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LARGE VOLUME LIPOSOMES BY AN ETHER VAPORIZATION METHOD

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Summary

Liposomes, in the size range of microsomal vesicles, are formed when ether solutions of a variety of lipids are injected into warm aqueous solutions. The liposomes are osmotically active, most are unilamellar, and the volume trapping efficiency is approximately ten times that of sonicated and hand-shaken preparations.

Liposomes are widely used as a model membrane system. Hand-shaken preparations, originally introduced by Bangham et al. [1] have been employed to measure permeability parameters of various ions and molecules across lipid bilayers (for review, see Bangham [2]). Sonication and gel filtration steps have since been introduced which provide more uniform preparations composed of single lipid bilayer membranes [3].

Both preparation methods have certain drawbacks. The original method has the advantage of simplicity but produces multilamellar structures of a variety of sizes. Since a major fraction of the lipid is present in multilamellar form, the volume sequestered per mole of lipid or trapping efficiency, is low. Sonication followed by gel filtration produces uniform vesicles composed of single bilayers, but has the disadvantage that the vesicles are much smaller than typical biological structures. Other methods, such as those of Reeves and Dowben [4] and Papahadjopoulos [5] work only under fairly specialized conditions. The alcohol injection method of Batzri and Korn [6] produces vesicles similar in size to sonicated preparations.

An ideal method for producing liposomes would form unilamellar vesicles of uniform size, would be applicable to most lipid mixtures, and could efficiently trap ions, metabolites and high molecular weight molecules. The diameter of the vesicles should be within the range of 0.1 to 1.0 μm to

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resemble natural membranes and produce high trapping efficiency. We have employed a novel method for liposome production based upon a solvent evaporation method described by Watkins (Papahadjopoulos and Watkins [7]) which involves injection of an ether-lipid solution directly into the aqueous solution to be sequestered. Our rationale was that the most effective trapping would occur if the lipids were not permitted to form multilamellar structures prior to mixing with the aqueous phase. Relatively gentle conditions (not sonication) should be employed to assure formation of large vesicles. It was therefore reasoned that if solutions of lipids in ether were injected into an aqueous phase at a temperature above the boiling point of the ether, these conditions might be met and the lipid would coalesce into large vesicles. In a previous study, Robinson [8] used nitrogen to evaporate ether/lipid solutions mixed with an aqueous phase, but in our hands this technique often formed numerous multilamellar structures.

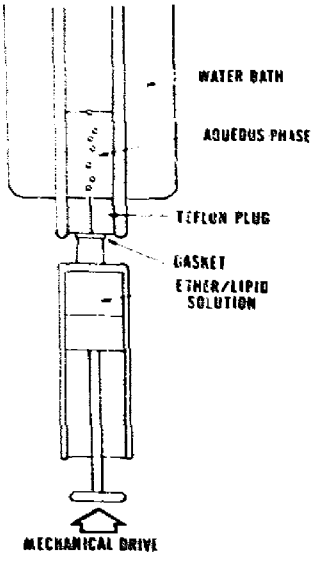

Fig. 1. Diagram of ether injection apparatus. (See methods for details).

In testing the method, it was necessary to control the temperature of the aqueous phase, the concentration of the lipid in the ether, and the injection rate. Fig. 1 shows a schematic illustration of an apparatus which permits control of these parameters. In this apparatus, a glass syringe contains the lipid solution in ether, the solution was injected into the aqueous phase through a fine gauge needle; the needle tip barely penetrated the aqueous phase, being thermally protected along its length by a Teflon plug to prevent vaporization of the ether in the needle shaft. The aqueous phase was heated by a water jacket, and in practice we found it convenient to carry out the preparation in a Liebig condenser, with the jacket of the condenser connected to a hot water circulator. The syringe plunger was driven upwards by a mechanical drive, such as a screw driven infusion pump. As the ether came into contact with the aqueous phase, a steady stream of ether vapor rose to the surface, producing an unstable foam which filled but did not overflow

the vessel. Several variables were optimized for maximum volume-trapping as listed below:

Lipid concentration in ether	2 μmol·ml^{-1}
Rate of injection	0.25 ml·min^{-1}
Temperature of aqueous phase	55—65°C
Volume of upper phase	4 ml

Lipids which were successfully processed included egg lecithin, dipalmitoyl lecithin, phosphatidic acid prepared from egg lecithin, mixtures of lecithin and phosphatidic acid, lecithin-cholesterol (50 mol percent) human erythrocyte lipids and lecithin containing 2 mol percent chlorophyll *a*. Aqueous systems included water, sucrose solutions, and various ionic solutions of sodium, potassium, chloride, sulfate, phosphate, acetate and fluoride. Normally 1 mM EDTA was included in the aqueous phase.

We also developed a system which permitted sequestration at lower temperatures (30°C) but which did not permit precise control over injection rates. In this method the condenser column was stoppered and a vacuum was drawn by mechanical pump. The ether/lipid solution was introduced through the top of the column via a fine glass capillary tube into aqueous solutions. Since the vacuum was sufficient to permit rapid vaporization of the ether at the lower temperature, the method could be used to trap temperature-sensitive molecules such as proteins. Trapping efficiencies were similar to those obtained at higher temperatures and atmospheric pressure.

At the end of the initial injection phase, the aqueous phase contained some vesicles large enough to be seen by light microscopy; these were routinely removed by filtration through a 1.2 μm Millipore filter, and amounted to 25% of the original lipid. For volume-trapping studies, the final step was a filtration through Sephadex G-50 gel column (1 × 20 cm) to remove residual ether and the non-trapped marker solute.

Volume-trapping was estimated after gel filtration by measuring trapped ions, colorimetrically. Typical ions were phosphate (as 0.1 M phosphate buffer) and 0.1 M chromate, the latter being measured directly by absorbance at 380 nm. Gel filtration was carried out in equiosmolar potassium sulfate, so that only trapped phosphate or chromate was measured. From knowledge of the quantity of trapped anion, a simple calculation gave volume trapping efficiency as microliters per micromole lipid.

Thin-layer chromatography was carried out on the purified egg lecithin before and after it was used to form liposomes by ether injection. We were unable to detect any evidence of degradation in the form of lysolecithin or fatty acid on the thin-layer chromatography plates, but in general this test should be performed on any new lipid mixture to which the method is applied.

Liposomes were prepared from lecithin/10% phosphatidic acid mixtures by hand-shaking, sonication or ether injection, followed by light and electron microscopic examination. A typical aqueous solution for these experiments was 0.1 M sodium phosphate, pH 7.0. In the polarizing microscope, liposomes prepared by hand-shaking were highly birefringent, indicating that the major lipid fraction was in the form of tightly packed, multilamellar structures. In unfiltered ether preparations, some of the lipid formed vesicles large enough to be seen, but significantly, these vesicles were not birefringent. After

Millipore filtration, no vesicles at all were visible in the light microscope even though filtration removed only a fraction (20—30%) of the lipid present. We conclude that the major fraction of lipid processed by ether injection forms liposomes too small for light microscopic resolution.

Fig. 2 shows uranyl acetate, ammonium molybdate and freeze-fracture

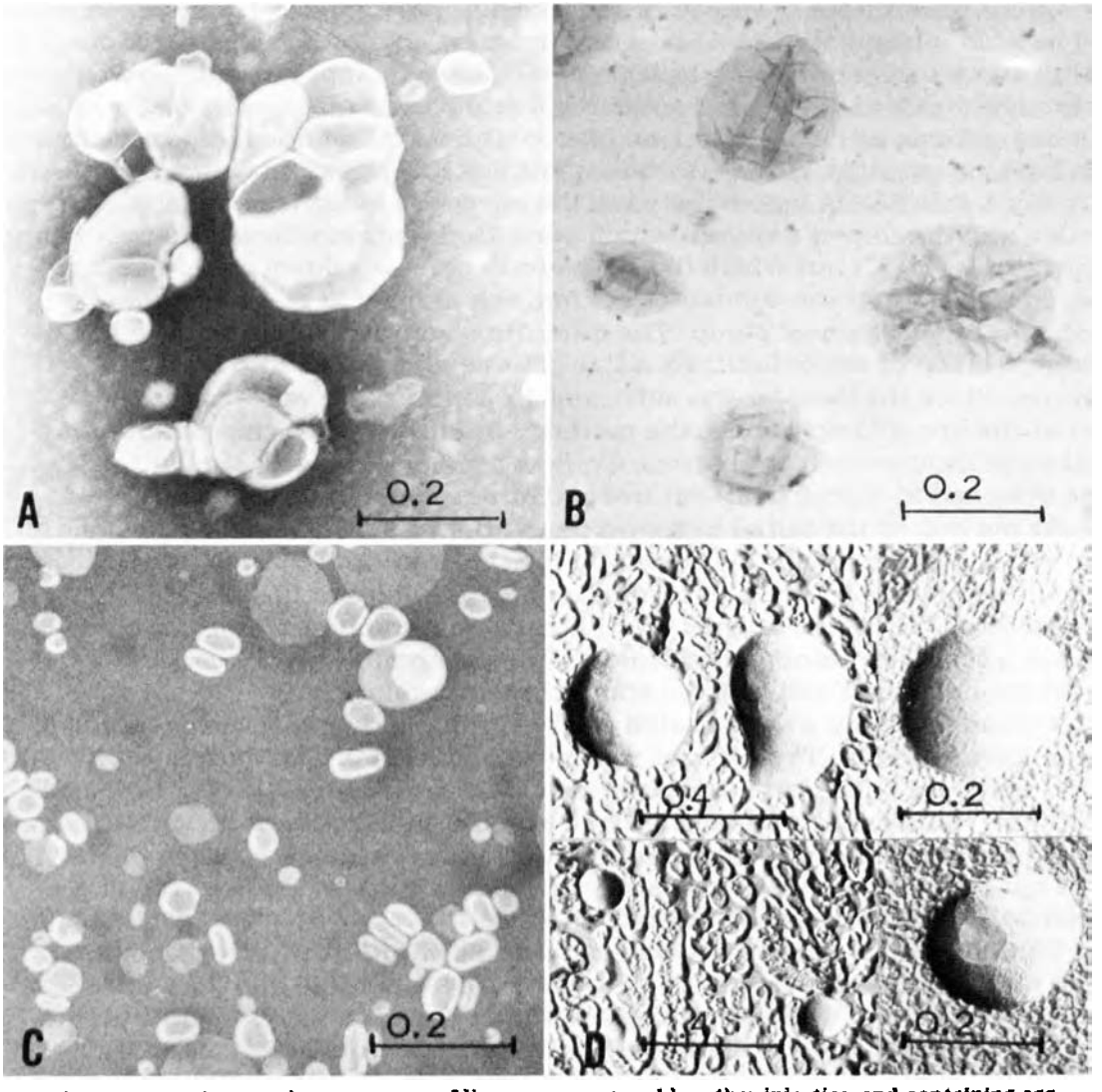

Fig. 2. Electron microscopic appearance of liposomes prepared by ether injection and containing egg lecithin/10% phosphatidic acid. A. This appearance is typical of fields seen in several different preparations stained with 2% ammonium molybdate. The aggregation may be an artifact of molybdate staining, since individual vesicles were more usually observed by uranyl acetate staining or by the freeze-fracture method. B. A liposome preparation stained with 1% uranyl acetate. The uranyl cation apparently produces a more rigid membrane which folds during drying. C. The liposome preparation shown in A above was sonicated for one minute in a Biosonik III sonicator set at half power, then stained with 2% ammonium molybdate. The resulting vesicles were considerably smaller and all appeared to be unilamellar. D. Ether-injection liposomes visualised by freeze-fracture microscopy were seen individually, and several are shown here as a collage. Glycerol (20%) was present as a cryoprotectant. The freeze-fracture method tends to display larger vesicles, since the fracture plane is more likely to strike such a vesicle. Bars show 0.2 and 0.4 μm.

micrographs of lecithin/phosphatidic acid liposomes prepared by ether injection and, for comparison, after sonication. Note that the filtered ether preparations were primarily composed of unilamellar vesicles in the size range of 0.13 ± 0.06 μm diameter (Fig. 2 A and B). In an unfiltered preparation, occasional multilamellar vesicles could be found (not shown) but the lamellae were not tightly packed as in hand-shaken preparations. Instead, loose concentric bags were seen, a result consistent with their lack of birefringence.

In other experiments, ether injection preparations (lecithin/10% phosphatidic acid) were subsequently sonicated in order to produce known unilamellar vesicles for comparison, by negative staining. Under these conditions smaller vesicles were formed (Fig. 2C) which otherwise did not vary markedly in appearance from ether injection liposomes. Freeze-fracture electron microscopy was also carried out on ether-injection liposomes and revealed roughly spherical vesicles in the same size range as those visualized by negative staining (Fig. 2D). Multilamellar liposomes showing double or multiple fracture planes were occasionally visualised by freeze-fracture analysis, but did not account for more than 5% of the total vesicles observed.

The trapped volume of the three preparative methods were compared in 4 separate experiments using lecithin/10% phosphatidic acid liposomes. Sonicated liposomes were least efficient, containing 0.8 ± 0.3 μl per μmol lipid. Hand-shaken preparations were slightly more efficient, but since most of the lipid was multilamellar, the improvement was minimal (1.8 ± 0.6 μl per μmol). Liposomes prepared by ether injection showed a dramatic increase in trapping volume, with typical values of 14 ± 6 μl per μmol lipid for filtered preparations. In this regard, it is interesting to calculate the expected value for volume trapping for a single size of liposome. For instance, if we assume that the average diameter of the negatively stained material is $d = 0.13$ μm and that each phospholipid molecule has a packing area of 5.5 nm^2 [9] the expected trapping value is 36 μl per μmol lipid. The greatest measured value in our experiments was 22 μl per μmol, and typical values ranged around 14. The discrepancy is most likely due to errors introduced by the negative stain measurements, since the distribution of particle sizes is probably skewed toward smaller particles which are likely to be overlooked. The occasional multilamellar vesicle would also contribute to the discrepancy.

Naturally it was important to consider whether residual ether could

TABLE I

RELATIVE PERMEABILITY OF LIPOSOMES TO SMALL MOLECULES

The light scattering changes monitored for the osmotic response were too rapid to be measured in lecithin/phosphatidic acid liposomes. Therefore 50 mol percent cholesterol was added to the lipid mixture to slow the response by decreasing water permeability of the liposome membranes. This is the same lipid mixture as that used by Hill and Cohen [10].

	Present study (4 experiments, 20°C)	Hill and Cohen [10]	
		15°C	30°C
Erythritol	1.0	1.0	1.0
Malonamide	4.2 ± 0.4	4.0 ± 0.3	3.2 ± 0.3
Urea	8.4 ± 1.5	9.5 ± 0.6	7.6 ± 1.2

affect permeability and other properties of the liposomes prepared by this technique. It seemed appropriate, therefore, to measure a parameter such as osmotic permeability to various small molecules and compare the results with similar data from hand-shaken preparations. We made such a comparison according to the method of Hill and Cohen [10] and our results are shown in Table I. It is clear that the ether preparations are osmotically active, and that their permeability characteristics to small uncharged molecules resemble that of hand-shaken liposomes. In a second paper, we will show that the ether preparations also resemble hand-shaken preparations in their permeability to sodium ions.

We conclude that ether injection produces a useful system of lipid bilayer membranes, approximately the size of microsomes, their most important property being that they are primarily unilamellar vesicles with a usefully large sequestered volume.

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