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FUSION AND PROTEIN-MEDIATED PHOSPHOLIPID EXCHANGE STUDIED WITH SINGLE BILAYER PHOSPHATIDYLCHOLINE VESICLES OF DIFFERENT DENSITY

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SUMMARY

A novel method has been developed for the study of phospholipid exchange and fusion of phospholipid vesicles. Two homogeneous populations of single bilayer phosphatidylcholine vesicles of similar size but markedly different density have been prepared. "Dense" vesicles were made from brominated dioleoyl phosphatidylcholine. "Light" vesicles were prepared from dioleoyl phosphatidylcholine. The two populations were easily separated by density gradient centrifugation. Phosphatidylcholine exchange protein from beef liver was used to promote lecithin exchange between the vesicle populations. Only the lecithin of the external monolayers of the vesicles was available for exchange by exchange protein, implying that flip-flop of vesicle phosphatidylcholine did not take place at a detectable frequency. No spontaneous intervesicle phosphatidylcholine exchange was observed. However, the dense and light vesicles did spontaneously fuse, over several hours, to produce particles of hybrid density.

INTRODUCTION

Phospholipid exchange proteins are soluble catalysts of phospholipid exchange between membranes [1]. The phosphatidylcholine exchange protein from beef liver [2] acts by a one-for-one exchange process [4] to randomize the various species of phosphatidylcholine present among substrate membranes, without changing their total lecithin content. However, only the lecithin of the external monolayer of the membrane bilayer can participate in the exchange process [5, 6]. The sidedness of protein-mediated exchange has led to the use of exchange proteins to study transmembrane phospholipid asymmetry and flip-flop [7] in natural [8–10] and artificial [5, 6] membranes.

Abbreviation: dibromosteoroyl phosphatidylcholine, *bis*-9,10-dibromosteoroyl phosphatidylcholine.

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Several studies of phospholipid exchange between populations of unilamellar vesicles have been reported [11–13]. Any such exchange experiment clearly requires that the vesicle populations be separated after the exchange process. This has been accomplished in the past by including in only one vesicle population a nonexchangeable marker which forms the basis for the separation. Thus Enholm and Zilversmit [11] included the Forssman antigen in one vesicle population which was selectively precipitated by specific antibody after incubation with non-antigenized vesicles. Hellings et al. [12] and van den Besselaar et al. [13] included large amounts of phosphatidic acid or phosphatidylinositiol in one population, thereby permitting their selective adsorption to columns of DEAE-cellulose. The other vesicle population, containing much less negatively charged phospholipid, does not bind to the resin. Although these techniques are useful for exchange studies, they do not lend themselves to the investigation of other properties of phospholipid vesicles, such as fusion. Thus, fusion of the two types of vesicles will produce hybrid particles which possess the separation marker; for example, the fusion product of an antigenized and a non-antigenized vesicle will coprecipitate with the unfused, antigenized population, and will consequently not be detected.

In this report we propose a novel method for the study of vesicle-vesicle interactions which permits the detection of possible hybird particles. Two populations of phosphatidylcholine vesicles, which differ in density but not size, are allowed to interact and then are separated by density gradient centrifugation. The density difference is achieved by the use of a lecithin with brominated fatty acid chains in the formation of one vesicle population. The possibility of uisng brominated fatty acids to impart density shifts in membranes was first pointed out by Fox et al. [14]. We have used this method to study exchange protein-dependent intervesicle lecithin exchange and vesicle fusion.

Synthesis of phosphatidylcholines

^{3}H-labeled dioleoylphosphatidylcholine, (25 Ci/mol), was synthesized as previoudly described [5]. (*bis*-9,10-Dibromostearoyl)-phosphatidylcholine (dibromostearoyl phosphatidylcholine) was prepared by brominating dioleoyl phosphatidylcholine according to the procedure described [15] for the bromination of diglycerides. Briefly, 2 mol of Br_2 were added dropwise at around -15 °C to 1 mol of dioleoyl phosphatidylcholine dissolved in petroleum ether, and the reaction proceeded with continuous stirring at this temperature for several hours. The lecithin product was purified by silicic acid column chromatography using increasing amounts of methanol in chloroform as the eluant. Dibromostearoyl phosphatidylcholine co-migrated with dioleoyl phosphatidylcholine on silica gel thin-layer plates (with chloroform/methanol /water (65 : 25 : 4, v/v) as the solvent). Methyl esters [15] of the fatty acids of dibromostearoyl phosphatidylcholine were analyzed by gas-liquid chromatography as described previously [17]. A single species was found whose retention time was identical to that of an authentic sample of the methyl ester of 9,10-dibromostearic acid (Nu Chek Prep., Inc., Elysian, Minn.).

Preparation and characterization of phosphotidylcholine vesicles

Dibromostearoyl phosphatidylcholine was lyophilized from a benzene stock solution, dispersed in 0.1 M KCl, 10 mM Tris · HCl pH 7.4, and sonicated as described

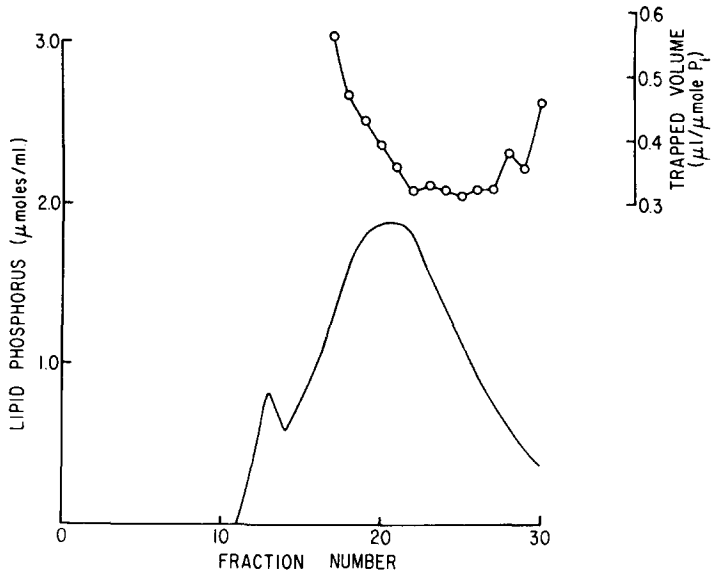

Fig. 1. The elution profile of sonicated dibromostearoyl phosphatidylcholine from Sepharose 4B. 50 mg dibromostearoyl phosphatidylcholine was lyophilized from a benzene stick solution, swollen in 5 ml of 0.1 M KCl, 10 mM Tris · HCl pH 7.4 containing 200 μCi/ml of [^{3}H]sucrose (New England Nuclear), and then sonicated and chromatographed as described earlier [5]. Phosphorous content of the fractions was determined by the Bartlett procedure [19]. The trapped aqueous volume per phosphatidylcholine in column fractions was calculated from the amount of [^{3}H]sucrose in the fraction as compared to the unfractionated sonicated suspension, and the known phosphatidylcholine concentration in the fraction. The insert at the top right shows the trapped volume for the eluted fractions. The apparent increase of trapped volume beyond fraction 28 is due to the overlap of the end of the vesicle with the beginning of the free sucrose peak in these fractions.

[5]. In most experiments, a trace (0.05 Ci/mol dibromostearoyl phosphatidylcholine) of [^{14}C]cholesteryl oleate (New England Nuclear) was colyophilized with dibromostearoyl phosphatidylcholine; the [^{14}C]ester served as a marker for dibromostearoyl phosphatidylcholine vesicles since it does not redistribute (Fig. 2).

The elution profile of sonicated dibromostearoyl phosphatidylcholine from Sepharose 4B (Fig. 1) is similar to that of dioleoyl phosphatidylcholine or egg lecithin [18]. The sonicated material is largely in the form of unilamellar vesicles because an aqueous volume of 0.32 l/mol phosphatidylcholine is trapped in the Fraction II region [18] of the Sepharose 4B elution profile, as compared to 0.22 l/mol phosphatidylcholine for dioleoyl phosphatidylcholine (Dawidowicz, E. A. and Lanni, F., unpublished). For comparison, multilamellar vesicles from the Fraction I region [18] of a Sepharose 4B elution for egg lecithin have a trapped volume of 0.80 l/mol phosphatidylcholine (Dawidowicz, E. A. and Thompson, T. E., unpublished). The similarity of the trapped volumes and Sepharose 4B elution profiles of dibromostearoyl phosphatidylcholine and dioleoyl phosphatidylcholine vesicles shows that they are of similar size, since an increase in vesicle radius of only 15 % accounts for this disparity in trapped volumes. Sonicated dibromostearoyl phosphatidylcholine was fractionated on a Sepharose 4B column prior to exchange experiments. The descending portion of the vesicle peak (for example, Fractions 23–28 in Fig. 1) was used.

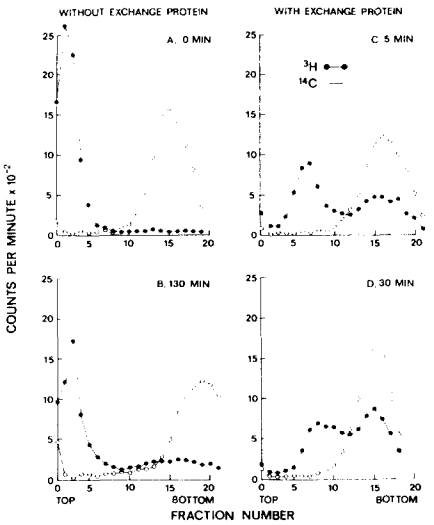

Fig. 2. Sucrose gradient analysis of phospholipid exchange. Sedimentation is from left to right. The exchange reaction had per ml 1.4 μmol dibromostearoyl phosphatidylcholine vesicles (containing [^{14}C]cholesteryl oleate), 0.02 μmol ^{3}H-labelled dioleoyl phosphatidylcholine vesicles, 0.1 mg bovine serum albumin (Pentex), and 3.4 units (as defined for the microsome-vesicle assay [2]) of exchange protein. The final buffer was 90 mM KCl, 2.5 mM citrate, 5 mM Na_2HPO_4, 9 mM Tris–HCl pH 7.4, 11 mM β-mercaptoethanol. The citrate, Na_2HPO_4, glycerol and some of the β-mercaptoethanol are components of the buffer in which the exchange protein is stored [2]. Albumin was included to prevent the slow loss of exchange activity which otherwise occurs. Under these conditions the exchange protein retains full activity after 24 h at 37 °C. The control reaction was identical to the exchange reaction except that the exchange protein was omitted. The reactions were conducted at 37 °C under N_2 to retard autoxidation of dioleoyl phosphatidylcholine. At appropriate times 0.2 ml aliquots were removed, and the reaction was stopped by adding to the aliquot 20 μl of 2 mg pronase (Calbiochem) per ml of 0.1 KCl 10 mM $CacCl_2$, 10 mM Tris–HCl pH 7.4. After 5 min at 37 °C, the sample was chilled on ice. 10 μl of sonicated, unfractionated dibromostearoyl phosphatidylcholine (50 mg/ml) was added as carrier and the sample was layered atop a linear 5–20 % (w/v) linear gradient of sucrose in 0.1 M KCl, 10 mM Tris–HCl pH 7.4. The gradients were centrifuged at 5 °C for about 50 min in a Beckman SW50.1 rotor at 49 000 rev./min. Fractions were collected from the bottom and counted. A, control reaction at 0 min; B, control reaction at 130 min; C, exchange reaction at 5 min; D, exchange reaction at 30 min. ○–○, [^{14}C]cholestryl oleate; ●–●, ^{3}H-labeled dioleoyl phosphatidylcholine. Note that the gradient in panel B was centrifuged separately from the other three and for a longer time, causing the dense vesicle peaks of panel B to appear farther down the gradient.

The trapped volume per phosphatidylcholine was constant in this region of the elution profile, suggesting that these vesicles were homogeneous. Egg lecithin vesicles from this region also have a constant trapped volume per phosphatidylcholine (Dawidowicz, E. A. and Thompson, T. E., unpublished) and are known to be homogeneous by other criteria [18].

^{3}H-labelled dioleoyl phosphatidylcholine was sonicated and fractionated on Sepharose 4B as previously described [5]. Only vesicles from the Fraction II region were used.

Separation of dense and light vesicles by gradient centrifugation

Although vesicles prepared from dioleoyl phosphatidylcholine and dibromo-

stearoyl phosphatidylcholine are of similar size, they are of markedly different density. This permits their separation on linear 5 to 20 percent sucrose gradients, as illustrated in Fig. 2A. Here, the dense (dibromostearoyl phosphatidylcholine) vesicles, labelled with [^{14}C]cholesteryl oleate, sediment much faster than the light (^{3}H-labelled dioleoyl phosphatidylcholine) vesicles, which are not sufficiently dense to enter the gradient. The separation is based on sedimentation velocity, and not equilibrium, since prolonged centrifugation causes the dense vesicles to pellet.

Protein-mediated phospholipid exchange between dense and light vesicles

When light vesicles are incubated with a 70-fold excess of dense vesicles in the presence of phosphatidylcholine exchange protein (purified 2000-fold from beef liver as described [2]), a rapid redistribution of phosphatidylcholine species occurs (Figs. 2C, D) confirming the lack of acyl chain specificity noted by others [3]. ^{3}H-labelled dioleoyl phosphatidylcholine appears in the denser peak. The lighter peak sediments faster, presumably because unlabelled dibromostearoyl phosphatidylcholine is incorporated into the light vesicles in exchange for dioleoyl phosphatidylcholine. The denser peak does not change; its composition is not altered appreciably by the exchange process since the dense vesicles are present at 70-times the concentration of the light species. The rate of redistribution depends on the amount of exchange protein added (unpublished data). When exchange protein is omitted from an otherwise identical incubation, little redistribution of label in the gradient takes place (Fig. 2B) during the brief time period (minutes) when protein-mediated exchange occurs. The small redistribution that is observed does not represent exchange, but corresponds to vesicle fusion, as will be discussed below.

The amount of exchange can be quantitated as follows. Since the [^{14}C]ester does not redistribute, the ratio of ^{3}H/^{14}C in the dense vesicles divided by the ratio ^{3}H/^{14}C in the unfractionated exchange incubation mixture gives the fraction of total dioleoyl phosphatidylcholine present in the dense vesicles after the reaction. As exchange proceeds, the lighter peak approaches the denser peak, making the ^{3}H/^{14}C

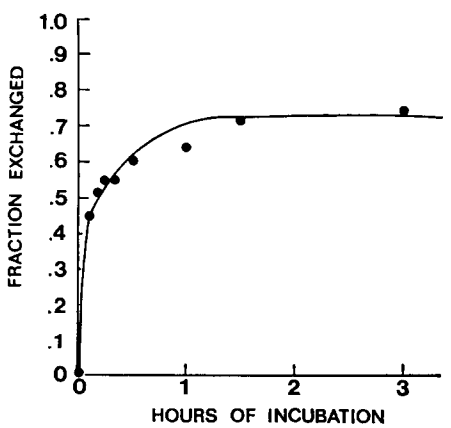

Fig. 3. Time course of protein mediated phospholipid exchange. The isotope ratio in the dense peak was used to quantitate exchange, which is represented as the fraction of total dioleoyl phosphatidylcholine introduced, by exchange for dibromostearoyl phosphatidylcholine, into dense vesicles. The data are from the same exchange reaction as Fig. 2.

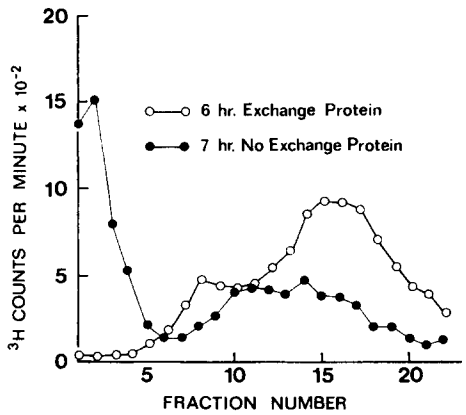

Fig. 4. Density gradient resolution of light vesicle peak from fusion peak after prolonged incubation with exchange protein. Note symbols are different from other figures. ●–●, ^{3}H-labelled dioleoyl phosphatidylcholine profile after 7 h incubation in the absence of exchange protein; the data from Fig. 6 was used. ○–○, ^{3}H-labeled dioleoyl phosphatidylcholine profile after 6 h incubation with exchange protein as described in the legend to Fig. 2. The ^{14}C-profiles have been omitted for clarity.

ratio of dense vesicles difficult to ascertain. To circumvent this problem, the ratio of the isotopes across the dense peak was plotted, and the average value over the region in which the ratio appeared constant was used (usually Fractions 16–20). Also the "fusion" peak (see below) does not make a significant contribution to the isotope ratio up to about 3 h., as can be seen from the fraction of total ^{3}H cpm which are in the right side of the dense peak in Fig. 2B.

The fraction of dioleoyl phosphatidylcholine exchanged as a function of time, for the experiment represented in Fig. 2, is shown in Fig. 3. It is seen that at most 70 % of the dioleoyl phosphatidylcholine is exchanged. This represents all the dioleoyl phosphatidylcholine on the outer surface of the light vesicle [5]. The remainder of the dioleoyl phosphatidylcholine remains inaccessible to the exchange protein, as expected from the extremely long flip-flop time for phosphatidylcholine in vesicles [5, 6].

An alternate, but less precise, means for quantitating the exchange process is from the ratio of the sedimentation constants of the two vesicle populations. As shown in the Appendix, this is approximately the same as the ratio of the number of the peak fraction of the light vesicle band to the number of the peak fraction of the dense vesicle band if the sedimentation is isokinetic, as expected for 5–20 % sucrose density gradients. Empirically, the gradients are isokinetic. Thus, the ratios in Fig. 5 from Experiment II were obtained from gradients in which the crest of the dense band was one fraction from the bottom of the gradient, whereas the dense vesicles in Experiment III of Fig. 5 only sedimented about half-way through the gradient. The exchange protein-independent band arising from fused vesicles (see below) does not interfere with the determination of this ratio, whereas the fusion peak does interfere with the isotope ratio method after a few hours incubation. For example, in Fig. 4 it can be seen that the crest of the light vesicle peak is readily discernable even after 6 hr incubation.

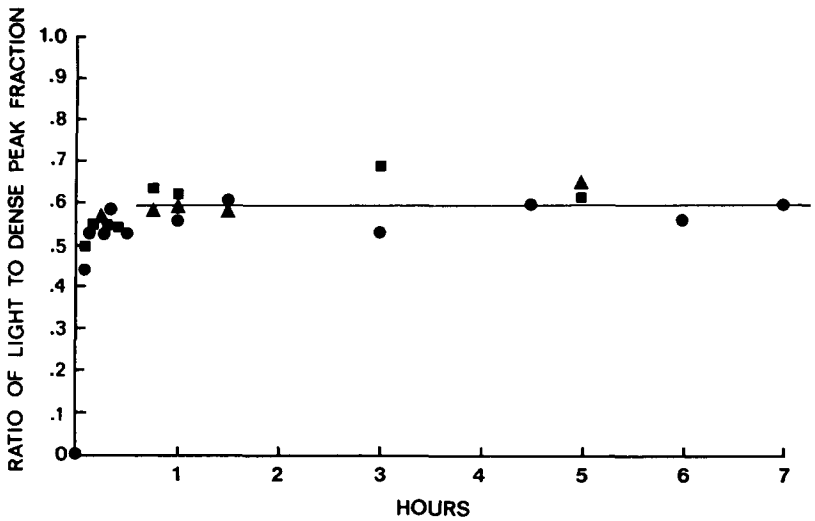

Fig. 5. Time course of protein mediated phospholipid exchange. The ratio of the peak fraction of the light vesicle band to the peak fraction of the dense vesicle band was used to quantitate the exchange. This ratio is approximately proportional to the fraction of phosphatidylcholine in the light vesicles which is dibromostearoyl phosphatidylcholine (see Appendix). Three independent exchange reactions are presented, all at 37 °C. The mean value for the ratio for all data points after 45 min is 0.60 ± 0.04. A horizontal line at this ratio is drawn in the figure. The expected error in the ratio due to the inherent inaccuracy of locating the peak fraction of the vesicles (plus or minus one-half fraction) is also ± 0.04. A ratio of 0.60 is consistent with 70 % exchange. Exp. I (●): the same experiment as in Figs. 2 and 3. Exp. II (▲): This reaction had per ml, 4.2 μmol dibromostearoyl phosphatidylcholine, 0.08 μmol ^{3}H-labelled dioleoyl phosphatidylcholine, 0.1 mg serum albumin, 8.5 units exchange protein. The final buffer composition was 75 mM KCl, 6 mM citrate, 12 mM Na_2HPO_4, 12 % glycerol, 7 mM Tris–HCl pH 7.4, 14 mM β-mercaptoethanol. The reaction was stopped with pronase, carrier was added and the sample centrifuged as described under Fig. 2. Exp. III (■): the composition of this reaction was the same as Exp. I, but the reaction was stopped by separating the vesicles from the exchange protein by rapid gel filtration. At appropriate times 0.2 ml. aliquots of the reaction were taken and chilled to 0 °C. 50 μl of a Dextran blue solution was added, and the mixture was passed through a small Sephadex-G 75 column (about 3 ml bed volume, presaturated with sonicated egg lecithin) which was eluted with 0.1 M KCl, 10 mM β-mercaptoethanol, 10 mM Tris–HCl pH 7.4 at 4 °C. The vesicles were collected in the void volume of about 0.3 ml which was located by the blue color of the high molecular weight dextran. The exchange protein (mol. wt. 21 300 [2]) elutes after the vesicles. This separation required about 5 min. Carrier dibromostearoyl phosphatidylcholine was added to the vesicles, and the sample was centrifuged through a sucrose gradient and analyzed as described in Fig. 2.

A plot of the peak fraction ratio versus time for three different experiments is shown in Fig. 5. Exchange proceeds until the ratio reaches 0.60, and does not increase detectably thereafter. Although this method does not provide a reliable absolute value for the amount of exchange, it does permit this process to be monitored for changes over extended periods of time. The isotope ratio method depends on the transfer of ^{3}H-labelled dioleoyl phosphatidylcholine to dense vesicles. The peak fraction ratio method depends on a density increase in the light fraction which is almost certainly caused by a transfer of dibromostearoyl phosphatidylcholine to light vesicles. The

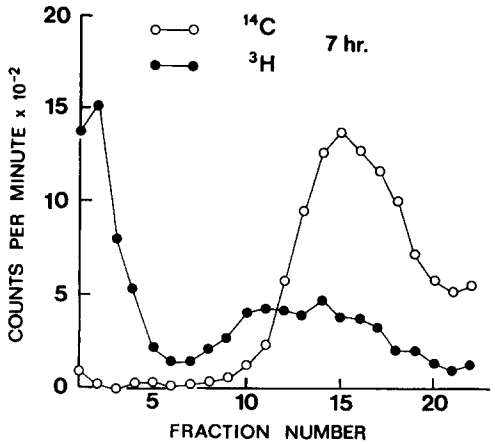

Fig. 6. Fusion of dense with light vesicles. Shown is a sucrose density gradient analysis of a mixture of dense with light vesicles incubated for 7 h under the same conditions as the control reaction of Fig. 2, in the absence of exchange protein. ○—○, [^{14}C]cholesteryl oleate, ●—● ^{3}H-labelled dioleoyl phosphatidylcholine.

agreement between the two methods therefore confirms the one-for-one nature of the exchange protein-mediated process [4].

Fusion of dense with light vesicles

As shown in Fig. 2B, there exists a slow process, independent of protein-mediated exchange, in which dense and light vesicles coalesce to form material which sediments as a broad zone slower than, but close to, the dense vesicles. This zone becomes more prominent with prolonged incubation (Fig. 6); by 7 h, it contains about half of the total dioleoyl phosphatidylcholine. The zone results from fusion of dense with light vesicles. It contains both dioleoyl phosphatidylcholine and dibromostearoyl phosphatidylcholine; dioleoyl phosphatidylcholine by itself could not have sedimented into the gradient unless it were associated with higher density material. The rate of formation of the zone increases with vesicle concentration in a manner consistent with a two particle vesicle-vesicle collision process. For example, when the product of the concentrations of dense and light vesicles was increased about 6-fold, the rate of zone formation increased about 7-fold. The position of the center of the zone in the gradient relative to dense vesicles is consistent with that calculated (by the methods used in the Appendix) for the binary fusion product of a dense with a light vesicle. The zone does not result from phospholipid exchange because (1) it cosediments with neither of the principal vesicle populations, and (2) at least 40 % of the ^{3}H-labelled dioleoyl phosphatidylcholine can be present in the zone without the light vesicles entering the gradient, indicating that a corresponding amount of dibromostearoyl phosphatidylcholine is not present in the light vesicles. If dense vesicles were to fuse with themselves, the expected products would have pelleted under the conditions of centrifugation used in these experiments, and would therefore not have been detected. If light vesicles were to fuse with themselves, they would not have been able to enter the gradient, and thus would not have separated from unfused light vesicles.

In this report we have demonstrated the usefulness of density labelling for the study of interactions of populations of phosphatidylcholine vesicles. The technique can be extended to other lipids in artificial membranes. It is hoped that the use of exchange proteins to introduce dense phospholipids may prove helpful in the isolation and study of more complex natural membranes.

APPENDIX

Monitoring exchange from ratio of peak fractions

The ratio, R, of sedimentation constant of the light vesicles, S_L, to that of the dense vesicles, S_D, is given by

$$R = \frac{S_L}{S_D} = \frac{m_L(1-\bar{v}_L\rho_s)f_D}{m_D(1-\bar{v}_D\rho_s)f_L} \tag{1}$$

where m, $\bar{v}$ and f are the masses, partial specific volumes, and frictional coefficients of dense (D) and light vesicles (L) respectively, ρ_s is the density of the solvent which varies throughout the gradient. However, since sedimentation through 5–20 % sucrose is empirically isokinetic (see text and Fig. 5), the density of solvent at the top of the gradient, ρ_0, may be used in place of ρ_s, whose variation need not be considered.

Because dense vesicles are in great excess over light ones, neither m_D, $\bar{v}_D$ or f_D changes as equilibration by one-for-one exchange of dense with light phosphatidylcholine takes place.

Eqn. 1 can therefore be rewritten

$$R \propto m_L(1-\bar{v}_L\rho_0)\frac{f_D}{f_L} \tag{2}$$

if v_L is the volume of the light vesicle, then

$$R \propto (m_L - v_L\rho_0)\frac{f_D}{f_L} \tag{3}$$

The mass and volume of light vesicles m_L and v_L will change in direct proportion to the amount of dense phosphatidylcholine introduced into light vesicles by exchange. Let E denote the fraction of total dioleoyl phosphatidylcholine transferred to dense vesicles. This is the same as the fraction of total phosphatidylcholine in the light vesicles which is brominated. Thus

$$R = k_1\left(E\frac{f_D}{f_L}\right) + k_2\frac{f_D}{f_L} \tag{4}$$

where k_1 and k_2 are constants.

The ratio of the frictional coefficients is the inverse of the ratio of the vesicle diameters. Since the diameters of the dense and light vesicles differ by only about 15 % (see text), the ratio f_D/f_L is effectively a constant, independent of E.

It can be concluded from Eqn. 4, that to a good approximation, R is linearly proportional to E. Deviation from linearity is determined by the degree to which f_D/f_L is independent of E; an upper limit of about 15 % deviation can thus be set.

It is obvious that when $E = 1$, $R = 1$. Also, when $E = 0$, it is seen that R is very nearly zero (Fig. 2A). Therefore, the constant of proportionality of E with R must be nearly unity, so that the amount of exchange is given, approximately, by R.

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