Lipid Model Membranes. Characterization of Mixed Phospholipid Vesicles†

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ABSTRACT: Mixed phospholipid vesicles, formed by ultrasonic irradiation, were characterized with respect to homogeneity of size and composition in order to extend the range of this model system apropos of simulating the complex mixture of phospholipids found in biological membranes. A study of the mole fraction dependence of the incorporation of phosphatidylethanolamine into phosphatidylcholine vesicles shows a slight deviation from linearity, whereas a similar study of the incorporation of phosphatidylinositol into phosphatidylcholine vesicles demonstrated linear behavior over the entire mole fraction range. The linearity of trapped volume measurements and consistency of composition determinations for fractions taken from the trailing side of the internal volume peak of a Sepharose 4B column indicate that the size and compositional distribution are constant for these fractions. The breadth of these distributions, as reflected by the heterogeneity in the sedimentation coefficient, has been measured by analysis of the boundary spreading in sedimentation velocity experiments; these results indicate that the heterogeneity in the phosphatidylcholine–phosphatidylinositol vesicles is less than 5.2% over the entire mole fraction range, while in the phosphatidylcholine–phosphatidylethanolamine system, the degree of heterogeneity increases from 2.8 to 22% for samples containing 0.1 and 0.5 mole fraction phosphatidylethanolamine, respectively. To demonstrate the versatility of this approach, we have prepared a ternary vesicle from phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine, whose mole fraction composition simulates that of the major phospholipid components of the retinal rod outer segments; this preparation exhibits the same consistency of size and compositional distribution found in the binary vesicle systems.

A wide variety of research into the structure and function of biological membranes has been stimulated by the development of appropriate models for the lipid portion of these membranes (Chapman and Dodd, 1971; Yost *et al.*, 1971; Thompson and Henn, 1970; Sessa and Weissman, 1968). Several physical techniques, such as X-ray diffraction (Engelman, 1972; Wilkins *et al.*, 1971; Levine and Wilkins, 1971), spin labeling (Kornberg and McConnell, 1971; Huang *et al.*, 1970; Hubbell and McConnell, 1968), calorimetry (Blazyk and Steim, 1972; Steim *et al.*, 1969), and freeze etching (Pinto da Silva and Branton, 1970) have correlated results from model membrane systems and biological membranes to demonstrate the presence of regions of phospholipid bilayer in these membranes. The functional aspects of membranes, *i.e.*, active transport, are presumed to reside with the protein portion of the membrane; the bilayer region of the membrane is thought to function primarily as a diffusion barrier and matrix into which the mosaic of different membrane proteins is imbedded (Singer, 1971). The phospholipid dependence of the activity of several membrane-derived enzymes indicates that a more subtle role may be played by the phospholipid, *i.e.*, one of mediating conformational changes in membrane-bound enzymes. The availability of a convenient model bilayer system would greatly enhance one's ability to study protein–lipid interactions. Huang (1969) has demonstrated the feasibility of preparing an ordered lipid structure in the form of a phosphatidylcholine vesicle, bounded by a single bilayer wall; this preparation was demonstrated to have a high degree of size homogeneity. An important extension of phospholipid vesicles as models for lipid bilayers in biological membranes would be achieved if they could be formed from mixtures of phospholipids in known mole fraction ratios and could be shown to be characterized by a narrow distribution of both size and composition. To accomplish this objective, we have undertaken the study of binary and ternary mixtures of phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserine in vesicles. In this paper we report the preparation and characterization of binary vesicles formed from phosphatidylcholine–phosphatidylethanolamine and phosphatidylcholine–phosphatidylinositol and a ternary vesicle formed from phosphatidylcholine–phosphatidylethanolamine–phosphatidylserine.

Experimental Section

Phospholipid Preparation. Crude phosphatidylcholine and phosphatidylethanolamine were obtained from fresh egg yolks by extraction with 2:1 chloroform–methanol (Folch *et al.*, 1957). A partial purification was obtained by three successive acetone precipitations from petroleum ether. Crude phospholipid was loaded (1 g/25 g of Unisil) as a chloroform solution on a silicic acid column (Unisil, 200–325, mesh, Clarkson Chemical Co.) which had been chemically activated by the method of Hirsch and Ahrens (1958). Phospholipids were eluted using increasing volume fractions of methanol in a chloroform–methanol mixture (Ansell and Hawthorne, 1964). The samples were pooled according to their purity as judged by thin layer chromatography. The thin layer plates were developed in chloroform–methanol–water (65:25:4) and the lipids visualized by exposure to iodine vapors. The samples were then taken to dryness on a flash evaporator under an inert atmosphere. Crude phospholipid (25 g) generally yielded about 4 g of pure phosphatidylethanolamine and 11 g of pure phosphatidylcholine. Phosphatidylinositol was prepared from

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soybean phosphatides by scaling up the procedure of Colacicco and Rapport (1967). Crude phosphatidylserine was obtained as a brain extract from Sigma Chemical Co. and purified by silicic acid chromatography.

Phospholipid Determination. Phospholipid concentrations were determined as inorganic phosphate by a modification of the procedure of Bartlett (1959). A phospholipid sample containing from 0.1 to 1 μmol of lipid phosphorus was added to a digestion tube along with 0.4 ml of concentrated H_2SO_4. Samples were digested in an aluminum block at 300° for 20 min. The charred solutions were cleared by addition of 3–5 drops of 30% H_2O_2 and continued digestion for 20 min. The tubes were cooled and 8.8 ml of distilled water was added to each tube. Addition of 0.4 ml of 0.04 M ammonium molybdate solution and 0.4 ml of Fiske–SubbaRow reagent was followed by thorough mixing. The samples were then heated for 7 min at 100°, cooled, and read at 660 nm.

Phospholipid Determination for Samples Containing Silica Gel. Samples to be analyzed for lipid composition were lyophilized overnight and redissolved in 1 ml of chloroform. Aliquots of 100–300 μl of the chloroform solution were applied as a band, in triplicate, to a 0.25 mm thick silica gel H plate. The samples were developed in chloroform–methanol–ammonia (14:6:1) in the case of phosphatidylcholine–phosphatidylinositol dispersions and in chloroform–methanol–acetic acid–water (50:25:8:4) in the case of phosphatidylcholine–phosphatidylethanolamine dispersions.

Separation of the phospholipid components in the phosphatidylcholine–phosphatidylethanolamine–phosphatidylserine ternary system required the use of two-dimensional thin layer chromatography. A single sample aliquot was applied to the lower left-hand corner of each plate. The plates were first developed in the chloroform–methanol–ammonia solvent, air dried for 10 min after development, and run in an anhydrous ethyl ether tank to ensure removal of water. After air drying for 10 min, the plates were rotated 90°, and developed in the chloroform–methanol–water–acetic acid solvent system.

Phospholipid ratios were determined as follows. Inorganic phosphate analysis was carried out by scraping into digestion tubes uniform areas of silica gel, corresponding to the various spots on the plate. Blanks were obtained by taking silica gel samples from a lane of the plate which had been washed by the eluting solvent, but which had not been spotted. After addition of 0.4 ml of concentrated H_2SO_4, the samples were digested in an aluminum block at 300° for 1 hr, after which the charred samples were treated with H_2O_2 as in the standard procedure. The cooled samples were diluted to 8.8 ml and allowed to stand overnight; they were then developed in the standard way, centrifuged, and read at 660 nm.

In order to determine the recovery levels in these experiments, standard mixtures of phospholipids were applied to thin layer plates and the spots were recovered from both developed and undeveloped plates; solution concentration determinations of these mixtures were employed as controls. The total phospholipid recovered ranged from 95 to 100% of the solution concentration and no systematic variation in recovery of individual phospholipids was observed. Recovery levels in the experiments reported here ranged from 90 to 95% as compared to solution concentration determinations. All column fractions were analyzed in triplicate and the ratios quoted are the average of a minimum of four column fractions analyzed per dispersion. The standard deviation for these measurements ranged from ±1 to ±5%.

Determination of the Ratio of Phosphatidylethanolamine in the Outer Vesicle Surface to the Total Phosphatidylethanolamine Content by Reaction with TNBS.[1] Reagents used were: 1.8% aqueous sodium bicarbonate, pH 8.5; 1.8% sodium bicarbonate in 50% propanol, pH 8.5; 2% TNBS in distilled water (Sigma Chemical Co.)—this solution is prepared fresh each week and stored at 4° in the dark; 0.5 N HCl in 96% propanol; and 0.5 N HCl in 50% propanol.

A standard curve for the TNBS analysis was obtained by analyzing aliquots (75–500 μl) of phosphatidylethanolamine stock solutions (whose concentrations had been determined by the phosphate method) according to the procedure described below for the determination of total phosphatidylethanolamine content. The reaction reached completion by 70 min after addition of TNBS solution and yielded identical absorbance values for aliquots allowed to react up to 160 min. After acidification the absorbance remained constant for at least 140 min. The TNBS reaction yielded a linear absorbance *vs.* concentration curve in the range from 0 to 1.75 μM/ml with a slope of 1.391 Å/μM and a correlation coefficient of 0.9995.

Determination of Phosphatidylethanolamine in the Outer Vesicle Surface. Aliquots (100–500 μl) of phosphatidylethanolamine containing vesicle solutions were diluted to a final volume of 2 ml with the aqueous bicarbonate buffer. Addition of 50 μl of the TNBS solution was followed by mixing and storage in the dark for 2 hr at room temperature. After the development time, 2 ml of the acidic 96% propanol solution was added with immediate mixing. Samples were stored in the dark until read. Prior to reading, each sample was loaded into a ground glass stoppered cuvet and spun in a desk top centrifuge for 1 min to eliminate air bubbles. The sample was read at 410 nm.

Determination of Total Phosphatidylethanolamine Content. Aliquots (100–500 μl) of vesicle solution were diluted to a final volume of 2 ml with the bicarbonate–propanol buffer. Subsequent addition of 50 μl of the TNBS solution was followed by mixing and storage in the dark for 2 hr at room temperature. After development, 2 ml of acidic 50% propanol solution was added followed by immediate mixing. The samples were read as described above.

Vesicle Preparation. Stock solutions of the individual lipids were mixed in a volume ratio so as to produce the desired mole fraction of the individual phospholipid components; this solution, containing about 300 μM of lipid phosphorus, was then lyophilized. After lyophilization, the sample was hydrated in 12 ml of the desired buffer and transferred to a jacketed sonication vessel. The sample was subjected to ultrasonic irradiation for 1.5 hr and was maintained at 4°, under an inert atmosphere (either N_2 or Ar) for the entire process. The phospholipid dispersion was then centrifuged at 100,000*g* for 30 min to remove undispersed lipid and titanium particles from the probe; this sample was then applied to a 2.5 × 40 cm jacketed, Sepharose 4B column and 5-ml fractions were collected. The column was monitored by recording the scattering due to the phospholipid vesicle at 300 nm and was maintained at 4° throughout the run.

Trapped Volume Experiments. Phospholipid vesicles were prepared in the standard way except that the sonication medium included 0.5 M glucose. The exact glucose concentration was determined spectrophotometrically. After sonication and centrifugation, the sample was loaded on a 2.5 × 40 cm jacketed Sepharose 4B column, which was preequilibrated

[1] Abbreviation used is: TNBS, 2,4,6-trinitrobenzenesulfonic acid.

with the glucose buffer. Column fractions falling in the linear region of the scattering *vs.* inorganic phosphate curve were selected for analysis. Aliquots (2 ml) of a sample were loaded on a 0.9 × 22 cm jacketed Sephadex G-50 column, the void volume being determined for each run by monitoring the vesicle scattering in the eluent. In each case the void volume was collected and stored at 0° until glucose was assayed, with pentachlorophenol added as an antibacterial agent. Each sample was run over a freshly washed column. The columns were thoroughly washed with buffer after each series of samples. All solutions and columns were maintained at 4° or lower to prevent loss of trapped glucose by diffusion out of the vesicle and deter bacterial growth.

Glucose Determination. Glucose determinations in trapped volume experiments were carried out by a modification of the procedure of Park and Johnson (1949). The phospholipid vesicle sample was suspended in 1% sodium dodecyl sulfate, before addition of subsequent reagents; this served to solubilize the lipid so as to free trapped glucose and ensured solubilization of the Prussian Blue formed in the test. Samples were allowed to develop for 20 hr, since the presence of the increased levels of sodium dodecyl sulfate required longer development time in standard glucose tests. Samples were read at 690 nm. Each glucose value is the average of at least four determinations.

Molecular Sieve Experiments. A 0.9 × 25 cm jacketed column was packed with Sepharose 4B and equilibrated with the desired salt concentration. Unchromatographed vesicle solution (200 μl) was loaded onto the column. Column parameters were obtained by using glycylglycine as a marker for the internal volume, V_i, and both india ink and the excluded portion of the vesicle preparation to determine the void volume, V_0; these parameters were measured for each salt concentration employed. The column elution position, V_e, for the various vesicle preparations was expressed in terms of the partition coefficient, σ, as calculated from the following equation (Ackers, 1967).

$$V_e = \sigma V_i + V_0 \tag{1}$$

Column flow rates were maintained between 0.14 and 0.30 ml/min by use of a peristaltic pump. The column temperature was maintained at 4°.

Ultracentrifuge Measurements. Sedimentation velocity experiments were carried out in a Spinco Model E ultracentrifuge equipped with a temperature control unit and schlieren optics. Measurements on phospholipid vesicles were performed at a rotor speed of 42,040 rpm, in a double-sector, capillary type synthetic boundary cell with a 12 mm length at 20 ± 0.05° with the exception of phosphatidylinositol vesicle runs, which were made at a rotor speed of 20,410 rpm. Schlieren patterns were recorded on metallographic plates, and read on a two-dimensional microcomparator, Nikon Shadowgraph, Model 6. The sedimentation coefficient, s, was calculated from the slope of a plot of ln r *vs.* t, where r is the position of the maximum ordinate of the schlieren peak at time, t. The concentration dependence of s is expressed by the following equation

$$s_{20} = s^0_{20}(1 - kc) \tag{2}$$

where c is the lipid concentration expressed as micromoles of P_i per milliliter, and s^0_{20} represents the value of the sedimentation coefficient at infinite dilution (Schachman, 1959).

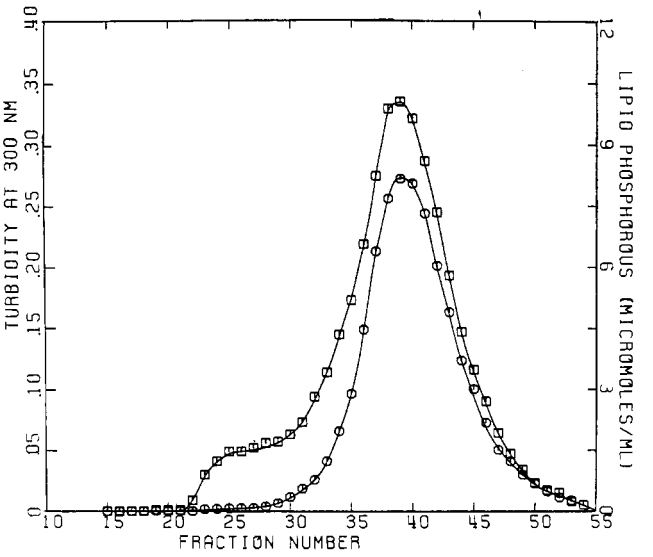

FIGURE 1: Elution profile of a 4:1 phosphatidylcholine–phosphatidylinositol vesicle preparation run on a Sepharose 4B column in 0.1 M NaCl–0.01 M Tris–HCl at pH 8.5: lipid concentration expressed as inorganic phosphate *vs.* fraction number (○); turbidity 300 nm *vs.* fraction number (□).

The heterogeneity of the vesicle preparations was analyzed in terms of the heterogeneity parameter, p (Fujita, 1962), by use of the following equation

$$\frac{\sigma^2(r_0/r)}{2t} = D + \frac{(p\omega^4 r_0)rt}{2} \tag{3}$$

where σ is the second moment of the gradient curve, r is the boundary position as measured by the position of the maximum ordinate of the gradient curve at time t, r_0 is the initial boundary position, D is the diffusion coefficient, and ω is the angular velocity.

The second moment was evaluated by use of the following relationship

$$\sigma^2 = \frac{1}{2\pi}\left[\frac{A}{KH_{max}}\right]^2 \tag{4}$$

where A is the integrated area under the schlieren peak, K is the magnification factor, and H_{max} is the height of the schlieren peak relative to the base line as measured at the position of maximum ordinate. Area and height were measured by projecting the schlieren image onto graph paper with an enlarger, cutting out the traced image of the schlieren peak, weighing the paper, and converting to area *via* an appropriate calibration procedure.

Results

Compositional Analysis and Gel Filtration. The characteristic chromatographic profile obtained upon molecular sieve chromatography of mixed phosphatidylcholine–phosphatidylinositol phospholipid vesicle preparation is shown in Figure 1 and is analogous to that obtained for pure phosphatidylcholine vesicles (Huang, 1969). The elution profile is monitored by determining the lipid concentration, as expressed in micromoles of inorganic phosphate (P_i), and by monitoring the turbidity due to the scattering of the lipid vesicles. The void volume fraction is easily seen in the turbidity plot due to the

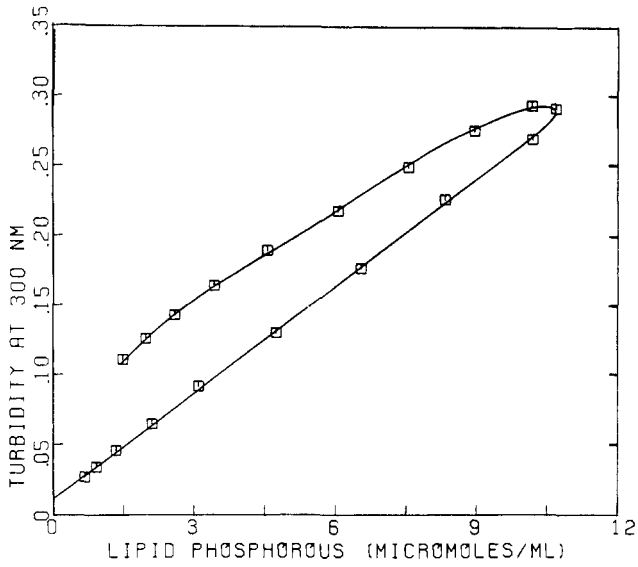

FIGURE 2: Variation in the turbidity of Sepharose 4B column fractions at 300 nm with the lipid concentration.

sensitivity of the scattering measurement; however, inspection of the concentration plot shows that the void volume contains no more than 3% of the total lipid sonicated. Extensive hydrodynamic studies such as boundary spreading analysis in sedimentation velocity experiments (Huang, 1969) have shown that phosphatidylcholine fractions which yield vesicles with a high degree of size homogeneity are those in which the turbidity is proportional to the lipid concentration; this can be tested by plotting the turbidity at 300 nm as a function of lipid phosphorus; such a plot is shown in Figure 2 for a 0.5 mole fraction phosphatidylcholine–phosphatidylinositol vesicle preparation. It is seen that a region of fractions with a constant scattering factor indeed exists; these fractions correspond to the backside of the internal volume peak of the Sepharose 4B column, in good agreement with the results obtained on the phosphatidylcholine system and indicative of a sample region of high size homogeneity.

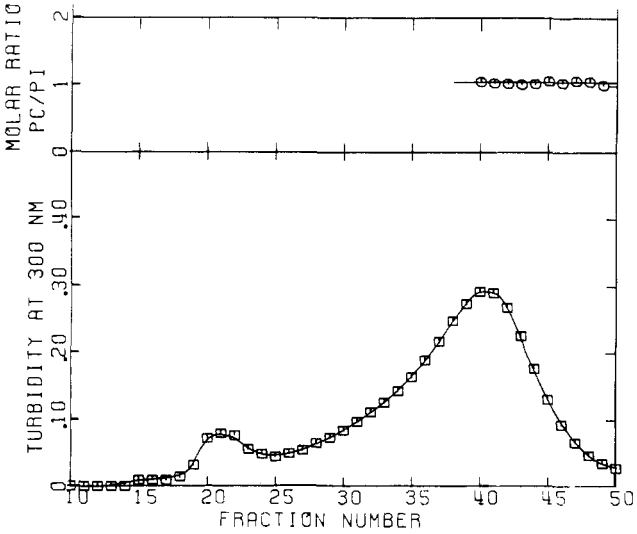

FIGURE 3: Comparison of the elution profile of a 1:1 phosphatidylcholine–phosphatidylinositol vesicle preparation (lower figure) with the molar ratio of phosphatidylcholine (PC) to phosphatidylinositol (PI) found in each column fraction (upper figure).

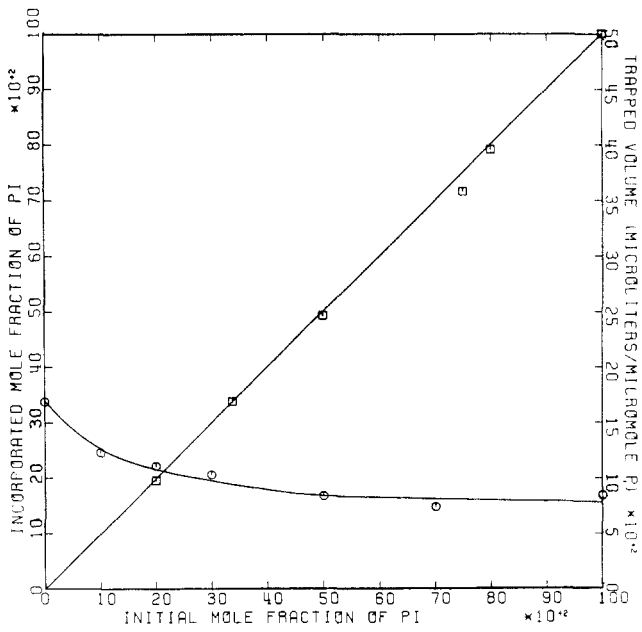

FIGURE 4: The dependence of the incorporated mole fraction of phosphatidylinositol (□) and the trapped volume (○) on the initial mole fraction of phosphatidylinositol.

Linear turbidity *vs.* lipid phosphorus curves were obtained for all preparations reported herein.

In order to determine the relationship between the mole fraction of lipid initially lyophilized and that actually incorporated into the phospholipid vesicle, we studied the lipid composition of the vesicles formed in sonication. To establish the compositional distribution of these mixed vesicle systems within a given dispersion, the phospholipid mole fraction composition was determined for each fraction of the eluent from a molecular sieve column. The results of such an analysis for a 0.5 mole fraction phosphatidylcholine–phosphatidylinositol vesicle preparation is shown in Figure 3. The chemical composition in this system is constant over the range of fractions tested. This result is typical of the systems we have studied and indicates that there is no size-dependent variation in chemical composition in these preparations. Further characterization of these systems is achieved by studying the relationship between the initial mole fraction sonicated and the incorporated mole fraction. Figure 4 shows the results of such a study for the phosphatidylcholine–phosphatidylinositol system; here we see there is a linear relationship for the incorporation of phosphatidylinositol into phosphatidylcholine. Figure 5 shows similar results for the phosphatidylcholine–phosphatidylethanolamine system, where a negative deviation from ideal incorporation occurs.

In the later stages of this work, a technique was developed for the spectrophotometric determination of the phosphatidylethanolamine concentration by formation of the trinitrophenyl derivative of the primary amine group of phosphatidylethanolamine. This procedure served the dual purpose of providing an alternative method for establishing the phosphatidylcholine–phosphatidylethanolamine ratio in mixed vesicles and allowed us to determine the distribution of phosphatidylethanolamine in the outer surface of the vesicle relative to the total amount of phosphatidylethanolamine in the vesicle. The latter ratio was established by forming the trinitrophenyl derivative in an aqueous buffer system, in which the vesicles were intact and only the surface phosphatidyl-

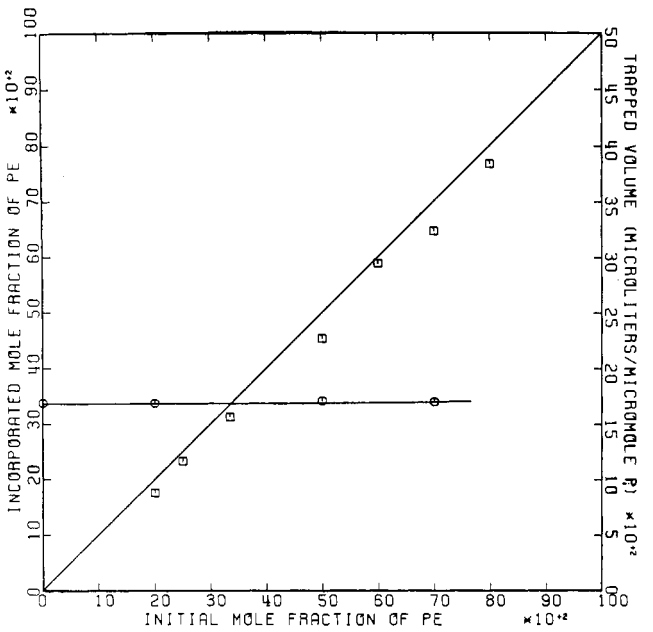

FIGURE 5: The dependence of the incorporated mole fraction of phosphatidylethanolamine (□) and the trapped volume (○) on the initial mole fraction of phosphatidylethanolamine.

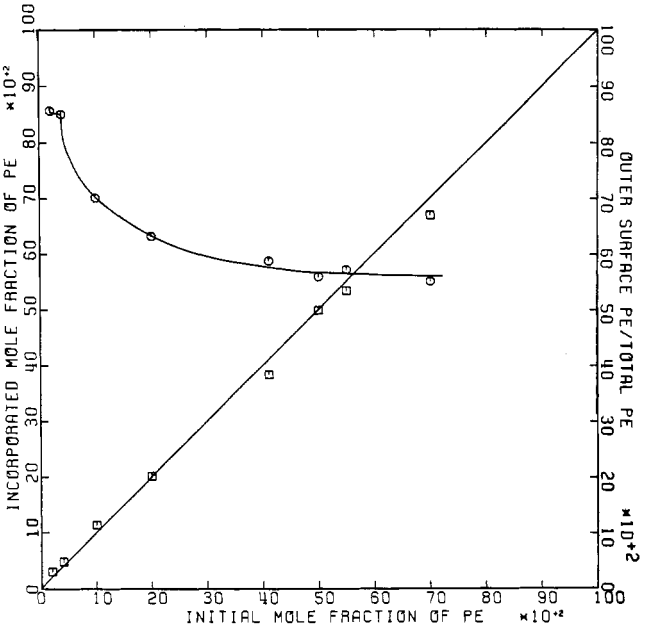

FIGURE 6: The dependence of the incorporated mole fraction of phosphatidylethanolamine (□) and the ratio of the outer vesicle surface phosphatidylethanolamine to the total vesicle phosphatidylethanolamine (○) on the initial mole fraction of phosphatidylethanolamine (as determined by formation of the trinitrophenyl derivative of phosphatidylethanolamine).

ethanolamine was reactive, and in a propanol buffer mixture in which the vesicles were disrupted and all the phosphatidylethanolamine was accessible for reaction. The relationship observed for the incorporated mole fraction of phosphatidylethanolamine relative to the initial mole fraction of phosphatidylethanolamine is similar to that obtained from analysis of the mixed phospholipid vesicles by thin layer chromatography. Above an initial phosphatidylethanolamine mole fraction of 0.2, the system shows a slight negative deviation from ideality, while below this value the system shows ideal behavior (Figure 6). The lack of ideal behavior is also expressed by the variation of the surface distribution of phosphatidylethanolamine in the vesicle. At very low phosphatidylethanolamine concentrations the ratio of phosphatidylethanolamine in the outer surface total phosphatidylethanolamine is 0.83; this ratio is seen to fall as the mole fraction of phosphatidylethanolamine is increased, leveling off at a value of 0.56 at a mole fracton of 0.4 (Figure 6).

Trapped Volume. Determination of the trapped volume of mixed phospholipid vesicles was undertaken as an additional means of characterizing these systems. Figure 7 shows the results of a series of trapped volume experiments for the phosphatidylcholine–phosphatidylinositol vesicle system. Here the trapped volume of glucose per milliliter of vesicle solution is plotted *vs.* lipid concentration for several preparations of differing mole fractions of phosphatidylinositol; these data demonstrate the linear dependence of the trapped volume on the lipid concentration for each mole fraction studied. The variation of the slope of these lines with mole fraction demonstrates the composition dependence of the trapped volume when expressed on a per micromole of phospholipid basis. The high degree of linearity of these plots, whose points represent fractions from a Sepharose 4B column, provides evidence for a high degree of size and compositional homogeneity. The phosphatidylcholine–phosphatidylethanolamine system shows no dependence of the trapped volume on the mole fraction of phosphatidylethanolamine (Figure 8) and the value obtained over the whole mole fraction range studied is identical with that found for phosphatidylcholine. The results of the trapped volume studies are summarized in Figures 4 and 5.

Analytical Molecular Sieve Chromatography. It was previously noted that the trapped volume decreased with increasing phosphatidylinositol mole fraction in a mixed phosphatidylcholine–phosphatidylinositol vesicle. In order to clarify the relationship between trapped volume and vesicle size, analytical molecular sieve chromatography experiments were carried out. Vesicle samples containing 0.0, 0.5, and 1.0

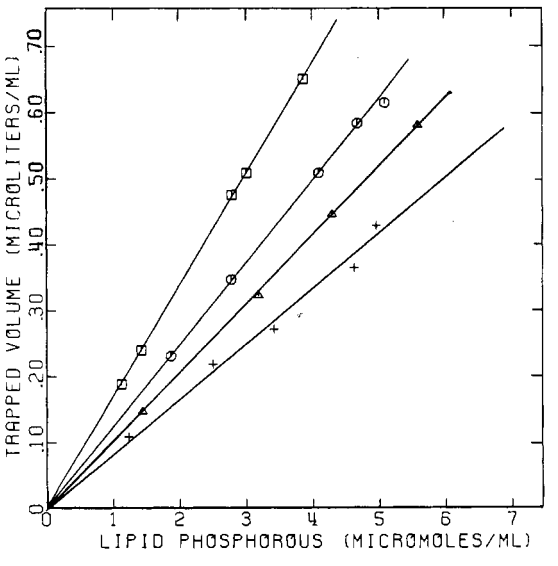

FIGURE 7: Variation in trapped volume of phosphatidylcholine–phosphatidylinositol vesicles with lipid phosphorus for mole fractions of phosphatidylinositol equal to 0 (□), 0.1 (○), 0.3 (△), and 0.5 (+).

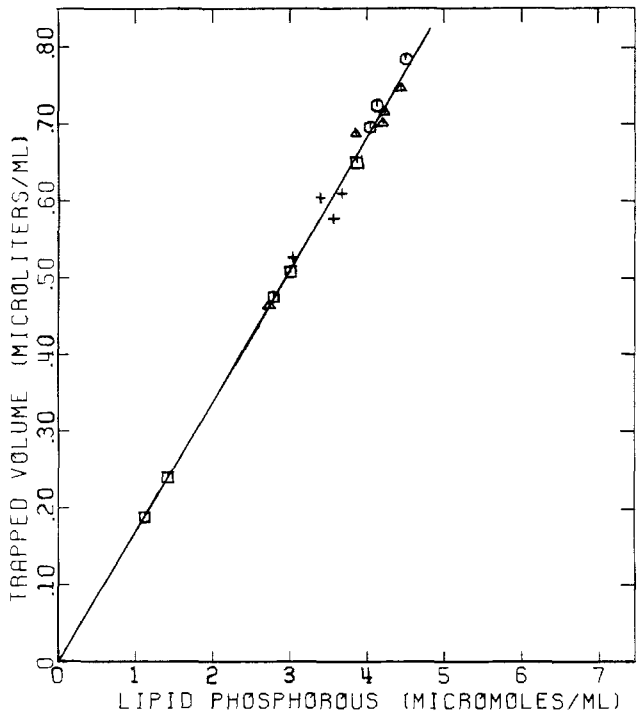

FIGURE 8: Variation in the trapped volume of phosphatidylcholine–phosphatidylethanolamine vesicles with lipid phosphorus for mole fractions of phosphatidylethanolamine equal to 0 (□), 0.2 (○), 0.5 (△), and 0.7 (+).

mole fraction of phosphatidylinositol were chromatographed on a Sepharose 4B column equilibrated with 0.1, 0.2, and 0.3 M NaCl. The results, summarized in Table I, show that the molecular sieve partition coefficients for 0.0 and 0.5 mole fraction phosphatidylinositol are identical and show little dependence on the NaCl concentration. In contrast to this result, the partition coefficient for 1.0 mole fraction phosphatidylinositol vesicles is different from the previous samples and shows a marked dependence on the salt concentration. The partition coefficient increases with increasing ionic strength. Measurements could not be made above 0.3 M NaCl due to precipitation of the lipid at these salt concentrations.

Analytical Ultracentrifugation. Sedimentation velocity experiments were employed as a means of estimating the breadth of the distribution in size and composition of the mixed phospholipid vesicles. The results of these measurements, as summarized in Table II, show that the sedimentation coefficient of mixed vesicles increases with increasing mole fraction of both phosphatidylethanolamine and phosphatidylinositol. Boundary spreading analysis results demonstrate that the heterogeneity in the sedimentation coefficient is less than 5% over the whole range of phosphatidylinositol mole fraction. In contrast, the heterogeneity increases with increasing mole fractions of phosphatidylethanolamine, ranging from 3 to 22% for phosphatidylethanolamine mole fraction of 0.1 and 0.5, respectively.

Ternary Vesicles. To demonstrate the potential of the approach adopted in this study, we prepared a ternary phospholipid vesicle whose mole fraction composition would simulate that of the retinal rod outer segment, *i.e.*, 0.54, 0.38, and 0.08 mole fraction for phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine, respectively. These three lipids were colyophilized in the aforementioned mole fraction ratio, subjected to sonication in the usual way, run

TABLE I: Dependence of the Partition Coefficient of Phospholipid Vesicles on the NaCl Concentration.

Mole Fraction of Phosphatidylinositol	Partition Coefficient		
	0.1 M NaCl	0.2 M NaCl	0.3 M NaCl
0.0	0.392	0.388	0.394
0.5	0.390		0.387
1.0	0.228	0.261	0.341

over a molecular sieve, and analyzed for the incorporated mole fraction of lipid. The results obtained are summarized in Table III. The average mole fraction values obtained were 0.66, 0.28, and 0.06 for phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine, respectively, demonstrating a marked nonideality in the incorporation of phospholipid in the ternary system. Scattering curves and chromatographic elution profiles obtained for this system are similar to those observed for the binary systems. In addition, the trapped volume measured is identical with that of phosphatidylcholine. A linear dependence of the trapped volume of glucose on the lipid concentration is also found in this system.

Discussion

Of the several methods for preparing lipid bilayer systems for use as models for biological membranes that have become available for use over the last several years (Huang, 1969; Papahadjopoulas and Miller, 1967; Bangham, 1968), that of Huang has produced the best characterized model for the lipid region of biological membranes; this system has been shown to consist of phosphatidylcholine vesicles, *i.e.*, structures formed of a single bilayer wall bounding an exchangeable volume of solution. These vesicles can be prepared by molecular sieve chromatography so as to have a high degree of size homogeneity. A major advancement in the development of models for the lipid region of biological membranes would be attained by preparing vesicles composed of binary and ternary mixtures of phospholipids; our aim has been to demonstrate the feasibility of preparing such vesicles with a known chemical composition and a well-defined size.

A study of the incorporation of phosphatidylinositol and phosphatidylethanolamine into phosphatidylcholine vesicles as a function of the initial mole fraction of these phospholipids shows that in the case of phosphatidylinositol ideal mixing occurs, as evidence by the linear correlation between the initial and the incorporated mole fraction. Stable phosphatidylcholine–phosphatidylinositol vesicles can be prepared over the entire mole fraction range. Phosphatidylethanolamine shows ideal incorporation below an initial mole fraction of 0.2 and a slight negative deviation from ideality at higher mole fractions. Initial mole fractions of phosphatidylethanolamine above 0.7 yield unstable preparations which precipitate on standing.

In any multicomponent vesicle preparation one might expect that the chemical composition of the system would be represented by some distribution about an average composition, a high degree of chemical homogeneity implying that this distribution is narrow and well represented by the average chemical composition. Chemical analysis of Sepharose 4B column fractions has demonstrated the consistency

TABLE II: Sedimentation Coefficients and Heterogeneity Parameters for Phospholipid Vesicles.

System	Mole Fraction	s^0_{20} ($\times 10^{13}$ sec)	k ($\times 10^8$ ml/μmol)	p ($\times 10^{13}$ sec)	p/s_{20} ($\times 100$)
Phosphatidylcholine–phosphatidylethanolamine[a]	0.0	2.1[c]		0.05[c]	
	0.1	2.58	6.16	0.066	2.8
	0.3	3.17	3.91	0.45	15
	0.5	5.04	9.28	1.03	22
Phosphatidylcholine–phosphatidylinositol[b]	0.23				4.6
	0.5				1.3
	1.0	16.7	0.541	1.24	5.2

[a] Mole fractions of phosphatidylethanolamine are given. [b] Mole fractions of phosphatidylinositol are given. [c] Huang, 1969.

of the average composition over the internal volume peak of the column. The linear dependence of the scattering of the mixed phospholipid vesicles on the phospholipid concentration is suggestive evidence for size homogeneity in the trailing side of the internal volume peak of the Sepharose 4B column; this conclusion is strongly supported by the linearity of the trapped volume *vs.* lipid phosphorus plots for the mixed vesicle systems. Furthermore, the consistency of the trapped volume with variation in the mole fraction of phosphatidylethanolamine shows that the volume accessible to the trapped glucose is not composition dependent and by implication that the vesicle size is not a compositional dependent parameter. By contrast, the phosphatidylcholine–phosphatidylinositol system shows a decrease in trapped volume with increasing concentration of phosphatidylinositol up to a mole fraction of 0.5 and remains constant thereafter; since the trapped volume of this system is compositional dependent, one would expect that any variation in composition in the trailing edge of the internal volume peak would show up as a heterogeneity in trapped volume. No such variation is observed; we can, therefore, conclude that the size distribution and the distribution in chemical composition in this system are constant in the trailing edge of the internal volume peak.

A method of measuring the breadth of the distribution of the size and composition in these systems is provided by analysis of the boundary spreading in sedimentation velocity experiments (Fujita, 1962); this analysis allows the separation of the boundary spreading into two contributions, one due to diffusion and one due to heterogeneity in the sedimentation coefficient. Since the sedimentation coefficient is a function of the vesicle size, any distribution of size in the vesicle sample will result in a corresponding distribution in the sedimentation coefficient; the breadth of this distribution will be reflected by the value of the heterogeneity factor, p (Huang, 1969). Since the partial specific volume of phosphatidylcholine is very close to unity, as is the solution density, the buoyancy term, $(1 - \bar{v}\rho)$, in the Svedberg equation (Svedberg and Pedersen, 1940) becomes a very sensitive factor in determining the sedimentation coefficient of the phospholipid vesicle. Huang and Charlton (1971) demonstrated the sensitivity of the sedimentation coefficient of phosphatidylcholine vesicles to the solution density. If the partial specific volume of the components of mixed phospholipid vesicles varies, then the partial specific volume of these vesicles will depend on the incorporated mole fraction of phospholipid. Hence, if there exists a spectrum of composition within a vesicle preparation, one would expect to find an equivalent distribution in the partial specific volume of the vesicles; this will produce a distribution in sedimentation coefficients due to the sensitivity of the buoyancy term; the breadth of the distribution will be reflected by the size of the heterogeneity factor.

The partial specific volume for the individual phospholipids can be estimated by using the determined partial specific volume of 0.9814 (Huang and Charlton, 1971) and an average mol wt of 775 for phosphatidylcholine, and estimating the partial specific volume of the various head groups by the method of summation of atomic volumes (Cohn and Edsall, 1943). Contributions to the partial specific volume of phosphatidylcholine are divided on a weight fraction basis into the contribution due to the choline group and that due to the acyl side chains and glycerol phosphoryl backbone. Assuming that the glycerol phosphoryl backbone and acyl side-chain contributions are the same for all three phospholipids, one can sum this value with the contribution of the ethanolamine and inositol groups, on a weight fraction basis, to obtain the partial specific volume of phosphatidylethanolamine and phosphatidylinositol, respectively. These results, summarized in Table IV, show a wide variation in partial specific volume for the phospholipids employed in this study, supporting the contention that compositional heterogeneity will contribute to the heterogeneity factor. In addition, the variation in calculated partial specific volume is in full agreement with the trend in sedimentation coefficients demonstrated in Table II, *i.e.*, the lower partial specific volume of phosphatidylethanolamine and phosphatidylinositol relative to phosphatidylcholine contributes to an increase in sedimentation coefficient for the mixed vesicles. A comparison of the par-

TABLE III: Composition and Trapped Volume of Ternary Phospholipid Vesicles.[a]

Fraction No.[b]	Incorporated Mole Fraction of Phospholipids			Trapped Vol (μl/μmol of P_i)
	χ_{PC}	χ_{PE}	χ_{PS}	
19	0.65	0.29	0.06	
20	0.66	0.27	0.07	
22	0.64	0.30	0.06	0.168
23	0.67	0.28	0.05	0.167
24	0.65	0.28	0.07	0.169
25	0.67	0.29	0.05	0.166
26	0.65	0.28	0.07	0.168

[a] Initial mole fraction of phospholipids: $\chi_{PC} = 0.54$, $\chi_{PE} = 0.38$, and $\chi_{PS} = 0.08$. [b] Fractions from Sepharose 4B column, 0.1 M NaCl.

TABLE IV: Calculated Partial Specific Volumes.[a]

Compound	$\bar{v}$ Based on $\bar{v}_{PC}$ (cm^3/g)	$\bar{v}$ Based on s^0_{20} (cm^3/g)
Ethanolamine	0.7938	
Inositol	0.7043	
Choline	0.9380	
Glycerol phosphoryl backbone and acyl side chains	0.9881	
Phosphatidylcholine[a]	0.9814	
Phosphatidylethanolamine	0.9719	
0.5 mole fraction phosphatidylethanolamine vesicle	0.9768	0.9573
Phosphatidylinositol	0.9283	0.8945
0.23 mole fraction phosphatidylinositol vesicle	0.9683	0.9535

[a] The $\bar{v}$ values calculated using $\bar{v}_{PC}$, as determined in D_2O–H_2O, represent the actual partial specific volume of the individual phospholipids; this is to be contrasted with the values based on the sedimentation coefficients determined in 0.1 M NaCl, which yields an effective specific volume containing a contribution due to preferential interaction of the vesicles with water (Huang and Charlton, 1971).

tial specific volumes obtained above with values calculated employing the measured sedimentation coefficient, the diffusion coefficient of the phosphatidylcholine vesicle (Huang, 1969), and molecular weights appropriately modified for the composition of the vesicles is shown in Table IV. Given the variation in partial specific volume for the component phospholipids, it follows that both the distribution in size and chemical composition will contribute to the heterogeneity factor, p. As seen in Table II, heterogeneity is less than 5.2% for all mole fractions of phosphatidylinositol and increases from 2.8 to 22% for phosphatidylethanolamine mole fractions of 0.1 and 0.5, respectively. Given the linearity of the trapped volume experiments and the independence of the trapped volume with respect to phosphatidylethanolamine mole fraction, it is thought that the compositional distribution is the major source of heterogeneity in the phosphatidylcholine–phosphatidylethanolamine system.

Pure phosphatidylethanolamine will not form stable vesicles, nor will phosphatidylcholine–phosphatidylethanolamine mixtures above 0.7 mole fraction phosphatidylethanolamine. Phosphatidylethanolamine, upon swelling with water at room temperature, is known to exist as a mixture of lamella and type II hexagonal (H_{II}) phases (Tinker and Pinteric, 1971; Junger and Reinauer, 1969; Husson, 1967), whereas phosphatidylcholine exists as a pure lamella phase at room temperature (Luzzati, 1968). The H_{II} phase is characterized by a hexagonal array of cylinders which are formed with the phosphatidylethanolamine head groups located at the inner cylinder surface and the hydrocarbon side chains directed radially out from this surface; water fills the central core of the cylinder. Formation of the hexagonal phase is favored by both increasing temperature and higher phosphatidylethanolamine concentration; the instability of pure and mixed phosphatidylethanolamine vesicles may be a reflection of the formation of a hexagonal phase, which is incompatible with the lamella structure of the vesicle wall. The packing requirements of phosphatidylethanolamine relative to phosphatidylcholine appear to manifest themselves in another way. The concentration of phosphatidylethanolamine in the outer surface of the vesicle, relative to the total vesicle concentration, can be determined by reaction with TNBS in aqueous and propanol solutions respectively. Using a value of 105 Å for the Stokes radius of the phosphatidylcholine vesicle, determined by diffusion measurements in the analytical ultracentrifuge (Huang and Lee, 1973), and a wall thickness of 40 Å, determined from X-ray diffraction studies (Engelman, 1972), to calculate the ratio of the outer surface area to the total surface area of the vesicle, one obtains a value of 0.73; this is in qualitative agreement with the value of 0.83 obtained for the distribution ratio of phosphatidylethanolamine at a very low mole fraction. Figure 6 demonstrates the drop in this ratio with increasing mole fraction of phosphatidylethanolamine. These results can be interpreted as a demonstration of the formation of an asymmetric bilayer in the vesicle wall, produced by nucleation of the phosphatidylethanolamine on the inner surface of the vesicle. The small negative radius of curvature of the inner surface of the bilayer is more analogous to the head group packing one finds in the H_{II} phase and may be the driving force for the process. This interpretation is supported by the findings of Luzzati and Husson (1962) in their study of the phase diagrams of mixed brain and mitochondrial phospholipids; they found that in regions of the phase diagram where only one phase was present, a homogeneous distribution of phospholipids was present. However, in regions where both hexagonal and lamella phases coexisted, separation of the different species was observed; the composition of each phase depended on the phase requirements of the different lipids in the mixture. Since phosphatidylcholine does not exist in a stable hexagonal phase, a means of satisfying the packing demands of both lipids would be to shift more phosphatidylethanolamine to the inner surface of the vesicle, thereby having a head group packing more analogous to the H_{II} phase, while the phosphatidylcholine packing requirements of a lamella type surface would be well satisfied on the outer vesicle surface.

Papahadjopoulos and Weiss (1969) observed a very low reactivity of TNBS with suspensions of phosphatidylethanolamine and an increased reactivity in suspensions containing 0.9 mole fraction phosphatidylcholine; these results were interpreted in terms of a reduced reactivity of the primary amino group of phosphatidylethanolamine due to intramolecular hydrogen bonding between an undissociated proton of the phosphate group and the amino group. This explanation was based on the results of infrared studies of organic solutions of phospholipids by Abramson *et al.* (1965), who interpreted their data as evidence for the presence of hydrogen bonding between the amino and phosphate groups in phosphatidylethanolamine. However, subsequent high resolution nuclear magnetic resonance and infrared studies (Chapman and Morrison, 1966) do not support the hydrogen-bonded structure and are interpreted to favor an ionic dipolar structure for phosphatidylethanolamine. Our own results indicate that only 25% of the primary amino groups are reactive in pure phosphatidylethanolamine suspensions. An alternative explanation of the low reactivity of pure phosphatidylethanolamine suspensions may lie in the gross structure of these systems rather than in the molecular interactions of the amino and phosphate groups. Both electron microscope and X-ray diffraction measurements demonstrate that phosphatidylethanolamine exists as a mixture of lamella and hexagonal phases at room temperature. The cylinders of the H_{II} structure have been reported to be 56 Å in diameter; assuming a

bilayer thickness of 40 Å, only a 16-Å central core is accessible to water. Structured water at the charged head group interface would further reduce the free volume of the central core of the cylinder. This small entrance cross section of the H_{II} cylinder may provide enough steric hindrance to exclude the TNBS reagent and mask a large proportion of the amino groups. Our studies indicate that TNBS does not diffuse across the bilayer into the interior of the vesicle. Since a large portion of the lamella phase is present as concentric bilayers with only one surface exposed to the bulk solution, the majority of the phosphatidylethanolamine in this form would be inaccessible to the coupling reagent. In the experiments described in this paper, we are dealing with a selected population of vesicles whose internal volume is bounded by a single bilayer wall; hence the only inaccessible amino groups are those on the inner vesicle surface. The TNBS measurements can, therefore, be expected to give a real measure of the surface distribution of amino groups in this system.

The trapped volume of phosphatidylcholine–phosphatidylethanolamine vesicles was shown to be independent of the phosphatidylethanolamine mole fraction, providing evidence that the size of the phosphatidylcholine–phosphatidylethanolamine vesicles is the same as that of phosphatidylcholine. The phosphatidylcholine–phosphatidylinositol system shows a systematic reduction in trapped volume up to 0.5 mole fraction phosphatidylinositol, at which point it levels off at a value of 0.081 μl/μmol of P_i; these results imply that the size of the vesicle is decreasing with increasing phosphatidylinositol content. Molecular sieve chromatography experiments were undertaken in order to clarify the relationship between trapped volume and vesicle size. An additional complication arises in the determination of the partition coefficient of the phosphatidylinositol vesicle; values obtained in 0.1 M NaCl imply a larger size for the phosphatidylinositol vesicle than for the phosphatidylcholine vesicle. In addition, the phosphatidylinositol vesicle has a smaller partition coefficient than the 0.5 mole fraction vesicle, although they have identical trapped volumes. This paradox is resolved by the observed increase in the partition coefficient of the phosphatidylinositol vesicle with increasing ionic strength. The high negative surface charge density of the pure phosphatidylinositol vesicle causes it to be partially excluded from the internal volume due to electrostatic repulsion; as the ionic strength is increased the partition coefficient increases, approaching that found for phosphatidylcholine. Although Sepharose is presumed to be an uncharged molecular sieve matrix, charge effects on the partition coefficient of certain proteins have been reported (Marrink and Gruber, 1969). Since the partition coefficients for 0.5 mole fraction phosphatidylinositol and phosphatidylcholine vesicles are equal, and the trapped volumes of 0.05 mole fraction and 1.0 mole fraction phosphatidylinositol vesicles are identical, we feel that the size of the phosphatidylinositol vesicles is the same as that of the phosphatidylcholine vesicles.

A possible explanation for the variation in trapped volume with mole fraction of phosphatidylinositol can be obtained by considering the structuring of solvent at the highly charged interface of the vesicle. The trapped volume measurement of 0.169 μl/μmol of P_i for phosphatidylcholine vesicles is smaller than the value of 0.239 μl/μmol of P_i one would predict for a sphere of radius 105 Å and bilayer wall thickness of 40 Å. A partial explanation of this deviation can be obtained by considering the volume contained by the water of hydration associated with the phospholipid (Huang and Charlton, 1971). One would expect the degree of ordering of water at the highly charged interface of the phosphatidylinositol vesicles to be much higher than for that observed for phosphatidylcholine. Since the trapped volume is being measured by a neutral marker, exclusion of the marker by electrostatic repulsion cannot be the explanation for the reduced trapped volume. Hence, one can conclude that the reduction in the volume accessible to the marker is caused by an ordering or increased binding of water at the highly charged phosphatidylinositol interface.

To demonstrate the approach made available by these systems, a ternary vesicle system of phosphatidylcholine–phosphatidylethanolamine–phosphatidylserine was formed in a mole fraction ratio which simulates the major phospholipid composition of the retinal rod outer segment. In this system one observes a marked nonideality, as evidenced by the alteration of the ratio of lipids, relative to the initial mole fraction ratio. Proper characterization of the phase diagram of this system will provide for the same kind of compositional flexibility as is obtainable in the binary systems.

It is clear that these systems provide for a versatile approach to simulating lipid membrane surfaces and to the study of protein–lipid interactions. The observations on the phosphatidylcholine–phosphatidylethanolamine system and the ternary lipid system indicate that deviations in the incorporated mole fraction of phospholipid can range from a minor perturbation to a major alteration. The results reported here demonstrate that in order to work with a well-defined model system one must not only characterize the incorporation curve over the mole fraction range of interest, but must also employ physical techniques capable of estimating the degree of heterogeneity of the resulting preparation.

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References

Abramson, M. D., Norton, W. T., and Katzman, R. (1965), *J. Biol. Chem. 240*, 2389.

Ackers, G. K. (1967), *J. Biol. Chem. 242*, 3237.

Ansell, G. B., and Hawthorne, J. N. (1964), Phospholipids, New York, N. Y., Elsevier, Chapter 4.

Bangham, A. D. (1968), *Progr. Biophys. Mol. Biol. 18*, 31.

Bartlett, G. R. (1959), *J. Biol. Chem. 234*, 466.

Blazyk, J. F., and Steim, J. M. (1972), *Biochim. Biophys. Acta 266*, 737.

Branton, D. (1966), *Proc. Nat. Acad. Sci. U. S. 55*, 1048.

Chapman, D., and Dodd, G. H. (1971), *in* Structure and Function of Biological Membranes, Rothfield, L., Ed., New York, N. Y., Academic Press, Chapter 2.

Chapman, D., and Morrison, A. (1966), *J. Biol. Chem. 241*, 5044.

Cohn, E. J., and Edsall, J. T. (1943), Proteins, Amino Acids, and Peptides, New York, N. Y., Reinhold, Chapter 7.

Colacicco, G., and Rapport, M. M. (1967), *J. Lipid Res. 8*, 513.

Engelman, D. M. (1972), *Chem. Phys. Lipids 8*, 298.

Folch, J. M., Lees, M., and Sloane-Stanley, G. H. (1957), *J. Biol. Chem. 266*, 497.

Fujita, H. (1962), Mathematical Theory of Sedimentation Analysis, New York, N. Y., Academic Press.

Hirsch, J., and Ahrens, E. H. (1958), *J. Biol. Chem. 233*, 311.

Huang, C. (1969), *Biochemistry 8*, 344.
Huang, C., and Charlton, J. P. (1971), *J. Biol. Chem. 246*, 2555.
Huang, C., Charlton, J. P., Shyr, C. I., and Thompson, T. E. (1970), *Biochemistry 9*, 3422.
Huang, C., and Lee, L. J. (1973), *J. Amer. Chem. Soc. 95*, 234.
Hubbell, W. L., and McConnell, H. M. (1968), *Proc. Nat. Acad. Sci. U. S. 61*, 12.
Husson, F. (1967), *J. Mol. Biol. 25*, 363.
Junger, E., and Reinauer, H. (1969), *Biochim. Biophys. Acta 183*, 304.
Kornberg, R. D., and McConnell, H. M. (1971), *Biochemistry 10*, 1111.
Levine, Y. K., and Wilkins, M. H. F. (1971), *Nature (London), New Biol. 230*, 69.
Litman, B. J. (1971), *Fed. Proc., Fed. Amer Soc. Exp. Biol. 30*, 301.
Luzzati, V. (1968), *in* Biological Membranes, Chapman, D., Ed., New York, N. Y., Academic Press, Chapter 3.
Luzzati, V., and Husson, F. (1962), *J. Cell. Biol. 12*, 207.
Marrink, J., and Gruber, M. (1969), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 2*, 242.
Papahadjopoulos, D., and Miller, N. (1967), *Biochim. Biophys. Acta 135*, 624.
Papahadjopoulos, D., and Weiss, L. (1969), *Biochim. Biophys. Acta 183*, 417.
Park, J. T., and Johnson, M. J. (1949), *J. Biol. Chem. 181*, 149.
Pinto da Silva, P., and Branton, D. (1970), *J. Cell. Biol. 45*, 598.
Schachman, H. K. (1959), Ultracentrifugation in Biochemistry, New York, N. Y., Academic Press.
Sessa, G., and Weissman, G. (1968), *J. Lipid Res. 9*, 310.
Singer, S. J. (1971), *in* Structure and Function of Biological Membranes, Rothfield, L., Ed., New York, N. Y., Academic Press, Chapter 4.
Steim, J. M., Tourtellote, M. E., Reinert, J. C., McElaney, R. N., and Radar, R. L. (1969), *Proc. Nat. Acad. Sci. U. S. 63*, 104.
Svedberg, T., and Pedersen, K. O. (1940), The Ultracentrifuge, London, Oxford University Press.
Thompson, T. E., and Henn, F. (1970), *in* Membranes of Mitochondria and Chloroplasts, ACS Monograph No. 165, Racker, E., Ed., New York, N. Y., Van Nostrand Reinhold, Chapter 1.
Tinker, D. O., and Pinteric, L. (1971), *Biochemistry 10*, 860.
Wilkins, M. H. F., Blaurock, A. E., and Engelman, D. M. (1971), *Nature (London), New Biol. 230*, 72.
Yost, P., Waggoner, A. S., and Griffith, O. H. (1971), *in* Structure and Function of Biological Membranes, Rothfield, L., Ed., New York, N. Y., Academic Press, Chapter 3.