Formation of Unilamellar Lipid Vesicles of Controllable Dimensions by Detergent Dialysis†

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ABSTRACT: Vesicles are formed by solubilizing mixtures of phosphatidylcholine and cholesterol with sodium cholate and removing the detergent by rapid (hollow fiber) dialysis [e.g., Goldin, S. M. (1977) *J. Biol. Chem. 252*, 5630–5642]. Characterization of the vesicle size distribution by agarose gel filtration, and determination of the intravesicular aqueous compartment, demonstrates that the vesicles are relatively homogeneous in size and are primarily unilamellar. The mean diameter of the vesicles can be varied from 340 to 1280 Å by varying the conditions under which they are formed; increasing the mole fraction of cholesterol and lowering the pH of the dialysate tend to produce larger vesicles. The gentle detergent treatment required for vesicle formation and the ability to control vesicle size distribution reproducibly may make this method particularly useful in studies of reconstitution of membrane proteins and in use of vesicles as vehicles for delivery of materials to living cells.

Several areas of cell membrane research have fruitfully employed artificial lipid vesicles. Such vesicles have been useful as reconstitution vehicles for the identification (Racker, 1972; Racker & Eytan, 1975; Goldin, 1977) and purification (Kasahara & Hinkle, 1977; Goldin & Rhoden, 1978) of proteins that catalyze transmembrane transport processes and in studies of the permeability of the lipid bilayer (Bangham et al., 1965; Papahadjopoulos et al., 1971; Toyoshima & Thompson, 1975). Lipid vesicles have recently been employed to deliver a variety of materials to cells (Gregoriadas, 1977; Poste & Papahadjopoulos, 1976; Rahamimoff et al., 1978) as a consequence of vesicle fusion with the cell membrane; this technique can cause specific modification of various aspects of the physiological or metabolic behavior of the cell.

Unilamellar lipid vesicles of reasonably homogeneous dimensions have been prepared by sonication of aqueous lipid dispersions (Huang, 1969; Barenholz et al., 1977), by injection of solutions of lipid in organic solvent into aqueous medium (Kremer et al., 1977), and by removal of detergent from detergent-solubilized lipid suspensions (Goldin, 1977; Milsmann et al., 1978). The use of mild detergents, such as the bile salt sodium cholate, makes this latter technique particularly useful when one wishes to reconstitute membrane components into the bilayer or to entrap soluble proteins for delivery to cells; the possible denaturing effect of intense sonication or of organic solvents on the biological activity of the protein of interest is avoided.

Control of the dimensions of the artificial vesicles one creates is desirable. For example, relatively small (≤500 Å) unilamellar vesicles may be more useful when reconstitution is used as a physical tool for identification (Goldin, 1977) and/or purification (Goldin & Rhoden, 1978) of membrane transport proteins (for this purpose one would ideally like a vesicle preparation containing, at most, one or a few membrane proteins per vesicle). Larger vesicles are more efficient for use as delivery vehicles to cells, for they can entrap more material per mass of lipid. The ability to form vesicles of controlled and relatively uniform size may be of particular utility in studies of the passage of vesicles through anatomical barriers in conjunction with their use as delivery vehicles (Gregoriadas, 1977).

This paper reports that one can create fairly uniform, primarily unilamellar vesicles of controllable dimensions by solubilizing phosphatidylcholine or phosphatidylcholine/cholesterol mixtures with sodium cholate and subsequently removing the detergent by rapid dialysis. The mean diameter of the resulting vesicle dispersion can be varied from 340 to 1280 Å by varying the mole fraction of cholesterol and by varying the pH of the dialysate employed for vesicle formation.

Experimental Procedures

Materials. Cholesterol was from Fisher Scientific Co., cholic acid was from Sigma Chemical Co., and D-[^{3}H]glucose was from New England Nuclear. Scintillation fluid consisted of Triton X-100 (Rohm and Haas) and Scintiverse (Fisher Chemical) in a 10:90 (v/v) ratio. Bio-Gel A-150m, β-mercaptoethanol, and Bio-Fiber 50 minitubes were from Bio-Rad Laboratories. Sephadex was from Pharmacia, and egg phosphatidylcholine (>99% pure) was from Avanti Biochemical Co. (catalogue no. 830051, shipped in dry ice under acetone/N_2). Standards for calibration of the Bio-Gel A-150m column included various sizes of "Uniform Latex Particles", Dow Diagnostics, Indianapolis, IN.

Preparation of Lipid. Egg phosphatidylcholine was prepared as previously described (Goldin, 1977) and stored as a 50–80 mg/mL solution in 20:1 (v/v) decane/ether under liquid N_2. β-Mercaptoethanol (1 mM) was used as an antioxidant in all solvents used for lipid preparation and storage. Commercial egg phosphatidylcholine (>99%) prepared by Avanti Biochemicals could be substituted for the above lipid preparation. Cholesterol was recrystallized twice from acetone/water (Goldin & Rhoden, 1978) and stored as a 47 mg/mL solution in benzene under liquid nitrogen. Lipid phosphate was determined by the method of Ames (1966).

Preparation of Vesicles. Phosphatidylcholine and cholesterol stock solutions were mixed to give the desired molar ratios. The volatile solvents were evaporated under an N_2 stream. The mixture was then frozen and lyophilized for 2.5–3 h followed by resuspension by vortexing under N_2 with 0.25 M sucrose, 0.55 mM EDTA, 5 mM Tris, 10 mM imidazole, 5 mM 2-mercaptoethanol, 0.235 M KCl, and 35 mg/mL cholic acid,

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pH 7.4. The final phospholipid concentration was 15 mg/mL in all cases. Unclarified phosphatidylcholine/cholesterol mixtures were stored at −70 °C.

One to two milliliters of lipid mixture under N_2 in a 10 × 75 mm tube was immersed in a boiling water bath for 30 s; the clarified suspension was immediately chilled on ice. Vesicles were formed by dialyzing the phosphatidylcholine/cholesterol mixture for 16 h in Bio-Fiber 50 minitubes as described (Goldin, 1977). As there currently appear to be problems in supply of these minitubes, it should be noted that a similar unit, "artificial capillaries", catalogue no. 3P10, is available from Amicon, Lexington, MA. Furthermore, thin-film dialysis, an older technique for providing a high membrane surface/volume ratio for rapid solute removal (Stewart, 1977), is a viable alternative. "Reconstitution dialysate" was 20 mM imidazole, 5 mM 2-mercaptoethanol, 0.1 mM EDTA, 125 mM NaCl, 0.02% sodium azide, and 0.1 mM D-glucose. pH was adjusted to pH 6, 7, or 8 at room temperature. [^{3}H]Glucose, 0.3 mCi/L, was added to the dialysate for determination of the intravesicular aqueous compartment. Thus, during the process of cholate removal by dialysis, the vesicles form in the radioactive dialysate so that the intravesicular aqueous compartment contains the same concentration of [^{3}H]glucose as outside the vesicle. Use of [^{3}H]cholate in parallel experiments demonstrated >98.5% removal of detergent by 16 h of hollow fiber dialysis.

Intravesicular aqueous compartment determinations were made by separation of the vesicles from medium in Sephadex G-50M columns formed on Pasteur pipets (Goldin, 1977). The Sephadex columns were equilibrated and eluted with the nonradioactive reconstitution dialysate, pH 7. Each fraction was collected in 0.5-dram cappable vials, and 1.6 mL of scintillation fluid was added. Total lipid phosphate was determined on each original vesicle preparation.

The vesicles are not assayed for intravesicular [^{3}H]glucose content by Sephadex filtration until >98% of the cholate has been removed by dialysis. Permeability measurements as in Goldin & Rhoden (1978) demonstrate that the vesicles, regardless of cholesterol content, are sufficiently sealed to glucose to insure that (within a few percent) all the intravesicular [^{3}H]glucose is retained during the 7 min at 4 °C required to separate the vesicles from the extracellular medium by gel filtration. The Sephadex elution profiles yield two well-defined and separated peaks of [^{3}H]glucose: a small peak in the void volume containing the vesicles, followed by a larger peak of extravesicular [^{3}H]glucose in the column volume. If an aliquot of vesicle-free dialysis buffer containing similar amounts of [^{3}H]glucose is eluted on the column, a single peak of [^{3}H]-glucose is detected; the void volume region counts for ^{3}H at the background level. The above observations demonstrate that accurate estimates of the intravesicular aqueous compartment by this technique have been obtained.

Agarose Gel Filtration. Four-hundred microliters of vesicles was placed on a column of agarose A-150m (21 × 0.94 cm). The column was equilibrated and eluted at 4 °C with nonradioactive reconstitution dialysate, pH 7, at a 10-cm aqueous head (about 1 mL/h). Fractions of ~0.4 mL were collected. Fifty microliters of each fraction was used for lipid phosphate assay, and 100 μL was used for scintillation counting of [^{3}H]glucose to determine the elution positions of phospholipid and [^{3}H]glucose. For calibration of the column with "Uniform Latex Particles", the column was preequilibrated and then eluted with 1% Triton X-100 in reconstitution dialysate, pH 7; latex spheres were diluted into 400 μL of this solution for loading on the column. The use of Triton X-100 prevented aggregation of the latex beads during operation of the column.

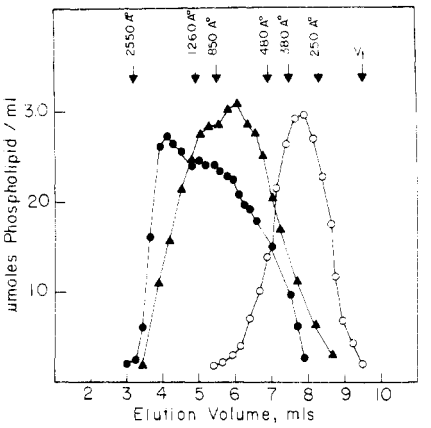

FIGURE 1: Agarose A-150m profiles of vesicles prepared by cholate/hollow fiber dialysis. Three batches of vesicles, two of which were formed from a 1.0:0.42 molar ratio of phosphatidylcholine/cholesterol at pH 6 (●) and at pH 8 (○) and the other of which was formed from a 1.0:0.21 phospholipid/cholesterol ratio at pH 6.0 (▲), were separately eluted on the column. The column was calibrated, as depicted by arrows, with four sizes of Dow "Uniform Latex Particles": 380 Å (±75 Å SD), 850 (±55) Å, 1260 (±43) Å, and 2550 (±22) Å. Also employed in calibration were phosphatidylcholine vesicles formed as for Na,K ATPase reconstitution (Goldin, 1977), 480 Å, and 250-Å phosphatidylcholine vesicles formed by prolonged sonication (Huang, 1969). Details of vesicle formation and column elution procedures are given under Experimental Procedures.

Sonicated vesicles, 250 Å in diameter, were prepared precisely as described by Huang (1969) and employed as a column calibration standard.

Results and Discussion

The size distribution of vesicles produced by cholate/hollow fiber dialysis can be estimated by agarose gel filtration (Goldin, 1977). Size estimates obtained by this technique have been shown, for phosphatidylcholine vesicles, to correlate closely with electron microscopic and alternative biophysical methods for determination of vesicle dimensions (Huang, 1969). Figure 1 shows agarose A-150m elution profiles for three separate vesicle preparations. Vesicles formed at pH 6 from a 1.0:0.42 molar ratio of phosphatidylcholine/cholesterol elute at a mean diameter (defined as the point at which half the lipid phosphate has eluted) of 1280 Å. Use of the same phospholipid/cholesterol ratio but alteration of the pH of the dialysate to 8.0 results in vesicles of much smaller size—330-Å mean diameter. Maintenance of dialysate pH at 6.0 results in smaller vesicles if the phospholipid/cholesterol ratio is increased; at a ratio of 1.0:0.21, vesicles of 700-Å mean diameter form.

Information on the breadth of the size distribution of these vesicle preparations can be obtained by comparing their elution profiles with those of highly uniform, monodiperse polystyrene latex spheres (Bangs & Kenney, 1976) (Figure 2). The width/height ratio of the lipid peak at half-maximum peak height is, for the vesicles of 330-Å mean diameter (Figure 1), 0.23. This is identical with the ratio for the absorbance peak (Figure 2) of uniform latex spheres of comparable size (380 Å). The width/height ratios of the 700- and 1280-Å mean diameter vesicle preparations are respectively 0.40 and 0.52, substantially broader than the 0.30 width/height ratio of 850-Å polystyrene latex spheres. This illustrates the general observation that alteration of the vesicle formation parameters toward production of larger vesicles tends to broaden the resulting size distribution. Nonetheless, the peak dimensions of all preparations studied suggest, on the basis of the calibration profile of the column, that most of the vesicles in any

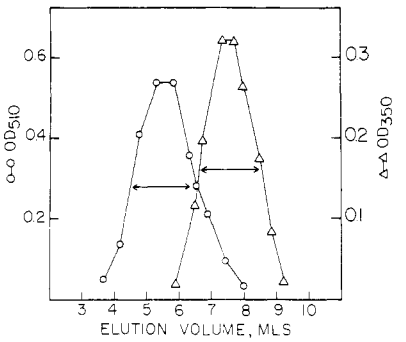

FIGURE 2: Agarose A-150m profiles of 850 (±55) Å (○) and 380 (±75) Å (△) Dow "Uniform Latex Particles". The position of 850-Å particles was determined by OD_{510} measurements. The position of 380-Å particles was determined by OD_{350} measurements. Arrows (↔) denote peak widths at half the peak height. Details are given under Experimental Procedures.

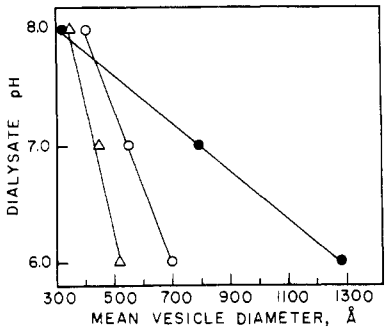

FIGURE 3: Effect of dialysate pH and mole fraction of cholesterol on the resulting mean size of vesicles (as determined by agarose gel filtration). The pH dependence of vesicle size is illustrated for phosphatidylcholine/cholesterol vesicles of 0.0 (△), 0.174 (○), and 0.296 (●) mol fractions of cholesterol. Details of experimental procedures are given in the text.

given preparation are within a factor of 2 of one another in diameter.

Figure 3 illustrates the effect of changes in cholesterol content and dialysate pH on vesicle size. One generalization that results is that lowering dialysate pH tends to increase vesicle size. A second is that, at pH values at or below neutrality, increasing the amount of cholesterol employed during vesicle formation increases vesicle size. At pH 8, variation of cholesterol content has no significant effect on mean vesicle size. The pH dependence of vesicle size was more pronounced at higher cholesterol content.

The intravesicular aqueous compartment can be measured by trapping a water-soluble, relatively impermeable radioactive marker (such as [^{3}H]glucose) in the vesicles during dialysis and separating external from intravesicular marker on small Sephadex columns (Goldin, 1977). Figure 4 illustrates the relationship between the volume of the intravesicular aqueous compartment and the measured mean vesicle diameter. As shown, increasing vesicle size monotonically increases the volume of the intravesicular aqueous compartment; the relationship between mean vesicle size and the volume of the intravesicular compartment is relatively independent of cholesterol content.

Are the vesicles primarily unilamellar or multilamellar? This can be determined by comparing the size of the intravesicular aqueous compartment with that expected for unilamellar vesicles of the same mean dimensions (Table I). Q is the experimentally determined ratio of the total volume of intravesicularly trapped aqueous fluid to the total volume of lipids. This is compared with Q' and Q'', the values for this ratio calculated if it is assumed that a vesicle of the experimentally determined mean diameter is bounded by a single lipid bilayer or by two lipid bilayers, respectively.

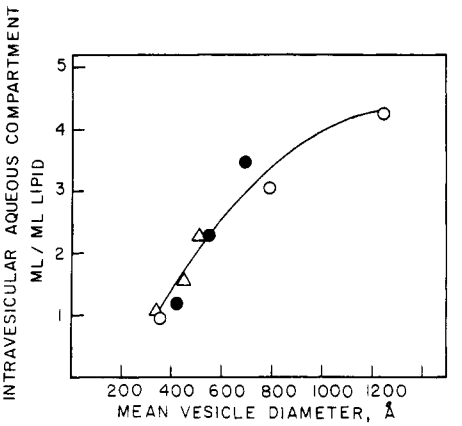

FIGURE 4: Relationship between the intravesicular aqueous compartment and mean vesicle size for phosphatidylcholine/cholesterol vesicles formed at pH 6, 7, or 8 from 0.0 (△), 0.174 (●), and 0.296 (○) mol fractions of cholesterol. Details of intravesicular aqueous compartment determinations are given under Experimental Procedures.

Table I: Comparison of the Measured Intravesicular Aqueous Compartment with That Predicted for Unilamellar and Bilamellar Vesicles of the Same Mean Diameter

mol fraction of cholesterol	pH of vesicle formation	measd mean vesicle diameter (Å)	Q (mL/mL of lipid)[a]	Q' (mL/mL of lipid)[b] (deviation from measd value)	Q'' (mL/mL of lipid)[c]
0	6	518	2.25	1.67 (−24%)	0.676
0	7	450	1.55	1.40 (−10%)	0.529
0	8	340	1.06	0.903 (−15%)	0.279
0.174	6	694	3.47	2.31 (−33%)	1.004
0.174	7	550	2.35	1.72 (−27%)	0.688
0.174	8	424	1.18	1.17 (−1%)	0.313
0.296	6	1278	4.27	4.55 (+7%)	2.06
0.296	7	796	3.06	2.55 (−17%)	1.13
0.296	8	326	0.944	0.704 (−25%)	0.183

[a] Measured aqueous compartment. [b] Aqueous compartment predicted for unilamellar vesicles of same mean diameter. [c] Aqueous compartment predicted for bilamellar vesicles of same mean diameter.

These ratios are calculated as follows:

$$Q' = \frac{[r - (t + \delta)]^3}{r^3 - [r - (t + \delta)]^3}$$

and

$$Q'' = \frac{[r - (2t + \delta)]^3}{r^3 - [r - (2t + \delta)]^3}$$

where t is the thickness of the hydrophobic lipid bilayer, δ is the thickness of the hydrophilic region of each side of the bilayer, both as determined by X-ray diffraction studies of hydrated egg phosphatidylcholine/cholesterol multilayers of variable composition (Small, 1967; Lecuyer & Dervichian, 1969; Levine & Wilkins, 1971; Franks, 1976; Milsmann et al., 1978), and r is the vesicle radius. This calculation assumes that the effective diameter of the vesicle for its elution on agarose columns includes the thickness of the hydrophilic region occupied by the lipid head groups; it further assumes that the radioactive glucose is not excluded from the hydrophilic region of the bilayer lining the inner surface of the

vesicle and (in the case of a bilamellar structure) that it is not excluded from the hydrated space separating the two hydrophobic bilayer regions.

As seen in Table I, there is much better agreement between experimentally determined Q and theoretically derived Q' than between Q and Q'' (the bilamellar value). The mean departure of Q' from Q is only −16%, whereas Q'' averages 3.2-fold below Q. The assumption that [^{3}H]glucose can occupy the hydrophilic portion of the bilayer and the interlamellar space is a conservative one, for if it is not made the result would be to further widen the disagreement between Q'' and Q. Thus, it is demonstrated that, throughout the range of mean vesicle sizes produced by varying the lipid and dialysate composition, the resulting preparations are primarily of unilamellar structure. However, one cannot rule out the possibility that a small fraction of the lipid is in the form of multilamellar vesicles or sheetlike structures.

The fact that Q is greater than Q' by an average of 16% deserves attention. This discrepancy is not likely to be due to aggregation of vesicles during elution on the agarose column; if this occurred it would tend to make the intravesicular aqueous compartment smaller rather than larger than that calculated for unilamellar spheres of the diameter determined by their elution position. An idealization in the theoretical calculations is that the vesicles are homogeneous in size, when in fact there is clearly some size heterogeneity. It can be demonstrated mathematically that the observed degree of heterogeneity, as estimated from the width of the agarose column peak, does not appear to be great enough to account for this discrepancy. The most likely reason for the discrepancy is a small systematic error in estimation of the mean vesicle size by agarose filtration. If the mean radius of the preparation were actually 15% larger than that determined by the column profile, the discrepancy between Q and Q' would be accounted for. A 15% radius deviation corresponds to a shift of only 280 μL on the elution position of the vesicles on the column. This deviation could be caused by a mild adsorptive interaction of the vesicles with the agarose gel matrix. Huang (1969) noted this adsorptive phenomenon on Sepharose; although we have pretreated our agarose (as he recommended) to minimize adsorption, the problem may not have been entirely eliminated. Huang (1969) showed that alternative methods of estimating his vesicle size yielded values between 220 and 300 Å. This variability is even greater than the magnitude of our discrepancy between Q and Q' and apparently reflects the inherent level of uncertainty in such estimates of vesicle size.

In summary, it has been shown that alteration of the phospholipid/cholesterol ratio and the pH of the dialysate regulates the mean size of the relatively uniform, primarily unilamellar vesicles that result from cholate/hollow fiber dialysis. We have also observed that raising the ionic strength of the dialysate tends to increase vesicle size (preliminary data, not shown). A rigorous explanation of why alteration of these parameters regulates vesicle size cannot be advanced at this point; such understanding requires further investigation of the mechanism of vesicle formation by this technique.

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