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# PREPARATION OF MULTIVESICULAR LIPOSOMES

SINIL KIM *, MITCHELL S. TURKER, EMIL Y. CHI, SHIFRA SELA and GEORGE M. MARTIN

*Division of Genetic Pathology and Center for Inherited Diseases, University of Washington, Seattle, WA 98195 (U.S.A.)*

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**A novel type of liposome, named here multivesicular liposomes, was prepared by evaporation of organic solvents from chloroform-ether spherules suspended in water. Within each spherule were numerous water droplets that contained solutes to be trapped in liposomes upon solvent evaporation. Liposome preparations of different average diameters were made, varying from 29 ± 10 μm to 5.6 ± 1.7 μm. The liposomes were morphologically characterized by light microscopy and transmission electron microscopy. Materials successfully trapped within the liposomes ranged in molecular size from glucose to nucleic acids. Extremely high percentages of encapsulation (up to 89%) were achieved.**

## Introduction

We have recently described a relatively simple method of making cell-size unilamellar lipsomes [1]. We now report a modification of the procedure which yields liposomes of entirely different morphology that we propose to call multivesicular liposomes.

## Materials and Methods

### *Lipids and other materials*

Soy phosphatidylcholine (PC), dioleoylphosphatidylcholine (DOPC), crude phosphatidylcholine (cPC, Sigma Type IV-S), dipalmitoylphosphatidylcholine (DPPC), cholesterol (C), phosphatidylethanolamine (PE), cardiolipin (CL), trioctanoin (TC), triolein (TO), bovine superoxide dismutase, poly(adenylic acid), and Arsenazo III dye were purchased from Sigma, St. Louis, MO. Phosphatidylglycerol (PG), dimyristoylphosphatidic acid (DMPA), dipalmitoylphosphatidylglycerol (DPPG), and phosphatidylserine (PS) were from CalBiochem, La Jolla, CA. Chloroform was from MCB reagents, Norwood, Ohio and diethyl ether was from J.T. Baker Co., Phillipsburg, NJ. Ethylenediaminetetra[2-$^{14}$C]acetic acid (EDTA), [3′,5′,7-$^{3}$H]methotrexate, poly([8-$^{3}$H]adenylic acid), [5-$^{3}$H]cytosine arabinoside (ara-C), and [U-$^{14}$C]sucrose were from Amersham, Arlington Heights IL. [*methyl*-$^{3}$H]Thymidine 5'-triphosphate, [U-$^{14}$C]glucose, [1,2-$^{3}$H]cholesterol, di[1-$^{14}$C]oleoylphosphatidylcholine, and Triton X-100 were from New England Nuclear, Boston, MA. Ponceau S dye was from Reeve Angel Ltd., London; methotrexate was from Lederle, Wayne, NJ; Ara-C was from UpJohn, Kalamazoo, MI; and 4(5)-carboxyfluorescein was from Eastman Kodak, Rochester, NY. All phospholipids were kept at −20°C under nitrogen in glass vials with aluminum-lined screw caps until use. Glassware was used exclusively for all steps involving lipids and organic solvents. All commercially available materials were used without further purification. [*methyl*-$^{3}$H]Thymidine-DNA and unlabelled DNA

* Present address: University of California Irvine Medical Center, Department of Internal Medicine, 101 City Drive, Orange, CA 92668, U.S.A.

were isolated from a HeLa cell line by the method of Pellicer et al. [2]. 1-dram vials (with screw caps) were from Acme Glass Co., Los Angeles, CA. The mechanical shaker was a vortex mixer ('Vari-whirl') purchased from Van Waters and Rodgers Inc. Millipore filters were from Millipore Corp., Bedford, MA.

*Preparation of water-in-lipid emulsion*

The lipid phase was made by combining the amphipathic lipids (see Table I) totaling 10 $\mu$mol plus a small amount of a neutral oil (triolein or trioctanoin) in a 1-dram glass vial (1.4 cm $\times$ 4.5 cm in external dimensions) fitted with a screw cap lined with aluminum foil, and then adding chloroform and diethyl ether in a 1 : 1 ratio to bring the volume to 1 ml.

The aqueous phase was prepared by dissolving the material to be trapped in 0.15 M aqueous sucrose solution or in salt solution (see items 9 and 10 in Table II). 1 ml of the aqueous phase was added slowly (over a period of 5 s) into the lipid phase while the vial was being gently hand-shaken. The vial was capped, shaken vigorously by hand several times, and flushed with nitrogen gas. The vial was then attached to the head of a standardized vortex machine with an adhesive tape (see caption for Fig. 4) and shaken for 9 min to form the water-in-lipid emulsion.

*Preparation of chloroform-ether spherules*

A Pasteur pipet with a narrowed tip was prepared by drawing out the pipet into a fine capillary over a flame, and then breaking off the capillary so that the tip aperture was approx. 0.2 to 0.4 mm in diameter. 2.5 ml of 0.2 M sucrose solution containing no other solute was placed in each of two new 1-dram vials. 1-ml aliquots of the water-in-lipid emulsion, prepared as above, were drawn into a Pasteur pipet with the narrowed tip and placed rapidly into each of the two vials containing 0.2 M sucrose. Each vial was attached to the head of the standardized vortex machine and shaken for 10 s or for various durations in order to make liposomes of various sizes (Figs. 4, 6 and 7). Two vials with exactly the same volumes were used in this step for every preparation in order to keep the parameters of mechanical shaking constant.

*Evaporation of chloroform and ether to form liposomes*

The two vials of chloroform-ether spherules suspended in 0.2 M sucrose were layered on the bottom of a 250 ml Erlenmeyer or filtration flask (bottom diameter, 8 cm). A stream of nitrogen gas at 8 l/min was introduced into the flask through a piece of glass tubing protruding 5 cm into the mouth of the flask. The flask was gently agitated continuously to prevent the spherules from settling. During the organic solvent evaporation, the flask was kept in a warm water bath (30–35°C). After about 5 min, the turbidity of the suspension decreased, indicating near completion of solvent evaporation. The evaporation was than allowed to proceed for a few more minutes. No odor of chloroform or ether was detectable at this time. (See Fig. 1 for a schematic representation of multivesicular liposome preparation).

For the separation of liposomes from untrapped material and lipid debris, an equal volume of a 5% glucose (w/v) solution or of phosphate-buffered saline (0.15 M, pH 7.4) was added to the liposome preparation. The liposomes were pelleted gently in a clinical centrifuge at 600 $\times$ *g* for 5 min. The supernate was removed and the liposomes were resuspended in 5% glucose or in phosphate-buffered saline.

Capture efficiencies were calculated from measurements of the amounts of material in the supernates and in the pellets. The captured volume was determined as the volume of the original aqueous solution trapped per unit quantity of the total lipids initially used. Percent encapsulated is the proportion of the original aqueous solution that is trapped within the liposomes.

*Gas chromatography*

For quantitation of ether and chloroform in the final liposome preparation, a Hewlett/Packard 5840 B gas chromatograph apparatus was used. A 30 meter J & W 0.25 mm internal diameter Carbowax fused silica capillary column employed helium as carrier gas at 20 cm/s. The initial temperature of the column was 35°C, maintained for 1 min, then elevated by 5 K/min. For standardization, 1 $\mu$l of saturated vapor from the solvents was used.

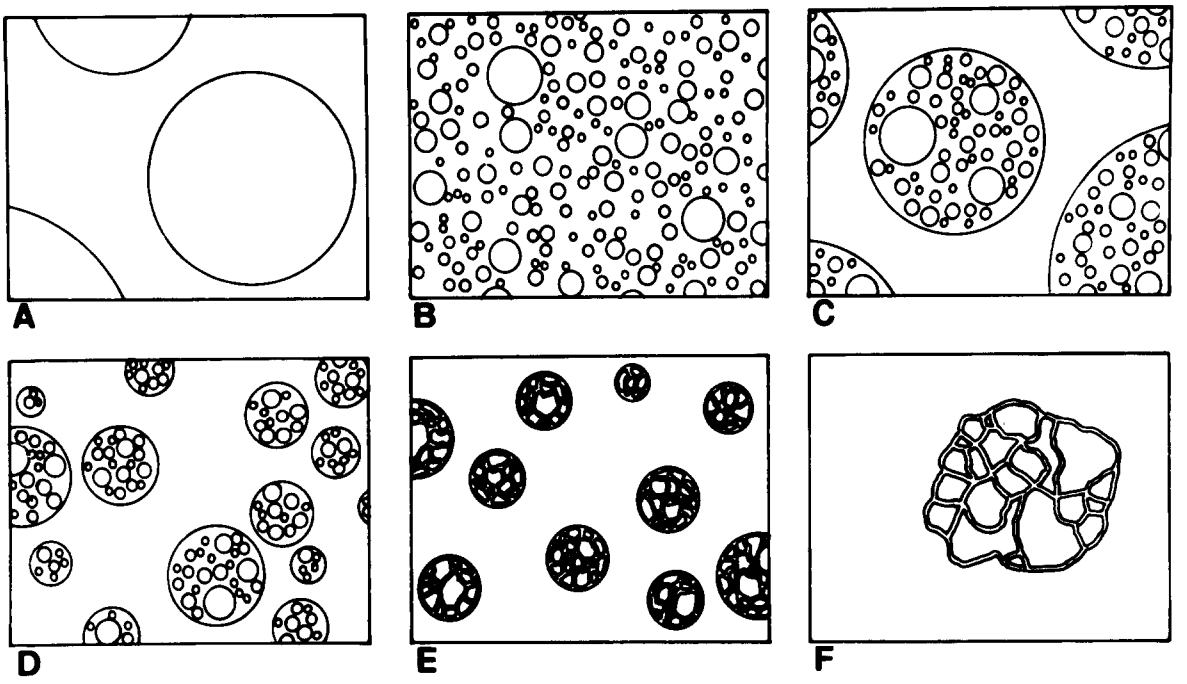



Fig. 1. Schematic representation of multivesicular liposome formation. Panel A depicts the initial water-in-lipid emulsion. B shows the water-in-lipid emulsion after 9 min of mechanical shaking. C shows the initial formation of chloroform-ether spherules by addition of the water-in-lipid emulsion to a 0.2 M sucrose solution. D shows the establishment of spherule size by the second mechanical shaking. E represents liposome formation via evaporation of the chloroform and ether, resulting in thinning of the lipid phase to a bilayer membrane as the solvents are removed. F is an enlarged represention of the final multivesicular liposome product, obtained by tracing an actual electron micrograph. Each line represents a monolayer of amphipathic lipids; those in panels A–D represent lipid monolayers at the interphase separating water and lipid phases, and those in panels E and F represent monolayers of bilayer unit membrane.

## Results

Many different lipid combinations successfully formed liposomes (Table I), including crude lipid extracts. In those preparations in which purer lipids were used, four lipid types were needed in each preparations. An amphipathic lipid with a net neutral charge, one with a negative charge, cholesterol, and a triacylglycerol, were all necessary for high capture efficiency. The concentration of the triacylglycerol, e.g. triolein, could be reduced down to a mole fraction of 0.01 in PC/C/CL/TO(4.5 : 4.5 : 1 : $X$) lipid combinations, but at lower fractions the capture efficiency suffered markedly (Fig. 2).

The effect of the amount of lipid (while keeping the ratio of lipids constant) on capture efficiency was studied in crude PC/C/TC (4.1 : 1.9 : 1, in weight ratio) lipid combinations. As the amount of lipid was increased, the percent encapsulated increased until it reached a plateau, while the captured volume ($\mu$l/mg) decreased steadily (Fig. 3).

The amount of the two major lipids in the initial lipid mixture DOPC/C/PS/TO (4.5 : 4.5 : 1 : 1, $\mu$mol per preparation) that became a part of the liposome was estimated by including $^{14}$C-labelled DOPC and tritiated cholesterol in the lipid mixture. Results showed 62% of labelled DOPC and 52% of tritiated cholesterol in the liposome pellet; 34% and 48% of the respective tracer lipids were in the supernate. This indicates that a significant amount of the original lipids used was lost in the supernate as lipid debris. We have not excluded the amount of lipids in the debris for the calculation of captured volume; doing so would have given higher captured volumes. For example, DOPC/C/PS/TO system gave captured volume of approx. 106 l/mol when

TABLE I

LIPOSOMES OF VARIOUS LIPID COMPOSITION

Capture efficiencies expressed in two ways, as the captured volume and as the percent encapsulated, were measured by trapping Ponceau S dye (0.1%). The standard procedure described in the text was followed, using the amount of lipids indicated in the table. Captured volume is expressed as μl/mg and/or l/mol, and is the volume of the original aqueous solution trapped per unit quantity of the total lipids initially used. Percent encapsulated is the proportion of the original aqueous solution that is trapped within liposomes.

| Lipids | mg or μmol of respective lipids used per preparation | Captured volume | | Percent encapsulated |
|---|---|---|---|---|
| | | μl/mg | l/mol | |
| cPC/C/TC | 4.1/1.9/1 [a] | 119 | – | 84 |
| cPC/C/TC | 4.1/1.9/6.6 [a] | 65 | – | 82 |
| PC/C/CL/TO | 4.5/4.5/1/1 | 100 | 72 | 80 |
| PC/C/CL/TC | 5/4.5/0.25/1 | 84 | 52 | 56 |
| PC/C/PG/TC | 5/4/1/1 | 113 | 71 | 78 |
| PC/C/DPPG/TC | 5/4/1/1 | 101 | 66 | 72 |
| DOPC/C/CL/TO | 4.5/4.5/1/1 | 101 | 73 | 80 |
| DOPC/C/PS/TO | 4.5/4.5/1/1 | 98 | 63 | 69 |
| DPPC/C/CL/TO | 4.5/4.5/1/1 | 15 | 10 | 11 |
| PE/C/CL/TO | 4.5/4.5/1/1 | 109 | 76 | 84 |
| PE/C/PS/TO | 4.5/4.5/1/1 | 127 | 79 | 87 |
| PC/C/PS/TO | 4.5/4.5/2/1 | 102 | 68 | 81 |
| PC/C/DMPA/TO | 4.5/4.5/1/1 | 86 | 54 | 59 |

[a] Units in mg, all subsequent units in μmol.

the lipids in the debris were excluded from calculation, in comparison to 63 l/mol (Table I) when the lipids in debris were not excluded.

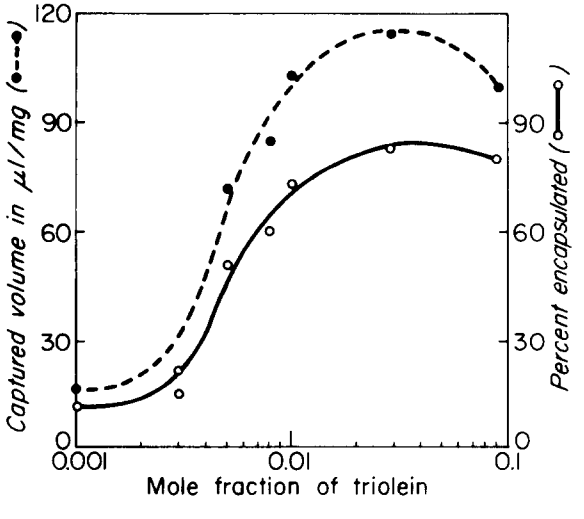



Fig. 2. Effect of triolein mole fraction upon captured volume and percent encapsulated. The lipid combination of PC/C/CL/TO in amounts of 4.5:4.5:1:$X$ μmoles, respectively, was used. Note log scale of abscissa.

A wide variety of substances was incorporated into liposomes, ranging from small molecules to macromolecules (Table II). We were able to trap an enzyme, bovine superoxide dismutase, with retention of 100% of its enzyme activity.

Items 9 and 10 in Table II show the effect of

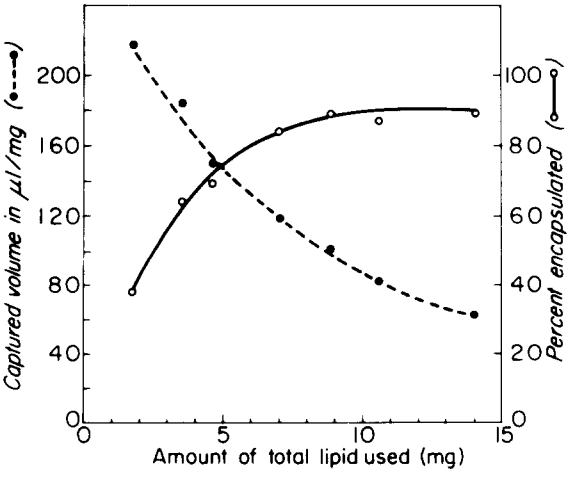



Fig. 3.Effect of varying the amount of total lipids used. ●, Captured volume in μl/mg; ○, percent encapsulated.

TABLE II

VARIOUS MATERIALS INCORPORATED INTO LIPOSOMES

Lipid combinations of PC/C/CL/TO (4.5 : 4.5 : 1 : 1, μmol, respectively) were used in each preparation. The standard method described in the text was used except for item 10, where the liposomes were centrifuged up to the top of the centrifuge tube through a 0.5 cm thick layer of solution containing 100 mM sucrose and 140 mM glucose. 4(5)-Carboxyfluorescein, Arsenazo III, and Ponceau S dyes were quantitated by measuring the absorbance at 470 nm, 560 nm, and 510 nm, respectively, after clarification with 0.8% Triton X-100. For 4(5)-carboxyfluorescein, the liposome and supernate samples were diluted so that the absorbance readings were approximately the same for both samples. Superoxide dismutase was assayed by the method of Marklund and Marklund [24] after disrupting the liposome membranes with distilled water and filtering through 0.22 μm Millipore filters. Radioactive tracers were used for quantitation of other materials. PBS, phosphate-buffered saline.

| Materials trapped | Concn. | Captured volume | | Percent encapsulated |
|---|---|---|---|---|
| | | μl/mg | l/mol | |
| 1 Glucose | 30 mM | 73 | 53 | 58 |
| 2 Sucrose | 150 mM | 81 | 58 | 64 |
| 3 Ara-C | 1 mg/ml | 26 | 19 | 21 |
| 4 Methotrexate | 1 mg/ml | 67 | 48 | 53 |
| 5 Thymidine triphosphate | tracer | 81 ± 3 | 59 ± 2 | 65 ± 3 |
| 6 4(5)-Carboxy-fluorescin | 40 mM | 69 | 50 | 55 |
| 7 Arsenazo III | 0.02% | 86 | 62 | 69 |
| 8 Ponceau S | 0.1% | 100 ± 8 | 72 ± 6 | 80 ± 6 |
| 9 Ponceau S | 0.1% (in 25 mM PBS & 100 mM sucrose) | 103 | 75 | 82 |
| 10 Ponceau S | 0.1% (in 75 mM PBS) | 67 | 48 | 53 |
| 11 EDTA | 0.015 M | 47 | 34 | 37 |
| 12 Superoxide dismutase | 1 mg/ml | 38 | 27 | 30 |
| 13 Poly(adenylic acid) | 0.1 mg/ml | 83 | 60 | 66 |
| 14 DNA, human | 0.1 mg/ml | 103 | 74 | 81 |

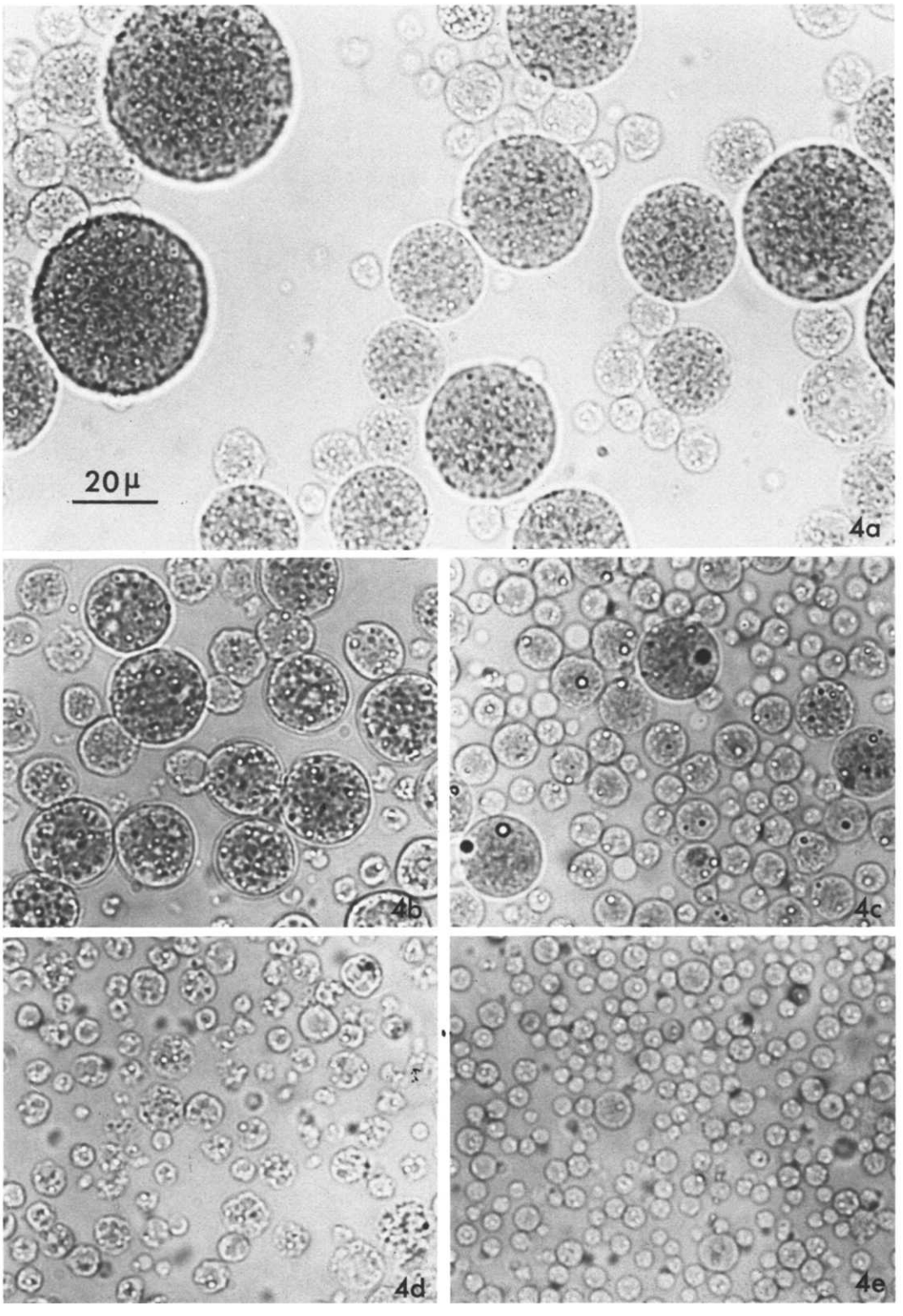
ionic strength on capture efficiency. It is possible to use salt solutions to form liposomes, although at reduced capture efficiency.

Morphologically, the liposomes are spheroids with granular internal structures under the light microscope (Fig. 4). The details of the membrane structure are seen best with transmission electron microscopy (Fig. 5). A bilayer forms the outermost membrane and the internal space is divided up into numerous compartments by bilayer septums.

The average size of liposomes can be varied by changing the size of chloroform-ether spherules, which in turn is controlled by the duration of the mechanical shaking. Fig. 4 shows the micrographs and Fig. 6 depicts the volume-adjusted size distributions for liposome preparations of various average sizes. Fig. 7 show the relationship between duration of mechanical shaking, average size, and the capture efficiency.

Osmosensitivity was studied by measuring the change in the average size of liposomes in various concentrations of glucose (Fig. 8). The data points fit fairly well with the ideal curve, in which the average volume is inversely proportional to osmotic pressure.

The liposomes are quite stable. We have kept liposomes PE/C/PS/TO, (4.5 : 4.5 : 1 : 1, μmoles) containing Ponceau S dye in a refrigerator at 3°C with retention of 95% of the dye within liposomes and preservation of liposome morphology after 9


20μ
4a
4b
4c
4d
4e

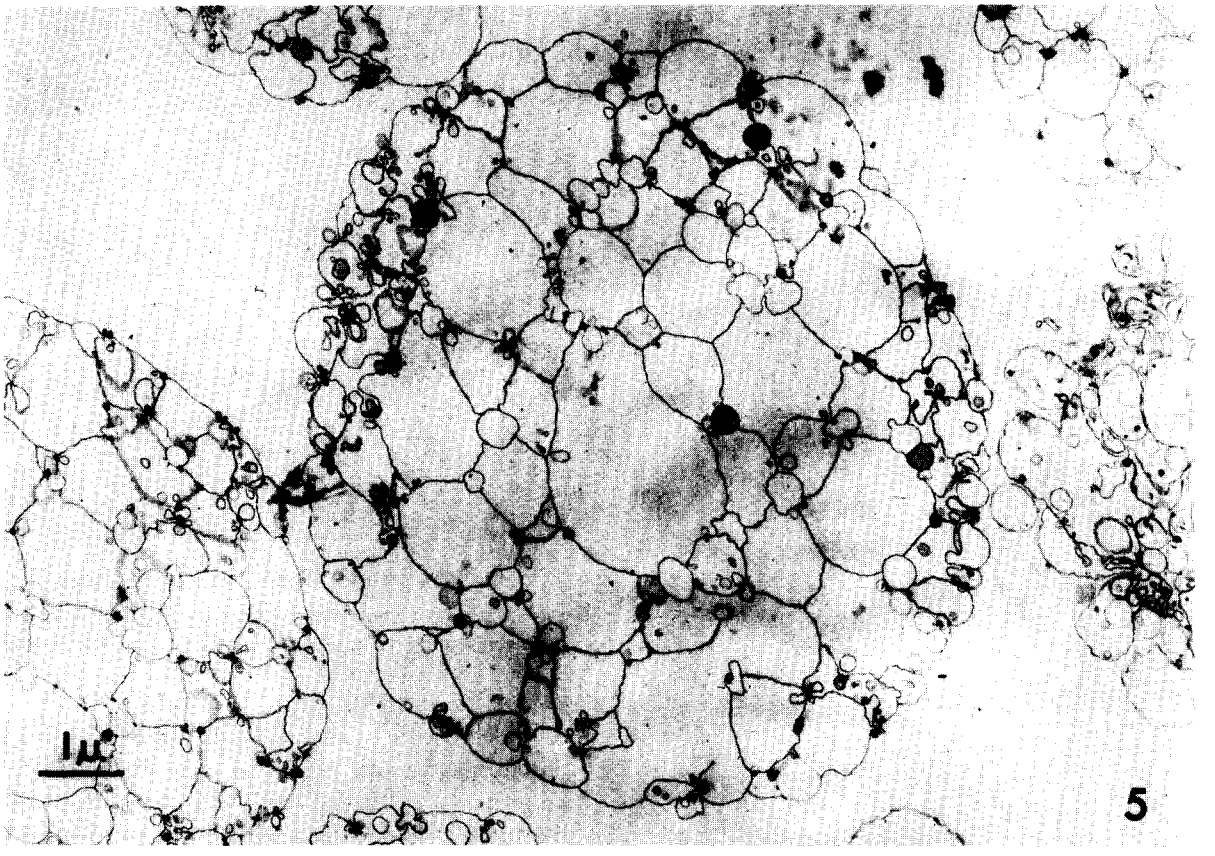



Fig. 5. Thin-section transmission electron micrograph. The liposomes were fixed with osmium tetroxide in 5% dextrose, stained with uranyl acetate, embedded in 2% agar, dehydrated with ethanol, and then re-embedded in epon. Scale bar, 1 μm.

days of storage.

In a typical liposome preparation, no chloroform or ether was detectable by gas chromatography, indicating concentrations of less than 1 part per million (ppm) for ether and less than 2 ppm for chloroform.

## Discussion

Since Bangham first made liposomes [3,4], many types of liposomes and methods of preparation have been reported [5–22]. Prior to this report, all previous liposomes were categorized into two major classes, the unilamellar and multilamellar liposomes. As the names indicate, the unilamellar liposomes have a single bilayer membrane enclosing an aqueous volume, and the multilamellar liposomes have numerous concentric membranes. Multivesicular liposomes fit into neither of these two types and are in a class of their own. Multivesicular liposomes are also distinct from unilamellar liposomes attached to each other in a manner, for

Fig. 4. Light micrographs of multivesicular liposomes of various average sizes. PC/C/CL/TO (4.5 : 4.5 : 1 : 1, μmoles) were used in each preparation. The duration of the second mechanical shaking on a standardized vortex machine was 1 s, 3 s, 10 s, 45 s, and 180 s for a, b, c, d, and e, respectively. The intensity of mechanical shaking on the vortex mixer was standardized with a 1-dram vial containing 0.1 ml chloroform and 0.33 μmol of lipids (PC/C/CL/TO in 4 : 4 : 1 : 1 molar ratio) and 4 ml of 0.2 M sucrose in water. The vial was attached horizontally to the machine head with a piece of adhesive tape. The machine was set to that after 60-s shaking of the standardization vial, chloroform particles of $7.8 \pm 2.9$ μm diameter (volume adjusted) were produced. Scale bar is 20 μm. The letters correspond to those in Fig. 6 and photomicrographs like these were used for determinations of size distribution.

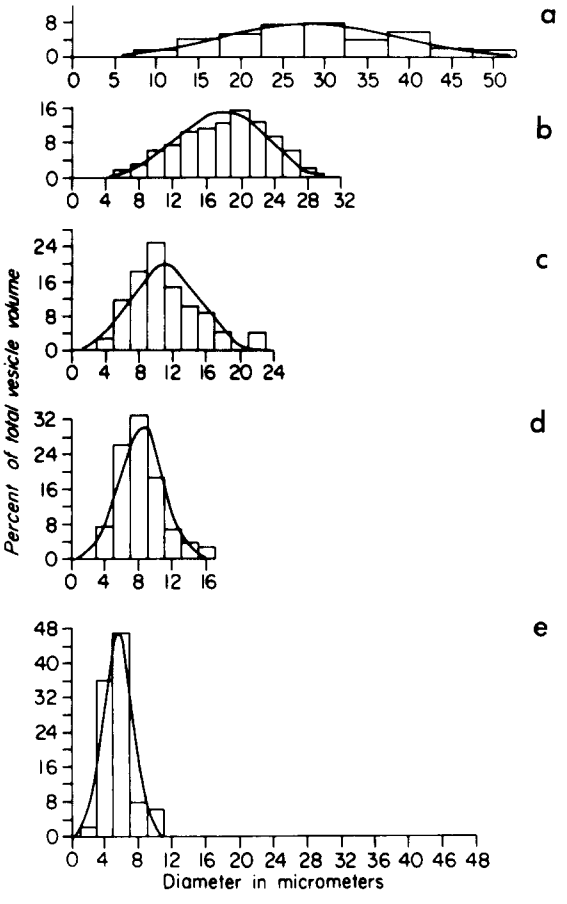



Fig. 6. Size distributions of five separate preparations of liposomes. Light photomicrographs of liposomes in 5% glucose medium, wet mounted on a hemocytometer, were taken on a Zeiss photomicroscope. The magnification was obtained from hemocytometer grid lines. Diameters of 500 liposomes were measured from micrographs of each preparation, and the liposomes were assigned to the nearest 5 $\mu$m (a) or 2 $\mu$m (b–e). The number of liposomes in each size group was multiplied by the cube of the diameter to obtain relative volumes and then divided by the sum of the relative volumes to obtain percent of total volume. From this volume-adjusted size distribution, the mean and standard deviation was calculated. Gaussian function curves were obtained from the means and the standard deviations for comparison.

example, analogous to embryonic blastulae; the wall separating one internal aqueous compartment from another is a single bilayer in multivesicular liposomes rather than two bilayers in close contact.

The method of multivesicular liposome preparation described in this report is a modification

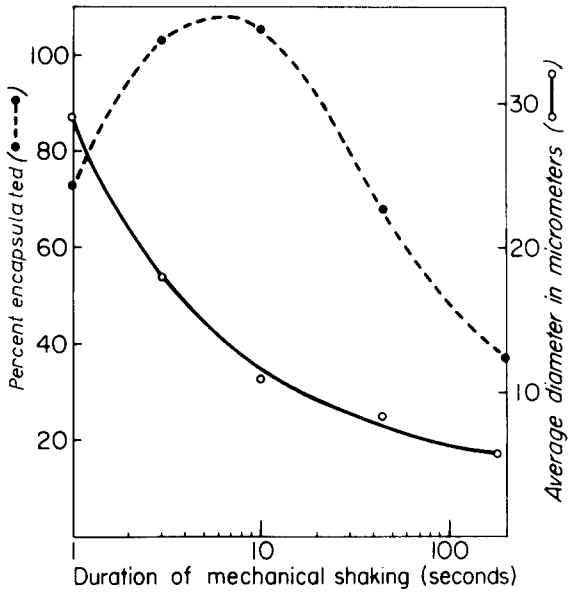



Fig. 7.The relationships between duration of mechanical shaking, average diameter of liposomes and the capture efficiency.

of the procedure for the preparation of cell-size unilamellar vesicles previously described from our laboratory [1]. In essence, the duration of the mechanical shaking to make the water-in-lipid emulsion was lengthened to make the water droplets in the emulsion comparatively small, so that multiple droplets were included within the chloroform-ether spherule; when the solvents were evaporated, multiple compartments within liposome resulted. In the original procedure [1], in contrast, the size of the internal water droplet approximated the size of the overall chloroform-ether spherule.

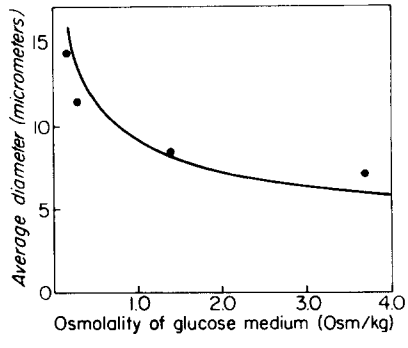



Fig. 8. The effect of osmolality of the medium on the average size of a liposome preparation.

Conceivably, it would be possible to achieve 100% encapsulation by this method of liposome preparation since all the initial aqueous volume is 'trapped' within the lipid phase when the water-in-lipid emulsion is first prepared. The losses in percent encapsulated occur at several points in the preparation. The first major loss occurs when the water-in-lipid emulsion is placed in the 0.2 M sucrose and mechanically shaken to make the chloroform-ether spherules. Some of the internal aqueous compartments burst through the lipid phase and join the external aqueous phase, carrying with them the solute intended to stay within liposomes. The loss in percent encapsulated at this point is minor, as 97.7% of the initial aqueous volume containing Ponceau S dye is found within chloroform-ether spherules when they are centrifuged. A more significant reduction in percent encapsulated occurs during evaporation of solvents when the lipid phase separating the internal aqueous from external aqueous compartment from external aqueous phase thins to a bilayer membrane. At this step the percent encapsulated decreased to a final value of 85.5% in a typical preparation using PC/C/CL/TO (4.5 : 4.5 : 1 : 1, $\mu$moles).

As one would expect, the lengthening of the duration of mechanical shaking (to make smaller liposomes) increases the probability that internal aqueous compartments will burst; this explains the descending portion of the capture efficiency curve in Fig. 7. It also explains the higher percent encapsulated for multivesicular liposomes as compared to cell-size unilamellar vesicles, since those internal aqueous compartments located away from the surfaces of liposomes are protected from bursting. It is expected from the fact that much of the lipid is located in the interior of multivesicular liposomes, that the captured volume (excluding lipids in debris) for multivesicular liposomes would be in a much smaller range than that for cell-size unilamellar vesicles. Comparing with previously published figure for cell-size unilammelar vesicles, a typical captured volume of 106 l/mol (see results section) for multivesicular liposomes is indeed in a much smaller range than 243 l/mol for the cell-size unilamellar vesicles [1].

The lipid debris in the supernate excluded from liposomes are lipid particles that contain no significant internal aqueous compartments. These particles arise from those chloroform-ether spherules that happen to contain no internal aqueous compartments or that have lost all of their internal aqueous compartments during mechanical shaking or solvent evaporation. In this report, we made liposomes pelletable in a clinical centrifuge by using the fact that sucrose solutions have higher specific gravity relative to glucose or saline solutions at comparable osmolality. Therefore, the lipid debris did not sink with the liposomes during centrifugation because they did not contain enough aqueous volume (sucrose solution) to make them denser than the surrounding medium.

It is not certain why there is a difference in the incorporation of DOPC and cholesterol into liposomes (66% versus 52%). We speculate that this is due to a difference in partition coefficients between the monolayer phase and the organic solvent phase when the water-in-lipid emulsion is made; hence, liposomes that have retained the monolayer would have a different composition compared to the lipid debris. If this explanation is correct, we would also expect the inner and the outer monolayers of the liposome membrane to be of slightly different composition.

It is not completely clear why a small amount of a neutral oil is needed to form liposomes. Perhaps they become a part of 'corners' or 'edges' where membranes meet each other and thus stabilize membrane boundaries in a manner analogous to planar black lipid membranes that require a neutral lipid [23]. Indeed, some of the corners in the electron micrograph (Fig. 5) do show osmiophilic 'buttons' that probably are collections of triacylglycerols.

## Acknowledgements

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