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TRANSBILAYER MOVEMENT OF CHOLESTEROL IN PHOSPHOLIPID VESICLES UNDER EQUILIBRIUM AND NON-EQUILIBRIUM CONDITIONS

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Summary

1. The exchange of [^{3}H]cholesterol between phospholipid : cholesterol vesicles and an excess of red cell ghosts is examined.

2. Using a number of different phosphatidylcholines, only the cholesterol thought to be associated with the outer half of the bilayer (about 70%) is available for exchange, suggesting that at least at equilibrium the transbilayer movement of cholesterol or "flip-flop", occurs very slowly, if it occurs at all.

3. The rate of exchange of cholesterol between the vesicles and the ghosts is dependent on the nature of the fatty acid chain of the phospholipids, being a function of both the fatty acid chain length and the degree of unsaturation.

4. Under non-equilibrium conditions, when cholesterol is being both exchanged and depleted from the lipid vesicles to red cell ghosts, the previously non-exchangeable vesicle cholesterol becomes available for exchange, suggesting that under these conditions "flip-flop" can occur.

Introduction

The localization of cholesterol in biological membranes is not precisely known and the mechanisms of exchange of cholesterol between membranes are not understood. While it is generally accepted that cholesterol plays an important role as a regulator of membrane fluidity (for reviews see Jain [1]; Bruckdorfer and Graham [2]), the mechanism that governs the distribution of cholesterol in membranes is unknown. The question of how much of the red blood cell cholesterol is exchangeable is still a matter of controversy, with several investigators allowing for only a single pool of cholesterol [3—6], while others have observed significant non-exchangeable portions, leading to the conclusion that membrane cholesterol may exist in two or more distinguishable pools [7,8].

A number of studies have now been attempted to measure the transmembrane movement, or "flip-flop", of cholesterol using a variety of systems. Smith and Green [9] reported a half-time of 70 min at 30°C for the flip-flop of the cholesterol analogue, sterophenol. More recently, Poznansky and Lange [10] and Lenard and Rothman [11] have shown that in dipalmitoyl lecithin : cholesterol sonicated vesicles and in influenza virus membranes, respectively, the half-time for flip-flop of cholesterol is in excess of 6 days, if it occurred at all. In this paper, we examine the effect of varying fatty acid chain length and degree of unsaturation of the phospholipid in lipid vesicles on the transbilayer movement of cholesterol and on the exchange rate of cholesterol between the vesicles and erythrocyte ghosts. Finally, the question of equilibrium and non-equilibrium asymmetries of lipid distribution is approached. Following 1 h of sonication of a preparation of pure phosphatidylcholine and cholesterol, it is assumed that an equilibrium distribution of cholesterol has been established. Under these conditions, the degree of exchangeability of cholesterol is measured. A non-equilibrium situation is then produced by selective depletion of cholesterol from the lipid vesicles, and then the transmembrane distribution of cholesterol is re-examined.

Materials and Methods

[^{3}H]Cholesterol, [^{14}C]cholesterol-oleate and ^{14}C-labelled phospholipids were purchased from New England Nuclear (Boston, Mass.). Cholesterol and synthetic phospholipids, including dipalmitoyl phosphatidylcholine, distearoyl phosphatidylcholine, dimyristoyl phosphatidylcholine and dioleoyl phosphatidylcholine were purchased from Sigma Chemicals (St. Louis, Mo.). Egg lecithin was prepared in our laboratory by silicic acid chromatography according to the technique of Litman [12], and the purity of all phospholipids was checked by thin-layer chromatography. All phospholipids gave a single spot following thin-layer chromatography on precoated silica gel plates in chloroform/methanol/H_2O (65 : 25 : 4, v/v). Dioleoyl phosphatidylcholine was also prepared in our laboratory using the technique described by Robles and van den Bergh [13].

Preparation of lipid vesicles. Lipid vesicles were prepared according to the technique of Huang [14]. 10.6 μmol of phospholipid and 10 μmol of cholesterol were co-lyophilized with 50 μCi of [^{3}H]cholesterol and 10 μCi of [^{14}C]cholesterol-oleate (or 10 μCi of ^{14}C-labelled phosphatidylcholine) and then added to 10 ml of a potassium/Tris buffer (100 mM KCl, 10 mM Tris · HCl) at pH 7.4. The ^{14}C labels were used as non-exchangeable markers. The lipid dispersion was sonicated for 1 h under nitrogen in a temperature-controlled cell at 4°C using a Branson W185 sonicator with a standard probe and a power output of 65 W. The sonicate was then centrifuged for 30 min at 35 000 $\times g$ to remove titanium particles coming off the probe. To obtain completely homogeneous preparations, the vesicles were "sized" by gel chromatography on Sepharose 4B. Over 90% of the lipid phosphorous in the sonicate was made up of "fraction II" homogeneous vesicles [14] and therefore sonicates were occasionally used without further sizing. The results were virtually identical whether sized or unsized vesicles were used. All of the radioactivity present in the sonicate eluted with the vesicles.

Preparation of red cell ghosts. Red blood cell ghosts were prepared according to the technique of Dodge et al. [15] with no attempt being made at resealing. Cholesterol-depeleted ghosts were made by incubating ghosts with a 60-fold excess of egg lecithin (phosphatidylcholine) vesicles for 24 h at 37°C in the presence of cholesterol-depleted plasma [16] and 10^{-6} M streptomycin sulphate. The ghosts were then washed 3—4 times in a KCl/Tris buffer at pH 7.4. ^{14}C-labelled phospholipid or ^{14}C-labelled cholesterol-oleate were added to the vesicles as a non-exchangeable marker. Vesicles and ghosts were easily separated by centrifugation at 15 000 × *g* for 5 min. Following washings, no detectable radioactivity from the non-exchangeable marker was detected in the ghost preparation. Lipids were extracted by chloroform/methanol from both the vesicle and ghost preparations. Lipid phosphorous was determined by standard procedures [17] and cholesterol was measured by digitonin precipitation [18] or else by enzymatic determination using cholesterol oxidase (Calbiochem, Palo Alto, Calif.).

Exchange of [^{3}H]cholesterol between lipid vesicles and red cell ghosts. 100 μl of vesicles (containing 0.10 μmol of cholesterol, 0.106 μmol of phospholipid, 0.5 μCi of [^{3}H]cholesterol and 0.10 μCi of [^{14}C]cholesterol-oleate in KCl/Tris buffer, pH 7.4) were incubated with 5 ml of red cell ghosts (containing 5.25 μmol of cholesterol and 5.84 μmol of phospholipid) at 37°C in a shaking water bath. At appropriate time intervals 0.3-ml aliquots were taken and centrifuged for 6 min at 15 000 × *g* in an Eppendorf 3200 centrifuge. 0.1 ml of supernatant was counted for ^{3}H and ^{14}C in Bray's solution and the pellet was washed three times with potassium/Tris buffer. The pellet was dispersed and the radioactivity determined. Only trace amounts of non-exchangeable [^{14}C]-cholesterol-oleate was ever detected in the ghost pellet, and the disappearance of [^{3}H]cholesterol from the vesicles in the supernatant always closely paralleled the appearance of ^{3}H label in the ghost pellet. The degree of sticking of the vesicles to the ghosts was always less than 5%.

Results

We have previously shown that only about 72% of cholesterol present in unilamellar dipalmitoyl phosphatidylcholine : cholesterol vesicles is available for exchange with an excess of acceptor membrane [10]. This correlates well with the outside : inside distribution of phospholipid molecules in dipalmitoyl phosphatidylcholine : cholesterol vesicles [19,20] and strongly suggests that the cholesterol on the inner half of the bilayer is not available for exchange. An analysis of the kinetics of exchange yielded the result that the transmembrane movement or "flip-flop" of cholesterol in dipalmitoyl phosphatidylcholine : cholesterol vesicles is an extremely slow process ($T_{1/2}$ in excess of 6 days), if it occurs at all * [10]. Fig. 1 indicates that the non-exchangeability of a fraction

* In a previous preliminary report (see Poznansky and Lange (1976) Nature 259, 420—421) we used a separate technique to demonstrate a non-exchangeable cholesterol pool in lipid vesicles in order to avoid the long-term co-incubation of ghosts and vesicles. This involved labelling the entire vesicle uniformly with [^{3}H]cholesterol during its production and subsequently pulse labelling the formed vesicles with [^{14}C]cholesterol from labelled red cells. Analysis of ^{3}H/^{14}C ratios of cholesterol exchanging from vesicles to unlabelled ghosts indicated a 30% non-exchangeable ^{3}H fraction corresponding to the inner half of the vesicle bilayer. This confirmed the results with simple exchange methods.

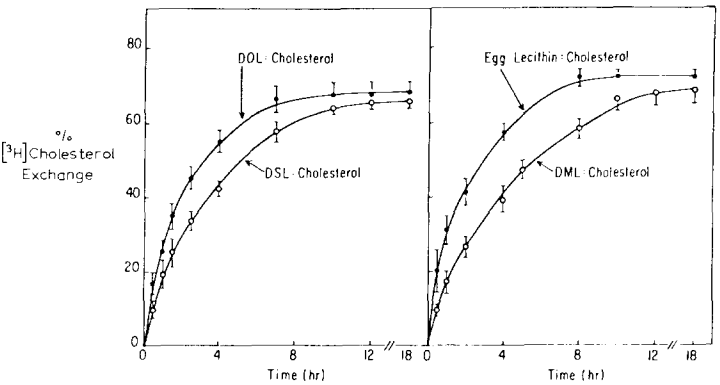

Fig. 1. Exchange of [^{3}H]cholesterol from dioleoyl phosphatidylcholine (DOL) : cholesterol, distearoyl phosphatidylcholine (DSL) : cholesterol, dimyristoyl phosphatidylcholine (DML) : cholesterol and egg lecithin : cholesterol vesicles into red blood cell ghosts. Percent cholesterol exchanged refers to the amount of [^{3}H]cholesterol transferred to the ghosts (acceptor membrane) which were present in a 55-fold excess. No net movement of cholesterol is observed either to or from the vesicles. No significant increase in percent cholesterol exchanged takes place between 18 and 48 h. The disappearance of [^{3}H]-cholesterol from the vesicles in the supernatant is exactly paralleled by its appearance in the ghost pellet.

of vesicle cholesterol also holds for phospholipid : cholesterol vesicles made with dioleoyl phosphatidylcholine, distearoyl phosphatidylcholine, dimyristoyl phosphatidylcholine and egg lecithin. In all cases only 66—72% of the vesicle cholesterol is exchangeable. While this is in excellent agreement with the outside to inside phospholipid distribution of these lecthins, it contradicts the calculations of de Kruijff et al. [20] used to determine the outside : inside distribution of cholesterol in a number of phospholipid vesicle systems. Their calculations assume that the inner half of the bilayer can assume a lipid composition with a mol ratio of cholesterol to phospholipid far in excess of 1. While this type of asymmetric distribution of cholesterol has not been ruled out, it has been assumed for some time that artificial bilayer membranes cannot easily be made with cholesterol : phospholipid mol ratios in excess of 1 : 1 [21]. In recent years there have been reports [22,23] that under certain conditions it may be possible to produce artificial membranes with cholesterol : phospholipid mol ratios as high as 2 : 1. Fig. 1 and Table I tend to support the hypothesis that the distribution of cholesterol on either side of the bilayer closely follows that of the phospholipid, at least when they are present in equimolar amounts.

Two pools of cholesterol only one of which is readily exchangeable with ghosts, could result from inhomogeneities in the vesicle population. Therefore we fractionated the sonicated vesicles by gel filtration on Sepharose 4B. A profile of one of the fractionations is shown in Fig. 2. Over 90% of the lipid phosphorous in the sonicate was made up of "fraction II" homogeneous vesicles [14]. 95% of the ^{3}H counts present in the sonicate eluted with the "fraction II" vesicles. The results of the exchange experiments were virtually identical whether sized or unsized vesicles were used, and it can be concluded that the non-exchangeable cholesterol pool was not a manifestation of vesicle inhomogeneity or the presence of multilamellar liposomes.

TABLE I

EXCHANGE OF CHOLESTEROL BETWEEN PHOSPHOLIPID VESICLES AND RED CELL GHOSTS

$T_{1/2}$ for exchange of cholesterol and percentage of exchangeable cholesterol between cholesterol : phospholipid vesicles and red cell ghosts as a function of fatty acid chain length and degree of unsaturation.

Phospholipid	$T_{1/2}$ * (h)	Exchangeable cholesterol (%)
Dimyristoylphosphatidylcholine	3.25	68 ± 2
Dipalmitoylphosphatidylcholine	3	72 ± 2
Distearoylphosphatidylcholine	2.5	66 ± 3
Dioleoylphosphatidylcholine	1.5	68 ± 4
Egg lecithin	1.25	72 ± 4

* In all reported experiments the ratio of vesicle lipid to ghost lipid was 1 : 55 and the concentration of ghosts per ml incubation mixture was kept constant. No net movement of cholesterol to or from the vesicles was detected. The $T_{1/2}$ for exchange represents the time required for 50% of the exchangeable cholesterol to move from vesicles to ghosts. In all experiments the ratio of vesicle phospholipid to cholesterol was 1.06 : 1.00 while the ghost ratio was 1.10 : 1.00. Human erythrocyte ghosts were used throughout these experiments.

While all phospholipid : cholesterol vesicles studied exhibited a pool of non-exchangeable cholesterol, the rates of exchange between vesicles and ghosts varied significantly as a function of fatty acid composition of the phospholipid. Exchange from vesicles containing unsaturated fatty acids was much faster than exchange from vesicles containing saturated fatty acids, and amongst the phospholipids containing saturated fatty acid side chains there was a distinct correlation between chain length and exchange rate. The exchange from dimyristoyl phosphatidylcholine : cholesterol vesicles was the slowest, followed by dipalmitoyl phosphatidylcholine and distearoyl phosphatidylcholine vesicles. The differences in exchange rates are illustrated in Table I.

In an attempt to better understand the mechanism of exchange, the temperature dependence of cholesterol exchange between vesicles and ghosts was examined. Fig. 3 shows Arrhenius plots for [^{3}H]cholesterol exchange between dioleoyl phosphatidylcholine : cholesterol vesicles and ghosts and between dipalmitoyl phosphatidylcholine : cholesterol vesicles and ghosts. Not only do the exchange rates differ significantly, the apparent activation energies differ by a factor of 2. While this indicates that the saturated fatty acid chains produce a greater barrier for the exchange process from the vesicles, it offers little in the way of explanation for the exchange process (see Bruckdorfer and Graham [2]). This correlates well with other studies involving saturated fatty acid chains in either myelin phospholipids [24] or lipoproteins [25].

Singer [26] has discussed the origin of lipid asymmetries and questioned whether these represent equilibrium or non-equilibrium states. For phospholipids this is an important question in attempting to ascertain the origin of phospholipid asymmetries. In the case of cholesterol where the evidence for asymmetric distribution is not so strong [27,28], it may be that the cholesterol is in an equilibrium distribution without the need for or presence of any flip-flop. To test this hypothesis we attempted to deplete dioleoyl phosphatidylcholine : cholesterol vesicles of cholesterol by incubating them with cholesterol-depleted ghosts. In Fig. 4 the exchangeable pool of [^{3}H]cholesterol was first exchanged from dioleoyl phosphatidylcholine : cholesterol vesicles by incuba-

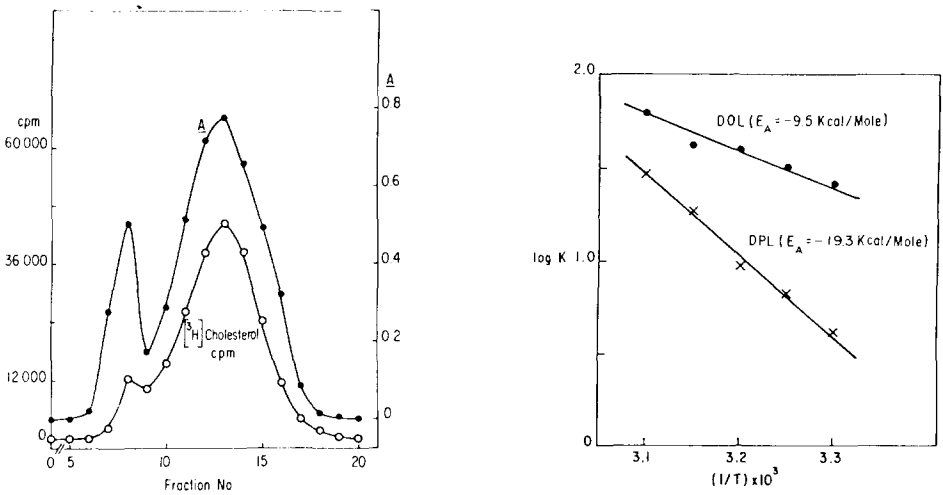

Fig. 2. Gel chromatography of dioleoyl phosphatidylcholine : cholesterol vesicles on Sepharose 4B. Absorbance (●———●) is monitored at 280 nM and ^3H counts (○———○) are determined in Brays solution. The void volume is determined using Dextran Blue and occurs at Fraction No. 7. "Fraction II" vesicles [14] are those falling between Fractions Nos. 12 and 16.

Fig. 3. Arrhenius plot for determination of E_A or energy of activation for the cholesterol exchange process between cholesterol : phospholipid vesicles (●———●, dioleoyl phosphatidylcholine (DOL)), (X———X, diplamitoyl phosphatidylcholine (DPL)) and red blood cell ghosts. The log of the exchange rate is plotted as a function of the inverse of the temperature and the activation energy derived from a form of the Arrhenius equation: $\log k_2/k_1 = E/2.303\ R \cdot (T_2 - T_1)/(T_1 \cdot T_2)$. The exchange rate is defined as the percent [^{3}H] cholesterol exchanged from the vesicles in 1 h. The mol ratio for phospholipid to cholesterol is identical for both dioleoyl phosphatidylcholine and diplamitoyl phosphatidylcholine vesicles (1.06 : 1.00) and the ghost phospholipid is present in a 55-fold excess in both cases.

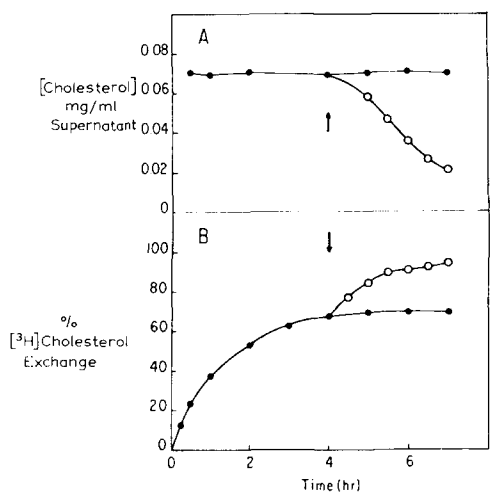

Fig. 4. The exchange of [^{3}H]cholesterol from dioleoyl phosphatidylcholine : cholesterol vesicles into red cell ghosts is measured as a function of time (B). The net movement of cholesterol is also monitored by measuring vesicle cholesterol concentration in the supernatant (A). At 4 h the vesicles were separated from the ghosts and re-incubated with either fresh ghosts (●———●) or with cholesterol-depleted ghosts (○———○) as described in the text. Cholesterol exchange and vesicle cholesterol content were monitored. These curves represent one experiment typical of three. The percentage of cholesterol drop in the supernatant correlated well with the exchange of the previously non-exchangeable cholesterol.

tion with normal ghosts having a cholesterol : phospholipid mol ratio of 1.00 : 1.06. The vesicles were then removed from the normal ghosts and incubated with cholesterol-depleted ghosts having a cholesterol : phospholipid ratio of 0.25 : 1.0. Two important observations can be made: (1) the vesicles have a net loss of cholesterol amounting to about 75% of the total cholesterol, and (2) a significant portion of the previously non-exchangeable cholesterol was removed from the vesicles. If the vesicles were re-incubated with normal ghosts instead of cholesterol-depleted ghosts, no net loss of vesicle cholesterol was observed, and no additional exchange of [^{3}H]cholesterol from the vesicles could be detected.

Discussion

In this paper the observation [10] that only 70% of the cholesterol in sonicated dipalmitoyl phosphatidylcholine : cholesterol vesicles is available for exchange has been extended to a series of lecithins varying in fatty acid composition. These results suggest that at equilibrium, in the absence of any net movement of cholesterol between vesicles and ghosts, there is a non-exchangeable pool of cholesterol that corresponds to the cholesterol on the inner half of the bilayer. Locating this non-exchangeable pool on the inner half of the bilayer assumes that the cholesterol : phospholipid mol ratios on either side of the bilayer are identical when mol ratios are maintained close to unity. Our results indicate, however, that once cholesterol is removed from the vesicles by incubation with cholesterol-depleted ghosts, previously inaccessible cholesterol becomes available for exchange. This suggests that the transbilayer movement or "flip-flop" of cholesterol can occur under non-equilibrium conditions.

The dependence on fatty acid composition of the exchange rate of cholesterol between vesicles and ghosts is consistent with the difference in the interaction between cholesterol and phospholipids of different fatty acid composition [25]. de Kruijff et al. [29] first described the non-random distribution of cholesterol in phosphatidylcholine bilayers by showing that cholesterol interacted preferentially with phospholipids containing shorter-chain fatty acids. Because the phase transition of the phospholipid bilayers as detected by differential scanning colorimetry is abolished when the amount of cholesterol exceeds 35 mol %, it was not possible to predict any preferential interaction at 50 mol %. In fact, at this mol ratio of cholesterol : phospholipid, it may not be possible for phase separations to occur if we assume that under normal conditions the mol ratio does not exceed 1 : 1. The hydrophobic interactions between cholesterol and phospholipids may still depend on the nature of the fatty acid component and thus affect exchange rates.

The fact that exchange of cholesterol between membranes appears to occur much more readily than transmembrane movement is somewhat puzzling. Based on polarity arguments alone, one might expect that it would be easier to solubilize a single hydroxyl group and allow for transbilayer movement or "flip-flop" than it would be to get the entire hydrophobic core of the cholesterol molecule to leave the bilayer, enter into an aqueous phase and exchange into another membrane. The lack of cholesterol flip-flop suggests that forces other than polarity prevent transbilayer movement of cholesterol and it

may be that hydrophobic interactions with neighbouring phospholipids, in addition to the positioning of the hydroxyl group, may provide this restriction. This view is further supported by the large difference in activation energies for exchange of cholesterol from dioleoyl phosphatidylcholine vesicles to ghosts as compared to dipalmitoyl phosphatidylcholine vesicles. To better understand the process of cholesterol exchange between membranes, several control experiments were performed. In order to rule out the possibility that some carrier molecule was involved, we put the vesicles and ghosts in individual compartments separated by a Millipore filter with a pore diameter of 100 Å. The two chambers were monitored over a 96-h period and no [^{3}H]cholesterol from the vesicle pool appeared in the ghosts. This suggests that in order for cholesterol exchange to take place, contact must be made between the vesicle and ghost membranes. Furthermore, in other experiments it was found that the addition of excess buffer to the incubation medium of vesicles and ghosts decreases the rate of cholesterol exchange to a degree that can be accounted for by a decrease in collision frequency. The addition of heat-treated plasma (to destroy enzyme activity) or albumin is without effect on the rate of exchange. We have previously reported the use of albumin to avoid problems of vesicles sticking to the red cell ghosts [10]. This was found to be unnecessary in this series of experiments as loss of non-exchangeable marker from the supernatant was consistently under 5%. Control experiments were also carried out to determine whether the vesicles remained intact during the incubation with ghosts. Cholesterol : phospholipid vesicles were prepared containing either [^{14}C]-glucose or [^{14}C]glycerol phosphate and then incubated with ghosts for varying times. The leakage of entrapped marker from the vesicles was determined by separation on Sephadex G-50. The glucose leaked out of the vesicles with the expected time constant [30], whereas all of the glycerol phosphate remained with the vesicles even after a 12-h incubation. This is strong evidence indicating the integrity of the vesicles during the incubation and cholesterol exchange experiment.

In summary, our experiments indicate that: (a) under conditions of simple cholesterol exchange and in the absence of any net movement of cholesterol, only the cholesterol from the outer half of the bilayer is available for exchange, implying that flip-flop of cholesterol occurs extremely slowly, if at all; (b) the process of cholesterol exchange requires the two membranes to come into close contact and is dependent on the nature of the fatty acid chains with respect to chain length and degree of unsaturation; and (c) if cholesterol is removed from the outer half of the vesicle then flip-flop of cholesterol can occur from the inner half in response to this perturbation.

In addition to suggesting that cholesterol flip-flop can occur under non-equilibrium conditions, the results suggest that caution must be used in interpreting exchange experiments to determine pool sizes. Unless care is taken to assure that the equilibrium condition is maintained by avoiding net movement of cholesterol, non-exchangeable pools of cholesterol may go unrecognized. The significance of phospholipid or cholesterol flip-flop or the lack thereof in small lipid vesicles deserves consideration. It appears that under equilibrium conditions lipids do not easily undergo transbilayer movement in these vesicles [31—33]. On the other hand, phospholipids and cholesterol have been shown

to undergo transmembrane movement in red cells [34—36] although in each case the measurement required some sort of membrane perturbation in the form of ghost formation or phospholipase treatment.

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