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Transposition and Distribution of Cholesterol in Rat Erythrocytes (39975)

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Introduction. Phospholipids of the erythrocyte membrane are distributed asymmetrically (1) and appear to be in a dynamic equilibrium across the bilayer (2). Less is known about the location of free cholesterol in the erythrocyte membrane. Experiments *in vitro* indicate that all the cholesterol of intact rat erythrocytes or erythrocyte ghosts can be exchanged with free cholesterol of low-density lipoproteins or phosphatidylcholine-cholesterol sonicated vesicles (3-5). Similar results were found with human red blood cells (6), but not with swine (7). However, none of these studies provides information about the distribution of cholesterol within the erythrocyte membrane or the rate of cholesterol transposition across the bilayer.

Materials and methods. Lipids. Glycerol tri[9,10-^{3}H]oleate (specific activity 142 mCi/mmole) was from New England Nuclear Corp. (Boston, Mass.). It was purified by silica gel H thin-layer chromatography with hexane:diethyl ether:acetic acid (60:40:1, v/v/v). The triolein band was eluted with chloroform and stored at −20°. [4-^{14}C]Cholesterol (specific activity 60 mCi/mmole) was from Amersham/Searle (Arlington Heights, Ill.). It was purified by silica gel H thin-layer chromatography with hexane:diethyl ether (50:50, v/v) as solvent. The cholesterol band was eluted with chloroform, diluted with unlabeled cholesterol to a specific activity of 2700 dpm/μg, and stored at −20° in the dark. Upon rechromatography with the same solvent system and scraping of the cholesterol area, the purity was found to be greater than 99%. Unlabeled cholesterol (Sigma Chemical Co., St. Louis, Mo.) was purified as the dibromide derivative and crystallized in methanol. Butylated hydroxytoluene (Nutritional Biochemical Corp., Cleveland, Ohio) was dissolved in chloroform and used without further purification. Soy phosphatidylcholine (Nattermann, Koln, Germany) was purified by neutral alumina chromatography (8) and stored in chloroform at −20°. Egg phosphatidylcholine (grade I) was obtained from Lipid Products Inc. (Surrey, England). Both preparations were more than 99% pure as judged by phosphorus distribution on silica gel H thin-layer plates developed with chloroform:methanol:acetic acid:water (25:15:4:2, v/v/v/v). The lysophosphatidylcholine content was less than 0.5% and did not increase during the preparation of vesicles by sonication.

Buffer solution. The same buffer was used for preparation of sonicated vesicles, gel filtration through Biogel A_{50}, washing of red blood cells, and incubation of sonicated vesicles with erythrocytes or erythrocyte membranes. The buffer contained 118 m*M* NaCl, 13.5 m*M* $NaHCO_3$, 5 m*M* KCl, 4.2 m*M* NaH_2PO_4, 1.7 m*M* Na_2HPO_4, 2.4 m*M* $CaCl_2$, 1.2 m*M* $MgCl_2$, and 10 m*M* glucose, and was adjusted to pH 7.4 with NaOH. A small precipitate was removed by filtration. Streptomycin sulfate (10 mg%) and benzylpenicillin (sodium salt, 6 mg%) were added to the solution.

Preparation and fractionation of sonicated vesicles. For preparation of sonicated vesicles with a phosphatidylcholine/cholesterol molar ratio of 1.0/0.8, 10 mg of phosphatidylcholine, 4.2 mg of [^{14}C]cholesterol, 0.10 mg of butylated hydroxytoluene, and a small amount of [^{3}H]triolein (0.2 mole%) were evaporated under N_2. The lipids were

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redissolved in a few milliliters of diethyl ether and redried under N_2. Two milliliters of buffer were added and bubbled with N_2 before capping the tube. After brief agitation in a Vortex mixer, the lipid suspension was sonicated in a bath-type sonifier (Laboratory Supply Co., Hicksville, N.Y.) for 2 hr. The water in the bath was changed several times during sonication to maintain the temperature between 0 and 10°. After sonication, the vesicles were centrifuged at 4° for 15 min at 40,000*g* and a small pellet was discarded. The vesicles were fractionated by gel filtration through an agarose column (Biogel A_{50}, 100–200 mesh, Bio Rad Laboratories, Richmond, Calif.). The column (26 × 3.5 cm) was operated at 4° and 4- to 5-ml fractions were collected every 15 min. Fractions were monitored by measurement of ^3H and ^{14}C. The smallest vesicles, contained in the trailing half of the second peak, were pooled and concentrated by dialysis against dry dextran (average molecular weight 264,000, clinical grade, obtained from Sigma Chemical Co., St. Louis, Mo.). The concentrated vesicles were centrifuged (15 min at 40,000*g*) and used within 4 days. They were kept under N_2 in the dark, at 4°.

Isolation of red blood cells. Blood was obtained by heart puncture from nonfasted male albino rats (400- to 600-g body weight) (Sprague–Dawley strain, Blue Spruce Farms, Altamont, N.Y.). Na_2EDTA, 100 m*M* (1 ml/10 ml of blood), was used as anticoagulant. The blood was centrifuged for 15 min at 2500*g* and the plasma and white cells were discarded. The red blood cells were washed three or four times with 3–5 vol of buffer. After each centrifugation the top portion of the red cells was discarded. The cells were finally suspended in an equal volume of buffer and used within 24 hr of isolation. The cholesterol content was measured in each of the preparations and ranged between 1.39 and 1.45 mg/ml of packed cells.

Incubations. Packed red blood cells (0.3 ml) and phosphatidylcholine/cholesterol vesicles containing 200 μg of [^{14}C]cholesterol and [^3H]triolein were incubated with gentle agitation at 37° in a final volume of 2 ml. The incubation mixture contained 5 mg/ml of bovine serum albumin (fraction V powder, fatty acid poor, Miles Laboratories, Inc., Kankakee, Ill.). Aliquots (80 μl) of the incubation mixture were taken at the beginning of the experiment (zero-time point) and at hourly intervals thereafter up to 12 hr. In some experiments, aliquots were taken each 30 min during the first 4 hr of the experiment. Several samples were taken between 24 and 33 hr. The aliquots were centrifuged (2 min, 8000*g*) and 50 μl of the supernatants were counted in 10 ml of liquid scintillation medium (9).

The amount of labeled cholesterol removed from the vesicles was calculated as the diminution in the $^{14}C/^3H$ ratio of the supernatant and expressed as percentage decrease from the zero-time ratio. Release of hemoglobin in the supernatants was measured by absorbance at 416 nm and was less than 2% after 12 hr of incubation and less than 7% after 30 hr.

Results. Figure 1 shows that when sonicated egg phosphatidylcholine/[^{14}C]cholesterol vesicles (molar ratio 1:0.8, 50 μg of cholesterol) are incubated with red blood cells containing 700 μg of cholesterol, the cholesterol is completely exchangeable with a half-time of 3.1 hr. Sonicated vesicles of soy phosphatidylcholine also show a single exponential exchange curve with a half-life of 1.8 hr. It appears that an inverse relation exists between the degree of phospholipid unsaturation and the half-life for phospholipid exchange (10). The cholesterol in phospholipid/cholesterol vesicles thus behaves as a single pool. In the experiments described below we incubated sonicated vesicles containing labeled cholesterol with

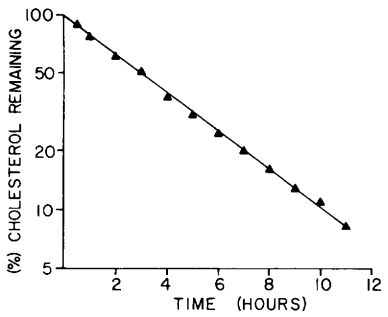

FIG. 1. Extensive exchange of cholesterol between sonicated egg phosphatidylcholine/[^{14}C]cholesterol (1.0/0.8, mole/mole) vesicles and an excess of rat erythrocytes.

intact red blood cells containing about twice the amount of unlabeled cholesterol so that isotopic equilibration could take place. The decrease in [^{14}C]cholesterol in the vesicles with time was measured. The recovery of sonicated vesicles was quantitative, as judged by the recovery of [^{3}H]triolein, a nonexchangeable marker (11). In one experiment recovery was monitored by a colorimetric determination of cholesterol in the supernatant containing the sonicated vesicles. Up to 24 hr of incubation, the cholesterol in the supernatant varied within 5% of the zero-time value.

Curve A in Fig. 2 shows that equilibration of the label takes place after 24–30 hr of incubation. The mathematical treatment of closed systems has been used for the analysis of the curve (12). When the asymptotic value of equilibration is subtracted, the newly generated curve (curve B, Fig. 2) cannot be described by a single exponential function. Computerized curve peeling shows that the data can be fitted by a three-pool closed system in which curves C and B added to the asymptotic value of curve A give the solid line of curve A. Since the cholesterol in the vesicles behaves like one pool, it appears that the cholesterol in the red blood cell is distributed in two pools. Figure 2 shows the three-pool model and the rate constants. The average constants obtained from five separate experiments are shown in Table I. The half-time of equilibration of cholesterol between the two erythrocyte pools, calculated as $0.693/(k_{bc} + k_{cb})$, was found to be 2.3 ± 0.6 hr. Knowing the amount of cholesterol in the sonicated vesicles and the rate constants, the relative size of the pools of cholesterol in the erythrocyte can be calculated. The distribution of cholesterol between pools b and c was found to be 57 and 43% of the total, respectively. From knowledge of the amount of cholesterol and of the decrease in labeled cholesterol in the sonicated vesicles, one can also calculate the theoretical value for (b + c). When this was expressed as a fraction of the measured total erythrocyte cholesterol, the mean for five experiments was 0.99 ± 0.05 (Table 1).

It is tempting to conclude from these results that cholesterol in the erythrocyte is nearly equally divided between the inner and outer portions of the bilayer. One might counter that in principle the two pools of labeled cholesterol could both be present on the outside of the red cell membrane. This hypothesis was rejected on the basis of an experiment in which erythrocytes were isolated for 24 hr after a rat was injected intravenously with [^{3}H]cholesterol in ethanol diluted with saline. The cells were washed and hemolyzed for the preparation of resealed ghosts and inside-out erythrocyte vesicles (2). Upon incubation with sonicated

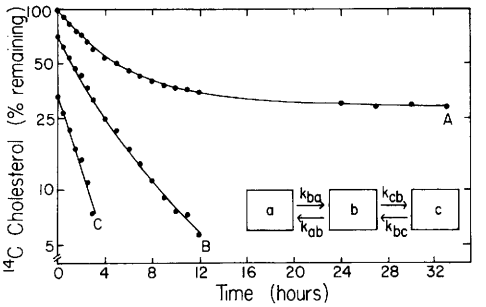

FIG. 2. Equilibration of [^{14}C]cholesterol between sonicated phosphatidylcholine/cholesterol vesicles and a twofold excess of cholesterol in rat erythrocytes. Curve A: [^{14}C]cholesterol remaining in the vesicles as a function of time. Curves B and C were generated by successive curve peeling. Inset: three-pool closed model and rate constants used in Table I.

TABLE I. POOL SIZES AND RATE CONSTANTS FOR CHOLESTEROL DISTRIBUTION IN THE ERYTHROCYTE MEMBRANE.

	Rate constants (hr^{-1})					Pool sizes[a]	
	k_{ab}	k_{ba}	k_{bc}	k_{cb}	$t_{1/2}$ (hr)	(b + c)	b/(b + c)
Average (five experiments)	0.156	0.215	0.178	0.131	2.3	0.99	0.57
Standard deviation	0.009	0.015	0.066	0.036	0.6	0.05	0.09

[a] The value for (b + c) derived from kinetic measurements is expressed as a percentage of the total erythrocyte cholesterol determined chemically.

phospholipid/cholesterol vesicles we found that in 5 hr the resealed ghosts lost 53% of the labeled cholesterol and that the inside-out vesicles lost 37% of the labeled cholesterol. Thus, labeled cholesterol was present on both sides of these membranes.

The rate of cholesterol transfer between these erythrocyte membranes and sonicated vesicles was rather slow; the monoexponential losses of labeled cholesterol from resealed ghosts and inside-out vesicles show rate constants of 0.15 and 0.074 hr^{-1}, respectively. These constants are smaller than those for the equilibration of the two cholesterol pools in the intact erythrocytes and do not allow a quantitative compartmental analysis. However, in a qualitative sense it is possible to conclude that labeled cholesterol is present on both sides of the erythrocyte membrane, since the membranes were closed, as judged by their retention of hemoglobin or by their impermeability to acetylthiocholine (2), and since exchange of labeled cholesterol was demonstrated from both sides of the erythrocyte membrane.

Discussion. Several approaches have been used in trying to determine the localization of cholesterol in the erythrocyte membrane. Murphy (13) used radioautographs of erythrocytes containing [^{3}H]cholesterol to show that the sterol was concentrated around the periphery of the biconcave disk. Higgins *et al.* (14) prepared osmate ester of cholesterol and incorporated it into sonicated vesicles of phosphatidylcholine. The osmate ester was found to exchange between the vesicles and intact red blood cells. Electron microscopy of the membranes suggested that the osmate esters of cholesterol are dispersed in groups throughout the thickness of the red cell membrane. Fisher (15) used membrane splitting by freeze-fracture of monolayers of human erythrocytes bound to cationized glass. The procedure seems to leave, bound to the glass, membrane fractions enriched in outer "halves." Cholesterol was determined by microanalysis. The results indicated that more cholesterol is present on the exterior side of the membrane. However, the degree of asymmetry was strongly dependent upon the buffer used for the suspension of the red blood cell. The above studies indicate that cholesterol is distributed heterogeneously in the erythrocyte membrane, but it is not yet known whether the cholesterol on two sides of the bilayer behaves kinetically as two separate pools of the sterol. The results presented in this paper indicate that, indeed, the cholesterol is distributed in two pools in the erythrocyte membrane and that cholesterol exchanges rapidly between these pools with a half-time of somewhat more than 2 hr.

We have carried out similar experiments to those described here with intact red blood cells from man, sheep, and swine. In these three species, 90 to 100% of the red blood cell cholesterol had exchanged with the sterol of the sonicated vesicles after 24–30 hr of incubation. These results indicate that the transmembrane movement of cholesterol in the erythrocyte membrane is rapid in the three species, in spite of the wide differences in their phospholipid composition (16).

A transbilayer distribution of cholesterol in the erythrocyte membrane is not unique. By means of filipin binding, Bittman and Rottem (17) have provided evidence that 50 and 66% of the sterol is located in the outer half of the membrane of *Mycoplasma gallisepticum* and *Mycoplasma capricolum*, respectively. Lenard and Rothman (18) studied the cholesterol distribution in the membrane of influenza virus. They found that only 50% of the sterol was available for exchange with sonicated vesicles, and that the half-time of equilibration between the two sides of the bilayer averaged 13 days. Erythrocyte and influenza virus are the only natural membranes in which the transbilayer movement of cholesterol has been investigated. The results obtained indicate that in the former, the sterol rapidly equilibrates throughout the membrane, while in the second it does not.

Summary. Unilamellar vesicles of soy phosphatidylcholine and [^{14}C]cholesterol were incubated with rat erythrocytes for up to 33 hr. From the kinetics of cholesterol equilibration between vesicles and erythrocytes we have calculated that erythrocyte cholesterol is present in two pools that equilibrate with a half-time of 2.3 ± 0.6 hr.

The evidence suggests that the two pools, which are of comparable size, reflect the cholesterol present in the inner and outer portions of the lipid bilayer.

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