Comments to the Editor

How Slow Is the Transbilayer Diffusion (Flip-Flop) of Cholesterol?

Garg et al. (1) recently used time-resolved small-angle neutron scattering to analyze the rate of passive transfer of cholesterol between phospholipid bilayer vesicles of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine made invisible by contrast matching. This elegant approach has the advantage of using the natural form of the sterol in a nonperturbing assay system. Their values for the half-time of intermembrane cholesterol transfer (namely, 88 min at 50°C) and its enthalpy of activation (101 kJ/mol) are in good agreement with earlier studies using other techniques. However, the surprising—indeed, newsworthy (2)—feature of this report was their inference that the transverse (i.e., transbilayer or intramembrane) diffusion (flip-flop) of cholesterol in the bilayer has a half-time of 200 min at 50°C. This value disagrees with those obtained by other techniques, where upper bounds for the half-time were on the order of minutes, seconds, and even milliseconds, estimates limited by the time-resolution of the experimental method (3–5). Molecular dynamics simulations also predict an upper bound of milliseconds (6). We therefore offer some thoughts as to why 200 min may not be an accurate estimate of the half-time of cholesterol flip-flop in phospholipid bilayers.

The slow values for the flip-flop step inferred by Garg et al. (1) were obtained by fitting their time courses for intervesicle cholesterol transfer to a two-process model. The authors do not justify using a two-exponential model except to say that it "provides a much better description of the data" than a single exponential rate process. However, any fit of rate process data might well be improved by employing a higher exponential expression. Presenting a single exponential fit in the figure for visual comparison or giving a statistical treatment would have been reassuring, because it is not clear from the presentation that there is actually more than one detectable cholesterol pool.

The authors reasoned that "If the flipping rate is very fast compared to the exchange rate one would only measure one decay process. However, if it is much slower, the cholesterol in the outer monolayer will deplete before that in the inner monolayer can replenish the reservoir leading to two distinct decay times." However, evidence for two distinct decay times does not necessarily mean that one of them corresponds to cholesterol flip-flop. Cholesterol flip-flop could be too fast to detect by their method, and the second, slow process they observe could reflect heterogeneity in intermembrane transfer kinetics.

Some of their data undercut the authors' inferences regarding a slow flip-flop rate for cholesterol:

1. Except for cholesterol sulfate, where very slow flip-flop seems plausible, the fast and slow transfer processes were similar, consistent with their both reflecting intermembrane transfer processes.
2. Their inference that cholesterol sulfate traverses the hydrophobic barrier of the bilayer at about the same rate as cholesterol seems inconsistent with the large difference in the polarity of the two headgroups.
3. It also seems puzzling that the energy of activation for flip-flop should be almost as great as that for intermembrane transfer. Values for the activation energy of cholesterol flip-flop are not available because of the difficulty in measuring such a fast process at convenient temperatures. However, one recent simulation study predicts that the energy of activation is much lower than the authors suggest (6). So, the two high activation energy values may both reflect intermembrane cholesterol transfer processes.
4. Similarly, it seems less likely that cyclodextrin speeds cholesterol flip-flop than that it facilitates intermembrane cholesterol transfer.

A two-component donor population could mean that a fraction of the vesicles were multilamellar. This would explain why the fast and slow transfer processes varied in parallel in most of the tests, because both of these processes involve intermembrane transfer reactions. To avoid multilamellar donors, small unilamellar vesicles could be made by sonication.

Another possible mechanism for a nonfirst-order time course is that the rate constant for intervesicle cholesterol transfer might decrease with the donor cholesterol/phospholipid ratio during the transfer process because the chemical activity of the donor cholesterol declined with its mole fraction in the bilayer. This effect has been observed (7). It is conceivable that such slowing could be prevented by adding deuterated cholesterol to the acceptor vesicle compartment so that the chemical activity of donor cholesterol is maintained by replenishment during the transfer reaction from "invisible" cholesterol in the acceptor. However, this might be technically difficult.

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The formulation used by the authors to extract rate constants from their experiments stipulates that, at equilibrium, the cholesterol is distributed equally between the two bilayer leaflets in both the donor and the acceptor. This constraint prevents them from estimating the relative abundance of cholesterol in the two donor bilayer leaflets. We wonder whether the two putative leaflet pool sizes in the donor vesicles and the apparent first-order exponents for the two transfer steps might be obtained by fitting the data to a simple biexponential expression, because this would apply whether or not the two processes proceed in parallel or sequentially (see pages 114–117 in (8)). Significant differences in the measured sizes of the two cholesterol donor pools and/or substantial variance in these relative sizes among experiments would suggest that the slow donor compartment does not correspond to the inner leaflet of the bilayers.

The authors do not comment on the frequent reports that cholesterol and related sterols introduced into the outer leaflet of plasma membranes move on a timescale of minutes to intracellular compartments such as mitochondria and endoplasmic reticulum (9). This observation is at odds with their inference of a half-time for its bilayer flip-flop of >3 h at 50°C. If the authors are right, their findings would make a very important prediction: that there might exist facilitators for cholesterol transport across the plasma membrane bilayer. The rapid flip suggested by other recent studies obviates the need for such a facilitator.

Thus, the authors' data might signify that cholesterol flip-flop is very rapid and was therefore not detected by their approach. In that case, the inferred biexponential process could reflect two parallel intervesicle transport steps.

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