Biochimica et Biophysica Acta, 941 (1988) 165–175
Elsevier

BBA 74021

Characterization of cholesterol hemisuccinate and α-tocopherol hemisuccinate vesicles

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(Received 19 October 1987)
(Revised manuscript received 3 March 1988)

Key words: Cholesterol hemisuccinate; α-Tocopherol hemisuccinate; Liposome; Vitamin E

Cholesterol hemisuccinate (CHS) and α-tocopherol hemisuccinate (α-THS) were found to be capable of forming liposomes of multi- or single lamellar character. Such vesicles formed spontaneously, did not require the use of organic solvents and yielded high trapping efficiencies and captured volumes. Both CHS and α-THS systems greatly restricted the motion of intercalated spin labelled fatty acids, yet were more osmotically responsive than similar vesicle types comprised of phosphatidylcholine. Small angle X-ray diffraction measurements were consistent with vesicles possessing extremely weak interlamellar forces. CHS vesicles were found to remain intact in vivo, yet followed a pattern of distribution dissimilar to phosphatidylcholine vesicles.

Introduction

Most diacyl phospholipids spontaneously adapt the bilayer organization when dispersed in excess water to form closed vesicular structures or liposomes. The importance of such structures as models for biological membranes or as drug delivery vehicles is well documented [1–7]. Such in vitro and in vivo applications rely heavily on a number of techniques allowing control of the size, lamellarity, trapped volume, and solute distribution of the resulting vesicle [8]. Another important variable concerns lipid composition. Liposomes can be prepared from a variety of phospholipids resulting in systems with varying degrees of charge, acyl chain saturation and stability. The great variation possible in phospholipid vesicles, both in terms of chemical and architectural properties, is responsible in large part for their great utility.

Although compounds other than phospholipids, such as digalactosyl diacylglycerols, diacyl surfactants and single-chain amphiphiles have been shown to form closed vesicles under certain conditions [9–12], a systematic investigation of the physical properties of nonphospholipid liposomes is still in its early stages. Such liposomes, not unexpectedly, exhibit unique physical behavior

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Abbreviations: CHS, cholesterol hemisuccinate; α-THS, α-tocopherol hemisuccinate; PC, phosphatidylcholine; MLV, multilamellar vesicle; LUVET, large unilamellar vesicles by extrusion techniques; SPLV, stable plurilamellar vesicle.

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quite different from the more well-known phospholipid systems. Thus single component multilayered liposomes consisting of cholesterol sulfate or 3-*O*-methoxyethoxyethoxyethylcholesterol (cholesterol-PEG) behave as ideal osmometers but collapse at lower pressure differentials than do phosphatidylcholine bilayers. Unlike phospholipid vesicles, these systems have been reported not to form single lamellar liposomes, even after extensive sonication [13]. On the other hand, vesicles prepared from triethoxycholesterol apparently form single lamellar systems spontaneously which convert to multilayered systems only following sonication. The low (1%) encapsulation efficiencies reported for these vesicles would, however, presumably limit their utility [14].

Recently, liposomes comprised only of cholesterol hemisuccinate (CHS) or α-tocopherol hemisuccinate (α-THS) have been described [15–18]. Although both these compounds form liposomes readily and without the necessity of any other membrane-stabilizing component, they have been studied principally in mixed systems with phospholipids. Such systems have been used to elucidate the role of bilayer contact in destabilization of phosphatidylethanolamine vesicles and to provide insights into cholesterol and α-tocopherol interactions with PC [17,19,20]. A characterization of single component cholesterol hemisuccinate and α-tocopherol hemisuccinate liposomes, however, has to our knowledge heretofore not been reported and is done so here.

Materials and Methods

Phospholipids. Egg phosphatidylcholine and egg phosphatidic acid were obtained from Avanti Polar Lipids, Birmingham, AL.

Preparation of cholesterol hemisuccinate (Tris). The Tris salt of cholesterol hemisuccinate was either purchased from Sigma Biochemicals and used without purification or synthesized by mixing 30 ml of a 3.3 M solution of aqueous Tris base with 1.5 liters of a 67 mM solution of cholesterol hydrogen succinate (ICN, Cleveland, OH) in diethyl ether, evaporating the ether and lyophilizing the remaining wet residue for 12 h. The resulting cholesterol hemisuccinate Tris (CHS-Tris) was recrystallized three times from ethyl acetate. Residual ethyl acetate was removed by heating to 56° C under vacuum (0.1 mmHg).

Preparation of α-tocopherol hemisuccinate (Tris). The Tris salt of α-tocopherol hemisuccinate was prepared by dissolving 5 gm of α-tocopherol hydrogen succinate (Sigma) in 100 ml diethyl ether. 1.14 g Tris base (Fisher, Fair Lawn, NJ) dissolved in 5 ml H_2O was added in 0.5 ml portions to the solution while stirring or shaking. The solution was rotoevaporated to dryness and then further dried under high vacuum.

Vesicle preparation. CHS or THS multilamellar systems were prepared by hydrating appropriate amounts of material in 0.01 M Tris-HCl buffer/0.14 M NaCl (pH 7.3). Generally, hydration was accomplished without difficulty in seconds, although occasionally vortexing with glass beads was required. To make single lamellar systems, multilamellar systems were (1) sonicated to clarity in a bath-type sonicator operating at room temperature at 80 W, (2) passed through a French pressure cell (at 40000 lb/in^3), or (3) passed ten times through stacked 30–100 nm Nucleopore filters at 300–400 lb/in^2 using a LUVET apparatus as described previously [21]. Egg PC and egg PC-egg phosphatidic acid (8 : 2, mol : mol) MLVs were made following the method of Bangham [22]. SPLVs made from egg PC were prepared as previously described [23]. In all cases hydration, extrusion and sonication were performed at ambient temperature.

Freeze-fracture electron microscopy. Samples for freeze-fracture were prepared by sandwiching 0.1–0.3 μl aliquots of the specimen between a pair of Balzers (Nashua, NH) copper support plates which were rapidly plunged from 23° C into liquid propane.

Samples were fractured and replicated on a double replicating device in a Balzers freeze-fracture unit at a vacuum of $2 \cdot 10^6$ mbar or better and at −115° C. Replicas were floated off in 3 M HNO_3, followed by washing in a graded series of clorox solutions. These were finally cleaned in distilled water and picked up on 300 Hex mesh copper grids (Polysciences, PA). Replicas were viewed on a Philips 300 electron microscope at magnifications of 7000 to 22000-times.

Size histograms from freeze-fracture electron

micrographs were obtained by the method of Weibol and Bolender [24].

Encapsulation efficiencies. CHS and α-THS vesicles were prepared by hydrating various amounts of material in 5.0 ml of 0.01 M Tris-HCl buffer/0.14 M NaCl (pH 7.3) containing ^{51}Cr (1 μCi/ml). The preparations were allowed to stand at room temperature for 2 h, resulting in suspensions of vesicles containing entrapped isotope. Samples of each preparation were pipetted into dialysis bags (Thomas Scientific, M_r 12000 cut-off) that had been boiled three times in distilled water and assayed for ^{51}Cr. Unentrapped ^{51}Cr was removed by dialysis for 20 h against buffer free of ^{51}Cr. Encapsulation efficiencies were calculated as the ratio of cpm after and before removal of the unentrapped ^{51}Cr. To insure that the ^{51}Cr was being adequately removed in control experiments, ^{51}Cr was added to empty vesicles and dialysis was effected as above. In other experiments, encapsulation efficiencies were determined by forming the vesicles in buffer containing [^{3}H]inulin and removing the unentrapped inulin by successive washing in [^{3}H]inulin-free buffer employing low speed centrifugation. The two techniques produced comparable results.

Osmotic swelling. CHS, α-THS, EPC and egg PC-egg phosphatidic acid (8 : 2) MLVs were prepared by hydrating 65 μmol of lipid in 2.0 ml 0.01 M Tris-HCl/0.1 M KCl (pH 7.3). After gentle vortical mixing, each suspension of MLVs was allowed to stand at room temperature for 2 h in the preparatory buffer. A 20 μl aliquot of each preparation was then added to 2.0 ml of a series of 0.01 M Tris-HCl/KCl buffers with KCl concentrations ranging from 0.055 M to 0.5 M. After equilibration for 0.5 h, light scattering was determined by measuring the absorbance of the samples at 550 nm. If care was not taken to gently hydrate the CHS and α-THS, systems were formed which have been shown to result in non-ideal behavior [15,16].

Spin labeling. Flexibility profiles were obtained by labeling the vesicles with a series of doxyl stearic acids where the doxyl reporter group was present at different positions along the fatty acid chain. Labeling was effected by drying 0.65 μmol of the spin probe from ethanol to a thin film on the side of a test-tube to which was added 65 μmol of CHS or α-THS in 2.0 ml buffer. The suspensions were vortexed and the resulting liposomes washed twice. In the case of egg PC, a thin film containing 65 μmol of lipid and 0.65 μmol probe was hydrated in 2.0 ml of buffer and washed twice. ESR spectra were recorded on an IBM Instruments ER100D ESR spectrometer with nitrogen gas flow temperature regulation. An external calibrated thermistor probe (Omega Engineering, Stamford, CT) was used to monitor the temperature of the sample. ESR spectra were recorded at a microwave power of 10 mW and a microwave frequency of 9.11 GHz with a field sweep of 100 G and a 100 kHz field modulation amplitude of 0.32 G. The order parameter was calculated from the maximum hyperfine splitting (A_{max}) as previously described [25].

Differential scanning calorimetry. DSC measurements were carried out on a Micro Cal MC-1 Unit from Micro Cal, Amherst, MA. Sample volumes of 0.70 ml containing 5–9 mg of suspension were injected into the sample cell, with the same volume of buffer used in the reference cell. Samples were heated either at 26 C°/h or 37 C°/h. Duplicate runs of the same sample with the same history gave onset and completion temperatures reproducible to 0.2°C.

X-ray diffraction. X-ray diffraction was performed using the two-dimensional image-intensified X-ray detector apparatus described elsewhere [26–28]. CHS and α-THS dispersions were held in 1.5 mm glass X-ray capillaries sealed with epoxy plugs. Specimens were hydrated, as indicated in the text, either gently or vigorously. For gentle hydration, the buffer was layered via a syringe onto dry CHS or α-THS in the bottom the X-ray capillary. The capillary was then momentarily centrifuged in a table-top centrifuge to eliminate air bubbles from the lipid water paste. The capillaries were then sealed and allowed to equilibrate for at least 4 h at 5°C. Vigorous hydration was accomplished by vortexing dry CHS or α-THS, buffer, and two glass mixing beads in a test tube. An aliquot was then transferred to an X-ray capillary.

Tissue distribution. Tissue distribution of CHS MLVs and EPC SPLVs were determined by preparing the liposomes in buffer containing ^{51}Cr and removing the unencapsulated radioactivity by

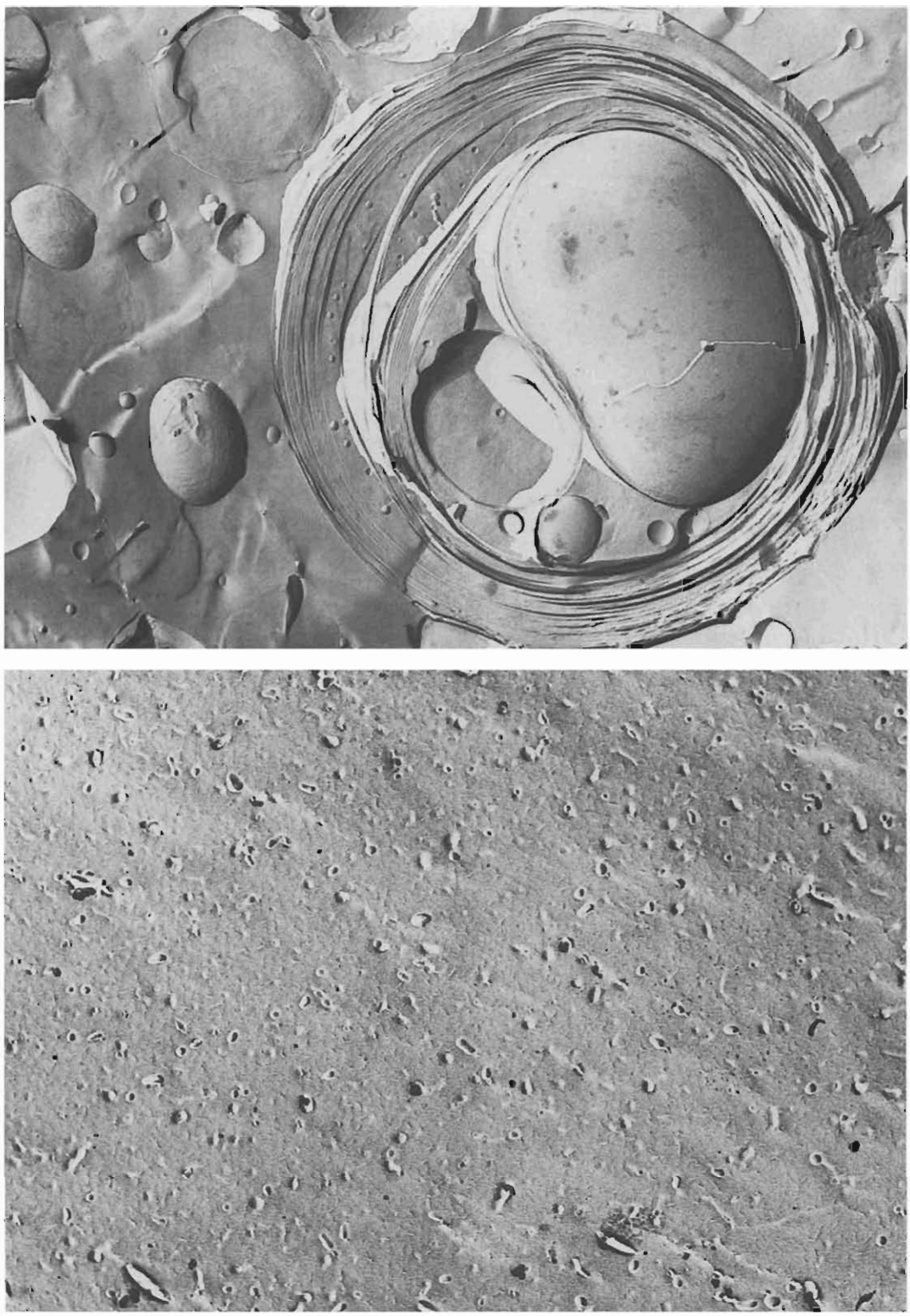

Fig. 1. Freeze-fracture electron micrographs of CHS (left) and α-THS (right) vesicles before (top) and after (bottom) extrusion through 30 nM polycarbonate filters at 100–200 lb/in^2. Bar = 78 nm.

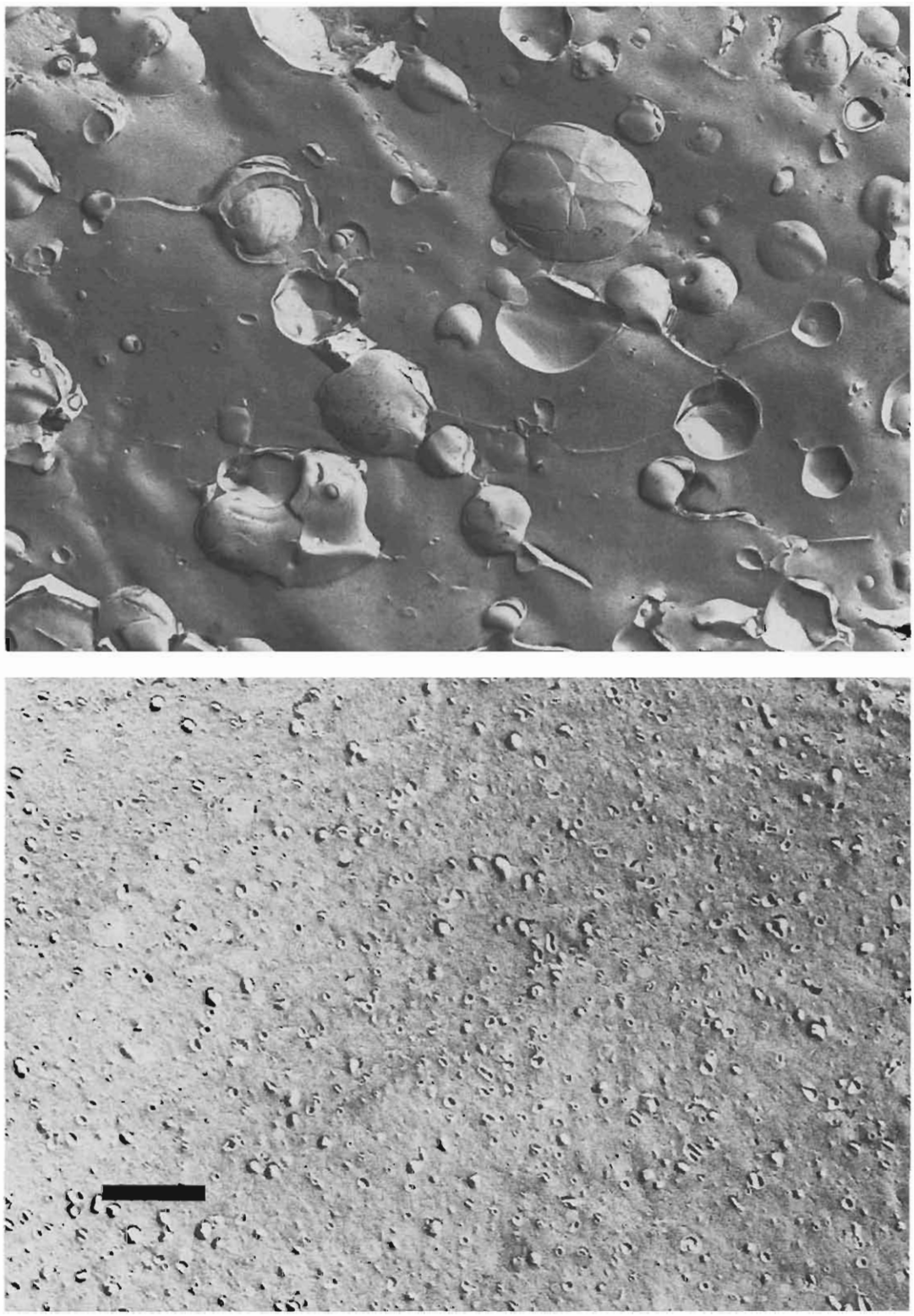

Fig. 1. (continued).

successive washings in isotope free buffer. Restrained, conscious male Swiss Webster mice each received 0.1 ml intravenous injections of either CHS or SPLV encapsulated ^{51}Cr or free ^{51}Cr via the tail vein at 165 μmol lipid/ml. 1, 2, 5 and 24 h post-administration, three mice from each group treated with a liposome preparation and four mice from the group treated with unencapsulated ^{51}Cr were killed by cervical dislocation. The organs were removed, rinsed with 0.9% saline, weighed and assayed for ^{51}Cr.

Results

Upon hydration in buffer, both cholesterol hemisuccinate and α-tocopherol hemisuccinate resulted in milky white suspensions reminiscent of multilayered liposomes. These suspensions cleared appreciably when extruded through stacked 30 nM polycarbonate filters at 100–200 psi or through a French pressure cell at 40 000 lb/in^2. Extensive sonication also resulted in less opaque preparations. Fig. 1 shows freeze-fracture replicas of the hydrated salts both before and after passage through polycarbonate filters. Clearly, both CHS and THS resulted in multilamellar vesicles, although α-THS systems seemed to cross-fracture less frequently than CHS systems. On extrusion through the polycarbonate filters, both CHS and α-THS preparations were converted to relatively homogeneous unilamellar systems with average diameters of approx. 0.065 μm. The mean diameter of the pre-extruded ('unsized') vesicles was found to be 0.3 μm (Fig. 2). Vesicles prepared by the French press procedure (see Materials and Methods) were extremely small (average diameter 0.025 μm or less, data not shown).

Fig. 3 shows the trapping efficiencies and captured solute profiles of CHS and THS multilayered vesicles prepared by hydrating various amounts of material in 5.0 ml of buffer containing ^{51}Cr. Both CHS and α-THS vesicles exhibited trapping efficiencies of about of 10% at low concentrations of membrane forming material. While trapping efficiencies increased linearly with concentration, the solute occluded from buffer per micromole of membrane forming material declined rapidly and reached constant values above 35 μmol/ml in both systems.

The properties of both CHS and α-THS were further explored using electron spin resonance spectroscopy. Vesicles comprised of either molecule were labeled with a series of positional isomers of doxyl stearic acid. The effective order parameter, S^{eff}, was calculated from the resultant ESR spectra by the method of Griffith and Jost [25]. Fig. 4 shows a plot of S^{eff} as a function of the position of the spin label reporter group on the fatty acid chain. S^{eff} decreased in value only at positions close to the terminal methyl end of the

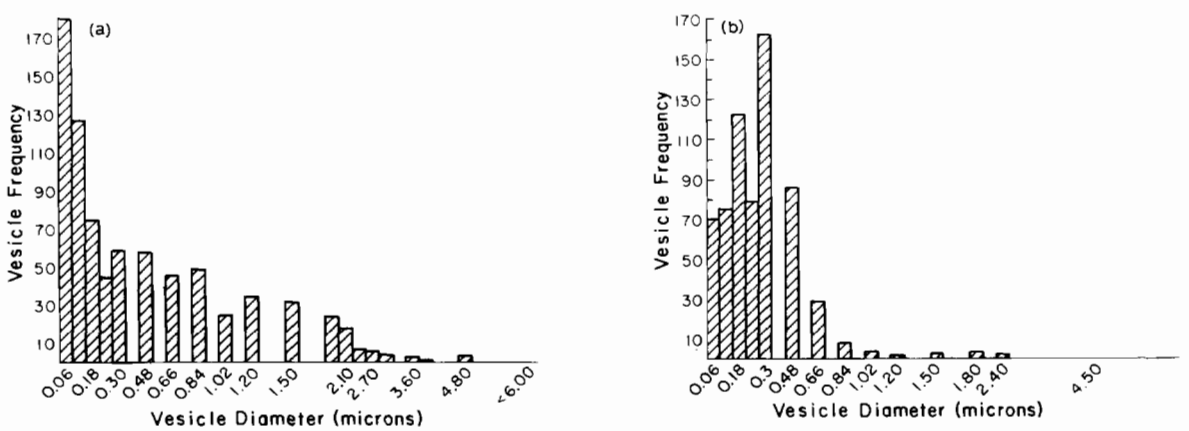

Fig. 2. Size distribution histograms of a random sampling of CHS (a) and α-THS (b) systems hydrated at 13 μmol/ml. Vesicles were not extruded or sonicated. The mean diameter in both systems was 0.3 μm. The ordinate is the frequency of occurrence of vesicles of a given size in arbitrary units.

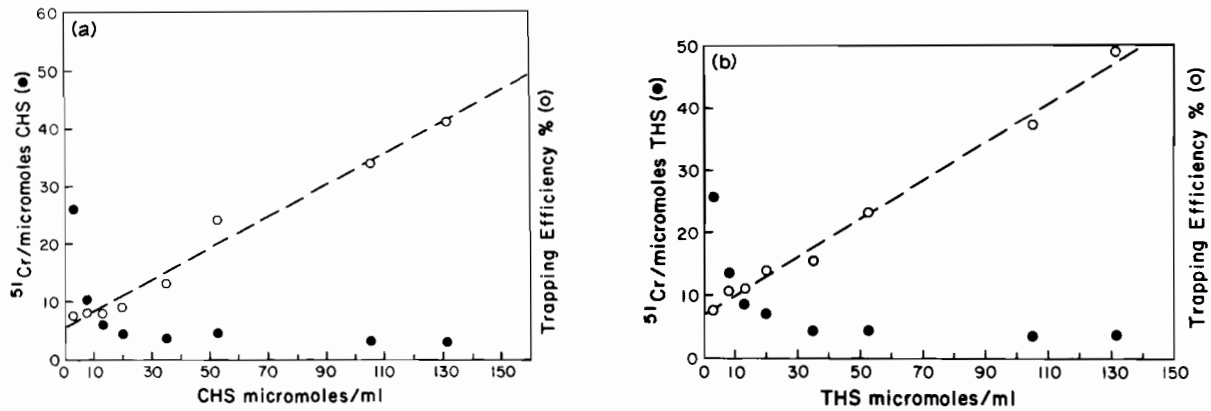

Fig. 3. Captured solute and trapping efficiency profiles of spontaneously formed CHS (a) and THS (b) multilamellar vesicles.

chain. This "flexibility profile" is characteristic of bilayers that are motionally restricted at locations close to the polar apolar interface. Thus, 1:1 mixtures of hydrogenated soy PC and cholesterol result in almost identical flexibility profiles (Janoff and Jablonski, unpublished observations).

The thermotropic behavior of both vesicle types was investigated by DSC. Both CHS and α-THS multilayered systems failed to exhibit detectable endotherms between 12 and 90 °C, consistent with what has been reported for CHS and THS mixed PC systems [17].

The osmotic properties of CHS and α-THS multilayered vesicles are shown in Fig. 5. The osmotic swelling of EPC and EPC:EPA MLVs are shown for comparison. Each vesicle type behaved as an osmometer (i.e., a closed system). CHS and α-THS were, surprisingly, more responsive than phospholipid bilayers to pressure differentials. In order to study this behavior further, we turned to low-angle X-ray diffraction.

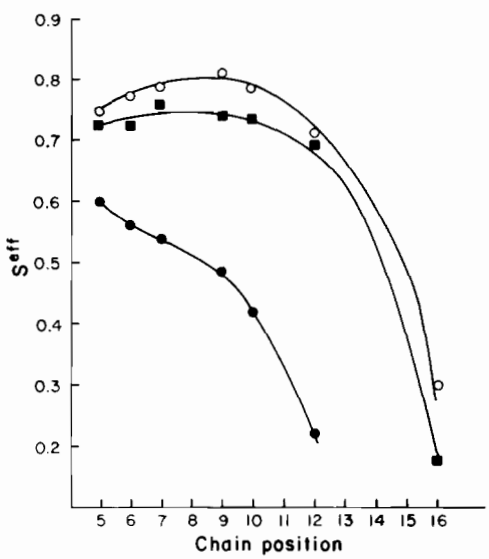

Fig. 4. Flexibility profiles of EPC (●), CHS (○) and α-THS (■) multilayered vesicles. The order parameters were obtained by spin labeling the vesicles with a series of doxylstearic acids where the doxyl reporter group was present at different positions along the fatty acid chain. Spectra obtained showed no evidence of unincorporated label.

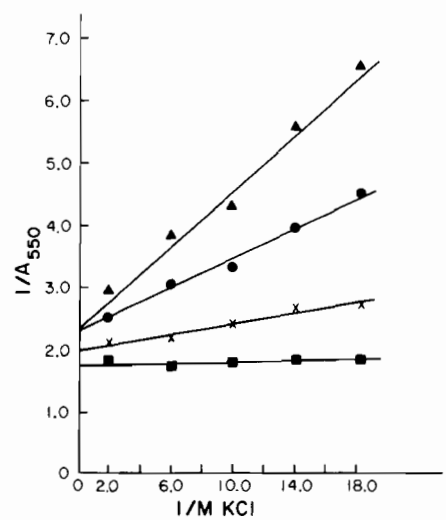

Fig. 5. Isotonic swelling profiles in aqueous KCl of egg PC MLVs (■), egg PC-egg phosphatidic acid (8:2) MLVs (×), CHS MLVs (●) and α-THS MLVs (▲).

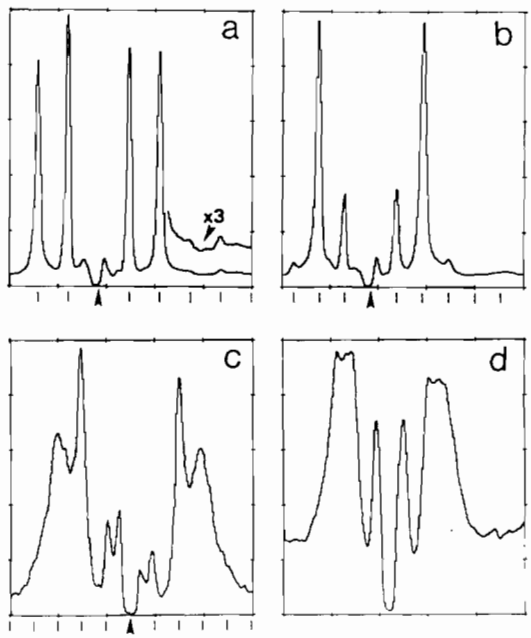

Fig. 6. The small-angle X-ray diffraction intensity (ordinate: arbitrary units) vs. scattering angle (abscissa) for CHS-buffer dispersions. The position of the incident beam (behind the beam stop shadow) is indicated by the arrow near the center of the abscissas. (a) 69% CHS (w/w) exhibits four orders which index well as a lamellar structure with a 68 Å repeat. The expected positions of the orders are indicated by thick marks below the graph. Likewise, 59% CHS (b) yields lamellar diffraction with a 80 Å repeat. However, when buffer is gently added to an overall CHS concentration of 20%, there is a clearly visible pool of excess water on top of the lipid. The resulting diffraction (c) does not index well as a single lamellar lattice. The tick marks indicate the expected positions for a 86 Å lamellar lattice. If, instead, a 20% CHS sample is prepared by vortexing, the diffraction is broad (d). Note that on an absolute intensity scale (not shown), patterns (a) and (b) are strong, pattern (c) is weak and pattern (d) is very weak. $T = 30\,^{\circ}C$.

X-ray diffraction confirmed that both hydrated CHS and α-THS formed multilamellar structures. Fig. 6a and b show the low angle diffraction which resulted from gently hydrated CHS specimens (see Materials and Methods) which were 69 and 59% CHS (by weight). Up to four equally spaced orders of diffraction were visible, consistent with multilamellar arrays of 68 Å and 80 Å, repeats, respectively. The orders were sharp and well-resolved, indicating that the lattice contained very little disorder. These concentrated CHS specimens were of a uniform paste-like appearance with no visible excess buffer. At very high aqueous concentrations, gently hydrated CHS specimens exhibited a clearly visible pool of excess buffer solution on top of the hydrated lipid. The diffraction from such a sample (20% CHS by total weight) is shown in Fig. 6c. Note the broadening of the higher angle diffraction peaks, indicative of considerable disorder in the lattice. The disorder in the lattice made a definitive lattice assignment difficult, but if a lamellar fit was made, the repeat was about 86 Å, suggestive of large aqueous spaces between the lipid layers. If, instead of using gentle hydration, a 21% CHS specimen was prepared via vortexing of the dry lipid with the buffer, as shown in Fig. 6d, the low-angle diffraction exhibited a broad band of scatter with little evidence of a sharply defined lattice. This diffraction signature would be expected from a multilamellar system in which the interlamellar aqueous widths varied widely.

Qualitatively similar behavior was observed with α-THS. When gently hydrated, relatively

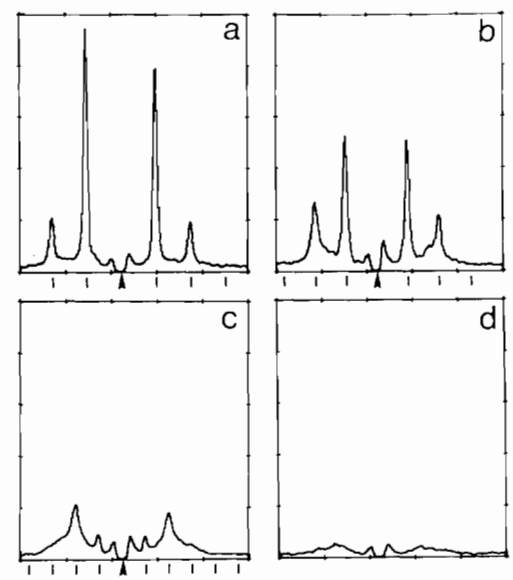

Fig. 7. The small-angle X-ray diffraction from α-THS-buffer dispersions are shown at various concentrations and mixing conditions. The axes and the tick marks are as in Fig. 6, except that the ordinates are to the same arbitrary scale to indicate the relative strengths of the diffraction. (a) Gently hydrated, 70% α-THS by overall weight. Tick marks are for a lamellar spacing, d_L, of 56 Å. (b) Gently hydrated, 59% α-THS, $d_L = 62$ Å. (c) Gently hydrated, 20% α-THS, $d_L = 84$ Å. (d) 20% α-THS prepared by vortexing. Temperature, 25 °C for (a)–(d).

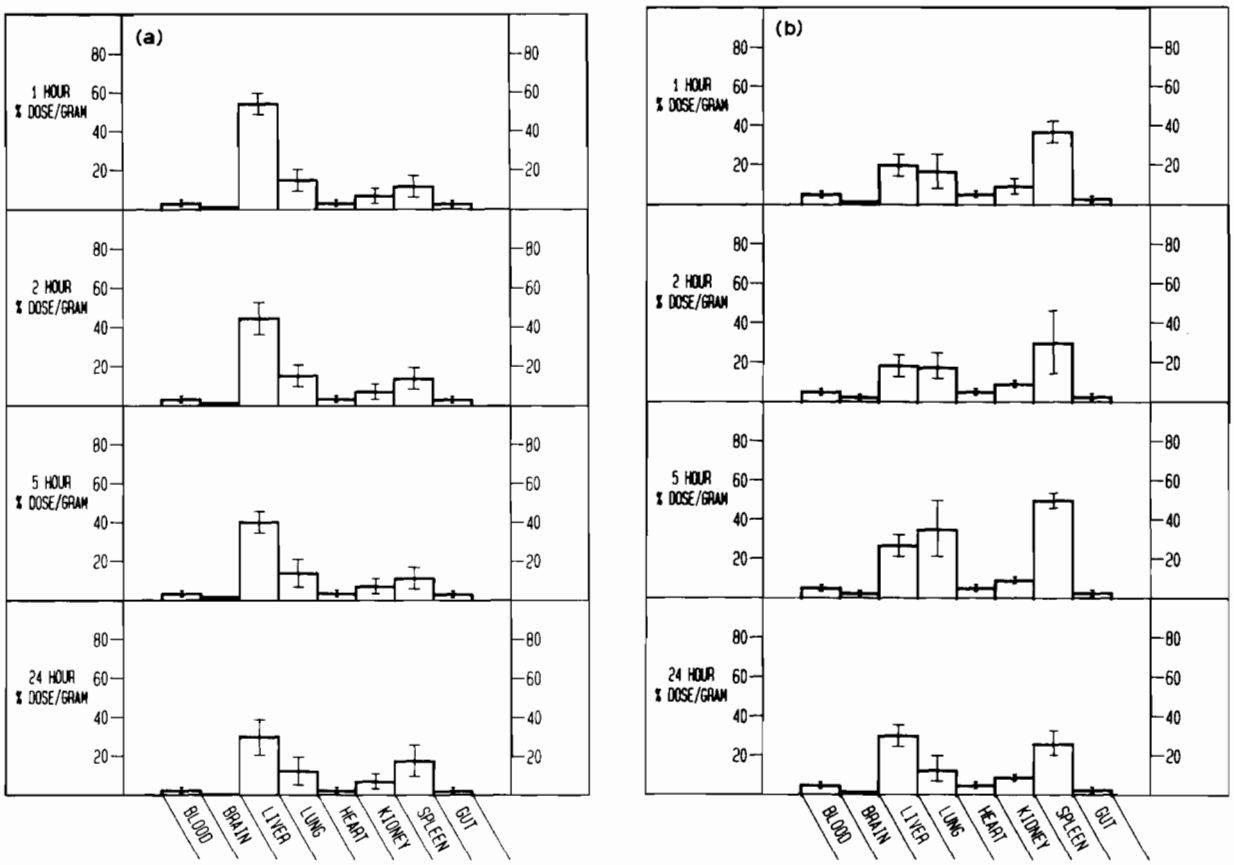

Fig. 8. Mouse tissue distribution of ^{51}Cr-labeled CHS-MLVs (a) and EPC-SPLVs (b) 1, 2, 5 and 24 h after intravenous administration.

sharp, uniformly spaced peaks were observed at α-THS concentrations of 70, 59 and 20% by total weight (Fig. 7a–c). This is consistent with lamellar lattices. However, if a 20% α-THS specimen was prepared by vortexing, only weak, broad X-ray scatter was seen.

Finally, in order to determine whether CHS vesicles remained intact when administered in vivo, the organ distribution of CHS entrapped ^{51}Cr was compared to that of free ^{51}Cr after intravenous injection in mice. The organ distribution of egg PC entrapped ^{51}Cr was determined for comparison. Unentrapped chromium was excreted rapidly and did not concentrate in any of the organs assayed (data not shown). As shown in Fig. 8, both CHS and EPC encapsulated chromium remained at measurable levels 24 h after injection. EPC vesicles accumulated primarily in the spleen and to a lesser extent into the liver and lung following a typical pattern of distribution [29]. On the other hand, CHS vesicles prepared at equimolar concentrations accumulated to a much greater extent in the liver.

Discussion

We have shown that CHS and α-THS are capable of forming liposomes of multi or unilamellar character. Such vesicles form easily and do not require the use of organic solvents during preparation. Multilamellar systems can be prepared such that encapsulation efficiencies approach 50%. These values are almost a factor of 10 greater than those that can be achieved by dispersing similar amounts of EPC in buffer. While encapsulation efficiencies of PC vesicles can be enhanced by freeze thaw cycling or by incorporating charged

lipid into the bilayer, such manipulations are largely effective because they yield wide interlamellar aqueous spacings [30], a situation that spontaneously arises, as we have shown in CHS and α-THS systems.

Multilamellar α-THS vesicles yielded quite different size histograms than did CHS systems. Although the mean diameter for both vesicle types was about 0.3 μm, α-THS systems were more narrowly distributed around this value. By comparison, MLVs and SPLVs produced from PC have been reported to have a mean diameter almost double the value of those reported here [23].

Interestingly, both CHS and α-THS multilamellar systems were less effective in capturing ^{51}Cr per μmol of membrane-forming material as concentration was increased. Such concentration-dependent phenomena have been described for phospholipid systems [31] and have been attributed to a nonspecific adsorption of the solute to exposed sites on the bilayer. In our hands, adherence of ^{51}Cr to the surface of the vesicles could not explain this phenomenon. Further, size histograms indicated little dependence of vesicle size on concentration. The possibility that CHS and α-THS systems exclude solute [23] and that this exclusion is enhanced at higher concentrations is not unlikely given the nature of multilamellar systems and is currently being explored.

Flexibility profiles of CHS and α-THS dispersions were quite different than those typically obtained for PC systems [32]. Unlike PC, CHS and α-THS were highly restrictive at spin-label positions C5–C10. In fact spectra of these environments being near to the limit of motional sensitivity, displayed only a small dependence on spin label position. Comparison of the two systems, however, suggested that α-THS provided a generally less restrictive environment for the stearic acid probe than did CHS.

Isotonic swelling profiles revealed that both CHS and α-THS behaved as osmometers, remained intact over a large range of pressure differentials, and in fact seemed more elastic than egg PC systems. This is in contrast to liposomes made of other sterol esters such as cholesteryl sulfate *o*-methoxyethoxyethoxyethylcholesterol and cholesterol phosphocholine, which are less elastic than phospholipid liposomes and collapse or leak at lower pressure differentials [13]. It must be noted, regarding the CHS system, that occasionally nonideal isotonic swelling was observed. These results have been reported previously [15,16] and most likely resulted from inhomogenities during vesicle hydration. In fact, such nonideal behavior was also observed if CHS was first hydrated to a paste prior to further dilutions (data not shown). If care was taken to ensure gentle, adequate and uniform hydration, however, the ideal response (which is shown in Fig. 6) was consistently obtained.

The great osmotic responsiveness seen in CHS and α-THS vesicles most likely arises because of the large aqueous spaces sequestered by these systems. In fact, the X-ray diffraction and freeze fracture replicas of dilute CHS and α-THS dispersions are most simply interpreted as arising from multilamellar systems in which the interlamellar forces are weak. For other lipid systems, such as dilute egg phosphatidylcholine dispersions, X-ray diffraction indicates a sharply defined lamellar lattice which is, by weight, mostly lipid. This well-defined lattice repeat is a result of a relatively sharp minimum in the lattice potential as a function of the lipid layer separation. If the potential vs. distance curve has only a shallow well, then one would expect weak interlamellar forces and considerable lattice disorder. This is the case, for example, for phosphatidylcholine bilayers containing a small fraction of charged lipid [33]. Similar behavior was exhibited by CHS and α-THS. In fact, the way these liposomes were hydrated affected the final distribution of aqueous widths. Gently hydrated specimens formed different structures than did vigorously hydrated samples.

Finally it must be mentioned that the enhanced accumulation of CHS liposomes into the liver cannot be explained because of the smaller size of these systems compared to PC systems. Size has been shown to have little impact in this regard [34]. CHS liposomes, however, might be expected to differ in several important rheological characteristics from phospholipid systems resulting in altered biodistribution. A remaining possibility concerns the fact that cholesterol is metabolized in the liver. Thus the introduction of cholesterol liposomes to this milieu could result in biochemically enhanced uptake.

Acknowledgements

We thank Peter So for assistance in acquiring the X-ray data and R. Mendelsohn for acquiring the DSC data. S.M.G. is supported by NIH grant GM32614 and DOE grant DE-FG02-87ER60522.A000.

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