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PREPARATION OF UNILAMELLAR LIPOSOMES OF INTERMEDIATE SIZE (0.1—0.2 μm) BY A COMBINATION OF REVERSE PHASE EVAPORATION AND EXTRUSION THROUGH POLYCARBONATE MEMBRANES

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Summary

Liposomes can be prepared by a combination of reverse phase evaporation and sequential extrusion through polycarbonate membranes. The vesicles have diameters in the range 0.05—0.5 μm and are mostly unilamellar as indicated by electron microscopy, capture volume, and availability of reactive groups to periodate oxidation. Sequential extrusion leads to a decrease in the encapsulation efficiency by 2—4-fold, depending upon the lipid composition. The inclusion of cholesterol at a 1 : 1 molar ratio of cholesterol-to-phospholipid increases both the mean size and the size heterogeneity of the liposomes as measured by negative-stain electron microscopy. The mean size of vesicles with an equal molar ratio of cholesterol-to-phospholipid after extrusion through a 0.1 μm membrane is 0.140 μm. Vesicles composed of phosphatidylglycerol/phosphatidylcholine (1 : 4) have a mean size of 0.08 μm after extrusion through a 0.1 μm membrane. The intermediate-size (0.1—0.2 μm) vesicles formed by this process have an aqueous space-to-lipid ratio of 3 : 5 and capture between 12 and 25% of the aqueous phase. The procedure is relatively simple, rapid, and yields almost quantitative recovery of vesicles that encapsulate a large percentage of the total aqueous space.

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Abbreviation: SDS, sodium dodecyl sulfate.

Introduction

The preparation of liposomes of defined and variable size is an important objective for the continued development of liposomes as drug carriers and model membranes. The original preparation of liposomes produces multilamellar vesicles of varying size with diameters in the range 0.2—5 μm [1,2]. Sonication of such preparations produces small unilamellar vesicles [3] which are very homogeneous in size distribution [4] and have been used extensively as a model membrane system [5,6]. However, their relatively small enclosed aqueous space produces serious limitations for their potential use as a drug carrier. Methods using detergent dialysis [7] and ether injection [8] have been described that produce vesicles of intermediate size, but these preparations have low efficiency of entrapment of aqueous space (for review, see Ref. 2). A recent method producing vesicles with very high efficiency of encapsulation [9] yields a vesicle-size distribution of the order of 0.4 μm. There is a need, therefore, for methods of liposome preparation which produce vesicles of intermediate size (0.05—0.2 μm) with relatively high efficiency of entrapment.

We have previously demonstrated that liposome preparations extruded through polycarbonate membranes contain smaller vesicles of a more homogeneous size distribution [9,10]. In this report, we describe in detail the conditions for a convenient and reproducible preparation of intermediate-size unilamellar liposomes by the use of reverse phase evaporation followed by extrusion through polycarbonate membranes.

Materials and Methods

Lipids and other reagents

Cholesterol was purchase from Fluka, A.B. (Buch, Switzerland). Phosphatidylcholine was purified from egg yolk and phosphatidylglycerol was synthesized from egg phosphatidylcholine by Mr. Thomas Isac as described previously [11]. All above lipids were finally purified by high-pressure liquid chromatography [12], shown to be pure by thin-layer chromatography and stored in $CHCl_3$ in sealed ampules under N_2 at −50°C until use. [^{14}C]Cholesterol was obtained from New England Nuclear, Boston, MA. [5-^{3}H]Cytosine β-D-arabinoside was purchase form Amersham-Searle, Arlington Heights, IL. All solvents were obtained from Fisher Scientific. Electron microscopy grids 400 mesh (3 mm) composed of copper and rhodium made by Graticules, Ld., were obtained from E.F. Fullman, Inc. (Schenectady, NY). Polycarbonate membranes and membrane holders were obtained from Nucleopore, Inc., Pleasanton, CA. Bacitracin was purchased from Sigma, St. Louis, MO. Ultrafiltration cells were obtained from Millipore Corp., Bedford, MA. Silicon monoxide and latex beads (0.357 μm) were purchased form Polyscience, Warrington, PA. Phosphate-buffered saline contained 137 mM NaCl, 2.6 mM KCl, 6.4 mM Na_2HPO_4, 1.4 mM KH_2PO_4 (pH 7.4). Water was twice distilled through an all-glass apparatus. All other chemicals were of reagent grade.

Preparation and extrusion of liposomes through membranes

Liposomes were prepared by the reverse phase evaporation technique [9].

The lipid mixture (60 μmol total lipid) in chloroform solution were deposited on the sides of a round-bottom flask by removal of the organic solvent by rotary evaporation (water aspirator). The lipid was then redissolved in 3 ml of diethyl ehter which had been redistilled in the presence of $NaHSO_3$ immediately prior to use. 1 ml of an aqueous soluiton of cytosine β-D-arabinoside (74 mg/ml) in a 1/10 dilution of phosphate-buffered saline was added to the solution of phospholipids and the mixture was sonicated in a bath-type sonicator (Lab. Supplies, Hicksville, NY), under N_2 at 20° C for 5 min. The mixture was then placed on a rotary evaporator and the organic solvent was removed under vacuum (water aspirator) in two stages: evaporation at 400 mmHg until the suspension became a gel, followed by brief vortex mixing and then continued evaporation at 730 mmHg until a homogeneous suspension was obtained. The preparation was either diluted to 12 μmol lipid/ml with the original aqueous buffer and extruded through the polycarbonate membrane in a Nucleopore 24 mm holder or extruded at 60 μmol/ml in the Millipore 25 mm ultrafiltration cell in which the stainless-steel screen was replaced with a polyester drain disc (Bio-Rad, Richmond, CA). The latter modification is necessary to achieve reproducible extrusions without clogging the membrane. The first extrusion was through a 0.4 μm membrane. The process was repeated through 0.2, 0.1 and 0.08 μm membranes. When the ultrafiltration cell was used, N_2 at pressures from 40 to 100 lb/inch^2 was applied to extrude liposomes through the membranes.

After each extrusion, an aliquot of the liposome preparation was dialyzed at 4° C against 100 vols. of phosphate-buffered saline changed five times within a 24 h period. The lipid concentration was determined with [^{14}C]cholesterol and the entrapped cytosine β-D-arabinoside with [5-^{3}H]cytosine β-D-arabinoside by liquid scintillation counting [10]. The size distribution of the liposome preparation was determined by negative-stain electron microscopy following a 1 h dialysis against 100 vols. of 0.15 M ammonium acetate/0.5 mM EDTA (pH 7.0).

Electron microscopy and determination of size distribution of liposomes

Negative-stain electron micrographs were prepared as previously described [10]. Carbon-coated grids were covered with a 0.1 mg/ml solution of bacitracin and blotted dry. Liposomes at a concentration of 0.5—3 μmol lipid/ml were applied onto the grid and drawn off with filter paper. A drop of 2% (w/v) $(NH_4)_2MoO_4$ solution (prefiltered through a 0.1 μm membrane) was immediately applied to the grid, drawn off with a piece of filter paper and allowed to dry for at least 30 min, but never longer than 6 h before being examined. A Siemens 101 electron microscope, at 80 kV, was used. All photographs were printed at 2.5 × the negative enlargements. The diameters of individual vesicles were measured with a caliper and assigned to a specific size interval. Twice the surface area of the disc was assumed to represent the equivalent surface area of a collapsed sphere; the diameter of the disc times 0.707 is then equal to the diameter of the equivalent sphere. The number of vesicles in each interval was determined, multiplied by the equivalent volume for that interval, and then summed to obtain the total apparent encapsulated volume. The fraction of the volume encapsulated by vesicles in each interval was then

plotted against the vesicle diameter. For each extruded fraction at least 200 vesicles were measured in three separate experiments, or when only one preparation was measured at least 500 vesicles were measured for each extruded fraction. In some experiments, 0.357 μm diameter latex particles were included as an internal calibration control.

The freeze-fracture electron micrographs of the vesicle preparations were performed as previously described [10].

The size distribution of the unextruded vesicles and vesicles extruded through the 0.1 and 0.08 μm membranes was also examined by negative-stain electron microscopy of vesicles spread on silicon monoxide supports and stained with 2% $(NH_4)_2MoO_4$ as described by Larrabee et al. [13].

Oxidation of phosphatidylglycerol-containing vesicles by periodate

We have used periodate because of its ability to oxidize vicinal hydroxyl groups under mild conditions, releasing 1 mol HCHO/mol available phosphatidylglycerol [14]. Vesicles were prepared from 10 μmol phosphatidylglycerol or 20 μmol phosphatidylglycerol/cholesterol (1 : 1). The lipid was resuspended in 1 ml redistilled diethyl ether, and 0.3 ml of 10 mM acetate, 60 mM NaCl (pH 5.5) was added prior to sonication. All manipulations were performed in 12 × 120 mm screw-capped culture tubes. Solvent evaporation was performed by placing the tube inside a larger boiling tube which could be connected to a rotary evaporator. The larger tube contained a small amount of water to aid thermal contact between the inner tube and the water bath.

The vesicles were diluted to approx. 10 μmol phosphatidylglycerol per ml with buffer and then to 5 μmol phosphatidylglycerol per ml with either buffer or 20% (w/v) sodium dodecyl sulfate (SDS). 1 ml of the vesicles was then mixed with 1 ml of 10 mM acetate, 60 mM $NaIO_4$ (pH 5.5.) to start the reaction. Tubes were kept in the dark and duplicate 50-μl aliquots were removed at intervals and mixed with 50 μl of 0.5 M $Na_2S_2O_5$ to stop the reaction. The HCHO released by periodate oxidation was measured with chromotropic acid [15]. 2 ml of chromotropic acid reagent were added to each sample, the tubes capped and placed in a boiling-water bath for 30 min. Samples were cooled and mixed with 0.2 ml of 12% (w/v) thiourea. Centrifugation at 600 × *g* for 10 min separated out an oily interfering material and the clear purple solution was removed from underneath and read at 570 nm. The measurement was standardized with periodate-oxidized samples of ethylene glycol [16].

The extent of oxidation was estimated either by comparing the number of moles of HCHO released to the phosphorus content or by comparing HCHO content in the presence and absence of SDS. Where both methods were applicable, they agreed to within 3%.

Results

Reverse phase evaporation vesicles can be reduced in diameter by sequential extrusion through polycarbonate membranes. As shown in Table I, extrusion produces a decrease in the mean diameter and a more homogeneous distribution of vesicles as measured by the size range that encompasses 12.5—87.5%

TABLE I

SIZE OF REVERSE PHASE EVAPORATION LIPOSOMES FOLLOWING SEQUENTIAL EXTRUSION AS MEASURED BY NEGATIVE-STAIN ELECTRON MICROSCOPY

Vesicles of the indicated composition were extruded as detailed in Materials and Methods through the indicated polycarbonate membranes. Mean vesicle diameter, in μm, as detailed in Materials and Methods. Values from three different preparations agreed to within 10% and were pooled to obtain the values in this table. 75% volume limit is the size range in μm that entails 12.5—87.5% of the vesicle volume. n = number of vesicle profiles measured. Vesicle diameter in μm calculated as previously described [9] where diameter = 12 captured volume/(surface area of lipid): for PG/PC/chol (1:4:5); 1 mol lipid = $2.57 \cdot 10^{25}$ Å^2; PG/PC (1:4) and PG, 1 mol lipid = $4.3 \cdot 10^{25}$ Å^2. PG, phosphatidylglycerol; PC, phosphatidylcholine; chol, cholesterol.

Polycarbonate membrane	Mean vesicle diameter	75% volume limits	n	Calculated vesicle diameter
PG/PC/chol (1:4:5)				
none	0.50	0.15—0.87	986	0.38
0.4	0.32	0.13—0.60	846	0.26
0.2	0.19	0.09—0.45	1771	0.17
0.1	0.14	0.08—0.25	1431	0.12
PG/PG (1:4)				
none	0.16	0.07—0.24	1624	0.16
0.2	0.11	0.06—0.17	1458	0.13
0.1	0.08	0.06—0.13	1848	0.11
0.08	0.07	0.05—0.09	583	0.09
PG				
none	0.19	0.09—0.31	887	0.17
0.2	0.11	0.06—0.21	466	0.13
0.1	0.08	0.05—0.15	1171	0.12
0.08	0.07	0.04—0.11	1522	0.11

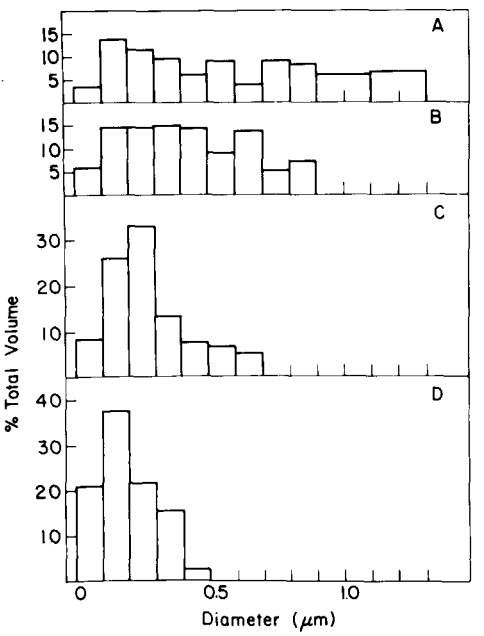

Fig. 1. Size distribution determined by negative-stain electron microscopy of phosphatidylglycerol/phosphatidylcholine/cholesterol (1 : 4 : 5) liposomes. Vesicles prepared as detailed in Materials and Methods. The number of vesicle profiles measured is given in Table I. A, unextruded; B, extruded through 0.4 μm membrane; C, extruded through 0.2 μm membrane; D, extruded through 0.1 μm membrane.

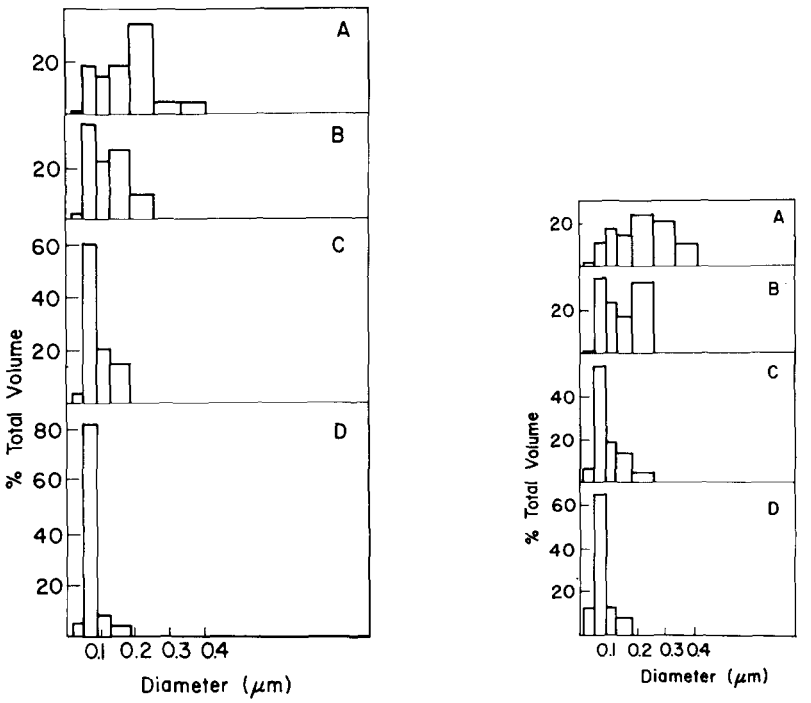

Fig. 2. Size distribution determined by negative-stain electron microscopy of phosphatidylglycerol/phosphatidylcholine (1 : 4) liposomes. Vesicles prepared as detailed in Materials and Methods. The number of vesicle profiles measured is given in Table I. A, unextruded; B, extruded through 0.2 μm membrane; C, extruded through 0.1 μm membrane; D, extruded through 0.08 μm membrane.

Fig. 3. Size distribution determined by negative-stain electron microscopy of phosphatidylglycerol liposomes. Vesicles prepared as detailed in Materials and Methods, A, unextruded; B, extruded through 0.2 μm membrane; C, extruded through 0.1 μm membrane; D, extruded through 0.08 μm membrane.

of the encapsulated volume. Phosphatidylglycerol/phosphatidylcholine/cholesterol (1 : 4 : 5) vesicles have the largest mean diameter (0.5 μm) and exhibit the greatest heterogeneity of the lipid compositions examined (Fig. 1). After extrusion through the 0.1 μm membrane, the mean diameter was reduced by 3-fold to 0.14 μm, which approaches the pore diameter of the polycarbonate membrane used for the extrusion.

Preparations without cholesterol form a smaller, more uniform population of liposomes before extrusion (Table I, Figs. 2 and 3), which become relatively homogeneous after sequential extrusion through the 0.08 μm membrane. The mean size and size distribution of phosphatidylglycerol/phosphatidylcholine (1 : 4) vesicles and phosphatidylglycerol vesicles become remarkably similar after the first extrusion through the 0.2 μm membrane (Table I, Figs. 2 and 3) forming a population of vesicles of about 0.1 μm diameter.

Examination of 200 of the phosphatidylglycerol/phosphatidylcholine/cholesterol (1 : 4 : 5) vesicles following extrusion through the 0.1 μm membrane by freeze-fracture electron microscopy indicated a mean diameter of 0.15 μm. Examination of 200 of the phosphatidylglycerol/phosphatidylcholine (1 : 4) vesicles by freeze-fracture after extrusion through the 0.08 μm mem-

brane indicated a mean diameter of 0.095 μm.

As an additional confirmation of the values obtained by negative-stain electron microscopy on carbon-coated grids, we used negative-stain electron microscopy on silicon monoxide supports [13]. The value obtained for the phosphatidylglycerol/phosphatidylcholine/cholesterol vesicles extruded through the 0.1 μm membrane was 0.14 μm. The mean diameter obtained for the phosphatidylglycerol/phosphatidylcholine vesicles was 0.14 μm before extrusion and 0.10 μm after extrusion through the 0.08 μm membrane. Representative micrographs of these vesicles on silicon monoxide supports are shown in Fig. 4.

As the mean size of the vesicle preparation was reduced, the encapsulation efficiency, i.e., the percentage of the initial aqueous phase that becomes entrapped in liposomes also was reduced. The phosphatidylglycerol/phosphatidylcholine/cholesterol vesicles show and encapsulation efficiency of 48% before extrusion, which decreases to 12% after the final extrusion through the 0.1 μm membrane. The capture volume, L/M (in l aqueous space per mol lipid), which is a measure of the amount of aqueous space per mole of lipid, is initially 8.2 L/M and decreases to 2.7 L/M after the final extrusion (Table II). To verify that this loss of aqueous space is due to a reduction of the vesicle size and not to rupture of the bilayer and dilution of the entrapped cytosine β-D-arabinoside by the phosphate-buffered saline used to dilute the preparation prior to extrusion, phosphatidylglycerol/phosphatidylcholine/cholesterol vesicles were extruded at 60 μmol lipid/ml without dilution. This preparation had an encapsulation efficiency of 41.7%, that decreased to 25.2% after the first extrusion, to 19.0% after the second extrusion, and to 15.0% after the final extrusion through the 0.1 μm membrane. The capture volumes were 9.0, 4.8, 4.0, and 3.1 L/M, respectively. These compare quite favorably with the values for the diluted preparations (Table II) and confirm that the extrusion process most probably brings about a decrease in vesicle size without dilution of the vesicle contents.

Vesicles made without cholesterol are initially smaller in size (Table I), and have a smaller capture volume (Table II). Following extrusion, all three preparations have similar capture volumes and encapsulation efficiencies (Table II). As one might also expect from their smaller size, vesicles lacking cholesterol extrude at a much lower N_2 pressure through the 0.2 and 0.1 μm polycarbonate membranes.

The recovery of lipid following multiple extrusions was between 80 and 90% for all of the preparations (Table II). The loss of lipid was primarily due to losses in the dead space of the membrane holders, although some lipids remain associated on the polycarbonate membranes.

Calculations of the expected diameter of the resulting vesicles based on the capture volume and the total surface area of the lipid can be made by assuming that the vesicles are unilamellar and form a uniform population of spherical vesicles [9]. For the phosphatidylglycerol/phosphatidylcholine/cholesterol composition we used a molecular area of 72 $Å^2$ for the phosphatidylglycerol [6], 55 $Å^2$ for the phosphatidylcholine [17], and 28 $Å^2$ for the cholesterol [17]. For the other two compositions we used a molecular area of 72 $Å^2$. The calculated diameter of the unextruded phosphatidylglycerol/phospha-

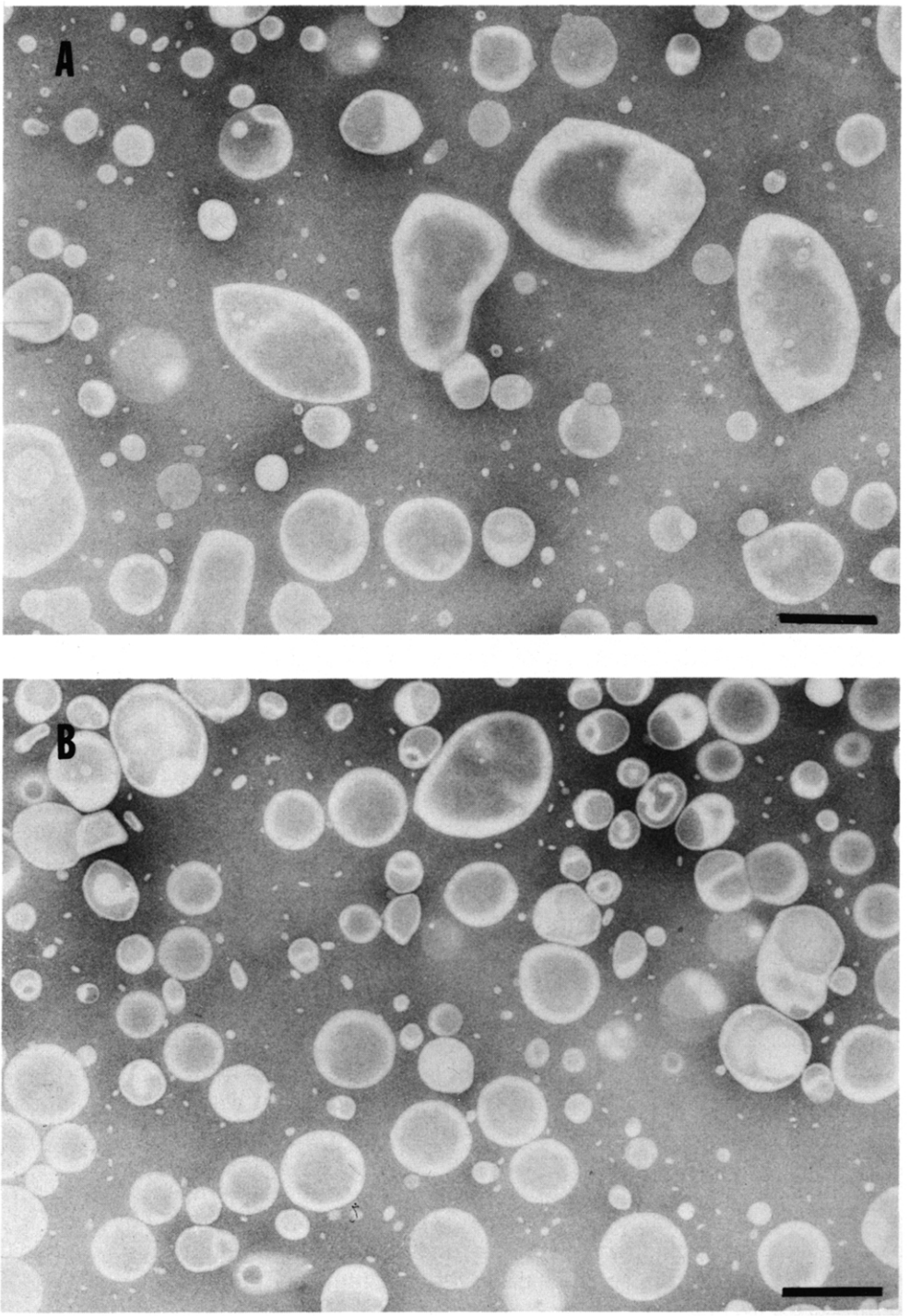

Fig. 4. Negative-stain electron micrographs of extruded liposomes. A, unextruded phosphatidylglycerol/phosphatidylcholine/cholesterol (1 : 4 : 5), Bar = 0.4 μm. B, phosphatidylglycerol/phosphatidlylcholine/cholesterol extruded through 0.1 μm membrane, bar = 0.2 μm. C, unextruded phosphatidylglycerol/phosphatidylcholine (1 : 4), bar = 0.2 μm. D, phosphatidylglycerol/phosphatidylcholine extruded through 0.08 μm membrane, bar = 0.2 μm.

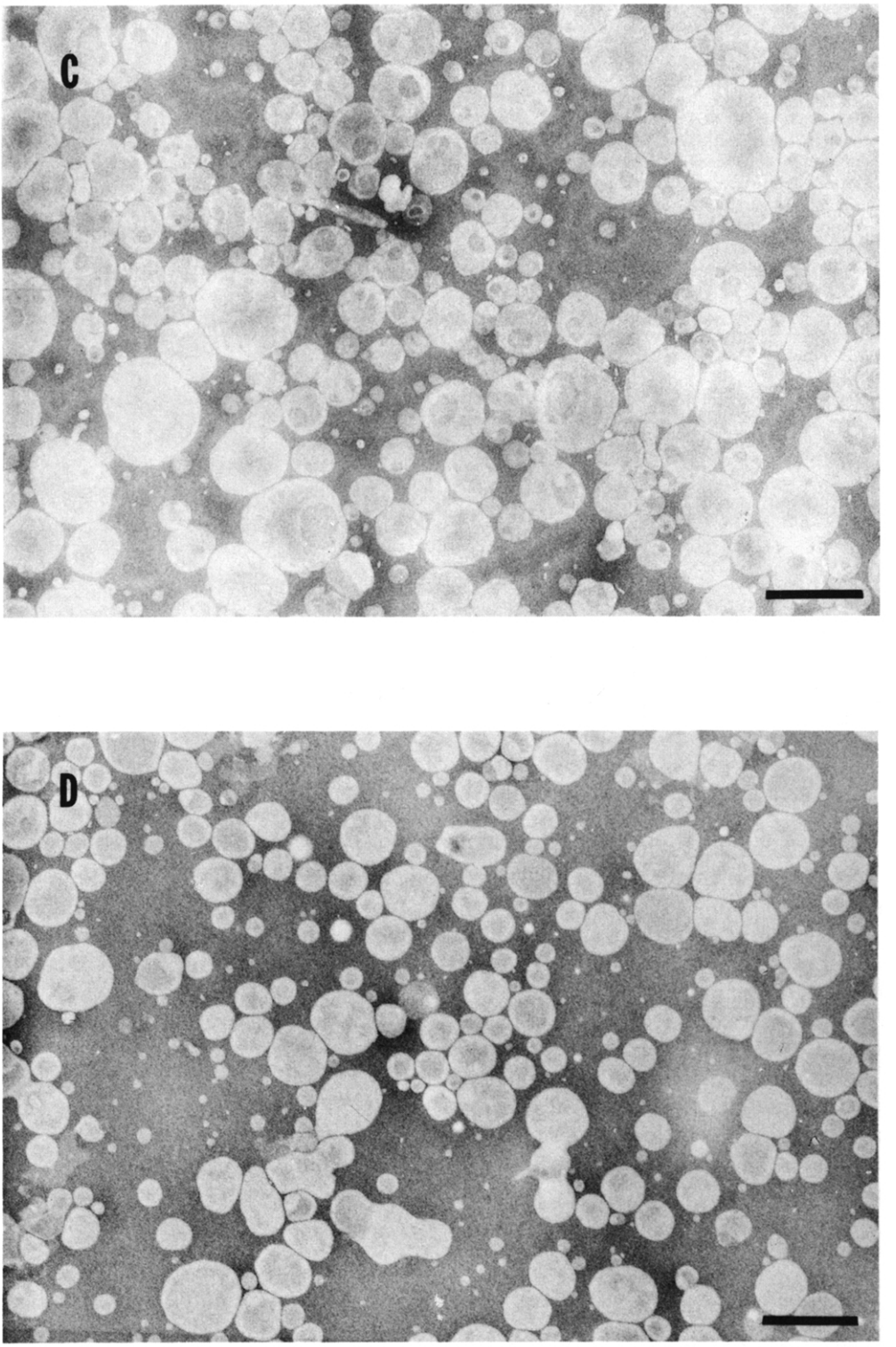

Fig. 4C, D.

TABLE II

ENCAPSULATION EFFICIENCY AND CAPTURE VOLUME OF REVERSE PHASE EVAPORATION LIPOSOMES FOLLOWING EXTRUSION

Vesicles were prepared at a lipid concentration of 60 μmol/ml and diluted to 12 μmol/ml in phosphate-buffered saline prior to extrusion. The values presented are the mean ± the S.D. for three or four separate experiments. Pore size of polycarbonate membranes used for extrusion expressed in μm. Encapsulation efficiency expressed as percentage of initial cytosine β-D-arabinoside that becomes vesicles associated. Each value of % recovery of lipid is compared to the lipid concentration in the unextruded preparation. Capture volume in aqueous space per mol lipid assuming cytosine β-D-arabinoside is distributed only in the aqueous space of the liposomes and calculated on the basis of the cytosine β-D-arabinoside and lipid recovered in each step of the preparation. PG, phosphatidylglycerol; PC, phosphatidylcholine; chol, cholesterol.

Liposome composition	Polycarbonate membrane	Encapsulation efficiency	% recovery of lipid	Capture volume (L/M)
PG/PC/chol	none	47.8 ± 3.7	—	8.2 ± 0.84
(1:4:5)	0.4	29.9 ± 5.1	90.8 ± 0.58	5.6 ± 0.93
	0.2	17.4 ± 2.8	82.3 ± 5.8	3.7 ± 0.38
	0.1	12.1 ± 1.6	77.3 ± 6.8	2.7 ± 0.05
PG/PC (1:4)	none	34.8 ± 0.88	—	5.8 ± 0.14
	0.2	24.3 ± 4.6	91.1 ± 4.9	4.6 ± 0.86
	0.1	18.7 ± 5.4	83.0 ± 6.3	3.9 ± 0.92
	0.08	16.0 ± 5.6	78.5 ± 4.4	3.4 ± 1.06
PG	none	36.3 ± 2.4	—	6.3 ± 0.70
	0.2	25.3 ± 6.6	93.2 ± 7.1	4.6 ± 1.01
	0.1	23.8 ± 3.9	90.5 ± 3.5	4.4 ± 0.53
	0.08	21.2 ± 3.6	90.7 ± 5.8	4.0 ± 0.45

tidylcholine/cholesterol vesicles is 0.383 μm (Table I, column 5) which compares reasonably well with the mean diameter of 0.5 μm observed by electron microscopy (Table I). After extrusion of the vesicles, the comparison between the calculated and observed values becomes very good (0.126 μm calculated versus 0.140 μm observed for the 0.1 μm extrusion). For the phosphatidylglycerol/phosphatidylcholine and phosphatidylglycerol liposomes the comparisons between the calculated 0.16 μm versus observed 0.16 μm (phosphatidylglycerol/phosphatidylcholine) or 0.17 μm calculated versus 0.19 μm observed (phosphatidylglycerol) was excellent. Upon extrusion, the calculated diameter in these two preparations, although in reasonable agreement with the observed diameter, was consistently larger.

The close correspondence of the calculated and observed vesicle diameter indicates that the large majority of the vesicles produced by this procedure are unilamellar. In order to evaluate directly the extent to which these vesicles are unilamellar, we have determined the amount of phosphatidylglycerol available for oxidation by periodate in both phosphatidylglycerol and phosphatidylglycerol/cholesterol (1 : 1) preparations. Fig. 5 shows the oxidation of phosphatidylglycerol vesicles as measured by the release of HCHO. The reaction proceeds rapidly but stops after 30 min, when only 57% of the lipid is oxidized. No further oxidation occurs for up to 4 h, suggesting that 43% of the phosphatidylglycerol is not available for periodate oxidation, and presumably located in the interior of the vesicle. Oxidation in the presence of SDS proceeds to 100% values, indicating that when the vesicle integrity is destroyed,

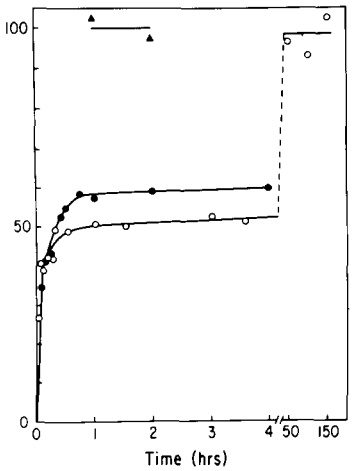

Fig. 5. Periodate oxidation of phosphatidylglycerol-containing vesicles. Vesicles were oxidized with 30 mM periodate as described. The percentage of phosphatidylglycerol (PG) oxidized is calculated assuming the mean values for oxidation in the presence of SDS to be 100% and agrees to within 3% for phosphatidylglycerol oxidation calculated from formaldehyde/phosphorus X 100. Closed circles, phosphatidylglycerol vesicles; open circles, phosphatidylglycerol/cholesterol vesicles; closed triangles, oxidation in the presence of SDS.

all the phosphatidylglycerol is available for oxidation. Similar experiments with vesicles composed of phosphatidylglycerol/cholesterol (1 : 1) indicate that oxidation reaches a maximal value after 30 min at 50 ± 1% and remains stable at that level for 4 h at 25°C. Control experiments with vesicles containing an oxidizable compound entrapped in the vesicle interior (glycerol 1-phosphate) showed conclusively that periodate does not enter the vesicle interior during the experimental time. All the above results indicate that the vesicles produced by our procedure are predominantly unilamellar, since for large unilamellar vesicles (diameter $\geqslant 0.1$ μm) it would be expected that 50% of the lipid would be located in the external monolayer and therefore would be available for oxidation.

Discussion

The reproducible preparation of liposomes of defined size with high encapsulation efficiencies and capture volumes per mole of lipid is a necessary objective for the continued development of liposomes as drug carriers. We first reported [9] that following formation, extrusion through polycarbonate membranes produces a smaller, more homogeneous population of liposomes. We have extended these observations to develop a method [10] for preparing a homogeneous population of multilamellar vesicles. However, multilamellar vesicles have a relatively low capture volume per mole of lipid and in a number of situations this requires that a large amount of lipid be administrated to an animal in order to obtain a therapeutically effective dosage. Furthermore, multilamellar vesicles are quite difficult to extrude through the 0.1 μm polycarbonate membranes, making it hard to obtain a preparation in the 0.1 μm size range.

This study documents that reverse phase evaporation liposomes can be extruded through small-pore polycarbonate membranes to form a homogeneous, intermediate-size population of unilamellar vesicles with both high capture efficiencies and high capture volumes per mole lipid. These conclusions are based on three electron-microscopic techniques which substantiate that the extrusion method yields liposomes the diameters of which approximate the diameter of the polycarbonate membrane pore. Problems with electron-microscopic methods for determining vesicle size have been previously discussed [5,10,13] and these make it difficult to determine the exact particle-size distribution of liposome preparations. However, the close correspondence between the mean diameters observed by the electron-microscopic techniques and the calculated vesicle diameters based on the total surface area of the lipids and capture volumes, lends added support to the values reported herein and to the effectiveness of the extrusion process in reducing these diameters. This agreement between the observed and calculated diameters along with our findings on the percentage of phosphatidylglycerol available for oxidation in intact vesicles also supports our previous observation that the reverse phase evaporation liposomes are predominantly unilamellar vesicles [9]. Moreover, unlike filtration of liposomes through cellulose acetate membranes which results in the removal of a substantial amount of lipid [18], even multiple extrusions through the polycarbonate membranes, which have straight-through pores, results in only a small loss of lipid.

Although there have been only a few studies on tissue distribution of liposomes where the size has been varied, small unilamellar vesicles were observed to have a considerably longer half-life in circulation compared to multilamellar vesicles [19,20]. Large-size multilamellar vesicles were removed from circulation even faster than extruded multilamellar vesicles and accumulated to a greater extent in lung tissue [21]. These results agree with the extensive literature on the effect of particle size on the in vivo circulatory life times and tissue distributions of various colloids [22,23]. However, pharmacological and pharmacokinetic investigations of these properties with liposomes have been hampered by the inability to prepare homogeneous-size liposomes that span the size range of interest. The combination of techniques for forming intermediate-size liposomes reported here should prove of value for exploring size intervals not covered by previous studies.

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