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CHARACTERISATION OF SMALL UNILAMELLAR VESICLES PRODUCED IN UNSONICATED PHOSPHATIDIC ACID AND PHOSPHATIDYLCHOLINE-PHOSPHATIDIC ACID DISPERSIONS BY pH ADJUSTMENT

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As the pH of phosphatidic acid and mixtures of phosphatidylcholine and phosphatidic acid dispersed in water is raised vesicles are formed. These vesicles are stable when the pH is returned to neutrality. Dispersions of phosphatidic acid transiently raised to pH 10–11 have been resolved by Sepharose 4B column chromatography into large structures (> 100 nm), which elute at the void volume, and small vesicles (< 60 nm), which elute at 0.44–0.48 column volumes (Hauser, H. and Gains, N. (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 1683–1687). A more detailed characterisation of these vesicles has now been made by including $^{22}Na^+$, $^{35}SO_4^{2-}$ and the fluorochromes, pyranine and harmaline, in the aqueous dispersion before the pH was raised. The encapsulated and unencapsulated ions were then separated, with the vesicles, by Sepharose 4B column chromatography. The material eluting in the void volume consists mainly of unilamellar vesicles with an encapsulated water phase of approx. 15 l per mole of phospholipid. The small vesicles formed from phosphatidic acid encapsulate approx. 0.5 l/mol. Their size is increased by increasing the concentration of sodium chloride in the initial dispersion medium and decreased by both increasing the maximum pH to which the dispersion is raised and increasing the rate of the pH change. Rapidly (in about 1 s) raising the pH to 9 or above results in more than 70% formation of small vesicles. This percentage is decreased in the presence of sodium chloride and increased by both increasing the maximum pH to which the dispersion is raised and increasing the rate of the pH change. The sodium salt of dilauroylphosphatidic acid does not form small vesicles (less than 60 nm) when the pH is rapidly raised to 11, unless the sodium ions have been removed by acid washing. The half-time of efflux of $^{22}Na^+$ and $^{35}SO_4^{2-}$ from these vesicles is of the order of days.

Introduction

Unilamellar phospholipid vesicles are of interest both as models for biological membranes and as vesicles for the potential delivery of encapsulated drugs to specific sites in the body. The latter, if the targetting can be sufficiently refined, should allow the therapy of individual organs and tissues or even the therapy, or destruction, of individual diseased cells [1–5]. Both from the view of model membranes and of drug carriers, it is important to characterise the vesicles as to their size, ability to encapsulate ions and uncharged molecules of various sizes, and as to their subsequent permeability to the encapsulated compounds. The efficiency of entrapment is an important factor in drug encapsulation, for uncharged molecules the concentration and size of the vesicles are the major considerations. However, small vesicles may in some cases be more efficiently taken up by certain biological targets [4], and further they are

Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid.

eliminated less rapidly from the circulation [6]. With charged drugs, as has been found with $^{22}Na^+$ [7], a higher efficiency of encapsulation should result if phospholipids of the opposite charge are used.

The methods of making small and large vesicles have recently been reviewed [8,9]. Of the methods that have been used to make small unilamellar vesicles only sonication [10–13] and extrusion through a French press [14,15] give good encapsulation efficiencies. In methods involving detergent removal [16–22] all or a part of the drug is liable to be lost before sealed vesicles are formed. Similarly, with vesicles of larger diameters only those formed by calcium-induced fusion [23] and reverse phase evaporation [24] efficiently encapsulate drugs. The relative merits of these procedures for drug encapsulation are summarised in Ref. 9.

It has been shown by Sepharose 4B column chromatography, NMR and electron microscopy that phosphatidic acid dispersions when exposed transiently to a pH of 10 or more form a mixture of small unilamellar vesicles, with a diameter of less than 60 nm, and larger particles [25]. The structural form of these larger particles, which are eluted from Sepharose 4B at the void volume, was uncertain. The main aims of the work in this paper were: to find conditions that optimised the formation of the small unilamellar vesicle population and thus minimised the formation of the larger particles; to measure the efficiency with which the small unilamellar vesicles encapsulated ions; and, to measure the permeability of the small unilamellar vesicles to the encapsulated ions. In the course of this characterisation it became apparent that the larger particles were mainly large unilamellar vesicles.

Materials and Methods

Materials. Egg phosphatidic acid and egg phosphatidylcholine were purchased from Lipid Products (South Nutfield, U.K.). Dilauroylphosphatidic acid (disodium salt) was from R. Berchtold (Biochemistry Laboratory, Berne, Switzerland). When necessary the disodium salt was converted to the acid form by acid washing, essentially using the procedure of Folch et al. [26]: 9.0 mg of the disodium salt were dissolved in 2 ml of chloroform/methanol/water (38 : 19 : 1, v/v), 0.2 ml of 0.1 M HCl was added and two phases were formed; the upper phase was discarded and the lower phase was washed four times with chloroform/methanol/0.2 M HCl (3 : 48 : 47, v/v); the lower phase was then rotary evaporated and the dilauroylphosphatidic acid was dissolved in chloroform/methanol (2 : 1, v/v); this solution was used immediately to prepare vesicles, as described below. Sepharose 4B was purchased from Pharmacia Fine Chemicals AB (Zürich, Switzerland). ^3H-labelled dipalmitoylphosphatidylcholine was tritiated by EIR (Würenlingen, Switzerland).

Preparation of vesicles. A solution of phospholipid in chloroform/methanol (2 : 1, v/v) was rotary evaporated and the phospholipid film was dried under high vacuum. Either phosphatidic acid or a mixture of this and phosphatidylcholine was used to which, where appropriate, ^3H-labelled dipalmitoylphosphatidylcholine was added as a marker. The dry phospholipid film was dispersed in water or a NaCl solution containing both $^{22}Na^+$ (about 3 nM) and $^{35}SO_4^{2-}$ (about 20 nM) or one of the fluorochromes, harmaline (1 mM) and pyranine (1 mM). Dispersion was aided by shaking by hand with glass beads. Vortex mixing was not, as previously reported [25], used as occasionally the glass beads break the round bottomed flask and scatter the contents. For the dispersion of phosphatidic acid alone it was also necessary to freeze-thaw about six times (from ice/methanol at about -15°C to about 5°C).

For phosphatidylcholine-phosphatidic acid mixed dispersions the initial pH was raised to a maximum value of 7–8 by slowly (in about 1 min) titrating NaOH, delivered from an Agla Syringe (Wellcome, Beckenham, U.K.), into a stirred dispersion. For phosphatidic acid alone the initial pH was increased, unless otherwise stated, with NaOH delivered rapidly (in about 1 s) from a micropipette into a stirred phosphatidic acid dispersion. The pH was reduced from its maximum value to 7.6 by adding HCl. The exposure of the phospholipids to high pH was short (less than 2 min), this is not sufficient to cause detectable degradation.

Column chromatography. Phospholipid dispersions were chromatographed at room temperature (20 ± 2°C) on a Sepharose 4B column (40–50

cm × 0.9 cm) with a flow rate of 2.5–3.0 ml/h. The elution buffer contained 0.02% sodium azide, 2 mM Hepes plus NaOH to pH 7.55. Before loading the samples onto the column they were centrifuged in 1.5 ml tubes for 3 min at 12000 × *g*. This pellets more than 97% of the material in an unsonicated dispersion of phosphatidylcholine (at 10 mg/ml) in distilled water. Routinely, 0.3 ml fractions were collected in scintillation vials using an automatic fraction collector. The concentration of the eluted reagents (Figs. 1 and 6) is expressed as a percentage (p) of their initial concentration in the sample applied to the column. It should be noted that Σp for all the fractions is equal to the (sample volume × 100)/(fraction volume).

In order to obtain reproducible elution profiles it was necessary to saturate the Sepharose 4B with the appropriate phospholipid. Reproducible elution profiles and a 92 ± 9% ($n = 13$) recovery of the ^{3}H-labelled phosphatidylcholine marker were achieved after 2–4 elutions of phospholipid vesicles (each of 8 mg in 1 ml) prepared as described above.

Fluorescence measurements. Fluorescence was measured in an Aminco Bowman Spectrofluorimeter. Over the concentration range used fluorescence was, within ±1%, directly proportional to fluorochrome concentration. 20 or 200 μl aliquots of the eluted fractions (450 μl) from column chromatography were dissolved in 2 ml of the elution buffer containing 2% sodium dodecyl sulphate. Pyranine was excited at 385 nm and the fluorescence monitored at 510 nm, harmaline was excited at 385 nm and monitored at 485 nm; 5 nm slit-widths were used.

Phosphate determination. Phosphate was determined by using a modification of the procedure of Chen et al. [28] suitable for small volumes containing little organic material (approx. 800 g per mol P).

Results

Chromatography of phosphatidic acid vesicles on Sepharose 4B

The elution profile of a phosphatidic acid dispersion eluted from a Sepharose 4B column is shown in Fig. 1. The phosphatidic acid was dispersed in distilled water containing $^{22}Na^+$ and

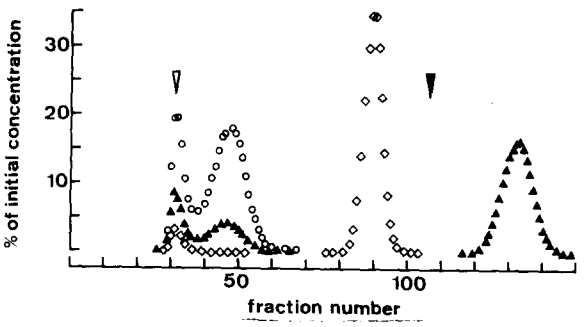

Fig. 1. Gel filtration of phosphatidic acid vesicles on Sepharose 4B. The phosphatidic acid (12 mM), containing ^{3}H-labelled phosphatidylcholine (○) as a marker, was dispersed in distilled water containing $^{22}Na^+$ (▲) and $^{35}SO_4^{2-}$ (◇), the pH was then raised rapidly (in about 1 s) to 9.8 by the addition of NaOH (18 mM) and returned to pH 7.6. The void volume is indicated by ▽ and the total column volume by ▼.

$^{35}SO_4^{2-}$, the pH was then raised from 2.8 to 9.8 by the rapid addition (in about 1 s) of NaOH and was then readjusted to 7.6 with HCl. The [^{3}H]phosphatidylcholine (○), used as a marker for the phosphatidic acid, is seperated into two peaks, one eluting at the void volume and the other at 0.43 of the total column volume. The phosphatidic acid in these peaks is in the form of vesicles, this is shown by the coelution of both $^{22}Na^+$ (▲) and $^{35}SO_4^{2-}$ (◇) with the [^{3}H]phosphatidylcholine marker (Fig. 1, although the $^{35}SO_4^{2-}$ does elute with the second peak it is too diluted to be seen in the figure). As evidence is given below that the vesicles in both peaks are unilamellar, the peak at the void volume is called the large unilamellar vesicle peak and that at about 0.45 of the total column volume the small unilamellar vesicle peak. The $^{22}Na^+$ (▲) elutes in three peaks, two associated with [^{3}H] phosphatidylcholine marker and the third at 1.24 column volumes. The $^{35}SO_4^{2-}$ (◇) elutes in two main peaks, one at the void volume and the other at 0.84 column volumes.

The separation of the unencapsulated $^{22}Na^+$ and $^{35}SO_4^{2-}$ from one another is caused by adsorption of phosphatidic acid onto the Sepharose 4B giving it the characteristics of a cation exchanger. For this reason the phosphatidic acid vesicles in the second peak are likely to elute at a smaller volume than uncharged vesicles of the same size. Vesicle size cannot therefore be de-

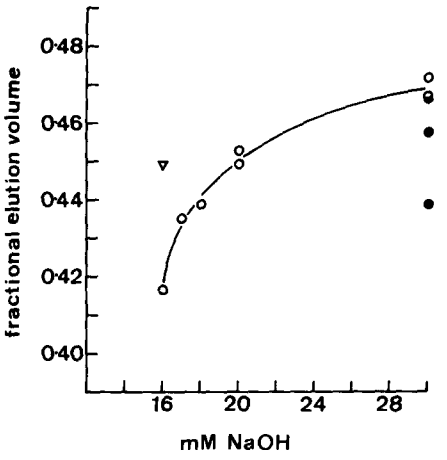

Fig. 2. Elution volume of the small unilamellar vesicle peak from a Sepharose 4B column, expressed as a fraction of the total column volume, plotted as a function of the NaOH concentration used to increase the pH to its maximum value. The samples were prepared and chromatographed, essentially, as for Fig. 1. The curve is drawn through data derived from samples of phosphatidic acid dispersed in water to which NaOH was rapidly (in about 1 s) added (○). For ● the initial dispersion was in 3, 30 and 150 mM NaCl, in descending order. For ▽ the Na^+ concentration was raised after the addition of NaOH to 30 mM by the addition of 14 mM NaCl.

termined with any degree of accuracy from the fractional elution volume.

Effect of pH and sodium ion concentration on elution volume and vesicle size

In Fig. 2 the fractional elution volume of the small unilamellar vesicle peak (derived from elution profiles similar to that in Fig. 1) is plotted against the concentration of NaOH that was added to raise the pH to its maximal value. The curve is drawn through the values (○) for the fast addition of NaOH to phosphatidic acid dispersed in water. For these dispersions the fractional elution volume depends on the maximal pH value and on the sodium ion concentration. The latter can be seen by comparing the two 16 mM NaOH values, the one with the larger fractional elution volume (▽) contained a further 14 mM NaCl added after the pH had been changed. This value is however smaller than those of samples containing the same Na^+ concentration but to which 30 mM NaOH was added. It therefore follows that vesicle size decreases as the maximum pH value, to which the sample was raised, is increased and that the fractional elution volume of the vesicles (as also that of the unentrapped $^{35}SO_4^{2-}$, data not shown) decreases as the Na^+ concentration in the sample is increased.

Although the addition of NaCl after vesiculation increases the fractional elution volume this is decreased if the phosphatidic acid is dispersed directly in water containing NaCl (●; 3, 30, 150 mM, in descending order) and the pH is then increased. This decrease in fractional elution volume is progressive as the NaCl concentration is increased and indicates that, for a given maximal pH value, the vesicle size is increased by increasing the sodium ion concentration in the dispersion medium. Further, the disodium salt of dilauroylphosphatidic acid does not form small

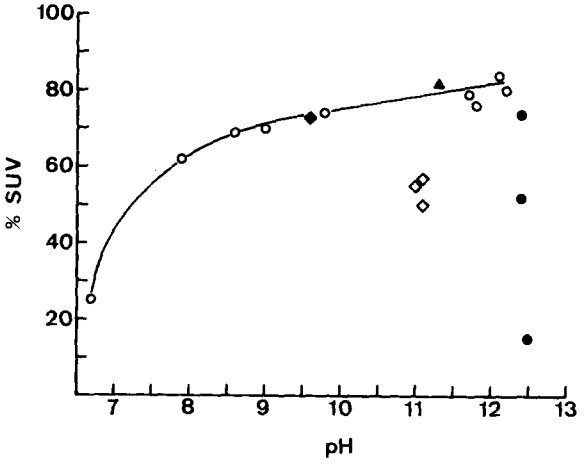

Fig. 3. The % of small unilamellar vesicles (SUV) formed plotted against the maximum pH to which the phosphatidic acid dispersions were raised. The % of small unilamellar vesicles formed was derived from the area under the small unilamellar vesicle peak of Sepharose 4B elution profiles (similar to that in Fig. 1). This % is calculated with reference to the ^3H-labelled phosphatidylcholine marker present before centrifugation (12000 × *g* for 3 min) and column chromatography. The average loss of ^3H-labelled phosphatidylcholine on centrifugation was < 1% except for the samples dispersed in 30 and 150 mM NaCl where the loss was 7 and 64%, respectively. The curve is drawn through data (○) from samples of egg phosphatidic acid dispersed in water to which NaOH was rapidly (in about 1 s) added. The following samples were prepared in the same way except that: for ● the initial dispersion was in 3, 30 and 150 mM NaCl, in descending order; for ▲ KOH was used; for ◆ dilauroylphosphatidic acid was used; and for ◇ the pH was raised slowly (in about 2 min).

vesicles when the pH is raised rapidly (in about 1 s) to 10. The vesicles formed elute at the void volume. However, if the acid form is used then the same procedure gives small unilamellar vesicles (see Fig. 3).

Effect of pH, rate of pH change and NaCl concentration on the percentage of small unilamellar vesicles formed

In Fig. 3 the percentage of small unilamellar vesicles formed (calculated from the area under the small unilamellar vesicle peak of elution profiles similar to that in Fig. 1) has been plotted against the maximum pH to which the dispersion was raised. The curve is drawn through the data (○) derived from the rapid (~ 1s) addition of NaOH to phosphatidic acid dispersed in water. Using KOH (▲) instead of NaOH (○), or using dilauroylphosphatidic acid (◆) instead of egg yolk phosphatidic acid (○), does not noticeably affect the percentage of small unilamellar vesicles formed. In contrast, slow addition (in about 2 min) of NaOH (◇) results in a decrease in the percentage of small unilamellar vesicles. This can also be seen for the rapid addition of NaOH to phosphatidic acid dispersed in NaCl (●); the percentage of small unilamellar vesicles decreases as the NaCl concentration in the dispersion medium is increased (3, 30, 150 mM, in descending order).

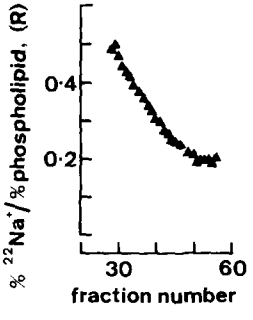

Fig. 4. Encapsulation of $^{22}Na^+$ by phosphatidic acid vesicles. The data from Fig. 1 are plotted as the ratio (R) of the % of the total $^{22}Na^+$ to that of phosphatidic acid (derived from the ^{3}H-labelled phosphatidylcholine marker) against fraction number. Under the conditions used here this ratio should be the same as the ratio of the number of phosphatidic acid molecules on the inside of the membrane to the total number present in the membrane (see text).

Encapsulation of $^{22}Na^+$ and $^{35}SO_4^{2-}$ as an indicator of vesicle size

From the data in Fig. 1 it is possible to calculate the ratio (R) of the % Na^+ to the % ^{3}H-labelled phosphatidylcholine (as a marker for the % phosphatidic acid) in any given fraction eluted from the column. This ratio is plotted against fraction number in Fig. 4; it decreases, from a value of 0.48 for the large unilamellar vesicle peak, with increasing fraction number to a limiting value of 0.194. These values and those for other phosphatidic acid samples raised to different maximum pH values are given in Table I. The total amount of $^{22}Na^+$ that is encapsulated is very high (average 41%, see Table I). This indicates that in the initial dispersion virtually all the $^{22}Na^+$ (approx. 3 nM) is bound to the phosphatidic acid (approx. 13 mM) and further that the fraction of $^{22}Na^+$ bound to the phosphatidic acid that is destined to form the inner half of the vesicle bilayer remains bound during vesiculation. Under these conditions it can be assumed that the ratio (R) of % $^{22}Na^+$ to % ^{3}H-labelled phosphatidylcholine marker will be the same as the ratio of the number of phosphatidic acid molecules on the inside of the vesicle bilayer to the total number in the bilayer. A ratio of 0.5 or less would therefore indicate that the vesicles are unilamellar. This is found if the maximum pH value, used to vesiculate the phosphatidic acid dispersion, is 9.8 or less. Above this pH value the ratio is higher but only for the vesicles that elute near the void volume (Table I), this indicates that some of these vesicles are not unilamellar.

If it is assumed that the surface area per phosphatidic acid molecule is the same on both sides of the membrane, then the relationship between the ratio (R) of the number of phosphatidic acid molecules on the inside of the vesicle bilayer to the total number in the bilayer and the external radius (r) is given by,

$$R = (r-d)^2/\left((r-d)^2+r^2\right)$$

where d is the membrane thickness. This equation was used to generate a standard curve of R against external radius (r) assuming a membrane thickness of 4 nm [7,29]. The ratio, R, derived from the $^{22}Na^+$ encapsulation (Table I) by large unilamellar vesicles is 0.5; for ratios between 0.45 and 0.5

TABLE I

ENCAPSULATION OF $^{22}Na^+$ AND $^{35}SO_4^{2-}$ BY PHOSPHATIDIC ACID VESICLES

The pH of a phosphosphatidic acid dispersion in water was raised to the indicated value by the rapid addition of NaOH and then returned to pH 7.6 with HCl. The parameters given below were obtained by replotting the data from an elution profile, such as that in Fig. 1, as has been done in Figs. 4 and 5. LUV (large unilamellar vesicles) peak, peak eluting at void volume, SUV (small unilamellar vesicles) peak, peak eluting at about 0.45 column volumes. The limiting values are presented as mean ± S.D., in parentheses the number of data.

Maximum pH	$^{22}Na^+$ encapsulation					$^{35}SO_4^{2-}$ encapsulation			
	% $^{22}Na^+$/% [^{3}H]phosphatidylcholine (R)			% Encapsulation		l of $^{35}SO_4^{2-}$ containing water per mole of phosphatidic acid (V)			% Encapsulation
	LUV peak	SUV peak	Limiting values	SUV peak	Total	LUV peak	SUV peak	Limiting values	SUV peak
7.9	0.50	0.31	0.254 ± 0.005 (7)	19	41	18	0.60	0.23 ± 0.10 (7)	0.37
8.6	0.52	0.30	0.228 ± 0.008 (7)	21	43	15	0.50	0.50 ± 0.21 (14)	0.33
9.0	0.53	0.35	0.313 ± 0.025 (11)	25	43	14	0.51	0.51 ± 0.17 (10)	0.49
9.8	0.48	0.22	0.194 ± 0.008 (7)	16	34	15	0.23	0.23 ± 0.14 (14)	0.23
11.8	0.63	0.30	0.263 ± 0.008 (7)	23	43	30 [a]	0.75	0.75 ± 0.11 (8)	0.75
12.2	0.63	0.29	0.256 ± 0.008 (7)	23	40	57 [a]	0.85	0.85 ± 0.12 (14)	0.70
Average		0.30	0.251	21	41	15		0.51	0.48

[a] Not included in the average.

the diameter cannot be accurately determined, but must be at least 100 nm. For the small unilamellar vesicles the limiting value of the same ratio is 0.251 (Table I), this gives an external diameter of 21 nm.

The data in Fig. 1 can also be used to calculate the encapsulated aqueous volume (V), if it is assumed that the % encapsulation of water is the same as that of $^{35}SO_4^{2-}$; this is plotted in Fig. 5 against fraction number. The encapsulated volume decreases with increasing fraction number from a value of 15 for the large unilamellar vesicle peak to a limiting value of 0.23 litre of $^{35}SO_4^{2-}$-containing water per mole of phosphatidic acid for the small unilamellar vesicle peak. These data and those for other phosphatidic acid dispersions raised to different maximum pH values are given in Table I. If it is assumed that the molecular packing density is equal in both halves of the vesicle bilayer, then the relationship between the encapsulated aqueous volume (V, in l/mol) and the external radius of the vesicles, in nm, is given by,

$$V = A(r-d)^3/3\left((r-d)^2 + r^2\right)$$

where A is the area (in km^2) occupied by one mole of phospholipid. This equation was used to generate a standard curve of V against external radius, assuming a value for A of 0.42 km^2 [8], the area occupied by one mole of phospholipid at 0.7 nm^2 per molecule, and a bilayer thickness of 4 nm [7,29]. The average volume encapsulated by the

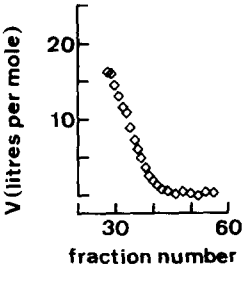

Fig. 5. Internal aqueous volume of phosphatidic acid vesicles determined by $^{35}SO_4^{2-}$ encapsulation. The internal volume (V), in l/mol, is plotted against fraction number. V is calculated from the data in Fig. 1 by dividing the % of the initial $^{35}SO_4^{2-}$ present in a given fraction by the % of the initial ^{3}H-labelled phosphatidylcholine present, this ratio is then divided by the initial phosphatidic acid concentration (in mol/l).

large unilamellar vesicles (15 l/mol, Table I) indicates a vesicle diameter of 440 nm and that encapsulated by the small unilamellar vesicles (0.51 l/mol) one of 29 nm. These diameters are in reasonable agreement with those derived from the $^{22}Na^+$ encapsulation data (> 100 nm and 21 nm, respectively.

Encapsulation of $^{22}Na^+$ and $^{35}SO_4^{2-}$ by phosphatidylcholine / phosphatidic acid vesicles

Mixed dispersions of phosphatidylcholine and phosphatidic acid (from 6 : 1 to 20 : 1, w/w) were prepared in water containing $^{22}Na^+$ and $^{35}SO_4^{2-}$ by slowly (in about 1 min) raising the pH to 7.6. The dispersions were then chromatographed as for Fig. 1. However, in contrast to dispersions of phosphatidic acid alone that have been raised rapidly to pH 10 (Fig. 1) the ^3H-labelled phosphatidylcholine marker elutes in one peak at, or near, the void volume. (This is also found for phosphatidylcholine/phosphatidic acid mixed dispersions at ratios of 6 : 1 (w/w) and above when the pH is raised both slowly (in about 2 min) and rapidly (in about 1 s) to a value above 10.) The $^{35}SO_4^{2-}$ encapsulation gave a value for the entrapped aqueous phase from 4.4 l/mol of phospholipid at a ratio of 20 : 1 to 1.2 l/mol at 6 : 1 (phosphatidylcholine/phosphatidic acid, w/w). The respective vesicle size being approx. 140 and 50 nm.

Permability of the vesicles to encapsulated $^{22}Na^+$ and $^{35}SO_4^{2-}$

Phospholipid dispersions were prepared and chromatographed as for Figs. 1 and 6. However, the total column volume was collected in 21.4, as opposed to 107, fractions and the efflux of $^{22}Na^+$ and $^{35}SO_4^{2-}$ was measured from the vesicle containing fractions. The half-times, derived by plotting the log of the relative efflux against time, are given in Table II. The half-time of the efflux of $^{22}Na^+$ from phosphatidic acid vesicles formed by rapidly (in about 1 s) raising the pH to 11.4 decreases (from 30 to 7 days) with increasing fraction number (Table II). This decrease in half-time is presumably dependent on the decrease in average vesicle size with increasing fraction number and is to be expected; as the ratio of the surface area to the internal volume increases with decreasing vesicle size. The permeability of these vesicles to $^{35}SO_4^{2-}$ was too small to be detected ($t_{1/2} > 70$ days). The half-time of the efflux of $^{22}Na^+$ and $^{35}SO_4^{2-}$ from vesicles formed by slowly raising the pH of a mixed dispersion of phosphatidylcholine and phosphatidic acid (6 : 1, w/w) is similar to those of pure phosphatidic acid raised to pH 11.4 (Table II).

Encapsulation of harmaline and pyranine

These fluorochromes were selected for encapsulation because their intense fluorescence means

TABLE II

EFFLUX OF $^{22}Na^+$ AND $^{35}SO_4^{2-}$ FROM PHOSPHATIDIC ACID AND PHOSPHATIDYLCHOLINE/PHOSPHATIDIC ACID VESICLES

The fractions were dialysed, at 16°C, against column buffer from which duplicate samples were taken at timed intervals. For fraction numbers see text.

Lipid composition	maximum pH	Ion	Half-time (days) in fraction number			
			7	8	10	11
Phosphatidic acid	11.4 [a]	$^{22}Na^+$	30	15	7	5
		$^{35}SO_4^{2-}$	> 70	> 70	> 70	> 70
Phosphatidylcholine /phosphatidic acid (6 : 1, w/w)	7.6 [b]	$^{22}Na^+$	16	14		
		$^{35}SO_4^{2-}$	67	63		

[a] the pH was raised rapidly (in about 1 s) and then returned to 7.6.
[b] the pH was raised slowly (in about 1 min).

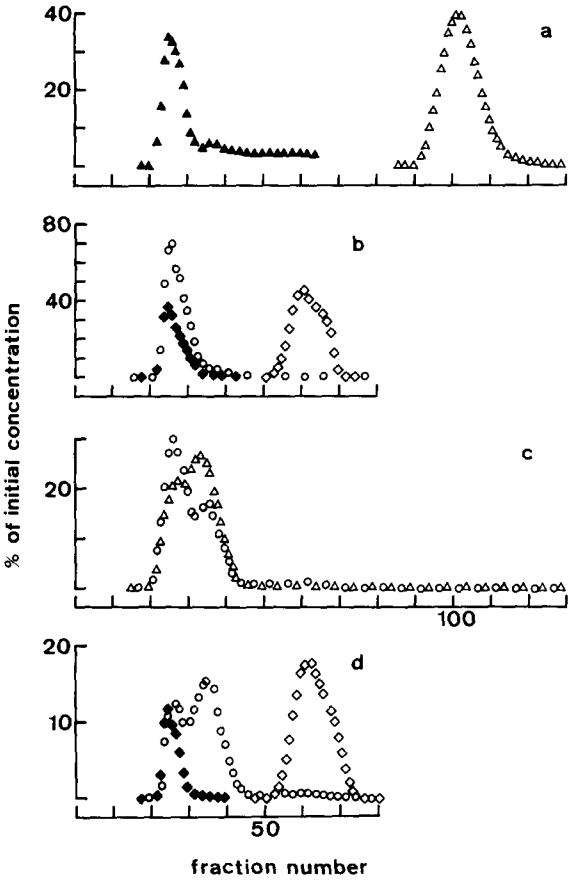

Fig. 6. Elution of phosphatidylcholine/phosphatidic acid (6 : 1, w/w) vesicles (a and b) and phosphatidic acid vesicles (c and d) from Sepharose 4B. Samples a and c were dispersed in 1 mM harmaline (△, ▲), b and d in 1 mM pyranine (◇, ◆). The pH of the phosphatidylcholine/phosphatidic acid dispersion was raised slowly (in about 1 min) to pH 7.6, the pH of the phosphatidic acid dispersion was raised rapidly to about 11 and then returned to pH 7.6. The open symbols correspond to the ordinate scale; the filled symbols in a and d correspond to one tenth of the ordinate scale and in b to one fiftieth. Phosphate (○) was determined by a modification of the method of Chen et al. [28].

that they can easily be detected at low concentrations, because they are oppositely charged and because their size is similar to that of many drugs. In Fig. 6 it is shown that these fluorochromes are encapsulated both by phosphatidylcholine/phosphatidic acid (6 : 1) dispersions (Figs. 6a and 6b) and by phosphatidic acid dispersions (Figs. 6c and 6d). As would be expected the positively charged harmaline (Figs. 6a and 6c) is more efficiently encapsulated than the negatively-charged pyranine (Figs. 6b and 6d). However, as can be seen in Fig. 6c, where all the detectable harmaline elutes with the phosphatidic acid, it is not possible to distinguish between harmaline encapsulation and its binding to the outside of the vesicles. As in the case of $^{35}SO_4^{2-}$, pyranine is almost exclusively encapsulated by the larger vesicles, which elute at or near the void volume of the column (Figs. 6b and 6d). A comparison of the elution profile of phosphatidic acid vesicles shown in Figs. 6c and 6d with that in Fig. 1 indicates that the presence of both pyranine (1 mM), which contains three moles Na^+ per mole of pyranine, and harmaline (1 mM) in the initial dispersion medium affects vesicle formation. In the case of pyranine this may, as discussed above, result from the presence of Na^+ affecting the size of the vesicles formed; however, in the case of harmaline it would seem, from a qualitative assessment of Fig. 6c, that the amount of vesicles eluting in the small unilamellar vesicle peak is reduced.

Discussion

Formation of small unilamellar vesicles occurs efficiently (60–80%) when the pH of the phosphatidic acid dispersion is raised abruptly from approx 3 to above 8, but only in the presence of a low concentration of added salt (Fig. 3). Although the optimal conditions for the formation of larger vesicles were not investigated, it should be possible to vary the size systematically from small unilamellar vesicles to large unilamellar vesicles. Vesicle size can be increased, by decreasing the rate of the pH change (data not shown), by using a mixture of phosphatidic acid with phosphatidylcholine (compare Figs. 1, 6c and 6d with Figs. 6a and 6b) and by starting from a dispersion that contains Na^+ as a counterion (Fig. 2).

The larger vesicles could be suitable for the encapsulation of drugs and possibly of macromolecules. They are relatively impermeant to ions (Table II) and can be made with a surface charge from that of pure phosphatidic acid to that of a phosphatidylcholine/phosphatidic acid mixture of 20 : 1. Surface charge seems to play an important role in the fusion of vesicles with cell membranes [3] and vesicles containing 5–10% phosphatidic

acid are frequently used for delivery of encapsulated drugs to cells (see references cited in Ref. 30). Further, when the presence of unencapsulated drug is not critical it should be possible to prepare phosphatidic acid or phosphatidylcholine/phosphatidic acid vesicles immediately prior to use. This could be done, using standard sterile techniques, by injecting one or two buffered solutions into an ampoule containing the predried phospholipid film. The whole procedure, including centrifugation or filtration to remove poorly dispersed material, should require only a few minutes and further requires no sophisticated apparatus or specialised skills.

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