Probing Red Cell Membrane Cholesterol Movement with Cyclodextrin

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ABSTRACT We probed the kinetics with which cholesterol moves across the human red cell bilayer and exits the membrane using methyl-β-cyclodextrin as an acceptor. The fractional rate of cholesterol transfer (% s^{-1}) was unprecedented, the half-time at 37°C being ~1 s. The kinetics observed under typical conditions were independent of donor concentration and directly proportional to acceptor concentration. The rate of exit of membrane cholesterol fell hyperbolically to zero with increasing dilution. The energy of activation for cholesterol transfer was the same at high and low dilution; namely, 27–28 Kcal/mol. This behavior is not consistent with an exit pathway involving desorption followed by aqueous diffusion to acceptors nor with a simple one-step collision mechanism. Rather, it is that predicted for an activation–collision mechanism in which the reversible partial projection of cholesterol molecules out of the bilayer precedes their collisional capture by cyclodextrin. Because the entire membrane pool was transferred in a single first-order process under all conditions, we infer that the transbilayer diffusion (flip-flop) of cholesterol must have proceeded faster than its exit, i.e., with a half-time of <1 s at 37°C.

INTRODUCTION

Sterols are major constituents of eukaryotic plasma membranes. They reduce the permeability of the membrane, increase its mechanical strength and help to organize its constituents laterally into domains such as rafts and caveolae (Barenholz, 2002; Simons and Ikonen, 2000). Despite repeated study, some basic features of the disposition of sterols in plasma membranes remain uncertain. It has been variously suggested that cholesterol may be mostly in the outer leaflet (Fisher, 1976), mostly in the inner leaflet (Brasaemle et al., 1988; Schroeder et al., 1991) or nearly equally distributed across the bilayer (Lange and Slayton, 1982; Muller and Herrmann, 2002). (See also Clejan and Bittman, 1984; Schroeder et al., 1996.) Furthermore, consensus is lacking as to whether cholesterol diffuses across bilayers on the time scale of seconds or less (Lange et al., 1981; Muller and Herrmann, 2002), minutes (Rodrigueza et al., 1995; Schroeder et al., 1996; Haynes et al., 2000; Leventis and Silvius, 2001), or hours (Brasaemle et al., 1988; Rodrigueza et al., 1995). Also unsettled is the mechanism by which cholesterol is passively transferred from membranes to acceptor particles, an issue of relevance to the physiological role of plasma lipoproteins and the pathophysiology of atherosclerosis (Brown, 1992; Rothblat et al., 1999). These issues also bear on the intra- and intermembrane dynamics of other bilayer lipids, amphipathic metabolites, and drugs (Brown, 1992).

β-Cyclodextrins selectively bind sterols to form water-soluble complexes. They have proven to be excellent vehicles for the rapid delivery and extraction of membrane sterols (Ohtani et al., 1989) and have been used to examine both intra- and intermembrane cholesterol movements (Yancey et al., 1996; Rothblat et al., 1999; Haynes et al., 2000; Leventis and Silvius, 2001; Hao et al., 2002). We now extend that work, documenting unprecedented rates of transfer of red cell membrane cholesterol to methyl-β-cyclodextrin (MBCD) and presenting evidence for an activation–collision pathway for this movement. Our data also show that transbilayer cholesterol diffusion is fast compared to the kinetics of extraction and thus must have a half time of less than 1 s.

MATERIALS AND METHODS

Materials

[1α,2α-^{3}H]cholesterol was from Amersham Pharmacia Biotech (Piscataway, NJ). Hydroxypropyl-β-cyclodextrin and MBCD were from Research Plus, Inc. (Bayonne, NJ) or Sigma (St Louis, MO). Blood was obtained from a healthy volunteer donor or outdated from the Rush-Presbyterian-St Luke's Blood Bank. Although all of the data presented were obtained using fresh blood, there were no systematic differences between these two sources. Plasma and buffy coat were separated from the cells, which were then washed twice with 5 volumes of PBS (150 mM, NaCl-5 mM, NaPi, pH 7.4).

Labeling donor cells

Approximately 1 μCi of [^{3}H]cholesterol was dried from ethanol under N_2 and taken up in 3 μl hydroxypropyl-β-cyclodextrin (200 mg/ml water) by warming at 37°C for 3 min. 40 μl PBS was added and the sample incubated for an additional 3 min. 20 μl of this complex was then mixed with one ml of washed, packed red cells, and the mixture incubated for 20–60 min at 37°C to allow the probe to equilibrate between the bilayer leaflets (see below). The labeled cells were then washed thrice with 3 ml PBS.

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Optimization of [^{3}H]cholesterol transfer to cholesterol-MBCD complexes

Aqueous solutions of ~5 mg cholesterol/100 mg MBCD were prepared in water as described (Klein et al., 1995). Before each experiment, these optically clear solutions were made isotonic with PBS. Sufficient MBCD/cholesterol was used to assure the transfer of >90% of red cell [^{3}H]cholesterol at equilibrium. Because both increasing and decreasing the mass of cholesterol in the red cells by incubation with MBCD/cholesterol caused significant hemolysis, we loaded the acceptor MBCD with an optimized amount of unlabeled cholesterol, ~5 mg/100 mg MBCD. This maintained red cell cholesterol content at a nearly normal level during the transfer reaction. Under these conditions, the release of hemoglobin was generally <1% and never >8%.

Transfer of [^{3}H]cholesterol from red cells to MBCD

Time courses were carried out in small hand-swirled beakers containing one ml of reaction mixtures. To start the reaction, MBCD/cholesterol complexes were added to the red cell suspension. Both the donor and the acceptor were in PBS and were pre-equilibrated at the specified temperature. At specified times, 80–100 μl aliquots of the reaction mixture were transferred to microfuge tubes containing 500 μl ice-cold stopping solution. (A metronome guided sampling in rapid time courses.) The acceptor was immediately separated from the donor cells by a 5-s spin in a microcentrifuge. For experiments involving single time points, 100-μl reactions were carried out in microfuge tubes. Stopping solution was then added, and the suspension centrifuged as described above.

The stopping solution contained 10 mg MBCD/ml PBS to prevent the [^{3}H]cholesterol in the extract from precipitating from the dilute, cold quenching buffer and entering the pellet nonspecifically. We showed in control assays that the stopping solution did not extract red cell cholesterol appreciably under the ice-cold conditions used. For zero time points, labeled red cells were mixed with the stopping solution before the addition of the MBCD—cholesterol. This background value amounted to ~5% of total counts.

After centrifugation, cell pellets were washed once with 1 ml ice-cold PBS and extracted for 10 min at room temperature with 20 volumes isopropanol. Aliquots of both the donor and acceptor were counted for tritium and the values corrected for quenching. Time courses were fit with SigmaPlot to an equation of the form: $y = y_o + a(1 - e^{-bt})$. The value of R^2 for these fits invariably exceeded 0.95 (see Fig. 1). Transfer rates are expressed as fractional velocity with units of % donor [^{3}H]cholesterol per s, or simply s^{-1}.

Other assays

Cholesterol mass was determined by HPLC, as described (Lange, 1991). Hemolysis was determined from the optical absorbance at 412 nm of dilutions of the supernatant, pellet, and input.

RESULTS

As previously reported for the exit of cholesterol from lipid vesicles (Leventis and Silvius, 2001), >90% of the [^{3}H]cholesterol probe transferred from red cells to an excess of MBCD with first-order kinetics. Half of the cholesterol left the membrane in ~1 s at 37°C (Fig. 1). This value was corroborated in related experiments in which multiple 1-s time points were taken.

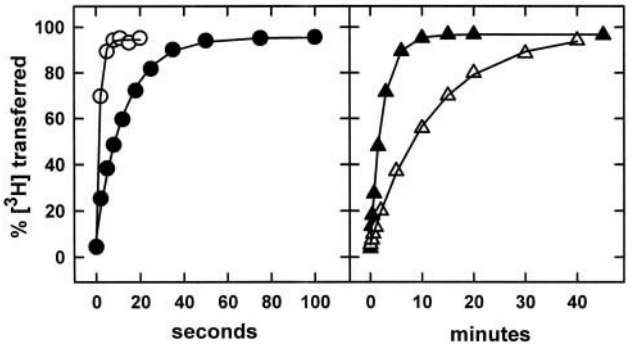

FIGURE 1 Time course of transfer of [^{3}H]cholesterol from red cells to MBCD. Red cells labeled with [^{3}H]cholesterol were vigorously mixed with MBCD–cholesterol in PBS at the stated temperatures. The reaction mixtures contained 100 μl red cells plus 72 mg MBCD/3.9 mg cholesterol acceptor in 1 ml final volume. At intervals, 80–100-μl aliquots were analyzed. Each curve shows a single experiment, representative of three, fit by a first-order exponential expression. *Left*: 37°C (O) and 25°C (●). *Right*: 10°C (▲) and 0°C (△).

The fractional rate of the transfer reaction did not vary with the abundance of the donor red cells at a standard level of acceptor MBCD (Fig. 2) but increased linearly with the abundance of the acceptor at a standard level of donor (Fig. 3). [A similar direct dependence on cyclodextrin concentration was reported for lipid vesicle donors (Leventis and Silvius, 2001).] When the ratio of donor to acceptor was held constant, the fractional rate of transfer decreased hyperbolically with increasing aqueous volume (Fig. 4). Extrapolation of the solid line in Fig. 4 suggests that the transfer velocity approaches zero at infinite dilution.

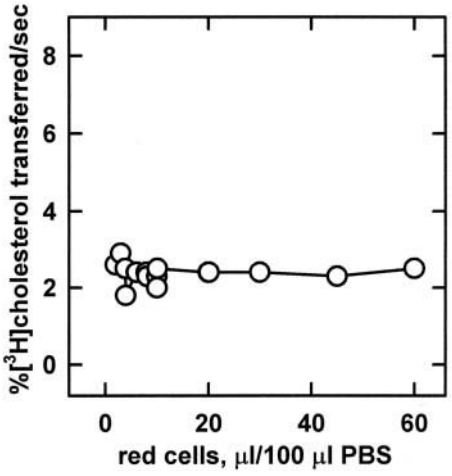

FIGURE 2 Transfer of [^{3}H]cholesterol from red cells to MBCD–cholesterol complexes; dependence on donor concentration. Increasing volumes of [^{3}H]cholesterol-labeled red cells were placed in a series of microfuge tubes at 25°C containing PBS to make a final volume of 100 μl. Transfer to acceptor (13 mg MBCD/0.7 mg cholesterol) was for 5 s at 25°C. Values are pooled from three separate experiments.

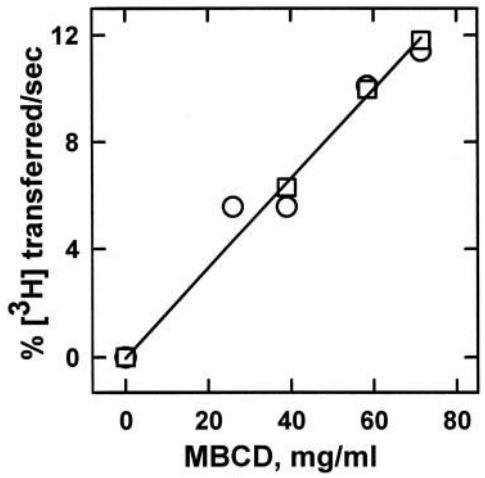

FIGURE 3 Transfer of [^{3}H]cholesterol from red cells to MBCD–cholesterol complexes; dependence on acceptor concentration. [^{3}H]cholesterol-labeled red cells (10 μl) were placed in a series of microfuge tubes at 25°C with PBS added to make the final volume 100 μl. Transfer to varied acceptor (with 55 μg cholesterol per mg MBCD) was for 3 s. Values are from two separate experiments.

The rate of transfer of [^{3}H]cholesterol from red cells to MBCD was highly temperature dependent (Fig. 5). At a high concentration of reactants (◇), half times varied from 460 s (~8 min) at 0°C to ~1 s at 37°C. The corresponding first-order rate constants are 1.5×10^{-3} s^{-1} and 0.7 s^{-1}. The energy of activation obtained from the slope of this

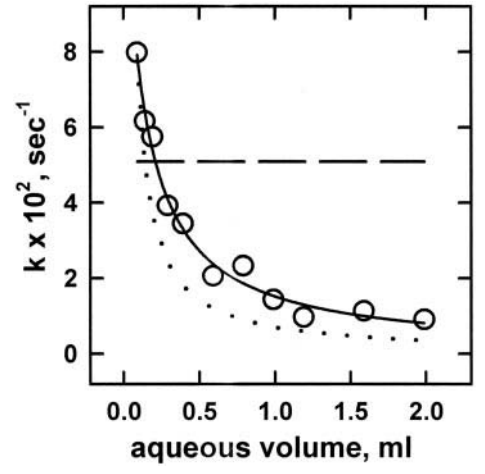

FIGURE 4 Transfer of [^{3}H] cholesterol from red cells to MBCD–cholesterol complexes; dependence on aqueous volume. Various volumes of [^{3}H]cholesterol-labeled red cells were placed in microfuge tubes at 25°C containing various volumes of PBS. Transfer was initiated by the addition of acceptor at a constant ratio to the donor, namely, 10 mg MBCD/0.525 mg cholesterol for each 10 μl of cells. The reaction was for 5 s at 25°C. Apparent rate constants were determined by fitting the kinetic curves to a first-order exponential expression. The abscissa gives aqueous volume per 10 μl packed red cells. For purposes of analysis, the data were fit to each of the three terms in Eq. A18: $y = a/x$ (*dotted line*), $y = a$ (*dashed line*), and $y = a/(b + x)$ (*solid line*). This experiment is representative of three replicates.

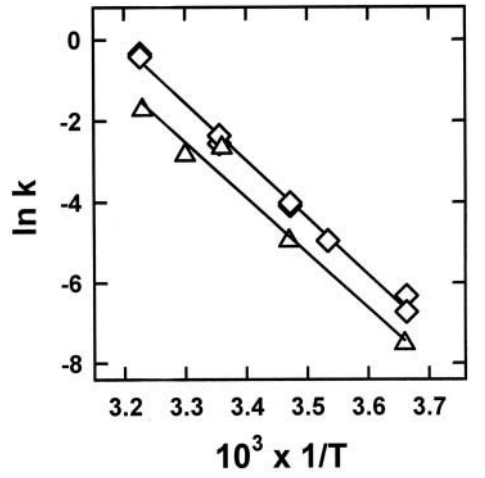

FIGURE 5 Arrhenius plot. Upper curve (◇), high concentration of reactants: donor (100 μl labeled red cells) plus acceptor (72 mg MBCD/3.9 mg cholesterol) in 1 ml final volume. Lower curve (△), low concentration of reactants: donor (100 μl red cells) plus acceptor (19 mg MBCD bearing 1 mg cholesterol) in 10 ml final volume. Reactions were carried out between 0 and 37°C as in Fig. 1. Time courses were fit to a first-order exponential expression and the derived kinetic constants plotted against inverse temperature. Energies of activation were estimated from the Arrhenius expression, $\ln k = E_{act}/RT$.

Arrhenius plot was 28 Kcal/mol. The temperature dependence of [^{3}H]cholesterol transfer was also examined at low reactant concentrations (Fig. 5, △). The fractional transfer rates were about one-fifth of those seen in Fig. 1, and the Arrhenius plot ran parallel to that found at high reactant levels, yielding an energy of activation of 27 Kcal/mol.

We also used red cells fixed with glutaraldehyde as donors because they resist lysis. We found that the rate of [^{3}H]cholesterol transfer out of fixed, washed red cells was about four times more rapid than in unfixed cells at both 0 and 15°C (not shown). Finally, we investigated whether the exogenous [^{3}H]cholesterol faithfully represented the cholesterol mass, because, for example, if there were negligible flip-flop, the probe might simply have remained in the outer bilayer leaflet. We found in three experiments that the first 20–35% of the [^{3}H]cholesterol transferred to MBCD had specific activities of 1.04 ± 0.004, 1.16 ± 0.02 and 1.09 ± 0.02 ($n = 3$) times that remaining in the cells. Thus, the cholesterol label distributed closely with cholesterol mass. Furthermore, we showed that the exit of cholesterol mass from fixed cells was as rapid and complete as was the [^{3}H]cholesterol probe (not shown).

DISCUSSION

The rate of transfer of cholesterol from red cells to MBCD observed here was the fastest yet reported, with half of the cholesterol transferred in one second at 37°C (Fig. 1). Extrapolation of the best-fit curve in Fig. 4 suggests that the maximal velocity at infinite concentration could be ~50% greater than the largest experimental value. These kinetics

are more than three orders of magnitude faster than from red cells to various lipid acceptors and nearly five orders of magnitude faster than from some cultured cells to synthetic vesicles (Phillips et al., 1987; Rothblat et al., 1999). The fractional transfer rate also showed a high energy of activation and a strong dependence on aqueous volume. These features are best interpreted in the context of our general understanding of the passive transfer of membrane lipid, as follows.

Cholesterol exited to acceptors with perfectly first-order kinetics in our system as in many others (Fig. 1). This feature indicates that there is a single donor pool, whereas other studies have suggested the presence of two donor cholesterol pools in a slow exchange equilibrium (Bar et al., 1986; Yancey et al., 1996; Haynes et al., 2000; Simons and Ikonen, 2000). In any case, it is not the time course but rather the dependence of transfer kinetics on donor and acceptor concentrations that points to the mechanism of the transfer reaction.

As described in Eq. A1 of the Appendix, three general models for the transfer of lipids between membrane compartments have been considered. These are a simple collisional mechanism, an aqueous diffusion pathway, and an activation–collision pathway. The first, transfer during collisions without an intervening activation step, was not favored in early studies (e.g., Backer and Dawidowicz, 1981; Phillips et al., 1987) but has been inferred more recently for some circumstances (Jones and Thompson, 1989, 1990; Thurnhofer and Hauser, 1990; Wimley and Thompson, 1991; Yang and Huestis, 1993). Our data do not conform well to this model (Fig. 4, *dotted line*). Most interpretations of lipid transfer data have favored an aqueous diffusion pathway in which lipid monomers desorb from the donor and diffuse in the medium until captured by collision with an acceptor (Nichols and Pagano, 1981; Phillips et al., 1987). Our data are clearly not consistent with this mechanism (Fig. 4, *dashed line*). In the third model, activation–collision, lipid monomers do not desorb from the membrane but rather enter an activated state, such as partial projection from the bilayer, from which they are either captured by collision with acceptors and other (*trans*) donor particles or else return to the ground state (Steck et al., 1988). As argued in the Appendix, our data are in accord with this model (Fig. 4, *solid line*).

In general, more water-soluble membrane intercalators are likely to be transferred by aqueous diffusion, whereas natural membrane lipids might require collisional capture. Although these mechanisms are distinct, a middle-ground is sometimes envisioned in which fully desorbed monomers are nevertheless sequestered at the surface of the donor and must be captured by collision with acceptors (Ferrell et al., 1985; Wimley and Thompson, 1991; Davidson et al., 1995a,b).

Unlike simple collisional transfer, both the aqueous diffusion and the activation–collision pathways show competition between donor and acceptor particles for the activated monomers, i.e., the fully or partially desorbed lipid. This is the significance of the expressions $k_3[A]/(k_3[A] + k_2[D])$ and $K_a[A]/(k_6 + K_a[A] + K_d[D])$ in the second (aqueous diffusion) and third (activation–collision) terms of Eq. A14. It is this competition that leads to the saturation isotherms for donor and acceptor concentrations seen in many lipid transfer experiments (Nichols and Pagano, 1981; Lange et al., 1983; Ferrell et al., 1985; Phillips et al., 1987). The concentration dependence that arises from competition between donor and acceptor compartments can complicate the kinetic analysis. However, this is easily dealt with by holding the ratio of donor to acceptor constant while varying the aqueous volume; see, for example, our Fig. 4 and Nichols and Pagano (1981), Steck et al. (1988), and Yang and Huestis (1993).

The activation–collision mechanism is distinguished kinetically from an aqueous diffusion pathway in that competition arises not only from distal (*trans*) donor particles (steps 11–13 in Eq. A1) but also from the return of emergent monomers to the parent membrane (step 6 in Eq. A1). This *cis* recapture is the sole difference in the form of these two kinetic processes: compare the second and third terms of Eq. A16. It is this step that confers volume-dependence upon activation–collision kinetics (Eq. A18). This is because, in the aqueous diffusion mechanism, the volume of the medium has precisely the same effect on the transfer of activated monomers to *trans* donor particles as to acceptor particles. In the activation–collision mechanism, however, it is competition between the volume-independent relaxation of monomers back to the *cis* donor and volume-dependent collisional capture that confers sensitivity to aqueous volume upon the kinetics.

The indifference of fractional transfer rate to donor concentration in Fig. 2 is consistent with both aqueous diffusion and activation–collision pathways when *trans* donor particles are weak competitors (see Eq. A16). In our system, weak donor competition arises from the very slow rate of transfer of cholesterol from red cell to red cell (Lange et al., 1983; Steck et al., 1988) relative to rapid transfer from red cells to MBCD (Fig. 1). The aqueous diffusion model predicts that transfer rates will be independent of acceptor concentration when donor competition is negligible (see Eq. A16). However, this is not observed here (Fig. 3). Rather, Fig. 3 is consistent with an activation–collision pathway in which $K_a[A] \ll K_d[D] + k_6$. In that case, the data in Fig. 3 would presumably fall on the nearly linear take-off of a hyperbolic saturation curve. Because we inferred above that $K_d[D] \ll K_a[A] + k_6$, it follows that $K_a[A] < k_6$. However, it is also clear from Fig. 4 that $K_a[A]$ is of a magnitude similar to k_6 at high particle concentrations.

Because acceptors can capture monomers at different degrees of projection, an activation–collision mechanism can lead to varied kinetics. Activation–collision will resemble the volume-independent aqueous diffusion pathway when the fall-

back rate constant, k_6, is relatively small; that is, the third term will approximate the form of the second term in Eq. A17. Activation–collision kinetics can also look like those of simple collision when the fall-back rate constant, k_6, is relatively large. Then, the third term will approximate the form of the first term in Eq. A17. In systems where the acceptor accesses an abundant population of low-lying monomers, large rate constants for both transfer (k_5) and *cis* capture (k_6) could lead to rapid, volume-dependent kinetics. This is the behavior seen in the transfer to cyclodextrins of membrane cholesterol (present data and those of Yancey et al., 1996; Rothblat et al., 1999; Haynes et al., 2000; Leventis and Silvius, 2001) and phospholipids (Tanhuanpaa and Somerharju, 1999; Tanhuanpaa et al., 2001).

In principle, a given acceptor can capture a given lipid at more than one degree of projection from the membrane, producing coexistent volume-dependent and volume-independent transfer kinetics in the same system. Such compound behavior has been observed both for membrane cholesterol (Steck et al., 1988) and phospholipids (Jones and Thompson, 1989, 1990; Wimley and Thompson, 1991; Yang and Huestis, 1993). The biphasic acceptor dependence of cholesterol transfer in other studies might have the same significance (Davidson et al., 1995a,b). Whether the volume-dependent process involves simple collision, as some suggest (Jones and Thompson, 1990; Yang and Huestis, 1993), or a two-step activation–collision pathway (Steck et al., 1988) can be approached by testing for competition by *trans* donor particles, because this is a feature of the latter mechanism but not the former.

In contrast to the rapid kinetics observed here, the transfer of plasma membrane cholesterol from intact cells to various donors is very slow. It has been argued that an aqueous diffusion mechanism obtains, but that the desorbed monomers are poorly accessible to bulky acceptors because of an unstirred water layer, a glycocalyx, or other physical barriers (Phillips et al., 1987). However, poor access of large acceptors to the surfaces of donor cells does not explain why desorbed cholesterol molecules would not rapidly diffuse to acceptors in the bulk solution. Furthermore, unstirred boundary water should slow the dispersion of released cholesterol in the solvent by only a matter of seconds and not hours, as observed. In addition, small uncharged lipid vesicles have negligible unstirred water layers and lack protein coats but nevertheless show slow cholesterol transfer kinetics (Backer and Dawidowicz, 1981; Phillips et al., 1987). Also arguing against rate-determining diffusional barriers is the rapid transfer to acceptors of numerous polar membrane lipids and sterol derivatives (e.g., Phillips et al., 1987; Steck et al., 1988; Butko et al., 1990; Kan et al., 1992; Rodrigueza et al., 1995; Bojesen and Bojesen, 1996). Because there is no strong rationale for why desorbed monomers would remain associated with the cell-surface, it is worthwhile considering that the observed slow exit kinetics from whole cells reflects an activation–collision process in which the fruitful interaction of various bulky acceptors with partially projecting monomers is slow compared to their *cis* capture.

Membrane lipid transfer systems are generally characterized by a high energy of activation, ascribed to the insolubility of monomers in the aqueous space (McLean and Phillips, 1984; Nichols, 1985; Phillips et al., 1987). Typical activation energies for cholesterol transfer are 10–20 Kcal/mol or more (Jonas and Maine, 1979; Poznansky and Czekanski, 1979; Gottlieb, 1980; McLean and Phillips, 1981; McLean and Phillips, 1982; Slotte and Lundberg, 1983). These values are similar to those found for synthetic phospholipids with 16–20 effective methylene units (Nichols and Pagano, 1981; McLean and Phillips, 1984; Nichols, 1985). The high energy of activation found in the present study, 27–28 Kcal/mol (Fig. 5), suggests that the transition form of the cholesterol taken up by MBCD could be comparable to that captured by the large and particulate acceptors studied earlier. Presumably, in both cases, it is the nearly fully projecting membrane monomer. The similarity of the energies of activation for the two curves in Fig. 5 suggests that the fall in transfer rate with dilution in this system does not reflect a shift in pathway. This was also the conclusion of a similar study on phospholipids (Jones and Thompson, 1990). We note in passing that very low energies of activation for cholesterol transfer from cells and model membranes to cyclodextrin have been reported; namely, 7 and 2 Kcal/mol (Yancey et al., 1996). The reason for the difference between those values and ours is not evident.

Finally, our data bear on the kinetics of cholesterol diffusion across the membrane. Because β-cyclodextrins are unrivalled in the extraction of cholesterol from bilayers, it was our hope that the rate of cholesterol exit could be made fast compared to that of transbilayer flip-flop. In that case, biphasic time courses would be obtained, and the rate constant for transbilayer movement could then be inferred from the slow limb of the curve. Such studies would also provide estimates of the fraction of the cholesterol residing in each leaflet. We found, however, that the entire membrane [^{3}H]cholesterol pool was exchanged with strictly first-order kinetics at temperatures between 0 and 37°C. Could all of the exogenous [^{3}H]cholesterol probe have been retained in the outer bilayer leaflet? This was not the case, because the specific activity of the extracted [^{3}H]cholesterol was nearly the same as that remaining in the cells. Furthermore, cholesterol mass exited as rapidly and completely as the [^{3}H]cholesterol probe.

Because there is no doubt that appreciable cholesterol resides in both leaflets (Muller and Herrmann, 2002), these data strongly suggest that the movement of sterols across the bilayer must be fast relative to the observed time scale of extraction. An upper bound on the time constant for flip at 37°C can be inferred from Fig. 1 to be ~1 s. A similar conclusion was previously drawn using cholesterol oxidase as a probe (Lange et al., 1981). However, in that case, the cholestenone reaction product could have perturbed the membrane (Brasaemle et al., 1988).

Although there are several reports of slow cholesterol flip-flop (see Schroeder et al., 1996; Muller and Herrmann, 2002), a time constant of <1 s is not unreasonable. Compared to phospholipids, where the flip-flop time is measured in hours or days, sterols contain only a single polar (oxygen) atom. Furthermore, far more polar amphipaths, such as fatty acids (Bojesen and Bojesen, 1996; Kleinfeld et al., 1997; Hamilton et al., 2001), porphyrins (Kuzelova and Brault, 1994), bilirubin (Zucker et al., 1999), and amphipathic drugs (e.g., Regev and Eytan, 1997), passively traverse the bilayer in seconds or less. Even lysophosphatides, with large polar head-groups, cross the membrane on the time scale of minutes (Bergmann et al., 1984). Presumably, the rapid flip-flop of sterols evolved to confer benefits; otherwise, their transbilayer diffusion could have been dramatically slowed by the addition of a few more polar atoms (Kan et al., 1992; Muller and Herrmann, 2002).

In conclusion, our results suggest that the transfer of red cell cholesterol to cyclodextrin proceeds via an activation–collision pathway and strengthen the case for the generality of this mechanism. As suggested (Jones and Thompson, 1989; Steck et al., 1988), a collisional mechanism is better suited than aqueous diffusion to mediate the specific transfer of lipids among membranes, hence their targeting in cells (Simons and Ikonen, 2000; Hao et al., 2002).

APPENDIX

By Ferenc J. Kézdy (Protein Science, Pharmacia Corp., Kalamazoo MI), Theodore L. Steck, and Yvonne Lange

Eq. A1 provides a general reaction scheme for three mechanisms relevant to the transfer of cholesterol from a donor particle, DC:

$$\begin{array}{ccccc} & & & & \mathrm{DC}+\mathbf{D} \\ & & & & \uparrow 13 \\ \mathrm{DC} & & & & \mathbf{D}\mathrm{C}'\mathrm{D} \\ \uparrow 2 & & & & 11 \Uparrow 12 \\ +\mathrm{D} & & & & +\mathrm{D} \\ \mathrm{C} & \xleftarrow{1} & \mathbf{D}\mathrm{C} & \underset{6}{\overset{5}{\rightleftarrows}} & \mathbf{D}\mathrm{C}' \\ +\mathrm{A} & & +\mathrm{A} & & +\mathrm{A} \\ 3 \Uparrow 4 & & \downarrow 14 & & 7 \Uparrow 8 \\ \mathrm{AC} & & \mathrm{AC}+\mathbf{D} & & \mathbf{D}\mathrm{C}'\mathrm{A} \\ & & & & 9 \Uparrow 10 \\ & & & & \mathrm{AC}+\mathbf{D} \end{array} \qquad \text{(A1)}$$

The pathway at the left depicts the *aqueous diffusion* mechanism in which cholesterol (C) is released into solution in a first-order step and is then taken up by donor (D) or acceptor (A) particles with second-order kinetics. The pathway in the center represents a *particle collision* mechanism, whereby nonactivated cholesterol is directly transferred through a second-order collision of donor with acceptor. The scheme at the right is the *activation–collision* mechanism. In this pathway, the membrane-associated activated intermediate, C′, can not only be transferred to another donor particle or to an acceptor but can also be recaptured by the parent donor particle (step 6).

Initially, when [AC] = 0, the condition for steady state in [DC′D] is

$$k_{11}[\mathrm{DC}'][\mathrm{D}] = (k_{12} + k_{13})[\mathrm{DC}'\mathrm{D}]. \qquad \text{(A2)}$$

The condition for steady state in [DC′A] is

$$k_7[\mathrm{DC}'][\mathrm{A}] = (k_8 + k_9)[\mathrm{DC}'\mathrm{A}]. \qquad \text{(A3)}$$

The condition for steady state in [DC′] is

$$k_5[\mathrm{DC}] + k_8[\mathrm{DC}'\mathrm{A}] + k_{12}[\mathrm{DC}'\mathrm{D}] = (k_6 + k_7[\mathrm{A}] + k_{11}[\mathrm{D}])[\mathrm{DC}']. \qquad \text{(A4)}$$

Rearranging Eq. A3 yields

$$[\mathrm{DC}'\mathrm{A}] = \frac{k_7[\mathrm{A}][\mathrm{DC}']}{(k_8 + k_9)}. \qquad \text{(A5)}$$

Rearranging Eq. A2 yields

$$[\mathrm{DC}'\mathrm{D}] = \frac{k_{11}[\mathrm{D}][\mathrm{DC}']}{(k_{12} + k_{13})}. \qquad \text{(A6)}$$

By substitution into Eq. A4, we obtain

$$k_5[\mathrm{DC}] = [\mathrm{DC}']\left\{k_6 + k_7[\mathrm{A}]\left(1 - \frac{k_8}{k_8 + k_9}\right) + k_{11}[\mathrm{D}]\left(1 - \frac{k_{12}}{k_{12} + k_{13}}\right)\right\}. \qquad \text{(A7)}$$

Solving for [DC′] yields

$$[\mathrm{DC}'] = \frac{k_5[\mathrm{DC}]}{k_6 + k_7k_9[\mathrm{A}]/(k_8 + k_9) + k_{11}k_{13}[\mathrm{D}]/(k_{12} + k_{13})}. \qquad \text{(A8)}$$

The condition for steady state in [C] is

$$k_1[\mathrm{DC}] = [\mathrm{C}](k_2[\mathrm{D}] + k_3[\mathrm{A}]). \qquad \text{(A9)}$$

Rearrangement yields

$$[\mathrm{C}] = \frac{k_1[\mathrm{DC}]}{k_2[\mathrm{D}] + k_3[\mathrm{A}]}. \qquad \text{(A10)}$$

The global initial rate of cholesterol transfer to acceptors by all three mechanisms (simple particle collision, aqueous diffusion, and activation–collision, respectively) is

$$\frac{\mathrm{d}[\mathrm{AC}]}{\mathrm{d}t} = k_{14}[\mathrm{A}][\mathrm{DC}] + k_3[\mathrm{A}][\mathrm{C}] + k_9[\mathrm{DC}'\mathrm{A}]. \qquad \text{(A11)}$$

Substitution from Eq. A5 results in

$$\frac{d[AC]}{dt} = k_{14}[A][DC] + k_3[A][C] + \frac{k_7k_9[A][DC']}{k_8 + k_9}. \tag{A12}$$

Substitutions from Eqs. A8 and A10 give the global initial rate of transfer to acceptor particles as

$$\frac{d[AC]}{dt} = k_{14}[A][DC] + \frac{k_1k_3[A][DC]}{k_3[A] + k_2[D]} + \frac{k_5k_7k_9[A][DC]/(k_8 + k_9)}{k_6 + k_7k_9[A]/(k_8 + k_9) + k_{11}k_{13}[D]/(k_{12} + k_{13})}. \tag{A13}$$

With the definitions $K_a = k_7k_9/(k_8 + k_9)$ and $K_d = k_{11}k_{13}/(k_{12} + k_{13})$, Eq. A13 takes the form

$$\frac{d[AC]}{dt} = k_{14}[A][DC] + \frac{k_1k_3[A][DC]}{k_3[A] + k_2[D]} + \frac{k_5K_a[A][DC]}{k_6 + K_a[A] + K_d[D]}. \tag{A14}$$

If DC′A and DC′D are not true complexes but only transition states, then K_a and K_d represent the second-order rate constants of the overall processes, according to the simplified reaction scheme,

$$\begin{array}{ccccc} DC & & & & DC+\mathbf{D} \\ \uparrow 2 & & & & \uparrow K_d \\ +D & & & & +D \\ C & \xleftarrow{1} & \mathbf{D}C & \underset{6}{\overset{5}{\rightleftarrows}} & \mathbf{D}C' \\ +A & & +A & & +A \\ 3 \Downarrow \Uparrow 4 & & \downarrow 14 & & \downarrow K_a \\ AC & & AC+\mathbf{D} & & AC+\mathbf{D} \end{array} \tag{A15}$$

From Eq. A14, the initial fractional rate, v_o, is given as

$$v_o = \frac{d([AC]/[DC]_o)_o}{dt} = k_{14}[A] + \frac{k_1k_3[A]}{k_3[A] + k_2[D]} + \frac{k_5K_a[A]}{k_6 + K_a[A] + K_d[D]}. \tag{A16}$$

Because concentrations equal quantity, Q, divided by volume, V, Eq. A16 may be rewritten as

$$v_o = \frac{k_{14}Q_a}{V} + \frac{k_1k_3Q_a}{k_3Q_a + k_2Q_d} + \frac{k_5K_aQ_a}{Vk_6 + K_aQ_a + K_dQ_d}, \tag{A17}$$

or

$$v_o = \frac{k_{14}Q_a}{V} + \frac{k_1k_3Q_a}{k_3Q_a + k_2Q_d} + \frac{k_5K_aQ_a/k_6}{V + K_aQ_a/k_6 + K_dQ_d/k_6}. \tag{A18}$$

According to Eq. A16, the mechanism of transfer can be inferred from the dependence of v_o on donor and acceptor concentrations. Of particular value are experiments that hold donor and acceptor quantities constant and vary the aqueous volume. Then, a nonlinear least squares analysis of v_o versus V according to Eq. A18 will suggest which of these transfer mechanisms contributes significantly to the overall reaction rate.

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