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# OUTSIDE-INSIDE DISTRIBUTIONS AND SIZES OF MIXED PHOSPHATIDYLCHOLINE-CHOLESTEROL VESICLES

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## SUMMARY

(1) The effect of the incorporation of cholesterol upon the distribution of various molecular species of phosphatidylcholine across the bilayers of mixed sonicated liposomes (vesicles) has been studied with $^{31}$P-NMR.

(2) The outside-inside ratio of both saturated and unsaturated phosphatidylcholine species was not much affected by the incorporation of up to 30 mol% cholesterol. Above 30 mol% cholesterol the outside-inside ratio strongly increased for phosphatidylcholines with *cis* unsaturated fatty acid chains. In contrast the outside-inside ratio of *trans* unsaturated and fully saturated phosphatidylcholine species was either not affected or decreased by the incorporation of more than 30 mol% cholesterol.

(3) A simple relationship between the size of the vesicle and the linewidth of the $^{31}$P-NMR resonance is described. From the measured linewidths the sizes of the various cholesterol containing vesicles have been obtained. It is found that incorporation of 0–30 mol% cholesterol does not significantly affect the size of the vesicle whereas above 30 mol% cholesterol the size of all phosphatidylcholine vesicles sharply increases. The increase in size is the largest for the more saturated phosphatidylcholine species.

(4) From the outside-inside ratio and the size of the vesicle the composition of the outer and inner layer of the mixed vesicles could be obtained. Below 30 mol% cholesterol the composition of outer and inner layer is nearly identical. Above 30 mol% cholesterol the distribution of lipid across the bilayer of all vesicles becomes assymetric with a disproportionately larger amount of cholesterol present in the inside monolayer.

## INTRODUCTION

The distribution of phospholipids across the membrane have been determined for various models [1–3] and some biological [4–6] membranes. Nothing is known,

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however, about the distribution of cholesterol across membranes. The methods which have been used for the measurements of the distribution of phospholipids across the membrane are less suitable for the determination of the sideness of cholesterol. This is because the polar head group of cholesterol is more difficult to attack chemically or enzymatically than the polar head group of the phospholipids. Furthermore, it is well established that even small changes in the chemical structure of the polar head group of cholesterol strongly influence the interaction with phospholipids in the membrane [7–10]. Proton NMR which has been successfully used for the determination of the sideness of phospholipids in model membranes is also unsuitable, because no signals of the polar head group region of cholesterol can be observed in mixed cholesterol/phospholipids vesicles [11].

$^{31}$P-NMR has proved to be a very usefull technique for the elucidation of the factors determining the sideness of phospholipids across bilayers of sonicated liposomes (vesicles) [2, 3, 12, 13]. Cholesterol cannot of course be observed directly by $^{31}$P-NMR. However, it was shown recently that by measuring the distribution of phosphatidylcholine across the bilayers of mixed cholesterol/phosphatidylcholine vesicles the distribution of cholesterol could be calculated [13]. The lipid distribution across the bilayer of 18 : $1_c$/18 : $1_c$-phosphatidylcholine/cholesterol (1 : 1) vesicles was shown to be assymetrical with a disproportionately large amount of cholesterol present in the inside layer. Also in a $^1$H-NMR study an increased outside-inside ratio for phosphatidylcholine in egg phosphatidylcholine/cholesterol (1 : 1) vesicles was noticed [14].

In this study we extend these observations by studying systematically the effect of incorporation of increasing amounts of cholesterol on the outside-inside distribution of phosphatidylcholine for vesicles prepared from various molecular species of phosphatidylcholine differing in fatty acid chain length and unsaturation.

A simple relationship exists between the size of a vesicle and the linewidth of the $^{31}$P-NMR resonance. This relation has been used to obtain the size of the cholesterol containing vesicles. The distribution of cholesterol across the membrane may then be calculated from the measured outside-inside ratio obtained via $^{31}$P-NMR.

## MATERIALS AND METHODS

1,2-dilauroyl-*sn*-glycero-3-phosphorylcholine (12 : 0/12 : 0-phosphatidylcholine), 1,2-dimyristoyl-*sn*-glycero-3-phosphorylcholine (14 : 0/14 : 0-phosphatidylcholine), 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylcholine (16 : 0/16 : 0-phosphatidylcholine), 1,2-distearoyl-*sn*-glycero-3-phosphorylcholine (18 : 0/18 : 0-phosphatidylcholine), 1,2-dioleoyl-*sn*-glycero-3-phosphorylcholine (18 : $1_c$/18 : $1_c$-phosphatidylcholine), 1,2-dielaidoyl-*sn*-glycero-3-phosphorylcholine (18 : $1_t$/18 : $1_t$-phosphatidylcholine) and 1,2-dipalmitoleyl-*sn*-glycero-3-phosphorylcholine (16 : $1_c$/16 : $1_c$-phosphatidylcholine) were synthesized as described previously [15]. Egg phosphatidylcholine was isolated from hen eggs according to established procedures. All the above lipids were kindly donated by the biomembranes group of the department of Biochemistry (Utrecht, The Netherlands). Soya phosphatidylcholine (main fatty acid linoleic acid) was a gift from Dr. H. Eikermann of Natterman and Cie, Köln, G.F.R. Cholesterol was obtained from Fluka (Buchs, Switzerland) and was recrystallized twice fro absolute ethanol. Soya phosphatidylcholine contained approximately 3 % of lyso-

phosphatidylcholine, but all other lipids were at least 99 % pure as measured by thin-layer chromatography. $^2H_2O$ was obtained from Ryvan, Southampton, U.K. Neodynium nitrate from Koch-Light, Colnbrook, Bucks, U.K.

*Preparation of vesicles*

A chloroform solution containing 50 μmol phosphatidylcholine (100 μmol in the case of 12 : 0/12 : 0-, 14 : 0/14 : 0- and 16 : $1_c$/16 : $1_c$-phosphatidylcholine) and the appropriate amount of cholesterol was dried down under a stream of $N_2$ and stored overnight under high vacuum. The lipids were dispersed in 1.5 ml of $^2H_2O$ containing 25 mM Tris/acetic acid p$^2$H 7.0 and 0.2 mM EDTA by vortexing above the transition temperature of the lipid. Vesicles were formed by sonication of the dispersion under $N_2$ at power setting 2 of a Dawe soniprobe until the mixture became translucent, which usually took 3–5 min. During sonication the vial containing the lipid dispersion was kept in a water bath at a temperature of 5 °C above the transition temperature of the lipid. For the unsaturated phosphatidylcholines the vial was placed in an ice-water bath. After the sonication the translucent vesicle solution was kept at the same temperature as used for the sonication and was used within 30 min for the $^{31}$P-NMR experiments. The 30–50 mol% cholesterol containing phosphatidylcholine vesicle solutions were always more turbid than vesicle solutions containing less cholesterol, which already indicates their larger size (see Results). No chemical degradation of the lipids as evidenced by thin-layer chromatography occurred during sonication and subsequent NMR experiments.

*Nuclear magnetic resonance*

For most measurements a Bruker WH-90 spectrometer operating in the Fourier transform mode at 36.4 MHz for $^{31}$P was used. The spectrometer was interfaced with a Nicolet B-NC-12 computer and equipped with temperature control, broad band proton decoupling and field stabilisation via a deuterium lock. Accumulated free induction decays were obtained from 500 transients (100 in case of 12 : 0/12 : 0-, 14 : 0/14 : 0- and 16 : $1_c$/16 : $1_c$-phosphatidylcholine vesicles) with a interpulse time of 4 s. For some experiments a 129 MHz $^{31}$P-NMR spectrometer built in this laboratory was used [16]. This spectrometer was similarly equipped as the lower field machine but had no broad band proton decoupling. Triphenylphosphine (in chloroform) in a central capillary insert in the 10 mm diameter NMR sample tube containing 1 ml of vesicle solution was used as an external standard.

To get complete separation of the $^{31}$P resonances arising from the phosphatidylcholine molecule present in the outside and inside layer of the vesicle membrane an aliquot of a 100 mM $Nd(NO_3)_3$ solution in $^2H_2O$ was added (see Fig. 1, ref. 13) such that the final $Nd^{3+}$ concentration was 8 mM (16 mM in case of the 12 : 0/12 : 0-, 14 : 0/14 : 0- and 16 : $1_c$/16 : $1_c$-phosphatidylcholine vesicles). The reason for the increased lipid and $Nd^{3+}$ concentration and the shorter accumulation time for the latter vesicles is to minimize any inward leak of $Nd^{3+}$ during the data accumulation which would decrease the separation between the resonances from the outside and inside facing phosphatidylcholine molecules.

Computer integration of the resulting spectra gives a measure of the intensity of the outside resonance relative to that of the inside resonance. To get the ratio of the number of phosphatidylcholine molecules on the outside to that on the inside of the

vesicle ($R_{o/i}$) the measured outside-inside signal ratio had to be increased by 1.25 to account for saturation effects and the Nuclear Overhauser Enhancement of the resonance from the inside phosphatidylcholine molecules [13].

## RESULTS

In sonicated phosphatidylcholine vesicles the outer layer contains about twice as many phosphatidylcholine molecules as the inside layer [13]. The effect of cholesterol incorporation on the outside-inside distribution of phosphatidylcholine in vesicles prepared from various unsaturated phosphatidylcholine species is shown in Fig. 1. At cholesterol concentrations up to 30 mol% the outside-inside distribution is not much affected. However, above 30 mol% cholesterol there is a marked increase in $R_{o/i}$ for all *cis* unsaturated phosphatidylcholine vesicles. In contrast $R_{o/i}$ of the *trans*-unsaturated 18 : $1_t$/18 : $1_t$-phosphatidylcholine vesicles is not significantly different at 30 and 50 mol% cholesterol concentration. It was not possible to produce stable cholesterol/soya phosphatidylcholine (1 : 1) vesicles. The lipid dispersion stayed turbid even after prolonged sonication and gave no high resolution $^{31}$P-NMR spectrum, indicating that only large lipid aggregates were present. It should be noted that under the experimental conditions used, between 90 and 100% of the phosphorus atoms present in the various vesicle preparations were observed even in the presence of $Nd^{3+}$. As soon as vesicle aggregation occurs (for instance after storing the sample for days, or after freezing and thawing the sample) the intensity of the narrow (8–35 Hz width) $^{31}$P-NMR signal from the vesicles strongly decreases [13]. As was noted before no stable vesicles could be formed from pure 12 : 0/12 : 0-phosphatidylcholine [13, 17]. Vesicles containing 15 mol% cholesterol were also unstable, as aggregation occured within minutes after stopping the sonication. Furthermore only one down-

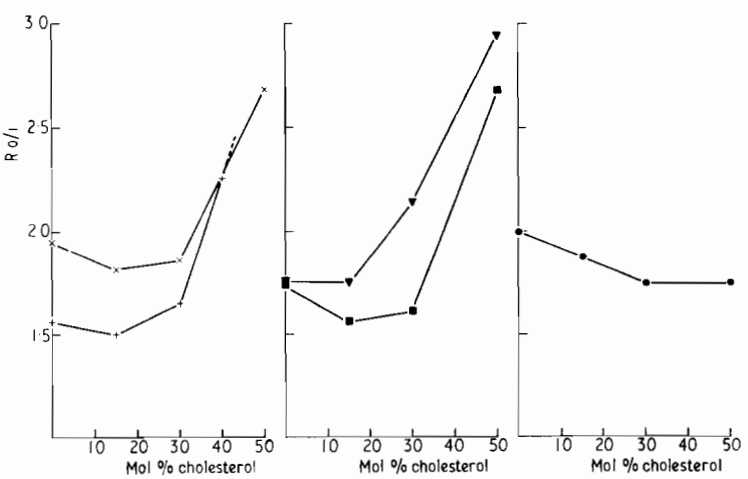


Fig. 1. Outside-inside distributions ($R_{o/i}$) of mixed unsaturated phosphatidylcholine/cholesterol vesicles. ×, egg phosphatidylcholine; +, soya phosphatidylcholine; ▲, 18 : $1_c$/18 : $1_c$-phosphatidylcholine; ■, 16 : $1_c$/16 : $1_c$-phosphatidylcholine; ●, 18 : $1_t$/18 : $1_t$-phosphatidylcholine. The outside-inside distributions of the vesicles were measured at 25 °C.

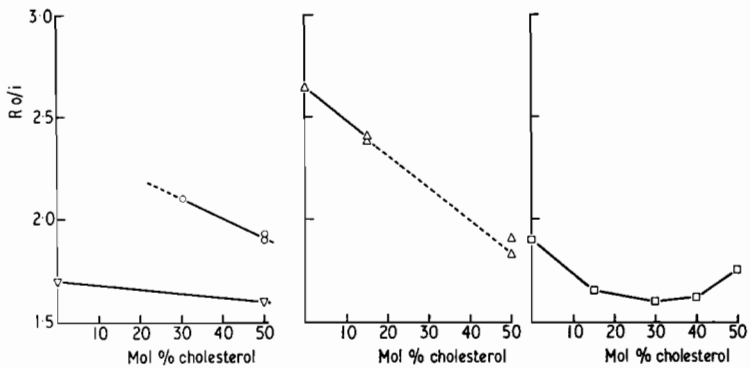


Fig. 2. Outside-inside distributions ($R_{o/i}$) of mixed saturated phosphatidylcholine/cholesterol vesicles. ○, 12 : 0/12 : 0-phosphatidylcholine; ▽, 18 : 0/18 : 0-phosphatidylcholine; △, 14 : 0/14 : 0-phosphatidylcholine; □, 16 : 0/16 : 0-phosphatidylcholine. The outside-inside distributions of the vesicles were measured at 25 °C, with the exception of 16 : 0/16 : 0-phosphatidylcholine and 18 : 0/18 : 0-phosphatidylcholine vesicles, which were measured at 45 °C and 60 °C, respectively.

field shifted resonance was present in the $^{31}$P-NMR spectrum after the addition of shift reagent demonstrating that these "vesicles" are permeable to $Nd^{3+}$. The 30 and 50 mol% cholesterol containing 12 : 0/12 : 0-phosphatidylcholine vesicles were stable at least for the time the measurements lasted. Vesicles prepared from cholesterol and longer chain saturated phosphatidylcholines were stable (no visible aggregation, relative $^{31}$P-NMR signal intensity 90–100 %). However, for the 30 mol% cholesterol containing 14 : 0/14 : 0-phosphatidylcholine species $R_{o/i}$ could not be determined because after the addition of $Nd^{3+}$ strong aggregation and loss of $^{31}$P-NMR signal occured.

For the saturated phosphatidylcholine vesicles $R_{o/i}$ tends to decrease with increasing amounts of cholesterol (Fig. 2). This is in strong contrast to the *cis* unsaturated phosphatidylcholine vesicles where incorporation of more than 30 mol% cholesterol increase $R_{o/i}$ (Fig. 1).

The width of the $^{31}$P-NMR signal from phospholipid vesicles is affected by the size of the vesicle (ref. 3, see also Appendix). The linewidth of $^{31}$P-NMR spectra of phosphatidylcholines at 45 °C is remarkably insensitive to the incorporation of up to 30 mol% cholesterol, demonstrating that the size of these vesicles is not significantly influenced by cholesterol (Figs. 3, 4). A sharp increase in linewidth occurs between 30 and 50 mol% cholesterol for all vesicles. Similar results were obtained for the linewidths measured at 30 °C (except for 16 : 0/16 : 0-phosphatidylcholine vesicles which are in the gel state at this temperature and therefore have a larger linewidth [13, 18]). Also for 18 : 0/18 : 0-phosphatidylcholine vesicles the linewidth at 60 °C was increased 2-fold by the incorporation of 50 mol% cholesterol. There are two possible explanations for this result. The first possibility is that cholesterol decreases the motion of the phosphate group of phosphatidylcholine. $^{31}$P-NMR studies at 129 MHz of unsonicated phosphatidylcholine liposomes however demonstrated that the motion of the phosphate group is not significantly affected by the presence of 50 mol% cholesterol [19]. The other possibility is that the vesicles become larger on the

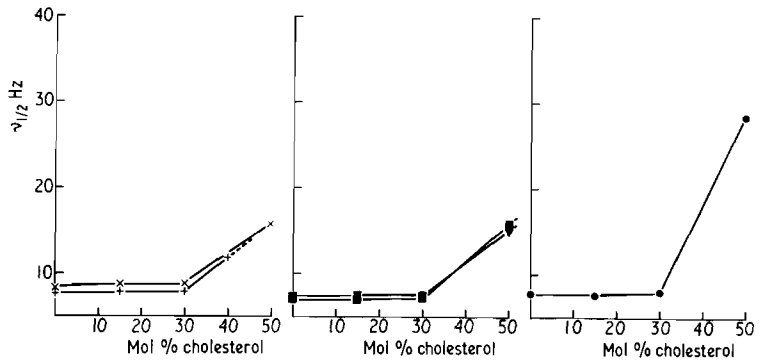


Fig. 3. Full width at half height ($\nu_{1/2}$) of 36.4 MHz $^{31}$P-NMR spectra of mixed unsaturated phosphatidylcholine/cholesterol vesicles at 45 °C. Symbols as Fig. 1.

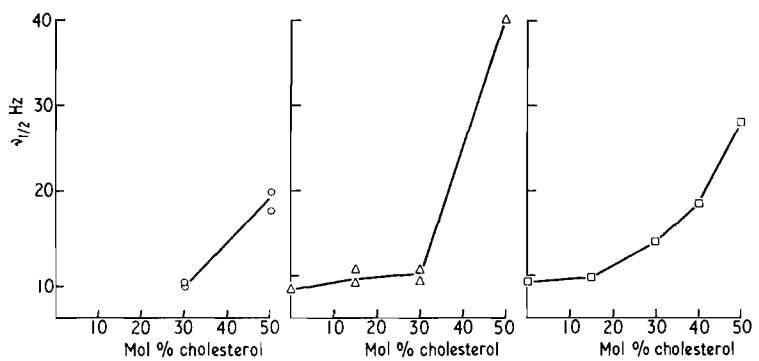


Fig. 4. Full width at half height ($\nu_{1/2}$) of 36.4 MHz $^{31}$P-NMR spectra of mixed saturated phosphatidylcholine/cholesterol vesicles at 45 °C. Symbols as Fig. 2.

incorporation of 50 mol% cholesterol which indeed has been demonstrated for egg and soya phosphatidylcholine vesicles in ultracentrifugation [20, 21] and gel filtration studies [22]. That the vesicles do not become multilayered on the incorporation of 50 mol% cholesterol is shown by the $R_{o/i}$ measurements. Consider a two-layered vesicle with the inner vesicle having an outer diameter of 125 Å [12, 20, 23] and the outer layer of the inner vesicle in direct contact with the inner layer of the outer vesicle. It can be calculated then that $R_{o/i}$ of such a vesicle as measured with $^{31}$P-NMR and a nonpermeating shift reagent would be 0.85 which is much less than the actual $R_{o/i}$ values measured (Figs. 1, 2). Introducing more layers into the vesicle or spacing the outer and inner vesicle would make $R_{o/i}$ even less.

As is shown in the Appendix the vesicle size can be calculated from the measured linewidth (Table I). All vesicles become substantially larger on the incorporation of 50 mol% cholesterol, the increase in size being more marked for the *trans* unsaturated and fully saturated phosphatidylcholine species.

Because both $R_{o/i}$ and the size of the *cis* unsaturated phosphatidylcholine vesicle increase the lipid distribution across the bilayer must be assymetric. For a symmetrical distribution $R_{o/i}$ would decrease if the vesicle size increased. Although

TABLE I

EFFECT OF CHOLESTEROL UPON THE SIZE OF PHOSPHATIDYLCHOLINE VESICLES

| Phosphatidylcholine species | Outer radius (Å) vesicles without cholesterol* | Cholesterol/phosphatidylcholine vesicles (1 : 1) | |
|---|---|---|---|
| | | $\nu_{1/2}$ at 45 °C (Hz) | Outer radius (Å) |
| Egg phosphatidylcholine | 120 | 16 | 180** |
| Soya phosphatidylcholine | 118 | 12*** | 160*** |
| 18 : $1_c$/18 : $1_c$ phosphatidylcholine | 131 | 15 | 173 |
| 16 : $1_c$/16 : $1_c$ phosphatidylcholine | 110 | 25 | 209 |
| 18 : $1_t$/18 : $1_t$ phosphatidylcholine | 123 | 28 | 218 |
| 12 : 0/12 : 0 phosphatidylcholine | - | 19 | 189 |
| 14 : 0/14 : 0 phosphatidylcholine | 84 | 40 | 243 |
| 16 : 0/16 : 0 phosphatidylcholine | 112 | 28 | 218 |

* Radius obtained from outside-inside measurements of phosphatidylcholine vesicles [13].
** Ref. 20.
*** Vesicles containing 40 mol % cholesterol Outer radius as measured in ref. 22.

for the *trans* unsaturated and fully saturated phosphatidylcholine vesicles there may be a slight decrease in $R_{o/i}$ between 30 and 50 mol% cholesterol, the bilayer of these vesicles is also expected to be assymetrical because the size of these vesicles is even larger than the size of the *cis* unsaturated phosphatidylcholine/cholesterol (1 : 1) vesicles. The extent of assymetry can be calculated from $R_{o/i}$ and the size of the vesicles as is shown in the Appendix. For all vesicles containing 50 mol% cholesterol the bilayer is found to be assymetric with a relatively larger amount of cholesterol on the inside. The inside monolayer then contains more than 50 mol% cholesterol in all cases. For flat liposomal bilayers of egg phosphatidylcholine it has been shown that only 50 mol% cholesterol can be incorporated [24]. Any excess of cholesterol forms a separate crystalline cholesterol phase. It can therefore be argued that the inside monolayer of the vesicle could only contain 50 mol% cholesterol, the calculated excess forming a separate cholesterol phase. That this is highly unlikely can be seen from the measured $R_{o/i}$ values. The cholesterol concentration in the outside monolayer of a vesicle will be at least 30 mol% because this amount of cholesterol can be incorporated in the vesicle bilayer without much affecting the size and outside-inside distribution of phosphatidylcholine. For egg phosphatidylcholine/cholesterol (1 : 1) vesicles it can then be calculated that the maximum value of $R_{o/i}$ that can be obtained is 1.75 (corresponding to the situation where the cholesterol concentration in the outside and inside layer is 30 and 50 mol% respectively). Since the measured value of $R_{o/i}$ is much higher (Fig. 1) the phosphatidylcholine concentration in the inside layer must be lower and therefore the cholesterol concentration is higher than 50 mol%.

## DISCUSSION

The size of a sonicated vesicle is determined by various factors. One important parameter is the flexibility of the hydrophobic part of the lipid molecule which is needed to fit the molecule in a curved bilayer without too much loss of van de Waals

interaction. This was clearly demonstrated for the sizes of various phosphatidylcholine vesicles. Increasing the fatty acid chain length and thereby increasing the chain interaction between the molecules leads to larger vesicles [13].

The limiting area of cholesterol is determined by the rigid ring system of the sterol molecule [7]. It can be expected therefore that the incorporation of cholesterol would lead to a larger vesicle. Incorporation of up to 30 mol% cholesterol does not significantly affect the size of vesicles, therefore it can be concluded that the flexible fatty acid chains of the various phosphatidylcholines can accommodate the cholesterol molecules in the curved bilayer. Apparently the increased van de Waals interaction between the fatty acid chains and cholesterol (condensing effect of cholesterol) compensate for the loss in interaction due to placing a rigid hydrophobic molecule in a curved bilayer. Above 30 mol% cholesterol-incorporation a sharp increase in vesicle size occurs. This increase is higher for 18 : $1_t$/18 : $1_t$ and the fully saturated phosphatidylcholines as compared to the *cis* unsaturated phosphatidylcholine vesicles.

Engelman and Rothman [25] have proposed a model for the lipid sterol interaction in bilayers (based on X-ray and differential scanning calorimetry data and an analysis of molecular models) in which each cholesterol molecule is surrounded ("solvated") by 7 fatty acid chains for cholesterol concentrations, up to 33 mol% and no cholesterol-cholesterol interactions occur. Above 33 mol% cholesterol there are not enough fatty acid chains available to "solvate" each cholesterol molecule and therefore cholesterol-cholesterol interactions occur. We propose that the increase in vesicle size above 30 mol% cholesterol is associated with the occurence of cholesterol-cholesterol interactions in the bilayer. In order to optimize the van de Waals interaction between the ring systems of two cholesterol molecules the bilayer must be as flat as possible. If the ring systems of cholesterol is thought to be cylindrical with a length of 11 Å and a radius of 3.5 Å it can be calculated that the van de Waals interaction between two cholesterol molecules present on the inner monolayer of a vesicle with outer radius of 180 Å (egg phosphatidylcholine/cholesterol (1 : 1) vesicles) is 20% higher than in the inner monolayer of a vesicle of outer radius 120 Å (egg phosphatidylcholine/cholesterol (10 : 3) vesicles). In this calculation only the interaction energy between the ring system of the cholesterol molecules is considered, and it is assumed that at the 3$\beta$-OH site of the ring system the axes of the cylinders are 7 Å apart.

The outside-inside distribution of phosphatidylcholine and the size of the vesicles is not much affected by the incorporation of up to 30 mol% cholesterol. It can be concluded therefore that the composition of the inner and outer layer of the vesicle bilayer is very similar. Above 30 mol% cholesterol the lipid distribution across the bilayer becomes assymetrical, for both saturated and unsaturated fatty acyl constituents of the phosphatidylcholine molecule. This strongly suggests that the cholesterol distribution is mainly determined by the occurence of cholesterol-cholesterol interactions. It should be noted that the most curved monolayer, the inside monolayer, is calculated to contain more than 50 mol% cholesterol. This result appears in contradiction with the finding that when cholesterol-cholesterol interactions occur the size of the vesicle increases and our conclusion that the vesicle size increases because the van de Waals interaction between the rigid ring systems is of main importance. However, the outside-inside distribution of cholesterol is also determined to a great extent by the exact shape of the ring system. A possible explanation for the observed assymetry would be that the shape of the ring system of the cholesterol

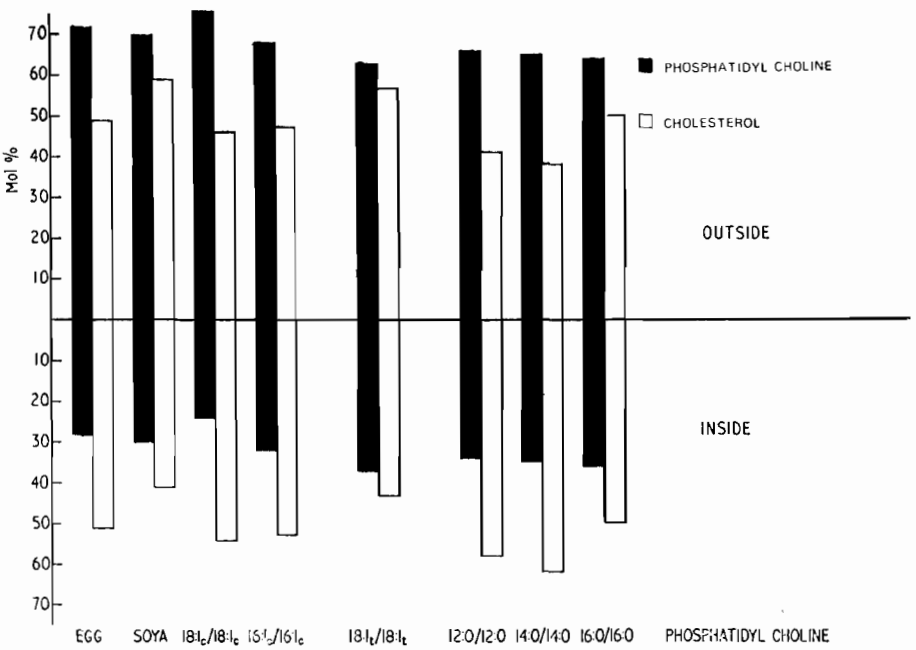


Fig. 5. Calculated outside-inside distributions of mixed phosphatidylcholine/cholesterol (1 : 1) vesicles.

molecule is slightly tapered towards the hydroxyl end of the molecule. To verify this with molecular models is rather difficult because in the presence of rotation along the long axis of the cholesterol molecule in the membrane the actual average shape of the molecule will be determined by the exact orientation of the axis of rotation.

Many biological membranes contain more than 30 mol% cholesterol. Our findings directly imply that in those membranes in highly curved regions the cholesterol concentration will be lower than in more flat membrane parts. Possible "meniscus" formation around proteins embedded in these membranes would also tend to reduce the local concentration of cholesterol around the protein molecule. In this respect a recent study on the sarcoplasmic reticulum ATPase is relevant. For this enzyme it has been demonstrated that the enzyme is fully active when reconstituted in a bilayer with 50 mol% cholesterol [26]. When the phospholipids in the first shell of the lipid bilayer surrounding the protein are replaced by cholesterol however there is a complete and reversible inactivation of ATPase activity. The phospholipid annulus therefore excludes cholesterol [26] possibly via a mechanism as indicated above. As the annular phospholipids are more rigid or immobilized than the lipid bilayer the exclusion of cholesterol from the annulus might also be caused by the preference of cholesterol for the more fluid component in mixed membranes [26, 27].

## APPENDIX

In a previous work [28] it has been shown that the $^{31}$P-NMR linewidths of the rapidly tumbling vesicles depend on the rotational correlation time $\tau_R$ and the applied magnetic field $H_0$ according to

$$\Delta\nu = a_1 H_0{}^2 \tau_R + a_2 H_0{}^2 + a_3 \tau_R + a_4 \quad (1)$$

where the $a_i$ are constants characteristic of chemical shift anisotropy and dipolar interactions experienced by the phosphate phosphorus. Thus at a particular frequency

$$\Delta\nu = b_1 \tau_R + b_2 \quad (2)$$

If the main source of line narrowing is vesicle tumbling, (and lateral diffusion of the phospholipid in the plane of the bilayer is slow enough) the rotational correlation time may be calculated through Stokes law to be $\tau_R = 4\pi a^3 \eta / 3kT$, where $a$ is the vesicle radius and $\eta$ is the viscosity of the aqueous medium. Thus it would be expected from Eqn. 2 that $\Delta\nu$ is directly proportional to the viscosity, which has been observed for 16 : 0/16 : 0-phosphatidylcholine vesicles [28]. Then, at a particular temperature Eqn. 2 may be rewritten as

$$\Delta\nu = ca^3 + d \quad (3)$$

where $d$ may be estimated as $\leqslant$ 2Hz at 36.4 MHz [28].

The constant $c$ may be estimated from the observed values of $\Delta\nu$ for vesicle systems with known values of $a$ (see Table I). It is estimated that $c = 2.9 \pm 0.2 \cdot 10^{-6} \cdot s^{-1} \cdot Å^{-3}$ at 36.4 MHz and 45 °C whereas $c = 22 \cdot 10^{-6} \cdot s^{-1} \cdot Å^{-3}$ at 129 MHz. Thus the size of the other cholesterol-containing vesicles may be obtained from measures of the halfwidth $\Delta\nu$ (see Table I). It should be noted that more accurate results would be expected at higher frequencies as a larger part of the observed linewidth then depends on $\tau_R$ (and therefore on the radius $a$) [28]. We have therefore checked the 36.4 MHz results by sizing the vesicles at 129 MHz, obtaining results which agree within 2 %. It is to be noted that $^{31}$P-NMR provides a fast and accurate method of determining vesicle sizes, and may similarly be used to size sonicated biological membrane fragments and other small phospholipid containing systems, as long as lateral diffusion does not provide an effective relaxation mechanism.

It is assumed in the above analysis that the sizes of the vesicles are relatively homogenous. If there is a substantial population of larger vesicles it would be expected that the signals of these larger vesicles would experience substantial broadening (for example, a 10 % increase in the radius produces a 30 % increase in the linewidth) and the net line-shape obtained would have appreciably more intensity in the wings as opposed to the expected Lorentzian line. However, as noted in Fig. 6 the spectrum arising from 16 : 0/16 : 0-phosphatidylcholine/cholesterol (1 : 1) vesicles fit a Lorentzian line-shape very closely, suggesting that the sizes of the vesicles are very homogeneous. On the basis of various spectral simulations assuming a Gaussion distribution of vesicle sizes it may be estimated that the square root of the second moment of the distribution is $\leqslant$ 30 Å for the 16 : 0/16 : 0-phosphatidylcholine/cholesterol (1 : 1) vesicles.

The calculation of the distribution of cholesterol across the membrane of the phosphatidylcholine/cholesterol (1 : 1) vesicles from the measured outside-inside ratio $R_{o/i}$ and vesicle size requires some additional information. Firstly, it is assumed that the vesicles maintain a spherical shape. This assumption is supported by recent freeze-etch electron microscopy results (Forge, A., Knowles, P. F. and Marsh, D. unpublished) which show that egg phosphatidylcholine/cholesterol vesicles remain spherical for cholesterol concentrations up to 50 mol%. Secondly, the thickness of the vesicle membrane is required. The membrane thickness of egg phosphatidylcholine

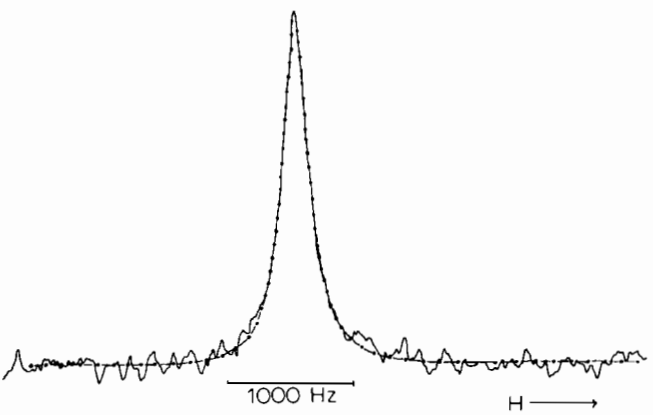


Fig. 6. 129 MHz $^{31}$P NMR spectrum of 16 : 0/16 : 0-phosphatidylcholine/cholesterol (1 : 1) vesicles at 50 °C. The dotted curve is a Lorentzians lineshape.

cholesterol (1 : 1) bilayers is reported as 45 Å [20]. For the other cholesterol containing vesicles no measurements of the membrane thickness have been done. We have therefore used for these vesicles membrane thicknesses obtained in the absence of cholesterol, which has been reported as 37 Å for 16 : 0/16 : 0-phosphatidylcholine [29] from which thickness of 28 and 32 Å for 12 : 0/12 : 0 and 14 : 0/14 : 0-phosphatidylcholine may be extrapolated. The membrane thickness of egg phosphatidylcholine bilayers is reported as 35 Å [30] from which the thicknesses of 16 : $1_c$/16 : $1_c$, 18 : $1_c$/18 : $1_c$, 18 : $1_t$/18 : $1_t$ and soya phosphatidylcholine bilayers may be estimated to be 28, 32, 36 and 35 respectively. It may be noted that the cholesterol/phosphatidylcholine distribution calculated from these data are not particularly sensitive to the membrane thickness (a change of 10 % in membrane thickness for the egg phosphatidylcholine/cholesterol (1 : 1) vesicles causes $< 5$ % change in the calculated cholesterol/phosphatidylcholine distribution). Finally, it is assumed that the packing densities of the lipids are equal on both sides of the vesicle bilayer, and that the areas of the various liquid-crystalline phosphatidylcholines and phosphatidylcholine/cholesterol complexes are equal to 72 Å$^2$ [30] and 85 Å$^2$ [30] respectively. The area per cholesterol molecule is reported as 38 Å$^2$ [7]. With the above information two sets of simultaneous equations may be obtained corresponding to the situation when there is more cholesterol than phosphatidylcholine on the outer monolayer or in the inner monolayer respectively. The relevant set of equations for the latter situation are given by:

$$x_1/x_2 = R_{o/i}$$
$$x_1 + x_2 = Y_1 + Y_2$$
$$(x_1 - Y_1)a_l + Y_1 a_{lc} = A_1$$
$$(Y_2 - x_2)a_c + x_2 a_{lc} = A_2$$

where $x_1$ = number of phosphatidylcholine molecules on the outside of the vesicle. $x_2$ = number of phosphatidylcholine molecules on the inside of the vesicle. $Y_1$ = number of cholesterol molecules on the outside of the vesicle. $Y_2$ = number of cholesterol molecules on the inside of the vesicle. $a_l$ = area of the phosphatidylcholine

molecule. $a_{lc}$ = area of the phosphatidylcholine/cholesterol complex. $a_c$ = area of the cholesterol molecule. $A_1$ = area of outside layer of the vesicle. $A_2$ = area of inside layer of the vesicle.

The distribution of the cholesterol and phosphatidylcholine molecules may be simply obtained on solving these equations (see Fig. 5).

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