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Stability and Stoichiometry of Bilayer Phospholipid–Cholesterol Complexes: Relationship to Cellular Sterol Distribution and Homeostasis

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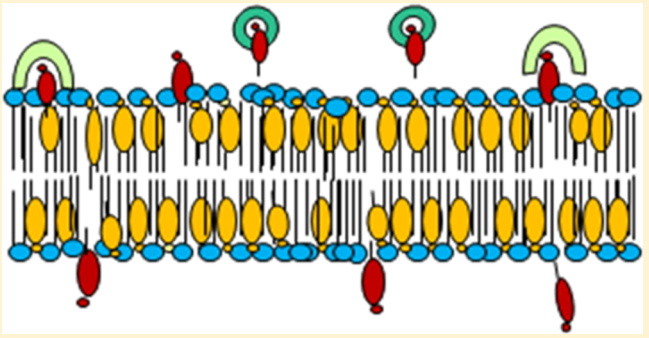
ABSTRACT: Is cholesterol distributed among intracellular compartments by passive equilibration down its chemical gradient? If so, its distribution should reflect the relative cholesterol affinity of the constituent membrane phospholipids as well as their capacity for association with the sterol. We examined this issue by analyzing the reactivity to cholesterol oxidase of large unilamellar vesicles (LUVs) containing phospholipids and varied levels of cholesterol. The rates of cholesterol oxidation differed among the various phospholipid environments by roughly 4 orders of magnitude. Furthermore, accessibility to the enzyme increased by orders of magnitude at cholesterol thresholds that suggested cholesterol:phospholipid association ratios of 1:1, 2:3, or 1:2 (moles:moles). The accessibility of cholesterol above these thresholds was still constrained by its particular phospholipid environment. One phospholipid, 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphatidylserine, exhibited no threshold. The analysis suggested values for the stoichiometries of the putative cholesterol–phospholipid complexes, their relative stabilities, and the fractions of bilayer cholesterol not in complexes at the threshold equivalence points. Predictably, the saturated phosphorylcholine species had the lowest apparent stoichiometric ratios and the strongest associations with cholesterol. These results are in general agreement with the equilibrium distribution of cholesterol between the various LUVs and methyl-β-cyclodextrin. In addition, the behavior of the cholesterol in intact human red blood cells matched predictions made from LUVs of the corresponding composition. These results support a passive mechanism for the intracellular distribution of cholesterol that can provide a signal for its homeostatic regulation.



Sterols and phospholipids are nonuniformly distributed among the organelles of eukaryotic cells.[1,2] Cholesterol is most enriched in the plasma membrane where it serves to condense and order the polar lipids, thereby thickening, stiffening, and strengthening the bilayer and reducing its passive permeability to small molecules even while increasing its fluidity.[3−7] The lipids in the membranes along endocytic pathways resemble those of the plasma membrane because they share its bilayer constituents through vesicular traffic to-and-fro. The intracellular membranes, and the ER in particular, are demonstrably sterol-poor.[1,8,9] Cholesterol circulates within the cell on a time scale of several minutes, presumably mediated by a combination of simple diffusion, collisional transfers, and carrier proteins.[10−13] The means by which cholesterol is apportioned to the organelles is not known. It could be distributed by passive equilibration down its chemical potential gradient or by energized, targeted transport.[10,13−15] In the former case, the localization of cell cholesterol would depend upon its affinity for the diverse organelle phospholipids. These affinities are a function of the length of the phospholipid apolar chains, their degree of unsaturation, and, to a lesser extent, the polar headgroups.[4,16−24] There is also evidence that at least some phospholipids associate dynamically with sterols in specific ratios; these molecular associations have been described as stoichiometric complexes, a terminology we adopt here.[20,25−27] Such complexes might additionally associate into higher-order oligomers of varied size[26,27] and be the basis for the formation of microdomains or rafts.[20,28]

The apparent stoichiometries of the putative sterol–phospholipid complexes are on the order of ∼1:1 to 1:3, i.e., CMFs of 0.50−0.25. Cholesterol in excess of this complexing capacity would remain dissolved in the bilayer with a weaker phospholipid affinity, i.e., a higher chemical activity, leaving tendency, and/or reactivity, which we term simply its activity.[10,16,26,29] The consequent increased projection of the super-threshold sterol into the aqueous compartment would enhance its accessibility to soluble ligands and probes.[13,16,30,31]

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This heightened exposure presumably underlies the sharp rise in the rate of exit of membrane sterols when their level exceeds a threshold taken to be stoichiometric equivalence with the phospholipids.[29,32−34] Also consistent with this premise is the observation that the binding of the bacterial toxin perfringolysin O to membranes increases dramatically when their cholesterol content exceeds a sharp threshold.[35−37]

Cells rigorously maintain their overall cholesterol levels through diverse feedback pathways. Super-threshold cholesterol in the ER and mitochondria could be the signal that elicits homeostatic responses through associations with regulatory proteins therein.[13,16,29] However, our knowledge of the abundance, composition, cholesterol binding affinity, and stoichiometry of the organelle bilayer phospholipids is not sufficient to establish whether the intracellular distribution and homeostasis of cholesterol are governed by the passive thermodynamic mechanism mentioned above. Furthermore, most of the support for the concept of complexes comes from experiments using monolayer films of a few saturated phospholipids at low temperature and surface pressure, hence, of uncertain applicability to biological systems.

We have therefore examined the behavior of cholesterol in bilayers of several relevant phospholipids. In one approach, we inferred the relative affinity of phospholipids for the sterol from its equilibrium distribution between LUVs and MBCD. In addition, we used cholesterol oxidase to probe cholesterol-containing LUVs because the activity of this enzyme is highly sensitive to the molecular environment of its substrate (see Discussion and refs 38−41). In particular, it appears that the association of cholesterol with bilayer phospholipids limits its availability and that the enzyme acts preferentially on sterol molecules not complexed with membrane phospholipids.[16,32,42−47] Supporting this supposition are observations that a variety of membrane-intercalating amphipaths stimulate cholesterol oxidase activity, apparently by displacing the sterol from its association with phospholipids.[48−50]

This study addressed the following questions. (a) Does cholesterol associate with all types of bilayer phospholipids in characteristic ratios? (b) What are the stoichiometries and the relative stabilities of these putative complexes? (c) Does the accessibility of the cholesterol at the surface of bilayers increase when the cholesterol exceeds the complexing capacity of the phospholipids? (d) Do the stoichiometries and affinities of the complexes support a passive distribution of cholesterol in cells? (e) Could cholesterol in excess of the capacity of the phospholipids serve as a homeostatic feedback signal?

EXPERIMENTAL PROCEDURES

Materials. Cholesterol was obtained from Steraloids. Phospholipids were from Avanti Polar Lipids, and [4-^{14}C]-cholesterol was from Perkin-Elmer. HPCD (average MW = 1396), MBCD (average MW = 1310), and Taloxapol (Triton WR-1339) were purchased from Sigma-Aldrich. The Amplex Red Cholesterol Assay kit was from Life Technologies. Cholesterol oxidase (EC 1.1.3.6, *Streptomyces* sp., reported activity of 43.9 units/mg of protein) was obtained from EMD; it was dissolved in PBS (pH 6.6) and stored frozen in aliquots that were thawed once before being used. The BCA protein assay kit was from Pierce. Avanti Polar Lipids supplied the Mini-Extruder and its holder/heating block as well as the Nuclepore extrusion filter membranes (200 nm, Whatman catalog no. 800281) and 10 mm filter supports (catalog no. 610014). Centrifugal filters (Amicon Ultracel, 0.5 mL, 100 K) were used without prior washing.

LUV Preparations. Organic solutions of phospholipids and cholesterol were mixed, dried under N_2, dissolved in ethanol at 37 °C, suspended in HBS to 1.0−1.6 mM total lipid by vigorous vortex mixing at 37 °C, incubated for 30 min at a temperature ≥10 °C above the melting temperature of the phospholipid, and passed 13 times through a pair of filters in an LUV extruder kept on a heating block at the corresponding temperature.[51] Large particulates were cleared by centrifugation.

RBC Preparation. Fresh human blood was stored on ice, and aliquots of erythrocytes were washed in HBS.[52] We determined that 1 μL of packed cells contained ∼1.0 × 10^7 cells with ∼2.5 nmol of cholesterol and ∼3.1 nmol of phospholipid P. To increment their cholesterol content, red cells were incubated at 37 °C for 30 min with 5% (w/v, ∼43 mM) HPCD carrying various amounts of cholesterol (typically, 0.003−0.01 mol/mol) and then washed. The HPCD−cholesterol complexes were kept at room temperature to prevent cholesterol precipitation.

General Analytical Procedures. Cholesterol was determined with a cholesterol oxidase-Amplex Red assay kit or by HPLC.[53] Phospholipid P was determined by phosphomolybdate colorimetry on organic extracts.[54] Protein was analyzed using the BCA method with bovine serum albumin as the standard. All assays were conducted in at least duplicate.

Cholesterol Oxidase Kinetics. The effect of the cholesterol content of membranes on the activity of cholesterol oxidase was assayed in 100 μL of PBS (pH 6.6) at 37 °C in microtiter wells. Mixtures contained 36 μM Amplex Red, 1.2 units of horseradish peroxidase, cholesterol oxidase, and LUVs bearing ∼1−6 nmol of phospholipid and varied proportions of cholesterol. Because the reactions proceeded at widely different specific rates, the amount of cholesterol oxidase was varied between 0.0002 and 2 IU to achieve half-times in the range of 5−60 min. The product was measured at intervals in a Victor 3 spectrophotofluorimeter. We assume that cholesterol flip-flop was fast on this time scale and therefore not rate-determining.[55] First-order oxidation of cholesterol went rapidly to completion in some systems, indicating that the LUVs were unilamellar. We verified that the reaction rate in representative experiments varied linearly with the concentration of enzyme and phospholipid and was not limited by the Amplex Red cocktail. Relative accessibilities of cholesterol (RA, expressed in arbitrary units) were calculated from reaction rates according to eq 6 in Appendix 1.

The cholesterol oxidase reaction for intact erythrocytes variously enriched in cholesterol was performed as described for the LUVs, except for the following. Reactions were performed with 0.2 IU of cholesterol oxidase for 10 min at 37 °C on 12.5−25 μL of packed cells (∼30−60 nmol of phospholipid P) in final volumes of 100 μL of PBS (pH 6.6) in microfuge tubes. Reactions were stopped with 1 mL of 2-propanol; this precipitated the proteins and extracted the lipids. HPLC was used to analyze residual cholesterol and the reaction product, cholest-4-en-3-one. From the release of hemoglobin to supernatants (measured by its optical absorbance at 415 nm) in control experiments, we showed that the extent of hemolysis was <5%.

Equilibrium Distribution of Cholesterol between LUVs and MBCD. We adapted a published procedure to estimate the relative cholesterol affinity of the phospholipids.[19] Reaction mixtures contained 10 mM MBCD bearing 0.02−0.8 mM [^{14}C]cholesterol. The LUV phospholipid was varied between 0.1 and 0.8 mM. We included 0.3% Triton WR-1339 to reduce the

adsorption of [^{14}C]cholesterol-MBCD to the filters; the agent did not disrupt the LUV or alter the partition of cholesterol. Equilibrations were performed in HBS at 25 or 37 °C for 0.5 h. Separation of mixtures was performed by centrifugal filtration close to the incubation temperature for 10 min at 12000*g* (25 °C) or 14000*g* (37 °C). The distribution of the sterol was calculated from the radioactivity in the filtrate and input mixtures. On the basis of controls lacking LUV, we corrected for a fairly constant ~10% loss of MBCD-[^{14}C]cholesterol to the filter; on the basis of controls lacking MBCD, we corrected for a fairly constant ~2% escape of LUV [^{14}C]cholesterol into the filtrate. (We assumed that the binding of MBCD-[^{14}C]cholesterol to the LUV was negligible.) In a few cases, we verified the radioisotope distribution method by directly determining chemically the cholesterol:phospholipid mass ratio in the retained LUV fraction.

The distribution of [^{14}C]cholesterol between MBCD and erythrocytes was determined using the method described above with the following changes. The MBCD (10 mM) was labeled with a trace of [^{14}C]cholesterol and incubated at 37 °C for 1 h with erythrocytes (~100−400 μL of packed cells containing ~0.3−1.2 μmol of phospholipid P) enriched to contain ~0.25−1.0 μmol of cholesterol. After centrifugation, the radioactivity and leaked hemoglobin in the supernatant were determined. The pelleted cells were washed and extracted with 2-propanol to precipitate the proteins and recover the radioactivity, cholesterol, and phospholipid for analysis. The extent of hemolysis was <4%.

RESULTS

Reaction of Cholesterol Oxidase with LUV Cholesterol. Representative time courses of the oxidation of the cholesterol in various phospholipid LUVs are shown in Figure 1. The relative accessibility to the enzyme (RA) of the cholesterol in each LUV preparation was calculated from the initial rates of reaction according to eq 6 of Appendix 1. RA values for 10 phospholipids are plotted as a function of LUV CMF in Figure 2. RA values were found to range over 6 orders of magnitude, the slowest being for DPPC LUV with a low cholesterol content and the fastest for DOPC LUV with a high cholesterol content. The fastest rates observed for DPPC at high CMF were an order of magnitude lower than the lowest rate for DOPC and POPS. In all but one case, a threshold was observed at a characteristic CMF above which the cholesterol accessibility rose by ~2−4 orders of magnitude. The exception was SOPS, for which oxidation rates increased linearly with CMF from an *x*-axis intercept close to zero.

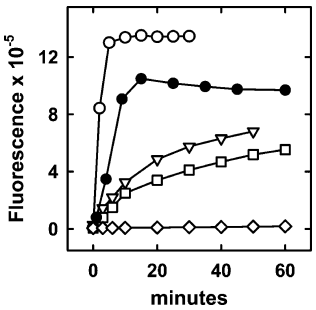

Figure 1. Examples of time courses of oxidation of LUV cholesterol. Values for the LUV phospholipid (P_t), cholesterol mole fraction (CMF), and cholesterol oxidase (CO) were varied as follows. POPC (P_t = 0.62 mM, CMF = 0.43, CO = 1 unit/mL) (○). DOPC (P_t = 0.71 mM, CMF = 0.26, CO = 0.04 unit/mL) (●). PSM (P_t = 0.25 mM, CMF = 0.61, CO = 10 units/mL) (▽). DMPC (P_t = 0.40 mM, CMF = 0.52, CO = 2 units/mL) (□). Minus LUVs (CO = 2 units/mL) (◇).

We fit the data in Figure 2 to a simple model as described in Appendix 2. For this purpose, we tested small integer cholesterol:phospholipid stoichiometries for the presumed complexes based upon the thresholds of these "*J* curves". Values for K_a and the fractions of complexed and uncomplexed sterol were obtained by a nonlinear least-squares Monte Carlo curve fitting procedure (see Appendix 2). These values were used to match theoretical curves to the data by choosing, through trial and error, a maximal RA for the totally uncomplexed cholesterol in each phospholipid system. The resulting curves are shown in Figure 2, and the corresponding values for the four parameters are listed in Table 1. We used E/N values to gauge the goodness of fit; see Appendix 2 and the legend of Figure 2. The best fitting theoretical curves were to SOPC (E/N = 0.14), DMPE (E/N = 0.14), and POPS (E/N = 0.15); the worst fit was to DMPS (E/N = 1.46).

Assuming stoichiometries of 1:1, 2:3, and 1:2 for the putative cholesterol−phospholipid complexes gave good fits. However, a cholesterol:phospholipid stoichiometry of 1.2:1 gave a statistically better fit for PSM than a 1:1 stoichiometry. The fit for POPC was better with a 1:1 stoichiometry than with a 2:3 stoichiometry, and a stoichiometry for DMPS of 2:3 was better than a 1:1 stoichiometry (Figure 2).

The RA values for complexed cholesterol are not considered to be very accurate because the enzyme reactions were generally close to the baseline sensitivity. The RA values for uncomplexed cholesterol fell into two distinct classes: all of the saturated phospholipids had RA values of <50, while all the unsaturated phospholipids had RA values of >1000. From the largest RA value and the scaling factor for each phospholipid, we calculated that the highest cholesterol accessibilities reported here ranged from ~3% (for SOPC) to ~63% (for POPS) of their projected maximal values. The inferred association constants, K_a in Table 1, suggested that the rise preceding the thresholds in the curves in Figure 2 was mostly due to the emergence of uncomplexed cholesterol with an elevated enzyme accessibility.

Equilibrium Distribution of [^{14}C]Cholesterol between LUVs and MBCD. This method has been used to compare the cholesterol affinities of various phospholipids.[19,24] Those studies assumed a simple partition equilibrium between two ideal compartments, that is

$$K_p = ([CP]/[P_t])/([CM]/[M_t]) \quad (1)$$

where CP is the cholesterol associated with phospholipid, P_t is total LUV phospholipid, CM is the cholesterol associated with MBCD, and M_t is total MBCD. However, if the LUVs or the cyclodextrin bind cholesterol stoichiometrically, the distribution of the sterol will reflect an equilibrium reaction dependent on the abundance of the free reactants, C and P. In that case, for 1:1 stoichiometries

$$K_e = ([CP]/[P])/([CM]/[M]) \quad (2)$$

Indeed, some of the cholesterol−phospholipid complexes appear not to be unimolecular (Table 1), and MBCD apparently binds cholesterol as a dimer.[56] Affinity constants for high-order reactions would then have different units than 1:1 reactions and not be directly comparable. Finally, K_e would presumably change above the stoichiometric equivalence point; we worked at low cholesterol levels to avoid that issue.

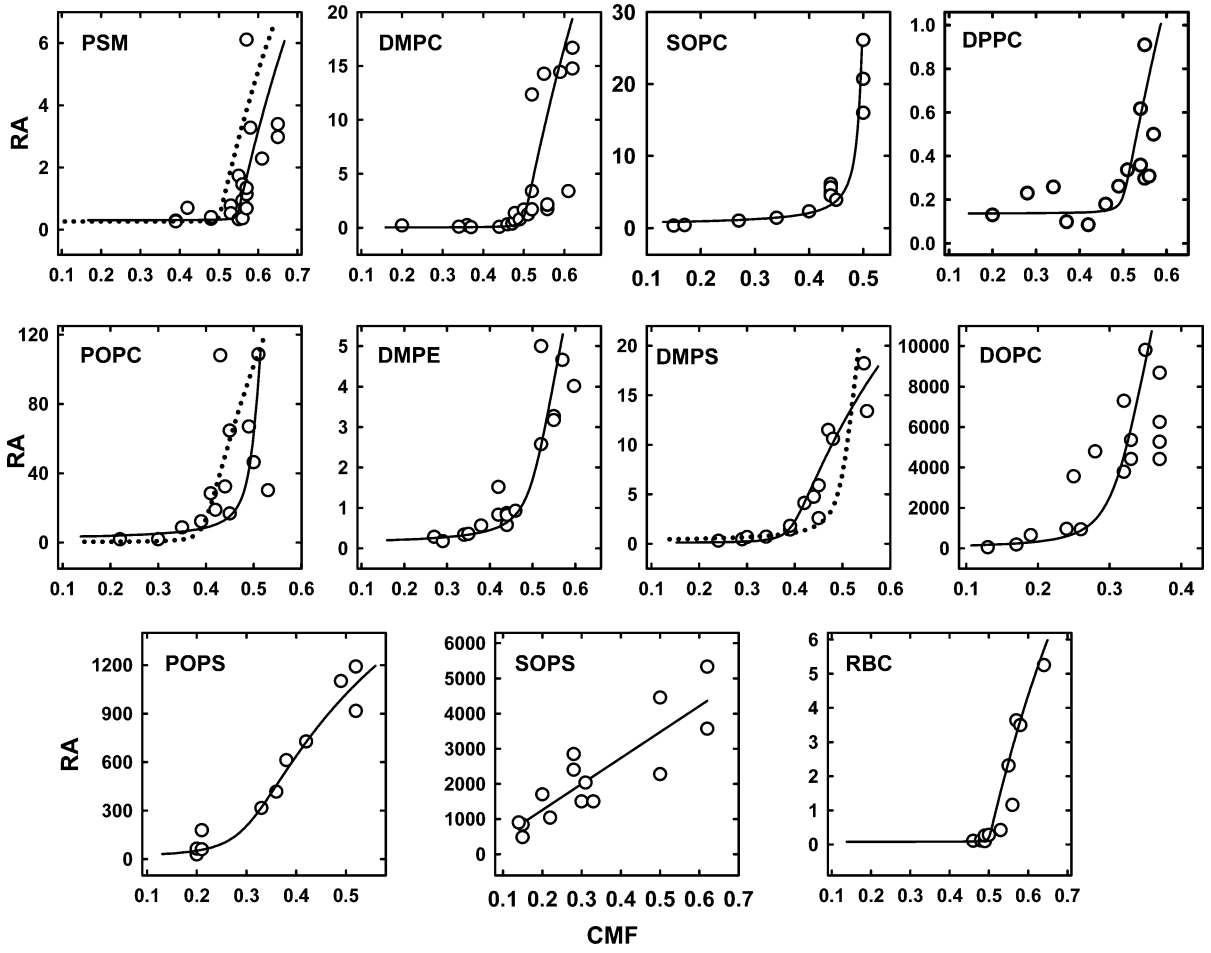

Figure 2. Dependence of the relative accessibility (RA) of cholesterol to cholesterol oxidase on membrane cholesterol mole fraction (CMF). Data points were calculated according to eq 6 of Appendix 1. Solid lines are best fits according to Appendix 2 using the values in columns 1−4 of Table 1; however, the calculated curve for DPPC had 0.1 added to the baseline. The dotted line for PSM was obtained by assuming a stoichiometry of 1:1; its goodness of fit E/N value was 1.17, clearly less good than the E/N of 0.36 for a 1.2:1 stoichiometry. The dotted line for POPC is for a stoichiometry of 2:3 (E/N = 1.86), clearly not as good a fit as for a 1:1 stoichiometry (E/N = 0.80). The dotted line for DMPS is for a stoichiometry of 1:1; its E/N value was 2.00, not as good a fit as for a 2:3 stoichiometry (E/N = 1.46).

Table 1. Values for Cholesterol−Phospholipid Complexes[a]

lipid	stoichiometry[b]	K_a[c]	RA of complexed cholesterol[d]	RA of uncomplexed cholesterol[d]	% uncomplexed cholesterol at the equivalence point[e]	Q[f]
PSM	1.2:1	5.8×10^3	0.35	15	2.0	35.3
DMPC	1:1	2.4×10^3	0.0026	50	1.9	7.2
SOPC	1:1	2.3×10^3	0.014	1600	2.6	4.8
DPPC	1:1	1.2×10^3	0.034	3	2.8	13.6
POPC	1:1	4.8×10^2	0.0024	1400	4.5	4.7
DMPE	1:1	1.3×10^2	0.011	20	8.5	2.3[g]
DMPS	2:3	6.6×10^5	0.0077	35	6.3	17.9
DOPC	1:2	9.3×10^2	0.34	60000	10.7	3.5
POPS	1:2	2.1×10^2	9.2	1900	17.4	3.3
SOPS				no threshold detected		5.1
RBC	1:1	7.5×10^3	0.0030	13	1.0	10.5[h]

[a]Values in columns 1−4 were used to generate the curves in Figure 2; see Appendix 2. [b]Cholesterol:phospholipid ratios (moles:moles). [c]Association constants for cholesterol−phospholipid complexes in arbitrary units; see eq 8. [d]Relative accessibilities of complexed and uncomplexed LUV cholesterol in arbitrary units. [e]Calculated using values in columns 1−4. [f]Cholesterol equilibrium distribution coefficient (see eq 3). Reactions contained 10 mM MBCD, 0.8 mM phospholipid, and 0.1 mM cholesterol at 37 °C. n = 2−4. For exceptions, see footnotes *g* and *h*. [g]Assayed with 10 mM MBCD, 0.6 mM phospholipid P, and 0.08 mM cholesterol at 25 °C. [h]Assayed with 10 mM MBCD, 1−1.6 mM phospholipid P, and 0.8−1.3 mM cholesterol at 37 °C. The corresponding K_e value is 19; see eq 2.

Because of these complexities, we did not infer either partition or association constants but rather expressed the equilibrium distribution of cholesterol between the various phospholipids and MBCD as a coefficient, namely

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$$Q = ([CP]/[P_t])/([CM]/[M_t]) \quad (3)$$

(Thus, although Q and K_p have the same form, these designations have different meanings.) Ratios of Q values then give estimates of the distribution of cholesterol between different phospholipid compartments; this, rather than their association constants, is the parameter of interest here. Employing relatively small CMFs allowed the approximations $[M] \cong [M_t]$ and $[P] \cong [P_t]$, so that the distribution coefficients, Q, were only a few percent smaller than the corresponding equilibrium constants, K_e, for unimolecular reactions. Except for the experiments shown in Figure 4, we also kept the proportions of the reactants constant, so that $[M_t]/[P_t]$ would be the same (and $[M]/[P]$ nearly so) for all the LUV systems, allowing direct comparison of their Q values.

Estimates of Q varied over about an order of magnitude for the phospholipids tested, with species bearing phosphorylcholine headgroups and saturated acyl chains having the highest relative cholesterol affinity (Table 1). In fact, the Q values for the various phospholipids fell into high and low classes that paralleled their thermal transition temperatures (Figure 3). (We included DLPC here to test a saturated phospholipid with a T_m well below the assay temperature.) Also shown in Figure 3, the Q values for most of the phospholipids were somewhat lower at 37 °C than at 25 °C. A similar phenomenon has been reported for POPC.[56]

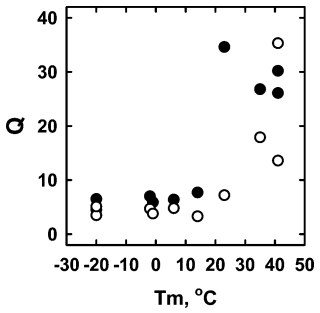

Figure 3. Cholesterol distribution coefficients (Q) as a function of the thermal transition temperature, T_m, of the phospholipids. Q values (given in Table 1) were obtained using these mixtures: 10 mM MBCD, 0.8 mM LUV phospholipid, and 0.1 mM cholesterol. Incubations were conducted for 0.5 h at 25 °C (●) (n = 3–4) and 37 °C (○) (n = 2–3). T_m values were obtained from textbooks and, mostly, the Avanti Polar Lipids catalog. From left to right, the phospholipids are DOPC, SOPS, POPC, DLPC, SOPC, POPS, DMPC, DMPS, DPPC, and PSM, respectively.

There was an additional reason to express equilibrium cholesterol distributions as Q rather than K_p values. Namely, seven high-affinity phospholipids showed a progressive ~2–4-fold increase in apparent cholesterol affinity as a function of CMF, while three low-affinity phospholipids did not (Figure 4). (A similar nonideal dependence of apparent affinity on CMF has been reported for POPC.[56]) The fact that the curves were not sigmoidal suggested that this cholesterol dependence was not cooperative. This phenomenon was observed for phospholipids with thermal transition temperatures both above and below the assay temperature (25 °C) and for DLPC, a saturated phospholipid with a T_m well below the assay temperature. Q values for DMPC rose up to CMF ~ 0.2 and fell 2-fold thereafter; other Q values seemed to approach a plateau at high CMF.

Characterization of RBC Membrane Cholesterol. We measured the accessibility to cholesterol oxidase of cholesterol in the membranes of intact human erythrocytes after increasing their sterol concentration by equilibration with MBCD-cholesterol. (To avoid hemolysis, we did not deplete the cells of cholesterol.) The results are given in Figure 2 and Table 1. The parameters for red cells resembled those for the saturated phosphorylcholine systems. We also determined the distribution of cholesterol between the red cells and MBCD. That Q value is presented in Table 1 as 10.5. This figure presumably underestimates the RBC cholesterol affinity, because the high level of their cholesterol makes $[P] \ll [P_t]$. The K_e for the RBC, estimated to be 19, is therefore a more appropriate value for comparison with the Q values for the LUVs.

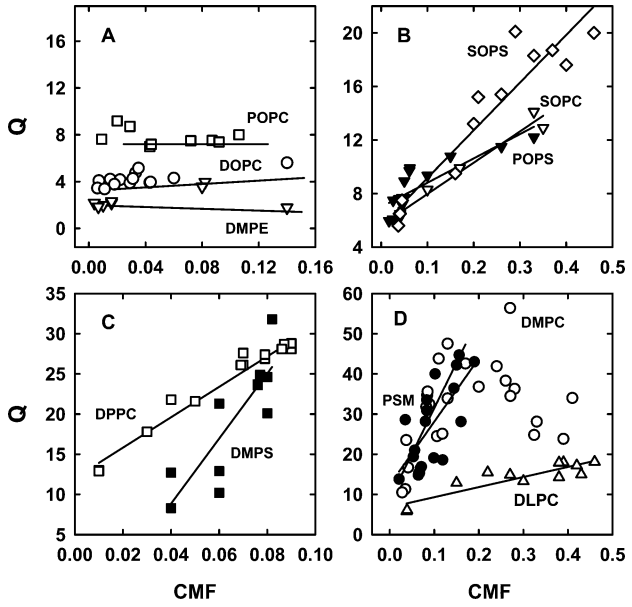

Figure 4. Cholesterol distribution coefficients (Q) as a function of cholesterol mole fraction (CMF). Multiple experiments were performed for each curve by incubating 10 mM MBCD bearing 0.02–0.10 mM [^{14}C]cholesterol with 0.1–1.0 mM LUV phospholipid for 0.5 h at 25 °C. Solid lines are linear least-squares fits: (A) POPC (□), DOPC (○), and DMPE (▽), (B) SOPS (◇), SOPC (▽), and POPS (▼), and (C) DPPC (□) and DMPS (■), and (D) DLPC (△), PSM (●), and DMPC, with a linear fit to the portion with a positive slope (○).

DISCUSSION

Considerable scatter was encountered in the data for the relative accessibility of cholesterol to cholesterol oxidase because each calculation entailed dividing the initial rate of reaction by the concentrations of phospholipids, cholesterol, and the enzyme (the activity of which can vary over time). Furthermore, each curve was composed of data from multiple experiments gathered over a period of months. The interpretation of the data is, of course, model-dependent, so we used the simplest and most appropriate formulation of which we know: that bilayer cholesterol is in a stoichiometric association equilibrium with phospholipids and that cholesterol in excess of this equivalence point has a greater accessibility than that in the complexes (see the introductory section and Appendix 2). While the RA data were reasonably well fit by statistical analysis, the values we inferred are not considered precise. Nevertheless, they provide novel and useful information regarding cholesterol–phospholipid associations and the use of the probes, cholesterol oxidase and β-cyclodextrins, as follows.

Relative Accessibilities. Consistent with earlier reports, the reaction of cholesterol with cholesterol oxidase varied greatly from one phospholipid environment to another and rose

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dramatically when LUV cholesterol was incremented above a threshold.[32,40,45,46,57] Polar headgroup species did not seem to exert a strong influence; in contrast, the RA values above threshold were orders of magnitude greater for unsaturated than for saturated chains. The large differences in reaction kinetics among the LUVs probably do not represent variations in K_d, the dissociation constant for the binding of cholesterol oxidase, given that the enzyme binds superficially to the surface of the vesicles so that the K_d is rather insensitive to vesicle composition.[38,42,43] Thus, while the various phosphorylcholine phospholipids should bind the enzyme similarly, they had widely divergent RA values.

It seems likely that the rate of the cholesterol oxidase reactions reflected the fractional occupancy by its substrate of the binding pocket of the membrane-bound enzyme. This, in turn, should reflect the free energy change associated with the partition of the substrate between the bilayer and the active site. RA should therefore be constrained to some degree by the strength of cholesterol−phospholipid associations.[38,42,58] On the other hand, the thermodynamic stability of cholesterol−phospholipid associations may not be the major factor limiting the cholesterol oxidase reactions, given that the RA values for both complexed and uncomplexed cholesterol varied over several orders of magnitude for the various phospholipids while their cholesterol affinities (Q values) varied by only ∼10-fold. We therefore imagine that a cholesterol molecule held with the same affinity by different phospholipids might assume a variety of dispositions that strongly affect its exposure at the aqueous interface. This could also explain why the rates of exit of cholesterol from different phospholipid vesicles have a much larger spread than their corresponding sterol affinities.[59] Other sterol-directed proteins might similarly sense the subtleties of sterol presentation reported by cholesterol oxidase.

The fact that oxidation rates varied by orders of magnitude above the thresholds of the different phospholipids suggests that the uncomplexed sterol is not free of their specific influences. Rather, the "free" cholesterol seems to be constrained by interactions resembling those experienced by the complexed sterols in that bilayer, albeit to a far smaller degree. This premise seems reasonable, given that the complexed and uncomplexed sterol molecules reside in the same phospholipid environment. It is important to note that the factors that determine the velocities of the enzyme reactions should not affect the values we obtained for cholesterol:phospholipid stoichiometries and affinities, discussed below.

Stoichiometries. While not definitive, the cholesterol dependence J curves in Figure 2 were consistent with small integer stoichiometries for cholesterol−phospholipid associations of ∼1:1, ∼2:3, and ∼1:2 (moles:moles). These values are in fair agreement with those reported for perfringolysin O binding, the rate of sterol transfer from bilayers to β-cyclodextrin, and the visualization of phase coexistence as well as average molecular area measurements on mixed monolayers.[26,27,29,32−36] Introducing oligomerization of the complexes in our model did not improve the fits to the data.

We considered some alternative explanations for the observed thresholds. Lateral phase heterogeneity would not occur in these bilayer systems, given that they should be homogeneous liquids at 37 °C[23,60,61] (but see ref 62). Furthermore, the Michaelis, dissociation, and catalytic constants (K_m, K_d, and k_{cat}, respectively) of the enzyme should not vary appreciably with the cholesterol content of the bilayer.[38,39,42,43] We also considered whether the observed thresholds might reflect the limited solubility of cholesterol in phospholipid bilayers, given that sterols in excess of a sharp threshold form crystals.[61] However, the observed thresholds for all the phosphatidylcholines occurred at cholesterol concentrations significantly below the well-documented solubility limit of CMF = 0.66.[61] In addition, we found that POPC and DOPC had lower thresholds than DPPC and DMPC, while the solubility limit (headgroup "umbrella coverage") for cholesterol is thought to be the same for all phosphatidylcholine bilayers.[61] Finally, crystallization is unlikely to account for the enhanced accessibility of superthreshold cholesterol observed with the probes cholesterol oxidase and perfringolysin O.

Stabilities of Complexes. The K_a values listed in Table 1 give estimates of the relative strength of association of cholesterol with phospholipids in the same bilayer, while previous studies gave the relative cholesterol affinities of different phospholipids; e.g., refs 19 and 24. The K_a values for complexes with different stoichiometries cannot be directly compared, because they describe different orders of reaction and, therefore, have different units. However, the calculated values for the fraction of cholesterol free at its equivalence point offer a comparison of the stabilities of all of the phospholipid complexes. These estimates suggest that on the order of 80−98% of bilayer cholesterol would be complexed at the equivalence points of the various phospholipids, with saturated phospholipids having the least free cholesterol at that point (Table 1). An implication of these results is that the sterol in essentially all eukaryotic membranes mostly resides in phospholipid complexes.

SOPS was the exception. It showed no apparent threshold in the cholesterol dependence of cholesterol oxidase reaction rates and, thus, no evidence of complex formation. This inference is supported by the high accessibility of the cholesterol in SOPS LUVs; its RA exceeded that of the other phospholipids at CMF < 0.2 where complexation should be the strongest (Figure 2). The fact that SOPS had a lower relative accessibility to cholesterol oxidase than did DOPC at high CMF is consistent with the premise, mentioned above, that the activity of uncomplexed sterol molecules is constrained by their bilayer environment in a phospholipid-specific fashion. SOPS is a prevalent phospholipid in biological membranes, where it might have the effect of lowering (by dilution) the overall threshold of the bilayer in which it resides, i.e., the CMF at the bend in the J curve for cholesterol accessibility. Any number of other, unexamined phospholipids could behave similarly.

***Q* Values.** The equilibrium distribution of cholesterol between LUVs and MBCD provided a measure of the relative affinity of cholesterol for the various phospholipids (Table 1). Our results are in accord with and extend reports in the literature.[19,24,56,60,63] The saturated phospholipids tested generally had a higher cholesterol affinity than the unsaturated species, just as indicated by cholesterol oxidase accessibility (see Table 1 and Figure 3). The low Q value for SOPS is consistent with the cholesterol oxidase data suggesting a weak affinity for cholesterol. Why SOPS had no threshold (Figure 2) yet showed a rising Q value (Figure 4) is worthy of investigation.

The Q values describe the distribution of cholesterol between different LUVs and a reference sink (MBCD) and thereby provide a comparison of the cholesterol affinities of the various phospholipids.[19,24] In contrast, K_a values describe the relative strength of the association of cholesterol distributed between its phospholipid complexes and its uncomplexed form in the same bilayer. Consequently, the two parameters need not agree. However, as shown in Table 1, the span of the K_a values for phospholipids making ∼1:1 complexes (namely, 32-fold)

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resembles that of their Q values (namely, 15-fold). Indeed, the K_a and Q values for these six ~1:1 species are well correlated; namely, $R^2 = 0.77$. Furthermore, the values listed in Table 1 for K_a, the RA of uncomplexed cholesterol, the percent of cholesterol free of complexes at the stoichiometric equivalence point, and the Q values, all of which relate to the relative strength of the association of cholesterol with the various bilayer phospholipids, are in good mutual agreement (Figure 5).

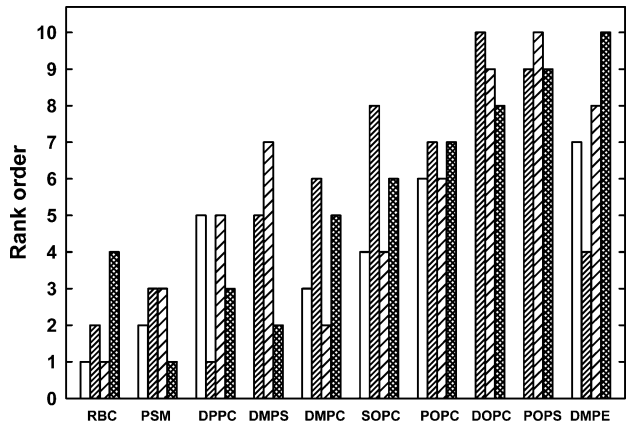

Figure 5. Correlations of four measures of the strength of association between cholesterol and phospholipids. The four bars give the relative rankings according to Table 1 for the respective values for the K_a, the RA of uncomplexed cholesterol, the percent free cholesterol at the equivalence point, and Q. Rank 1 denotes the strongest association for each parameter. Values are not given for the K_a of the three phospholipids with stoichiometries of 1:2 and 2:3.

Implications. The data we present bear on the question of whether the distribution of cholesterol among cellular membranes is driven by its association equilibrium with their constituent phospholipids. Key factors would then be the relative abundance of the different phospholipid species in each compartment, the relative stability of their cholesterol complexes, and their complexation stoichiometries, above which cholesterol affinity would fall. While much more quantitative lipidomic data on purified organelles are needed, we know that plasma membrane bilayers are rich in sphingolipids, phosphatidylcholines, and phosphatidylserines bearing saturated alkyl chains, while the nonendocytic cytoplasmic membrane phospholipids have more unsaturated fatty acid chains and polar headgroups other than phosphorylcholine and phosphorylserine.[1,2] Indeed, the cholesterol:phospholipid ratio in plasma membranes is typically close to 0.8 (i.e., CMF = 0.44), approaching the threshold stoichiometry of the high-affinity plasma membrane-type phospholipids shown in Table 1; see also ref 16. In the case of cultured human fibroblasts, the plasma membrane contains approximately 80% of the cell cholesterol and 50% of the phospholipid phosphorus.[64,65] The endocytic pathway might contain another 10% of the cell cholesterol through its traffic with the plasma membrane, leaving ~10% of the cholesterol in the other cytoplasmic membrane compartments. Thus, the ~5−10-fold difference in the cholesterol:phospholipid ratio of the fibroblast plasma membranes and cytoplasmic membranes is consistent with the relative affinity and abundance of the phospholipids in those compartments; see Table 1 and refs 1, 19, and 24. Passive equilibration of cholesterol among the organelle phospholipid bilayers is also consistent with the lack of evidence of energized cholesterol pumping by any of the transfer proteins identified thus far.[10−12,15]

With regard to cholesterol homeostasis, our data support the following scenario.[13] Cholesterol would tend to accumulate as phospholipid complexes in the various bilayer compartments up to their stoichiometric equivalence points. Cholesterol above those thresholds would increasingly exit, as has been reported.[29,32,34] Indeed, the cholesterol in the plasma membrane, ER, and mitochondria does seem to reside at such thresholds.[8,13,32,63,67] Once the large pool of high-affinity, high-threshold phospholipids in the plasma membrane is fully complexed, further increments in cholesterol will increasingly partition to the cytoplasmic membranes. The relatively weak cholesterol affinity of those bilayers will diminish further as their low complexing thresholds are exceeded. When the cholesterol in the ER bilayer passes its equivalence point, the excess sterol therein will become available to and activate resident homeostatic proteins. These effectors will then mediate the downregulation of cell cholesterol accretion.[13] A parallel mechanism occurs in mitochondria. There, excess cholesterol is converted to 27-hydroxycholesterol, a manifold feedback inhibitor of cholesterol accretion.[13] In this scenario, therefore, the overall steady-state level of membrane cholesterol in cells will depend on the magnitude of the phospholipid compartments of the diverse organelles, their net cholesterol thresholds, and their relative cholesterol affinities both below and above those thresholds.

(The threshold for ER cholesterol has been reported to be ~0.05 mol/mol of phospholipid, far below any stoichiometric equivalence point thus far reported; see Results and refs 8, 13, 35−37, and 63. It is implausible that this stoichiometry signifies complexes with very high phospholipid to cholesterol ratios. Instead, certain ER phospholipids, like SOPS, might bind cholesterol very weakly or not at all, so that their presence reduces the overall equivalence point of that bilayer. Alternatively, the presence of intercalating amphiphiles that bind to phospholipids could reduce the ER threshold by displacing cholesterol.[55−57])

Finally, to test the biological relevance of this characterization of cholesterol−phospholipid interactions, we examined human red blood cell membranes. As seen in Table 1, the cholesterol oxidase accessibility of cholesterol in intact red blood cells closely resembled that of the saturated phosphorylcholine phospholipid LUVs in their 1:1 threshold, their high K_a values, the relatively low RA of their cholesterol both below and above their threshold, and the fraction of cholesterol free at the threshold. These data are in accord with the abundance of phospholipids with saturated chains and phosphorylcholine headgroups in the outer leaflet of the RBC bilayer.[66−68] The relatively high cholesterol oxidase sensitivity of the cytoplasmic leaflet of this membrane (not tested here) also agrees with its phospholipid composition.[41,69−71] The apparent Q value for the RBC (namely, 10.5) was lower than that predicted from the total phospholipids in their two leaflets. However, one should use K_e rather than Q for this estimate, because the high cholesterol in this system makes [P] significantly smaller than [P_t]. The value then obtained for K_e is 19; this scales well with the Q values for PSM and DPPC (Table 1).

These results bear on how the level of cholesterol in RBCs is maintained over their ~4 months in circulation, given that these cells lack the homeostatic elements mentioned above. The pool of cholesterol in RBCs is not static but exchanges with the plasma lipoproteins on a time scale of hours.[72] Nevertheless, human red

cell membrane cholesterol rests at stoichiometric equivalence with its phospholipids, as evidenced by the thresholds in both its susceptibility to cholesterol oxidase and its rate of transfer to MBCD; see Figure 2 and refs 32 and 57. It seems plausible that the bilayer cholesterol in the RBC is maintained at its equivalence point by passive equilibration with the plasma lipoproteins. If so, it could be that the chemical activity of cholesterol in the plasma lipoproteins is itself set physiologically to maintain the level of red cell plasma membrane cholesterol (and perhaps that in plasma membranes in general) at their equivalence points. How this might occur is not known.

APPENDIX 1. ANALYSIS OF CHOLESTEROL OXIDASE KINETICS

To estimate the relative accessibility (RA) of bilayer cholesterol in each cholesterol oxidase assay, we assumed a simple rate expression for the reaction: $v = (E_e k_{cat} C_a)/(C_a + K_m)$, where v is the initial velocity of the enzyme reaction (in micromoles per minute), E_e is the effective (i.e., membrane-associated) fraction of the total enzyme (E_t), K_m is the Michaelis constant, C_a is the bilayer cholesterol available to the enzyme (all in micromoles), and k_{cat} is the first-order rate constant for the catalytic step (in inverse minutes). Because the cholesterol concentration was well below the K_m for the cholesterol oxidase, the velocity of the reaction was assumed to vary directly with its availability, C_a.[42] In that case, $v \cong (E_e k_{cat} C_a)/K_m$. In addition, the effective enzyme concentration, E_e, is a small fraction of total cholesterol oxidase. Under our conditions, the abundance of these enzyme−bilayer complexes is far below their dissociation constant, K_d.[42] Consequently, the effective enzyme concentration is given by $E_e \cong (E_t P_t)/K_d$, where P_t is the total phospholipid concentration. Then

$$v \cong (k_{cat} E_t P_t C_a)/(K_d K_m) \quad (4)$$

Because k_{cat}, K_d, and K_m are assumed not to vary under our experimental conditions

$$v \propto E_t P_t C_a \quad (5)$$

Hence, $C_a \propto v/(E_t P_t)$. The relative fraction of total LUV cholesterol accessible to the enzyme (RA = C_a/C_t) was therefore estimated as

$$\mathrm{RA} \propto v/(E_t P_t C_t) \quad (6)$$

in arbitrary units.

APPENDIX 2. INTERPRETATION OF CHOLESTEROL OXIDASE DATA

We assumed an ideal equilibrium association reaction between cholesterol (C) and phospholipids (P) as

$$n\mathrm{C} + m\mathrm{P} \leftrightarrow \mathrm{C}_n\mathrm{P}_m \quad (7)$$

$$K_a = [\mathrm{C}_n\mathrm{P}_m]/([\mathrm{C}]^n[\mathrm{P}]^m) \quad (8)$$

where n/m denotes the stoichiometry of a given complex and K_a is its association constant. To model the binding curves, we set $P_t = 1$ and varied C_t; reactants were expressed as mole fractions. We assumed that the relative accessibility of the cholesterol to cholesterol oxidase in a given system is

$$\mathrm{RA} = qn[\mathrm{C}_n\mathrm{P}_m] + r[\mathrm{C}] \quad (9)$$

where q and r are the fractional accessibilities of the cholesterol in the complexed and uncomplexed states, respectively. We fit the data in Figure 2 using eqs 7−9, assigning various small integers for n and m and iterating values for K_a, q, and r in a Markovian Monte Carlo scheme such that the cost function, E, converged to a minimum. E is the sum of the squares of the differences between the logs of data points and the logs of the corresponding fits. The goodness of fit is E/N, where N is the number of data points in the curve. Using logs of the values made the comparisons independent of the scale of RA and gave better fits at the bends in the curves (sensitive indicators of K_a). Lower E/N values signify better fits. The calculated best fit RA values were scaled to the data using an empirical factor that corresponds to the RA of the completely uncomplexed cholesterol in each phospholipid system.

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SYMBOLS USED

C, uncomplexed cholesterol (see eqs 7−9); C_a, LUV cholesterol available to the enzyme (see eqs 4 and 5); CM, cholesterol−MBCD complex (see eq 2); CP, cholesterol−phospholipid complex (see eq 2); C_t, total cholesterol (see eq 6); E_e, effective enzyme concentration, i.e., that bound to LUV; E_t, total enzyme concentration (see eq 4); K_a, stability constant for cholesterol−phospholipid association (see eq 8); k_{cat}, first-order rate constant for the cholesterol oxidase reaction (see eq 4); K_d, dissociation constant for the cholesterol oxidase−LUV binding reaction; K_e, equilibrium constant for the cholesterol association with LUVs and MBCD (see eq 2); K_m, Michaelis constant (see eq 4); K_p, partition coefficient for cholesterol equilibrium between LUVs and MBCD (see eq 1); m, stoichiometry of cholesterol complexed with phospholipid (see eq 7); M_t, total MBCD (see eq 3); n, stoichiometry of cholesterol complexed with phospholipid (see eq 7); P, uncomplexed phospholipid (see eq 2); P_t, total phospholipid (see eq 3); Q, cholesterol equilibrium distribution coefficient (see eq 3); q, fractional accessibility of cholesterol in complexes (see eq 9); r, fractional accessibility of uncomplexed cholesterol (see eq 9); RA, relative accessibility of cholesterol in cholesterol oxidase experiments (see eq 6); v, initial velocity of cholesterol oxidase reactions (see eqs 4−6).

ABBREVIATIONS

CH, cholesterol; CMF, bilayer cholesterol mole fraction [i.e., (moles of cholesterol)/(moles of cholesterol + moles of phospholipid)]; DLPC, 1,2-dilauroyl-*sn*-glycero-3-phosphatidylcholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine; DMPE, 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylethanol-

amine; DMPS, 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylserine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine; DPPC, 1,2-dipalmitoyl-*sn*-glyco-3-phosphatidylcholine; ER, endoplasmic reticulum; HBS, 150 mM NaCl and 5 mM Hepes-NaOH (pH 7.4); LUVs, large unilamellar vesicles; MBCD, methyl-β-cyclodextrin; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylserine; PSM, *N*-palmitoyl-D-*erythro*-sphingosylphosphorylcholine; RA, relative cholesterol accessibility; RBC, red blood cell; SOPS, 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphatidylserine; SOPC, 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine. For a list of the symbols used in the equations, see Symbols Used.

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