A Simple Method for the Preparation of Homogeneous Phospholipid Vesicles†

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ABSTRACT: A new method is described for the preparation of homogeneously sized, single-lamellar phospholipid vesicles. This method, which is based on differential high-speed ultracentrifugation, has the advantages of a higher vesicle yield without dilution and rapidity of preparation when compared to the molecular-sieve technique. The homogeneity of vesicle dispersions, prepared by this new method, is examined by several physical techniques and found to be comparable to the best samples prepared by molecular-sieve chromatography.

Phospholipid vesicles are one of the most widely studied bilayer model systems. Aqueous dispersions of spherical vesicles, each comprised of a single continuous bilayer surrounding an internal aqueous compartment, can be prepared by several methods which can be reduced to two basic procedures: (a) ultrasonic irradiation of phospholipid dispersed in an aqueous medium (Saunders et al., 1962); (b) dilution of an organic solvent containing phospholipid in an aqueous buffer either by blowing off the solvent with an inert gas (Green and Fleischer, 1964) or by rapid injection of the phospholipid solution into the aqueous system (Batzri and Korn, 1973). All procedures yield an aqueous dispersion of vesicles, heterogeneous in size, usually ranging in diameter from 200 to 1500 Å. The heterogeneity of these dispersions is a serious disadvantage for two reasons. First, several important physical properties of phospholipids are known to be strongly dependent on the radius of curvature of the bilayer comprising the vesicle wall. Among these properties are molecular packing density and attendant molecular motions (Sheetz and Chan, 1972), the transbilayer distribution of lipids in multicomponent systems (Litman, 1973, 1974; Michaelson et al., 1973; Huang et al., 1974), and the thermotropic behavior of the component phospholipids (Suurkuusk et al., 1976; Lentz et al., 1976a,b). Second, the heterogeneity of the dispersion makes it virtually impossible to characterize accurately such important parameters as the mass of lipid per vesicle and the bilayer surface area and degree of hydration. Currently, the only satisfactory method for preparing a dispersion of single lamellar vesicles, homogeneous[1] in size, is that developed by Huang (1969). This procedure utilizes molecular-sieve chromatography to isolate a homogeneous vesicle fraction from an ultrasonically irradiated phospholipid dispersion. Although this method has been used successfully on a variety of systems (e.g., Litman, 1973; Newman and Huang, 1975), several disadvantages are encountered. (1) The amount of lipid in the homogeneous fraction is usually 40% or less of the total lipid. (2) The molecular-sieve chromatography and its attendant analyses are time consuming. This is not only often inconvenient, but also may permit relatively slow changes in vesicle structure to occur to an appreciable extent (Suurkuusk et al., 1976). (3) An approximate tenfold dilution of the vesicle dispersion necessarily accompanies the chromatography. The preparation can only be reconcentrated by ultrafiltration. (4) In the course of autocorrelation light-scattering studies, it became apparent that the molecular-sieve column shed materials which contributed to the low-angle scattering which were virtually impossible to eliminate.

The purpose of this paper is to describe a method based on high-speed ultracentrifugation by which homogeneous vesicle dispersions may be prepared without the disadvantages of molecular-sieve chromatography.

Materials and Methods

Phospholipid Preparation. Beef brain sphingomyelin and egg phosphatidylcholine were prepared and purified following the procedures of Shinitzky and Barenholz (1974). Tritiated phosphatidylcholine was prepared by the method of Gatt (1968). Mixtures of egg phosphatidylcholine and tritiated phosphatidylcholine were in a mole ratio of 19:1 in all experiments. No contaminating components including amino phosphatides were detectable in any preparation using both thin-layer silicic acid chromatography with the solvent $CHCl_3$-CH_3OH-H_2O 65:25:4 and two-dimensional thin-layer chromatography (Kates, 1972). Phosphatidylethanolamine prepared and purified as described by Litman (1973) contained no impurities detectable by these chromatographic systems. All phospholipid preparations were dissolved in $CHCl_3$ and stored under argon at −20 °C. The high purity of the preparations remained unchanged for 6 months under these storage conditions.

Preparation of Phospholipid Vesicles. Individual phospholipids or mixtures of phospholipids were first diluted in benzene and then lyophilized to remove all traces of organic solvent. In preparation for sonication, the dry phospholipid was suspended in either the 50 mM KCl or 100 mM NaCl. These solutions were prepared using extra-pure KCl or NaCl (Heico, Delaware Water Gap, Pa.) and deionized water, which was first distilled over an alkaline solution of $KMnO_4$ and then glass redistilled.

Lipid dispersions were sonicated using a Heat Systems

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[1] Homogeneity in these systems is understood to mean a vesicle perparation to which the major fraction of the mass of phospholipid is concentrated in a narrow size range, i.e., the mean vesicle radius ±15 Å. This point is discussed in detail in the paper by Goll and Stock (1977) and in the paper on vesicle size distributions by Barenholz et al. (1977).

Sonifier (W-350) under nitrogen in glass tubes with flat bottoms so designed that clearance between probe and tube wall did not exceed 5 mm. The volume of the phospholipid dispersion was 2–8 mL and the size of the tube and the probe position was adjusted to the dispersion volume. Sonication was carried out intermittently at 0 °C for 2–3 min, followed by a 1-min cooling period for a maximum sonication time of 30 min. Following sonication, the vesicle dispersion was centrifuged for 15–30 min at 100 000*g* (Ti-65 fixed angle rotor) to remove any probe particles as well as large multilamellar liposomes.

A homogeneous vesicle dispersion was prepared from this supernatant by additional centrifugation in the same rotor at 159 000*g* for a critical period of time which is dependent upon the lipid, buffer composition, and the temperature. The experimental details pertinent to the preparations studied in this work are described under Results and Discussion. The crucial step in obtaining a homogeneous vesicle preparation is the removal of the supernatant from the centrifugation tube. Three regions can clearly be observed in the centrifuge tube after the high-speed run: the pellet on the bottom side of the tube (region I), a zone immediately above the pellet, which usually extends to the upper boundary of the pellet and has a distinctly high level of light scattering (region II), and the clear or weakly scattering supernatant (region III). The only zone that contains a population of homogeneous vesicles is the clear supernatant (region III). The three regions of the tube are illustrated in Figure 1. The difference in vesicle homogeneity between regions II and III is discussed under Results and Discussion. Removal of the clear supernatant can be done either by a Pasteur pipet or by a peristaltic pump, but it is essential to remove region III only.

Turbidity Measurement. The specific turbidity is defined as the turbidity per micromole of phospholipid. The turbidity was measured in a 1-cm cell at 300 nm, using a Gilford 240 spectrophotometer.

Gel Chromatography. Gel filtration chromatography was carried out as described by Huang (1969) with the exception that Sepharose 2B was used instead of Sepharose 4B. All chromatography was carried out at 4 °C.

Sedimentation Velocity Measurements. Sedimentation velocity measurements were carried out at 44 000 rpm on samples of regions II and III obtained from a high-speed centrifuge run at 20 ± 0.05 °C in a Spinco Model E ultracentrifuge equipped with RTIC temperature control unit and a schlieren optical system fitted with a phase plate. In all measurements, a double-sector capillary-type synthetic boundary cell with a 12-mm optical path was used. Values of the sedimentation coefficient and boundary spreading parameter obtained for region III egg phosphatidylcholine vesicles were indistinguishable from the values obtained for similar vesicles prepared by the Huang procedure (Huang and Charlton, 1971).

Determination of Partial Specific Volume. Partial specific volumes were calculated from density measurements obtained using the Parr-Mettler cavity resonance type density meter Model DMA 02 C with temperature maintained at 20 ± 0.005 °C. The instrument was calibrated using sucrose solutions of known densities (National Bureau of Standards). The standard deviation on the slope and intercept of the curve describing density as function of concentration was better than ±2.0% with a correlation coefficient higher than 0.997. The values for the density of water and salt solutions were in excellent agreement with the values in the International Critical Tables.

NMR Measurements. Estimates of the ratio of the number of phospholipid molecules in the two opposing monolayers of the vesicle bilayer were made by NMR[2] spectroscopy for choline-containing lipids. *N*-methyl protons or ^{31}P nuclei on the inner and outer bilayer faces were differentiated from each other by using Pr^{3+} as a paramagnetic shift reagent (Huang et al., 1974). All studies were carried out on a JEOL PS-100 P/EC-100 Fourier transformed spectrometer. The desired temperature was obtained using a JEOL VT/3B temperature control unit. The temperature was measured to ±0.1 °C using a Yellow Spring Instrument Thermistor No. 44016 set in a NMR tube under conditions which were identical to experimental conditions. The instrument was locked on the D_2O. Typical conditions were a spectra width of 1 kHz using 4K data points in the frequency domain. At least 100–1000 pulses were averaged, depending on lipid concentration.

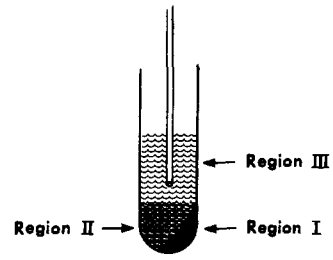

FIGURE 1: Schematic representation of the distribution of phospholipid in the centrifuge tube after a high-speed centrifuge run. Region I contains undispersed phospholipid and any probe particles. Region II contains large multilamellar structures and a broad size distribution of vesicles. Region III contains the population of homogeneous, minimal size vesicles.

Determination of Phosphatidylethanolamine in the Outer-Vesicle Surface. An aliquot of an aqueous vesicle solution (containing no more than 0.25 μmol of amino groups) was diluted to a final volume of 0.6 mL with the vesicle buffer solution. An addition of 0.2 mL of 0.8 M $NaHCO_3$ (pH 8.5) was made and the vesicle sample was mixed. A 20-μL aliquot of 1.5% 2,4,6-trinitrobenzenesulfonic acid (TNBS) was added to this solution. The sample was mixed and allowed to incubate in the dark for 30 min at room temperature. After the incubation period, 0.4 mL of 1.2% Triton X-100 in 1.5 N HCl was added to the sample, followed by mixing and storage in the dark. The absorbance at 410 nm was read within an hour after acidification.

Determination of the Total Phosphatidylethanolamine Vesicle Content. An aliquot of an aqueous vesicle solution (containing no more than 0.25 μmol of amino groups) was diluted to a final volume of 0.6 mL with the vesicle buffer solution. An addition of 0.2 mL of 1.6% Triton X-100 in 0.8 M $NaHCO_3$ (pH 8.5) was made and the sample mixed. A 20-μL aliquot of 1.5% TNBS was then added to this solution. The sample was mixed and allowed to incubate in the dark for 30 min at room temperature. After the incubation period, 0.4 mL of 0.4% Triton X-100 in 1.5 N HCl was added to the sample, followed by mixing and storage in the dark. The absorbance at 410 nm was read within an hour of acidification. The absorbance at 410 nm is linear with concentration of amino group to at least 1.5 absorbance units, with a slope of 4.52 absorbance units per micromole of amino groups.

Photon Correlation Spectroscopy Measurements. Photon correlation spectroscopy measurements were carried out as described by Goll and Stock (1977). The average radius of the vesicles was determined from Stokes' law using the diffusion

[2] Abbreviations used are: TNBS, 2,4,6-trinitrobenzenesulfonic acid; NMR, nuclear magnetic resonance.

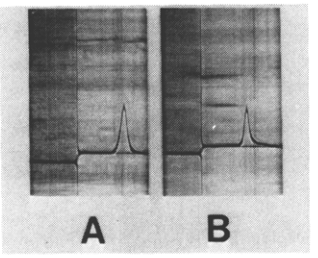

FIGURE 2: Sedimentation velocity patterns for phosphatidylcholine vesicles obtained from (A) region III and (B) region II of a centrifuge tube (Figure 1) after a high-speed centrifugation run. The sample was prepared by centrifugation at 100 000*g* for 20 min, followed by centrifugation at 159 000*g* for 195 min. Regions II and III samples were removed as described under Materials and Methods.

coefficient obtained from the photon correlation spectroscopy measurements and assuming the vesicles to be spheres (Carlson, 1975).

Results and Discussion

Figures 2 and 3A illustrate the characteristics of region II and III material prepared from egg phosphatidylcholine by the high-speed ultracentrifuge method. Figure 2 is a comparison of the schlieren pattern obtained in the analytical ultracentrifuge of region III (Figure 2A) and region II (Figure 2B) vesicles. The marked asymmetry of the peak in Figure 2B is in sharp contrast to the symmetry of the peak exhibited by region III vesicles in Figure 2A. The high degree of size homogeneity evidenced by the symmetry of the peak in Figure 2A is commensurate with the size homogeneity of vesicles prepared by molecular-sieve chromatography (Huang, 1969; Huang and Thompson, 1974). The heterogeneous region II material corresponds to similar material appearing in the void volume of the molecular-sieve column used in the Huang procedure.

A contrast between the Sepharose 2B chromatographic behavior of phosphatidylcholine vesicles prepared by low-speed centrifugation with a region III vesicle sample from a high-speed centrifugation preparation is shown in curves a and b, respectively, of Figure 3A. The chromatographic profile of the low-speed centrifugation sample displays both a void volume and internal volume peak, while the region III sample shows only an internal volume peak. The observed specific turbidity will be constant with elution position when the vesicle size distribution in the fractions is invariable. Hence, regions of changing size distribution in the elution profile of the two samples will be characterized by a nonlinear behavior of the specific turbidity. Curves c and d of Figure 3A show that, while the region of nonlinear specific turbidity for the low-speed centrifugation sample extends well into the peak in the internal volume profile, this region encompasses only the first three fractions of the leading side of the internal volume peak for the high-speed centrifugation sample. Ninety-seven percent of the sample prepared by high-speed centrifugation is within the region of constant turbidity, while only 60% of the material in the low-speed centrifugation sample falls into this region. The value of the specific turbidity in the plateau region for the high-speed centrifugation sample is somewhat lower than that observed for the low-speed centrifugation sample; this may reflect a narrower size distribution and smaller particles in the former sample relative to the latter one.

The improved yield of homogeneously sized vesicles prepared by high-speed centrifugation as compared to the chromatographic method is illustrated by the results of a typical experiment. Initially, 200 μmol of egg phosphatidylcholine was

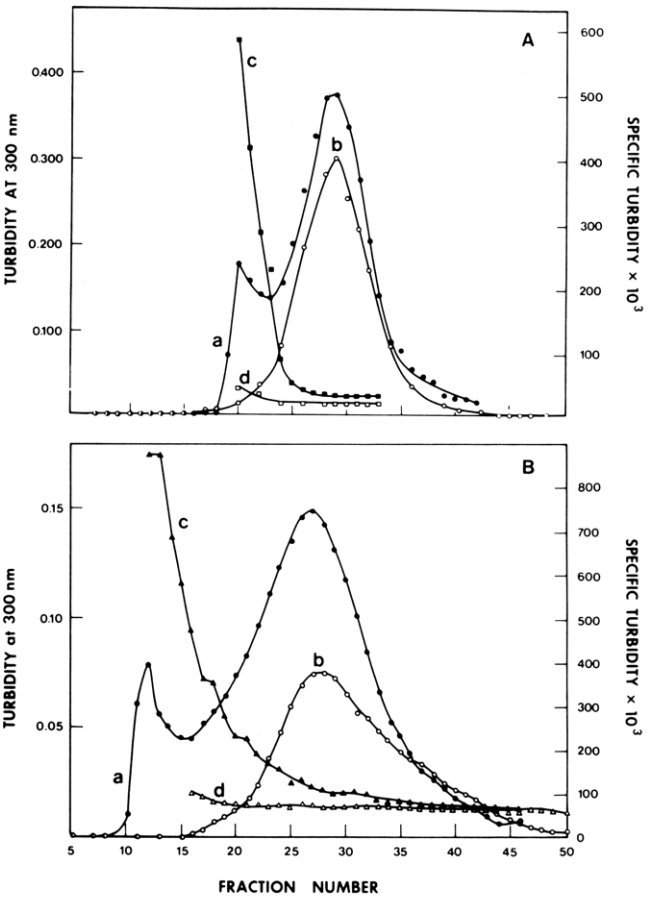

FIGURE 3: (A) The Sepharose 2B elution profile and specific turbidities of phosphatidylcholine vesicles prepared by the high- and low-speed centrifugation procedures. The low-speed sample was prepared by centrifugation at 100 000*g* for 20 min, while the high-speed, region III sample had an addition centrifugation at 159 000*g* for 195 min. The turbidity at 300 nm vs. fraction number: the low-speed sample, curve a; the high-speed sample, curve b. The specific turbidity at 300 nm vs. fraction number: the low-speed sample, curve c; the high-speed sample, curve d. (B) Similar information for sphingomyelin vesicles. The low-speed sample was prepared by centrifugation at 100 000*g* for 15 min, while the high-speed, region III sample had an additional centrifugation at 159 000*g* for 120 min. The turbidity at 300 nm vs. fraction number: the low-speed sample, curve a; the high-speed sample, curve b. The specific turbidity at 300 nm vs. fraction number: the low-speed sample, curve c; the high-speed sample, curve d.

sonicated. After the high-speed centrifugation, 120 μmol of phospholipid was recovered. Of this material, 97% was contained in fractions which were on the linear region of the specific turbidity curve (Figure 3A). The yield of homogeneous vesicles is on the order of 58%; this is in contrast to the yield of from 30 to 40% normally obtained by column chromatography.

A more stringent test of homogeneity is given by the determination of the diffusion coefficient of the vesicle sample by photon correlation spectroscopy. If all of the vesicles are Rayleigh scatters, then the calculated diffusion coefficient will be independent of the scattering angle. If, however, a high degree of size inhomogeneity exists in the sample, then a nonlinear dependence of the diffusion coefficient on the angle will be observed. Excellent linearity was observed for all preparations over a wide range of angles. This point will be discussed further in the case of sphingomyelin vesicles. The mean vesicle radius determined by photon correlation spectroscopy for phosphatidylcholine vesicles prepared by the high-speed centrifugation procedure was found to be 105 Å,

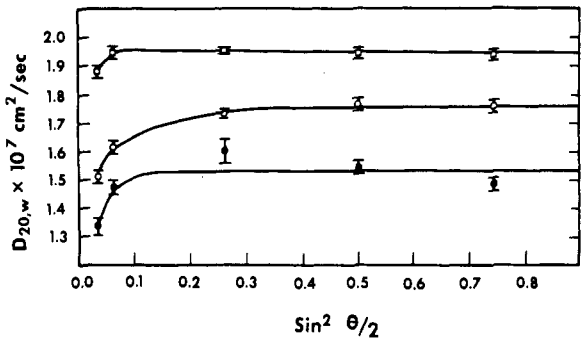

FIGURE 4: Dependence of sphingomyelin vesicle diffusion coefficients, $D_{20,w}$, on $\sin^2 \theta/2$, where θ is the scattering angle. Vesicles were prepared by centrifugation at 100 000g for 135 min (●); 159 000g for 135 min (○); and 159 000g for 150 min (□).

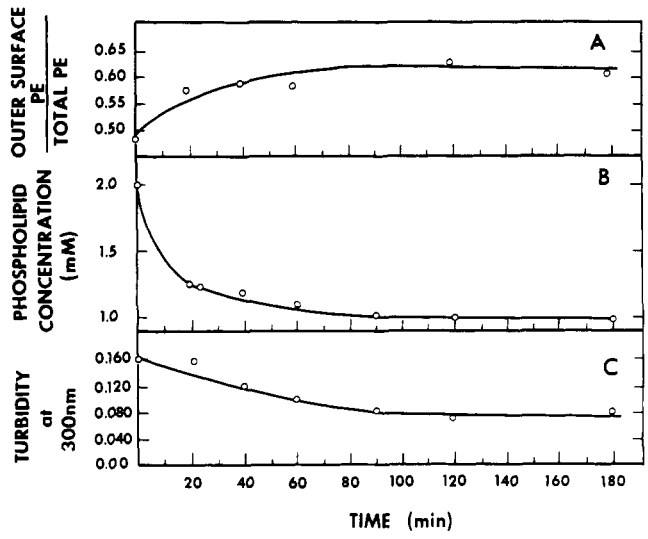

FIGURE 5: The dependence of several physical parameters of mixed phosphatidylethanolamine–phosphatidylcholine vesicles on the centrifugation time at 159 000g and 4 °C: (A) the phosphatidylethanolamine surface ratio; (B) the turbidity at 300 nm; and (C) the loss of phospholipid.

in good agreement with that determined by Huang and Lee (1973) for column chromatographed preparations.

In order to further demonstrate the range of usefulness of the high-speed centrifugation procedure, we report here on the preparation of both pure sphingomyelin vesicles and a 1/1 phosphatidylcholine–phosphatidylethanolamine mixed vesicle system. The chromatographic profiles of sphingomyelin vesicles prepared both by the low-speed centrifugation procedure and the high-speed centrifugation methods are shown in Figure 3B (curves a and b, respectively). The behavior of the sphingomyelin vesicles prepared by the high-speed centrifugation technique is like that of similarly prepared phosphatidylcholine vesicles, i.e., a single internal volume peak with greater than 95% of the phospholipid within the region of constant specific turbidity, as shown by curves (Figure 3B). This is in contrast to the sample prepared by the conventional low-speed centrifugation technique which shows both an internal and void volume peak, with only 15% of the phospholipid exhibiting a constant specific turbidity (curve c, Figure 3B). Additional evidence for the elimination of void volume material in the sphingomyelin preparation is given by the loss of the angular dependence of the diffusion coefficient in photon correlation spectroscopy measurements, with increased centrifugation time, shown in Figure 4. It can be seen that, while a sample centrifuged for 135 min at 102 000g still shows a marked angular dependence for the diffusion coefficient at low angle, an equivalent sample centrifuged at 159 000g for 150 min shows virtually no variation in the diffusion coefficient over the entire angular range studied; these results indicate the loss of non-Rayleigh scatters during the high-speed centrifugation.

Mixed phospholipid vesicles, composed of equimolar mixtures of phosphatidylcholine and phosphatidylethanolamine, show similar chromatographic behavior to phosphatidylcholine vesicles when they were subjected to high- and low-speed centrifugation. A useful parameter in evaluating the high-speed centrifugation procedure for the mixed phospholipid vesicles is to determine the surface ratio of phosphatidylethanolamine at various times of spinning. Initially, this value should be low due to the multilamellar nature of the void volume material which will result in a masking of the phosphatidylethanolamine in the inner bilayers. As the void volume material is eliminated, the ratio rises, reaching a constant value when the remaining vesicle population becomes stable with respect to size. Figure 5 shows the results of such an analysis as well as the loss of phospholipid, and turbidity of the vesicle sample as a function of centrifugation time at 159 000g. The phosphatidylethanolamine surface ratio rises with centrifugation time, reaching a constant value after about 90 min. This value is in good agreement with surface ratios obtained from identical mole fraction samples prepared by molecular-sieve chromatography. The turbidity data shows a continual reduction to about 90 min, and then a plateau. The behavior of both the surface ratio and turbidity data is indicative of an elimination of the large, multilamellar particles from the vesicle sample in the first 90 min of centrifugation, after which time the sample exhibits a constant size distribution.

TABLE I: Apparent Partial Specific Volumes and Centrifugation Times for Phospholipid Vesicle Systems.

Vesicle System	Apparent $\bar{v}$	Centrifugation Time (h)	Solvent
Phosphatidylcholine	0.98400[a]	3–4	50 mM KCl
Phosphatidylcholine/phosphatidylethanolamine (1/1)	0.97929[a]	1.5–2	100 mM NaCl
Sphingomyelin	0.96590[a]	1–1.5	50 mM KCl

[a] Standard deviation ±0.00003.

The mole fraction of phosphatidylethanolamine is unchanged during centrifugation, indicating that this property is the same for both the large and small vesicles. While there is a marked loss of phospholipid up to 90 min, after this time the loss of phospholipid is negligible. The loss of material in the high-speed centrifugation procedure is about 50% in the mixed phospholipid vesicle system.

In the case of both phosphatidylcholine and sphingomyelin vesicles, the ratio between the number of choline groups in the outer vesicles surface to the number of choline groups in the inner vesicles surface was determined using both proton and phosphorus NMR; both were in very good agreement. The fraction of homogeneous phosphatidylcholine vesicles obtained by gel filtration gave a ratio of 2.2 ± 0.2. The same ratio was obtained for homogeneous phosphatidylcholine vesicles prepared by the high-speed centrifugation procedure. However, after the first short centrifugation of 30 min at 100 000g the ratio was 1.7 ± 0.2, which demonstrates the contribution of

the larger vesicles. Similar results were obtained for sphingomyelin vesicles. In this case, ratio values of 1.9 ± 0.2 for the vesicles prepared by the high-speed centrifugation procedure and 1.5 ± 0.25 for the vesicles after the first centrifugation (15 min at 100 000*g*) were obtained.

The experiments described herein demonstrate that, in the case of phosphatidylcholine, sphingomyelin, and mixed phosphatidylcholine–phosphatidylethanolamine vesicles, the high-speed method of preparation is capable of yielding vesicle preparations of equivalent size homogeneity to that obtained by molecular-sieve chromatography. In addition, the high-speed procedure represents a rapid method capable of generating solutions of high phospholipid concentration. A major problem with the molecular-sieve chromatography procedure is that a large percentage of the homogeneous vesicles are mixed with larger vesicles of various sizes in the ascending side of the internal volume peak. This region is not useful for studies requiring homogeneous, minimal size vesicles. However, in the high-speed centrifugation procedure a much larger percentage of these "good" vesicles can be isolated. The flexibility of the procedure is demonstrated by the diverse preparations used in these experiments. The actual spinning time required for any particular system will be dependent on the partial specific volume of the vesicle sample and the density of the buffer solutions (Table I). Once the time of centrifugation has been determined, however, the procedure can be employed routinely for the rapid preparation of homogeneous vesicle samples.

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