Use of Cyclodextrins to Monitor Transbilayer Movement and Differential Lipid Affinities of Cholesterol

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ABSTRACT In view of the demonstrated cholesterol-binding capabilities of certain cyclodextrins, we have examined whether these agents can also catalyze efficient transfer of cholesterol between lipid vesicles. We here demonstrate that β- and γ-cyclodextrins can dramatically accelerate the rate of cholesterol transfer between lipid vesicles under conditions where a negligible fraction of the sterol is bound to cyclodextrin in steady state. β- and γ-cyclodextrin enhance the rate of transfer of cholesterol between vesicles by a larger factor than they accelerate the transfer of phospholipid, whereas, for α- and methyl-β-cyclodextrin, the opposite is true. Analysis of the kinetics of cyclodextrin-mediated cholesterol transfer between large unilamellar vesicles composed mainly of 1-stearoyl-2-oleoyl phosphatidylcholine (SOPC) or SOPC/cholesterol indicates that transbilayer flip-flop of cholesterol is very rapid (halftime $<$ 1–2 min at 37°C). Using β-cyclodextrin to accelerate cholesterol transfer, we have measured the relative affinities of cholesterol for a variety of different lipid species. Our results show strong variations in cholesterol affinity for phospholipids bearing different degrees of chain unsaturation and lesser, albeit significant, effects of phospholipid headgroup structure on cholesterol-binding affinity. Our findings also confirm previous suggestions that cholesterol interacts with markedly higher affinity with sphingolipids than with common membrane phospholipids.

INTRODUCTION

Cholesterol–lipid interactions have been widely studied in model systems to understand how cholesterol modulates the organization and dynamics of the lipid bilayer in animal cell membranes. The area-condensing and chain-ordering effects of cholesterol on diverse phospho- and sphingolipids have been characterized by a variety of methods in both mono- and bilayer systems (for reviews see Yeagle, 1988; Finegold, 1993). Much recent interest has focussed on the potential of cholesterol to promote the formation of liquid-ordered or other types of fluid domains in membranes (Bretscher and Munro, 1993; Rietveld and Simons, 1998; Brown, 1998; Brown and London, 1998; Huster et al., 1998; Mitchell and Litman, 1998; Polozova and Litman, 2000). This potential rests in part on the differential interactions of cholesterol with different phospho- and sphingolipid species, which have been investigated by a variety of physical techniques (Smaby et al., 1994, 1997; Huster et al., 1998; Mitchell and Litman, 1998; Huang et al., 1999; McMullen et al., 1999; Epand et al., 2000; and references therein).

To date, only a few direct measurements of the relative affinities of cholesterol for different lipid species have been reported (Lange et al., 1979; Nakagawa et al., 1979; Wattenberg and Silbert, 1983; Rujanavech and Silbert, 1986; Yeagle and Young, 1986). Although cholesterol can spontaneously transfer at measurable rates between different lipid vesicles, the rate of equilibration is relatively sluggish, particularly for vesicles with larger radii of curvature, like those characteristic of biological membranes. As a result, with one exception (Yeagle and Young, 1986), most previous measurements of cholesterol partitioning between different bilayers have focussed on cholesterol interactions with lipids in small (sonicated) unilamellar vesicles, which permit faster rates of cholesterol equilibration, but in which lipid–lipid interactions may be perturbed by the vesicles' high radii of curvature. Even in such systems, cholesterol equilibration typically requires times of several hours or longer, which may complicate the application of this approach in systems containing polyunsaturated or other sensitive lipid species.

β-Cyclodextrins are able to bind cholesterol in a water-soluble complex, and high concentrations of methyl- and 2-hydroxypropyl-β-cyclodextrin have been shown to extract cholesterol from mammalian cell membranes (Ohtani et al., 1989; Kilsdonk et al., 1995; Yancey et al., 1996; Atger et al., 1997; Haynes et al., 2000) and to donate cholesterol to cellular membranes under certain conditions (Christian et al., 1997). These findings suggested that cyclodextrins might also be able to serve as catalysts of cholesterol transfer between different lipid vesicles, ideally under conditions where they would not markedly perturb the equilibrium distribution of the sterol. In this report, we demonstrate the ability of cyclodextrins to mediate rapid intervesicular transfer of cholesterol and identify conditions in which such transfer can be achieved using cyclodextrins in a truly catalytic manner. We apply this capability to measure the relative affinities of cholesterol for a variety of different lipid species in large unilamellar vesicles, and to determine that, at physiological temperatures, cholesterol exhibits rapid transbilayer diffusion (halftime $<$ 1–2 min) in fluid lipid bilayers.

Received for publication 23 May 2001 and in final form 2 July 2001.

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0006-3495/01/10/2257/11 $2.00

MATERIALS AND METHODS

Materials

Cyclodextrins were obtained from Sigma/Aldrich Canada (Oakville, Ontario, Canada). [7-^{3}H(n)]Cholesterol (specific activity 5.0 mCi/μmol) was obtained from Amersham Pharmacia Biotech (Baie d'Urfé, Québec, Canada); in some experiments, [1,2-^{3}H(n)]-cholesterol (New England Nuclear, specific activity 48.3 mCi/μmol) was used instead, with identical results. Cholesteryl[1-^{14}C]oleate (specific activity 56.0 μCi/μmol) was obtained from Amersham Pharmacia. [^{3}H]Dipalmitoylphosphatidylcholine ([^{3}H]DPPC) was synthesized from 1-palmitoyl-lyso-phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) and [9,10-^{3}H(n)]palmitic acid (Amersham, specific activity 60 mCi/μmol) by the method of Mason et al. (1981). All other phosphatidylcholine (PC) species used in this study were purchased from Avanti; phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) species were synthesized from the corresponding PCs by phospholipase D-mediated transphosphatidylation (Comfurius and Zwaal, 1977). 1-Stearoyl-2-oleoyl-phosphatidylserine (SOPS) was prepared from the corresponding PC in a similar manner but using phospholipase D from Streptomyces sp. (Sigma). Lipid stock solutions were standardized using the assay of Lowry and Tinsley (1974).

Vesicle preparation and determination of vesicle lamellarity

Lipid samples were mixed in 2:1 CH_2Cl_2/CH_3OH and dried under a stream of nitrogen with warming to 40–45°C, then further dried under high vacuum for 3–12 h. For most experiments, the dried lipid mixtures were redispersed by vortexing under argon at 45°C (or at 85°C for cerebroside-containing samples) in buffer (25 mM KCl, 10 mM 3-[*N*-morpholino]propanesulfonic acid, 0.1 mM ethylenediaminetetraacetic acid, pH 7.0), then extruded through 0.1 μm pore-size polycarbonate filters (MacDonald et al., 1991). Recovery of lipid from the extrusion procedure was determined by liquid scintillation counting of small samples taken before and after the extrusion step. For specific experiments, as indicated, vesicles were prepared by one of the following alternative protocols. Sonicated/freeze–thawed vesicles were prepared by vortexing dried lipids in distilled water, bath-sonicating under argon to virtual clarity (for ~10 min), adding 0.11 volumes of 10× concentrated buffer, resonicating for 1 min and five times freeze–thawing in a dry ice/ethanol bath. Vortexed/freeze–thawed vesicles were prepared by vortexing dried lipids into buffer for 2 min, then five times freeze–thawing.

To estimate the lamellarity of vesicle preparations, parallel samples were prepared in an identical manner but including 10 mol% 1-stearoyl-2-oleoyl phosphatidylethanolamine (SOPE). The fraction of total vesicle PE exposed at the outer surface of the vesicles was then determined using the assay of Nordlund et al. (1981), based on the reaction of surface-exposed PE with the impermeant reagent trinitrobenezenesulfonic acid. Because, for large vesicles, PE is distributed symmetrically across the lipid bilayer (Nordlund et al., 1981), the fraction of total lipid present in the outermost lamellae of the vesicles was calculated as 1.95 times the fraction of surface-exposed PE, where 1.95 is the calculated ratio of total to outer-leaflet lipids in extruded vesicles prepared as described here (mean diameter ~120 nm [Rodrigueza et al., 1995 and our unpublished results]).

[^{3}H]Cholesterol and [^{3}H]DPPC transfer/partitioning assays

Mixtures of negatively charged donor vesicles (0.2–1.6 mM, as indicated, containing 1–2 μCi [^{3}H]cholesterol/μmol lipid) were incubated with neutral acceptor vesicles (0.4–1.6 mM, containing 0.2 μCi [^{14}C]cholesteryl oleate per μmol lipid) in buffer and under argon for mixtures containing polyunsaturated lipids. For kinetic measurements, a 10:1 ratio of acceptor to donor lipids was used, whereas, for cholesterol-partitioning measurements, incubation mixtures were prepared with four different acceptor/donor ratios, spanning at least an eightfold range. At various times 50-μl samples were withdrawn and applied to chilled minicolumns of DEAE-Sephacel (Amersham Pharmacia) which had been prerun at 25°C with 30 μl of 10 mM sonicated egg PC vesicles, then washed with 2 ml buffer to minimize nonspecific vesicle adsorption. The minicolumns were washed at 0°C with 1.25 ml of buffer, which typically eluted 85–90% of the acceptor vesicles while retaining the donor vesicles quantitatively (Jones and Thompson, 1989; Leventis and Silvius, 1993). The eluates were mixed with 12.5 ml Cytoscint (ICN, Montréal, Québec, Canada) and analyzed by liquid scintillation counting for ^{3}H and ^{14}C with appropriate spillover corrections. All data presented here were averaged from, or representative of, results obtained in at least two independent experiments. In some experiments using polyunsaturated phospholipids, in addition to the precautions against peroxidation described above, all buffers were purged with argon, and the antioxidant butylated hydroxytoluene was added to the lipid mixtures at 1:250 mol/mol lipid, with no discernible difference in the results obtained.

The nonexchangeability of the [^{14}C]cholesteryl oleate marker between vesicles was verified by incubating negatively charged vesicles (85:15 SOPC/SOPG, 0.4 mM), labeled with this marker, with uncharged vesicles (SOPC, 4 mM), for varying times at 37°C. After incubation, samples were applied to DEAE-Sephacel minicolumns and the uncharged vesicles eluted and counted for transferred [^{14}C]cholesterol oleate. By this criterion no significant transfer of labeled cholesteryl oleate (<0.05% of input) was observed from the anionic to the neutral vesicles, even after preincubation for up to 3 h at 37°C in the presence of 5 mM β-cyclodextrin or methyl-β-cyclodextrin.

RESULTS

In initial experiments, we examined the potential of β-methylcyclodextrin at varying concentrations to accelerate the transfer of [^{3}H]cholesterol between large unilamellar lipid vesicles (LUV). Cholesterol transfer was measured using the assay of McLean and Phillips (1982) as modified by Bar et al. (1986) and Jones and Thompson (1989). Negatively charged donor vesicles, incorporating [^{3}H]cholesterol (at tracer levels [0.01–0.1 mol%] except where otherwise indicated), were incubated with neutral acceptor vesicles incorporating the nonexchangeable marker [^{14}C]cholesteryl oleate. After incubation, vesicle mixtures were applied to DEAE-Sephacel minicolumns (which quantitatively retained the donor vesicles), and the ratio of eluted [^{3}H]cholesterol to [^{14}C]cholesteryl oleate provided a direct measure of the extent of [^{3}H]cholesterol transfer to the acceptor vesicles. Using this assay, the halftime for spontaneous transfer of [^{3}H]cholesterol from SOPC/SOPG (85:15) donor to SOPC acceptor LUV at 37°C was found to be 195 ± 13 min. This halftime for cholesterol transfer is similar to that reported for transfer between egg PC/cholesterol/cerebroside (75:10:15) vesicles of similar size (Rodrigueza et al., 1995) and roughly twofold longer than that observed using small unilamellar egg PC vesicles (McLean and Phillips, 1982; Bar et al., 1986).

As shown in Fig. 1, *A* and *B*, the rate of intervesicle transfer of cholesterol rises linearly with the concentration of β-cyclodextrin, such that at 1 mM β-cyclodextrin the

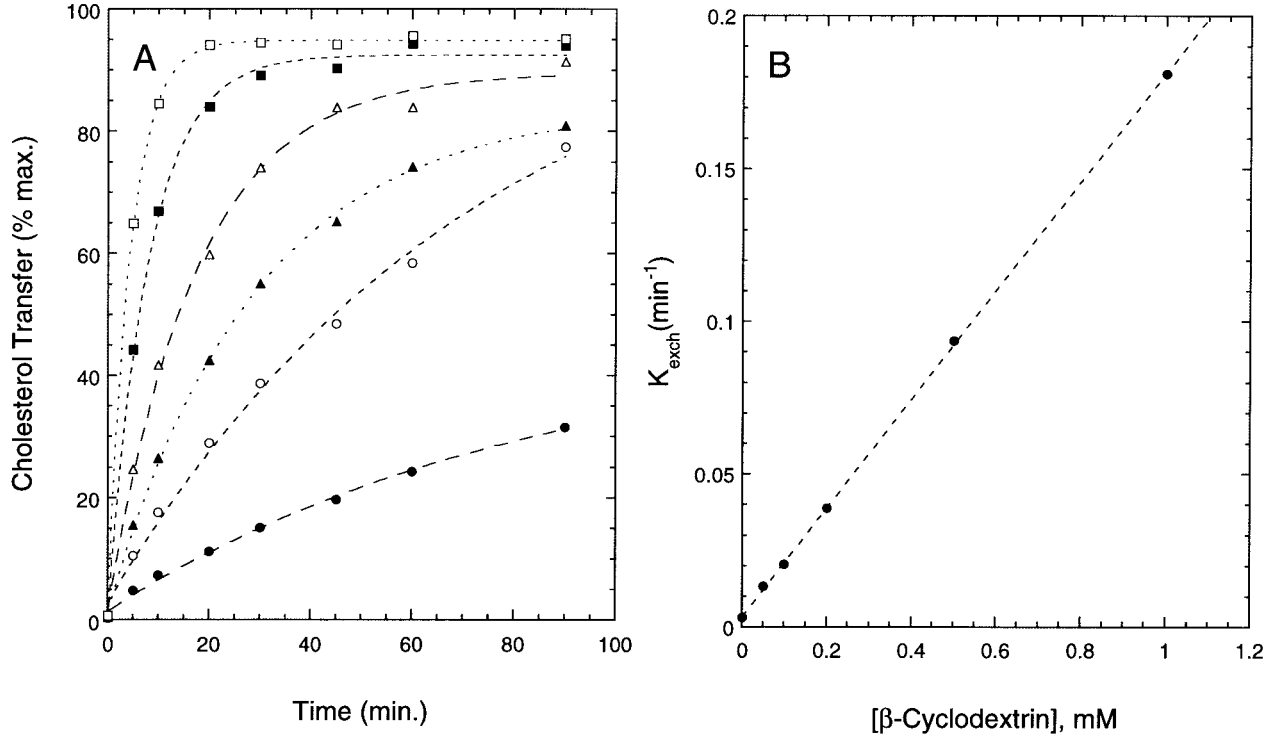

FIGURE 1 (*A*) Time courses of transfer of [^{3}H]cholesterol from SOPC/SOPG (85:15) donor LUV (0.4 mM) to SOPC acceptor LUV (4 mM) at 37°C in the presence of (●) 0 mM, (○) 0.05 mM, (▲) 0.1 mM, (Δ) 0.2 mM, (■) 0.5 mM and (□) 1 mM β-cyclodextrin. Details of vesicle preparation and assay of cholesterol transfer were as described in Materials and Methods. Curves fitted to each equation were of the form (% Transfer) = $K_1 - K_2\exp(-k_{exch}t)$, where K_1, K_2, and k_{exch} are fitting constants. The *y* axis values are scaled to the maximum value calculated assuming complete equilibration of cholesterol between the donor and acceptor vesicles (= 91% of cholesterol present in acceptor vesicles). (*B*) First-order rate constants (k_{exch}) for cholesterol transfer from donor to acceptor vesicles, determined from the curve fits shown in panel *A*, as a function of the concentration of β-cyclodextrin. Data shown are representative of results obtained in duplicate experiments.

halftime for cholesterol transfer is roughly 60-fold lower than that observed in the absence of cyclodextrin. Under the same conditions, methyl-β- and γ-cyclodextrins, but not α-cyclodextrin, also strongly accelerated the rate of cholesterol transfer between vesicles (e.g., by 53- and 64-fold respectively, compared to 63-fold for β-cyclodextrin, at 1 mM cyclodextrin in a representative experiment). As discussed in Materials and Methods, cyclodextrins did not promote intervesicle transfer of [^{14}C]cholesteryl oleate under the same conditions, indicating that the cyclodextrin-mediated transfer of [^{3}H]cholesterol between vesicles is not a consequence of vesicle fusion or hemifusion. The time courses of [^{3}H]cholesterol transfer are monophasic and approach the limit expected if all of the labeled cholesterol in the donor vesicles is readily available for exchange.

High concentrations of methyl-β-cyclodextrin can extract substantial amounts of cholesterol from membranes into a soluble cholesterol–cyclodextrin complex (Ohtani et al., 1989; Kilsdonk et al., 1995; Yancey et al., 1996; Atger et al., 1997; Christian et al., 1997; Haynes et al., 2000; Ohvo-Rekila et al., 2000). It was thus important to determine the fraction of cholesterol bound to cyclodextrin in steady state under the conditions of the above experiments. In control experiments, we found that negligible amounts of labeled cholesterol (<0.25% of input) were eluted from DEAE-Sephacel minicolumns after applying samples containing [^{3}H]cholesterol-labeled donor vesicles (0.4–4 mM) and β-cyclodextrin (up to 1 mM) but lacking neutral acceptor vesicles, even when the samples were preincubated for 2 h at 37°C. In complementary experiments, in which β-cyclodextrin was preincubated with neutral SOPC vesicles containing both [^{3}H]cholesterol and [^{14}C]cholesteryl oleate, a negligible decrease was observed in the ratio of [^{3}H]cholesterol to [^{14}C]cholesteryl oleate (<2%) when the vesicles were subsequently passed over DEAE-Sephacel. In both types of control experiments, the labeled cholesterol thus behaved as though it was essentially completely bound to the lipid vesicles, with at most a very small fraction present as a soluble complex with β-cyclodextrin. At high cyclodextrin/lipid ratios, particularly using methyl-β-cyclodextrin in place of β-cyclodextrin, significant extraction of cholesterol from vesicles was detected by the second type of experiment described above (i.e., by a decrease in the ratio of [^{3}H]cholesterol/([^{14}C]cholesteryl

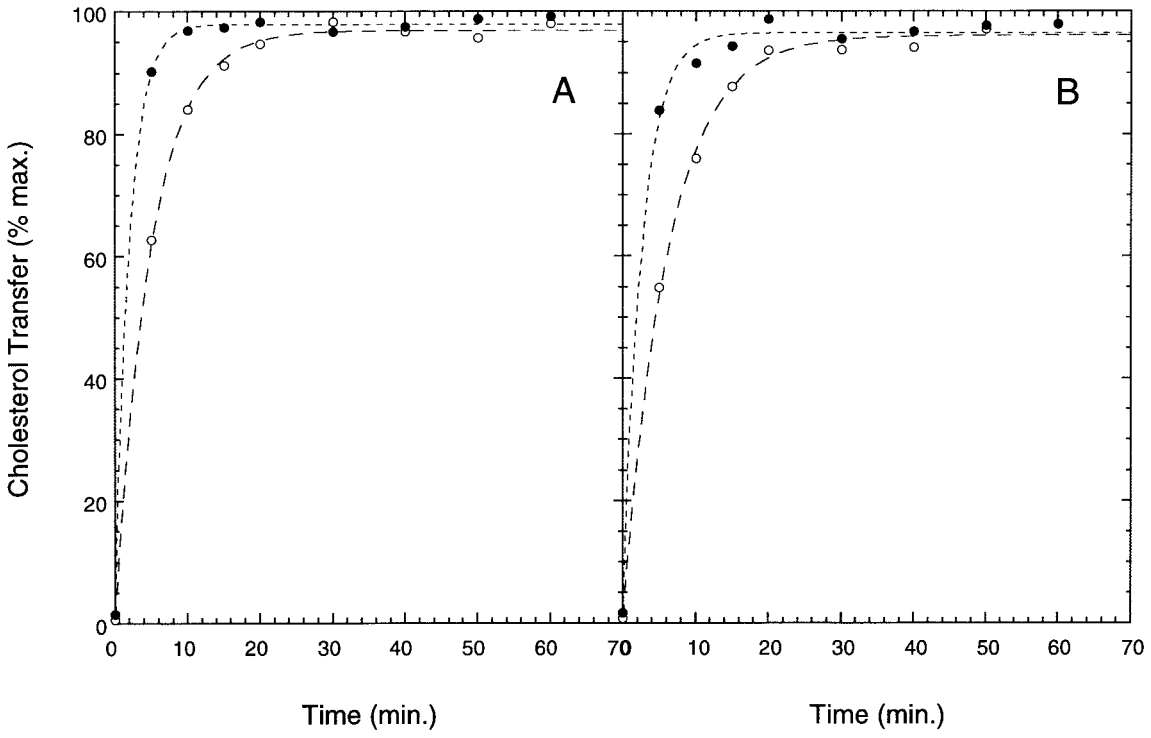

FIGURE 2 (*A*) Time courses of transfer of [^{3}H]cholesterol from SOPC/SOPG (85:15) donor LUV (0.4 mM) to SOPC acceptor LUV (4 mM) in the presence of (○) 1 mM or (●) 3 mM β-cyclodextrin. The *y* axis values are scaled as a fraction of the expected maximal extent of cholesterol transfer at equilibrium (= 91% of cholesterol present in acceptor vesicles). (*B*) Time courses of transfer of [^{3}H]cholesterol from SOPC/SOPG/cholesterol (85:15:50) donor LUV (0.4 mM) to SOPC/cholesterol (100:50) acceptor LUV (4 mM) in the presence of (○) 1 mM or (●) 3 mM β-cyclodextrin. Other details were as for panel *A*. Control measurements carried out as described in Materials and Methods indicated that the donor vesicle preparations used in these experiments were homogeneously unilamallar (98 ± 4% of total lipids in the external lamellae). Data shown are representative of results obtained in duplicate experiments.

oleate in SOPC vesicles after elution from DEAE-Sephacel). The above results are consistent with previous findings suggesting that the affinity of cholesterol for β-cyclodextrins is significantly weaker than its affinity for phospholipids (Kilsdonk et al., 1995). All experiments described below were carried out using lipid and cyclodextrin concentrations determined to give very small fractions of cholesterol bound to cyclodextrin (<2%) using the experimental approaches just described.

Measurements of the halftime for transbilayer diffusion (flip-flop) of cholesterol in fluid lipid bilayers have given widely varying results, ranging from seconds to hours (Smith and Green, 1974; Poznansky and Lange, 1976, 1978; Backer and Dawidowicz, 1979, 1981; Rodrigueza et al., 1995). The rapid intervesicle transfer of cholesterol catalyzed by β-cyclodextrin provided an opportunity to estimate a lower limit for the rate of cholesterol flip-flop in large unilamellar vesicles under equilibrium conditions, because flip-flop is required to render cholesterol from the inner vesicle monolayer available for transfer. As shown in Fig. 2 *A*, using 1 mM β-cyclodextrin, the estimated halftime for cholesterol transfer between SOPC/SOPG (85:15) vesicles at 37°C is ~3.4 min, and at 3 mM β-cyclodextrin, the estimated halftime is reduced to <1.5 min. A similar result is obtained using donor and acceptor vesicles containing 33 mol% cholesterol (Fig. 2 *B*); using 1 mM or 3 mM β-cyclodextrin the halftime for cholesterol transfer is 4.3 min and ~1.8 min, respectively. As was observed at lower concentrations of β-cyclodextrin, the progress of [^{3}H]cholesterol transfer can be well-described by a monoexponential time course, which closely approaches the plateau expected for complete equilibration of cholesterol between the donor and acceptor vesicles. Similar results were obtained using γ- or methyl-β-cyclodextrin in otherwise identical experiments (results not shown). As discussed below, under the same conditions, only ~50% of [^{3}H]-labeled phosphatidylcholine in identically prepared vesicles was available for rapid exchange, indicating that the cyclodextrins did not disrupt the integrity of the vesicle bilayer. From these results we can conclude that transbilayer flip-flop of [^{3}H]cholesterol is not rate-limiting for cholesterol transfer even at the highest cyclodextrin concentrations tested, and hence that, at 37°C, the halftime for this process is no more than 1–2 min in either SOPC/SOPG (85:15) or SOPC/SOPG/cholesterol (85:15:50) LUV.

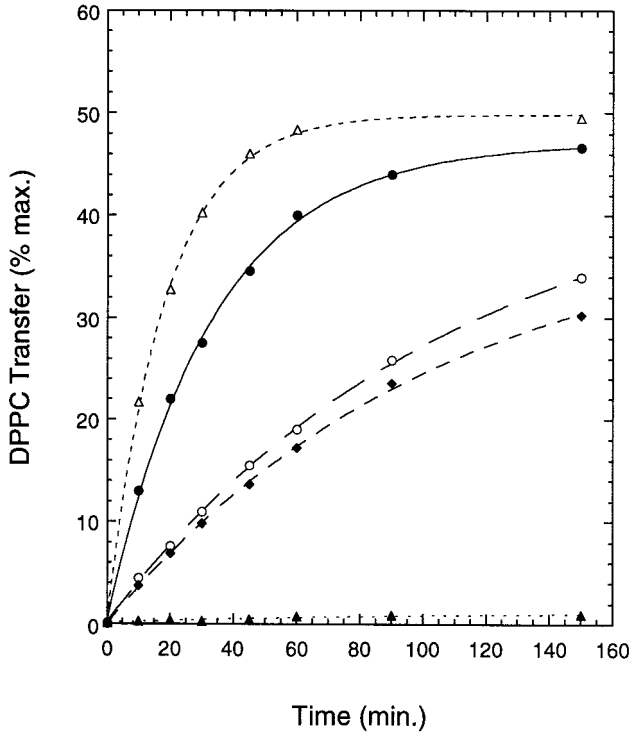

FIGURE 3 Time courses of transfer of [^{3}H]DPPC from SOPC/SOPG (85:15) donor vesicles to SOPC acceptor vesicles at 37°C in the presence of (●) α-, (○) β-, (Δ) methyl-β-, and (◆) γ-cyclodextrin (1 mM) or in the absence of cyclodextrin (▲). Other experimental details were as described for Fig. 1.

Cyclodextrin-mediated intervesicle transfer of phospholipids

Previous studies have shown that cyclodextrins can bind, and hence potentially enhance interbilayer transfer of, certain phospholipids and sterols (Debouzy et al., 1998; Tanhuanpää and Somerharju, 1999). This latter potential was first examined by measuring the ability of various cyclodextrins to enhance the transfer of [^{3}H]dipalmitoylphosphatidylcholine ([^{3}H]DPPC) between large unilamellar lipid vesicles. As shown in Fig. 3, at 37°C and at a concentration of 1 mM, various cyclodextrins indeed markedly accelerated the transfer of [^{3}H]DPPC between SOPC/SOPG (85:15) donor and SOPC acceptor vesicles. Methyl-β-cyclodextrin and α-cyclodextrin enhanced the transfer rate most strongly (by roughly 250- and 80-fold, respectively), and β- and γ-cyclodextrins enhanced the rate of transfer by smaller but still substantial factors (~35-fold). Increasing the concentration of cyclodextrin from 1 to 2 mM increased the transfer rate by almost exactly twofold for β-, methyl-β-, and γ-cyclodextrin but by nearly fourfold ([3.9 ± 0.3]-fold) for α-cyclodextrin (results not shown). Comparing these results to those presented above for [^{3}H]cholesterol transfer, it can be seen that methyl-β-cyclodextrin and α-cyclodextrin accelerate the transfer of DPPC by considerably larger factors than they accelerate the transfer of cholesterol, while the opposite is true for β- and γ-cyclodextrin.

The fraction of [^{3}H]DPPC that was available for rapid intervesicle transfer in the presence of cyclodextrins was estimated as 48 ± 2% of the total labeled DPPC incorporated in the vesicles. This figure agrees closely with the percentage of total lipids exposed at the outer surface of identically prepared vesicles containing 10 mol% SOPE (estimated as 50.6 ± 1.7% by measuring the fraction of surface-exposed PE [Nordlund et al., 1981]). It thus appears that only the fraction of [^{3}H]DPPC present in the outer leaflets of the vesicles is available for exchange on the time scale of our experiments (2–3 h), as expected given the very low rates previously reported for spontaneous transbilayer flip-flip of phosphatidylcholines (Rothman and Dawidowicz, 1975; Dawidowicz and Rothman, 1976; Dicorleto and Zilversmit, 1979). As noted above, this finding indicates that the ready intervesicle exchange of 100% of the cholesterol in LUV under similar conditions cannot be ascribed to a cyclodextrin-induced destabilization of the vesicle bilayer integrity.

For the measurements described below of cholesterol partitioning between vesicles with different compositions, it was important to ensure that significant redistribution of phospholipids did not occur on the time scale required for cholesterol to equilibrate between the vesicles. Two measures were taken to ensure this result. First, β-cyclodextrin was used to catalyze the intervesicle transfer of cholesterol in these assays, because at a given concentration, it accelerates transfer of cholesterol by a substantially larger factor than it enhances the transfer of phospholipid. Second, we directly estimated the rates of β-cyclodextrin-mediated transfer of various unlabeled lipid species between vesicles, using the following type of experiment. Phosphatidylglycerols (PGs) were prepared with the acyl-chain compositions of each of the phospholipids used in the cholesterol-partitioning assays discussed in the next section. Unlabeled vesicles (0.4 mM) combining small proportions of these PG species with SOPC were incubated for varying times at 37°C with [^{14}C]cholesteryl oleate-containing SOPC vesicles (0.4 mM) in the presence of β-cyclodextrin, and the samples were then passed through DEAE-Sephacel minicolumns and the recovery of the [^{14}C]cholesteryl oleate-labeled vesicles determined. As shown in Fig. 4, recovery of SOPC vesicles from DEAE-Sephacel falls sharply as small proportions of PG become incorporated into them, providing a sensitive means to monitor transfer of PG from the unlabeled to the [^{14}C]cholesteryl oleate-labeled vesicles. Transfer of SOPS or (with roughly tenfold lesser sensitivity) SOPE to SOPC vesicles could be monitored in the same manner (results not shown). Using this approach, we confirmed that, at 37°C and in the presence of 0.5 mM β-cyclodextrin, the halftimes for the transfer of the various PG species examined (and for SOPE and SOPS) were far longer than those for [^{3}H]cholesterol transfer (e.g., 740, 1600, and

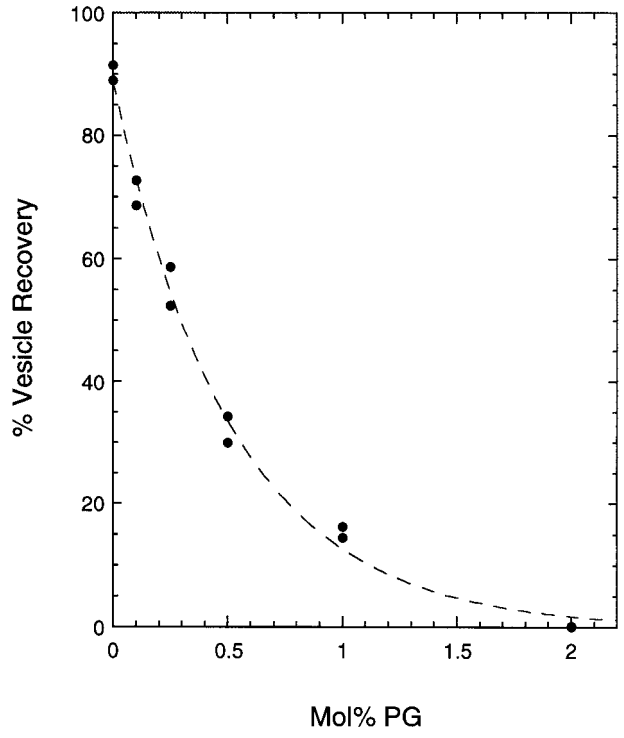

FIGURE 4 Efficiency of recovery of [^{14}C]cholesteryl oleate-labeled SOPC/SOPG vesicles, incorporating the indicated molar percentages of SOPG, from DEAE-Sephacel minicolumns. Vesicles were prepared and recovery from the minicolumns analyzed as described in Materials and Methods.

7400 min for transfer of DPPG, dioleoyl PG, and SOPG, respectively, compared to 7 min for transfer of cholesterol).

Combining the above results with those from our [^{3}H]DPPC-transfer experiments, we chose vesicle compositions and time courses for the cholesterol-partitioning studies described below to ensure <5% transfer of nonsterol components between vesicles on the time scale of the experiments. In these experiments, measurements of cholesterol transfer over periods as short as 15–45 min typically yielded estimates of the equilibrium (plateau) distribution that agreed very well with those obtained by extending the measurements to much longer times.

Equilibrium partitioning of [^{3}H]cholesterol between different lipid vesicles

To monitor the equilibrium partitioning of [^{3}H]cholesterol between different types of lipid bilayers, donor vesicles combining 15 mol% PG with neutral lipids and labeled with [^{3}H]cholesterol were incubated at 37°C with uncharged acceptor vesicles (labeled with [^{14}C]cholesteryl oleate) in different molar ratios, together with β-cyclodextrin (0.5 mM where not otherwise indicated). Time courses of [^{3}H]cholesterol redistribution between donor and acceptor vesicles, determined as in the kinetic experiments described above, were fitted to a monoexponential equation to determine the equilibrium distribution of cholesterol between the two vesicle populations, as illustrated in Fig. 5 *A*. In principle, each such time course provides an independent estimate of the partition coefficient K_p describing the relative affinity of cholesterol for the acceptor over the donor vesicles, according to the equation,

$$(\%\text{Cholesterol in acceptors}) = 100\% \cdot \left(\frac{(A/D)}{1 + K_p(A/D)}\right), \quad (1)$$

where (A/D) represents the ratio of concentrations of acceptor to donor vesicle lipids. In practice, however, data from time courses obtained using different acceptor/donor ratios were fit simultaneously to the above equation, as illustrated in Fig. 5 *B*. To avoid complications due to possible slow kinetics of cholesterol transfer from gel-state domains, all experiments were carried out using vesicle compositions predicted to give only fluid (liquid-ordered and liquid-disordered) phases at 37°C (Barenholz et al., 1976; Curatolo et al., 1985; Ahmed et al., 1997).

The application of Eq. 1 to experimental data is strictly appropriate only in the case where the donor or acceptor vesicles are purely unilamellar. For most types of vesicles used to measure cholesterol partitioning (including all types of donor vesicles used), the fraction of total lipid present in the outer lamellae, estimated as described in Materials and Methods, was essentially equal to unity (0.96–1.02). However, for certain types of (uncharged) acceptor vesicles, this fraction was significantly smaller (usually >0.80, but ranging as low as 0.64 in the case of SOPC/sphingomyelin vesicles). We therefore examined whether cholesterol could transfer readily between the external and internal lamellae of such vesicles on the time scale of our cholesterol-partitioning measurements. As shown in Fig. 6, when vesicles (SOPC/SOPE/SOPG, 75:10:15) incorporating [^{3}H]cholesterol are prepared with a significant fraction of their total lipid in internal lamellae, only a fraction of the labeled cholesterol is available for rapid transfer to other vesicles in the presence of 1 mM β-cyclodextrin (halftime = 3–4 min), whereas the rate of transfer of the remaining cholesterol is much slower (halftime = ~250 min). The fraction of rapidly exchangeable cholesterol in each preparation of vesicles (72% for sonicated/freeze–thawed and 65% for vortexed/freeze–thawed vesicles, respectively) agreed well with the estimated fraction of lipid present in the external vesicle lamellae (71 ± 2% and 61 ± 3%, respectively, estimated as discussed above). The times required to estimate the equilibrium distribution of cholesterol between donor and acceptor vesicles (typically 15–45 min) were therefore much shorter than the time scale required for cholesterol to redistribute substantially between external and internal lamellae of the acceptor vesicles. Accordingly, to analyze data from cholesterol-partitioning experiments

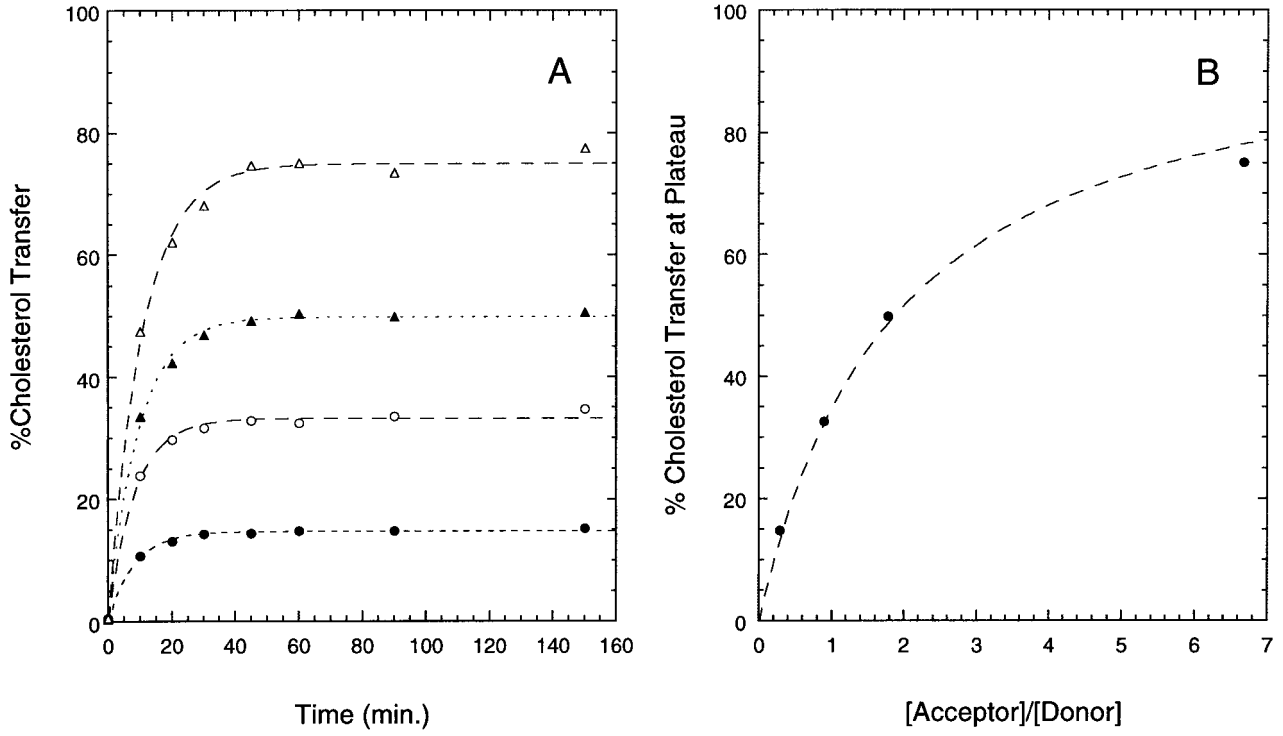

FIGURE 5 (*A*) Representative time courses of equilibration of [^{3}H]cholesterol, determined as described in Materials and Methods, between SOPC/SOPG/cholesterol (85:15:50) donor vesicles and (18:0/18:2-PC)/cholesterol (100:50) acceptor vesicles. Mixtures of donor vesicles containing [^{3}H]cholesterol and acceptor vesicles labeled with [^{14}C]cholesteryl oleate were incubated at four different concentration ratios (*bottom* to *top curves*: acceptor/donor ratios of 0.25:1, 1:1, 2:1, and 6.7:1) at 37°C in the presence of 0.5 mM β-cyclodextrin. Time courses of [^{3}H]cholesterol transfer were fitted to a monoexponential equation of the form described in the legend to Fig. 1, from which the extent of cholesterol transfer at the plateau was estimated for each curve. (*B*) Plateau values determined from the fitted curves in panel *A* were plotted as a function of the ratio of concentrations of acceptor to donor vesicle lipids. The resulting plot was fitted to Eq. 1 in the text, yielding an estimate of $K_p = 0.59 \pm 0.03$ for the experiment shown.

using Eq. 1, we equated the effective acceptor-vesicle lipid concentrations A to the estimated concentration of lipid present in the external lamellae of the acceptor vesicles.

In Table 1 are summarized the values of K_p estimated, using the approach outlined above, for the partitioning of [^{3}H]cholesterol between different types of donor and acceptor vesicles. To facilitate interpretation of the data, the final column of this table also indicates the calculated affinity of cholesterol for various bilayer compositions (indicated in boldface in the table) relative to that for pure SOPC bilayers. As expected, when both vesicle populations incorporate SOPC as the major lipid species, the measured partition coefficient is close to unity. Cholesterol shows a significantly lower affinity for multiply unsaturated lipids than for SOPC, the affinity decreasing with the total extent of unsaturation of the acyl chains. The presence of a saturated 1-position acyl chain significantly enhances interaction with cholesterol at a given level of total lipid unsaturation (compare the affinities listed in Table 1 for 18:0/20:4 PC versus 18:2/18:2 PC, or for 18:0/18:2 PC versus 18:1/18:1 PC). The preference of cholesterol for SOPC over a more highly unsaturated phospholipid (18:0/18:2 PC) is maintained when the donor and acceptor vesicles contain 33 mol% cholesterol rather than simply trace amounts.

Cholesterol shows a moderately higher affinity for SOPC vesicles than for SOPE/SOPS (85:15) vesicles (Table 1). By contrast, cholesterol shows a modest preference for SOPS over SOPC, both when PS comprises 100% of the bilayer lipid and when it is mixed in equimolar proportions with PC. These findings agree with previous conclusions that, for phospholipids with identical acyl chains, the relative affinity for cholesterol will vary in the order PS > PC > PE (van Dijck et al., 1976; Van Dijck, 1979; Yeagle and Young, 1986). However, the magnitudes of these differences in affinity based on headgroup structure alone appear to be relatively modest.

The β-cyclodextrin-promoted equilibration of [^{3}H]cholesterol in mixtures including pure sphingomyelin vesicles showed relatively slow kinetics, even at 45°C, in agreement with the extremely slow rates of cholesterol equilibration observed in similar systems without cyclodextrin (Yeagle and Young, 1986 and our unpublished observations). However, in the presence of 1 mM β-cyclodextrin and at 37°C, cholesterol equilibration proceeded at acceptably rapid rates (halftimes of 20–30 min or less) between SOPC/SOPG donor vesicles and acceptor vesicles containing 50 mol% or even 67 mol% sphingomyelin. In Table 1 are summarized the partition coefficients K_p determined from such experi-

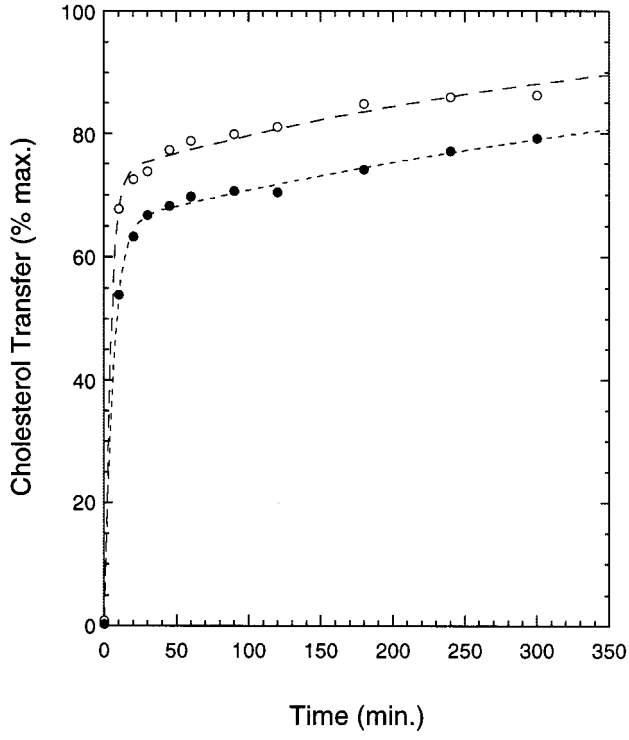

FIGURE 6 Time courses of [^{3}H]cholesterol transfer from SOPC/SOPE/SOPG (75:10:15) vesicles (0.4 mM), prepared by (○) sonication/freeze–thawing or (●) vortexing/freeze–thawing, to SOPC vesicles (4 mM) prepared by filter extrusion. Vesicles were incubated at 37°C in the presence of 1 mM β-cyclodextrin. *y* axis values are scaled as a percentage of the expected maximal extent of cholesterol transfer (91% present in acceptor vesicles), calculated assuming complete equilibration of cholesterol between the donor and acceptor vesicle populations. Curves shown were fitted to the data using the equation (% Transfer) = $100 - K_1\exp(-k_1t) - K_2\exp(-k_2t)$, where K_1 and k_1 represent the amplitude and rate constant for the rapid component of cholesterol transfer and K_2 and k_2 represent the corresponding parameters for the slow component.

ments. Sphingomyelin clearly exhibits a markedly higher affinity for cholesterol than does SOPC or even the disaturated species dipalmitoyl PC, which exhibits a similar transition temperature (Barenholz et al., 1976). Inclusion of 33 mol% cholesterol in the donor and acceptor vesicles only slightly modifies the observed preference of sphingomyelin-containing vesicles for cholesterol. Assuming a roughly linear variation of cholesterol affinity with bilayer sphingomyelin content, we can calculate that the affinity of cholesterol for bovine brain sphingomyelin in fluid bilayers is 3–3.5 times greater than that for SOPC. Acceptor vesicles containing a 1:1 mixture of sphingomyelin and total brain cerebroside showed an affinity for cholesterol similar to that observed for vesicles containing the equivalent amount of sphingomyelin (Table 1, last entry).

DISCUSSION

Cyclodextrins have been shown previously to enhance the rate of transfer of cholesterol between mammalian cell membranes and acceptors such as serum lipoproteins or lipid vesicles (Atger et al., 1997; Christian et al., 1999). However, the magnitudes of the rate enhancements observed in these systems have typically been relatively modest (fivefold or smaller) and the equilibration times correspondingly long (hours). By contrast, in the lipid–vesicle systems examined here, β-, methyl-β-, and γ-cyclodextrin can accelerate the rate of interbilayer transfer of cholesterol by large factors (of the order of 60-fold at 1 mM cyclodextrin) under conditions where only a very small fraction of cholesterol is cyclodextrin-bound in steady state. In the present study, we have exploited this potential to gain information about the rate of transbilayer diffusion of cholesterol in lipid bilayers and the relative affinity of cholesterol for a variety of phospholipids. The potential to use cyclodextrins catalytically to accelerate the interbilayer transfer of cholesterol may prove useful in a variety of other applications. For example, sterol-containing lipid vesicles and low concentrations of β-cyclodextrins may provide a useful alternative to sterol-cyclodextrin complexes (which can be tedious to prepare) as a means to introduce exogenous sterols into mammalian cells.

Our finding that cholesterol undergoes rapid flip-flop in fluid-phase large unilamellar vesicles with or without cholesterol (33 mol%) agrees with some previous reports that transbilayer movement of the sterol is rapid (halftimes of seconds to a few minutes) in small unilamellar vesicles (Backer and Dawidowicz, 1979, 1981; Kan et al., 1992), but not with other reports concluding that cholesterol flip-flop is very slow (halftimes of several hours or more) in small or in large unilamellar vesicles (Poznansky and Lange, 1976, 1978; Rodrigueza et al., 1995). It is not clear what factors account fully for these discrepancies. However, most previous evidence for slow transbilayer flip-flop of cholesterol has rested on the identification of rapid and slow components of sterol exchange between vesicles (reviewed in Rodrigueza et al., 1995; Schroeder and Nemecz, 1990), the origins of which may be attributable to sources other than cholesterol molecules in the inner versus outer bilayer leaflets. It should be noted that the rapid transbilayer movement of cholesterol described here is observed under essentially equilibrium conditions, as the amounts of labeled cholesterol transferred between vesicles are negligible on a mass basis (0.1–1 nmol labeled cholesterol per μmol lipid in the donor vesicles). It could be argued that cyclodextrins in some manner accelerate the transbilayer flip-flop of cholesterol. However, we suggest that this possibility is unlikely, for at least three reasons. First, as already noted, cyclodextrins do not promote detectable transbilayer flip-flop of [^{3}H]DPPC on the time scale of the experiments presented here, indicating that these agents do not perturb the integrity of the vesicle bilayers. Second, rapid transbilayer flip-flop of cholesterol is observed when any of the species β-, methyl-β-, or γ-cyclodextrin is used to promote intervesicle transfer of labeled sterol, in spite of substantial differences

TABLE 1 Partition coefficients (K_p) for cholesterol distribution between different donor and acceptor vesicles at 37°C*

Donor Vesicle Composition	Acceptor Vesicle Composition	K_p	K_p/K_p (SOPC)
SOPC/SOPG (85:15)	**18:2/18:2-PC**	0.28 ± 0.02	0.31†
SOPC/SOPG (85:15)	**18:0/20:4-PC**	0.41 ± 0.01	0.45†
SOPC/SOPG (85:15)	**18:1/18:1 PC**	0.46 ± 0.01	0.51†
SOPC/SOPG (85:15)	**18:0/18:2 PC**	0.60 ± 0.03	0.66†
SOPC/SOPG (85:15)	**SOPC**	0.91 ± 0.06	(1.00)†
SOPC/SOPG (85:15)	**SOPC/DPPC (50:50)**	1.52 ± 0.10	1.67†
SOPC/SOPG (85:15)	**SOPC/SM‡ (50:50)**	2.33 ± 0.17	2.56†
SOPE/SOPS (85:15)	SOPC	1.49 ± 0.02	0.67§
SOPC/SOPS (50:50)	SOPC	0.85 ± 0.04	1.18§
SOPS	SOPC	0.69 ± 0.09	1.45§
SOPC/SOPG/Chol (85:15:50)	18:0/18:2 PC/Chol (100:50)	0.60 ± 0.03	
SOPC/SOPG/Chol. (85:15:50)	SOPC/SM/Chol. (50:50:50)	2.02 ± 0.06	
SOPC/SOPG/Chol. (85:15:50)	SOPC/SM/Chol. (33:67:50)	2.31 ± 0.05	
SOPC/SOPG/Chol. (85:15:50)	SOPC/SM/Cereb/Chol. (50:25:25:50)	2.79 ± 0.53	

*Values shown were determined as described in the text as the average of 2–4 independent experiments, each using four different ratios of donor-to-acceptor vesicle concentrations. K_p values higher than unity indicate a net preference for the acceptor vesicles. Error estimates shown represent the greater of the standard deviation calculated from the K_p estimates in the replicate experiments, or the average of the standard errors of estimation of K_p determined from the curve fits in the replicate experiments.

†Calculated affinity of cholesterol for the indicated vesicles (composition shown in boldface) relative to that for pure SOPC vesicles (= K_p/K_p (SOPC acceptors) = $K_p/0.91$).

‡Special abbreviations used in the table: Cereb, bovine brain galactocerebroside; Chol, cholesterol; SM, bovine brain sphingomyelin.

§Calculated affinity of cholesterol for the indicated donor vesicles (composition shown in boldface) relative to that for pure SOPC vesicles (= $1/K_p$).

in the behavior of these three compounds in other assays (this study and Ohtani et al., 1989). Finally, cyclodextrins have been reported to show negligible binding to erythrocytes and not to penetrate into cholesterol monolayers (Ohtani et al., 1989), again suggesting that they do not exert significant perturbing effects on bilayer organization. The rapid rate of transbilayer diffusion that we observe here for cholesterol is consistent with previous reports of rapid transbilayer flip-flop for similarly hydrophobic species such as diacylglycerol (Ganong and Bell, 1984; Allan et al., 1978; Bai and Pagano, 1997).

Although cyclodextrin-mediated transfer of phospholipids between lipid bilayers was not the primary focus of this work, our results suggest that certain cyclodextrins may prove very useful for catalyzing phospholipid transfer between membranes. Two aspects of this phenomenon are worthy of particular note. The first is that methyl-β-cyclodextrin, in contrast to β- and γ-cyclodextrin, accelerates intervesicle transfer of [^{3}H]DPPC by a much greater factor than it does the transfer of cholesterol under the same conditions. High concentrations of methyl-β-cyclodextrin (1–10 mM or higher) are commonly used to deplete plasma-membrane cholesterol from mammalian cells in culture (for examples see Furuchi and Anderson, 1998; Ilangumaran and Hoessli, 1998; Ostermeyer et al., 1999; Rodal et al., 1999; Roy et al.; 1999; Sheets et al., 1999; Kabouridis et al., 2000). In the light of the present results, the possibility should be considered that this agent could also redistribute membrane phospholipids under certain conditions (e.g., between different cell-surface domains that do not normally allow rapid lipid exchange). A second striking result from our present studies is that α-cyclodextrin can strongly accelerate the interbilayer transfer of [^{3}H]DPPC even though it shows a negligible ability to transfer cholesterol. This curious finding may be explained by the findings of Debouzy et al. (1998), which suggest that an individual α-cyclodextrin molecule can complex a single chain of a phospholipid. Our observation that the rate of α-cyclodextrin-mediated DPPC transfer increases as the square of the α-cyclodextrin concentration would then suggest that DPPC molecules may transfer between different bilayers as a complex with two molecules of α-cyclodextrin.

Previous studies of cholesterol partitioning between different types of lipid vesicles (Lange et al., 1979; Nakagawa et al., 1979; Wattenberg and Silbert, 1983; Rujanavech and Silbert, 1986; Yeagle and Young, 1986) have suggested that sphingomyelin exhibits a higher affinity for cholesterol than do unsaturated phosphatidylcholines, which in turn interact with cholesterol more avidly than do unsaturated phosphatidylethanolamines. Our present results support these conclusions and provide more quantitative estimates of the effects of different aspects of lipid structure on cholesterol affinity. From our data we can estimate that sphingomyelin shows a 3–3.5-fold higher affinity for cholesterol than does SOPC, and a roughly fivefold higher affinity for cholesterol than does 1-stearoyl-2-linoleoyl PC. These differential affinities are moreover not markedly different whether cholesterol is present in trace amounts or in a roughly 2:1 ratio with respect to the vesicle lipids. Applying these findings to the context of the plasma membrane, we conclude that choles-

terol is expected to be significantly enriched in sphingolipid-rich "rafts" compared to the surrounding phospholipid-enriched (liquid-disordered) regions of the membrane bilayer. This result agrees with reports that cholesterol is enriched in the low-density raft fractions obtained by low-temperature detergent treatment from plasma membranes of mammalian cells and from lipid dispersions with similar compositions (Brown and Rose, 1992; Schroeder et al., 1994; Brown and London, 2000).

From our results, it is clear that cholesterol not only associates preferentially with saturated phospholipid species but also discriminates markedly among phospholipids with different degrees of acyl chain unsaturation. Cholesterol for example shows a 2.5-fold higher affinity for SOPC than for 18:0/20:4-PC, both of which are relatively common membrane constituents, and even stronger discrimination (roughly fourfold) between SOPC and 18:2/18:2-PC, a species with two polyunsaturated chains. The relatively weak affinity of cholesterol for the latter species is consistent with previous observations that cholesterol exerts very weak chain-ordering effects on phospholipids with two polyunsaturated chains and cannot suppress the gel-to-liquid-crystalline phase transition for such lipids (Kariel et al., 1991; Mitchell and Litman, 1998). Although phospholipids with two polyunsaturated chains are not common in most mammalian cell membranes, they occur at substantial levels in certain specialized cell types, including sperm, visual, and neural cells (Salem, 1989). The present results suggest that it may be of interest to examine further whether cholesterol can promote fluid–fluid phase separation in mixtures combining such phospholipids with more "conventional" phospholipids, as some previous studies have suggested (Huster et al., 1998; Mitchell and Litman, 1998).

This research was supported by an operating grant from the Canadian Institutes of Health Research (grant number MOP-7776) to J.R.S.

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