraliposomal NH_4^+ to phospholipids was 0.15, which corresponded to 7.6% encapsulation of the ammonium sulfate used in the hydration step. DXR was then added to a final concentration of 0.4 mM. The reaction mixture was incubated for 45 min at 60°C. 100% encapsulation of DXR was achieved as determined on aliquots of the loaded liposomes. The molar ratio between ammonium ion released from the liposomes and DXR uptake into the liposomes was 4:1. Under these conditions the DXR loading was much below the loading capacity of the liposomes. Increasing the level of DXR in the medium improved the loading. However, the lag in DXR uptake relative to ammonia release required prolonged incubation time, otherwise part of the DXR remained in the medium, and its removal by Dowex also removed ammonium ions which made the accurate ammonia determination impossible.

Stability of liposome components

We followed the chemical stability of DXR, PC and cholesterol of the liposomes loaded with DXR immediately after their preparation and after storage for two weeks and six months at 4°C. In 'fresh' preparations degradation of both lipids and DXR was below detection limits for all three components. Analyses after two weeks of storage indicate in some cases a little hydrolysis ($< 2.0\%$) of the PC, but no degradation of DXR and cholesterol or peroxidation of PC acyl chains could be detected. After six months of storage at 4°C the DXR and cholesterol degradation were still below detection. PC hydrolysis increased and varied among preparations; the highest value being less than 10%.

Effect of liposome type on ammonium-ion-gradient-dependent loading of DXR

Table I shows a comparison of DXR loading into 11 types of liposome. Preparations I, II and III are all multilamellar, composed of egg-PC/cholesterol liposomes. In all cases the gradient was obtained either by gel-exclusion chromatography or by dialysis. Loading was determined after 24 h incubation in the presence of DXR. For the various MLV preparations the maximal drug to lipid ratio is in the order SPLV > FTMLV > MLV (Table I). This order is related to the trapped volume of these three MLV types (reviewed in Ref. 2). Loading stability was determined after 100 h storage at 4°C (Table I). It is of interest that for egg-PC/ cholesterol multilamellar vesicles the MLV are leakier than the FTMLV or the SPLV, suggesting better annealing of the latter two types of MLV [2]. For liposomes composed of HPC the leakage at 4°C is below detection (Table I).

Effect of degree of hydrophobicity of the weak base on its loading

Our experiment with acridine orange, an amphipathic base chemically very different from DXR, suggests that the ammonium ion gradient acts as a driving force for the partitioning of other amphipathic weak bases into the liposomes (Fig. 2). The much faster permeation kinetics of acridine orange into the liposome interior in comparison to DXR (compare the inset of Fig. 2 and Fig. 3; uptake < 1 min, and 0.5–24 h, respectively) may be related to the fact that acridine orange is both more hydrophobic and a weaker base (pK_a 9.25 and 8.2 for acridine orange and DXR, respectively [30,32]. In order to separate these two

parameters we compared the behavior of three anthracyclines which have identical pK_a values of their amino group: DXR, epirubicin and daunorubicin (DNR). The chemical difference between the three anthracyclines is also reflected in the octanol/Tris buffer (pH 7) partition coefficients: DXR 0.5 < epirubicin 1.1 < DNR 1.5, respectively [37]. Using the same conditions for the uptake of all three anthracyclines we found that the rate of incorporation is in the order of the partition coefficient, for example for DNR the incorporation went to completion in less than 1 h compared with 24 h for DXR.

Aggregation of DXR inside the liposomes

In aqueous phase at a concentration higher than about 1 μM, DXR-HCl is known to form dimers and higher molecular weight aggregates [38] (reviewed in Ref. 5). The apparent concentration achieved in the intraliposomal medium in the 'ammonium sulfate' liposomes far exceeds this limit. Also, at a concentration of 10 mM, DXR in 0.9 mol% KCl or NaCl, or in 5% glucose or 10% sucrose forms a clear solution because the aggregates are very small. In ammonium sulfate the solution is very turbid and flocculates are formed with a gel-like appearance. We studied the physical state of the drug inside the vesicles using two independent methods.

(i) Absorbance characterization of entrapped DXR: The simplest physical parameter that changes by aggregation is the absorbance spectrum of the drug. The ratio of absorbance at 470 nm to the absorbance at 550 nm can give a semi-quantitative estimate of the state of aggregation of the drug (reviewed in Ref. 5). Table IV presents data on this ratio for various concentrations of DXR in solution and also for the DXR-loaded liposomes. The ratio in liposomes was obtained from a difference spectrum, in which the same liposomes before DXR loading served as a reference to correct for the light scattering of the liposomes. A substantial aggregation of the drug can be inferred from the data.

TABLE IV

Change in the ratio of absorbance at 470 nm to absorbance at 550 nm as a function of doxorubicin concentration

	Concentration (M)	A_{470}/A_{550}
Doxorubicin solution	$8.6 \cdot 10^{-6}$	5.15
	$8.6 \cdot 10^{-5}$	3.44
	$8.6 \cdot 10^{-4}$	2.85
Doxorubicin in DPPC/Chol liposomes [a]	$1.19 \cdot 10^{-1}$	1.90

[a] From Table III.

TABLE V

Self-quenching of doxorubicin fluorescence

Fluorescence emission was corrected for the inner filter effect as proposed by Lakowicz [39]. For more details see Results. The fact that the quenching is somewhat less than 100% may be related to slow leakage of DXR from the liposomes to the external medium.

	Concentration (M)	Quenching (%)
In solution	$5.0 \cdot 10^{-7}$	0
	$3.4 \cdot 10^{-5}$	16
	$6.9 \cdot 10^{-5}$	25
	$1.7 \cdot 10^{-4}$	33
In DPPC/Chol OLV	$1.19 \cdot 10^{-1}$ [a]	97±3
In egg-PC/Chol OLV	$1.15 \cdot 10^{-1}$ [a]	94±4

[a] From Table III.

(ii) Fluorescence studies of the entrapped DXR: We compared the emission fluorescence intensity of DXR in solution as a function of its concentration to the emission fluorescence intensity of the liposome-entrapped drug. DXR was diluted in saline to the concentrations indicated in Table V. In solution at concentrations higher than 10^{-5} M the drug fluorescence is partially quenched. The extent of quenching was calculated by comparison of the fluorescence at every concentration with the fluorescence of the same solution after dilution to a concentration of $5 \cdot 10^{-7}$ M, at which there is no quenching. However, in studying this self-quenching in solution one encounters the problem of the inner filter effect. Since the correction usually made is applicable only when the absorbance does not exceed 1–1.5 absorbance units [39], we could not extend the study to solutions of concentrations higher than $1.7 \cdot 10^{-4}$. The liposomes do not pose this problem, since the actual concentration inside the liposome is unrelated to liposome dilution; a dilute solution of loaded liposomes still has a concentrated solution of DXR within the liposomes. In Table V it can be seen that inside the liposomes DXR fluorescence is almost 100% quenched. This was calculated from the ratio between the fluorescence of the liposome preparations (preparations IV and V, Table I) and the fluorescence of the same mixture after solubilizing the liposomes by 10-fold dilution in isopropanol containing 10% 0.75 M HCl. This caused more than a 10^5-fold total dilution of DXR and therefore represents virtually 0% quenching conditions. A correction for the DXR fluorescence enhancement in acidified isopropanol solution relative to 0.15 M NaCl was determined from measurements of the fluorescence of 'free' drug ($1 \cdot 10^6$ M) in the two solutions. The small amount of unquenched DXR in the liposome samples is related to a small leakage of free drug out of the vesicles (Tables I and V).

TABLE VI

Leakage of doxorubicin from liposomes

Energy of activation (E_a) of the leakage process, as calculated from the two temperature points, assuming equal DXR concentrations in the two preparations (from Table III), and thus using the rate directly in the Arrhenius equation:

$$E_a = \frac{-2.303R(\log \text{rate}_2 - \log \text{rate}_1)}{1/T_2 - 1/T_1}$$

where R is the gas constant. For more details see text.

Liposome composition	Temperature (°C)	Initial leakage rate (% of total trapped/min)	Energy of activation (kcal/mol)
DPPC/Chol	24	0.034	12.3
	49	0.20	
Egg-PC/Chol	24	0.13	9.8
	49	0.53	

Leakage of DXR from the 'ammonium' liposomes

The high self-quenching of the fluorescence of DXR gives a direct and easy method for the determination of DXR leakage from the liposomes. Enhancement in the fluorescence intensity is a reflection of dequenching due to dilution, and therefore can be used to monitor the exact amount of released drug. Table VI summarizes the data obtained on the leakage rate from preparations IV and V (Table I) using this approach. The egg-PC/cholesterol liposomes (preparation IV) are 'leakier' than the DPPC/cholesterol liposomes (preparation V) throughout a broad temperature range, even well above the DPPC transition temperature (41.5°C; [22]). However, the difference between the two narrows at higher temperature, indicating a notable change in the physical state of the DPPC/cholesterol bilayer. This is reflected in the higher energy of activation for the DPPC/cholesterol liposomes (see Table VI). Table I demonstrates that even at 4°C, DXR leaks from the egg-PC/cholesterol liposomes. The extent of leakage is, however, dependent on the exact type of liposomes, though leakage during prolonged storage is slower than predicted from the initial leakage rate described in Table VI. DNR leaks faster than DXR (data not shown).

Finally, leakage increased quickly upon addition of nigericin under conditions described in Materials and Methods. Incubation for 1 h in the presence of nigericin and potassium ions at 37°C caused release of $\geq 75\%$ of the encapsulated DXR, even for liposomes composed of the 'solid' HPC.

Discussion

We describe and characterize the use of an ammonium sulfate gradient in liposomes as a means to obtain efficient and stable accumulation of amphipathic weak bases such as anthracyclines in the intraliposomal aqueous phase. Both the ammonium cation and the sulfate anion have important roles in the drug loading. We demonstrate that the ammonium-sulfate-gradient-dependent anthracycline loading can be successfully applied to liposomes which differ drastically in method of preparation and in their physical properties, such as size distribution, gel to liquid crystalline phase transition temperature (T_m), charge and headgroup hydrophilicity (Table I). The only requirement is that the lipid can form a permeability barrier. For example, no ammonium-ion gradient and no loading of DXR were obtained for liposomes containing peroxidized egg-PC. The rate, extent and stability of accumulation of the loaded molecule in the intraliposomal aqueous phase are dependent on many parameters. These can be classified into three categories of properties: those of the medium, the vesicles and the loaded molecules.

Medium-related properties

The main medium-related factor is the magnitude of the ammonium sulfate gradient. The theoretical limit to the loading is determined by the ratio $[(NH_4)_2SO_4]_{lip.}/[NH_4)_2SO_4]_{med.}$ The mechanism of accumulation is complex. There are two main explanations, both relying on similar principles. Removal of all or part of the ammonium sulfate from the extraliposomal medium of liposomes which were prepared in ammonium sulfate creates an ammonium sulfate gradient. This gradient, due to the large difference in the permeability coefficients through lipid bilayers of the various species is $(NH_4)_2SO_4 < SO_4^{2-} < NH_4^+ \ll H^+ \ll NH_3$ [40,41]. The very high permeability coefficient of NH_3 ($1.3 \cdot 10^{-1}$ cm/s) leads to fast diffusion of the neutral ammonia to the extraliposomal medium. For every NH_3 molecule which leaves the liposome, one proton is left behind, forming a pH gradient in which the inside of the liposome becomes more acidic than the external medium. The magnitude of this gradient is determined by the ratio $[NH_4^+]_{med.}/[NH_4^+]_{lip.}$ The acidification of the intraliposomal aqueous phase slows down the process by reducing the level of NH_3. One way to explain the influx of the amphipathic weak bases is the pH gradient. This mechanism is well-established (see Introduction). The accumulation of the protonated base inside the liposome will lead to elevation of internal pH which will increase the level of NH_3, and therefore reduces again the pH enabling more drug to come in. The process should stop after the release of all NH_4^+ out of the liposomes. Another alternative is that the leakage of the neutral ammonia is followed by slower though still high (approx. 10^{-5} cm/s) efflux of protons; therefore, an ammonium ion

diffusion potential is formed which acts as a driving force for the influx and accumulation of the amphipathic weak bases inside the liposomes. This may be part of the explanation why the kinetics of anthracycline accumulation is much slower than that of the liposome acidification. The relative contribution of each of these two factors to the loading is now under investigation. Indeed, release of ammonium ions was in the same order of magnitude as the uptake of DXR by the liposomes. More studies are required to assess the direct contributions of the membrane potential to this intraliposomal base accumulation.

Finally, the very low permeability coefficient of the sulfate ion through lipid bilayers is a crucial factor in obtaining a stable intraliposomal accumulation of the anthracyclines. Much smaller accumulations (which were further reduced with storage time) were obtained when anions of higher permeability coefficients such as chloride [41] were used (data not shown). An additional reason for having stable DXR accumulation is the lower solubility of the anthracycline sulfate salt formed in the intraliposomal aqueous phase, which is further reduced in the presence of ammonium sulfate in the solution. Probably, the salting-out effect of ammonium sulfate accelerates flocculation and gelation of the drug, therefore further improving the encapsulation and stabilizing it due to the mass action law.

The aggregation of DXR in the intraliposomal aqueous phase was demonstrated by changes in the DXR absorbance and the virtually complete fluorescence quenching (Tables V and VI); this is also supported by the lack of contribution of the encapsulated DXR to the internal osmotic pressure (data not shown).

The process can be summarized as an exchange of NH_3 efflux and DXR influx. DXR accumulates due to its precipitation in the intraliposomal aqueous phase.

(ii) Vesicle-related properties

(a) The physical state of the liposome bilayer. This is determined by its lipid composition, lipid quality and temperature (reviewed in Refs. 1,2). This combination determines many of the liposome properties, including their permeability to various solutes [41–43]. This can be demonstrated by using liposomes containing PC of different acyl-chain composition and cholesterol (2:1 molar ratio). For vesicles composed of egg-PC, DPPC or fully-saturated soy PC (HPC) loading to a DXR/lipid ratio > 0.1 was obtained at the following temperatures: 21°C, 45°C and 60°C, respectively. The temperature effect was also demonstrated for preparations IV, V and X at 21°C and 4°C. For egg-PC/cholesterol (preparation IV), maximum loading at 21°C was reached after 24 h, while at 4°C it was not obtained even after one week. Thus, transmembrane diffusion may become a rate-limiting factor which can be overcome by temperature elevation or manipulation of vesicle lipid composition. Similar data were obtained for preparations V and X, although to obtain a faster loading rate a temperature above the T_m of the PC was used (49°C and 60°C, respectively). A highly peroxidized egg-PC batch could not be used for the preparation of an intact loading system. This is a further proof of the importance of the intactness of the lipid bilayer for the efficient retention of solute inside liposomes. Indeed, the loaded drug probes the quality of the lipid, and retention of most of the loaded DXR in egg-PC/cholesterol vesicles (preparation IV) for more than two weeks at 4°C indicates stability of the lipid itself. Also, the lipid composition will determine shelf-life of a preparation. For example, having HPC as the main component prevents drug leakage and keeps a stable ammonium sulfate gradient at 4°C for at least six months.

(b) Trapped volume of the liposomes. The facts that the drug/lipid ratio is proportional to the trapped volume (Tables I and III) and that the DXR is not released upon liposome dilution [18] indicate that most of the DXR resides in the aqueous compartment of the liposomes. There the loaded drug reaches a concentration much above its concentration in the external medium and even above the solubility limit of DXR-HCl (60 mM) though the DXR is in an aggregated state (Tables IV and V). This is further supported by our recent studies by X-ray diffraction and cryoelectron microscopy which suggest that the DXR inside the liposomes is organized in a gel-like structure [44].

(c) Vesicle surface charge. It is known that addition of negatively-charged lipids to liposomes increases the ratio of membrane-associated DXR to lipid [5,19,45,46]. This ratio is dependent on the charge density and on the association constant of the DXR-liposome interaction [19,46]. From Table I it is clear that surface charge has almost no effect on drug loading, which suggests that most of the DXR loaded by the ammonium sulfate gradient is not bilayer-associated.

(iii) Loaded-molecule-related properties

Our experience so far suggests the involvement of three properties of the loaded molecule on the rate and extent of accumulation. Comparison between the loading of acridine orange, DNR, epirubicin and DXR indicates the contribution of two properties, the pK_a and the degree of hydrophobicity. The order of loading rate is acridine orange > DNR > epirubicin > DXR. The very high and very fast accumulation of acridine orange inside the liposome aqueous phase is a result of the amphipathy together with high pK_a (9.25) [30]). The three anthracyclines have a very similar pK_a [39], while they differ in their amphipathy as expressed by the octanol/Tris buffer (pH 7.0) partition coefficient. Their relative rates of accumulation parallel their partition coefficients (DNR > epirubicin > DXR. The

third property is the degree of solubility of the loaded molecules in the aqueous compartment of the liposomes. In the case of anthracyclines their aggregation prevents the system from reaching true equilibrium and stabilizes the intraliposomal accumulation.

Release of DXR from liposomes loaded using the ammonium sulfate gradient

The release rate of the loaded molecule from the liposome into the external medium should be controlled by the same parameters which defined the loading. Indeed, release rate was strongly related to the hydrophobicity of the loaded molecule (DNR leaked faster than DXR). Therefore, the liposomes loaded with amphipathic weak bases through the ammonium sulfate gradient can be used to obtain controlled release of the loaded molecules. For example, the DXR first-order rate constant (k_1) from egg-PC/cholesterol extruded OLV (preparation IV, Table I) at 37°C is $3 \cdot 10^{-3}$ min^{-1} and assuming first-order kinetics $t_{1/2}$ is 231 min. k_1 is increased by two orders of magnitude by addition of nigericin to the DXR liposomes loaded by ammonium sulfate gradients. The nigericin-dependent leakage rate was faster for acridine orange than for DXR. This can be explained by the higher pK_a and hydrophobicity of acridine orange.

Leakage rate was also dependent on lipid composition. A comparison of egg-PC/cholesterol, DPPC/cholesterol and HPC/cholesterol vesicles having identical DXR concentrations shows almost no release for HPC vesicles (Table I), a slower release rate for DPPC/cholesterol vesicles (preparation V), and a faster release rate for egg-PC/cholesterol vesicles (preparation IV, Tables I and VI). This difference is attributed to the physical properties, mainly the larger free volume of bilayers composed of egg-PC/cholesterol lipid compared to bilayers composed of disaturated PC. In all systems, leakage rate increased with increasing temperature. However, the energy of activation for egg-PC/cholesterol vesicles (preparation IV) was lower than for DPPC/cholesterol (preparation V, Table VI). Therefore, the difference between the two became smaller at the higher temperature, suggesting that at high temperature the liquid-ordered phases [47] of these two compositions have similar order and dynamics.

The effect of DXR being aggregated in a gel-like state [44] inside the vesicle on its release from the liposomes is not yet known. However, it is clear that almost all the encapsulated DXR is bioavailable and can be released despite its aggregation, as was accomplished by the ionophore nigericin in the presence of potassium ions. We recently took advantage of the ability of nigericin to induce remote release, demonstrating that it thus improves cytotoxicity of liposomes loaded with DXR by the ammonium sulfate gradient [48].

Other evidence for the bioavailability of the encapsulated DXR is our demonstration in a pilot clinical trial with Doxil™ [16] that urine and effusate obtained from the tumor by biopsy have a normal metabolite profile in humans. (Doxil (Liposome Technology) is almost identical to preparation VIII, Table I).

General features of liposomes having ammonium sulfate gradients

The use of an ammonium sulfate gradient as a means of loading of amphipathic weak bases into liposomes has some distinct features. The most important one, which is unique to this approach, is that the loading is a result of changing the internal aqueous phase of the liposomes, and not as done by others, by changing the external pH [49,50]. Another advantage is that the magnitude of the gradient is controlled very easily by dilution of the ammonium sulfate liposomes with an isoosmotic mixture of a nonpermeable solute such as NaCl, KCl, Na_2SO_4, glucose or sucrose. The loading cannot be explained by pH gradient only, since similar or even better loading was obtained when the pH gradient was ≤ 1 pH unit compared to 3 pH units. However, it is highly dependent on the ammonium sulfate gradient.

Another important aspect is the ease of applying this method. There is no need for buffer or pH titration during the whole procedure. Therefore, the method was used successfully for preparation of immunoliposomes loaded with anthracyclines, without inactivation of the antibody attached to the liposome by the low pH of the medium (Emanuel, Kedar, Bolotin, Smorodinsky and Barenholz, unpublished data).

One may also note that the method of preparation of the gradient implies that the liposomes are exposed to a low pH only for a very short time before loading, and only at their internal surface, while the pH of the medium does not have to be altered. As loading begins, the entrance of the amphipathic base into the aqueous compartment of the liposomes raises the pH.

This has an important consequence for the stability of the lipid vesicles, since the exposure of phospholipids to low pH for a prolonged time may lead to their degradation [51–53]. For clinical use of these liposomes it is important to stress that the liposomes having ammonium sulfate gradients are not toxic to mice after intravenous injection (data not shown).

Finally, we would like to elaborate on the relevance of the 'ammonium sulfate liposomes' to the accumulation of DXR inside cells. DXR enters cells via an as yet undefined mechanism. There are indications for active transport, passive passage across membranes and/or distribution according to electrochemical gra-

dients [54]. The ammonium sulfate system proves that the combination of a pH gradient, a membrane potential and the presence of a sink (DXR-sulfate aggregates) can alter the partitioning of the drug between the extra- and the intraliposomal medium.

A similar mechanism may operate in cells, the sink being nucleic acids [5,46].

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