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Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation

(drug delivery/encapsulation/lipid vesicles/encapsulated macromolecules)

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ABSTRACT **Large unilamellar and oligolamellar vesicles are formed when an aqueous buffer is introduced into a mixture of phospholipid and organic solvent and the organic solvent is subsequently removed by evaporation under reduced pressure. These vesicles can be made from various lipids or mixtures of lipids and have aqueous volume to lipid ratios that are 30 times higher than sonicated preparations and 4 times higher than multilamellar vesicles. Most importantly, a substantial fraction of the aqueous phase (up to 65% at low salt concentrations) is entrapped within the vesicles, encapsulating even large macromolecular assemblies with high efficiency. Thus, this relatively simple technique has unique advantages for encapsulating valuable water-soluble materials such as drugs, proteins, nucleic acids, and other biochemical reagents. The preparation and properties of the vesicles are described in detail.**

The use of phospholipid vesicles (liposomes) in biology and medicine, a promising new area of research (1), will depend to a large degree on technological improvements in the formation of vesicles of various sizes and properties. As more attempts are made to modify cellular physiology by introducing regulatory molecules into the cell or to improve chemotherapy in the whole animal, the need for a vesicle preparation that entraps a large percentage of the aqueous phase has become apparent.

The original liposome preparations of Bangham *et al.* (2), consisting of multilamellar vesicles (MLV), have been admirably suited in defining many membrane properties (3, 4) and were the basis for the development of the sonicated unilamellar vesicles (SUV) (5). However, both preparations show a relatively low volume of entrapped aqueous space per mole of lipid and restricted ability to encapsulate large macromolecules. This is because in MLV most of the lipid is participating in the internal lamellae, and the close apposition of the adjacent concentric bilayers restricts the internal water space. In SUV, which are single-compartment vesicles, the ratio of surface area to encapsulated volume is so large that only a small aqueous volume per mole of lipid can be attained. Attempts to circumvent these shortcomings (6–8) have been only partially successful. The ethanol injection method produces vesicles of about the same size as SUV with the same shortcomings. The ether infusion technique produces large unilamellar vesicles with high captured volumes per mole of lipid, but the efficiency of encapsulation is relatively low. Other useful techniques for preparing large volume vesicles either use specialized conditions (9) or are restricted to a single phospholipid (10). Methods based upon solvent evaporation have been attempted in the past but have resulted in the formation of multilamellar vesicles (6, 11, 12). A method designed to form asymmetric vesicles by centrifugation of a suspension of dense aqueous inverted micelles through an organic solvent/water interface has been reported (13). The report indicated that the internal volume was small and the vesicles themselves relatively unstable (13). Techniques based upon the removal of detergents yield vesicles slightly larger than SUV (14). These are suitable for membrane reconstitution experiments but, like the SUV, fail to encapsulate the aqueous phase efficiently. Recently a method that combines detergent dialysis and solvent evaporation has been described (15). This technique leaves an equal weight of detergent per phospholipid in the resulting vesicles, making them unsuitable for many biological applications (15).

Thus, no method has been described that can produce vesicles with the following desirable properties: (*i*) the ability to entrap a large percentage of the aqueous material presented; (*ii*) a high aqueous space-to-lipid ratio; and (*iii*) widely variable chemistry of the lipid components. We were particularly concerned with the first characteristic and reasoned that if total encapsulation of the material of interest could be achieved in an "inverted" micelle within an organic solvent, subsequent removal of the organic solvent might result in a high encapsulation. We have developed conditions that not only achieve a high percentage of encapsulation, but also produce vesicles with a large aqueous space-to-lipid ratio. This report describes the method and characteristics of the vesicles produced by the reverse-phase evaporation technique.

MATERIALS AND METHODS

Lipids and Other Materials. Cholesterol and palmitic acid were purchased from A. B. Fluka (Buch, Switzerland). Phosphatidylcholine (PtdCho), phosphatidylglycerol (PtdGro), and phosphatidylserine (PtdSer) were purified from egg yolk and bovine brain as described (16). Phosphatidic acid and dipalmitoyl phosphatidylcholine ($Pal_2PtdCho$) were synthesized as reported (16). All above lipids were finally purified on silicic acid columns, shown to be pure by thin-layer chromatography (17), and stored in chloroform in sealed ampules under nitrogen at −50°C until use. Sphingomyelin (bovine brain) was purchased from K and K Chemicals, Plainview, NY. Poly(A), [^{3}H]poly(A), ferritin, albumin, and rabbit IgG were purchased from Miles Research, Elkhart, IN. Porcine insulin was obtained from Lily, Indianapolis, IN. Alkaline phosphatase and ribonuclease (RNase) were purchased from Worthington, Freehold, NJ. ^{125}I-, ^{22}Na-, and ^{14}C-labeled sucrose were obtained from

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Abbreviations: ara C, 1-β-D-arabinofuranosylcytosine; LUV, large unilamellar lipid vesicles; MLV, multilamellar lipid vesicles; REV, reverse-phase evaporation lipid vesicles; SUV, sonicated unilamellar lipid vesicles; PtdCho, phosphatidylcholine; $Pal_2PtdCho$, dipalmitoylphosphatidylcholine; PtdGro, phosphatidylglycerol; PtdSer, phosphatidylserine; phosphate-buffered saline, 137 mM NaCl/2.6 mM KCl/6.4 mM Na_2HPO_4/1.4 mM KH_2PO_4; 1/10 phosphate-buffered saline, 1/10 dilution of phosphate-buffered saline in distilled water.

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New England Nuclear, Boston, MA. [^{3}H]Cytosine arabinoside (ara C) was purchased from Amersham-Searle, Arlington Heights, IL. 25S [^{32}P]RNA was a gift from E. Niles, State University of New York at Buffalo. All solvents were obtained from Fisher Scientific. For some preparations, diethyl ether was redistilled from sodium bisulfite immediately before use to remove any peroxides. There were no apparent differences between vesicles formed with or without distilled diethyl ether. Unipore filters were obtained from Bio-Rad Laboratories, Richmond, CA. All other chemicals were of reagent quality. The chloramine T method of Greenwood *et al.* (18) was used to iodinate proteins, and labeled proteins were separated from unincorporated ^{125}I by passage through a Sephadex G-25 medium (1 × 25 cm) column.

Preparation of Reverse-Phase Evaporation Vesicles (REV). Several phospholipids, either pure or mixed with other lipids such as cholesterol, long-chain alcohols, etc., can be used with similar results. The lipid mixture is added to a 50-ml round-bottom flask with a long extension neck, and the solvent is removed under reduced pressure by a rotary evaporator. The system is then purged with nitrogen and lipids are redissolved in the organic phase, in which the reversed phase vesicles will be formed. The solvents that have been most thoroughly investigated are diethyl ether and isopropyl ether, although halothane and trifluorotrichloroethane have been successfully used. When the lipid has low solubility in ether, chloroform or methanol can be added to increase their solubility. The detailed composition in each case is given in Table 1.

The aqueous phase is added at this point, the system is kept continuously under nitrogen, and the resulting two-phase system is sonicated briefly (2–5 min) in a bath-type sonicator (Lab Supply T-80-80-IRS) until the mixture becomes either a clear one-phase dispersion or a homogeneous opalescent dispersion that does not separate for at least 30 min after sonication. The degree of opalescence of the preparation at this point depends upon the solvent, phospholipid, and amount of aqueous phase in the preparation. The sonication temperature for diethyl ether and most lipids is 0–5°C unless otherwise stated. The mixture is then placed on the rotary evaporator and the organic solvent is removed under reduced pressure (water aspirator) at 20–25°C, rotating at approximately 200 rpm. Additional details are in Table 1.

Table 1. Effect of lipid composition on encapsulation of ara C and sucrose in REV*

Lipid composition†	Captured volume,‡ μl/mg	% encapsulation§ ara C	Sucrose
1. PtdGro/PtdCho/Chol (1:4:5)	13.7	55.0 ± 3.9	64.6
2. PtdGro/PtdCho (1:4)	9.2	24.2 ± 0.5	30.1
3. PtdGro/PtdCho (1:4)	8.1	42.8 ± 0.6	ND
4. SA/PtdCho (1:4)	15.6	59.7 ± 2.7	63.0
5. PtdGro	10.5	27.7 ± 2.4	18.7
6. PtdGro	8.7	46.7 ± 2.7	ND
7. Pal_2PtdCho	11.7	28.9 ± 2.7	35.5
8. PtdGro/PtdCho/Chol (1:4:5) MLV	4.1	16.5	ND
9. PtdGro/PtdCho/Chol (1:4:5) SUV	0.5	1.8	ND

* Preparations 1–6 contained the encapsulated compound in 1.5 ml of 1/10 dilution of phosphate-buffered saline in distilled water with 5 ml of diethyl ether. Preparation 7 contained the encapsulated compound in 1.5 ml of 1/10 phosphate-buffered saline with 5 ml of isopropyl ether and 5 ml of chloroform, sonicated and evaporated at 45°C. Preparations 8 and 9 (MLV and SUV) were prepared with ara C in 1/10 phosphate-buffered saline.

† All preparations contained 66 μmol of total lipid per ml of aqueous phase, except for 2, 5, and 7, which contained 33 μmol/ml. Chol, cholesterol; SA, stearylamine.

‡ Ratio of captured volume (μl) over weight (mg) of lipids. Captured volume was calculated from ara C encapsulation.

§ Based on ara C or sucrose capture; ara C or sucrose concentration was 3 mM in a 1/10 phosphate-buffered saline. Values for ara C are the mean ±SEM for at least three experiments; the values for sucrose capture are from a single experiment. ND, not done.

During evaporation of the solvent, the system froths. As the majority of the solvent is removed, the material first forms a viscous gel and subsequently (within 5–10 min) it becomes an aqueous suspension. At this point excess water or buffer can be added (but this is not necessary) and the suspension evaporated for an additional 15 min at 20°C to remove traces of solvent. When lipid mixtures lacking cholesterol are used at low concentrations (<7.5 μmol of lipid per ml of aqueous phase) the gel phase may not be apparent since the system rapidly reverts to a lipid-in-water suspension. The preparation is then either dialyzed, passed through a Sepharose 4B column, or centrifuged to remove nonencapsulated material and residual organic solvent.

A typical preparation contains 33 μmol of phospholipid and 33 μmol of cholesterol in 1.0 ml of aqueous phase (phosphate-buffered saline) and 3 ml of solvent. These ratios must be maintained for maximal captures, but they can be scaled down or up without any change in the characteristics of the resulting vesicles. When vesicles are formed from Pal_2PtdCho, an additional 3 ml of chloroform or 0.8 ml of methanol is added to the preparation, and the vesicles are allowed to remain at 45°C for at least 30 min after evaporation of the solvent.

To determine the amount of encapsulated small molecules such as sodium, sucrose, or [^{3}H]ara C, we dialyzed the vesicles overnight against 300 vol of phosphate-buffered saline (three changes) at 4°C. Encapsulated iodinated proteins are separated from unencapsulated proteins by column chromatography on Sepharose 4B (1.5 × 42 cm). Encapsulated [^{3}H]poly(A) can be separated from unencapsulated material by centrifugation at 100,000 × *g* for 30 min and resuspension of the pelleted vesicles in buffer (twice); the unencapsulated poly(A) remains in solution. Encapsulated 25S [^{32}P]RNA is separated from unencapsulated material by first treating with RNase (50 μg) and alkaline phosphatase (10 μg) and then separating the encapsulated material from the hydrolyzed RNA on a Sepharose 4B column as above. The unencapsulated RNA is totally degraded by this procedure. The latency of alkaline phosphatase is established by measuring enzyme activity (19) in the presence and absence of 0.1% Triton X-100.

Freeze fracture and negative stain electron micrographs were prepared as described (6, 10). The size of the vesicles was also determined by a dynamic light scattering technique as described (20). Permeability experiments and preparation of SUV and MLV were carried out as described (6). The nomenclature for the vesicle preparations adheres to recent recommendations (see p. 367 in ref. 1).

RESULTS AND DISCUSSION

The size and organization of the REV prepared from PtdGro/PtdCho/cholesterol (1:4:5 mole ratio) are illustrated in Fig. 1*A*. The inclusion of ferritin in the preparation can be seen as small (150 Å) particles within the aqueous space both internally and externally. Smaller vesicles can be found within the interior aqueous space. Some vesicles appear to consist of a few concentric bilayers with a large internal aqueous space, while some appear as unilamellar. Electron micrographs of negative-stained vesicle preparations are shown in Fig. 1 *B–D*.

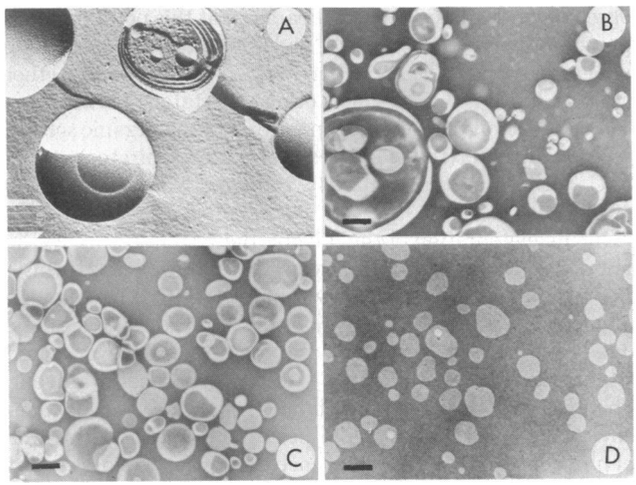

FIG. 1. (*A*) Freeze-fracture electron micrograph of PtdGro/PtdCho/cholesterol (1/4/5) REV containing ferritin. Caliper indicates 100 nm. (*B–D*) Negative-stain electron micrographs of REV prepared by the standard procedure: (*B*) PtdGro/PtdCho/cholesterol (1/4/5), a typical field of an unfiltered preparation; (*C*) PtdGro/PtdCho/cholesterol (1/4/5), a typical field of preparation filtered through a 0.2-μm Unipore filter; (*D*) PtdGro/PtdCho (1/4), a typical field of an unfiltered preparation. Bar indicates 200 nm.

Greater than 90% of REV composed of PtdGro/PtdCho/cholesterol (1:4:5) formed in 1/10 phosphate-buffered saline range from 200 to 1000 nm, with a mean size of 460 nm. Examination of the PtdGro/PtdCho/cholesterol REV containing carboxyfluorescein in the aqueous space under a fluorescent microscope revealed that the vast majority of the vesicles were under 1 μm, with only a rare larger structure observed. Filtration of these vesicles through a 200-nm Unipore filter gives a more uniform vesicle preparation of between 120 and 300 nm with no loss of lipid (Fig. 1*C*). Dynamic light scattering yielded a mean value of 300 nm for PtdGro/PtdCho/cholesterol REV formed in phosphate-buffered saline and 450 nm for vesicles of the same composition formed in 1/10 phosphate-buffered saline, in good agreement with the negative stain. These vesicles could be filtered through an 800-nm Unipore polycarbonate filter without loss of contents, while filtration through a 400-nm Unipore filter released 25–30% of the encapsulated (ara C) material, producing a more uniform sized vesicle preparation. All the lipid was recovered in the filtrate after these filtrations. These data, in conjunction with the freeze-fracture (Fig. 1*A*) and the negative-stain electron micrographs (Fig. 1*B*), suggest that REV composed of PtdGro/PtdCho/cholesterol (1:4:5) have a mean diameter between 200 and 500 nm. The size of the vesicles obtained by this method, however, seems to depend on the lipid composition and the solvent. REV formed by the standard technique and composed of PtdGro/PtdCho (1:4) range in size from 100 to 300 nm when viewed by negative-stain electron micrographs (Fig. 1*D*). REV composed of PtdGro have a mean diameter of 200 nm (obtained by negative-stain electron microscopy).

The ability of REV to encapsulate ara C depends on the ionic strength of the buffer (Fig. 2). As the ionic strength is increased, there is a decrease in both the percent encapsulation and the volume of encapsulated aqueous space per mole of phospholipid. High concentrations of sucrose, glycerol, or urea have no effect on the percentage encapsulation of ara C under similar conditions. We routinely encapsulate 30–45% of ara C from solutions containing 84 mg/ml in 15 mM NaCl buffer.

Varying the lipid concentration brings about an increase in the amount of capture up to a maximum of about 45% in 150

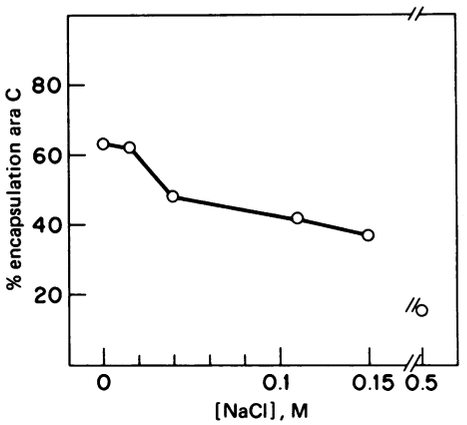

FIG. 2. Effect of ionic strength on encapsulation of ara C in PtdGro/PtdCho/cholesterol (1/4/5). REV were formed with 100 μmol of total lipid in 5 ml of diethyl ether and 1.5 ml of the appropriate ionic strength NaCl buffer containing 3 mM ara C.

mM NaCl (Fig. 3). Although the percentage of ara C encapsulated decreases with decreasing total lipid, the aqueous volume per mole of phospholipid is increased. It is 13.5 liters of water per mole of phospholipid at a total of 100 μmol of lipid and increases to 22.5 liters of water per mole of phospholipid when the total amount of lipid is reduced to 20 μmol. Table 1 gives the captured volume of aqueous phase per weight of total lipid, obtained for various preparations of REV and also of MLV and SUV. It is obvious from the values obtained with PtdGro/PtdCho/cholesterol that the REV capture a much larger volume of aqueous space (approximately 14 μl/mg) compared to MLV (approximately 4 μl/mg) and SUV (approximately 0.5 μl/mg) of comparable composition. These calculations on trapped volume are based on the specific activity of ara C. Under these conditions, ara C or sucrose added to preformed vesicles does not give any measurable binding after equilibrium dialysis against the initial buffer. Similar results on trapped volume are obtained with sodium and other nonpermeable molecules such as polynucleotides.

Calculations of the expected diameter of the PtdGro/PtdCho/cholesterol vesicles based on the entrapped values indicated in Table 1 give a value of 340 nm, which compares favorably with the value obtained from light-scattering mentioned earlier (450 nm). This calculation is based on the following: a phospholipid surface area of 72 Å^2 per molecule, condensed to 58 Å^2 in the presence of cholesterol (4); a cholesterol surface area of 38 Å^2; and a uniform population of spherical unilamellar vesicles of a diameter $D = 12\ V/A$, where

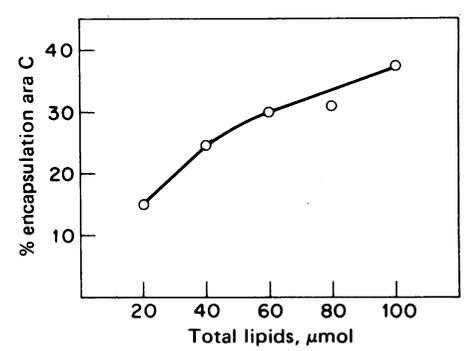

FIG. 3. Effect of lipid concentration on the encapsulation of ara C in PtdGro/PtdCho/cholesterol (1/4/5). REV were formed with the indicated concentration of lipid in 5 ml of diethyl ether and 1.5 ml of phosphate-buffered saline containing 3 mM ara C.

V is the total encapsulated volume and A is the total surface area of a lipid monolayer. We have neglected the actual thickness of the lipid bilayer and any difference in surface area between inside and outside monolayers. Similar calculations based on the capture of REV preparations of PtdGro (based on an area per molecule of 72 Å^2) gives a value of 200 nm, which is in good agreement with the value obtained by negative stain. The proximity of the calculated and observed diameter values indicates that most of the vesicles must be unilamellar.

Different lipid compositions at similar preparations of lipid/solvent/buffer have various effects on the percentage encapsulation (Table 1). The REV method as described here was developed for maximal encapsulation with PtdGro/PtdCho/cholesterol. However, stearylamine/PtdCho/cholesterol (Table 1) and PtdSer/PtdCho/cholesterol (1:4:5 molar ratio, data not shown) behave similarly in terms of capture of ara C and sucrose. The captured volumes obtained by PtdGro/PtdCho/cholesterol prepared as MLV and as SUV are also given for comparison. As can be seen in Table 1, the inclusion of cholesterol produces a significant increase in the captured volume compared to PtdGro/PtdCho vesicles and this is probably due to the increased size of the PtdGro/PtdCho/cholesterol vesicles (Fig. 1 *B* and *D*). The smaller captured volume obtained with pure PtdGro is also related to the smaller size vesicles produced with this lipid. Pure Pal_2PtdCho (35% encapsulation) and sphingomyelin (24% encapsulation) also show smaller captured volumes than PtdGro/PtdCho/cholesterol although it has not been determined whether this is also due to smaller vesicles. It is possible that variation of the lipid/solvent/buffer ratios or a different solvent can be used to maximize captured volume with each individual lipid.

Although we have not determined the amount of solvent remaining in REV after an overnight dialysis, Deamer (21) has investigated this in vesicles prepared by the ether infusion technique and could not detect any ether in the vesicles after dialysis. Examination of the permeability properties of REV to sodium and ara C reveals a similarity to previous studies on MLV and SUV for these compounds (Table 2) (6, 22).

The utility of these vesicles in entrapping various compounds is illustrated in Table 3. To achieve maximal encapsulation of aqueous space it is necessary to use a buffer of low ionic strength. Alkaline phosphatase was totally latent when encapsulated in this way. To observe activity it was necessary to solubilize the REV with 1% Triton X-100. The aqueous fluorescent probe carboxyfluorescein remained highly quenched when encapsulated in REV, but underwent a 55-fold enhancement when the REV was lysed by 1% Triton X-100. In preliminary experiments the REV procedure appears suitable for the encapsulation of particulate material such as bacteria and mammalian chromosomes.

Table 2. Permeability of REV composed of PtdGro/PtdCho/cholesterol (1:4:5) to low molecular weight compounds

	% entrapped material released per hr at		
	10°C	20°C	37°C
^{22}Na: 1st hr	0.069	0.143	0.353
2nd hr	0.043	0.096	0.199
3rd hr	0.041	0.087	0.151
Ara C: 1st hr	0.376	0.697	1.97
2nd hr	0.221	0.613	1.69
3rd hr	0.195	0.649	1.54

Mean of duplicate measurements that agree to within 10%. REVs, dialyzed overnight to remove unencapsulated material, were placed in dialysis bags and dialyzed against three consecutive 10-ml portions of phosphate-buffered saline for 1 hr each.

Table 3. Encapsulation of various molecules in PtdGro/PtdCho/cholesterol (1:4:5) REV

Encapsulated material	Buffer	% encapsulation
Sodium	PBS	42
Carboxyfluorescein	1/10 PBS	57
Poly(A)	PBS	24
Poly(A)	1/10 PBS	43
25S RNA	1/10 PBS	40
Insulin	PBS	34
Ferritin	1/10 PBS	54
Alkaline phosphatase	PBS	34
Albumin	PBS	38
IgG	1/10 PBS	28–40

The preparation contained the compound to be encapsulated in 1.5 ml of either phosphate-buffered saline (PBS) or a 1/10 dilution of phosphate-buffered saline, 5 ml of diethyl ether, and 100 μmol of total lipid. The concentrations of the encapsulated material were as follows: sodium, 0.15 M; carboxyfluorescein, 0.01 M; poly(A), 3 mg/ml; 25S RNA, 1 mg/ml; insulin, 1 mg/ml; and ferritin, alkaline phosphatase, albumin, and IgG, 10 mg/ml.

A schematic diagram of the processes that might be occurring during formation of REV is shown in Fig. 4. Although we have not investigated the properties of the system by any physical means, we suggest that the initial sonication of the buffered aqueous phase in the organic solvent in the presence of the amphiphatic phospholipid molecules produces small water droplets stabilized by a phospholipid monolayer (panel 3). Such droplets or "inverted micelles" are collapsed into a viscous gel-like state when the organic phase is removed by evaporation (panel 4). The critical point in this procedure is probably when the gel state collapses (panel 5). At this point some of the inverted micelles disintegrate, releasing their encapsulated material; the excess lipid contributes to a complete bilayer around the remaining micelles, resulting in the formation of vesicles (panel 6). If we add [^{14}C]sucrose to the preparation when it is in the gel state, only 5% is incorporated into the vesicles. This suggests to us that only material previously entrapped in inverted micelles is incorporated into the final vesicle. It is obvious that a large number of variables may be responsible for determining the final product in terms of vesicle size. These include the type of phospholipid and its solubility in the organic solvent, the interfacial tension between aqueous buffer and organic solvent, and the relative amounts of water phase, organic solvent, and phospholipids. The specific solvents and relative ratios of solvent/water/lipid reported here represent a system maximized for the highest possible capture efficiency with PtdGro/PtdCho/cholesterol. These optimal conditions were

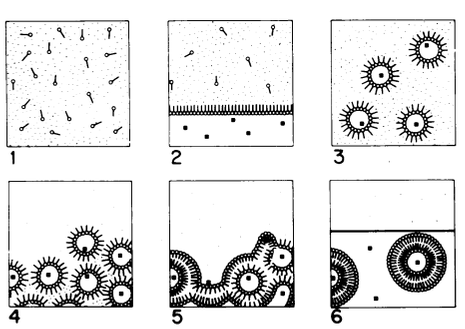

FIG. 4. Diagram of the formation of REV. Dissolved lipids in appropriate solvents, lipids indicated by lollipop structures; addition of aqueous phase containing compound to be encapsulated, indicated by filled square. See text for complete description.

Table 4. Summary of size, encapsulation efficiency, and internal volume of different liposome preparations

Liposomes	Encapsulation efficiency*	Captured volume,† μl/mg	Diameter,‡ nm (range)
REV (PtdGro/PtdCho/ Chol, 1:4:5)	35–65	13.7	200–1000
REV (PtdGro/PtdCho, 1:4)	30–45	8.1	100–300
MLV (PtdGro/PtdCho/ Chol, 1:4:5)	5–15	4.1	400–3500
SUV (PtdGro/PtdCho/ Chol, 1:4:5)	0.5–1	0.5	20–50
LUV (PtdSer)	5–15	9.1	200–1000

Values for this table were determined from the appropriate vesicle preparation described in Table 1 or for large unilamellar lipid vesicles (LUV) (10). Values given are for unfiltered preparations, at 66 μmol/ml.

* Encapsulation efficiency for water-soluble, low molecular weight compounds. The ratio of the amount of material remaining with the vesicles after separation from the unencapsulated material to the amount of material present for the encapsulation times 100.

† Captured volume per mg of lipid. The ratio of the equivalent volume of aqueous space containing the encapsulated compound to the weight of lipid in the preparation.

‡ Size range that included 90% of the vesicles; determined by negative-stain electron microscopy.

arrived at after systematic evaluation of the effect of various solvents and relative ratios of solvent/water/lipid.

The system we are describing produces large (200–500 nm, mean diameter) uni- and oligolamellar vesicles from a variety of phospholipids and other lipids, with a high capture efficiency for various solutes and macromolecules and ease of preparation. Furthermore, the process can be easily scaled up to large volumes and the vesicles can be sterilized by passage through a 200-nm filter. A summary of the size ranges, internal volumes, and reported encapsulation efficiencies for different liposome preparations is given in Table 4.

It is possible that exposure to organic solvents might denature proteins that are particularly labile under these conditions. Our experience with alkaline phosphatase indicates, however, that an appreciable (41%) amount of activity can be retained. Nucleic acids are often isolated in the presence of organic solvents such as phenol or ethanol. This technique might therefore be valuable for those interested in encapsulating either mRNA or DNA and introducing them into cells either *in vitro* or *in vivo*. In this regard previous attempts to encapsulate poly(I·C) in MLV (23) or in LUV (24) have resulted in only 5–10% encapsulation. A small virus has been encapsulated in LUV (25) with a 10% encapsulation efficiency, and a recent report has demonstrated a 5% entrapment of *E. coli* RNA in vesicles by the ether infusion technique (26). For comparison, using the REV we have been able to encapsulate 43% of poly(A) and 40% of a 25S rRNA. This is almost a 10-fold improvement in the efficiency of encapsulation. Such results suggest that this simple method for forming large uni- and oligolamellar vesicles (REV) with high capture efficiency could be an important technique for the introduction of various pharmacological and biochemical agents into biological systems *in vitro* and *in vivo*.

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