Unilamellar liposomes made with the French pressure cell: a simple preparative and semiquantitative technique

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Abstract A simple, rapid, and almost quantitative technique is described for the preparation of 1–40 ml of homogeneous unilamellar liposomes from dilute or concentrated aqueous suspensions of egg phosphatidylcholine. Aqueous suspensions of lipid are placed within the chamber of a French pressure cell at room temperature and rapidly extruded at 20,000 psi through the small orifice. A single pass transforms more than 70% of the extruded lipid into a homogeneous population of single-wall bilayer vesicles; more than 90% is transformed by recycling the lipid through the French pressure cell. About 95% of these liposomes range between 150–300 Å in diameter (mean 200 Å). The liposomes are stable for days to months when stored under nitrogen at 0–4°C and can be prepared at 0°, 25°, or 37°C. The liposomes appeared unaltered by repeated passages through the French pressure cell and no degradation of the phospholipid was detected after ten consecutive cycles at 20,000 psi in the absence of a nitrogen atmosphere. The method is especially useful for trapping small molecular weight substances because the concentration of both lipid and solute can be made quite high. Cholesterol up to 45 mole % can be incorporated into larger liposomes of egg phosphatidylcholine (mean diameter 315 Å). Other phospholipids and different lipid mixtures can also be transformed into unilamellar vesicles with this method which has the advantage that additional steps of ultracentrifugation, column chromatography, dialysis, and concentrating procedures are usually unnecessary. Multilayered liposomes of small size (980 Å mean diameter; > 95% between 500–1,500 Å) are produced at lower pressure (3,000 psi). The latter are separated by gel permeation chromatography from a second population of homogeneous vesicles of even smaller size (580 Å mean diameter; > 95% between 300–900 Å) that contain two bilayer shells.—**Hamilton, R. L., Jr., J. Goerke, L. S. S. Guo, M. C. Williams, and R. J. Havel.** Unilamellar liposomes made with the French pressure cell: a simple preparative and semiquantitative technique. *J. Lipid Res.* 1980. **21:** 981–992.

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In 1950, Milner, Lawrence, and French (1) described a novel apparatus for disrupting chloroplasts that retained much of the original photochemical activity. They suggested that the apparatus (subsequently named the French pressure cell) could be used for fine dispersion of many other biological materials. In 1972, we reported that the French pressure cell effectively released nascent plasma lipoproteins from within membrane compartments of intact Golgi apparatus isolated from rat liver (2). We also observed, but did not report, that the continuous membrane compartments that had formed the complicated structures of the intact Golgi apparatus (3, 4) were transformed into vesicles having the appearance of microsomes in the electron microscope. This observation suggested that multilayered suspensions of hydrated phospholipids might be dispersed into smaller liposomes by the French pressure cell.

In this report we describe a simple and nearly quantitative technique that we have used for several years to make single bilayer liposomes. We also report the effect of pressure, temperature, and time on the formation, size, and stability of unilamellar liposomes composed of egg phosphatidylcholine alone and with cholesterol. An accompanying report (5) shows that liposomes prepared with the French pressure cell exhibit the same characteristics of interaction with plasma lipoproteins as unilamellar liposomes prepared by a commonly used sonication procedure.

METHODS

Materials

Chloroform and methanol (analytical reagent, Mallinckrodt, St. Louis, MO) were redistilled just be-

Abbreviations: PC, phosphatidylcholine; psi, pounds per square inch.

fore use. Ethanol (absolute, IMC Chemicals, Agnew, CA) and salts (analytical reagents, Mallinckrodt) were used as supplied. Water was double-distilled from glass. Egg phosphatidylcholine (egg PC) (Sigma, St. Louis, MO) was treated with activated charcoal (Matheson, Coleman, and Bell, Norwood, OH) suspended in absolute ethanol to remove colored contaminants. The purity of the egg PC was evaluated by thin-layer chromatography. Two hundred μg of lipid was applied as a single spot on a 20 × 20 cm plate (Silica gel G, Analabs, Inc.). The plate was developed with chloroform–methanol–acetic acid–water 100:60:16:8 (v/v) and the lipid was visualized by spraying with a 0.1% 8-anilino-1-naphtholine sulfonic acid. Only a single spot corresponding to PC was evident in this system with a detection limit of about 1 μg lipid. Soya PC (gift of Dr. H. Betzing, A. Nattermann and Cie, Köln, West Germany) had a trace of lysophosphatidylcholine on thin-layer chromatograms. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (dipalmitoyl PC) (Fluka, Buchs, Switzerland) gave a single thin-layer spot as did the ether analog of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (dioleoyl PC) (Serdary, London, Ontario). Beef brain sphingomyelin (Serdary, London, Ontario) was used without testing for purity. Cholesterol (Nutritional Biochemicals, Cleveland, OH) was thrice recrystallized from ethanol just before use. [^{3}H]Inulin (New England Nuclear, Boston, MA) was used as received.

Analytical procedures and separations

Fatty acid methyl esters were separated on glass columns (180 × 0.40 cm) filled with phosphoric acid-stabilized 5% diethylene glycol succinate (Supelco, Bellafonte, PA) in a Bendix gas–liquid chromatograph (model 2500, Bendix, Los Altos, CA). Peak areas were measured with an on-line computer system.

Gel chromatography was performed on columns (95 × 2.5 or 95 × 1.2 cm) of 2% or 4% agarose (Biorad, Richmond, CA). Lipid phosphorus (6) and cholesterol (7) were measured in effluent fractions.

Thin-layer chromatography of phospholipids was performed on silica gel G plates (Analtech, Newark, DE) with chloroform–methanol–water–acetic acid 60:35:4.5:0.5 (by volume) as the developing solvent.

We prepared multilamellar liposomes by depositing lipids by vacuum evaporation from organic solvents on the walls of round-bottom flasks (8). The lipids (50 mg/ml) were suspended with glass beads in added aqueous media, and were usually diluted ten-fold before use.

We made single bilayer vesicles in three different ways. *1*) Vesicles were prepared with a standard French pressure cell (2.5 cm inner diameter 40 ml capacity; Aminco, Silver Spring, MD). A hand operated press was used to obtain pressure in the earlier studies but later experiments showed that homogeneous preparations of smaller liposomes were more routinely prepared with a motor-driven laboratory press (Aminco) that maintained more constant pressures. The flow rate was adjusted by opening the needle valve (1) so that the fluid was extruded at the maximum speed (~30–40 ml/min) without a drop in pressure. Many of the experiments were also done with a smaller pressure cell of 3-ml capacity (Aminco) with the same results. Both the stainless steel needle valve and the nylon ball valve proved satisfactory, although the latter was easier to control. Strict maintenance of the pressure cells was essential for obtaining reproducible liposome preparations. Thorough cleaning after each use with warm water and mild detergent followed by methanol and rapid drying with forced air was necessary to prevent lipid accumulation or rust in the small channels of the cells. Frequent replacement of nylon ball valves and damaged O rings improved reproducibility.

2) Vesicles were prepared by sonicating the ice-chilled multilayer suspensions under nitrogen with a probe sonicator at 80W (model W185, Branson Ultrasonics, Plainview, NY) for 30-sec bursts. Three minutes usually sufficed to reach the endpoint of a clear opalescent suspension. Samples were centrifuged 15 min at 1,000 *g* to remove metal particles and larger lipid aggregates.

3) Vesicles were also made from ethanol solutions of lipids by the method of Batzri and Korn (9).

Liposomes were prepared in a variety of aqueous media as described below. We observed no difference between liposomes prepared in distilled water or sodium chloride solutions (0.15–0.20 M) containing 0.04% EDTA, and 0.02% NaN_3, subsequently referred to as column buffer.

Electron microscopy

Liposomes were examined and photographed at 20,000 and 60,000 diameters at 80 KV in a Siemens 101 electron microscope (Siemens Medical/Industrial Groups, Iselin, NY) with a condenser aperture of 200 μm and an objective aperture of 60 μm.

Negative staining of liposomes often causes artifactual images resulting from disruption of small liposomes that subsequently form larger multilamellar structures (10). Although the mechanism of this process is not understood, it may be caused in part by the intense hydrophobicity of freshly evaporated carbon on grid surfaces. Our attempts to modify the

surface film of freshly prepared carbon-coated grids (200–300 mesh copper grids, Fullum, Schenectady, NY) by glow discharge, polylysine, or addition of albumin to sample or grid did not prove satisfactory. However, we have learned empirically that parlodion-covered grids with carbon films made with a Siemens evaporator (V13 G 500) permit even spreading of liposomes without apparent structural changes, provided that the grids have been aged 6–12 months on the shelf. Carbon rods for filming were obtained from Ringsdorff-Herke (Spektral Kohlen, Bonn-Bad Godesberg, West Germany). With such "matured" carbon-coated grids, liposomes were prepared for electron microscopy with 2% potassium phosphotungstate at pH 6.45–6.5 by the drop procedure described previously (11). We found that the lipid concentration in the sample was also important for preparing uniform spreads. Optimal results were more consistently obtained with samples containing 1–10 mg/ml PC. Improved results can be obtained for more dilute samples by increasing contact time of the sample on the grid to 2–3 min, and by using a tighter 400 mesh grid (12).

We also modified a method (10, 12) to obtain thin sections of liposomes from aqueous suspensions. Samples (0.2–1.0 ml) were fixed by adding 3% osmium tetroxide in sodium veronal acetate buffer pH 7.4 with gentle mixing by aspiration. Best results were obtained with concentrations of 0.5–2 mg PC diluted with fixative to 1.0–1.5 ml. Samples were left in fixative about 48 hr at 4°C. The lipid should turn yellow to brown within a few minutes to hours (depending on the number of double bonds) and should not form precipitates. For filtration, 0.1 ml of a freshly prepared 2% gelatin (Baker and Adamson, New York, NY) solution is added to 1 ml of fixed sample and gently mixed by aspiration. The bottom half of a stainless steel microanalysis filter holder (Hoefer Scientific Instruments, San Francisco, CA) is attached by clear Tygon tubing to a water aspirator and supported in a level position. A 13-mm millipore filter, 250 Å pore diameter, is placed shiny side up in the filter holder and moistened by aspirating 2–3 ml of water at low pressure. The top half of the filter is pressed firmly in place forming a water-tight seal and about 1.0 ml of sample is pipetted on top of the millipore filter. After 3–6 hr of gentle aspiration, the sample forms a moist brown to black uniform film on the millipore filter. Fully saturated lipids such as dipalmitoyl PC form faintly stained films. The millipore filter is transferred to 10 ml of 2% aqueous uranyl acetate and left in the dark at 37°C for 48 hr. The sample is dehydrated in 70, 80, 90% ethanol (5 min each), and 100% (three changes) for 10 min each followed by propylene oxide (three changes) 10 min each. The sample and filter curl up in the propylene oxide and can be separated with gentle agitation so that the millipore filter can be removed from the vial. Sample fragments are infiltrated with propylene oxide–Epon mixtures and embedded in Epon. Sections are made perpendicular to the thin sample edge so that the entire sample is viewed in the electron microscope. This technique prevents non-random spatial distribution usually seen in pellets of centrifuged particulates (10). The prolonged fixing and staining procedures were found necessary to obtain clear images of the trilaminar staining of phospholipid bilayers routinely. With this procedure, even PC samples that contain no unsaturated fatty acids can be stained successfully. Thin sections were stained sequentially with 5% aqueous uranyl acetate (10–60 min at 37°–60°C) followed by alkaline lead citrate for 5–20 min (12).

Measurements of particle size

Electron microscopic images taken at 20 or 60,000 diameters were enlarged precisely three times (electron microscope enlarger, Durst, Inc., Bolzano, Italy) with a calibrated negative carrier (Carlwen Industries, Rockville, MD). A minimum of 200 particles was measured on each 8 by 11 inch print by systematically traversing two rectangular paths at about 1 inch and about 2 inches from the print edge. Individual particles encountered on the rectangular path were measured in mm by a Bausch and Lomb measuring magnifier (cat. #81-34-35). The percentage distributions from all samples were calculated and plotted as histograms. Data in the text show both mean diameters and a range that includes more than 95% of particles of a given experiment.

RESULTS

The bulk of the data on liposomes produced by the French pressure cells is presented here in two ways. First, profiles of lipid phosphorus eluting from 2% and 4% agarose columns reflect the transformation of large multilayered liposomes (8) into lipid vesicles smaller than about 600 Å that enter the included volume; larger forms emerge in the void volume. Second, electron microscopic images of negatively stained and thin-sectioned preparations provide particle size measurements and also show the number of bilayer shells in the liposomes.

Effect of pressure

Operating pressure within the cell proved to be the single most important variable in producing the conversion of larger multilamellar precursors into unilamellar liposomes. The transformation of large multilamellar structures into smaller heterogeneous mixtures of multilamellar and unilamellar liposomes and further into smaller homogeneous unilamellar liposomes is depicted in **Fig. 1.** In this experiment, three samples of egg PC (5 mg/ml) in 0.04% EDTA pH 7.0 were each passed once through the pressure cell with a hand-operated press at about 2,000–3,000, 8,000–9,000 (not shown), and 14,000–16,000 psi. The top image of Fig. 1 shows the multilayered structures of the original aqueous suspension of egg PC. The middle image shows the heterogeneous mixture of multilamellar liposomes together with small and large unilamellar structures produced by a single pass at a pressure of about 2,000–3,000 psi. The major structures produced by raising the pressure to 14,000–16,000 psi, are unilamellar vesicles of a broad size range (~200–1,200 Å), together with scattered multilayered liposomes as shown at the bottom left of Fig. 1. The multilayered liposomes are almost all converted into single bilayer vesicles by repeated passage (two times) of the egg PC sample through the pressure cell at 20,000 psi (Fig. 1, bottom right).

The effect of preparing liposomes at three different pressures is demonstrated by the phospholipid elution profiles obtained from a 2% agarose column (**Fig. 2** left). Egg PC was passed in each case twice through the French pressure cell at the different pressures shown in Fig. 2 (left). At ~19,000 psi, all of the PC was converted into particles that entered the included volume of the gel, whereas at lower pressures, more of the lipid mass eluted with the void volume as larger particles which were found by electron microscopy to be multilayered liposomes of 500–1,500 Å diameter (Fig. 10). Liposomes in the included volume were small and unilamellar as shown by images obtained in negatively stained and thin-sectioned preparations (Fig. 5, top and middle).

Effects of lipid concentration and number of passages through the pressure cell

We routinely eliminate most of the air space within the chamber of the pressure cell before raising the pressure and expressing the lipid through the orifice. Thus, a small portion of the sample is not subjected to the procedure initially and is lost unless the sample is recycled. A residual volume (0.05–0.3 ml) of sample also remains within the piston chamber after the bulk has been processed. To reduce these potential losses and to ensure that all of the sample undergoes the procedure, we routinely recycle the sample through the pressure cell several times.

Fig. 2 (right) shows that there is little difference in elution profiles of egg PC passed either two to six times through the pressure cell at high pressures (20,000 psi). The amount of void volume material (i.e., multilamellar structures) is only slightly reduced by recycling the lipid more than twice and there is no apparent change in size of unilamellar liposomes contained in the included volume. These results were obtained for samples containing either 5 or 15 mg/ml of PC. At concentrations of egg PC greater than 25 mg/ml, more lipid eluted in the void volume. Passage of egg PC liposomes two, six, or ten times through the pressure cell caused no detectable hydrolysis of the PC, as assessed on 250-μm thin-layer plates loaded with as much as 175 μg PC and no detectable alteration of fatty composition as assessed by gas–liquid chromatography.

Measurements of liposome size

Because the elution profiles of included volume liposomes formed at 19,000 psi appeared constant, irrespective of the number of passages through the pressure cell, we measured the distribution of diameters from electron microscopic images of negatively stained samples from the ascending slope, peak, and descending slope of the liposome profile (**Fig. 3**).

In another experiment, the distribution of diameters of unilamellar liposomes (**Fig. 4**) in the included volume was estimated from the trapped volume of [^{3}H]inulin (10). The calculated diameter of unilamellar liposomes was 150–200 Å; a mean value of 187 Å was obtained from negatively stained preparations of the pooled fractions from the included volume.

The diameters of four preparations of liposomes made months apart from different samples of egg PC that was passed three to four times through the French pressure cell at 20,000 psi are shown in **Table 1.** The mean particle size and distribution were closely similar among three different negatively stained preparations. A slightly larger value was obtained in the fourth preparation, in which measurements were made from images of thin sections. Some of the measurements in this study were made from the images shown in **Fig. 5,** top and middle.

Effect of temperature

The pressure cell was immersed in ice or placed in a 38°C oven for 1 hr before liposomes were prepared. The lipid suspensions, at the corresponding temperatures, were passed three times through the pressure cell and subjected to chromatography at room temperature. Only a slight shift in elution pro-

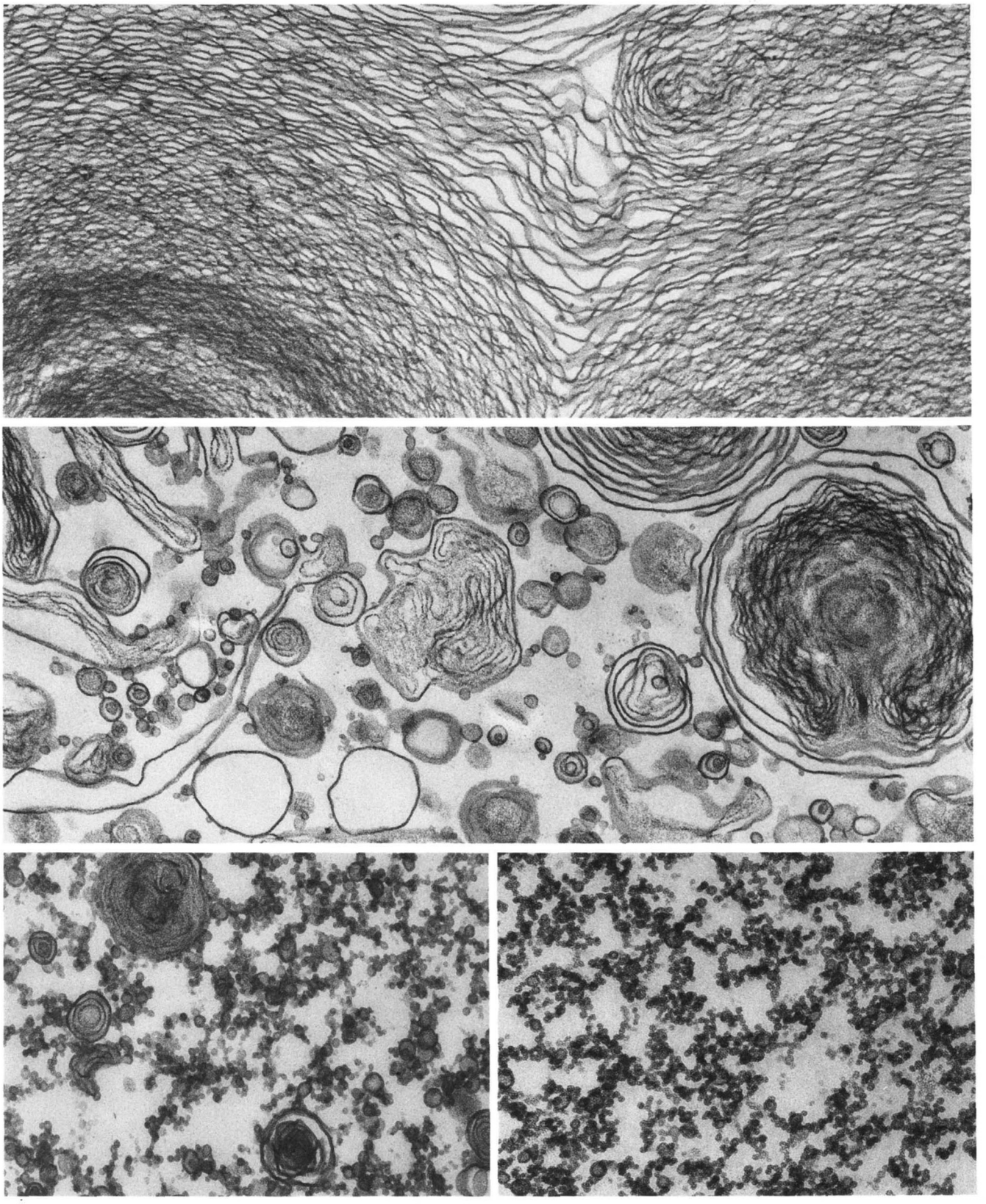


Fig. 1. Thin sections of liposomes at low magnification (×60,000). Top: Multilamellar structures of egg PC suspensions before processing with French pressure cell. Middle: Heterogeneous multi- and unilamellar liposomes produced by a single pass of egg PC through the French pressure cell at 2,000–3,000 psi. Bottom left: Unilamellar liposomes of broad size range (200–1,200 Å) together with scattered multilamellar structures predominate after two passes of egg PC through French pressure cell at about 15,000 psi. Bottom right: Only unilamellar liposomes appear after two passes of egg PC through the French pressure cell at 20,000 psi.

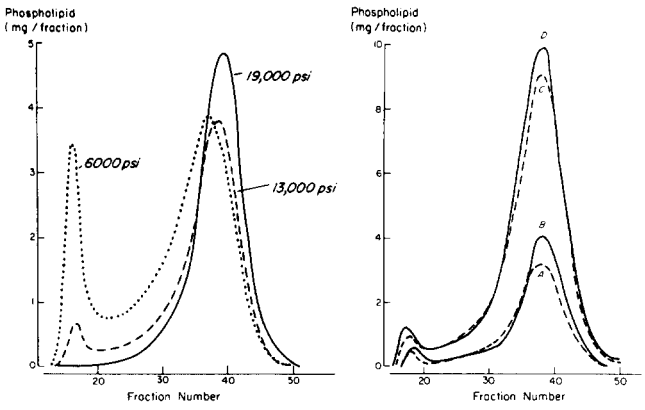


Fig. 2. Left: Elution profiles (from a 2% agarose column, 2.5 × 95 cm) of liposomes formed by passing hydrated suspensions of egg PC through a French pressure cell at different pressures. Sixty mg of lipids suspended in 10 ml of 0.04% EDTA, pH 7.0, were passed twice through the pressure cell at room temperature at each of the pressures indicated. Right: Elution profiles (from a 2% agarose column, 2.5 × 95 cm) of egg PC liposomes in 0.04% EDTA at pH 7.0, formed at ~19,000 psi, showing the effects of different lipid concentrations and different numbers of passages through the French pressure cell. *A*. 5 mg lipid/ml, six passages; *B*. 5 mg lipid/ml, two passages; *C*. 15 mg lipid/ml, six passages; *D*. 15 mg/ml, two passages.

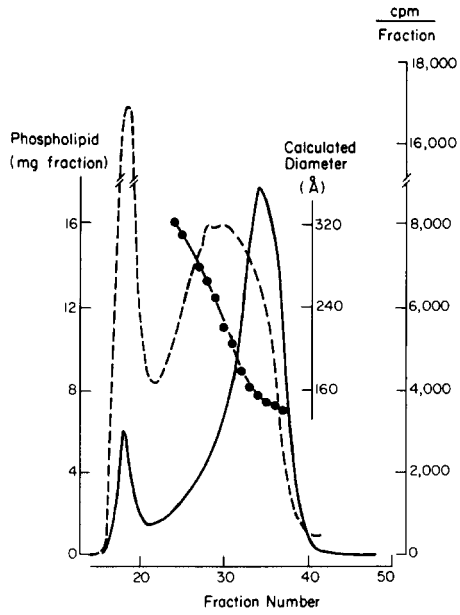


Fig. 4. Elution profiles (from a 4% agarose column, 2.5 × 95 cm) of liposomes formed by passing egg PC, 13 mg/ml, twice through the French pressure cell at ~19,000 psi. [^{3}H]inulin was trapped in the included volume as a non-diffusable marker. The broken line indicates the elution profile of ^{3}H as cpm and the solid line that of PC. The solid circles are the calculated vesicle diameters for liposomes in the included volume, assuming the structure of single walled, spherical vesicles formed by a bilayer of 43 Å thickness. By electron microscopy, a mean diameter of 187 Å was obtained from negative stains of pooled fractions 26–37.

file was observed for liposomes prepared at 0°C (**Fig. 6,** top left) as compared to that of vesicles formed at 37°C or at room temperature. By electron microscopy, three fractions of the included volume from each experiment at 0°C and 37°C appeared as unilamellar liposomes indistinguishable from those prepared at room temperature.

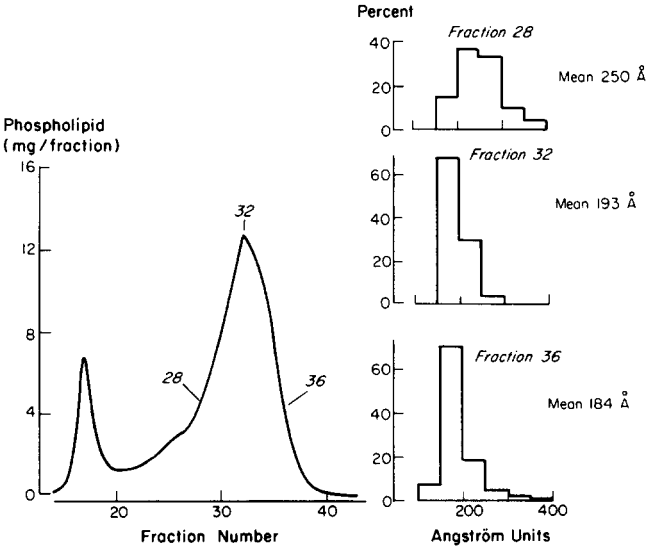


Fig. 3. Elution profiles (from a 4% agarose column, 2.5 × 95 cm) after a single pass of egg PC, 13 mg/ml, through a French pressure cell at ~19,000 psi. Seventy percent of the phospholipid recovered from the column is eluted between fractions 28 and 37. Measurements of particle size of three fractions were made from photographs of negatively stained preparations and are shown in the histograms.

Effect of centrifugation on void volume lipid

We found that 2–8% of the recovered lipid eluted in the void volume, except for one experiment (Fig. 2

TABLE 1. Diameters of unilamellar liposomes of egg PC[a] as determined from electron microscopic images of negatively-stained or thin-sectioned samples

Technique	Liposome Preparation	General Appearance[b]	Diameter
	(date)		*(Å)*
Negative stain	March 8, 1976	Oval	216 ± 42[c]
Negative stain	March 23, 1976	Round	188 ± 35
Negative stain	March 23, 1976	Oval	213 ± 46
Negative stain	November 1, 1976	Round	192 ± 48
Thin section	December 17, 1976		243 ± 96
Thin section	December 17, 1976		226 ± 69

[a] Liposomes were formed by passing egg PC suspensions (5–10 mg/ml in 0.04% EDTA 0.15 M NaCl) two to four times through a French pressure cell at about 20,000 psi (no chromatographic fractionation was done).

[b] In negatively-stained preparations, PC liposomes are usually seen as round or oval. In this experiment, diameters were measured from images which contained about 50% of oval particles (oval) or over 95% of round particles (round).

[c] Mean ± SD for 100 particles.

left) in which no lipid was detected in these fractions. Numerous attempts to reproduce this quantitative transformation of larger multilamellar liposomes into small single-wall liposomes were not fruitful. Ultracentrifugation of egg PC liposomes greatly reduced the void volume material, but did not completely eliminate it (Fig. 6, top right). The liposomes eluting in the void volume prior to ultracentrifugation at 100,000 *g* for 1 hr appeared by negative staining to be multilayered structures measuring between 500–1,500 Å in diameter, indistinguishable from those produced by low pressure (e.g., see Fig. 10). After ultracentrifugation, the small amount of PC remaining in the void volume (Fig. 6, top right) was in the form of liposomes with two bilayers similar to but somewhat larger (mean 690 Å; range 400–1,000 Å) than those shown in Fig. 10, bottom image.

Stability of liposomes

To evaluate liposome stability at 37°C, egg PC liposomes were prepared with the small (3.0 ml) pressure cell at 25°C at an initial concentration of 10 mg/ml. One-half of the sample was then incubated at 37°C for 4 hr, during which time the unincubated control half of the liposome fraction was chromatographed. The incubated liposome fraction was then chromatographed on the same column (95 × 1.2 cm). The elution profiles shown in Fig. 6 (bottom left) suggest that, at 37°C for 4 hr; some of the smaller unilamellar liposomes are transformed into larger structures that elute in the void volume.

Unilamellar liposomes prepared by passage through the French pressure cell formed a white precipitate during storage in the refrigerator at about 4°C. In the experiment shown in Fig. 6 (bottom right), one half of a liposome preparation was stored at 0–4°C in the refrigerator for 2 weeks before chromatography on 4% agarose gel. The refrigerated sample was first allowed to equilibrate at room temperature, but it remained opalescent. The sample was then warmed

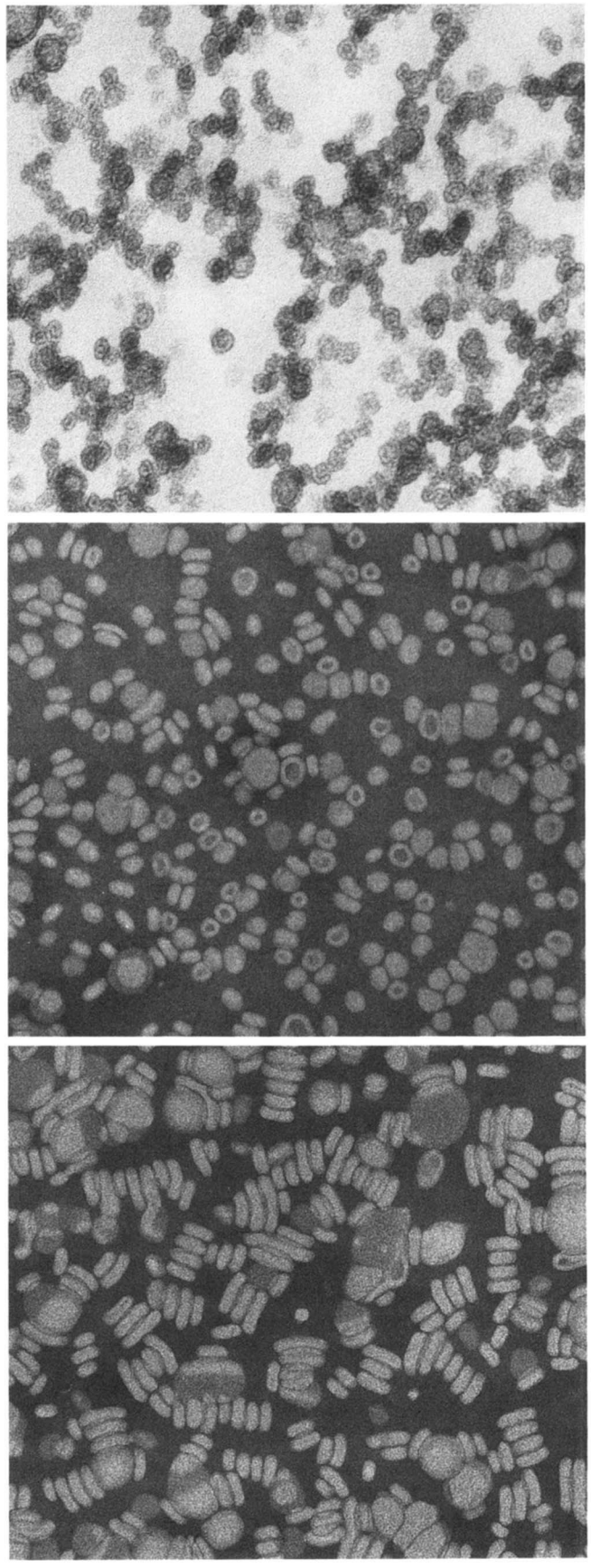


Fig. 5. Examples of electron microscopic images of unilamellar liposomes used to measure particle size (×180,000). (See Table 1). Top: Thin section of homogeneous unilamellar liposomes prepared by passing egg PC four times through the French pressure cell at 20,000 psi. This image illustrates the trilaminar staining of the head group regions of phospholipids, the spherical shape of the liposomes, and the trapped volume compartment. The few larger liposomes can be removed by ultracentrifugation or gel chromatography. Middle: Negative stain of same sample shown in top image. Images that contain many flattened (oval) vesicles give a slightly larger diameter than images of the same sample that show only round particles (see Table 1). Bottom: Negative stain of egg PC liposomes containing 45 mol % cholesterol. Although the mean particle size is much larger, (~375 Å), some particles as small as those shown above for egg PC alone are also present.

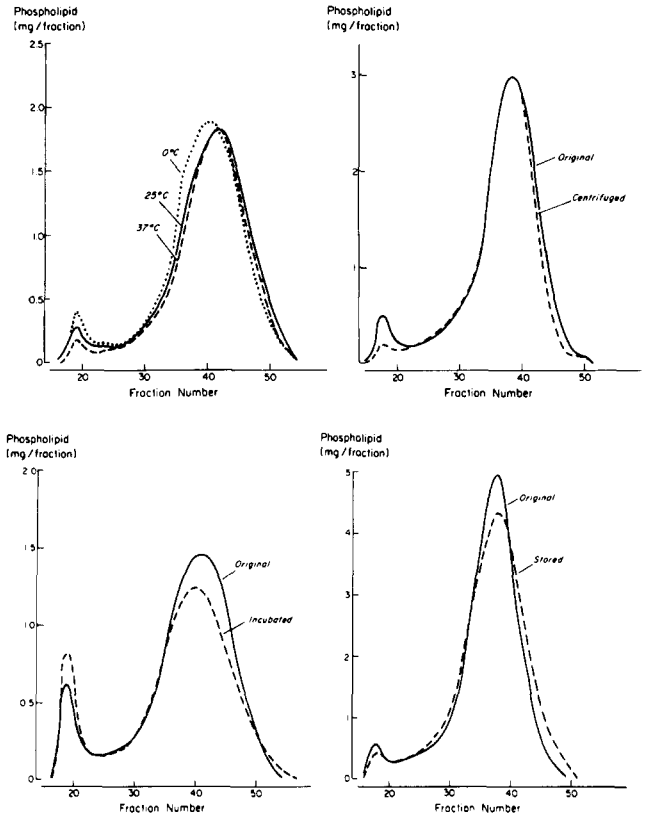


Fig. 6. Elution profiles (from a 2% agarose column, 1.2 × 95 cm) of Top left: Egg PC liposomes formed at 0, 25, and 37°C by passing each sample twice through a French pressure cell at the temperature shown. Each initial sample contained 25 mg PC in 3 ml of column buffer. Top right: Egg PC liposomes (solid line) and the supernatant fraction after ultracentrifugation (dashed line) of liposomes at 100,000 *g* for 1 hr. The liposomes were formed at 20,000 psi from a 10 mg/ml suspension in column buffer. Bottom left: Freshly prepared egg PC liposomes (solid line) and after liposomes were incubated at 37°C for 4 hr (dashed line). The liposomes were formed at 20,000 psi from a 20 mg/ml suspension of egg PC in 0.2 M aqueous 6-carboxyfluorescein at pH 7.7 (see following paper by Guo et al. ref. 5). Bottom right: Freshly prepared egg PC liposomes (solid line) and following storage (dashed line) at 0–4°C for 14 days. The liposomes were formed at 20,000 psi from a 10 mg/ml suspension of PC in column buffer.

at 37°C with partial clearing. However, its elution profile was virtually identical to the original one (Fig. 6, bottom right), showing that no transformation of unilamellar liposomes into larger or multilamellar structures had occurred. When egg PC liposomes, with or without cholesterol, were centrifuged at 100,000 *g* for 1 hr and then stored under nitrogen in the refrigerator, no precipitation was observed even after several months.

Effect of cholesterol

Inclusion of cholesterol in suspensions of egg PC increased the size of liposomes as shown by the elution profiles in **Fig. 7.** Although the initial mixture contained 30 mole % cholesterol, the resultant liposomes separated by gel chromatography contained somewhat less (Fig. 7, left). In a second experiment in which the initial ratio of the two lipids was 1:1, the proportion of cholesterol contained in the liposomes was increased twofold but remained less than equimolar with PC (Fig. 7, right). This higher initial content of cholesterol in the suspensions also increased the amount of PC eluted in the void volume. By electron microscopy, the liposomes in the included volume fractions 28, 36, and 44 in Fig. 7 right, had a mean diameter of 377 Å, 305 Å, and 277 Å, respectively, with a weighted mean of 316 Å diameter. The liposomes in the descending limb of the included volume contained less cholesterol and this corresponded to an increased number of smaller liposomes the same size as those containing only egg PC (see Fig. 5 bottom).

Liposomes from different phospholipids

Three different phospholipids (egg PC, dipalmitoyl PC, and sphingomyelin) were passed through the French pressure cell (at room temperature) three times each at 20,000 psi and subjected to chromatography on 2% agarose gel. As shown in **Fig. 8** (left), no dipalmitoyl PC eluted in the void volume and the included volume lipid was distributed in a symmetrical peak, indicating larger liposomes than for egg PC. By negative staining, these liposomes appeared as unilamellar particles with a size between 200 and 500 Å in diameter (mean 303 Å). Sphingomyelin, in contrast, formed more material that eluted in the void volume and the included volume liposomes

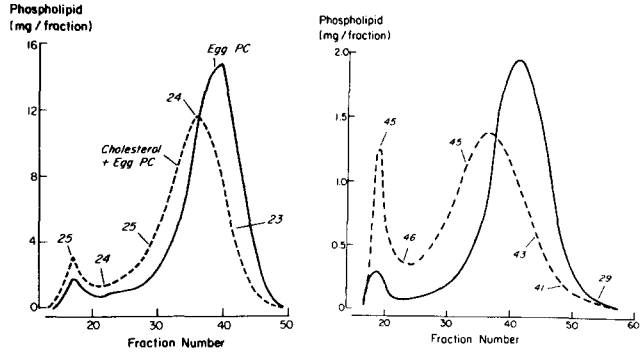


Fig. 7. Left: Elution profiles of liposomes (from a 2% agarose column, 2.5 × 95 cm) prepared from egg PC alone (solid line), and from a mixture of cholesterol and egg PC (30 mol %) after passing each sample twice through a French pressure cell at about 19,000 psi. Each initial sample contained 13 mg/ml of egg lecithin. The numbers in the figure are the measured mol % of cholesterol in the fractions indicated. Right: Elution profiles of liposomes (from a 2% agarose column, 1.2 × 95 cm) prepared from egg PC alone (solid line) and from a mixture of cholesterol and egg PC (50 mol %) after passing each sample twice through a French pressure cell at 20,000 psi. Each initial sample contained 9 mg/ml of egg PC.

appeared by electron microscopy to consist of a larger and more heterogeneous population of unilamellar liposomes (Fig. 8, left). Unilamellar liposomes were also successfully prepared from soybean PC, egg PC containing 10 mole percent lysolecithin, dimyristoyl PC, and 1-palmityl-2-oyleylether PC. Neither pure phosphatidyl-ethanolamine nor phosphatidylserine (in 0.2 M sodium chloride containing 0.04% EDTA at pH 7.0) formed vesicles when processed in the French pressure cell.

Comparison of unilamellar liposomes prepared by different methods

The elution profiles of unilamellar liposomes prepared with the French pressure cell at 20,000 psi were compared (by 2% agarose column chromatography) to those made by ultrasonic irradiation and by injection of ethanol-solubilized lipid into a stirred aqueous solution (9). The profiles of the included volumes for liposomes prepared by each method were closely similar (Fig. 8, right) indicating that the bulk of the egg PC was contained in liposomes of similar size. Liposomes prepared by the ethanol injection method were the most homogeneous and closely matched the pattern obtained with the French pressure cell except for the small amount of lipid in void volume

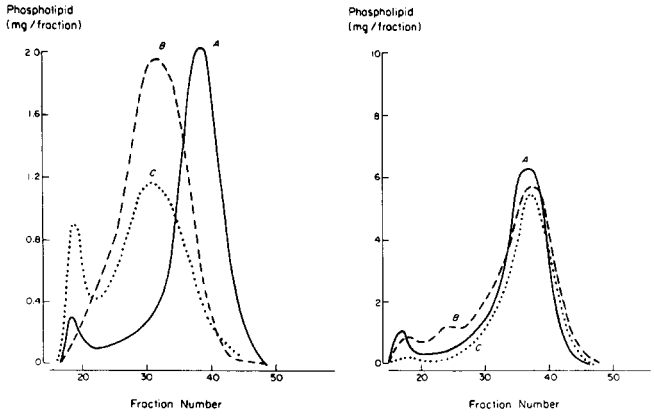


Fig. 8. Left: Elution profiles (from a 2% agarose gel column, 2.5 × 95 cm) of liposomes formed from various phospholipids. Three to five mg/ml of lipid suspension was passed three times through the pressure cell at room temperature and 20,000 psi. *A*. Egg PC; *B*. Dipalmitoyl lecithin; *C*. Beef brain sphingomyelin. Right: Elution profiles (from a 2% agarose gel column, 2.5 × 95 cm) of liposomes of egg PC formed by three different methods. *A*. Lipid suspension containing 6 mg/ml egg PC was passed twice through the French pressure cell at 20,000 psi. *B*. Lipid suspension containing 6 mg/ml egg PC was sonicated at 80W for 3 min (30-sec bursts) while chilled in ice and under N_2. The supernatant lipid was chromatographed following centrifugation at 100,000 *g* for 1 hr to remove titanium and large liposomes (14). *C*. An ethanol solution of PC (57.5 mg in 3.5 ml) was injected rapidly into 43 ml of water containing 0.04% EDTA. The mixture was then dialyzed and concentrated to 10 ml by ultrafiltration (9).

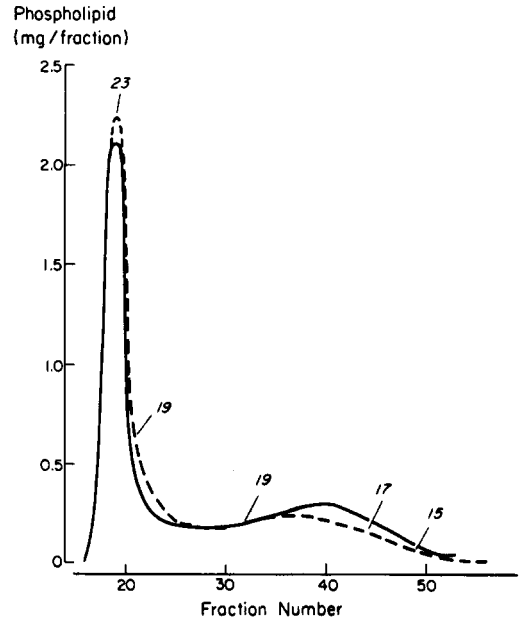
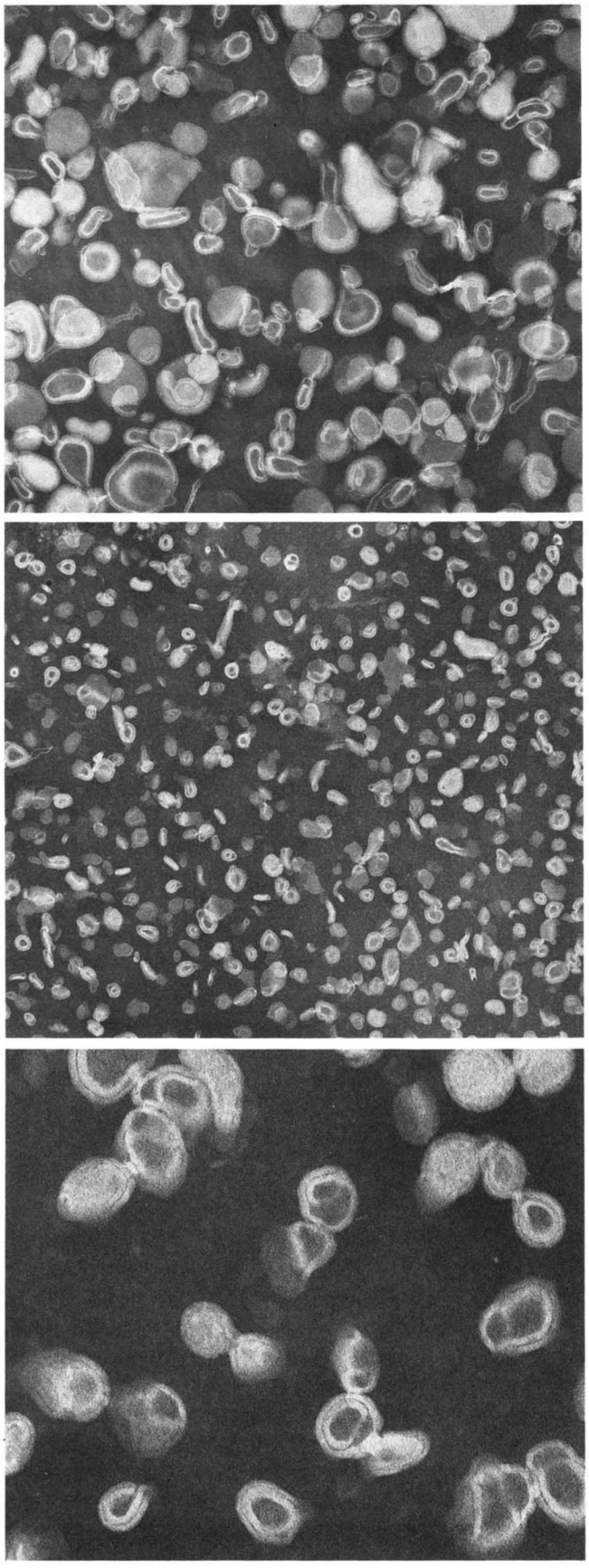


Fig. 9. Elution profiles of liposomes (from a 2% agarose column, 1.2 × 95 cm) of egg PC alone (solid line), and egg PC plus cholesterol (50 mol %) (dashed line) after passing each sample twice through a French pressure cell at the low pressure of 3,000 psi. Each sample contained 9 mg/ml of egg PC. The numbers in the figure are the measured mol % of cholesterol of the fractions indicated.

(Fig. 8, right). The pattern shown for the sonication method is not fairly represented because it was necessary to subject the sample to centrifugation to remove probe-metal particles and undispersed PC before column chromatography (13–15). Even after this centrifugation step, the remaining liposomes were apparently somewhat larger (Fig. 8, right). By electron microscopy, liposomes in the included volume fractions appeared to be unilamellar for each method of preparation.

Preparation of small (500–1,500 Å) multilamellar liposomes with low pressure

Egg PC and equimolar egg PC-cholesterol suspensions were passed through the French pressure cell twice at only 3,000 psi. The elution profiles and electron microscopic appearances of these suspensions were remarkably similar and much less cholesterol was incorporated into the liposomes at this lower pressure (**Fig. 9**). The multilamellar liposomes eluting in the void volume (**Fig. 10**) were indistinguishable from those remaining in the void volume after high pressure (see Fig. 6, top right). The mean diameter (980 Å) and size distribution (>95% between 500–1,500 Å) of these small multilamellar liposomes were the same with or without cholesterol. Most of the liposomes in the included volume (with and without



cholesterol) appeared, by negative staining, to have two bilayers (Fig. 10, middle and bottom). These double bilayer liposomes had a mean diameter of 570 Å with more than 95% between 300–900 Å.

DISCUSSION

Our studies show that aqueous suspensions of large multilayered liposomes of egg PC are easily, reproducibly, and almost quantitatively (>90%) converted into small unilamellar vesicles (~200 Å mean diameter) by passage through the French pressure cell at 20,000 psi. Large or small volumes (1 to 40 ml cell capacity) of concentrated (up to 25 mg/ml) PC can be made into unilamellar liposomes within 5–10 min at controlled temperatures of 0–37°C. Only 5–8% of the lipid remains in multilayered structures larger than 600 Å and most of this can be eliminated by centrifugation at 100,000 *g* for 1 hr. Either the metal needle valve or the nylon ball valve of the pressure cell and either a hand-operated or automatic press may be used. No evidence of chemical degradation of egg PC was observed even after ten consecutive passages through the pressure cell at 20,000 psi at room temperature without the use of argon or nitrogen atmosphere which is recommended for sonication procedures (13–15). Egg PC liposomes containing up to 45 mole % cholesterol are readily prepared with this method. These liposomes are larger (about 315 Å mean diameter) as is the case for liposomes prepared by ultrasonic methods (16) and by organic solvent injection techniques.[1] Other phospholipids such as dipalmitoyl PC, dimyristoyl PC, sphingomyelin, soya PC, as well as lipid mixtures such as egg PC, cholesterol, and lysophosphatidylcholine form unilamellar vesicles with this procedure. Unilamellar egg PC liposomes prepared alone or with 45 mole % cholesterol appear to be remarkably stable if stored

[1] Hamilton, R. L., J. Goerke, L. S. S. Guo, M. C. Williams, and R. J. Havel. Unpublished observations.

Fig. 10. Small multilamellar liposomes made with the French pressure cell. Top: (60,000×). The void volume fraction of egg PC liposomes following two passes through the French pressure cell at 3,000 psi (see Fig. 9). Diameters range between 500–1,500 Å (mean 980 Å). The same type of image is obtained from negative stains of the small amount of lipid in the void volume after 20,000 psi (see Fig. 6, top right). Middle: (60,000×). The included volume fraction from egg PC liposomes following two passes through the French pressure cell at 3,000 psi (Fig. 9). Diameter range from 300–900Å (mean 570 Å) and the majority of the liposomes appear to contain two lamellae. Bottom: (180,000×). This higher magnification of the above sample illustrates the double lamellae of these liposomes.

under nitrogen in the refrigerator for days to months. However, these stored liposomes precipitate by aggregation when stored at 0–4°C although they do not fuse to form larger multilayered structures. Centrifugation of the liposomes at 100,000 *g* for 1 hr prevents precipitation during storage, indicating that this phenomenon requires the presence of multilamellar liposomes. Sonicated liposomes of distearoyl PC reportedly increase in size with time if stored below their transition temperature (17). The remarkable stability of liposomes made with the French pressure cell may be due to storage of these liposomes above the transition temperature of egg PC. However, dipalmitoyl PC liposomes also appeared stable when stored in the refrigerator, suggesting that liposomes prepared with the French pressure cell are much more stable than those prepared by standard sonication procedures.

The described technique has several additional advantages over the commonly used method of sonication. Titanium particles from probe sonications are avoided and no aerosols are produced, thereby simplifying use of radioactive lipids. Protein denaturation is apparently less of a problem with the French pressure cell (1, 2), possibly because of the brief period the sample need be subjected to severe physical stress. Future work with individual proteins under specific conditions of treatment will be necessary to test this hypothesis. For most work, ultracentrifugation will be unnecessary for liposomes prepared with the French pressure cell whereas this is requisite after sonication (13–15).

The French pressure cell technique also has several advantages when compared to the ethanol injection method. Organic solvent and detergent methods (18) of preparing liposomes have the disadvantages of producing very dilute lipid samples and very dilute trapped solute (9). Dialysis is also necessary to reduce organic solvent or detergent, although it is doubtful that these contaminating molecules can be completely removed from liposomes by simple dialysis (18). Preparation of unilamellar liposomes by the French pressure cell avoids concentration and dialysis steps for most purposes.

Others (19) have recently described briefly an apparently similar procedure for making unilamellar liposomes with the French pressure cell. Our observations differ from theirs in several respects. We found, by four different methods (agarose gel chromatography, trapped volume measurements with [^{3}H]inulin, negative stain, and thin-section electron microscopy), a mean particle size of 200 Å for unilamellar egg PC liposomes prepared with the French pressure cell. Their report of a range of 315–525 Å diameter (~400 Å mean) for unilamellar liposomes of egg PC is even larger than we obtained for cholesterol-containing liposomes. These authors also state that cholesterol up to 48 mole % did not result in a different particle size (i.e., still 400 Å diameter). Unlike our observations (5), which show that the permeability of unilamellar liposomes prepared with the French pressure cell is the same as those formed by sonication methods, these authors reported that the leakage rate of 6-carboxyfluorescein differs greatly between the two liposomal preparations. We thought that these discrepancies might be explained by the difference in the temperature used to prepare liposomes (we routinely use room temperature whereas they use 4°C). Accordingly we tested this by preparing liposomes from egg PC at 0°C, 25°C, and 37°C and compared them by gel chromatography (Fig. 6A) and electron microscopy. The elution profile of PC liposomes prepared at 0°C, was only slightly shifted and cannot account for the twofold larger size reported by them.

We found that low pressure (3,000 psi) passages of egg PC through the French pressure cell could be used to produce two different forms of small multilayered vesicles that could be separated by gel chromatography. At this low pressure, the bulk (~60%) of the lipid appeared as multilayered liposomes between 500–1,500 Å in diameter which eluted in the void volume of 2% agarose gel columns. These small multilayered liposomes were indistinguishable from the 5–8% lipid not transformed into unilamellar liposomes by high pressure. The remaining one-third of the lipid eluting in the included volume after low pressure was characterized by small (300–900 Å) bilamellar liposomes. The multilayered liposomes produced with the French pressure cell with low pressures (3,000 psi) are much smaller than those produced by extrusion of lipid through polycarbonate membranes (20).

Milner, Lawrence, and French (1) called attention to the possible broad application of their apparatus for preparing colloidal dispersions of biological materials. We have used the apparatus to make phospholipid-stabilized emulsions of triglycerides as substrate for lipoprotein lipase. We suggest that this apparatus may be employed to form stable emulsions of many other simple and more complex lipid mixtures in future studies.

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