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THE EFFECT OF CHARGE AND CHOLESTEROL ON THE SIZE AND THICKNESS OF SONICATED PHOSPHOLIPID VESICLES

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SUMMARY

Equations are derived describing the sedimentation of hollow vesicles in the ultracentrifuge in terms of the external radius of the vesicles and the volume fraction which is at constant density. The equations were applied to liposomes composed of mixtures of phosphatidic acid, phosphatidylcholine and cholesterol, and enabled the vesicle size and bilayer thickness to be studied. Results for phosphatidylcholine correlated well with those quoted by previous authors from X-ray studies on myelins or small vesicles.

The vesicle size increased with charge unless the liposomes were prepared in 2 M salt, although the ionization of the phosphatidic acid inside was partially depressed.

10% cholesterol caused a 30% increase in the surface area of the phospholipid molecule, and a consequent decrease in bilayer thickness. As the molar proportion of cholesterol rose further the surface area of the bilayer per phospholipid molecule remained constant at 89 $\pm$ 4 Å^2, and the bilayer thickness increased.

INTRODUCTION

There is a shortage of simple methods of measuring the vesicle size and bilayer thickness of sonicated phospholipid vesicles. Wilkins *et al.*[1] published an X-ray analysis of sonicated phosphatidylcholine vesicles giving their bilayer thickness but not their size. Huang[2] measured the diffusion coefficient of purified phosphatidylcholine vesicles, giving their external radius. However, Huang's purification method was found to be unsuitable for mixed lipids due to chemical adsorption by the Sepharose 4B column. In any case sedimentation coefficients are more quickly and accurately measured than diffusion coefficients. Furthermore, sedimentation coefficients are proportional to the square of the particle radius, which immediately shows any heterogeneity in the vesicle preparation. Provided only a small proportion of larger vesicles are present the ultracentrifuge itself will separate these during a run; so the sedimentation coefficient for the majority can be calculated together with an estimate of how heterogenous the material is. A single liposome preparation can be used to find the sedimentation coefficient, bilayer density[3] and the aqueous trapped volume. If the liposomes are spherical these quantities will give a measure of the liposome radius and bilayer thickness which in turn can be used to calculate surface areas and vesicle weight.

The method allows the effect of charge on vesicle size to be studied, and also the effect of increasing proportions of cholesterol on vesicle size and bilayer thickness. Results indicate a probable correlation between biological membrane thicknesses and their phospholipid:cholesterol ratio.

The equations conventionally used to describe the sedimentation behaviour of particles in the ultracentrifuge are not well suited to hollow vesicles such as liposomes. These structures are exceedingly impermeable to cations, and it frequently happens that the density of the interior aqueous compartments differs both from that of the phospholipid and from the density of the solution in which the liposomes are suspended. When considering the sedimentation behaviour of such structures it is simpler to divide them into two or three volume proportions of constant density rather than to use the more conventional partial specific volume. The mathematical treatment is given below.

Acting on one liposome there is a force F, produced by the angular acceleration of the rotor such that

$$F = \Delta\rho v\omega^2 x \tag{1}$$

where $\Delta\rho$ is the difference in density between the liposome and the solution through which it sediments, v is the volume of the liposome and ω the angular velocity of the rotor. x is the distance of the liposome from the axis of rotation at time t. At any time t the particles are subject to a frictional resistance F_r such that

$$F_r = 6\pi\eta r_s \frac{dx}{dt} \tag{2}$$

where η is the viscosity of the solvent and r_s the Stokes' radius of the liposome.

Under steady conditions $F=F_r$, so by equating Eqns 1 and 2 we obtain

$$\frac{1}{\omega^2 x}\frac{dx}{dt} = \frac{\Delta\rho v}{6\pi\eta r_s}$$

The sedimentation coefficient for one liposome is taken to be s°, the sedimentation coefficient for the liposome suspension, concentration c_1, as $c_1 \to 0$

By definition,

$$s = \frac{1}{\omega^2 x}\frac{dx}{dt}$$

$$s^0 = \frac{\Delta\rho v}{6\pi\eta r_s} \tag{3}$$

The phospholipid is assumed to have a fixed density ρ_l and its volume proportion in the sedimenting or diffusing liposome is λ. The volume proportion of the central aqueous compartment, density ρ_b is λ_c and the volume proportion of any of the external solution sedimenting with the liposome is $1-\lambda-\lambda_c$. The density of the external solution is ρ_a.

$$\Delta\rho = \lambda(\rho_l - \rho_a) + \lambda_c(\rho_b - \rho_a) \tag{4}$$

If $\rho_b = \rho_a$

$$\Delta\rho = \lambda(\rho_l - \rho_a) \tag{5}$$

Substituting for $\Delta\rho$ in Eqn 3

$$s^0 = \frac{\lambda v(\rho_l - \rho_a)}{6\pi\eta r_s} \tag{6}$$

If the liposome is an ellipsoid of semi-axes a, b, c

$$v = \frac{4\pi abc}{3} \tag{7}$$

Substituting for v in Eqn 6

$$s^0 = \frac{2\lambda abc(\rho_l - \rho_a)}{9\eta r_s} \tag{8}$$

If the liposome is spherical and remains the same size during sedimentation $a=b=c=r_s$ Eqn 8 becomes

$$s^0 = \frac{2\lambda r_s^2(\rho_l - \rho_a)}{9\eta} \tag{9}$$

Due to the practical difficulty of estimating accurately the volume of external solution sedimenting with the liposomes, λ_i is easier to measure than λ.

$$\lambda_i = \frac{\text{Lipid volume}}{\text{Lipid + internal aqueous volumes}}$$

$$\lambda_i \geqslant \lambda$$

Let r_e be the distance from the centre of the liposome to the outside edge of the bilayer. Let h be the thickness of the external solution layer sedimenting or diffusing with the liposome, see Fig. 1.

$$r_e = r_s - h \tag{10}$$

$$\lambda/\lambda_i = (r_e/r_s)^3 \tag{11}$$

If r_c is the radius of the central aqueous compartment

$$(r_c/r_e)^3 = 1 - \lambda_i \tag{12}$$

The bilayer thickness,

$$\delta = r_e - r_c \tag{13}$$

MATERIALS

Phosphatidylcholine was extracted from egg yolk and phosphatidic acid prepared from it by enzymic hydrolysis according to Papahadjopoulos and Miller[4]. The cholesterol was Sigma's chromatographic grade. Isotopes came from the Radio-

chemical Centre, Amersham. All other reagents were analytical grade and the salts used to prepare solutions for the ultracentrifuge were dried before use. Water was twice distilled, the second time from $KMnO_4$ in borosilicate glass apparatus.

Preparation of the liposomes

A chloroform solution containing from 12 to 50 μmoles of mixed phospholipids with or without cholesterol was evaporated to dryness under reduced pressure. 0.6 ml of aqueous salt solution was added, and the phospholipid allowed to form liposomes under nitrogen. The liposomes were transferred to a 1.3 cm diameter flat-bottomed glass vial, and sonicated to clearness (0.5–1.5 h) under N_2 in a Kerry's ultrasonic cleaning bath type KB 80/1 at 80 kcycles/s, or in a Mullard E 759 DB at 10–25 kcycles/s. Both sonicators were cooled by running tap water at approx. 15 °C. The unopened vials were left to stand overnight. Previous experience had shown that this method avoided oxidation of the phospholipid[5], and no lysolecithin was detected by thin-layer chromatography in a Folch extract of a sonicated sample. The liposomes were then diluted to the concentration required for the ultracentrifuge. If necessary the concentration of phospholipid was checked by phosphate analysis, using McClare's method[6].

Measurement of sedimentation coefficients

Sedimentation coefficients were measured at 20.0±0.1 °C using a Beckman Model E analytical ultracentrifuge fitted with schlieren optics. The schlieren analyser angle was 50 °C. Unless otherwise stated, two sedimentation velocities were measured simultaneously using a plane window and a wedge window cell with standard 12 mm single sector centrepieces. The rotor speed was 60000 rev./min, giving an acceleration of $300000 \times g$. s was calculated from a plot of log x against t, and all such plots were linear. t was measured from the time the rotor reached its final speed.

Measurement of the volume proportion of bilayer in a liposome

Determination of the internal volume trapped per μmole of lipid phosphorus.

The liposomes were prepared in 10 mM [^{14}C]sucrose (or [^{14}C]glucose) and 148 mM ^{42}KCl or ^{24}NaCl. 0.01 ml portions of the swelling solution were retained as standards. After sonication and overnight equilibration, the liposomes were separated from the untrapped isotope by passage over a 30 cm column of coarse G-50 sephadex (anhydrous weight 3 g), which took about 2.5 min. Alternatively the liposomes were prepared in 0.1475 M ^{42}KCl solution and after sonication and equilibration dialysed against 0.1475 M KCl (5 times 250 ml) to avoid dilution of the sample which was to be used later in the ultracentrifuge. ^{42}K was counted immediately in a Packard scintillation counter by its Cerenkov radiation in aqueous solution. ^{24}Na was also counted immediately, either in 0.16 M NaCl or in Bray's solution. After 10 days the two short-life isotopes had disappeared, and the ^{14}C was counted in Bray's solution. Standards were prepared to contain the same amount of aqueous salt solution as their matching samples to avoid quench corrections and the ratio (count rate of sample/count rate of standard) was calculated, making allowance for decay corrections or the presence of a second isotope when necessary.

0.1-ml portions of the original sample containing about 0.15 μmole P were analysed for phosphorus by McClare's method[6]. This process involves digestion

in concentrated perchloric acid; it should be noted that samples containing much sugar explode unless they are left for at least 2 h at room temperature before heating.

V_c, the volume of isotopic solution trapped per μmole of lipid was found from the relation

$$\frac{\text{Count rate of sample} \times 0.01}{\text{Count rate of standard} \times \mu\text{mole phosphorus in sample}}$$

Determination of the volume of the bilayer per μmole of lipid phosphorus

To find the molecular weight of the phospholipid used the molar proportions of the fatty acids produced by the hydrolysis of the 4% phosphatidic acid–96% phosphatidylcholine stock solution were determined by gas chromatography of their methyl esters. The average molecular weight of phosphatidylcholine was calculated from the mean fatty acid composition *plus* the glycerol, phosphate and choline moieties. It was 780. Allowing for the missing choline groups on the phosphatidic acid, the mean molecular weight of the mixture was 776.5. The "phosphorus molecular weight" of a mixture of phospholipid and cholesterol was calculated as the gram molecular weight associated with one phosphorus atom. For example the phosphorus molecular weight of the mixture of 71% phosphatidic acid–phosphatidylcholine–29% cholesterol was calculated as $(776.5+(0.29/0.71)\times 386.6)=934$. Anhydrous molecular weights were used throughout. The density of the phospholipid with or without cholesterol was found to be 1.014 $g\cdot cm^{-3}$ (ref. 3). Hence the volume of the bilayer per μmole of phosphorus was

$$\frac{\text{phosphorus mol. wt} \times 10^{-6}}{1.014} = v_l$$

The volume fraction of phospholipid associated with the internal aqueous compartment is λ_i.

$$\lambda_i = v_l/(v_c + v_l)$$

RESULTS

Values of v_c and λ_i are shown in Table I. Except for the liposomes made with 33% phosphatidic acid the v_c cation:sugar ratios did not differ significantly ($\pm 10\%$) from 1.0. Results for the 4% phosphatidic acid–phosphatidylcholine liposomes in KCl are similar to those already published, Johnson and Bangham[7], but results for phosphatidylcholine are not similar to those of Kornberg and McConnell[8].

Sedimentation coefficients

Fig. 2 shows the sedimentation of 4% phosphatidic acid–phosphatidylcholine liposomes in 0.16 M KCl. The run lasted 5 h, and the plot of log x against t was linear with a correlation coefficient of 0.9999. Note the distortion of the KCl solvent baseline in the high gravitational field. It will be seen that the phospholipid peak has a leading edge, and is not quite symmetric. The mean of two pictures showed that 57.1% of the total area lay to the right of X_t, where X_t was the distance of the peak from the interface at time t. This suggests that the material contained about 14% of rather

TABLE I

Phosphorus mol. wt is the gram molecular weight of the mixed lipids associated with 1 atom of phosphorus; S.D. is the standard deviation. Phosphatidic acid–phosphatidylcholine refers to the standard 4% phosphatidic acid–96% phosphatidylcholine mixture.

Liposome molar proportions	*Solvent*	*10^4 $v_c \pm$ S.D. ($cm^3/\mu mole$)*	*No. of expts*	*Phosphorus mol. wt*	*λ_i*	*Cation vol.* / Sugar vol.*
Phosphatidylcholine	0.16 M KCl	2.6±0.1	2	780	0.746	0.99
4% Phosphatidic acid–phosphatidylcholine	0.16 M KCl	3.2±0.2	5	776.5	0.705	1.02
33% Phosphatidic acid–phosphatidylcholine	0.16 M KCl	11.4	1	751.6	0.394	1.27
33% Phosphatidic acid, 29% cholesterol phosphatidylcholine	0.16 M KCl	14.8	1	898	0.3745	1.41
10% Cholesterol phosphatidic acid–phosphatidylcholine	0.16 M KCl	5.8	1	819	0.584	
20% Cholesterol phosphatidic acid–phosphatidylcholine	0.16 M KCl	5.6	1	873	0.607	
29% Cholesterol phosphatidic acid–phosphatidylcholine	0.16 M KCl	6.3	1	934	0.593	
40% Cholesterol phosphatidic acid–phosphatidylcholine	0.16 M KCl	7.4	1	1034	0.580	
50% Cholesterol phosphatidic acid–phosphatidylcholine	0.16 M KCl	8.2±0.2	4	1163	0.583	
4% Phosphatidic acid–phosphatidylcholine	0.16 M NaCl	2.6±0.3	2	776.5	0.745	

* If the cation:sugar volume ratio > 1.10 the value of v_c quoted is the sugar volume.

larger material. There is no sign of smaller particles. The arrows on Fig. 2 represent the estimated positions of double and treble bilayer liposomes calculated for a bilayer spacing of 66 Å and the radius values given in Table II. It appears that the asymmetry in the single peak is caused mostly by single bilayer vesicles which are rather larger than average.

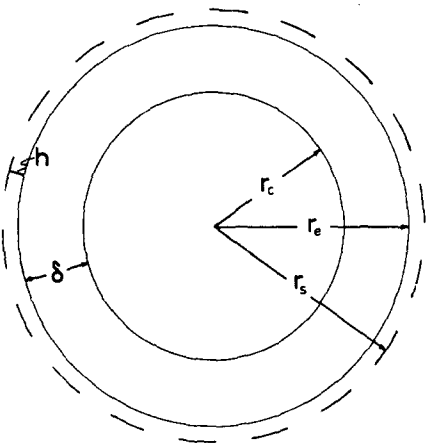

Fig. 1. Liposome radii.

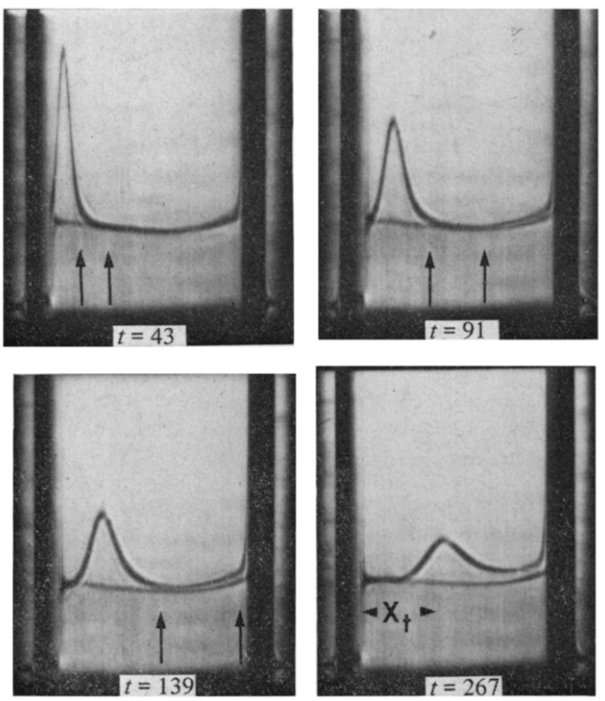

Fig. 2. Photographs of sedimenting 4% phosphatidic acid–phosphatidylcholine liposomes in 0.16 M KCl. t is time in min from the start of the run. Arrows represent calculated positions for double and treble bilayer liposomes.

TABLE II

Liposome mixture	*Solvent*	$10^{13} \cdot s^{\circ}$ (*s*)	r_e (*Å*)	δ (*Å*)	$10^{-6} \times$ *mol. wt bilayer*	*Phospholipid molecules in bilayer*	*Area/No. phospholipid molecules* ($Å^2$)	*Area phospholipid less Cholesterol* ($Å^2$)	*Molecules KCl inside vesicle*
Phosphatidylcholine	0.16 M KCl	1.29	102	37	2.0	2600	71	71	110
4% Phosphatidic acid–phosphatidylcholine	0.16 M KCl	1.60	117	39	2.9	3700	67	67	190
4% Phosphatidic acid–phosphatidylcholine	2 M KCl	−12.8	104						
4% Phosphatidic acid–phosphatidylcholine	0.16 M NaCl	1.60	109	40	2.5	3200	66	66	
4% Phosphatidic acid–phosphatidylcholine	2 M NaCl	− 9.46	106						
10% Cholesterol phosphatidic acid–phosphatidylcholine	0.1475 M KCl	1.67	124	31	2.9	3500	87	83	
20% Cholesterol phosphatidic acid–phosphatidylcholine	0.1475 M KCl	1.67	119	32	2.6	3000	91	82	
29% Cholesterol phosphatidic acid–phosphatidylcholine	0.1475 M KCl	2.06	131	34	3.4	3700	92	77	
40% Cholesterol phosphatidic acid–phosphatidylcholine	0.1475 M KCl	2.56	150	38	5.0	4800	91	67	
50% Cholesterol phosphatidic acid–phosphatidylcholine	0.16 M KCl	3.46	181	45	8.7	7500	85	49	
33% Phosphatidic acid–phosphatidylcholine*	0.16 M KCl		254	39	17	22000	63	63	4000
33% Phosphatidic acid 29% Cholesterol phosphatidylcholine*	0.16 M KCl		231	33.5	12	13000	88	73.5	3100
Estimated error		±1%	±4%	±6%	±12%	±12%	±4%	±4–6%	±10%

* δ was assumed to be that of the appropriate 4% phosphatidic acid–phosphatidylcholine mixtures with or without cholesterol, and r_e was calculated from Eqn 12.

In general the sedimentation coefficient decreased linearly as the lipid concentration increased. For the 4% phosphatidic acid–phosphatidylcholine liposomes in 0.16 M KCl, $s = 1.61\ (1 - 0.13\ c_l) \cdot 10^{-13}$ s. However, for some other KCl concentrations or different size of liposome s could be independent of liposome concentration or even increase, see Fig. 3.

$s°$ was usually calculated from the two s values obtained when two different concentrations of phospholipid were centrifuged at the same time. Of eight preparations of 4% phosphatidic acid–phosphatidylcholine liposomes swollen in 0.16 M KCl, five had $s° = 1.60 \pm 0.01 \cdot 10^{-13}$ s, two had values of $2.5 > s° > 2.0$ and one a value of $s° = 1.35 \cdot 10^{-13}$ s. Recent evidence (Johnson, S. M. and Miller, N., unpublished) suggest that one cause of the variation in size may have been due to fluctuation in the temperature of the sonicator cooling water. No difference in liposome size was noted between the two different sonicators. After the initial equilibration period the liposomes were kept at 4 °C, but not always under nitrogen. No deterioration was found until at least three days after preparation, when values of $s°$ began to rise. For example one liposome preparation (not kept under N_2) had an $s°$ of $1.34 \cdot 10^{-13}$ s the first day, 1.35 the second and 1.5 the third. A second preparation with an initial $s°$ of 1.60 and kept under nitrogen showed no deterioration until the 5th day when $s° = 2.1$. The rise in $s°$ was too small to be due to coagulation, nor did the preparations appear more heterodisperse, so presumably the phospholipid had deteriorated chemically, probably by oxidation of the unsaturated hydrocarbon chains to fatty acids.

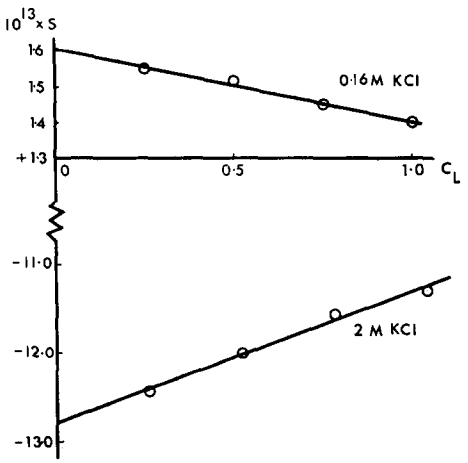

Fig. 3. Sedimentation coefficients of 4% phosphatidic acid–phosphatidylcholine liposomes in 0.16 M KCl and 2 M KCl as a function of liposome concentration. s in seconds.

Table II shows values of $s°$ for a variety of phospholipid vesicles. λ_i and $s°$ were measured on the same preparation for the phospholipid cholesterol mixtures.

Table IIIA and B show the densities and viscosities of the phospholipid mixtures and solutions used.

TABLE IIIa

Relative viscosities were calculated from the International Critical Tables (1925) 1st edn, McGraw Hill. Densities and the viscosity of water were calculated from the Handbook of Chemistry and Physics (1971) 51st edn, Chemical Rubber Company.

Solvent	*Density* ($g \cdot cm^{-3}$)	*Viscosity* (*P*)
H_2O	0.99823	0.01002
0.1475 M KCl	1.0052	0.009989
0.16 M KCl	1.0058	0.009989
2 M KCl	1.0903	0.009840
0.16 M NaCl	1.0048	0.01015
2 M NaCl	1.0771	0.01209

TABLE IIIb

The densities of the phospholipid mixtures were taken from Johnson and Buttress[3].

Phospholipid	*Density* ($g \cdot cm^{-3}$) ± 0.0003
4% Phosphatidic acid–phosphatidylcholine	1.0135
Phosphatidylcholine	1.0135
10% Cholesterol phosphatidic acid–phosphatidylcholine	1.0138
*20% Cholesterol phosphatidic acid–phosphatidylcholine	1.0142
30% Cholesterol phosphatidic acid–phosphatidylcholine	1.0145
*40% Cholesterol phosphatidic acid–phosphatidylcholine	1.0142
50% Cholesterol phosphatidic acid–phosphatidylcholine	1.0142

* Interpolated.

DISCUSSION

(*1*) *The problem of heterogeneity*

Huang[2] showed that passage of sonicated phosphatidylcholine liposomes over a Sepharose 4B column pretreated with lipid separated the material into two fractions. Fraction I contained large liposomes passing over in the void volume, but Fraction II contained liposomes that were smaller and behaved as though they were homogeneous in the ultracentrifuge. When a sample of the 4% phosphatidic acid–phosphatidylcholine liposomes in 0.16 M KCl used in these experiments was passed over a 1.5 cm diameter 40 cm long Sepharose 4B column at 0 °C which had been pretreated with lipid a small shoulder appeared in the void volume, followed by a larger single peak. This peak was interpreted as being equivalent to Huang's Fraction II. However severe "tailing" of this main peak occurred indicating that the material was still binding to the Sepharose, so the column appears to be unsuitable for the purification of mixed lipid vesicles because of the possibility of differential phospholipid adsorption.

(2) The problem of hydration and the method of calculating r_e and δ

The difficulty here is the estimation of the thickness of the external layer of solution which may be sedimenting with the liposome. Values of r_s for 4% phosphatidic acid–phosphatidylcholine liposomes prepared in 0.16 M KCl and NaCl are available. They are 120 ± 4 Å and 115 ± 7 Å where $\pm$ represents the 95% confidence limits[3]. By substituting these values in Eqn 9, together with the appropriate sedimentation coefficients, densities and viscosities from Tables II, IIIa and IIIb. λ was found to be 0.649 and 0.635 for the KCl and NaCl liposomes, respectively. Values of λ_i are given in Table I, and are 0.705 and 0.745.

$\lambda_c = [(1-\lambda_i)/\lambda_i] \times \lambda = 0.272$ and 0.217, and the volume proportion of the sedimenting liposome which is external water of hydration is 0.079 and 0.148. r_e was found from Eqns 11 and 10. The values of r_e are given in Table II, h is between 0–6 Å for the KCl liposomes and 0–12 Å for the NaCl liposomes. In the absence of further evidence an arbitrary value of 3 Å was taken for h in all other liposomes.

For liposomes for which r_s was not available an approximate value of r_e was calculated by substituting values of λ_i rather than λ in Eqn 9. A better value of λ was found by putting $r_s = r_e + 3$ in Eqn 11. Finally a more accurate r_e was obtained by substituting the λ from Eqn 11 into Eqn 9. r_c was found from Eqn 12 and δ from Eqn 13.

A variation of ± 3 Å in h gives an uncertainty of $\pm 1\%$ in δ and r_c. λ_i should be accurate to $\pm 2\%$. The most serious source of experimental error lies in the determination of $(\rho_l - \rho_a)$ and is estimated at $\pm 5\%$. The experimental errors and uncertainty in h leave an error of about $\pm 4\%$ in r_e, $\pm 5\%$ in r_c and $\pm 6\%$ in δ. The error in δ increases to $\pm 7\%$ for the larger liposomes (depending on whether r_c is 2 or 3 times δ). The uncertainty in δ is ± 2 Å, except for the 40 and 50% cholesterol liposomes where it is ± 3 Å. Liposome areas, calculated essentially from r_e^2, are accurate to $\pm 8\%$ and functions calculated from liposome volumes such as bilayer weight and the number of molecules per liposome to $\pm 12\%$.

Liposome shape

Johnson *et al.*[9] reported that the external radius r_e in Å for 4% phosphatidic acid–phosphatidylcholine liposomes in 0.16 M KCl was $115 \leqslant r_e \leqslant 132$, calculating the limits for a simultaneous $\pm 5\%$ variation in v_c, liposome external area, and δ the bilayer thickness. For 1 μmole $v_c = 3.04 \cdot 10^{-4}$ cm^3 and the surface area = 2830 cm^2. Using electron-micrographic observations it was calculated that $\delta = 44$ Å. The value of r_e was compared with the Stokes' radius of 120 ± 4 Å. As the two values of r agreed the liposomes were believed to be spherical.

Huang[2] concluded that his phosphatidylcholine liposomes were spherical from a freeze-etched electronmicrograph, and Chapman *et al.*[10] reported that freeze-etched sonicated samples of phosphatidylcholine vesicles below 800 Å diameter were spherical although larger ones were elongated. Attwood and Saunders[11] reported in 1965 that sonicated phosphatidylcholine liposomes had an axial ratio of approximately 2, but they based their results on light scattering and viscosity measurements from a heterogeneous preparation, and treated the particles as solid micelles containing only 0.16 g/g water of hydration. For phosphatidylcholine we obtain 0.3 g/g associated water, most of which is trapped passively inside, and suggest that a confusion between asymmetry and 'hydration' is the source of the discrepancy.

Kornberg and McConnell[8] also concluded that their phosphatidylcholine vesicles were asymmetric as they obtained a value of only $1 \cdot 10^{-4}$ cm^3/μmole for the disaccharide trapped volume. However they purified their sample over Sepharose 4B which is a lengthy procedure. As sucrose is about 18 times as permeable as potassium[3] some of the disaccharide may have been lost during the process, particularly in view of the adsorption of phospholipid by the Sepharose[2] which may well perturb the liposome structure.

We therefore believe that the phosphatidylcholine and 4% phosphatidic acid–phosphatidylcholine liposomes are spherical, and in the absence of contrary evidence assume that the liposomes containing varying proportions of cholesterol are spherical also.

The effect of charge on liposomes

In general it can be seen from Tables I and II that charge increases the liposome radius. It is interesting to note that the 4% phosphatidic acid–phosphatidylcholine liposomes in 2 M KCl have nearly the same value of r_e as the uncharged phosphatidylcholine liposomes in 0.16 M KCl, so the strong salt solution must effectively screen the charge. From figures in Table II it is possible to calculate that for singly ionised phosphatidic acid molecules the ($v_{c\ \text{cation}}/v_{c\ \text{sugar}}$) ratio should be 1.24 for the 4% phosphatidic acid–phosphatidylcholine, 1.76 for the 33% phosphatidic acid–phosphatidylcholine and 1.83 for the 29% cholesterol–33% phosphatidic acid–phosphatidylcholine. The experimental values are given in Table I and are appreciably lower, indicating suppression of ionization of the phosphatidic acid. The proportion of molecules on the inside of the bilayer was found from the ratio internal area/(internal + external area). The total number of phospholipid molecules in a liposome is given in Table II, from this it can be calculated that there are 46 molecules of phosphatidic acid inside a 4% phosphatidic acid–phosphatidylcholine liposome. As there are only 191 molecules of any 0.16 M solute the degree of ionisation of the phosphatidic acid must be determined by itself alone. It might reasonably be expected that the degree of ionisation of the phosphatidic acid would depend on the pH of the last washing solution used in its extraction, in this case approximately 7.0, at which it would be at least singly ionised. However, the results show that this is not so. The suppression of ionization may be due to the small radius of curvature of liposomes and the binding of phosphatidic acid in the surface.

Charged phospholipid multilamellar liposomes are more easily broken down to small vesicles by the sonicator. The 4% phosphatidic acid–phosphatidylcholine phospholipid mixture was used rather than pure phosphatidylcholine as phosphatidylcholine cholesterol liposomes are particularly difficult to disrupt.

Difference between sodium and potassium

The 4% phosphatidic acid–phosphatidylcholine vesicles in 0.16 M salt were marginally smaller in NaCl than in KCl. There are three pieces of evidence for this statement. Table I shows that the trapped volume per μmole lipid inside a 4% phosphatidic acid–phosphatidylcholine liposome in 0.16 M NaCl is smaller than that inside a 0.16 M KCl liposome. The difference is significant at a 95% confidence level. If corrections are made for the density and viscosity of the media, liposomes prepared in 0.16 M NaCl sediment less fast than those in 0.16 M KCl. If the s° for the liposomes

in NaCl is adjusted to KCl it becomes $1.44 \cdot 10^{-13}$ s which is significantly less than the $1.60 \pm 0.01 \cdot 10^{-13}$ s for the liposomes prepared in KCl.

The diffusion coefficient of the liposomes prepared in NaCl is marginally greater than that for the liposomes prepared in 0.16 M KCl but this difference is only significant at the 95% level if a one-sided *t*-test is used.

The effect of cholesterol on liposomes

λ_i and s° were measured on the same preparation of liposomes, and values of r_e, r_c and δ calculated from them. Fig. 4 shows the variation in δ and r_c as a function of molar liposome composition, and Table II gives values for the surface area per phosphorus molecular weight and per phospholipid molecule, taking the surface area of a cholesterol molecule as 35.5 Å^2. Fig. 4 also includes results calculated from two other preparations of 50% cholesterol liposomes, in this case the value of λ_i used was the mean value given in Table I.

NMR and ESR studies have shown a "stiffening" of the hydrocarbon chains of phosphatidylcholine in the presence of cholesterol, Levine[12]. This is reflected by the increasing values of r_e and r_c, the radii of curvature of the sonicated vesicles.

The most surprising feature of the results is the sudden increase in the surface area per phospholipid molecule when cholesterol is added. Furthermore the function

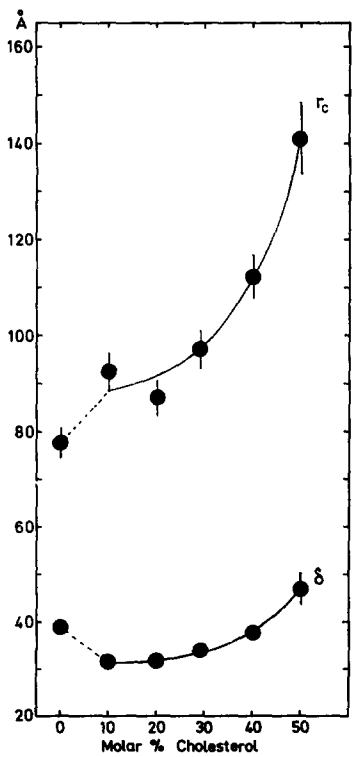

Fig. 4. The internal radius of curvature of the liposomes r_c and the bilayer thickness δ as a function of the molar cholesterol ratio. The functions are discontinuous between 0–10% cholesterol.

$$\frac{\text{total bilayer area}}{\text{number of phospholipid molecules}}$$

remains constant at 89 Å within the $\pm 4\%$ experimental error and does not increase as expected however much cholesterol is added.

It is suggested that the cause of this surface expansion of the phospholipid molecules in the presence of cholesterol is a reorientation of the phospholipid head groups which then require access to a larger area of water to become fully hydrated. When the proportion of cholesterol is low they can only do this by tilting the rest of the molecule sideways, so decreasing δ. However, as more cholesterol is put into the bilayer the heads of the phospholipid molecules can obtain their water of hydration by overlapping the cholesterol, and their hydrocarbon chains straighten out behind, thereby increasing δ.

The sudden decrease of δ with 10% cholesterol is not consistent with the X-ray phosphatidylcholine myelin results of Lecuyer and Dervichian[13], who found that the thickness of the hydrocarbon region alone increased linearly from 29 to 33 Å at 35% cholesterol and then stayed constant. However, their figures are for a maximum of only 40% w/w water, and their low value of 64 $Å^2$ for the surface area of a phosphatidylcholine molecule suggests that their mixture was not fully hydrated. The head group rearrangement may be prevented by lack of water.

The 37 ± 2 Å bilayer thickness of the phosphatidylcholine liposomes agrees with that of 38 Å obtained by Reiss-Husson[14] from X-ray diffraction studies on phosphatidylcholine multilamellar liposomes at 25 °C. It is also consistent with the value Wilkins *et al.*[1] calculated from the X-ray scattering of single bilayer phosphatidylcholine vesicles. They measured δ as 35 Å between the centres rather than the edges of the phosphate head groups on either side of the bilayer. The 72 $Å^2$ surface area per phosphatidylcholine molecule given by Small[15] and Reiss-Husson[14] from X-ray studies on fully hydrated phosphatidylcholine multilamellar liposomes can be compared with the value of 71 $Å^2$ quoted in Table II. There seems to be no detectable difference in the thickness of a phosphatidylcholine bilayer whether in a large multilamellar liposome or a 102 Å radius vesicle.

It appears that the phospholipid:cholesterol ratio is more important in determining membrane thickness than the nature of the phospholipid head groups. The value of $\delta = 40$ obtained by Gulik-Krzywicki *et al.*[16] for liposomes made of beef heart mitochondrial lipids, which contain only 2% cholesterol, can be compared to the 39 ± 2 Å thickness of the 4% phosphatidic acid–phosphatidylcholine liposomes. Again the thickness of the 50% cholesterol liposomes, 47 ± 3 Å, is similar to the thickness of the bilayer region of erythrocyte ghosts and nerve terminal membranes which also have large amounts of cholesterol. Wilkins *et al.*[1] quote values of 45 and 50 Å, respectively.

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