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Morphology of Egg Phosphatidylcholine-Cholesterol Single-Bilayer Vesicles, Studied by Freeze-Etch Electron Microscopy

A. Forge*, P.F. Knowles, and D. Marsh**

Astbury Department of Biophysics, University of Leeds, Leeds, England, and Max-Planck-Institut für biophysikalische Chemie, 34 Göttingen, Germany

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Summary. Homogeneous, small, single-bilayer vesicles were prepared from egg phosphatidylcholine with various concentrations of cholesterol by ultrasonic dispersion in 0.1 M KCl, 0.01 M Tris, pH 8.0, buffer, followed by gel chromatography. The shape and size distributions of the fractionated vesicles were investigated for preparations with cholesterol compositions from 0 to 50 moles/100 moles, using freeze-etch electron microscopy. The size distribution was estimated from the shadow width of vesicles which were exposed by etching and the vesicle shape was checked by comparing the images obtained by tilting the replicas. The widths of the vesicle diameter distributions were relatively broad, corresponding to standard deviations in the range 60–90 Å, but showing no systematic variation with cholesterol composition. In all cases it was found that 70 % of the vesicle diameters lay within 150 Å of the modal value. The apparent vesicle diameters remained constant for cholesterol compositions up to 20 moles/100 moles (modal diameter = 330 ± 20 Å, mean diameter = 350 ± 3 Å), but there was a sharp net increase in diameter at 30 moles cholesterol/100 moles reaching a model diameter of 430 ± 20 Å (mean diameter = 430 ± 3 Å) at 50 moles cholesterol/100 moles. Using the tilted microscope stage it was found that all vesicles were spherical at all cholesterol compositions studied, including those above 30 moles cholesterol/100 moles. The molecular mechanism by which cholesterol controls the vesicle size is discussed in terms of the asymmetric distribution of cholesterol across the vesicle bilayer.

Cholesterol is a major component of most mamallian cell plasma membranes, whereas it is either absent or present at considerably lower levels in the membranes of most intracellular organelles, except those thought possibly to be derived from the plasma membrane. One of the suggested roles of cholesterol is the stabilization of the plasma membrane against large fluidity changes (Ladbrooke, Williams & Chapman, 1968; Schreier-Muccillo *et al.*, 1973), and the molecular basis of this fluidity-

* *Present Address:* Department of Pathology, Institute Laryngology and Otology, 330/332 Grays Inn Road, London, U.K.

** *To whom reprint requests should be addressed at:* Max-Planck-Institut für biophysikalische Chemie, D-3400 Göttingen-Nikolausberg, Postfach 968, G.F.R.

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regulating mechanism has been studied in some detail (Schreier-Muccillo *et al.*, 1973; Marsh & Smith, 1973; Marsh, 1974). Since the plasma membrane is the limiting structure which defines the cell, a further possible role for cholesterol could be in controlling the cell membrane morphology as modulated by the internal cytoskeleton.

In the present paper we have investigated the effect of cholesterol on the shape and size of sonicated, single-bilayer egg phosphatidylcholine-cholesterol vesicles using freeze-etch electron microscopy[1]. The small, single-bilayer vesicles are a useful model system for morphological studies since they can be obtained as a relatively homogenous population using gel chromatography (Huang, 1969). Many of the techniques used to define the nature of such vesicle preparations (for example: analytical ultracentrifugation, analytical gel chromatography or light scattering) average over an assembly of vesicles. Freeze-etch electron microscopy has the advantage that it allows direct measurement of the size of individual vesicles and of the heterogeneity of the population. It is also one of the most direct and reliable means of determining vesicle shape.

The results of these studies suggest that cholesterol is capable of regulating or controlling lipid membrane morphology. It is found that, at above 30 moles/100 moles composition, addition of cholesterol causes the vesicles to increase in size whereas the vesicle shape and heterogeneity remains constant. In addition these studies constitute a valuable characterization of the vesicle preparations, which will be of use in other biophysical investigations (*see*, e.g., de Kruijff, Cullis & Radda, 1975).

Materials and Methods

Sonicated Egg Phosphatidylcholine-Cholesterol Vesicles

Approximately 150 mg of egg phosphatidylcholine, together with the appropriate amount of cholesterol, were dissolved in 10 ml benzene and freeze-dried. The freeze-dried material was suspended in 10 ml Tris/HCl buffer (0.1 M NaCl, 0.01 M Tris, pH 8.0) and sonicated under argon at 4 °C as described by Huang and Charlton (1972). Following centrifugation (105,000 × g for 30 min), the supernatant was concentrated by vacuum dialysis (Sartorius-Membranefilters, Göttingen, Germany) and chromatographed on Sepharose 4B (Pharmacia, Sweden) as described by Huang and Charlton (1972), except that the column eluant was monitored at 257 nm. In all cases good resolution of the vesicle peak from the

1 Negative staining would not be appropriate for such a study since this preparative procedure causes the vesicles to collapse to flattened discs (Marsh *et al.*, 1972). Thus the information on vesicle shape is lost and dimensional measurements, in particular the changes with cholesterol, may bear no direct relation to vesicle size in solution.

void-volume peak was obtained, and more prolonged sonication essentially made no difference to the chromatographic properties of the vesicles. The fractions in the vesicle peak were combined, concentrated by vacuum dialysis, and rechromatographed on Sepharose 4B. A single vesicle peak was then obtained with no void volume peak. All samples for the electron microscopic examination were taken and frozen at once from the fractions eluting immediately after the peak of the rechromatographed material (*cf.* Huang, 1969).

Freeze-Etching

Samples were placed on ridged copper or cardboard support discs and rapidly frozen in liquid Freon 22. Freeze-etching was performed in an NGN 680 apparatus under a vacuum of 10^{-5} torr. Specimens were fractured at $-100\,°C$ and the fractured surfaces etched by sublimation for 30 sec. Replicas were floated free on distilled water and cleaned in chloroform, acetone, 50% aqueous acetone and water. Photomicrographs of freeze-etch replicas have been photographically reversed so that shadows appear black.

Electron Microscopy

Grids were examined on a Phillips EM 200 electron microscope operating at 60 or 80 kV. Experiments to examine the shape of the vesicles were performed with a tilted goniometer stage. Magnification was calibrated using a carbon replica of a diffraction grating with 28,800 lines/inch and with polystyrene spheres of 0.109 μm diameter (both from E. Fullam, Inc., Schenectady, N.Y.).

Measurements

Measurements on the freeze-etch replicas were made from projected negatives at 20× magnification. The vesicle diameters were measured from the shadow widths. Only vesicles with convex fracture faces were measured, so that the true diameter and not a sub-equatorial section was exposed on etching.[2] For each sample at least 500 measurements were taken from at least two different replicas and sometimes from different preparations.

Results

Typical micrographs from refractionated vesicles with cholesterol contents of 0 to 50 moles/100 moles are given in Fig. 1. Vesicles are observed which have either been cleaved or (because of their relatively small size) are simply exposed by etching. Both convex and concave fracture planes are seen which reveal a continuous, organized hydrophobic region within the vesicle membrane. Only one fracture plane is ever observed per vesicle indicating a single bilayer structure. In all cases the vesicles appear to be

2 By measuring only vesicles which were clearly exposed (upon their ice pillar) by etching, the possibility of measuring super-equatorial sections was also eliminated.

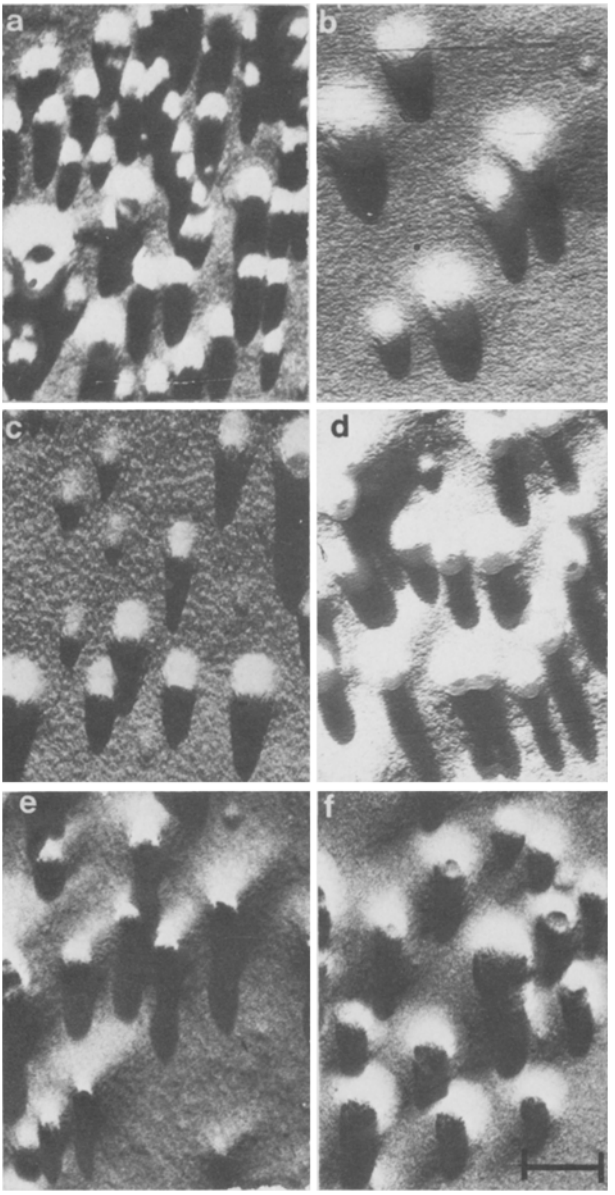

Fig. 1. Freeze-etch electron micrographs of egg phosphatidylcholine vesicle preparations with: (*a*) 0 mole cholesterol/100 moles, (*b*) 10 moles cholesterol/100 moles, (*c*) 20 moles cholesterol/100 moles, (*d*) 30 moles cholesterol/100 moles, (*e*) 40 moles cholesterol/100 moles, (*f*) 50 moles cholesterol/100 moles. Shadowing direction is from the top of the figure. Bar marker represents 0.1 μm

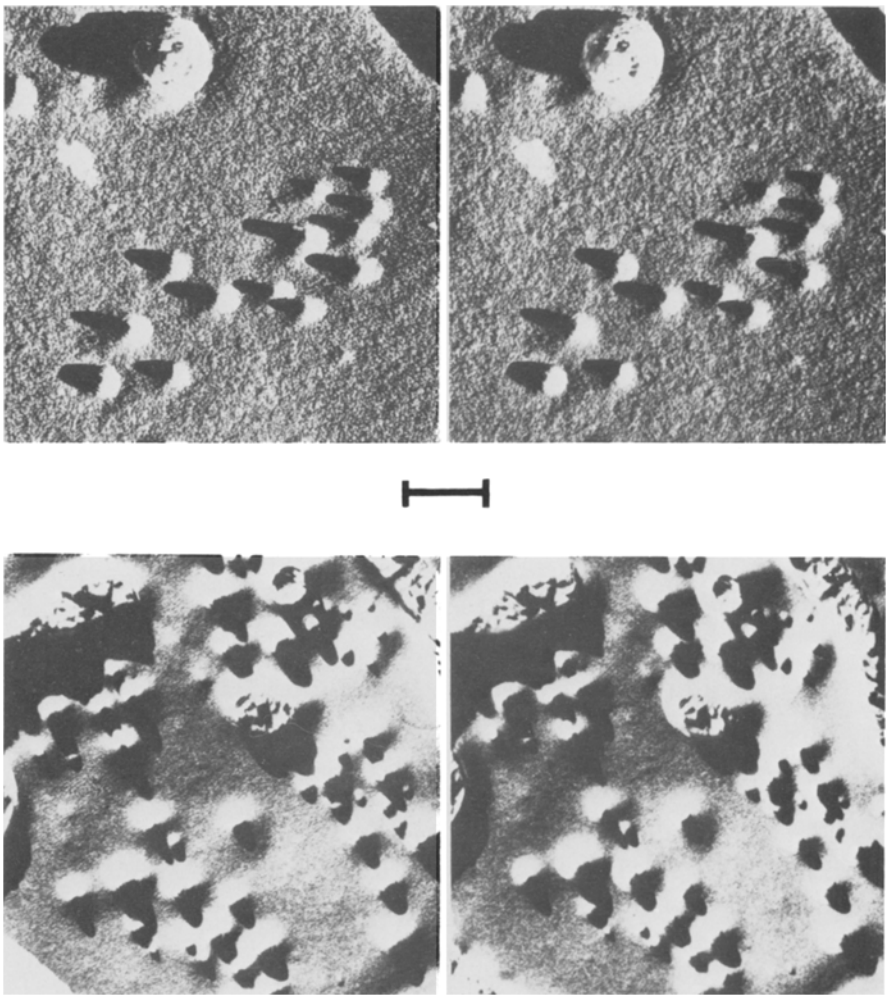

Fig. 2. Stereo-pair freeze-etch electron micrographs taken with the specimen at a tilt of $\pm 12°$. *Upper:* egg phosphatidylcholine +0 mole cholesterol/100 moles vesicles. *Lower:* egg phosphatidylcholine + 50 moles cholesterol/100 moles vesicles. Bar marker represents 0.2 μm

spherical in shape and this is confirmed in experiments made by tilting the specimen. Fig. 2*a* shows stereo-pair micrographs from replicas of egg phosphatidylcholine vesicles taken with the specimen tilted at $\pm 12°$ relative to the normal to the electron beam. The two micrographs appear identical, although corresponding to projections tilted by 24° relative to each other, clearly indicating that the vesicles are spherical. The vesicles remain spherical up to 50 moles/100 moles cholesterol content as is illustrated by the stereo-pair micrographs in Fig. 2*b*.

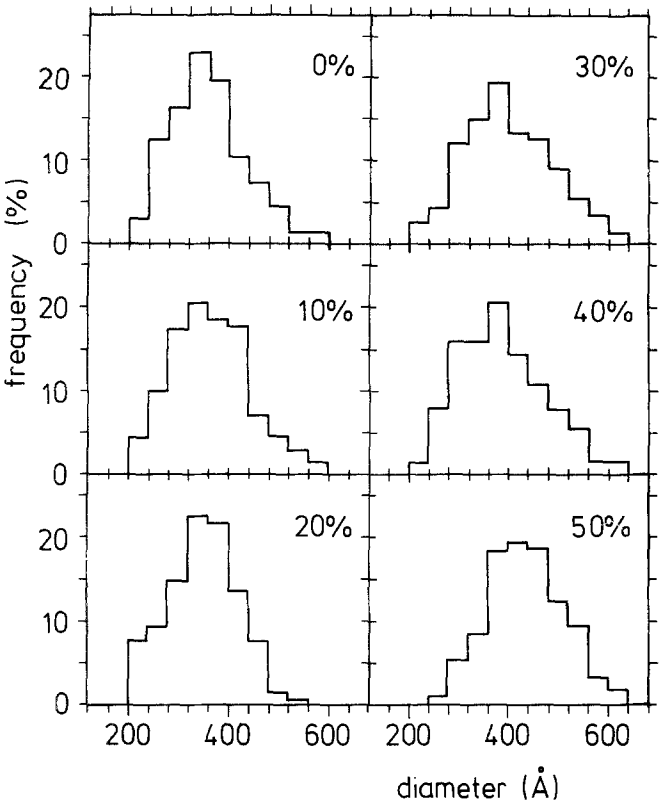

Fig. 3. Histograms of the diameter distributions of egg phosphatidylcholine vesicles. The percentage figures refer to the molar proportion of incorporated cholesterol. Each histogram summarizes more than 500 measurements

The electron microscope fields of view indicate that the vesicle populations are fairly homogeneous for all cholesterol contents up to 50 moles/100 moles. Histograms based on shadow diameter measurements are given in Fig. 3. Although all samples show a considerable range in vesicle size, approximately 70% of vesicles lie within 150 Å of the modal value at all cholesterol concentrations. Fig. 4 shows the variation in the modal vesicle diameter, the mean vesicle diameter and the standard deviation of the vesicle diameter distribution, as a function of vesicle cholesterol content. It should be emphasized that the vertical bars in Fig. 4*a* are not error bars, but the SD of the distributions. A sufficiently large number of vesicles was measured that the SEM is considerably smaller than this.

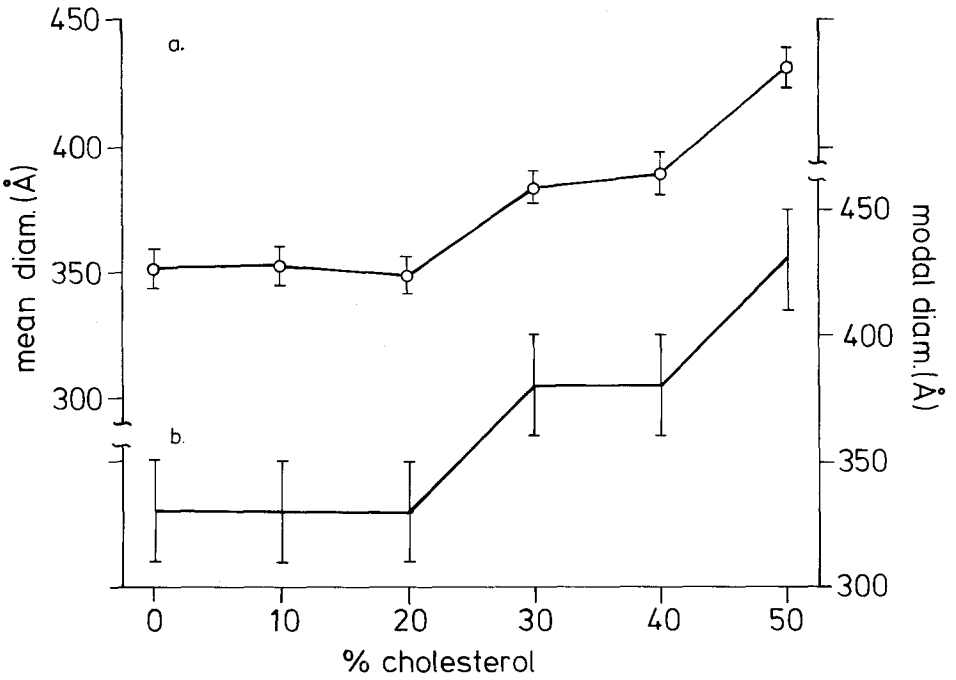

Fig. 4. Diameter of egg phosphatidylcholine vesicles as a function of molar percentage of incorporated cholesterol. (*a*) Mean diameters (SE = ±2.2–3.1 Å); vertical bars represent the SD of the diameter distribution (SE = ±1.6–2.2 Å). (*b*) Modal diameters; vertical bars represent the sampling interval

Discussion

The electron microscopy results clearly show that the phospholipid vesicles produced by the methods described here are spherical and composed of a single bilayer for all cholesterol concentrations studied. It is found that the vesicle diameters remain constant, independent of cholesterol concentration, up to 20–30 moles cholesterol/100 moles and then increase rapidly up to 50 moles cholesterol/100 moles. The value of 175 Å obtained for the mean radius of vesicles without cholesterol is considerably larger than the values obtained on similar preparations by analytical ultracentrifugation: 106 Å (Newman & Huang, 1975) and 125 Å (Huang, 1969). A similar discrepancy was found in a study of dimyristoyl phosphatidylcholine vesicles (Watts, Marsh & Knowles, 1978) and was attributed to the finite thickness of the replica and the formation of salt eutectic at the vesicle surface. Since these factors will remain constant for a given preparative procedure, they do not affect the intercomparison of

vesicle sizes within a given population[3], or between populations with different cholesterol content. Neither should they affect conclusions reached about the vesicle shape.

The present finding that the vesicles are spherical at all cholesterol concentrations up to 50 moles/100 moles is in contrast to the suggestion by Newman and Huang (1975) of vesicle asymmetry at cholesterol concentrations above 30 moles/100 moles. Their conclusions were based on the apparent discrepancies between the effective vesicle radii derived from independent viscosity and diffusion measurements. However, a detailed error analysis indicates agreement between their viscosity and diffusion data, to within experimental error, assuming the vesicles to be spherical. In addition, the Scheraga-Mandelkern β-function obtained by Newman and Huang (1975) for vesicles with 42 moles cholesterol/100 moles is fully consistent with the vesicles being spherical. Thus the present freeze-etch electron microscopy, taken together with the hydrodynamic data of Newman and Huang (1975), strongly suggests that the vesicles are spherical in solution, in the unfrozen state, at all cholesterol concentrations up to 50 moles/100 moles.

The finding that the vesicle radius remains approximately constant up to 20–30 moles cholesterol/100 moles and then increases rapidly up to 50 moles/100 moles has also been observed by gel chromatography (Gent & Prestegard, 1974) and by analytical ultracentrifugation (Newman & Huang, 1975; *see Appendix*) and also for vesicles containing phosphatidic acid (Johnson, 1973). It is thus clear that cholesterol is capable of exerting a controlling effect on the vesicle size. The molecular origin of this control lies in the condensing effect of cholesterol and in the asymmetric packing distribution of cholesterol in small, highly curved vesicles, as seen below.

The condensing effect, or reduction in lipid molecular area, by cholesterol is well known for both bilayers and monolayers of egg phosphatidylcholine (de Bernard, 1958; Shah & Shulman, 1967; Lecuyer & Dervichian, 1969; Marsh & Smith, 1973). It is clearly this condensation of molecular area which is responsible for the lack of increase in vesicle surface area in the low cholesterol regime, up to 30 moles/100 moles. The critical concentration of cholesterol, beyond which the vesicle surface area must increase, can be estimated from the maximal condensation observed in

3 Thus the width of the size distributions in Fig. 3 must be taken as a direct measure of the vesicle populations in solution. The vesicles obtained by rechromatography are highly homogeneous in the sense that their diameter distribution peaks very clearly about a well-defined mean diameter. However, their distributions do have a considerable width, even for vesicles with no cholesterol, as might be expected from the preparative procedure.

extended bilayers or monolayers (*c.f.* Huang *et al.*, 1974). The area per phospholipid molecule in egg phosphatidylcholine bilayers is 62.7 $Å^2$ in the absence of cholesterol and 46.7 $Å^2$ in the fully condensed state at 50 moles cholesterol/100 moles (Levine & Wilkins, 1971). The area/molecule for tightly packed cholesterol is 38 $Å^2$ (Demel, Geurts van Kessel & van Deenen, 1972). Thus the maximum mole ratio of cholesterol which can be incorporated by a fixed number of phosphatidylcholine molecules, without increase in vesicle size, is $16/38 = 0.42$, or 30 moles/100 moles cholesterol composition. Similarly in egg phosphatidylcholine monolayers, at a surface pressure of 20 dynes/cm, the area per phospholipid molecule is 72 $Å^2$ in the uncondensed state and 60 $Å^2$ in the cholesterol-condensed state (Shah & Shulman, 1967). Thus the maximum amount of cholesterol which can be incorporated without increase in surface area is 24 moles/100 moles. Both estimates agree rather well with the observed onset of increase in vesicle radius at 20–30 moles cholesterol/100 moles (*cf.* Fig. 4).

At cholesterol concentrations above the critical level, the observed increase in vesicle size cannot be accounted for solely by the increase in surface area arising from the additional cholesterol (*see Appendix*). It is shown in the Appendix that the explanation for the extremely rapid increase in vesicle size, above 20–30 moles cholesterol/100 moles, lies in the asymmetric packing of the cholesterol molecules between the outer and inner monolayers of the vesicle. The reason for this asymmetric packing can be seen from Fig. 5. Because of the high radius of curvature of the vesicle bilayer, the area/molecule for lipids in the outer monolayer is 30% *less* at the center of the bilayer than it is at the surface, whereas for lipids in the inner monolayer the area/molecule is 56% *greater* at the bilayer center than at the surface. The reduction in area/molecule in the outer monolayer means that no further cholesterol can be incorporated, beyond the critical value of maximum condensation, without cholesterol separating out. On the other hand, the increase in area/molecule in the inner monolayer allows further incorporation of cholesterol above the critical ratio. Thus above 20–30 moles cholesterol/100 moles, the cholesterol composition of the outer monolayer of the vesicle remains essentially constant, with all additional cholesterol being incorporated in the inner monolayer. These conclusions are fully supported by NMR measurements of the outside-inside distribution of phospholipid molecules across the bilayer of unsaturated phosphatidylcholine vesicles containing cholesterol (Huang *et al.*, 1974; de Kruijff, Cullis & Radda, 1975, 1976). This asymmetric packing of cholesterol means that the number of phosphatidylcholine molecules per vesicle must also increase with increasing cholesterol composition, in order not to have an

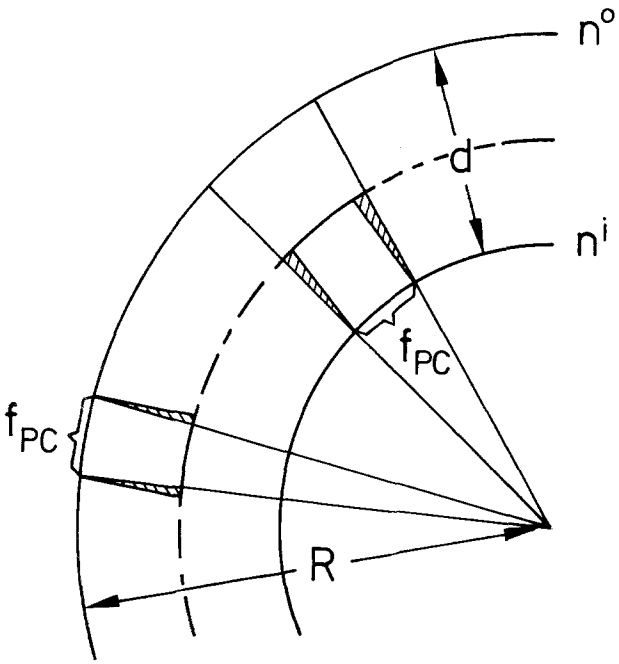

Fig. 5. Schematic diagram of the asymmetric molecular packing effects in small-diameter vesicles. The phosphatidylcholine molecules have a given molecular area, f_{PC}, defined at the bilayer surface

unrealistically high cholesterol/phospholipid ratio in the inner monolayer. This is, at least partly, the explanation for the rapid increase in vesicle size, as is outlined in the Appendix.

The reason why the cholesterol shows its full condensing effect in vesicles immediately at 25–30 moles/100 moles, rather than at 50 moles/100 moles composition as in extended planar bilayers, is also seen from Fig. 5. The 30% decrease in area/molecule at the center of the bilayer means that, even in the absence of cholesterol, the tail regions of the phospholipid molecules in the outer monolayer are already in the fully-condensed form. Further up the chains the molecular area is larger and still capable of condensation. Thus the cholesterol molecule, which is shorter than the phospholipids, can still insert between the chains, but is immediately required to exert its maximum condensation. A more detailed consideration of the asymmetric molecular packing in single-component phospholipid vesicles has been given by Chrzeszczyk, Wishnia and Springer (1977) and Huang and Mason (1978). A more simplified treatment is used in the Appendix to show that the increase in vesicle radius can be accounted for, at least semiquantitatively, by the asymmetric packing of cholesterol across the bilayer.

Thus, cholesterol is capable of exerting a molecular control of membrane morphology in areas with high radius of curvature. Such changes in morphology could be coupled to changes in lateral distribution of cholesterol (Shimshick & McConnell, 1973). Conversely, the asymmetric packing effect could give rise to the triggering of rapid transbilayer migration of cholesterol by changes in lateral segregation. This might be one possible mechanism by which regions of high radius of curvature could be centers of high biological activity, as suggested by Huang *et al.* (1974).

We would like to thank Miss Denise Ashworth for measurement of the electron micrographs and Dr. C. Huang for correspondence regarding vesicle shape.

Appendix

Model for the Control of Vesicle Size by Cholesterol

The model is based on three assumptions: (i) at up to 25 moles/100 moles content, cholesterol has no effect on the total bilayer surface area (condensing effect); (ii) at above 25 moles/100 moles total cholesterol content, the composition of the outer monolayer remains constant at 25 moles/100 moles, the remaining cholesterol all going into the inner monolayer (asymmetric packing effect, *c.f.* Fig. 5); (iii) the number of phosphatidylcholine molecules in the inner monolayer remains constant with increasing cholesterol composition. The third assumption is necessary because, if the total number of phosphatidylcholine molecules per vesicle were held constant, the number in the inner monolayer would decrease with increasing cholesterol concentration at above 25 moles/100 moles, as a result of the asymmetry effect. This would be an unfavorable situation since there would be a decreasing number of phosphatidylcholine molecules available to "solvate" a rapidly increasing number of cholesterol molecules, which would quickly lead to separation out of the cholesterol.

According to the model, the vesicle dimensions remain constant up to 25 moles cholesterol/100 moles. At higher concentrations the outer surface area of the vesicle (*c.f.* Fig. 5) is determined solely by the number of phosphatidylcholine molecules, n^o_{PC}, in the outer monolayer:

$$4\pi R^2 = n^o_{PC} \times f_{PC} \tag{1}$$

where R is the vesicle outer radius and f_{PC} is the area/phosphatidylcholine molecule (assumed constant). The number of cholesterol molecules in the

outer monolayer, n^o_{ch}, is also determined by the number of phosphatidylcholine molecules:

$$n^o_{ch} = 0.25/0.75 \times n^o_{PC}. \tag{2}$$

The area of the inner surface of the vesicle is determined both by the number of phosphatidylcholine molecules in the inner monolayer, n^i_{PC}, and the excess number of cholesterol molecules over the 25 moles/100 moles which have no effect on the bilayer surface area, thus:

$$4\pi(R-d)^2 = n^i_{PC} \times f_{PC} + (n^i_{ch} - 0.25/0.75\, n^i_{PC}) \times f_{ch} \tag{3}$$

where n^i_{ch} is the number of cholesterol molecules in the inner monolayer, f_{ch} is the area/cholesterol molecule and d is the bilayer thickness. The total number of cholesterol molecules in the vesicles is related to the total number of phosphatidylcholine molecules by the mole fraction of cholesterol, X_{ch}:

$$(n^o_{ch} + n^i_{ch}) = X_{ch}/(1 - X_{ch}) \times (n^o_{PC} + n^i_{PC}). \tag{4}$$

Using Eqs. (1)–(4) it is possible to calculate the vesicle radius as a function of mole fraction of cholesterol. Egg phosphatidylcholine vesicles with no cholesterol have an outer radius of 105.6 Å and mol wt of 1.88×10^6 (Newman & Huang, 1975) and the bilayer thickness is $d = 34$ Å (Levine & Wilkins, 1971). Thus there are a total of 2335 phosphatidylcholine molecules/vesicle in the absence of cholesterol, the area per molecule is $f_{PC} = 87.6$ Å^2, and the number of molecules/vesicle in the inner monolayer is $n^i_{PC} = 735$. The area per cholesterol molecule can be taken from monolayer studies to be $f_{ch} = 38$ Å^2 (Demel, Geurts van Kessel & van Deenen, 1972).

The calculated variation in vesicle diameter with cholesterol composition using the above parameters, is given in Fig. 6*a* (full line). The model predicts the observed sharp increase in diameter at 25 moles cholesterol/100 moles, and the calculated total increase in diameter of 84.0 Å between 25 and 50 moles cholesterol/100 moles is in good agreement with the measured value of 80 Å from Fig. 4. Values for the outer diameter obtained from the measured diffusion coefficients of Newman and Huang (1975), assuming the vesicles to be spherical, are also seen to be in good agreement with the predictions of the model. Comparison is also made in Fig. 6*a* with values calculated by assuming that there is no asymmetry in molecular packing across the bilayer and that the total number of phosphatidylcholine molecules/vesicle, $(n^o_{PC} + n^i_{PC})$, remains constant. The total vesicle surface area at 25 moles cholesterol/100 moles and above is then given by:

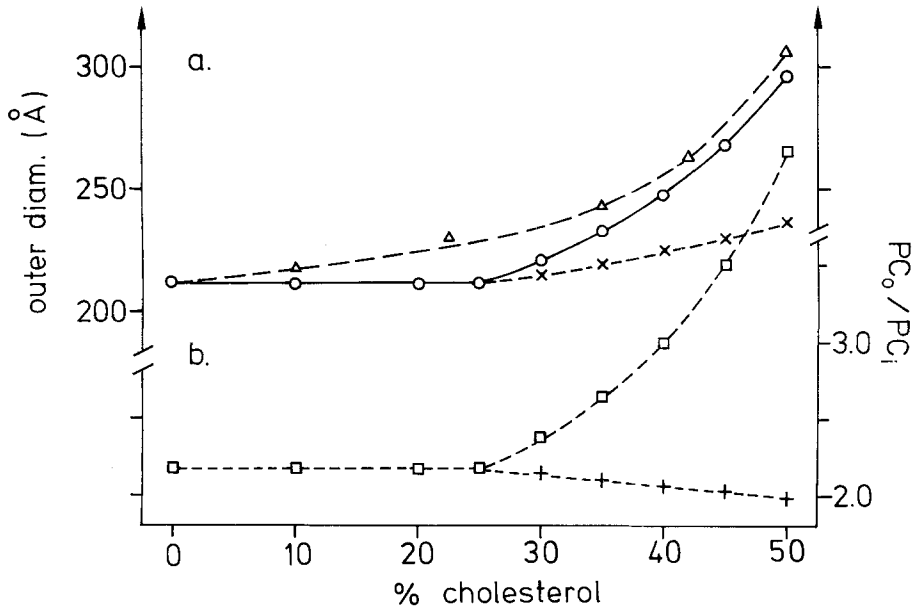

Fig. 6. Model for the change in vesicle dimensions as a function of mole proportion of cholesterol. (*a*) Outer diameter: (○) calculated values based on the model with asymmetric packing; (×) calculated values assuming no asymmetry and a fixed number of phosphatidylcholine molecules per vesicle; (△) Stokes' diameter calculated from the experimental diffusion coefficients of Newman and Huang (1975). (*b*) Calculated ratio of numbers of phosphatidylcholine molecules in outer and inner monolayers: (□) model with asymmetric packing; (+) assuming no asymmetry and a fixed number of phosphatidylcholine molecules per vesicle

$$4\pi[R^2+(R-d)^2]=(n^o_{PC}+n^i_{PC})\times\left[f_{PC}+\left(\frac{X_{ch}}{1-X_{ch}}-\frac{0.25}{0.75}\right)f_{ch}\right]. \quad (5)$$

Clearly this is insufficient to account for the observed increase in vesicle diameter, without the asymmetric packing effect of cholesterol and consequent increase in number of phosphatidylcholine molecules per vesicle.

The calculated ratio of the number of phosphatidylcholine molecules in the outer monolayer to the number in the inner monolayer is given in Fig. 6*b*, since this quantity is an important parameter of the asymmetric packing effect. This outside-inside ratio is seen to remain constant at a value of 2.18 up to 25 moles cholesterol/100 moles as is observed experimentally (Huang *et al.*, 1974; de Kruijff, Cullis & Radda, 1976), and then increase rapidly, with increasing cholesterol concentration. (In contrast, in the model without asymmetric packing the ratio actually decreases, exactly the opposite to the experimental situation). However the calculated ratio

increases to much higher asymmetries than are observed experimentally. The reason for this is that the model holds the number of phosphatidylcholine molecules in the inner monolayer constant, which leads to extremely high cholesterol/phosphatidylcholine ratios in the inner monolayer, e.g., 3.5:1 at 50 moles cholesterol/100 moles, whereas experimentally it has been suggested that 2:1 would be the maximum obtainable (Freeman & Finean, 1975). Apparently in the real vesicle situation the number of phosphatidylcholine molecules in the inner monolayer must increase so as not to give rise to unattainably high ratios of cholesterol/phosphatidylcholine. Nevertheless, it is clear that the present model, without elaboration to take account of this effect [nor more sophisticated treatment of the asymmetric packing of the phosphatidylcholine molecules (Chrzezczyk, Wishnia & Springer, 1977; Huang & Mason,1978)], contains the essential elements of the mechanism by which cholesterol exerts a control on the vesicle dimensions. These are the condensing effect at low cholesterol concentrations and the asymmetric packing effect at cholesterol concentrations of 30 moles/100 moles and greater.

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