Sterilization of Liposomes by Heat Treatment

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Autoclaving of liposomes composed of egg phospholipids or saturated phospholipids, the latter sometimes combined with cholesterol, was performed in an isotonic acetate buffer (pH 4.0) or Hepes buffer (pH 7.4). After a standard autoclaving cycle (15 min, 121°C), no change could be observed in pH, size, and extent of oxidation. Dependent on the experimental conditions, a minor or substantial increase in the fraction of hydrolyzed phospholipids was found. After a sterilization cycle, pronounced leakage was found for a water-soluble, encapsulated compound (calcein) and for an amphiphilic compound (doxorubicin). Lipophilic, liposome bilayer-associated compounds [*N*-trifluoroacetyldoxorubicin-14-valerate (AD-32) and α-tocopherol] remained in the liposomes after autoclaving. However, substantial degradation of AD-32 was observed. Under proper conditions liposomes without or with thermostable, lipophilic drugs can be sterilized by autoclaving. However, the hydrolysis of phospholipids can pose a problem, as hydrolysis kinetics depend on the pH used. In the chosen circumstances the autoclaving cycle caused massive loss of hydrophilic, nonbilayer interacting compounds; under those conditions "free" drug removal or drug encapsulation should be performed after the autoclaving step.

KEY WORDS: liposomes; autoclaving; heat sterilization; hydrolysis; leakage/retention; chemical and physical stability.

INTRODUCTION

Liposomes, (phospho)lipid vesicles, become more and more successful as drug carriers. One of the problems that still remain is the preparation of sterile liposomes. Production of liposomes under aseptic conditions is possible but complex and expensive. Thus, there is a need for a widely applicable sterilization method. In the sterilization process particle size and chemical composition of the liposomes should remain unchanged. Moreover, the encapsulated drug should not leak out of the liposomes.

Sterilization methods, which meet these requirements, are (i) filtration through the pores of a 0.22-μm membrane (1–3) or (ii) exposure of freeze-dried liposomes to chemical sterilizing agents as ethylene oxide (4). The limitations of these approaches are that filtration is not applicable to large liposomes (>0.2 μm) and that residues of the incompletely removed chemical agents (ethylene oxide and/or contaminants caused by ethylene oxide) can be toxic.

Although exposure to high temperature or various types of irradiation is thought to be unsuitable for liposome sterilization (5), several publications appeared over the years suggesting that autoclaving of liposomes was not as detrimental to the liposome formulations as was generally assumed by the "field" (6–8). However, none of these studies discussed the chemical (hydrolysis/oxidation) and physical (size, drug retention, etc.) stability in a comprehensive fashion.

Therefore, the aim of this study is to assess in more detail the possibilities and limitations of autoclaving of liposomes. Chemical degradation of lipid bilayer components and size changes of different types of liposomes and, in addition, the chemical degradation and leakage of a number of model compounds as a result of autoclaving were monitored. Charged liposomes composed of egg phospholipids or saturated phospholipids were autoclaved. In some cases the saturated phospholipids were combined with cholesterol. For leakage studies model compounds with different physicochemical characteristics were selected: the nonbilayer interacting calcein, the amphiphilic doxorubicin, the lipophilic derivative of doxorubicin *N*-trifluoroacetyldoxorubicin-14-valerate, and the lipophilic α-tocopherol. The buffer used was an isotonic acetate buffer (pH 4.0) or Hepes buffer (pH 7.4). To allow for monitoring size changes after liposome autoclavation, all liposomes were sized during their preparation process and had an average size of about 0.2 μm before heat treatment.

MATERIALS AND METHODS

Materials

Dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol (DPPG) were obtained from Nattermann GmbH (Cologne, FRG). Egg phosphatidylcholine (EPC) and egg phosphatidylglycerol (EPG; sodium-salt) were products of Lipoid (Ludwigshafen, FRG). As determined by Lipoid, the PC content of the EPC was 98.6% and contained traces of lysophosphatidylcholine (<0.2%), sphingomyelin (<0.5%), cholesterol (0.3%), and α-tocopherol (0.1%). The peroxide value and iodine value of EPC were 0 and 60, respectively. The purity of EPG was >98%. As determined by Lipoid, it contained lysophosphatidylglycerol (0.4%), EPC (0.2%), egg phosphatidic acid (1.0%), cholesterol (0.1%), and α-tocopherol (0.1%). The peroxide value and iodine value of EPG were 1 and 55.6, respectively. Cholesterol was purchased from Sigma (St. Louis, MO). Monopalmitoylphosphatidylcholine and egg lysophosphatidylcholine were obtained from Avanti Polar Lipids (Pelham, AL). Doxorubicin (DXR) was obtained from Pharmachemie B.V. (Haarlem, The Netherlands). *N*-Trifluoroacetyldoxorubicin-14-valerate (AD-32) was kindly provided by Dr. J. H. Beynen (Slotervaart Hospital/Netherlands Cancer Institute, Amsterdam, The Netherlands). Calcein and α-tocopherol were purchased from Fluka AG (Buchs, Switzerland). Dowex 50W-X4 was obtained from Baker Chemicals (Phillipsburg, NJ). All other chemicals were of analytical grade. The water was double-distilled before use.

Preparation of Liposome Dispersions

Liposomes were prepared by the "film" method. Ap-

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propriate mixtures of the (phospho)lipids were dissolved in chloroform/methanol (1:1) in a round-bottom flask. When α-tocopherol and AD-32 were incorporated, these compounds were codissolved. The concentration of these lipophilic compounds was about 0.9 mg α-tocopherol/15 mg phospholipid and 1.0 mg AD-32/15 mg phospholipid. The organic solvent was removed under vacuum by rotary evaporation. The thin film obtained was dried for at least 3 hr under reduced pressure. Then the film was hydrated with 10 m*M* Hepes buffer (pH 7.4) + 0.15 *M* sodium chloride (NaCl) or with 10 m*M* acetate buffer (pH 4.0) + 0.15 *M* NaCl. When calcein or DXR was incorporated, these compounds were added to the buffer before hydration. The concentrations of calcein and DXR in the buffer were about 5 and 4 m*M*, respectively. The liposome dispersions were sized with an extruder (Sartorius, Göttingen, FRG) through 0.6-μm-pore size and three times through 0.2-μm-pore size filters, respectively (Bio-Rad, Richmond, CA). The pH of the dispersion was measured and adjusted before and after extrusion, if necessary. The final phospholipid concentration was about 20 m*M*.

Removal of "Free" Compound

Nonentrapped calcein was separated from the liposomes by ultracentrifugation (30 min at 200,000*g* in a Beckman L70 ultracentrifuge (Beckman Instrument Inc., Palo Alto, CA) and removal of the supernatant (three times). Free DXR was removed by incubating the liposomes for 15 min with a Dowex 50W-X4 resin (in sodium form) in an extruder with an 8-μm filter (9).

Autoclaving

The liposome dispersions were filled into 2-mL ampoules. Unless otherwise stated, nitrogen was bubbled through for ±15 min to remove oxygen before closing the ampoules. The autoclaving cycle took place in a SANOclav, Type M-ECZ (Wolf, Geislingen, FRG). It took ±15 min to reach 100°C. After 4 min at 100°C, the autoclave was closed and the temperature increased. Subsequently, it took ±3 min to reach 121 ± 1°C. The ampoules stayed at this temperature for 15 min, before being cooled down within 3–4 min to ambient temperature.

Determination of Leakage of Encapsulated Compound from Liposomes

Free DXR or calcein was separated from the liposome dispersion by ultracentrifugation of an 11-times-diluted sample (30 min at 200,000*g*). The DXR or calcein content of the supernatant was determined as described below. The supernatant was checked for lack of phosphate content. In the case of AD-32 and α-tocopherol the ratio drug/phosphate in the supernatant was determined after 1 min of centrifugation at 2000*g* in a table centrifuge. If crystals are present, they were spun down; the liposomes remained in the supernatant.

Differential Scanning Calorimetry (DSC)

Liposomes were concentrated by ultracentrifugation at 200,000*g* for 30 min. The pellet was put into an aluminum pan. As a reference, an empty aluminum pan was used. Calorimetric scans from 30 to 60°C were performed on a Netzsch DSC 200 low-temperature DSC (Netzsch-Gerätebau, Selb, FRG). The scanning rate was 2°C/min. Each sample was measured twice. The amount of liposomes in the sample pan was estimated by phosphate determination of the content as described below.

Analytical Methods

Phospholipids were analyzed by HPLC as described earlier (10). The HPLC system consisted of a type 400 solvent delivery system (Kratos, Ramsey, NJ), a Kontron sampler MSI 660 (Kontron AG, Zürich, Switzerland), and a Waters 410 RI detector (Waters Associates, Milford, MA). Peak areas were measured with an integrator (3392A Hewlett-Packard, Avondale, PA). The separation of the phospholipids was carried out on a Zorbax aminophase column (25 cm × 4.6-mm I.D., 5-μm particle size; Du Pont Company, Wilmington, DE) at 35°C. An Adsorbosphere NH2 5μ guard column (Alltech Associates, Deerfield, IL) was connected before the Zorbax aminophase column. The mobile phase consisted of acetonitrile/methanol/10 m*M* ammonium dihydrogen phosphate solution, pH 4.8 (57/38/4, v/v). The flow rate was 1.5 mL/min.

Cholesterol was analyzed as described by Lang (11). The analysis was performed on a non-end-capped Spherisorb S-5 ODS-1 (25 × 0.46-cm I.D.) with a mobile phase of 100% methanol and a flow rate of 1.5 mL/min. The column temperature was ambient. The HPLC system consisted of a Type 400 solvent delivery system (Kratos, Ramsey, NJ), a WISP 710B automatic sampler (Waters Associates), and a Model 783A absorbance detector (Applied Biosystems, Kratos, Ramsey, NJ). Detection was carried out at 207 nm.

α-Tocopherol was analyzed basically as reported by New (12). The same HPLC system was used as described above. The only differences were that the detection was carried out at 214 instead of 207 nm and that the flow rate was 1 instead of 1.5 mL/min.

The analysis of DXR and AD-32 was performed as described earlier (13,14). The HPLC system for the analysis of DXR consisted of a Type 400 solvent delivery system (Kratos), a WISP 710B automatic sampler, and a Model 440 absorbance detector (Waters Associates). For the analysis of AD-32 the automatic sampler was replaced by a Kontron sampler MSI 660 (Kontron AG, Zürich, Switzerland). The stationary phase for both drugs was a Lichrosorb RP8-column (12.5 × 4.0-mm I.D., 5-μm particle size; Merck, Darmstadt, FRG). The mobile phase consisted of water/acetonitrile (50/50, v/v). Before mixing, the pH of the water phase was adjusted to 2.5 with phosphoric acid. The flow rate was 1.5 mL/min. The UV detector was operating at 254 nm.

Calcein was quantitated by fluorescence measurements, performed on an LS50 luminescence spectrometer (Perkin Elmer Ltd., Norwalk, CT), using an excitation wavelength of 491 nm and an emission wavelength of 511 nm (slits, 5 nm). Total encapsulated calcein was determined after solubilization of the liposomes in a ±1% Triton X-100 solution at 80°C.

Oxidation of the egg phospholipids was monitored through the formation of conjugated dienes (15) and the for-

mation of thiobarbituric acid (TBA)-reactive products as described by Ondrias *et al.* (16). For monitoring the generation of conjugated dienes, 20 μL of liposomes was dissolved in 2 mL of ethanol. The oxidation index was defined as the absorption ratio at 233/215 vs 300 nm. For monitoring the formation of TBA-reactive products, 150 μL liposomes and 250 μL buffer were mixed with 2.4 mL TBA-TCA solution [2.1 g TBA, 84 g trichloroacetic acid (TCA), 3.6 mL 37% hydrochloric acid diluted in water to 500 mL]. To prevent further oxidation, 0.24 mL of a solution of 1.5 mg/mL butylated hydroxytoluene in ethanol was added. The samples were incubated at 80°C for 15 min. After cooling the samples were centrifuged at 2000*g* for 5 min. The presence of TBA-reactive products was assessed by determining the absorption at 533 vs 600 nm. The measurements were performed with a double-beam spectrophotometer (Lambda 5, Perkin-Elmer Corp., Norwalk, CT).

The phospholipid content was measured by phosphate determination according to Fiske and Subbarow (17). Glycerophospho compounds, the (actual) end products of hydrolysis of phospholipids, were measured by phosphate determination in the supernatant of a Bligh & Dyer extraction (18) mixture.

The *Z*-average particle size and polydispersity index (p.d.) at 25°C were determined by dynamic light scattering with a Malvern 4700 system using a 25-mW He–Ne laser (NEC Corp., Tokyo) and the automeasure version 3.2 software (Malvern Ltd., Malvern, UK). For viscosity and refractive index, the values of pure water were used. The p.d. is a measure of the particle size distribution and ranges from 0.0 for an entirely homogeneous up to 1.0 for a completely heterogeneous size distribution.

RESULTS

The effects of autoclaving on different liposome dispersions were studied. Variables of investigation were the bilayer composition, pH, and presence of oxygen. The results are presented in Table I. None of the liposome dispersions showed a change in particle size and polydispersity (all about 0.2 μm with a p.d. of about 0.1; results not shown). Also, hardly any change in pH could be found ($\Delta pH < 0.1$; results not shown). These results indicate that no major changes occurred during autoclaving. An increase in hydrolysis after autoclaving could be found. At pH 7.4 this increase was a minor one (<1%). At pH 4.0 the increase was 3–7%. The percentage hydrolysis was defined as

$$\% \text{ hydrolysis} = 100\% - [PC] \times 100\%/\{[PC] + [LPC] + [GPC]\} \quad (1)$$

where [PC] is the concentration of phosphatidylcholine, [LPC] is the concentration of lysophosphatidylcholine (the first hydrolysis product of PC), and [GPC] is the concentration of glycerophospho compounds [the (actual) end hydrolysis products of PC]. Here the contribution of glycerophospho compounds from phosphatidylglycerol is neglected. No degradation of cholesterol could be found (results not shown). Because of the presence of double bonds in the fatty acids of egg phospholipids, oxidation could take place. But

Table I. Liposome Composition and Extent of Hydrolysis and Oxidation, Before and After Autoclaving[a]

Lipid composition	pH	Atmosphere in ampoules	Concentration of model compound in liposomes	% Hydrolysis		$A_{233\ nm}/A_{215\ nm}$		$A_{533\ nm}$	
				Before	After	Before	After	Before	After
DPPC/DPPG 10/1	4.0	Nitrogen		2.0 ± 0.2	9.5 ± 0.6	n.d.	n.d.	n.d.	n.d.
DPPC/DPPG/CHOL 10/1/4	4.0	Nitrogen		2.4 ± 0.6	7.3 ± 0.5	n.d.	n.d.	n.d.	n.d.
EPC/EPG 10/1	4.0	Nitrogen		1.3 ± 0.1	6.3 ± 0.1	0.13 ± 0.01	0.13 ± 0.01	−0.01 ± 0.01	−0.01 ± 0.01
EPC/EPG 10/1	4.0	Air		1.5 ± 0.4	5.0 ± 0.6	0.21 ± 0.01	0.207 ± 0.001	0.000 ± 0.003	0.020 ± 0.003
DPPC/DPPG/CHOL 10/1/4	4.0	Nitrogen	±0.3 m*M* DXR	2.1 ± 0.1	5.1 ± 0.5	n.d.	n.d.	n.d.	n.d.
EPC/EPG 10/1	4.0	Nitrogen	± 0.1 mg/mL AD-32	1.1 ± 0.1	4.1 ± 0.6	0.4 ± 0.2	0.4 ± 0.2	0.005 ± 0.003	0.007 ± 0.005
DPPC/DPPG 10/1	7.4	Nitrogen		0.2 ± 0.1	0.9 ± 0.1	n.d.	n.d.	n.d.	n.d.
DPPC/DPPG/CHOL 10/1/4	7.4	Nitrogen		0.2 ± 0.2	1.1 ± 0.1	n.d.	n.d.	n.d.	n.d.
EPC/EPG 10/1	7.4	Nitrogen		0.1 ± 0.1	0.4 ± 0.3	0.21 ± 0.01	0.24 ± 0.03	0.042 ± 0.002	0.045 ± 0.003
EPC/EPG 10/1	7.4	Air		0.4 ± 0.1	0.2 ± 0.1	0.16 ± 0.04	0.16 ± 0.03	0.001 ± 0.002	0.001 ± 0.001
EPC/EPG 10/1	7.4[a]	Air		—[b]	—[b]	0.19 ± 0.02	0.21 ± 0.05	0.001 ± 0.003	0.002 ± 0.002
DPPC/DPPG/CHOL 10/1/4	7.4	Nitrogen	±0.5 m*M* calcein	0.1 ± 0.2	1.0 ± 0.5	n.d.	n.d.	n.d.	n.d.
DPPC/DPPG/CHOL 10/1/4	7.4	Nitrogen	±2 m*M* α-tocopherol	0.3 ± 0.1	0.7 ± 0.1	n.d.	n.d.	n.d.	n.d.

[a] All experiments were done three times. n.d., not determined, because the phospholipids contained only saturated fatty acids.

[b] The Hepes buffer was replaced here by a 10 m*M* phosphate buffer. Therefore the percentage hydrolyzed phospholipids was not determined here, because the phosphate buffer interferes with the determination of GPC.

as a rule, no change in the oxidation parameters was found (see Table I), even in the presence of air. Only autoclaving of liposomes composed of egg phospholipids at pH 4.0 resulted in a very small increase in the formation of TBA-reactive products (monitored by absorbance at 533 nm).

At pH 4.0 changes in the chain melting properties of the liposomal bilayers were found after autoclaving of DPPC/DPPG 10/1 liposomes (see Fig. 1A). A shoulder appeared at the right-hand side of the main phase transition peak. A minor change of 16 ± 11% in the ΔH of the main transition was found after autoclaving. The pre-transition was not detectable anymore. After autoclaving the liposomes at pH 7.4 minor changes were found in the gel-to-fluid transition (see Fig. 1B). Here a shoulder appeared as well but, in this case, at the left-hand side of the main phase transition peak. No change in the enthalpy (ΔH) of the main phase transition could be found. Only a minor decrease of 20 ± 9% in the ΔH of the pre-transition was found after autoclaving.

A major problem with autoclaving liposomes can be the retention and, in some cases, the stability of the encapsulated agent. Several compounds varying in physical properties were tested. The results are shown in Table II. The water-soluble and amphiphilic compounds calcein and DXR, respectively, leaked out of the liposomes during autoclaving. The leakage of the amphiphilic DXR was less extensive than the leakage of the water-soluble calcein. In the case of DXR, degradation was observed (see Table II). When liposomal AD-32 was autoclaved, a pronounced degradation of AD-32 was found after autoclaving (see Table II). Bekers *et al.* described conversion of AD-32 to doxorubicinone-14-valerate under acid conditions (14). Doxorubicinone-14-valerate is not stable in acidic media and degrades further into doxorubicinone. The HPLC retention data for AD-32 and doxorubicinone-14-valerate were indeed comparable with the chromatograms published by Bekers *et al.* The concentration of doxorubicinone was too low to be detected by the HPLC protocol used. From its molecular structure one can derive that doxorubicinone-14-valerate is also lipophilic. Thus, as expected, no degradation product was found in the supernatant by HPLC after spinning down autoclaved AD-32 liposomes in the ultracentrifuge. Doxorubicinone-14-valerate presumably has an absorbance similar to that of AD-32. Calculations made on the basis of that assumption showed that neither AD-32 nor AD-32 derived compound left the bilayer (see Table II). The only successful sterilization by autoclaving in this study was achieved with α-tocopherol in DPPC/DPPG 10/1 liposomes. Neither degradation nor disappearance from the liposomes was found (see Table II).

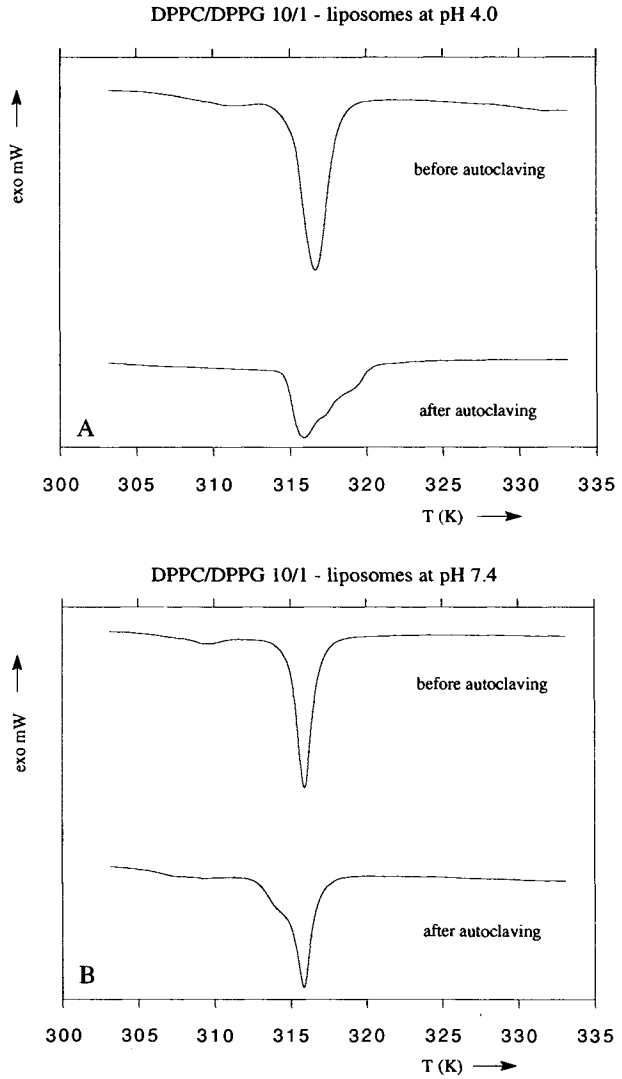

Fig. 1. Typical examples of calorimetric scans of DPPC/DPPG 10/1 liposomes at pH 4.0 (A) and pH 7.4 (B) before and after autoclaving.

Table II. The Percentage Leakage and Percentage Degradation of Encapsulated Agents After Autoclaving[a]

Lipid composition	pH	Encapsulated model compound	% leakage[b]	% degradation[c]
DPPC/DPPG/CHOL 10/1/4	4.0	DXR	20 ± 5	26 ± 8
EPC/EPG 10/1	4.0	AD-32	—[d]	39 ± 4
DPPC/DPPG/CHOL 10/1/4	7.4	Calcein	44 ± 4	—[e]
DPPC/DPPG/CHOL 10/1/4	7.4	α-Tocopherol	—	—[e]

[a] All experiments were done three times.
[b] For DXR and calcein, leakage was defined as percentage free compound/total compound.
[c] For the lipophilic compounds AD-32 and α-tocopherol the % degradation was deduced by comparing the ratio mg or m*M* agent/mol P before and after autoclaving after 1 min of centrifugation at 2000*g* in a table centrifuge. Control experiments without autoclaving showed no degradation.
[d] The degradation product of AD-32, doxorubicinone-14-valerate, also remained in the bilayer (see text). A disappearance of 4 ± 4 was calculated.
[e] No significant degradation or disappearance was observed.

DISCUSSION

The objective of this study was to evaluate the possibility to autoclave liposomes. Here we demonstrated that the liposomes under investigation were hardly degraded by autoclaving at neutral pH. No change could be found in pH, size, and extent of oxidation. Only at pH 4.0 was there a substantial increase in percentage hydrolysis (3–7%; see Table I).

In this study no aggregation of the charged liposomes

could be found upon autoclaving. Kikuchi *et al.* observed aggregation of uncharged and charged liposomes in a 0.9% sodium chloride solution (6). In the presence of 5% glucose or 2% propylene glycol, no aggregation was observed. They claimed that the absence of electrolytes was the reason for the stabilizing effect of the glucose and propylene glycol. Garelli and Vierling also found aggregation of liposomes after heat sterilization of lipophilic perfluoroalkylated bipyridine platinum and palladium complexes incorporated into uncharged liposomes composed of egg phospholipids (8). The size of their liposomes was about 50–100 nm. The absence of aggregation in our study may therefore be ascribed to the liposomal dispersion used (type, charge, and buffer) as described.

The lack of signs for oxidation in liposome dispersions composed of egg phospholipids in the presence of air was a surprising result (see Table I). This observation is in conflict with findings reported by Kikuchi *et al.* (6). The experimental conditions were different in the two studies, and this might be the reason for the discrepancy. Probably the time that the dispersion is kept at elevated temperatures is not long enough to induce oxidation of the egg phospholipids. Another explanation for the absence of signs of lipid oxidation could be the decreasing concentration of dissolved oxygen in water when the temperature of the dispersion is raised. Phosphate-buffered liposome dispersions (pH 7.4) gave the same results as Hepes-buffered dispersions (see Table I). Therefore, in this study no special protective effect against oxidation could be ascribed to Hepes buffer. Such a protective effect was found by Fiorentini *et al.* after ultrasonication of liposomes composed of egg phospholipids (19).

The only recorded damage of the phospholipids by the autoclaving process was a slight increase in the percentage hydrolyzed phospholipids after autoclaving (see Table I). The somewhat larger increase in percentage hydrolysis of the phospholipids in liposomes at pH 4.0 is caused by the reported proton- and hydroxyl-catalyzed hydrolysis of phospholipids (20,21). The order of magnitude of the degree of hydrolysis one could expect was derived from the Arrhenius plots published by Grit *et al.* (21). Hydrolysis follows pseudo-first-order kinetics. For the hydrolysis of aqueous liposome dispersions composed of saturated soybean phosphatidylcholine at pH 4.0, Grit *et al.* found an activation energy (E_a) of 58 kJ · mol^{-1} and a frequency factor (A) of 1.3 × 10^3 sec^{-1}. At pH 7.0 the E_a was 86 kJ · mol^{-1} and the A was 1.6 × 10^6 sec^{-1}. Taking the time and temperature for sterilization as 20 min (5-min correction for heating and cooling) and 121°C, respectively, the predicted percentages hydrolyzed phospholipids were 3% at pH 4.0 and 0.8% at pH 7.0. This corresponds well with our findings in this study.

Minor differences were found in the calorimetry scans (see Fig. 1) of liposomes at pH 7.4 before and after autoclaving; the differences were more pronounced in the calorimetry scans of liposomes at pH 4.0 before and after autoclaving. The disappearance of the pre-transition at pH 4.0 is an especially striking result. The pre-transition has been reported to be highly sensitive to perturbations of the lipid matrix (22). After autoclaving a shoulder appeared at the left-hand side (at pH 7.4) or at the right-hand side (at pH 4.0) of the main peak. The DSC scans did not change upon repeated scanning of the same sample from 30 to 60°C (two cycles), indicating that the observed changes in melting properties have a permanent character. The only differences between the two liposome dispersions are the pH and the percentage hydrolysis. Hydrolysis results in the presence of lysophospholipids and fatty acids in the bilayers. However, a study on the effect of the incorporation of both fatty acids and lysophospholipids in DPPC liposomes has never been done. It has been reported that up to 40 mol% incorporation of only 1-palmitoyl-*sn*-glycerol-3-phosphorylcholine in DPPC liposomes at pH 7.0 resulted in only one sharp transition, while the pre-transition was abolished (23). The sum of the ΔH of the pre-transition and the main transition showed only a linear decrease after incorporation of 20 mol% lysophosphatidylcholine (levels not reached after autoclavation). The addition of only palmitic acid to DPPC resulted in an increased transition temperature and a broadening and an increase in ΔH for the main transition, while abolishing the pretransition (24). The fatty acids formed by the hydrolysis of the phospholipids are in a different ionization state; they are noncharged at pH 4.0 and charged at pH 7.4 (25,26). Therefore, incorporation of fatty acids in DPPC liposomes was shown to have a pH-dependent effect on the phase transition (27). Only fully protonated fatty acids resulted in an increase in the phase transition temperature. The shoulder at the right-hand side of the main transition after autoclaving of DPPC/DPPG 10/1 liposomes at pH 4.0 may be explained by the presence of the fully protonated hydrolysis product: palmitic acid.

The intriguing issue of the relation between the hydrolysis of phospholipids in the bilayers and the physical stability of liposomes has not been investigated in great detail yet. With partially hydrogenated EPC/EPG 10/1 liposomes, Grit and Crommelin reported hardly any change in the permeability of calcein with increasing phospholipid hydrolysis up to a level of 15% (both fatty acids and lysophospholipids are present in the bilayer). The permeability of liposomes with only added lysophospholipids gradually increased with the lysophospholipid content (28). This 15% level is, by far, not reached after autoclavation as performed in the present study.

A major problem may be encountered when autoclaving a liposome dispersion with an encapsulated drug. Water-soluble or amphiphilic compounds leak out of the liposomes (see Table II). The selected bilayer composition for this experiment was DPPC/DPPG/CHOL 10/1/4; these liposomes have a low permeability at ambient temperatures and have no detectable phase transition in the experimental range. Apparently, the permeability of the bilayers strongly increased at the elevated temperatures used in this study. Leakage of the amphiphilic, positively charged DXR from negatively charged liposomes was less pronounced than leakage of the non-bilayer interacting calcein (20 ± 5 and 44 ± 4%, respectively; see Table II). Thus, the interaction between DXR and liposomes inhibited leakage. However, this level of drug loss is not acceptable for DXR liposome formulations to be used in therapy.

DXR was also chemically unstable; 26 ± 8% was degraded after autoclaving. Beynen *et al.* (29) found an E_a of 89.7 kJ · mol^{-1} and an A of 8.2 × 10^7 sec^{-1} for the pseudo first-order degradation of DXR at pH 4.0. If a sterilization time of 20 min (5-min correction for heating and cooling) and a temperature of 121°C were taken, 12% degradation of DXR was calculated. This is not in agreement with our finding (see

Table II). The discrepancy may be caused by the concentration dependence of the degradation process of DXR as reported by Janssen *et al.* at pH 7.4 (30).

The lipophilic agents AD-32 and α-tocopherol remained encapsulated after autoclaving (see Table II). For AD-32 the liposome composition was changed to EPC/EPG 10/1, because of the low encapsulation efficiency of AD-32 in the "standard" liposomes composed of DPPC/DPPG/CHOL 10/1/4 (result not shown). Garelli and Vierling also found complete retention after autoclaving of liposomes, composed of egg phospholipids, with lipophilic perfluoroalkylated bipyridine platinum and palladium complexes incorporated (8).

From this study it can be concluded that under neutral pH conditions, liposomes without encapsulated agents or with heat-stable, bilayer interacting (lipophilic) agents can be sterilized by autoclaving. Both saturated and unsaturated phospholipids showed a similar behavior as hydrolytic reactions, oxidation and leakage were negligible. For other pH's hydrolysis of the phospholipids could be a problem. Autoclaving of liposomes with water-soluble, non-bilayer interacting agents is an option only (i) if the agent is encapsulated after the autoclaving process (for instance, with "remote loading" techniques), (ii) if the free drug is removed after autoclaving, or (iii) if the presence of nonencapsulated material is not interfering with the desired therapeutic effect.

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