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PREPARATION OF LIPOSOMES OF DEFINED SIZE DISTRIBUTION BY EXTRUSION THROUGH POLYCARBONATE MEMBRANES

F. OLSON [a], C.A. HUNT [b], F.C. SZOKA [a], W.J. VAIL [c] and D. PAPAHADJOPOULOS [a,d,*]

[a] *Department of Experimental Pathology, Roswell Park Memorial Institute, Buffalo, NY, 14263;* [b] *Department of Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, CA 94143;* [c] *Department of Biology, Frostburg State College, Frostburg, MD and* [d] *Cancer Research Institute and Department of Pharmacology, School of Medicine, University of California, San Francisco, CA 94143 (U.S.A.)*

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Summary

Liposomes of defined size and homogeneity have been prepared by sequential extrusion of the usual multilamellar vesicles through polycarbonate membranes. The process is easy, reproducible, produces no detectable degradation of the phospholipids, and can double the encapsulation efficiency of the liposome preparation. Multilamellar vesicles extruded by this technique are shown by both negative stain and freeze-fracture electron microscopy to have mean diameters approaching the pore diameter of the polycarbonate membrane through which they were extruded. When sequentially extruded down through a 0.2 μm membrane, the resulting vesicles exhibit a very homogeneous size distribution with a mean diameter of 0.27 μm while maintaining an acceptable level of encapsulation of the aqueous phase.

Introduction

Liposomes (lipid vesicles) have emerged as promising new methodology for various biological applications including drug delivery in vivo [1]. However, the definition and control of particle size distribution within a liposome preparation is a difficult technological problem. In most of the studies on tissue distribution reported to date [2,3] various investigators have used either the

* Reprint requests to be sent to: Dr. D. Papahadjopoulos, Cancer Research Institute, School of Medicine, University of California, San Francisco, CA 94143, U.S.A.

initial liposome preparation of Bangham et al. [4] or brief sonication to reduce the size of the multilamellar vesicles. In either case, the liposome preparation consists of a heterogenous dispersion of vesicles in which the size distribution is dependent upon the method of preparation. This size heterogeneity has not been carefully examined.

In the few studies on tissue distribution where the size of the liposomes has been varied, small sonicated unilamellar vesicles were observed to have a considerably longer half-life in circulation than multilamellar vesicles [5,6]. Larger multilamellar vesicles have been observed to accumulate in lung tissue in relatively high amounts [16]. These results are in accord with the extensive literature on the effect of particle size on the in vivo circulation life times and tissue distribution properties of various colloids [7,8]. In general, however, pharmacological and pharmacokinetic investigations have been hampered by multiplicity of preparation methods and inability to specify the size distribution of the liposomes used.

These considerations point out the need for a quantitative examination of the size distribution of various liposome preparations. The size characteristics of the sonicated unilamellar vesicles [9] have been well-documented [10]; methodologies exist for their preparation in homogeneous populations [11]. However, these vesicles have a restricted size distribution and a relatively small internal aqueous volume. The importance of defining the size of liposome preparations has prompted us to devise a convenient method for producing multilamellar vesicles with reproducible, well-defined size distributions. To accomplish this we have employed sequential extrusion of large multilamellar vesicles through a series of polycarbonate membranes with fixed 'straight bore' pores of decreasing diameter. This report presents the exact conditions of the extrusion method, the size distribution of the resulting vesicles, and their efficiency in entrapping the aqueous volume.

Materials and Methods

Lipids and other reagents. Cholesterol was purchased from Fluka, A.B. (Buch, Switzerland). Phosphatidylcholine and phosphatidylserine were purified from egg yolk and bovine brain, respectively, as described previously [12]. All above lipids were finally purified on silicic acid columns, shown to be pure by thin-layer chromatography [12] and stored in chloroform in sealed ampules under nitrogen at −50°C until use. [^{14}C]Sucrose, and [^{14}C]cholesterol were obtained from New England Nuclear, Boston, MA. [^{3}H]Cytosine arabinoside was purchased from 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 Limited were obtained from E.F. Fullam, Inc. (Schenectady, NY). Polycarbonate membranes and membrane holders were obtained from Nucleopore, Inc., Pleasanton, CA. Bacitracin, U.S.P. was purchased from Upjohn, Kalamazoo, MI. All other chemicals were of reagent grade.

Preparation and extrusion of liposomes through membranes. Liposomes were prepared essentially by the technique of Bangham et al. [4]. The lipid used for all preparations was composed of phosphatidylserine, phosphatidylcholine and

cholesterol at molar ratios of 1 : 4 : 5. The lipid mixture in chloroform solution was deposited on the sides of a round-bottom flask by removal of the organic solvent on a rotary evaporator. Dried lipid was then hydrated with aqueous buffer containing the compound to be entrapped by dispersion into the aqueous phase at 37°C under a nitrogen atmosphere by either of two methods. The first consisted of vigorous shaking in a Vortex mixer for 20 s, allowing to stand for 30 s, and then repeating ten times. The preparation, referred to as the vortexed preparation, was then placed on a mechanical reciprocal shaker (two shakes/s, 5 cm amplitude), for 30 min at 37°C. The second method, referred to as the shaken preparation consisted of suspension of the lipid by gentle (two shakes/s, 1 cm amplitude) mechanical reciprocal agitation for either 2 or 20 h at 37°C. In most instances the lipid was suspended at a concentration of 60 μmol lipid/ml aqueous phase.

To determine the encapsulation efficiency, either [^{3}H]cytosine arabinoside or [^{14}C]sucrose was included in the aqueous phase as a labeled marker. The non-encapsulated material was removed by dialysis at 4°C against 100 vols. of phosphate-buffered saline changed five times. In certain instances [^{14}C]-cholesterol or [^{3}H]phosphatidylcholine was included in the vesicles to follow the recovery of lipid after extrusion through the nucleopore membranes.

For extrusion, the lipid concentration was usually adjusted to 10—12 μmol/ml by dilution in the same buffer used to prepare the liposomes and passed through the largest pore size membrane, (usually 3 μm) in a 25 mm holder. When the preparation was extruded at 60 μmol/ml, more pressure was required. The liposomes were then sequentially passed through membranes with pore diameters 1.0, 0.8, 0.6, 0.4, and 0.2 μm. Extrusion using a lipid concentration of 10—12 μmol/ml could be accomplished at a relatively low pressure until the 0.2 μm membrane was encountered, when approximately 50 pounds/square inch was required to extrude the vesicles. It was necessary to seal tightly the membrane holder, otherwise leaks would result. Samples were taken both prior and after each extrusion and non-encapsulated material was removed by dialysis.

Electron microscopy and determination of size distribution of liposomes. Negative stain electron micrographs were prepared using a modification of a previously described technique [9]. Carbon-coated grids were covered with a 0.1 mg/ml solution of Bacitracin and blotted dry. Liposomes that had been dialysed against 0.15 M ammonium acetate/0.5 mM ethylenediaminetetraacetic acid, pH 7.0, were applied onto the grid at concentrations between 0.6 μmol lipid/ml (unextruded liposomes) and drawn off with filter paper. A drop of a 2% (w/v) ammonium molybdate (prefiltered through a 0.1 μm membrane) was then applied to the grid, drawn off with a piece of filter paper and allowed to dry 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 times the negative enlargments. The diameters of individual vesicles were measured with a caliper and assigned to a specific size interval. Two times the surface area of the disc was assumed to represent the equivalent surface area of a collapsed sphere; the diameter of the disc times 0.75 was equated to the diameter of the equivalent sphere. The number of vesicles in each interval was determined, multiplied times 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 graphed 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.

The freeze-fracture electron micrographs of the vesicle preparations were done as previously described [13]. The negatives were printed at a final magnification ×10 000. The apparent vesicle diameters were measured and were assumed to represent the diameters of the equivalent spheres. At least 500 vesicles from each fraction were measured from a single experiment. The total apparent vesicle volume was calculated and the fraction of the volume in each interval was determined. Since the cleavage plane during the fracture was expected to cut randomly through the vesicles, the resulting frequency distribution would be shifted to smaller profile sizes compared to the diameter of whole vesicles. However, we estimate that the correction for this artifact must be relatively small because of the reasonable agreement between the size distribution obtained by the two techniques, negative stain, and freeze-fracture. The data presented in the results section therefore assumes that the clearage plane around each vesicle is located close to the equitorial position.

Results

Fig. 1A illustrates the size distribution of a multilamellar preparation dispersed (vortexed) at a lipid concentration of 2 μmol/ml buffer as observed by negative stain electron microscopy (Fig. 2A). The average liposome diameter based on the distribution of vesicle profiles is 1.3 μm with 75% of the total volume resulting from vesicles between 0.57 and 2.14 μm. Fig. 3A illustrates the size distribution from the freeze-fracture electron micrographs (Fig. 4A) of similarly vortexed preparations. The analysis of size distribution reveals a mean diameter of 1.6 μm with 75% of the total volume resulting from vesicles between 0.99 and 2.96 μm. Examination of Table I reveals a close agreement between the mean volume and 75% distribution limits of extruded vesicles determined by either negative stain or freeze-fracture electron microscopy. The size discrepancy between the two techniques decreases as the vesicles are passed through membranes of successively smaller pore diameter. Sequential extrusion of the vortexed preparation through polycarbonate membranes reduces the mean size and size heterogeneity of the liposomes as quantified from both negative stain (Fig. 1B—E) and freeze-fracture (Fig. 3B—D) electron micrographs. Representative negative stain and freeze-fracture electron micrographs are shown in Fig. 2B and C and Fig. 4B—D, respectively.

Table II shows the characteristic mean size and the range which accounts for 75% of the volume in the liposome preparation made and extruded at 2 μmol lipid/ml, along with the capture of aqueous volume/mol of lipid and the efficiency of encapsulation of cytosine arabinoside. A comparison with liposomes of the same composition prepared at 60 μmol/ml and extruded at 12 μmol/ml is also included.

The striking reduction in the mean size and heterogeneity is accompanied by an increase in the captured volume/mol lipid. In the case of cytosine arabinoside,

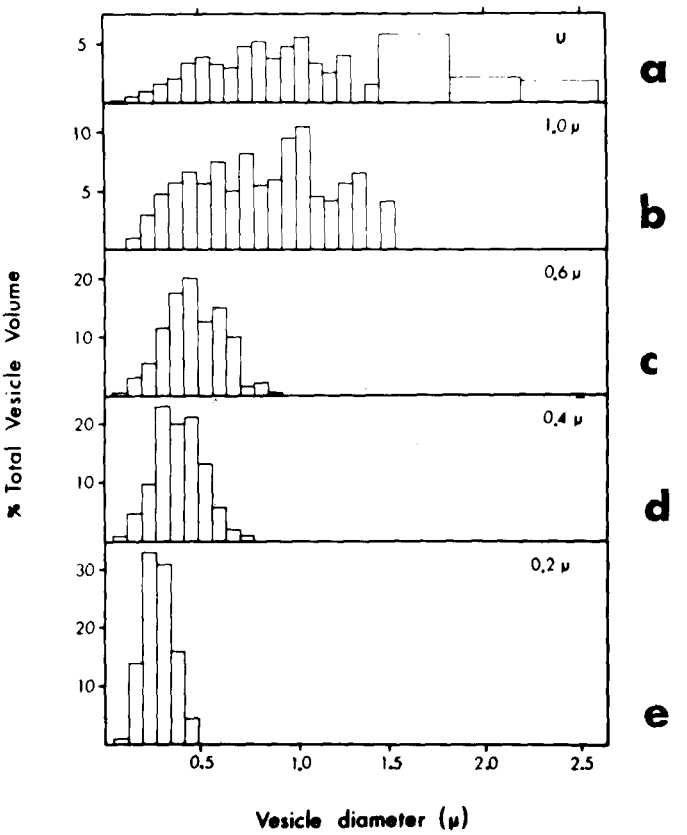

Fig. 1. Size distribution determined by negative stain electron microscopy of vortexed multilamellar vesicles sequentially extruded through polycarbonate membranes. Vesicles were prepared as detailed in the methods for the vortexed preparation. The data are pooled from three experiments that agreed to within 10%. The number of vesicle profiles measured for each extrusion can be found in Table I. (a) Unextruded preparation; (b) extruded through 1 μm membrane; (c) extruded through 0.6 μm membrane; (d) extruded through 0.4 μm membrane, and (e) extruded through 0.2 μm membrane.

the capture increases from 1.9 l/mol lipid for the unextruded preparation to 3.8 l/mol lipid for liposomes extruded through the 0.2 μm pore size membrane. This increase in aqueous volume/mol lipid is accompanied by an increase in the capture efficiency from 11.3% to 22.6% when going from the unextruded preparation to one extruded sequentially through the series of membranes. The preparation at the higher lipid concentration initially has a lower capture volume/mol lipid (1.89 l/mol versus 2.36 l/mol) but following the first extrusion the two preparations have similar capture volumes. With sequential extrusions the higher concentration lipid preparation continues to increase in captured volume while the lower concentration lipid preparation begins to show a decrease in capture volume after extrusion through the 0.4 and 0.2 μm membranes (Table II). At least 80% of the lipid is recovered after the multiple extrusions. The apparent loss of lipid is probably due to trapping in the dead space of the membrane holders. An important feature of this technique is that the liposomes prepared and extruded at 2 μmol lipid/ml of aqueous buffer have a

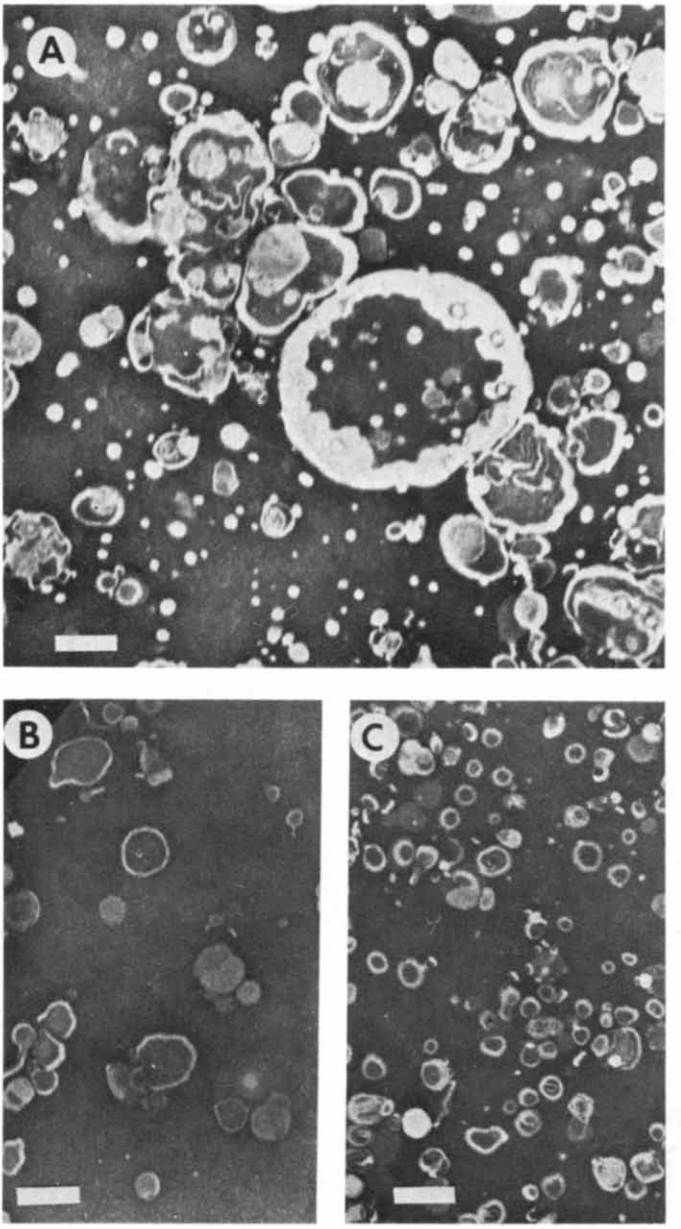

Fig. 2. Negative stain electron micrographs of extruded vortexed multilamellar vesicle preparations. The bar in the lower left corner indicates 0.5 μm. (a) Unextruded; (b) extruded through 0.4 μm membrane, and (c) extruded through 0.2 μm membrane.

size distribution similar to those prepared at 60 μmol/ml and extruded at 12 μmol/ml aqueous buffer.

Fig. 5 illustrates the size distribution of a liposome preparation of identical composition suspended by gentle mechanical shaking and composed of phosphatidylserine, phosphatidylcholine and cholesterol at molar ratio 1/4/5 as

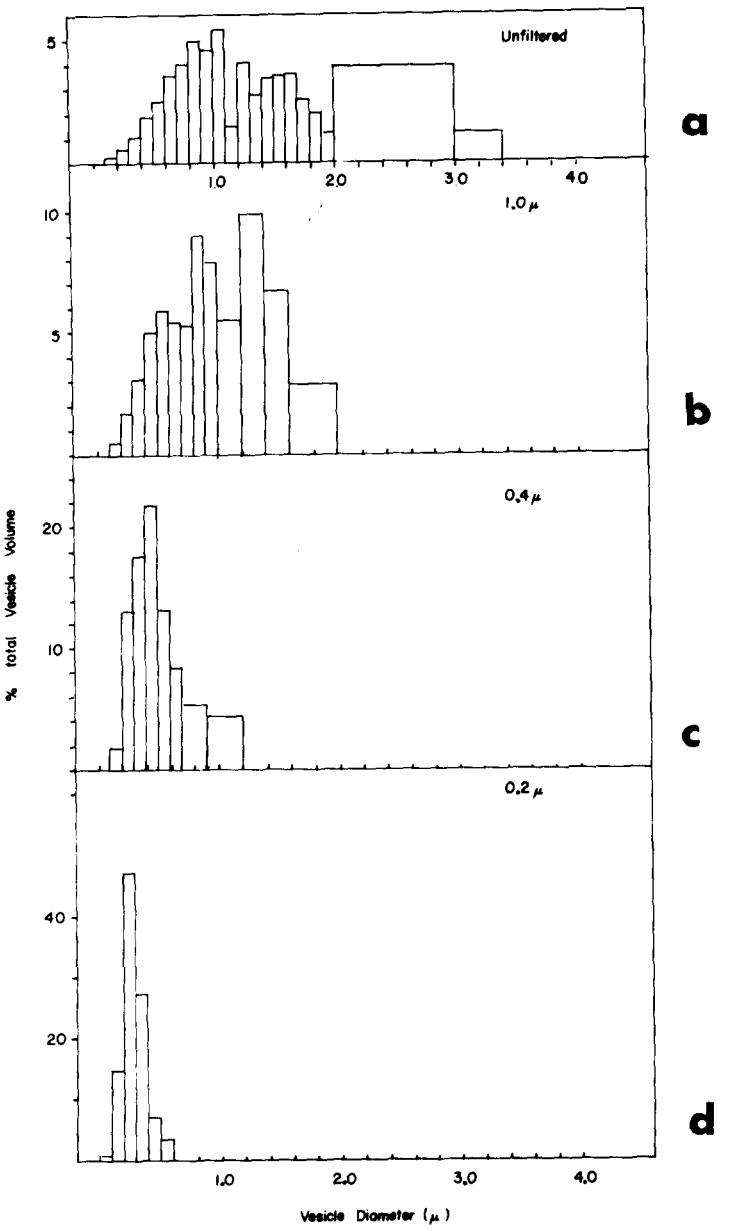

Fig. 3. Size distribution determined by freeze-fracture electron microscopy of vortexed multilamellar vesicles sequentially extruded through the polycarbonate membranes. Vesicles were prepared as detailed in Materials and Methods and the number of vesicle profiles measured for each extrusion can be found in Table I. (a) Unextruded; (b) extruded through 1 μm membrane; (c) extruded through 0.4 μm membrane, and (d) extruded through 0.2 μm membrane.

determined from negative stain electron micrographs. The size distribution of liposomes after 2 h of gentle shaking is similar to that following 20 h of similar shaking (data not shown). However, there is a slight increase in liposomes with a diameter greater than 2.0 μm. Sequential extrusion of this preparation through 3.0 μm and 1.0 μm membranes reduces both the mean size and the size

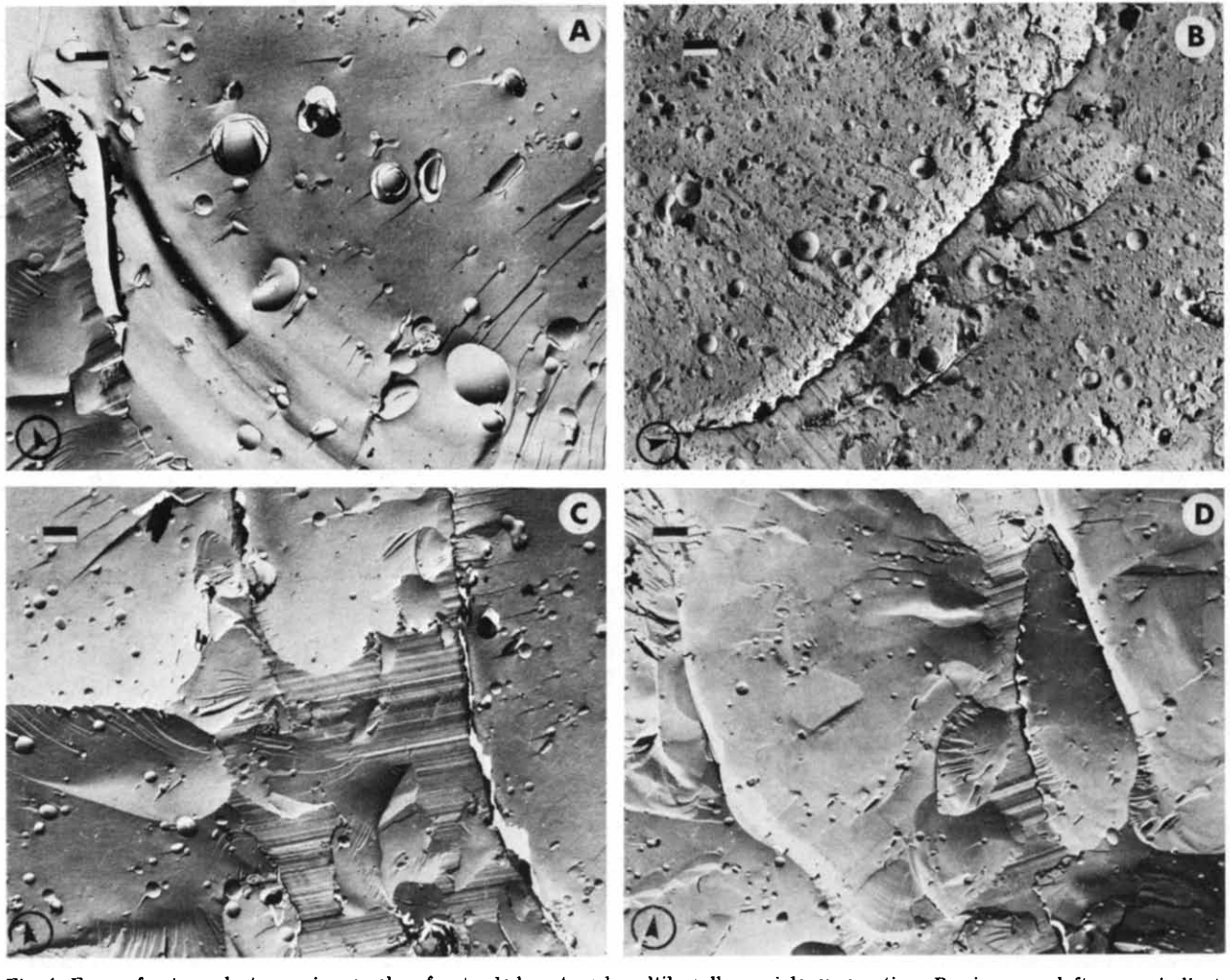

Fig. 4. Freeze-fracture electron micrographs of extruded vortexed multilamellar vesicle preparation. Bar in upper left corner indicates 1.0 μm. (a) Unextruded; (b) extruded through 1.0 μm membrane; (c) extruded through 0.4 μm membrane, and (d) extruded through 0.2 μm membrane.

TABLE I

COMPARISON OF THE SIZE DISTRIBUTION OF THE EXTRUDED VORTEXED MULTILAMELLAR VESICLES (MLV) DETERMINED BY EITHER NEGATIVE STAIN OR FREEZE-FRACTURE ELECTRON MICROSCOPY

For negative stain the initial vortexed MLV was prepared in 300 mM glucose, 1/10 phosphate-buffered saline [a], pH 7.4, at 37°C with 5 mM [^{14}C]sucrose as the aqueous space marker. The total lipid concentration was 2 μmol/ml (phosphatidylserine/phosphatidylcholine/cholesterol: 1/4/5) and the vesicles were extruded at this concentration. For freeze-fracture MLV were prepared in 300 mM cytosine arabinoside 1/10 phosphate-buffered saline, pH 7.4, at 37°C containing 3 mM [^{3}H]cytosine arabinoside as the aqueous space marker. The total lipid concentration was 60 μmol/ml (phosphatidylserine/phosphatidylcholine/cholesterol: 1/4/5) and the vesicles were diluted to 12 μmol/ml in the hydrating buffer prior to extrusion. Membrane, pore size in μm of the polycarbonate membrane that MLV were extruded through. 75% volume limits is the calculated diameter range in μm that contains 75% of the vesicles volume. *N*, number of vesicle profiles measured, n.d., not determined.

Membrane (μm)	Negative stain			Freeze-fracture		
	Mean vesicle * diameter	75% volume limits	*N*	Mean vesicle ** diameter	75% volume limits	*N*
None	1.32	0.57—2.14	1047	1.61	0.99—2.96	1254
1.0	0.85	0.36—1.29	822	1.03	0.44—1.59	841
0.8	0.62	0.33—1.02	1063	n.d.	n.d.	n.d.
0.6	0.44	0.36—0.65	631	n.d.	n.d.	n.d.
0.4	0.38	0.25—0.60	875	0.47	0.18—0.92	515
0.2	0.26	0.17—0.37	386	0.28	0.18—0.39	461

[a] Containing: 13.7 mM NaCl, 0.26 mM KCl, 0.64 mM Na_2HPO_4 and 0.14 mM KH_2PO_4.

* Mean vesicle diameter in μm as detailed in the methods for the negative stain preparation. The value is from the pooled data from three separate experiments that agreed to within 10%.

** Mean vesicle diameter in μm as detailed in the methods for the freeze-fracture preparation. The values are from a single experiment.

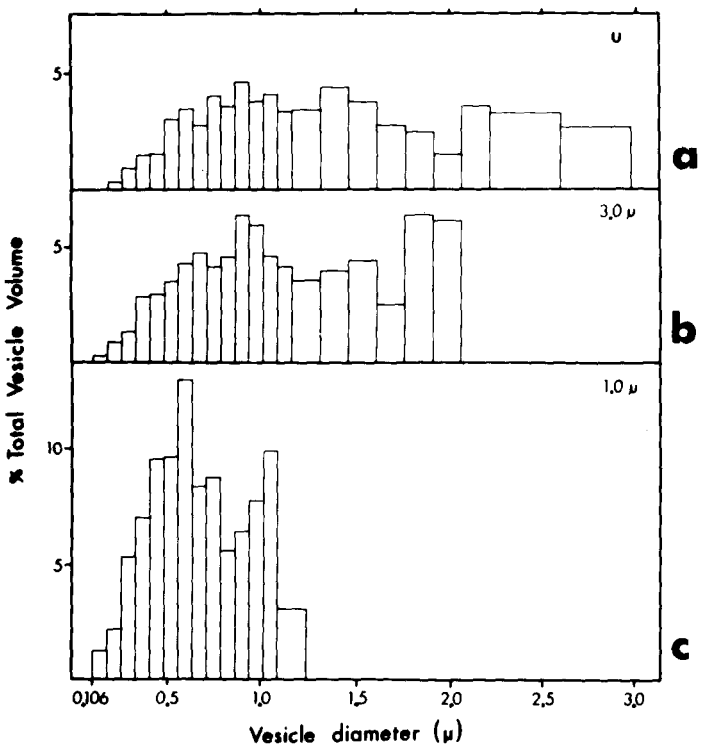

Fig. 5. Size distribution determined by negative stain electron microscopy of gently shaken multilamellar vesicle preparation. Vesicles prepared as detailed in Materials and Methods. The data are pooled from three experiments that agreed to within 10% at the 3.0 and 1.0 μm extrusions and within 50% for the non-extruded preparation. The number of vesicle profiles measured for each extrusion can be found in Table III. (a) Unextruded; (b) extruded through 3.0 μm membrane, and (c) extruded through 1.0 μm membrane.

TABLE II

CHARACTERISTICS OF THE VORTEXED MULTILAMELLAR VESICLES (MLV) SEQUENTIALLY EXTRUDED THROUGH POLYCARBONATE MEMBRANES OF DECREASING PORE DIAMETER AT DIFFERENT LIPID AND SALT CONCENTRATIONS

75% volume limits is the calculated diameter range in μm that contains 75% of the vesicle volume. *N*, number of vesicle profiles measured. n.d., not determined.

Membrane [c]	Lipid concentration							
	2 μmol/ml [a]				12 μmol/ml [b]			
	Mean vesicle [d] diameter	75% volume limits	N	Capture [e] volume	Mean vesicle [f] diameter	75% volume diameter	N	Capture [g] volume
None	1.32	0.57—2.14	1047	2.36	0.93	0.68—1.93	3144	1.80 ± 0.017
1.0	0.85	0.36—1.29	822	2.45	n.d.	n.d.	n.d.	2.43 ± 0.043
0.8	0.62	0.33—1.02	1063	2.63	n.d.	n.d.	n.d.	2.84 ± 0.024
0.6	0.44	0.36—0.65	631	2.81	n.d.	n.d.	n.d.	3.17 ± 0.026
0.4	0.38	0.25—0.60	875	2.75	0.47	0.34—0.69	381	3.66 ± 0.09
0.2	0.26	0.17—0.37	386	2.28	0.26	0.17—0.37	645	3.76 ± 0.047

[a] The MLV were prepared in 300 mM glucose, 1/10 phosphate-buffered saline, pH 7.4, at 37° C with 5 mM [^{14}C]sucrose as the aqueous space marker and [^{3}H]phosphatidylcholine in the bilayer. The total lipid concentration was 2 μmol/ml (phosphatidylserine/phosphatidylcholine/cholesterol: 1/4/5) and the MLV were extruded at this concentration.

[b] The MLV were prepared in 300 mM cytosine arabinoside in 1/10 phosphate-buffered saline, pH 7.4, at 37° C with [^{3}H]cytosine arabinoside as the aqueous space marker and [^{14}C]cholesterol in the bilayer. The total lipid concentration was 60 μmol/ml (phosphatidylserine/phosphatidylcholine/cholesterol: 1/4/5) that was diluted in the hydrating buffer to 12 μmol/ml prior to extrusion.

[c] Pore diameter in μm of polycarbonate membrane that vesicles were extruded through.

[d] Mean vesicle diameter in μm as detailed in the methods for the negative stain preparation.

[e] The sucrose-captured volume in l aqueous space/mol total lipid. This value is the mean of two separate experiments that agreed to within 10%.

[f] Mean vesicle diameter in μm. This value is from pooled data of three separate experiments that agreed to within 15%.

[g] Cytosine arabinoside-captured volume in l aqueous space/ml lipid. This value is the mean and S.E. from three separate experiments.

heterogeneity of this preparation (Fig. 5B and C). The results obtained by negative stain are confirmed in the size distribution determined from the freeze-fracture electron micrographs (Fig. 6A—C).

The captured volume, capture efficiency, and size range that includes 75% of the volume is tabulated in Table III for the 20 h 'shaken' preparation. As has been observed for the vortexed preparation, sequential extrusion results in an increase in the entrapped aqueous volume/mol lipid and an increase in the capture efficiency.

Examination of the entrapped volume/mol of lipid as a function of time shows that there is almost a 50% increase in the captured volume/mol lipid for the unextruded multilamellar vesicles after 20 h of gentle shaking compared to 2 h (Table III).

Attempts to obtain a more homogeneous preparation of larger multilamellar vesicles by pelleting the large vesicles at 100 000 × *g* for 30 min in an ultracentrifuge were unsuccessful. Following centrifugation, the liposomes

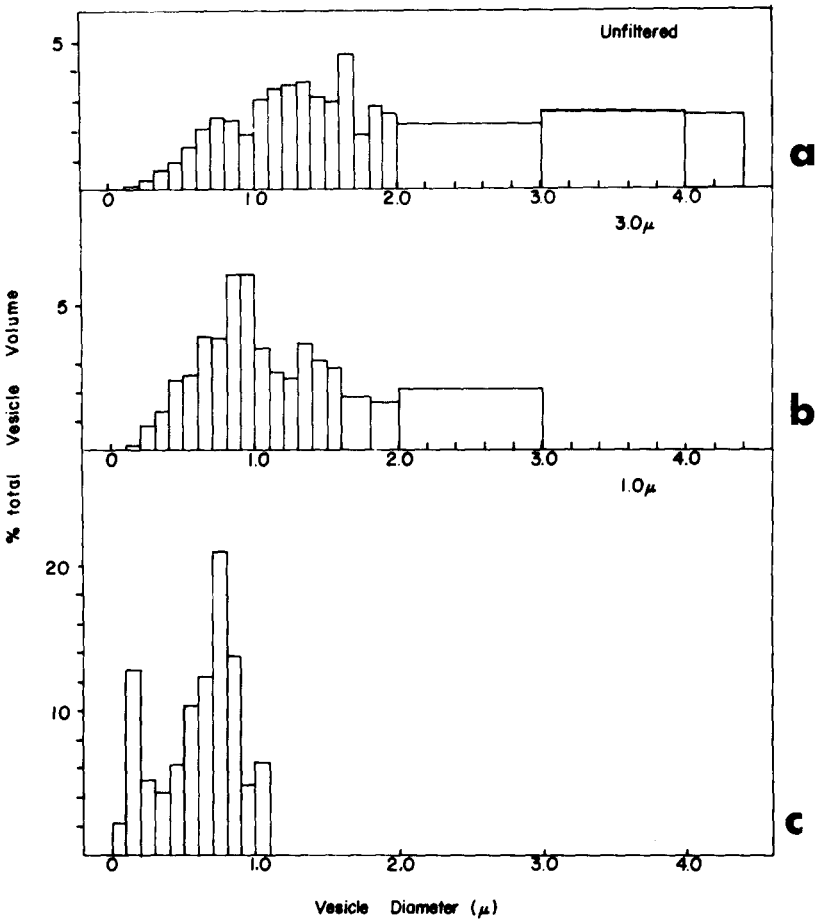

Fig. 6. Size distribution determined by freeze-fracture electron microscopy of gently shaken multilamellar vesicle preparation. Vesicles prepared as detailed in Materials and Methods. The data are from a single experiment and the number of vesicle profiles measure are given in Table III. (a) Unextruded; (b) extruded through 3.0 μm membrane, and (c) extruded through 1.0 μm membrane.

remaining in the supernatant had distinctly smaller diameters but the pellet had essentially the same distribution as the unseparated mixture (data not shown). The relative amount of lipid remaining in the supernatant increased as the vesicles were sequentially passed through the polycarbonate membranes and then centrifuged, but the size distribution of the lipids in the pellet appeared similar to that in the precentrifuged vesicle preparation. Furthermore, the aqueous space/μmol total lipid was then same in the pellet (2.50 ± 0.06 l/mol) as in the precentrifugation mixture (2.48 ± 0.08 l/mol).

When the phospholipids of extruded liposomes were analyzed by thin-layer chromatography [9] after sequential passage through five membranes of decreasing pore size, no detectable breakdown of the phospholipids or other impurity was observed, even at a loading of 500 μg/spot.

TABLE III

COMPARISON OF THE SIZE DISTRIBUTION MEASURED BY NEGATIVE STAIN OR FREEZE-FRACTURE ELECTRON MICROSCOPY OF THE GENTLY SHAKEN MULTILAMELLAR VESICLES (MLV) SEQUENTIALLY EXTRUDED THROUGH POLYCARBONATE MEMBRANES OF DECREASING PORE DIAMETER

For negative stain and freeze-fracture MLV composed of (phosphatidylserine, phosphatidylcholine and cholesterol: 1/4/5) were hydrated at a total lipid concentration of 60 μmol/ml of 300 mM cytosine arabinoside in 1/10 phosphate-buffered saline, pH 7.4, at 37°C. The preparation was diluted in the same buffer and extruded at a total lipid concentration of 12 μmol/ml. Membrane, pore size in microns of polycarbonate membranes that the MLV were extruded through. Mean vesicle diameter in μm determined as detailed in the methods for the negative stain preparation. The data are pooled from three separate experiments that at the 3.0 and 1.0 membrane agreed to within 10% and for the unextruded preparation agreed to within 50%. 75% volume limits is the calculated diameter range in μm that contains 75% of the total vesicle volume. *N*, number of profiles measured. Captured volume is the [^{3}H]cytosine arabinoside-captured volume in 1 aqueous space/mol total lipid (mean of two separate experiments that agreed to within 20%). n.d., not determined.

Membrane	Negative stain				Freeze-fracture		
	Mean vesicle diameter	75% volume limits	N	Capture volume	Mean vesicle diameter	75% volume limits	N
None [a]	1.41	0.64—2.25	881	3.25	2.56	0.92—3.75	936
3.0 [a]	1.15	0.59—1.84	633	3.40	1.09	0.63—2.52	824
1.0 [a]	0.68	0.40—1.04	1052	3.66	0.67	0.18—0.89	1436
None [b]	1.33	0.63—2.04	897	2.21	n.d.	n.d.	n.d.

[a] MLV gently shaken for 20 h.
[b] MLV gently shaken for 2 h.

Discussion

The extrusion of liposomes through polycarbonate membranes represents an initial attempt to produce a population of multilamellar vesicles with a defined and well-characterized size distribution. Extrusion is convenient, easily reproducible, does not introduce impurities into the vesicles, and does not induce breakdown of the phospholipids. Vesicles can be extruded through 0.2 μm membranes allowing production of a sterile preparation for in vivo injection. This technique can give an apparent increase in encapsulation efficiency when the extrusions are carried out in the original 'mother liquid'. Two electron microscopic techniques substantiate that the extrusion method produces a population of vesicles whose diameter approaches the diameter of the pore in the polycarbonate membrane.

Negative stain electron microscopy is recognized to have a number of difficulties introduced by drying. These include shrinkage and clumping of the vesicles, and the generation of irregular shapes during the drying procedure [14]. The latter can be particularly troublesome for the preparations of larger size vesicles. Drying artifacts are probably the principle cause for the discrepancy between the distributions observed by the negative stain and freeze-fracture techniques. Analysis of the freeze-fracture data involves the following assumptions: first, that the profiles are spherically shaped; second, that the vesicles are randomly distributed in the sample; and third, that the cleavage

plane intersects the vesicles in a random fashion. The first condition was fulfilled in the large majority of the profiles measured. In the non-extruded preparation some profiles were observed to be ellipsoid. For these the mean of the major and minor axis are considered to represent the area occupied by an equivalent sphere [15]. The effect of shadowing for the freeze-fracture, or the stain thickness in the negative stain preparations on the observed profile size, were not considered in these calculations but represent only a small contribution to the profile diameter. In spite of the potential artifacts, the observed distributions obtained by these two techniques are remarkably similar. Furthermore, the reproducibility between different preparations is excellent. Following extrusion through the 1 μm membrane, the mean diameter from five different preparations agreed to within 10%.

It is important to note the finding that vesicles formed by 'gentle shaking' have an appreciably larger mean diameter than those that have been formed by 'vortexing'. The difference in the encapsulation efficiencies between liposomes shaken for 2 and 20 h, although they result in similar size distributions, might be due to incomplete equilibration at the marker with the internal lamellae of the multilamellar vesicles during the shorter time period. Although this study was done with multilamellar vesicles composed of a specific mixture of lipids, we have observed that extrusion also affects similarly the size of liposomes of other lipid composition. Liposome preparations varying in their percentage of cholesterol and phosphatidylserine exhibit different turbidity and encapsulation efficiencies when prepared under the same initial conditions (indicative of differences in the size of the vesicles) but have similar size and encapsulation efficiencies following extrusion through the polycarbonate membranes.

These observations suggest that it is possible to obtain a reproducible protocol for the preparation of liposomes of defined size distribution for studies where the size may be an important parameter, such as tissue distribution and delivery of encapsulated drugs in vivo. Recent experiments on the effect of the size of multilamellar vesicles on their retention in lung tissue indicate the importance of this variable [16]. It is nevertheless necessary to examine the size distribution of the resulting liposomes when altering the surface properties of liposomes by the inclusion of various charged amphipatic molecules, carbohydrate-containing lipids, or hydrophobic drugs that insert into the bilayer. Although not superceding the need for examining the size distribution of liposomes, extrusion through polycarbonate membranes appears to be an effective way to insure size homogeneity.

At present, we have no direct evidence on the mechanism for liposome size reduction during passage through the membrane. We envisage the process of extrusion as pinching-off part of the liposome as it is pressed through a pore of a diameter smaller than its own. The vesicle can deform to a certain extent but presumably it reaches a point where the pressure differential on the opposite sides of the pore causes the bilayers to rupture. The ruptured bilayers would presumably anheal immediately forming smaller vesicles which can eventually all pass through the pore. The extrusion pressure may also bring about the interaction of the liposome bilayer with the walls of the polycarbonate membrane pore. Rupture should be more difficult for liposomes composed of phospholipids below their transition temperature. Indeed, multilamellar vesicles

composed of dipalmitoyl phosphatidylcholine can be extruded without difficulty at a temperature above their solid-to-fluid transition (42°C), but only with much greater difficulty at lower temperatures, usually clogging the polycarbonate membrane or resulting in its breakage (unpublished observation). That most of the bilayers rupture in this process is supported by the observation that sequential extrusion results in loss of most of the encapsulated marker if the unencapsulated marker is removed by dialysis prior to extrusion (unpublished observation). The substantial increase in capture of aqueous space/lipid that occurs during extrusion could be explained on the basis of an increased ratio of lipid participating in external versus internal bilayers in each liposome.

The reasonably good capture and the relatively mild conditions make the extruded vesicles an attractive carrier for compounds that cannot be exposed to the organic solvents which are used in the ether injection technique [17] or the reverse phase evaporation technique [18] for forming large uni- and oligolamellar vesicles. In addition to its use with multilamellar vesicles, the extrusion technique can also be used to produce a more uniform preparation of vesicles formed by the above methods [18].

In conclusion, the extrusion technique can be used to produce conveniently and reproducibly a homogeneous size distribution of vesicles with a mean size approaching the polycarbonate membrane pore used for the extrusion, with little loss of lipid and a reasonable capture of the aqueous space. Use of this method should prove useful in various experimental situations where the liposome size or total surface area need to be defined. It should be of special value for pharmacological and pharmacokinetic studies of liposomes as a drug delivery system.

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