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THE OSMOTIC INSENSITIVITY OF SONICATED LIPOSOMES AND THE DENSITY OF PHOSPHOLIPID–CHOLESTEROL MIXTURES

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SUMMARY

The cation permeability and size of sonicated phospholipid vesicles were found to be relatively resistant to osmotic strain. The density of 4% phosphatidic acid–96% phosphatidylcholine vesicles was found to be almost unaffected by the presence of cholesterol up to a molar ratio of 1:1, the mean value was 1.014. A possible water structure effect was detected in the difference in sign between the dependence of diffusion coefficient on lipid concentration in 0.16 M KCl and 0.16 M NaCl.

Huang[1] and Johnson *et al.*[2,3] have described the preparation of about 23-nm diameter single bilayer phospholipid vesicles. Johnson[4] has shown that when cholesterol and charged phospholipids are present these vesicles approach the size of the 50-nm nerve storage vesicles for acetylcholine and noradrenaline, Smith[5]. One interesting feature of these very small vesicles is their osmotic insensitivity, a property which is described in this paper.

Huang and Charlton[6] used a homogeneous preparation of phosphatidylcholine vesicles in an attempt to find a very accurate value for the partial specific volume of phosphatidylcholine. They obtained two values for the partial specific volume, depending on whether the measurement was done in aqueous solution or in increasingly concentrated salt solutions. However, Huang and Charlton calculated their results assuming that their liposomes were permeable to salt; but we have found that ours are not. We have therefore used a modified version of the Huang and Charlton method to remeasure the density of phospholipid vesicles, and to study the effect of increasing proportions of cholesterol.

MATERIALS

Phosphatidylcholine was extracted from egg yolk and phosphatidic acid prepared from it by enzymic hydrolysis according to Papahadjopoulos and Miller[7]. The pH of the last Folch wash in the preparation of the phosphatidic acid was approx. 7.0. The phospholipid mixture used contained 4% phosphatidic acid and 96% phosphatidylcholine, and is described in the text as 4% phosphatidic acid–phosphatidylcholine. The cholesterol was Sigma's chromatographic grade. All other reagents were analytical grade and the salts used to prepare solutions for the ultra-

centrifuge were dried before use. The radiochemicals came from the Radiochemical Centre, Amersham, England. Water was twice distilled, the second time from $KMnO_4$ in borosilicate glass apparatus. The ^2H_2O atomic purity 99.7%, came from Koch Light.

METHODS

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 swell and form liposomes under nitrogen. The liposomes were transferred to a 1.3 cm diameter flat-bottomed glass vial, and sonicated until the suspension was optically clear (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 ultrasonic disintegrator E 759 DB at 10–25 kcycles/s. The sonicators were cooled by running water at 15 °C. The unopened vials were left to stand overnight. The ultraviolet spectrum of the phospholipids was checked before and after sonication, as described by Klein[8], and no chemical deterioration was noted. The liposomes were not further purified, and were believed to consist of a homogeneous fraction similar to Huang's Fraction II, contaminated with about 14% of slightly larger vesicles. Details are given in Johnson.

Osmotic sensitivity measurements

Liposomes were prepared in 0.1475 M ^{42}KCl and separated after sonication from the untrapped isotope by passage over a 30 cm column of G-50 (coarse) Sephadex, 3 g anhydrous weight, equilibrated in 0.1475 M KCl. They were diluted with an equal volume of 0.1475 M KCl (controls) or water. 1 ml portions were placed in 8/32 Visking dialysis bags and dialysed either against distilled water or against 10 ml KCl at the same molar concentration as inside the bag. At the end of the experiment the dialysis bags were recovered, cut open into 9 ml KCl, and counted in a Packard scintillation counter by the Cerenkov radiation from the ^{42}K. Corrections for isotope decay were necessary. The isotope outside the dialysis bag which was lost from the liposomes during the experiment was also counted, and calculated as a % loss of total counts trapped.

Measurement of sedimentation and diffusion coefficients

Sedimentation and diffusion coefficients were measured at 20.0 ± 0.1 °C using a Beckman Model E analytical ultracentrifuge fitted with schlieren optics. Diffusion coefficients were measured in a valve type synthetic boundary cell at a rotor speed of 10000 rev./min about $7000 \times g$. D was calculated from the gradient of the linear plot of μ^2 against t, where t was the time from the formation of the boundary and μ one half the distance between the two inflexion points on the schlieren photograph of the diffusing liposomes. See Kabat and Mayer[9]. At this low rotor speed the baseline was horizontal. The schlieren curve remained Gaussian throughout the diffusion run.

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 $300000 \times g$. The sedimentation coefficient s was calculated from a plot of $\log x$ against t, where x was the distance of the liposome boundary from the axis of rotation at time t. All these plots were linear. s^0 was found by extrapolation to zero concentration. Further details of the measurement of s^0 are given in Johnson[4].

Determination of phospholipid density

The $^2H_2O/H_2O$ method used was based on that recommended by Huang and Charlton[6].

Two solutions were prepared. One was 0.16 molal KCl in H_2O, the other 0.16 molal KCl in 16.7570 g 99.7% ^2H_2O+34.7884 g H_2O. The density of this solution at 20 °C was calculated to be 1.0382 and was measured as 1.0379 ± 0.0002 (S.D.) using a pycnometer. The density of the 0.16 molal KCl solution was 1.0058. A 4% suspension of mixed phospholipid (with or without cholesterol) in 0.16 molal KCl in H_2O was sonicated to clearness as described previously. It was then diluted to 0.5 ml. 0.1 and 0.03 ml portions were added to three pairs of tubes containing the appropriate KCl $H_2O/^2H_2O$ mixtures to give final solvent densities of 1.0058, 1.0220 and 1.0340, respectively. The different density mixtures were prepared by weighing out the appropriate amounts of KCl H_2O and KCl $H_2O/^2H_2O$ solutions, and the s^0 values of the phospholipid in the three density mixtures were measured as described previously.

The viscosity values of the $^2H_2O/H_2O$ mixtures were calculated from data obtained by Lee in Huang and Charlton[6] making due allowance for the presence of KCl. A graph of ηs^0 against the density of solvent was plotted, see Fig. 1. When $\eta s^0 = 0$, the density of the phospholipid was equal to the density of the solvent.

RESULTS

Diffusion coefficients

Fig. 2 shows a plot of the diffusion coefficients of 4% phosphatidic acid–phosphatidylcholine liposomes prepared in 0.16 M KCl or 0.16 M NaCl as a function of liposome concentration. The equations of the two regression lines are given below:

$$D_{20,w} = 1.76 \cdot 10^{-7} \ (1 + 0.064\, c_1) \ \text{(0.16 M KCl)}$$

$$D_{20,w} = 1.86 \cdot 10^{-7} \ (1 - 0.063\, c_1) \ \text{(0.16 M NaCl)}$$

The diffusion coefficient $D_{20,w}$ is in $cm^2 \cdot s^{-1}$. c_1 is the phospholipid concentration in g/100 ml. One liposome preparation in 0.16 M KCl gave points at $c_1 = 1.57$ and 0.52, a second preparation giving the double point at $c_1 = 1.00$. A single preparation of liposomes in 0.16 M NaCl had an s^0 of $1.60 \cdot 10^{-13}$ s, and gave the diffusion coefficients shown on the graph, but points marked Δ at $c_1 = 1.14$ and 0.825 were obtained when liposomes from this preparation were suspended in 0.11 M and 0.08 M NaCl. These latter two samples were diluted with the appropriate amount of water some hours previous to the diffusion experiment, subjecting the liposomes to an osmotic shock. However, this apparently had no effect on their size or shape since these two points still lie on the line through the other three.

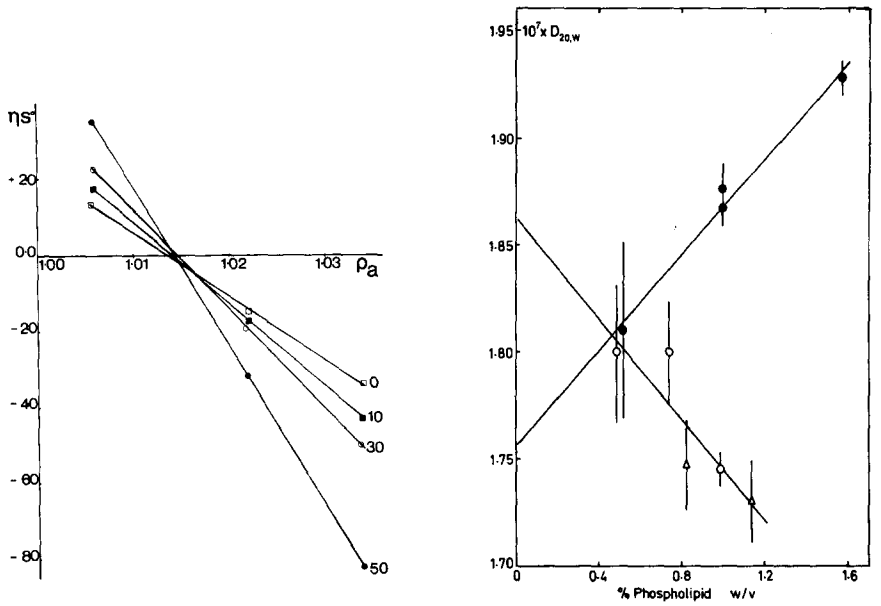

Fig. 1. $\eta s^0 \times 10^{15}$ P·s for 4% phosphatidic acid–phosphatidylcholine liposomes with varying molar proportions of cholesterol sedimenting through 0.16 molal KCl solutions in H_2O or $H_2O/{}^2H_2O$, density ρ_a g·cm^{-3}. The intercept values on the ρ_a axis give the liposome membrane density and are listed in Table (II). □—□, no cholesterol; ■—■, 10% cholesterol; ○—○, 30% cholesterol; ●—●, 50% cholesterol.

Fig. 2. Diffusion coefficients of 4% phosphatidic acid–phosphatidylcholine liposomes prepared in 0.16 M KCl or 0.16 M NaCl as a function of liposome concentration. ●, 0.16 M KCl liposomes diffusing in 0.16 M KCl; ○, 0.16 M NaCl liposomes diffusing in 0.16 M NaCl; △, 0.16 M NaCl liposomes diffusing in hypotonic NaCl. The error bars represent the standard error of the gradient of the plot from which D was calculated.

Resistance to osmotic lysis

Table I shows the results of the experiments to detect osmotic lysis. If there

TABLE I

LOSS OF ISOTOPE FROM HIGHLY SONICATED LIPOSOMES PREPARED IN 0.148 M KCl AND SUBJECT TO OSMOTIC SHOCK

Phosphatidic acid–phosphatidylcholine refers to the standard phospholipid mixture consisting of phosphatidylcholine with 4% phosphatidic acid.

Liposome composition	*Dialysis solutions outside*	*Temperature of dialysis*	*Time of dialysis (h)*	*% loss radioactivity*
Phosphatidylcholine	0.074 M KCl	21 } 4 }	6 } 12 }	2.5
50% Cholesterol phosphatidic acid–phosphatidylcholine	0.074 M KCl	21 } 4 }	6 } 12 }	3
Phosphatidic acid–phosphatidyl-choline	0.074 M KCl	25	18	6
Phosphatidic acid–phosphatidyl-choline	0.148 M KCl	25	18	5
Phosphatidic acid–phosphatidyl-choline	H_2O (2 × 1 l)	4	18	11

is no osmotic strain on the liposomes the loss of ^{42}K at 4 °C is almost impossible to detect, and well under 1% in 18 h.

Density measurements, results and discussion

The intercepts on the axis $\eta s^0 = 0$ which give the densities of the phospholipid mixtures are tabulated below, Table II. The errors are estimated from weighing errors in the preparation of the salt solutions of known density through which the liposomes sedimented. Errors in the determination of s^0 are of the order of 1% and are less important. It will be seen that cholesterol has remarkably little effect on phospholipid bilayer density, which can be taken as 1.014 for all the mixtures.

TABLE II

THE DENSITIES OF PHOSPHOLIPID-CHOLESTEROL MIXTURES AT 20 °C

Phospholipid	*Density g·cm^{-3}*
Phosphatidic acid–phosphatidylcholine	1.0135 ± 0.0003
10% Cholesterol phosphatidic acid–phosphatidylcholine	1.0138
30% Cholesterol phosphatidic acid–phosphatidylcholine	1.0145
50% Cholesterol phosphatidic acid–phosphatidylcholine	1.0142

Huang and Charlton[6] found a value of 1.0190 ± 0.0004 for the density of phosphatidylcholine vesicles prepared in 0.1 M NaCl (or KCl) and sedimented through $^2H_2O/H_2O$, and a value of 1.0118 ± 0.0002 when the vesicles were sedimented through increasing concentrations of KCl. The former is higher presumably due to the failure of the salt inside the liposomes to escape, and the latter low because the external salt could not penetrate the internal volume. Klein, R. (personal communication) found a value of 1.0137 ± 0.0005 for phosphatidylcholine vesicles sedimented for 3 days in a sucrose gradient at 20 °C. Since the rate of exchange of sucrose is approx. 5% per h (Johnson, S. M., unpublished) the figure should represent an equilibrium value.

Discussion of diffusion coefficients and osmotic sensitivity

The Stokes–Einstein diffusion equation predicts that at infinite dilution the diffusion coefficient

$$D^0 = \frac{kT}{6\pi\eta r_s} \tag{1}$$

where k is Boltzmann's constant, T the absolute temperature, η the viscosity of the solvent and r_s the Stokes' radius of the diffusing particle. r_s is a function of both the shape and size of the diffusing molecule. Freeze-etch photographs of highly sonicated liposomes suggest that they are spherical, Chapman *et al.*[10], Huang[1]. If this is so r_s becomes the geometric radius of the diffusing particle, and can be calculated from Eqn 1. For the liposomes in 0.16 M KCl $r_s = 120 \pm 4$ Å and for those in 0.16 M NaCl $r_s = 115 \pm 7$ Å, where ± indicates the 95% confidence limits. These values are comparable to that obtained by Huang[1] for his purified phosphatidylcholine liposomes in 0.1 M NaCl, $r_s = 114 \pm 5$ Å.

The difference in slope between the NaCl and KCl plots in Fig. 2 is striking, and it would be interesting to know the meaning of this on a molecular level since the two solutions approximate to the biological inter- and intra-cellular fluids.

A diffusing liposome appears to be slowed down by the presence of other liposomes in 0.16 M NaCl, but accelerated to the same degree by their presence in 0.16 M KCl. From the equation and phospholipid surface area figure in Johnson *et al.*[3] the 4% phosphatidic acid liposomes might be expected to have a surface charge of −9.75 mV at pH 6.0. This would induce a double layer of Na^+ or K^+ counter ions with a "thickness", $1/\varkappa$, of 8 Å. The surface potential and double layer is too small to produce a measurable electrical retardation in D, but the excess Na^+ or K^+ should affect the viscosity of the solvent in the region of the liposomes, Na^+ producing a net increase in water structure thereby raising the viscosity, and K^+ producing a net decrease in water structure and a decrease in viscosity. Figures taken from the 1st edition of the International Critical Tables[11] for 0.16 M bulk solutions at 20 °C show that the viscosity of water is increased by 0.13% for 0.16 M NaCl and decreased by 0.13% for 0.16 M KCl. However, the matter requires further quantitative investigation.

Osmotic insensitivity

The trapped volume of a 4% phosphatidic acid–phosphatidylcholine liposome in 0.16 M NaCl is 0.217 of the total liposome volume, Johnson[4]. If a 2-fold dilution of the salt solution produced a doubling of the internal volume, the diffusion coefficient should decrease by 7%. Fig. 2 excludes this possibility. On the other hand, if a momentary increase in internal volume produced a temporary lysis and resealing of the membrane, escape of trapped ^{42}K should be shown. For a 2-fold dilution this possibility is excluded by the figures in Table I. There is a small loss of ^{42}K only in the case of liposomes dialysed against distilled water. Two explanations for this apparent lack of osmotic sensitivity are considered. (1) The liposomes do not contain osmotically active salt or water, the "trapped volumes" representing adsorbed salt or water of hydration. (2) The excess osmotic pressure induced inside the liposomes by a dilution of the solution outside is not sufficient to overcome the tension in the phospholipid bilayer.

Bangham *et al.*[12] showed that the relatively large multilayered liposomes which are normally ellipsoidal will swell or shrink with variation in osmotic pressure. They found that the extrapolated limiting volume occupied by phosphatidylcholine under high osmotic pressure was $1.215 \cdot 10^{-3}$ $cm^3/\mu mole$ lipid. Using a mol. wt of 780 for phosphatidylcholine (Johnson[4]) and a density of 1.014 gives a value of 740 $Å^3$ of water per phospholipid molecule. As the surface area of liposome occupied by a phospholipid molecule is 66 Å (ref. 3), the thickness of the osmotically inactive water layer should be 11 Å. The thickness of the bilayer is 40 Å (Johnson[4]). This should leave a sphere in the centre of the liposome radius 58 Å, containing osmotically active water. Johnson[4] showed that the trapped cation: sugar volume ratio was 1.0 within an experimental error of ±10%, which does not support an adsorption theory as it is most unlikely that a cation and a sugar should be equally adsorbed.

The second possibility seems more consistent with the experimental evidence. Under normal circumstances the surface tension of a planar phospholipid bilayer is virtually zero, Haydon and Taylor[13]. The small radius of curvature of the vesicles

must put some strain on the almost cylindrical phospholipid molecules, therefore inducing a finite surface tension. The excess pressure P on the concave side of a curved surface is given by the relation $P=(2\gamma/r)$, where γ is the surface tension and r the radius of curvature. As the liposome has two surfaces the excess pressure inside will be $P=2[(\gamma_i/r_i)+(\gamma_e/r_e)]$ where γ_i, r_i, γ_e and r_e are the surface tension and radius of curvature of the internal and external surfaces. When the salt outside the liposomes is diluted, the excess osmotic pressure inside the liposomes should cause the liposomes to swell. However, if the interior volume of the liposome increases both surface areas must also increase, thereby partially exposing the hydrocarbon bilayer core, and enormously increasing the surface tension. Lack of information on the value of the original surface tension makes calculation difficult, but the small spherical bilayer should have an appreciable tensile strength which would antagonise the osmotic swelling. This hypothesis is consistent with a possible increased rate of loss of isotope from a liposome subject to osmotic strain rather than a sudden lysis resulting in almost total isotope loss.

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