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CHOLESTEROL TRANSFER FROM SMALL AND LARGE UNILAMELLAR VESICLES

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The rates of transfer of [^{14}C]cholesterol from small and large unilamellar cholesterol/egg yolk phosphatidylcholine vesicles to a common vesicle acceptor were compared at 37°C. The rate of exchange of cholesterol between vesicles of identical cholesterol concentrations (20 mol%) did not differ from the rate of transfer from donor vesicles containing 20 mol% cholesterol to egg yolk PC vesicles. Further, the rate of transfer of [^{14}C]cholesterol from vesicles containing 15 mol% dicetyl phosphate (to confer a negative charge) was not different from the rate of transfer from neutral vesicles. However, the half-time for transfer of [^{14}C]cholesterol from large unilamellar donor vesicles was about 5-times greater (10.2 h, 80 nm diameter) than from small unilamellar vesicles (2.3 h, 23 nm diameter). These data suggest that increased curvature in small unilamellar vesicles reduces cholesterol-nearest neighbor interactions to allow a more rapid transfer of cholesterol into the aqueous phase.

Introduction

The level of cholesterol in plasma cell membranes is determined by the rate of cholesterol synthesis within the cell and by the rate of cholesterol flux between the cells and the bathing medium [1]. We, and others, have shown that cholesterol exchange between cholesterol-phosphatidylcholine (PC) vesicles proceeds through the aqueous phase in the absence of added protein [2–4]. A similar mechanism applies to cholesterol exchange between cells and a variety of acceptors in the incubation medium [5–8]. An interesting observation in some of the latter studies on cellular cholesterol exchange is that the rate of cholesterol transfer from the cells to the medium depends on the type of cell serving as donor [5,6]. It is evident that the rate of cholesterol exchange from various bilayers and lipoproteins [9,10] depends on the molecular details of the local environment of the desorbing cholesterol molecule. Numerous physical studies of the acyl chain structure of sonicated and multilamellar vesicles suggest that acyl chain packing depends in part on the radius of curvature of the vesicle [11–13]. For this reason we have undertaken a comparative investigation of the rate of cholesterol transfer from large and small vesicles.

Bloj and Zilversmit [9] have shown that the rate of exchange of cholesterol from sonicated vesicles eluted in the void volume of an agarose column is 1.8–2.5-times slower than exchange from unilamellar vesicles of the same composition. We have performed similar experiments using large vesicles prepared by ether evaporation and extrusion [14] and found that the rate of cholesterol exchange is slower from large vesicles than from small vesicles (unpublished results). However, in our hands, these

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vesicles are largely multilamellar as judged by negative-stain electron microscopy. It is difficult to interpret the data for [^{14}C]cholesterol transfer from multilamellar vesicles because of at least three complicating factors: (1) the vesicles are highly heterogeneous in size and in the number of lamellae per vesicle, (2) the vesicles tend to precipitate during incubation, and (3) the kinetic pool size cannot be estimated accurately due to multiple pools of cholesterol in the various lamellae. To minimize these complicating factors, we have followed the rate of cholesterol exchange from vesicles prepared by dialysis of octyl glucoside [15]. These vesicles are largely unilamellar and are stable to flocculation over the course of the experiment. Here we demonstrate that the rate of cholesterol exchange from large unilamellar vesicles is much slower than from small sonicated unilamellar vesicles of identical compositions.

Materials and Methods

Materials. The sources and purity of all lipids used in this study have been described in detail elsewhere [2]. Octyl glucoside was used as supplied by Calbiochem-Behring (La Jolla, CA).

Preparation of vesicles. The preparation and characterization of small unilamellar vesicles has been described previously [2,16]. Large unilamellar vesicles were prepared by octyl glucoside dialysis according to the method of Mimms et al. [15]. Egg yolk PC, cholesterol and dicetyl phosphate were mixed in chloroform to give a molar ratio of 65 : 20 : 15. To this mixture was added 1.0 μCi of [4-^{14}C]cholesterol/mg of total lipid and 0.01% (w/w of egg PC) of butylated hydroxytoluene. The solutions were dried under N_2 to a thin film on the walls of a test tube and traces of remaining solvent were removed by drying in vacuo for 2 h at 40 °C. Octyl glucoside was added to give a 10 : 1 (w/w) detergent : lipid ratio. The mixture was dispersed in water to a final lipid concentration of 10 mg/ml. The clear dispersion was dialyzed against 20 mM sodium phosphate buffer, pH 6.0, for 36 h. The resulting turbid suspension was chromatographed on a Sepharose CL-2B column. The void volume was collected and stored under N_2.

Measurement of cholesterol exchange. Donor vesicles were either small or large unilamellar vesicles and contained 1.0 μCi of [4-^{14}C]cholesterol (spec. act. 55 mCi/mmol) per mg of total lipid. Neutral vesicles comprised either pure egg PC or 20 mol% cholesterol and 80 mol% egg yolk PC. A trace amount (0.1 μCi) of [7-^{3}H]cholesteryl oleate (spec. act. 55 mCi/mmol) per mg of total lipid was included to monitor neutral vesicle recovery. Charged vesicles comprised 20 mol% cholesterol, 65 mol% egg yolk PC and 15 mol% dicetyl phosphate. Charged and neutral vesicles were mixed and incubated under the conditions described in the figures. Rapid separation on ion-exchange columns was achieved in the absence of added protein as described in detail elsewhere [2]. With this method less than 1% of the negatively charged vesicles and 80–90% of the neutral vesicles were collected in the eluate following separation of the incubation mixture on DEAE-Sepharose columns.

Kinetic analysis. Kinetic data were analyzed by isotope exchange kinetics [2,17]:

$$kt = -X_\infty \ln(1 - X/X_\infty) \quad (1)$$

where k is the frequency of transfer of cholesterol, X is the fraction of labeled molecules transferred to the acceptor vesicle population at time t, and X_∞ is the fraction of labeled molecules transferred at equilibrium, $a/(a+b)$, where a is the concentration of lipid in the acceptor vesicle compartment and b is the concentration of lipid in the donor vesicle compartment. The value of k is based on exchange of cholesterol as a single pool [2].

Experimental fluxes were calculated from $J = ka$ where k is the pseudo-first-order rate constant for exchange and $a = (X_{chol}/N \cdot A) \cdot (SA_{outer}/(SA_{outer} + SA_{inner}))$. Here X_{chol} is the mol fraction of cholesterol in the donor vesicles (0.2), N is Avogadro's number, A is the average area per molecule of a 20 mol% cholesterol/egg yolk PC bilayer ($59 \cdot 10^{-16}$ cm^2/molecule, Ref. 18) and SA is the surface area of the outer and inner vesicle surfaces based on the mean vesicle diameter determined by negative-stain electron microscopy (see under Results) and a bilayer thickness of $33 \cdot 10^{-8}$ cm [18].

Other methods. Lipid vesicles were stained with 2% sodium phosphotungstate and observed at 80 000–160 000 × magnification with a Zeiss 10

high-resolution transmission electron microscope operating at 80 kV. Phospholipid concentrations were estimated by analysis for phosphorus [19]. Cholesterol was assayed with the Liebermann-Burchard reagent [20].

Results

To show the general applicability of the results to cholesterol transfer and exchange processes, the rate of [^{14}C]cholesterol transfer to acceptor vesicles with an without cholesterol was compared using negatively charged donor vesicles containing 20 mol% cholesterol (Fig. 1 and Table I). For small unilamellar vesicles, where both donor and acceptor vesicles contain 20 mol% cholesterol, more than 95% of the cholesterol exchanges as a single kinetic pool over 12 h at 37 °C [2]. The exponential curve fitted to these exchange data is reproduced in Fig. 1 as a solid line. The new data for transfer from donor vesicles containing 20 mol% cholesterol to egg yolk PC vesicles fit this line closely over an 8 h incubation at 37 °C. The rate constants for exchange and transfer (Table I) do not differ significantly at $P < 0.05$ (cf. Ref. 21). Similar data are obtained for 20 mol% cholesterol/egg yolk PC vesicles when the donor vesicles are neutral and the acceptor vesicles are negatively charged with 15 mol% dicetyl phosphate. The rate constants for neutral and negatively charged donor vesicles (Table I) do not differ significantly at $P < 0.05$.

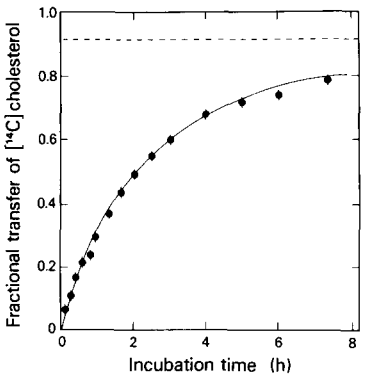

Fig. 1. Relative rates of cholesterol exchange and transfer at 37 °C. Donor vesicles comprising 20 mol% cholesterol, 65 mol% egg yolk PC, 15 mol% dicetyl phosphate and 0.1 μCi of [4-^{14}C]cholesterol/mg of lipid were incubated with acceptor vesicles of pure egg yolk PC containing 0.1 μCi of [7-^{3}H]cholesteryl oleate/mg of lipid. The donor vesicle concentration was 0.10 mg of lipid/ml; the acceptor vesicle concentration was 1.0 mg of lipid/ml. Separation was achieved on ion-exchange columns as described under Methods. Error bars represent the standard error of the mean for four separate measurements at each time point. The solid line is redrawn from Ref. 2 and gives the rate of cholesterol exchange under similar conditions where the donor vesicles were of identical composition to those in transfer experiments and the acceptor vesicles comprised 20 mol% cholesterol, 80 mol% egg yolk PC and 0.1 μCi of [^{3}H]cholesteryl oleate/mg of lipid. The dashed line corresponds to equal distribution of labeled cholesterol in donor and acceptor vesicles based on total lipid concentrations in each pool (X_∞ under Methods).

TABLE I

EFFECT OF VESICLE CHARGE ON THE RATES OF CHOLESTEROL EXCHANGE AND TRANSFER BETWEEN SMALL UNILAMELLAR CHOLESTEROL-EGG YOLK PC VESICLES

Vesicles were incubated at 37 °C and separated as described under Methods. D refers to the donor vesicle composition; A refers to the acceptor vesicle composition. All vesicles contained egg yolk PC. Charged vesicles contained 15 mol% dicetyl phosphate to confer a negative charge. The concentration of charged donors was 0.10 mg lipid/ml, and that of neutral acceptors 1.0 mg lipid/ml. In experiments with neutral donors, the donor and acceptor vesicle concentrations were 0.30 mg lipid/ml. The data are expressed as the average ± S.E. of the individual k and $t_{1/2}$ values from the indicated number of experiments.

Vesicle composition	k (s^{-1}) ($\times 10^5$)	$t_{1/2}$ (h)
D: 20 mol% cholesterol, charged A: 20 mol% cholesterol neutral [a]	8.9 ± 1.1 (5)	2.2 ± 0.3
D: 20 mol% cholesterol, charged A: pure egg PC, neutral	8.6 ± 1.0 (5)	2.2 ± 0.3
D: 20 mol% cholesterol, neutral A: 20 mol% cholesterol, charged	9.6 ± 1.9 (3)	2.0 ± 0.4

[a] From Ref. 2.

More than 90% of the large vesicles and 95% of the small vesicles show single bilayer structures under the transmission electron microscope in negatively stained preparations where the stain has penetrated into the interior of the vesicle. The size distributions of representative preparations of small and large unilamellar vesicles measured by negative-stain electron microscopy are compared in Fig. 2. The average diameter of the small vesicles was 21 ± 1 nm and of the large vesicles 83 ± 5 nm before incubation. After incubation for 6 h at 37 °C, the vesicles did not increase significantly in

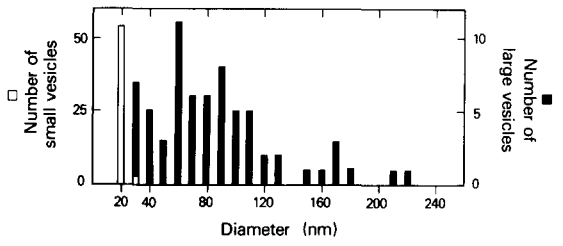

Fig. 2. Size distribution of small and large unilamellar vesicles before and after incubation. Vesicles were prepared and examined under the electron microscope after negative staining as described under Methods. The vesicle composition was 20 mol% cholesterol, 65 mol% egg yolk PC and 15 mol% dicetyl phosphate. The histogram is for 57 freshly prepared small unilamellar vesicles (open bars) and 60 freshly prepared large unilamellar vesicles (closed bars).

size. This is consistent with our previous results for small vesicles where gel filtration chromatography was also used to monitor vesicle size [23]. For large vesicles the average diameter was 83 ± 3 nm ($n = 152$) after incubation.

In Fig. 3, the rates of transfer of [^{14}C]cholesterol from large and small unilamellar vesicles of identical composition to acceptor vesicles of identical size and composition are compared. The exponential decrease in the fraction of [^{14}C-cholesterol in the donor vesicles has been corrected for the equilibrium distribution of radiolabeled molecules based on a single pool. It has been shown previously that cholesterol exchange from small vesicles proceeds from a single kinetic pool [2]. These data are reproduced in the figure for comparison. The data for large unilamellar vesicles fit a single pool model over the course of a 5 h incubation at 37 °C where more than 30% of the [^{14}C]cholesterol transfers to the acceptor vesicles. To determine whether more than 95% of the cholesterol pool exchanges from a single kinetic pool would require incubation for 5 half-times. Based on an experimental half-time for cholesterol exchange between large vesicles of 10.2 h ($k = 1.89 \cdot 10^{-5}$ s^{-1}), accurate pool size estimates would require incubations of more than 50 h. Over this period of time, the large vesicles are not stable to flocculation and coalescence, and increase in size. Therefore, pool size measurements were precluded because of the instability of the large vesicles. However, the kinetic analysis is considered appropriate, since transfer and exchange follow the same time course (Fig. 1) and the data fit a single pool model over the first 5 h of incubation; the vesicles remain largely unilamellar during this period.

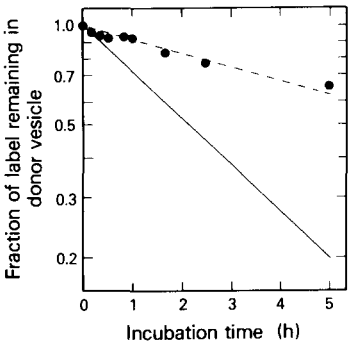

Fig. 3. Relative rates of cholesterol exchange from large and small unilamellar vesicles. The transfer of [4-^{14}C]cholesterol from large unilamellar vesicles comprising 20 mol% cholesterol, 65 mol% egg yolk PC and 15 mol% dicetyl phosphate to small unilamellar vesicles of 20 mol% cholesterol and 80 mol% egg yolk PC was followed as described under Methods at 37 °C. The concentration of donor vesicles was 0.10 mg of lipid/ml and the concentration of acceptor vesicles was 0.84 mg of lipid/ml. To facilitate comparison with data for cholesterol exchange from small unilamellar vesicles (solid line drawn from Ref. 2), the data are expressed as the fraction of cholesterol remaining in the donor vesicles corrected for the expected equilibrium distribution of [^{14}C]cholesterol based on a single pool of exchangeable molecules. Each experimental point is the mean of at least four separate measurements. The dashed line was fitted to the experimental data points by linear regression analysis using the origin as a fixed point.

Discussion

In this report we have utilized large vesicles prepared by octyl glucoside dialysis to measure the rate of cholesterol exchange from bilayers of low curvature and have compared these data with exchange data from highly curved bilayers. The large unilamellar vesicles used in this study are more suitable than multilamellar vesicles for this comparison, since the vesicles have a well-defined size distribution (Fig. 2) and are stable to aggregation over at least the time course of the experiment. In addition, a single kinetic pool of cholesterol is expected in single bilayer vesicles based on our studies of small unilamellar vesicles [2]. The data for cholesterol exchange from large unilamellar vesicles fit a single pool model over the 5 h time course of the experiment. The results for the large unilamellar vesicle composition used here are

probably applicable to large vesicles of different cholesterol concentrations (cf. Ref. 3), to both transfer and exchange of cholesterol (Fig. 1), and to neutral and charged donors (Table I) based on our results for small unilamellar vesicles.

Based on a half-time for cholesterol exchange from large vesicles of 10.2 h and from small vesicles of identical composition of 2.3 h (Ref. 2) at 37 °C, the flux of cholesterol from the surface of the large vesicles to the aqueous phase is $5.5 \cdot 10^{-16}$ mol · $cm^{-2} \cdot s^{-1}$, and from the small vesicles to the aqueous phase is $53 \cdot 10^{-16}$ mol · $cm^{-2} \cdot s^{-1}$ [2]. That is, the frequency of transfer of a cholesterol molecule from the vesicle bilayer to water is 10-times greater from highly curved vesicles. Two factors influence the rate of this desorption process: (1) the aqueous-phase concentration of the cholesterol molecules, and (2) the interaction energy between cholesterol and its nearest neighbors in the lipid-water interface [2]. The concentration of cholesterol present as monomer in equilibrium with dispersed droplets containing cholesterol is greater if the dispersion comprises small droplets rather than large ones [10,22]. For large and small vesicles, the dependence of the aqueous-phase concentration of cholesterol on the radius of the vesicle is not sufficient to account for the more rapid rate of cholesterol flux from small unilamellar vesicles into the aqueous phase. Apparently, molecular packing within the bilayer plays the major role in determining the overall rate of exchange from vesicles of differing radii of curvature.

A number of physical studies comparing the physical properties of highly curved (sonicated) vesicles with hand-shaken multilamellar vesicles have been interpreted in terms of differences in packing of the phospholipid hydrocarbon chains. Differential scanning calorimetric [11] and Raman spectroscopic [12,13] data are consistent with decreased interchain interactions within the PC bilayer of highly curved vesicles. Direct application of physical studies on PC bilayers to cholesterol exchange is difficult. However, the rate of cholesterol exchange from PC bilayers depends on the acyl chain packing because cholesterol exchange proceeds more slowly from bilayers which comprise saturated rather than unsaturated PC acyl chains [3,9]. The present data support a model for the high rate of cholesterol desorption from small vesicles where the increased curvature reduces cholesterol-nearest neighbor interactions. This decrease in interaction energy results in a decreased energy barrier for the desorption of the cholesterol molecules into the aqueous phase.

The physiological significance of a more rapid transfer of cholesterol from bilayers of high curvature is not clear. Regions of high local curvature in plasma membranes are known to occur in places such as endocytic vesicles and the highly invaginated surface of intestinal mucosal cells. One of the possible consequences of a more rapid transfer of cholesterol to the surrounding medium in such regions of high curvature could be the appearance of multiple kinetic pools of cholesterol in the membrane.

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