Why cholesterol should be found predominantly in the cytoplasmic leaf of the plasma membrane

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Abstract

In the mammalian plasma membrane, cholesterol can translocate rapidly between the exoplasmic and cytoplasmic leaves, and is found predominantly in the latter. We hypothesize that it is drawn to the inner leaf to reduce the bending free energy of the membrane caused by the presence there of phosphatidylethanolamine. Incorporating this mechanism into a model free energy for the bilayer, we calculate that approximately two thirds of the total cholesterol should be in the inner leaf.

1 Introduction

The importance of cholesterol in the regulation of the properties of mammalian cells is widely recognized, and it has been the subject of intense research (1, 2). Nonetheless, some very basic questions about it remain unanswered. Among these is its distribution between the two leaves of the plasma membrane. It is well known that cholesterol can translocate rapidly between these leaves (3–5). As a consequence, its distribution should be determined from the equilibrium requirement that the chemical potential of cholesterol be the same in both leaves. Given this, the well-known preference of cholesterol for sphingomyelin (SM) among phospholipids (6), and the fact that almost all of the SM is in the exoplasmic leaf of the plasma membrane (7), one might expect that the majority of cholesterol would also be found in that leaf. Indeed molecular dynamics simulations of some simple models of asymmetric bilayers incorporating SM and cholesterol do find the majority of cholesterol in the outer leaf (8, 9). But the results of experiment tell a different story; the majority of cholesterol is found in the cytoplasmic leaf (10–13). Why this should be is not understood.

In this paper we propose a simple mechanism that can account for the asymmetric distribution of cholesterol. It begins with the observation that the almost all of the phosphatidylethanolamine (PE) is in the cytoplasmic leaf (7). PE has a small head group, and thus a spontaneous curvature which is relatively large (14). Because of this curvature, PE forms inverted hexagonal phases at high temperatures, at which the entropy of its hydrocarbon tails dominates, and forms lamellar phases only at lower temperatures (15). Thus the free energy of bilayers containing PE in the inner leaf must encompass a significant amount of bending energy. Cholesterol is known to order the hydrocarbon tails of phospholipids (16), so we suggest that it is found predominantly in the cytoplasmic leaf because it reduces the bending energy caused by the inclusion of PE. This hypothesis is in accord with the effect of cholesterol on the temperature of transition of PE from the high-temperature hexagonal phase to the low-temperature lamellar one. Whereas the addition of small amounts of cholesterol decreases this transition temperature, amounts greater than 0.3 mol fraction increases it and stabilizes the lamellar phase (17, 18).

We incorporate this idea into a model of an asymmetric membrane consisting of phosphatidylcholine (PC), SM, and cholesterol in the outer leaf and phosphatidylserine (PS), PE, and cholesterol in the inner leaf. We take the ratios of SM to PC and of PE to PS to be given by experiment as well as the ratio of the total amount of cholesterol to the total amount of lipid.

Requiring that the chemical potential of cholesterol in the two leaves be the same, we determine the fraction of cholesterol in the inner leaf. For reasonable values of the interaction parameters and elastic constants, we find that approximately two thirds of the cholesterol is in the inner leaf.

2 Theoretical Model

2.1 Procedure

We consider a bilayer of which the outer, exoplasmic, leaf consists of N_{SM} molecules of sphingomyelin, N_{PC} molecules of phosphatidylcholine, and N_{C_o} molecules of cholesterol, and the inner, cytoplasmic leaf, consists of N_{PE} molecules of phosphatidylethanolamine, N_{PS} molecules of phosphatidylserine, and N_{C_i} molecules of cholesterol. We denote the total number of molecules in the outer leaf by N_o, the total number of molecules in the inner leaf by N_i, and the total number of molecules in the bilayer by N_{bi}. We assume that each leaf is an incompressible liquid. As a consequence, the areas of the outer leaf, A_o, and of the inner leaf, A_i, are directly related to their molecular compositions. If the area per molecule of the phospholipids be denoted by a and that of cholesterol by $r_a a$, then

$$\begin{aligned} A_o &= [N_{SM} + N_{PC} + r_a N_{C_o}]a = [N_o - (1 - r_a)N_{C_o}]a \\ A_i &= [N_{PE} + N_{PS} + r_a N_{C_i}]a = [N_i - (1 - r_a)N_{C_i}]a \end{aligned} \tag{1}$$

A further consequence of incompressibility is that the Helmholtz free energy of the bilayer, F_{bi}, depends only on the temperature, T, and the numbers of molecules of each component. As the free energy is an extensive quantity, it can be written in the form

$$\begin{aligned} F_{bi}(N_{SM}, N_{PC}, N_{C_o}, N_{PE}, N_{PS}, N_{C_i}, T) = \\ N_{bi} f_{bi}(x_{SM}, x_{PC}, x_{C_o}, x_{PE}, x_{PS}, x_{C_i}, T), \end{aligned} \tag{2}$$

where $x_{SM} \equiv N_{SM}/N_{bi}$, is the mol fraction of SM in the bilayer and similarly for the other components. By definition the sum of the mol fractions of all components is unity.

It will be more convenient for us to express quantities in terms of the mol fractions of a component in the inner or outer leaf rather than the mol fraction in the bilayer. Thus we introduce

$$y_{SM} = \frac{N_{SM}}{N_o} = x_{SM}\frac{N_{bi}}{N_o}, \qquad y_{PC} = x_{PC}\frac{N_{bi}}{N_o}, \qquad y_{C_o} = x_{C_o}\frac{N_{bi}}{N_o}, \tag{3}$$

$$y_{PE} = \frac{N_{PE}}{N_i} = x_{PE}\frac{N_{bi}}{N_i}, \qquad y_{PS} = x_{PS}\frac{N_{bi}}{N_i}, \qquad y_{C_i} = x_{C_i}\frac{N_{bi}}{N_i}. \tag{4}$$

By definition $y_{SM}+y_{PC}+y_{C_o}=1$ and $y_{PE}+y_{PS}+y_{C_i}=1$, so that only four of these mol fractions are independent. We shall assume that the areas of the two leaves, Eq. (1), are equal. From this condition the fractions N_{bi}/N_i and N_{bi}/N_o can be obtained, and the mol fractions x can be expressed in terms of the mol fractions y and *vice versa*. In particular, the total mole fraction of cholesterol in the bilayer, x_C, is given by

$$x_C = \frac{y_{C_i} + y_{C_o} - 2(1-r_a)y_{C_i}y_{C_o}}{2-(1-r_a)(y_{C_i}+y_{C_o})}. \tag{5}$$

The four independent mol fractions, then, are determined by the requirement that the chemical potentials of cholesterol in the two leaves be the same, that the ratios of SM to PC in the outer leaf, y_{SM}/y_{PC}, and of PS to PE in the inner leaf, y_{PS}/y_{PE} in the inner leaf be equal to their experimental values, and that the total mol fraction of cholesterol in the bilayer, Eq. (5), be equal to its experimental value. Then the distribution of cholesterol between leaves is determined.

We now turn to two models for the free energy of the bilayer. In the first, we consider each leaf to be described by a phenomenological, regular solution, free energy (19). There is no explicit coupling between leaves. In the second, we include a bending energy which couples the leaves and draws the cholesterol to the cytoplasmic leaf to reduce the amount of that energy caused by the presence of PE.

2.2 Regular Solution Free Energy

We take as the model free energy a simple sum of the free energies of the two leaves in the form

$$\begin{aligned}
&F_{bi}(N_{SM},N_{PE},N_{C_o},N_{PE},N_{PS},N_{C_i},T) = N_o f_o(y_{SM},y_{PC},y_{C_o},T) + N_i f_i(y_{PE},y_{PS},y_{C_i},T),\\
&f_i = 6\epsilon_{PS,PE}y_{PS}y_{PE} + 6\epsilon_{PS,C}y_{PS}y_{C_i} + 6\epsilon_{PE,C}y_{PE}y_{C_i} +\\
&\qquad k_BT(y_{PS}\ln y_{PS} + y_{PE}\ln y_{PE} + y_{C_i}\ln y_{C_i}), \qquad (6)\\
&f_o = 6\epsilon_{SM,PC}y_{SM}y_{PC} + 6\epsilon_{SM,C}y_{SM}y_{C_o} + 6\epsilon_{PC,C}y_{PC}y_{C_o} +\\
&\qquad k_BT(y_{SM}\ln y_{SM} + y_{PC}\ln y_{PC} + y_{C_o}\ln y_{C_o}). \qquad (7)
\end{aligned}$$

We have assumed an average of six nearest-neighbor interactions per molecule. From this free energy we calculate the chemical potential of the cholesterol in the inner and outer leaves.

$$\begin{aligned}
\mu_{C_i} &= \frac{\partial F_{bi}}{\partial N_{C_i}} = \frac{\partial N_i f_i(y_{PE}, y_{PS}, y_{C_i}, T)}{\partial N_{C_i}} \\
&= \frac{\partial f_i}{\partial y_{C_i}} + f_i - \sum_j \frac{\partial f_i}{\partial y_j} y_j, \qquad j = PE, PS, C_i, \qquad (8) \\
\mu_{C_o} &= \frac{\partial F_{bi}}{\partial N_{C_o}} = \frac{\partial N_o f_o(y_{SM}, y_{PC}, y_{C_o}, T)}{\partial N_{C_o}} \\
&= \frac{\partial f_o}{\partial y_{C_o}} + f_o - \sum_k \frac{\partial f_o}{\partial y_k} y_k, \qquad k = SM, PC, C_o. \qquad (9)
\end{aligned}$$

Again, to determine the six mol fractions, we equate these two chemical potentials, utilize the two constraints

$$\begin{aligned}
\sum_j y_j &= 1 \qquad j = PE, PS, C_i, \qquad (10) \\
\sum_k y_k &= 1 \qquad k = SM, PC, C_o, \qquad (11)
\end{aligned}$$

and set to their experimental values the ratios of SM to PC in the outer leaf, y_{SM}/y_{PC}, of PS to PE in the inner leaf, y_{PS}/y_{PE}, and the total mol fraction of cholesterol in the bilayer, x_C, Eq. (5).

We must now set the parameters of our model. For the ratio of the area per molecule of cholesterol to the area per molecule of the other phospholipids, we take $r_a = 0.6$ because the average area per molecule of phospholipids is on the order of $a = 0.7$ nm^2 and that of cholesterol is about 0.4 nm^2 (16, 20). For the binary interactions, we choose $\epsilon_{SM,C} = -0.58\, k_B T$, $\epsilon_{PC,C} = 0.2\, k_B T$, $\epsilon_{SM,PC} = 0.$, $\epsilon_{PS,C} = -0.06\, k_B T$, $\epsilon_{PE,C} = 0.28\, k_B T$, and $\epsilon_{PS,PE} = 0$. We discuss the selection of these values in the Appendix.

We must also specify the membrane. We assume that it is at a temperature $T = 37°C$. We take the ratios of the components from the data of Zachowski (21). There we find that the SM accounts for 0.22 of phospholipids in the outer leaf and 0.02 in the inner leaf, while PC accounts for 0.20 of the phospholipids in the outer leaf and 0.07 in the inner. For simplicity, we assume that all the SM and PC are in the outer leaf and take the ratio $y_{SM}/y_{PC} = 22/20 = 1.1$. Similarly PS accounts for 0.13 of phospholipids in the inner leaf and 0.02 in the outer, while PE accounts for 0.25 in the inner leaf and 0.08 in the outer. Assuming that all PS and PE are in the inner leaf, we take the ratio $y_{PS}/y_{PE} = 13/25 = 0.52$. Lastly we set the total mol fraction of cholesterol in the bilayer to be $x_c = 0.4$ (22).

It is now straightforward to carry out our program, and we find a single solution of our equations. By examining the matrix of second derivatives of the free energy, we have verified that this solution is stable. The ratio of the number of cholesterols in the inner leaf to the outer leaf is $x_{C_i}/x_{C_o} = 0.37$ so that only 27.2% of cholesterol is in the inner leaf. This is easy to understand from the Boltzmann distribution and the fact that the energy of cholesterol is reduced if it goes to the outer layer where it can interact favorably with the SM concentrated there. It is easy to understand, but clearly not in accord with experiment. What physics is missing?

2.3 Addition of the Bending Energy

It is our hypothesis that what is missing is that cholesterol can ameliorate the bending energy cost of having PE in the cytoplasmic leaf by increasing the order of its tails and thereby reducing its curvature. One knows, of course, that cholesterol increases the chain order of lipids (16), even those with only one unsaturated chain (23). Molecular dynamics simulations of asymmetric bilayers with cholesterol also show the increase in order of the chains (24). If this hypothesis were correct, one would expect that cholesterol would stabilize the lamellar phase of mixtures of PE and cholesterol, which would be reflected in an increase of the transition temperature from the high-temperature inverted hexagonal phase to the low-temperature lamellar one. Experiment shows that whereas the addition of small amounts of cholesterol stabilizes the inverted-hexagonal phase and lowers the transition temperature, the addition of larger amounts, about 0.3 mol fraction, does indeed stabilize the lamellar phase and increase the transition temperature (17). Thus there is an azeotrope in this system beyond which there is more cholesterol in the lamellar phase than there is in the co-existing inverted-hexagonal one.

We incorporate this hypothesis into our model by adding to the free energy of the flat bilayer a bending energy. Because the magnitude of the spontaneous curvatures of SM, PC, and PE are much smaller than that of PE (14), we consider the curvature only of the latter and write the bending energy as

$$\begin{aligned} F_b &= \left(\frac{A_0 + A_i}{2}\right)\frac{\kappa}{2} y_{PE} H_{PE}^2 \\ &= \frac{1}{2}[N_i + N_o - (1 - r_a)(N_{C_o} + N_{C_i})] f_b, \qquad (12) \\ f_b &= \frac{1}{2} a\kappa y_{PE} H_{PE}^2, \qquad (13) \end{aligned}$$

with κ the bending modulus. For the curvature of PE with added cholesterol, we take

$$H_{PE}(y_{C_i}) = H_0 - A y_{C_i} + B y_{C_i}^2, \tag{14}$$

with $H_0 = -0.316\text{nm}^{-1}$ (14), $A = 0.6\text{nm}^{-1}$, $B = 1\ \text{nm}^{-1}$. With these values, the magnitude of $H_{PE}(y_{C_i})$ increases with cholesterol mol fraction up to $y_{C_i} = 0.3$ at which it has a maximum and then begins to decrease.

Our model free energy is now

$$F_{bi} = N_o f_o + N_i f_i + \frac{1}{2}[N_i + N_o - (1 - r_a)(N_{C_o} + N_{C_i})] f_b, \tag{15}$$

with f_i and f_o given by Eqs. (6) and (7) and f_b by Eq. (13). We calculate the chemical potential of the cholesterol in the outer and inner layers. We then set equal the areas of the two leaves, Eqs. (1), after which one obtains

$$\mu_{C_o} = f_o + \frac{\partial f_o}{\partial y_{C_o}} - \sum \frac{\partial f_o}{\partial y_j} y_j + \frac{1}{2} r_a f_b, \qquad j = SM, PC, C_o, \tag{16}$$

$$\mu_{C_i} = f_i + \frac{\partial f_i}{\partial y_{C_i}} - \sum_k \frac{\partial f_i}{\partial y_k} y_k + \frac{1}{2} r_a f_b + r_a y_{C_i} \left[\frac{\partial f_b}{\partial y_{C_i}} - \sum_k \frac{\partial f_b}{\partial y_k} y_k \right], \qquad k = PE, PS, C_i \tag{17}$$

Requiring, as before, that the total mol fraction of cholesterol be given by experiment as well as the ratios of PS to PE in the inner leaf and SM to PC in the outer leaf, we set the chemical potentials of cholesterol in the two leaves to be equal. Taking a bending modulus $\kappa = 65\, k_B T$ (25), we now obtain three solutions of our equations. Of them, the one with the lowest free energy is stable, while the other two are not. The solution with lowest free energy corresponds to a bilayer in which $x_{C_i}/x_{C_o} = 2.23$ so that 69% of the cholesterol is in the inner leaf. The bilayer mol fractions of the other lipids are then $x_{SM} = 0.18$, $x_{PC} = 0.16$, $x_{PS} = 0.09$, and $x_{PE} = 0.17$.

3 Discussion

We have proposed that most of the cholesterol in the plasma membrane should be in the cytoplasmic leaf because that is where the phosphatidylethanolamine is; that by going there in sufficient quantity, the cholesterol reduces the bending energy penalty incurred by the presence of the PE. We incorporated this idea within a simple model and found that it predicts that

69% of the cholesterol should be in the inner leaf. The specific fraction depends, of course, on the interaction parameters that we have taken, but that the majority of the cholesterol is found in the inner leaf does not depend crucially on our particular choice. The fraction also depends upon the bending modulus of the membrane, as we show in Fig. 1, but again for any reasonable value, the majority of the cholesterol is in the inner leaf.

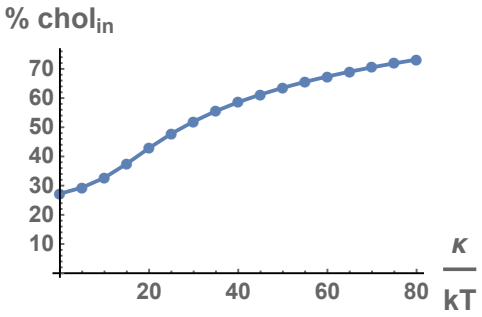

Figure 1: Percentage of the total cholesterol that is in the inner leaf as a function of bending modulus.

Of interest is the manner in which the fraction of the bilayer cholesterol that is in the inner leaf depends upon the total mol fraction of cholesterol in the membrane, x_C. This is shown in Fig. 2. It is clear that the percent of cholesterol in the inner leaf must approach 50% as x_C approaches unity. What is not *a priori* obvious is that the percentage should exhibit a maximum around $x_C = 0.34$. This is not very different from the observed total mol fraction of cholesterol in the plasma membrane, $x_C \approx 0.4$ (22). We also find that for the existence of our solution in which the fraction of cholesterol in the inner leaf is large, the total amount of cholesterol must exceed $x_C \approx 0.26$. This is related to the fact that for the cholesterol to decrease the curvature of the PE, it must be present in sufficient amounts in the inner leaf. This only happens for a sufficiently large total mol fraction of cholesterol, x_C. If x_C is decreased below 0.26, a first-order transition occurs to a solution in which the cholesterol and PE phase separate so as to reduce the bending energy cost. However in this regime, in which there is a tendency to form an inverted-hexagonal phase, additional physics, particularly involving the configuration of the lipid tails, comes into play, physics which is not encompassed by the regular solution theory we have employed (26, 27).

As noted earlier, the hypothesis presented here is consistent with experiments on the effect of cholesterol on the inverted-hexagonal to lamellar phase transition temperature in mixtures of cholesterol and POPE (17).

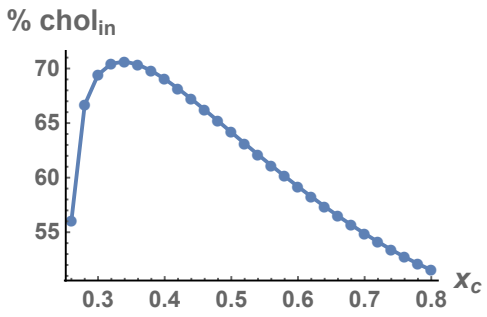

Figure 2: Percentage of the total cholesterol that is in the inner leaf as a function of the total mol fraction of cholesterol in the membrane. The bending modulus is $\kappa/k_BT = 65$.

There are some correlations clearly expected between cholesterol content and the specific form of PE in the membrane. Because the trans double bond in dielaidoylphosphatidylethanolamine is more easily ordered by cholesterol than is a cis double bond (17, 18), one expects the asymmetry in the cholesterol distribution to be less. It would be interesting to know how cholesterol affects a lipid with a polyunsaturated tail such as 18:0-20:4 PE as these polyunsaturated tails make up a non-negligible fraction, perhaps 18%, of the chains of PE in the plasma membrane (28). The effects of our hypothesis can, presumably, be tested in model asymmetric membranes which mimic the plasma membrane (29).

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5 Appendix

The free energy of a binary mixture of molecules of species A, and B, as obtained from regular solution theory is simply the mean-field approximation to the exact free energy obtained from a from a lattice-gas Hamiltonian of form

$$H = \sum_{<ij>} [E_{A,A} n_i^A n_j^A + E_{B,B} n_i^B n_j^B + E_{A,B}(n_i^A n_j^B + n_i^B n_j^A)] \qquad (18)$$

where $n_i^A = 1$ if there is a molecule of species A at the site i and is zero otherwise, and similarly for n_i^B. The sum is over all distinct nearest-neighbor pairs of molecules. This Hamiltonian is easily mapped to that of an Ising model

$$H = -J \sum_{<ij>} S_i S_j - B \sum_i S_i, \qquad S_i = \pm 1 \qquad (19)$$

via the relation $S_i = 2n_i^A - 1 = 1 - 2n_i^B$ so that the presence of an A molecule is related to an up spin, and a B molecule to a down spin. With this mapping, the interaction J of the Ising model is, then, $J = \omega_{A,B}/2$ with $\omega_{A,B} \equiv E_{A,B} - (E_{A,A} + E_{B,B})/2$. The exact transition temperature of the two-dimensional Ising on a triangular lattice is known to be $k_B T_c = 4J/\ln 3 = 2\omega_{A,B}/\ln 3$ (30). Thus if a physical system is known to undergo a phase separation at a critical temperature T, then the interaction parameter in a model including fluctuations should be positive and taken to be $\omega_{A,B} = (1/2)\ln 3 k_B T \approx 0.55 k_B T$.

Regular solution theory, however, does not include fluctuations. Within this theory, the free energy per particle of a binary mixture on a triangular lattice can be written

$$f = 6\epsilon_{A,B} y_A y_B + k_B T(y_A \ln y_A + y_B \ln y_B),$$

It yields a transition $k_B T_c^{rs} = 3\epsilon_{A,B}$. Therefore to obtain in regular solution theory the same transition temperature as the exact result one must choose $\epsilon_{A,B} = (2/3\ln 3)\omega_{A,B} \approx 0.6\omega_{A,B}$.

The values of the interaction parameters, $\omega_{A,B}$ for many pairs of lipids can be estimated from experiment and have been collected in a table by Almeida (31). In particular for the interactions between components of the outer leaf at $T = 37°$C, $\omega_{PC,C} = 0.33 k_B T$, $\omega_{SM,C} = -0.97 k_B T$, and $\omega_{SM,PC} = 0$. As $\omega_{PC,C}$ is positive, we use in our regular solution theory the estimate $\epsilon_{PC,C}/k_B T = 0.6\omega_{PC,C}/k_B T = 0.20$. If we take the ratios of the other interactions parameters to be the same as in the table, *i.e.* $\epsilon_{SM,C}/\epsilon_{PC,C} = \omega_{SM,C}/\omega_{PC,C}$, then $\epsilon_{SM,C}/k_B T = -0.58$ and similarly $\epsilon_{SM,PC}/k_B T = 0$.

The interactions relevant to the inner leaf, those between PE and cholesterol, between PS and cholesterol, and between PE and PS are not included in the table of Almeida. To obtain an estimate for them, we proceed as follows: Cholesterol and PE do not phase separate at $T = 37°$ so that we should

take $\epsilon_{PE,C}/k_BT < 1/3$. We choose $\epsilon_{PE,C}/k_BT = 0.28$. Next we estimate the interaction between PS and cholesterol. Niu and Litman (6) have measured the differences $\Delta_{SM} \equiv \omega_{SM,C} - \omega_{PC,C} = -1181$ cal/mol$=-1.92k_BT$ at 37°C, and $\Delta_{PS} = \omega_{PS,C} - \omega_{PC,C} = -0.65k_BT$ We assume that

$$\frac{\epsilon_{PS,C} - \epsilon_{PC,C}}{\epsilon_{SM,C} - \epsilon_{PC,C}} = \frac{\omega_{PS,C} - \omega_{PC,C}}{\omega_{SM,C} - \omega_{PC,C}} = \frac{0.65}{1.92} = 0.34,$$

so that $\epsilon_{PS,C}/k_BT = \epsilon_{PC,C} + 0.34(\epsilon_{SM,C} - \epsilon_{PC,C}) = -0.06$. Lastly because the tails of PE and PS lipids are similar, we take $\epsilon_{PE,PS}/k_BT = 0$. This completes the specification of the interactions.

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