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THE RAPID TRANSMEMBRANE MOVEMENT OF CHOLESTEROL IN SMALL UNILAMELLAR VESICLES

JONATHAN M. BACKER and ELIEZAR A. DAWIDOWICZ

Biophysical Laboratory, Harvard Medical School, Boston, MA 02115 (U.S.A.)

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Summary

The exchange of cholesterol between two populations of small unilamellar vesicles has been investigated using a new system. Uniformly sized egg lecithin-cholesterol vesicles containing [^{3}H]cholesterol and the glycolipid *N*-palmitoyl-DL-dihydrolactocerebroside were used as donors, whereas similar vesicles containing unlabelled cholesterol and no glycolipid were used as cholesterol acceptors. The two populations of vesicles were separated with the castor bean lectin *Ricinus communis*. It was found that greater than 90% of the cholesterol in the donor vesicle could be exchanged with a single time constant, the half-time for the completion of this exchange process being 1.5 h at 37°C. Therefore, the rate of transmembrane movement or flip-flop of cholesterol in these vesicles must be at least as fast as the intermembrane exchange process. Similar results were obtained using hemoglobin-free human erythrocyte ghosts as the acceptor membrane. If the molecular-sieve chromatography step used to fractionate the vesicles was omitted, a non-exchangeable pool of cholesterol was detected which was shown not to be due to the presence of multilamellar vesicles.

Introduction

An asymmetrical distribution of phospholipids between the two surfaces of the lipid bilayer in biological membranes was first suggested [1] and then demonstrated [2] by Bretscher and has since been shown to occur in several membranes [3]. The assembly and maintenance of these asymmetries are ques-

Abbreviations: KCl/Tris buffer, 100 mM KCl, 10 mM Tris-HCl, pH 7.4.

tions that have lead to the measurement of the transmembrane movement or 'flip-flop' of lipids across various membranes. Using techniques involving exchange proteins [4—6] and chemical labeling [7] it has been shown that the half-time for the flip-flop of phosphatidylcholine in small unilamellar lecithin vesicles is greater than 11 days at 37°C. In contrast to this are measurements with natural membranes. The flip-flop of phospholipids across rat erythrocyte membranes is relatively rapid, occurring with a half-time of 2—4 h [8,9] and is extremely rapid in the membranes of *Bacillus cereus* [10] and rat liver microsomes [11,12] where it takes place with a half-time of less than 10 min. In the presence of this fast transmembrane movement of phospholipids, it is presently unclear how the given asymmetry of these molecules across the membrane is maintained.

Although the distribution of cholesterol across biological membranes is not known, measurements of the flip-flop process have nevertheless been conducted. Bruckdorfer and Green [13] demonstrated in their investigation of cholesterol exchange between plasma and red cells that all of the cholesterol present in these two components took part in the exchange process. Their results clearly indicate the ability of cholesterol to traverse the erythrocyte membrane. Recently this transmembrane movement of cholesterol in erythrocytes has been measured and reported to have a half-time of around 2 h [14, 15].

However, a conflict has arisen concerning the flip-flop of cholesterol in small unilamellar vesicles composed of lecithin and cholesterol. Using exchange techniques, Poznansky and Lange [16,17] have concluded that cholesterol flip-flop is immeasurably slow. On the other hand, Bloj and Zilversmit [18], conducting essentially identical experiments on apparently similarly produced vesicles, conclude that the transmembrane movement of cholesterol across the bilayer is too rapid for them to measure. On the basis of polarity alone, one might predict a rapid transmembrane movement for cholesterol (see ref. 19). In fact, in the studies where this process has not been detected, the arguments presented [3, 17] would also rule out the possibility of cholesterol exchange between membranes, which is definitely known to occur. In order to determine whether cholesterol is capable of undergoing flip-flop in small unilamellar vesicles, we have used a new approach to follow cholesterol exchange between two populations of these vesicles.

Materials and Methods

Lipids. Egg lecithin was either purified from egg yolks by chromatography on silicic acid [20] or purchased from Makor Chemicals (Jerusalem, Israel) and stored as a stock solution in ethanol at —20°C. Both samples gave a single spot after thin-layer chromatography on Silica Gel G using $CHCl_3/CH_3OH/H_2O$ (65 : 25 : 4, v/v) as the eluting solvent, had a comparable Klein oxidation index [21] and were not contaminated with lysophosphatidylethanolamine as indicated by testing with ninhydrin. There were no detectable differences in the exchange experiments performed using the lecithin from these two sources. Cholesterol obtained from Sigma (St. Louis, MO) was recrystallized three times from absolute ethanol and stored in $CHCl_3$ at —20°C. The glycolipid, *N*-palmitoyl-DL-

dihydrolactocerebroside was purchased from Miles Laboratories (Elkhart, IN) and used without further purification. This was stored in $CHCl_3/CH_3OH$ (4 : 1, v/v) at −20°C.

Radioactivity. [oleate-1-^{14}C]cholesteryl oleate (50 Ci/mol) and [1,2-^{3}H]cholesterol (60 Ci/mmol) were obtained from New England Nuclear (Boston, MA). Radioactivity was determined using 10 ml Triton/toluene cocktail [22] containing 1 ml aqueous buffer. Appropriate cross-over corrections were made to obtain the amounts of ^{3}H and ^{14}C in a given sample.

Ghosts. Hemoglobin-free erythrocyte ghosts were prepared from outdated human blood according to the method of Hanahan and Ekholm [23]. They were finally washed into KCl/Tris buffer (100 mM KCl, 10 mM Tris-HCl, pH 7.4) and stored at −20°C. Prior to use, the ghosts were warmed to 37°C and used directly.

Lectin. Ricinus communis was purified using the procedure of Podder et al. [24]. The purified protein was concentrated to 3—6 mg/ml by removal of the buffer through dialysis tubing using Aquacide IIA obtained from Calbiochem and then stored at 4°C.

Preparation of vesicles. Homogeneous single-walled lipid vesicles were prepared by sonication followed by molecular-sieve chromatography on Sepharose 4B of appropriate lipid dispersions according to the method described by Huang and Thompson [25]. During the initial stages of this work the lecithin/cholesterol mixtures were lyophilized from benzene solution prior to dispersion in buffer. In order to reduce our exposure to benzene these lipids are currently stored in chloroform or ethanol at −20°C. Mixtures of the lipids and the appropriate radioactive compounds are now dried down in a rotary evaporator and placed under vacuum overnight. The dried lipids are then dispersed in KCl/Tris buffer prior to sonication at 4°C. We find no difference in our ability to form vesicles using this procedure when compared with lipid mixtures lyophilized from benzene.

(i) Donor vesicles. Donor vesicles containing glycolipid were prepared by mixing cholesterol and egg lecithin in a 1 : 1 molar ratio, with [^{3}H]cholesterol and a trace of the non-exchangeable marker [^{14}C]cholesteryl oleate, and 10% by weight glycolipid. After removing the organic solvent as described above, the lipids were dispersed in KCl/Tris buffer and sonicated for 1 h at 4°C under N_2, using a Branson Model W185 sonifier. The resulting dispersion was centrifuged to remove titanium shed by the probe and then chromatographed on Sepharose 4B. The elution profile from the column (Fig. 1) is similar to that described by Huang [26]. Those fractions from the descending portion of the second fraction, which have been shown to be homogeneous single-walled vesicles by several criteria [26], were then pooled and stored under N_2 at 4°C. These vesicles typically had a cholesterol : lecithin molar ratio of 0.8—0.9 : 1.

(ii) Acceptor vesicles. Homogeneous vesicles were prepared from a mixture of egg lecithin and cholesterol in a 1 : 1 molar ratio as described above. The resulting vesicles typically had a cholesterol : lecithin molar ratio of 0.8—0.9 : 1.

Analytical determinations. Phospholipid concentrations were determined from the inorganic phosphate content [27], and cholesterol was determined using the enzymatic assay kit from Boehringer-Mannheim. Protein assays were performed using the method of Lowry et al. [28].

Exchange experiments. (i) Vesicle-vesicle exchange. Donor and acceptor vesicles were mixed at 37°C. The ratio of donor to acceptor was 1 : 30, based on phospholipid content, to avoid any back flux of [^{3}H]cholesterol. Also included in the incubation was 0.5 mM NaN_3 as a bacteriocide, and bovine serum albumin at 5 mg/ml. It has been shown by Bloj and Zilversmit [18] that albumin at this concentration aids in the recovery of vesicles without effecting the extent or initial rate of cholesterol exchange. Similarly, Poznansky and Lange [16,17] have not reported any difference in their exchange experiments which were performed in the presence and absence of albumin. At various times a 200 μl aliquot was removed from the incubation mixture and added to 400 μl *R. communis* (2.5 mg/ml) at 19°C, so that the final mixture was at 25°C. This mixture was vortexed and centrifuged for 5 min in a Brinkmann 3200 centrifuge. 400 μl of the supernatant was then removed and the amount of ^{3}H and ^{14}C present was determined. To correct for any possible loss of total radioactivity during the experiment, an additional 100 μl aliquot was removed from the incubation mixture and the amount of radioactivity determined directly.

The percentage of [^{3}H]cholesterol exchanged from the donor was calculated using the expression

$$\% \text{ exchanged} \equiv \frac{H_e}{H_t} \cdot 100 = \frac{H_s - C_s R_v}{H_t - C_s R_v} \cdot 100$$

where in each aliquot removed from the exchange incubation: H_e, ^{3}H exchanged from donor vesicles; H_t, total ^{3}H counts in aliquot; C_t, total ^{14}C counts in aliquot; H_s, ^{3}H in supernatant after removal of donors; C_s, ^{14}C in supernatant after removal of donors, and R_v, H_t/C_t.

This expression takes into account any incomplete precipitation of the glycolipid donors, using the recovery of ^{14}C counts of the non-exchangeable marker cholesteryl oleate in the supernatant. The derivation is given in the Appendix.

(ii) Vesicle-ghost exchange. Human erythrocyte ghosts and donor vesicles were mixed in a 30 : 1 ratio, based on phospholipid content at 37°C, in the presence of 5 mg/ml bovine serum albumin and 0.5 mM NaN_3. The mixture was continuously shaken during the incubation to avoid any sedimentation of the ghosts. At various times 200-μl aliquots were removed and centrifuged for 5 min in a Brinkmann 3200 centrifuge. 100 μl of the supernatant was then removed for radioactive counting. The percentage of [^{3}H]cholesterol remaining in the vesicles was calculated from:

$$\% \text{ remaining} = \frac{H_s}{C_s} \cdot \frac{C_0}{H_0} \cdot 100$$

where H_0 and C_0 are the ^{3}H and ^{14}C counts in the original vesicles, and H_s and C_s are the counts left in the supernatant after pelleting the ghosts. The [^{14}C]-cholesteryl oleate serves simply as a marker for the recovery of the vesicles in this case.

Results

Formation and precipitation of glycolipid-containing vesicles

The elution profile from Sepharose 4B of a sonicated dispersion of egg lecithin, cholesterol and the lactocerebroside glycolipid (Fig. 1) is similar to that reported for sonicated dispersions of egg lecithin [26]. Careful characterization by Huang [26] has indicated that those fractions eluting in the descending portion of the second peak are single walled and homogeneous by several criteria. We take the similarity between the elution profiles to indicate that we have successfully produced single-walled vesicles from our mixture of lecithin, cholesterol, and the glycolipid. By analogy with egg lecithin, we have used only those fractions which elute on the descending portion of the second peak for our exchange experiments.

Curatolo et al. [29] had indicated that *R. communis* was capable of precipitating sonicated lecithin vesicles containing lactocerebroside. This suggested to us a method for separating two populations of lipid vesicles following the exchange of cholesterol between them. In the present study we were typically able to obtain between 85 and 95% precipitation of donor vesicles (in the concentration range 0.5—2 mM lipid phosphorous) after adding *R. communis* to a final concentration of 1.5 mg/ml. This amount of lectin was shown to be unable to precipitate vesicles which did not contain the glycolipid.

Exchange of [^{3}H]cholesterol between vesicles

Greater than 90% of the [^{3}H]cholesterol could be exchanged out of the glycolipid-containing vesicles with a single time constant (Fig. 2). The process was essentially completed in 8 h with a half-time of 1.5 h. In order to clarify a comparison of our results with those of other studies, we felt that it was necessary to examine possible artifacts that the inclusion of glycolipid in the donor vesicles could have on the transbilayer movement of cholesterol. We compared the exchange of [^{3}H]cholesterol from donor vesicles, prepared with and without glycolipid. Using an excess of red cell ghosts as acceptors we found that the

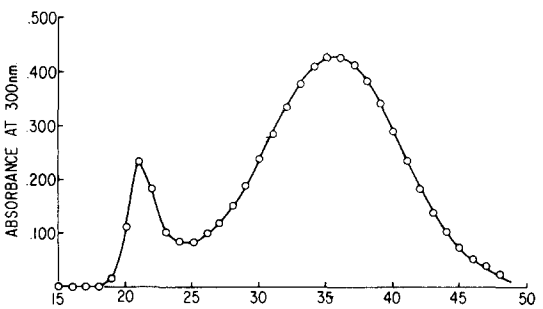

Fig. 1. Elution profile of donor vesicles. 66.5 μmol egg lecithin, 66.5 μmol cholesterol, 5.36 mg *N*-palmitoyl-DL-dihydrolactocerebroside, 1 μCi [^{14}C]cholesteryl oleate and 20 μCi [^{3}H]cholesterol were dried as described in Materials and Methods. The mixture was dispersed in 4 ml KCl/Tris buffer, sonicated and chromatographed on a Sepharose 4B column (2.5 × 35 cm). 4-ml fractions were collected. Fractions 37—44 were pooled and used in exchange experiments.

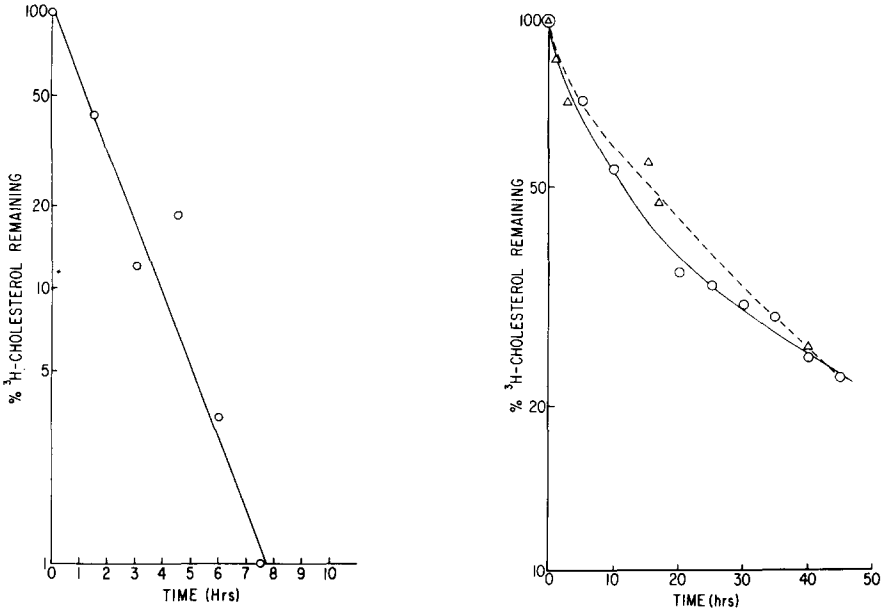

Fig. 2. Exchange of [^{3}H]cholesterol between fractionated vesicles. Donor vesicles (0.85 μmol phospholipid) were incubated at 37°C with acceptor vesicles (24.3 μmol phospholipid) in a final volume of 8.2 ml. Also included was 5 mg/ml bovine serum albumin and 0.5 mM NaN_3. The fraction of [^{3}H]cholesterol that was exchanged was then determined.

Fig. 3. Exchange of [^{3}H]cholesterol from fractionated donors with and without glycolipid. ○———○, donor vesicles without glycolipid (0.55 μmol phospholipid) were incubated at 37°C with erythrocyte ghosts (16.6 μmol phospholipid) in a final volume of 6.3 ml containing 5 mg/ml bovine serum albumin and 0.5 mM NaN_3; △———△, donor vesicles (0.72 μmol phospholipid) incubated at 37°C with erythrocyte ghosts in a final volume of 9 ml containing serum albumin and NaN_3 as above. The fraction of [^{3}H]-cholesterol that had exchanged was then determined.

presence of the glycolipid has no effect on the rate or extent of cholesterol exchange (Fig. 3). There is also no indication of a non-exchangeable pool of cholesterol in these experiments. However, the rate of cholesterol exchange in these vesicle-ghost incubations decreases with time, in contrast to the constant rate of cholesterol exchange between vesicles (Fig. 2). We therefore decided to examine vesicle-ghost exchange in more detail.

The applicability of erythrocyte ghosts as an acceptor in exchange studies depends on the ability to separate them from the donor vesicles by centrifugation. We have found that our preparations of erythrocyte ghosts tend to fragment or bleb during prolonged incubations at 37°C possibly due to increased fragility caused by freezing and thawing the ghosts (Matayoshi, E.D., personal communication). The resulting membrane fragments do not sediment under the conditions that we normally use to pellet ghosts, as indicated by protein determinations of the supernatant following a typical centrifugation. Since this fragmentation process appears to increase with time, it would manifest itself in an exchange experiment as a reduction in the amount of [^{3}H]cholesterol that should appear in the pellet. This would result in an apparent decrease in the rate of cholesterol exchange. These membrane fragments can however be pelleted after the addition of 0.5 mg poly-L-lysine (from Sigma) to an aliquot

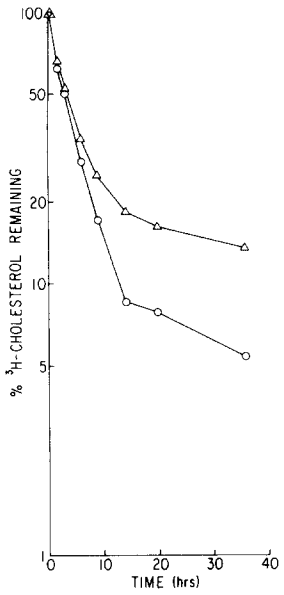

Fig. 4. Exchange of [^{3}H]cholesterol between fractionated glycolipid-containing vesicles and erythrocyte ghosts. Donor vesicles (0.88 μmol phospholipid) were incubated with ghosts (28 μmol phospholipid) in a final volume of 12.1 ml containing 5 mg/ml serum albumin and 0.5 mM NaN_3. Aliquots were removed and the fraction of [^{3}H]cholesterol that had been exchanged was determined. ○———○, aliquots centrifuged after the addition of 0.5 mg poly-L-lysine; △———△, aliquots centrifuged without poly-L-lysine. The apparent change in rate that occurs after more than 90% of the vesicle [^{3}H]cholesterol has been exchanged is probably due to the addition of an insufficient amoung of poly-L-lysine. It has no effect on the conclusions that we have drawn from this experiment.

removed from a vesicle-ghost incubation. Poly-L-lysine causes both ghosts and membrane fragments to precipitate as a result of the electrostatic interaction with the negatively charged membrane, and was shown to have no effect on the glycolipid-containing vesicles.

The gradual diminution in the rate of cholesterol exchange between vesicles and ghosts is again indicated in Fig. 4. This decrease in rate can be eliminated by using poly-L-lysine to precipitate membrane fragments (Fig. 4) as described above and is therefore an artifact produced by membrane fragmentation. Greater than 90% of the [^{3}H]cholesterol could then be exchanged from vesicles to ghosts with a single time constant. The half-time for this exchange process was approx. 3.5 h.

Thus the use of glycolipid in these experiments has introduced no significant anomalies into processes involving cholesterol exchange and has enabled us to follow the movement of cholesterol between two populations of lipid vesicles.

Discussion

The exchange experiments shown in Figs. 2 and 4 indicate that all of the cholesterol in fractionated lecithin-cholesterol-glycolipid vesicles behaves as a single pool in the exchange process. Since cholesterol is present on both surfaces of the vesicle bilayer [30], we infer that the rate of transmembrane move-

ment of cholesterol must be at least as rapid as the rate of intermembrane exchange reported here. These experiments therefore set an upper limit of 1.5 h for the half-time of cholesterol flip-flop at 37°C in small unilamellar vesicles, which is in agreement with the findings of Bloj and Zilversmit [18]. We realize that these results place us in disagreement with the reports by Poznansky and Lange [16,17] who were unable to detect the flip-flop of cholesterol in similar vesicles. In an effort to understand the factors that could account for these differences, we varied our procedure in the preparation of the donor vesicles.

A pronounced effect was achieved when exchange experiments were conducted between erythrocyte ghost acceptors and glycolipid-containing donor vesicles which had not been fractionated on Sepharose 4B. A small aliquot of this unfractionated dispersion was analyzed by molecular-sieve chromatography to insure that it displayed the same elution profile which characterized a successful vesicle preparation. After prolonged incubations of unfractionated donors with a 30-fold excess of ghosts at 37°C it was found that no more than 55% of the [^{3}H]cholesterol in the vesicles was exchangeable (Fig. 5). This limit on the exchange process differed from the decrease in rate caused by membrane fragmentation, in that it appeared to be an end point.

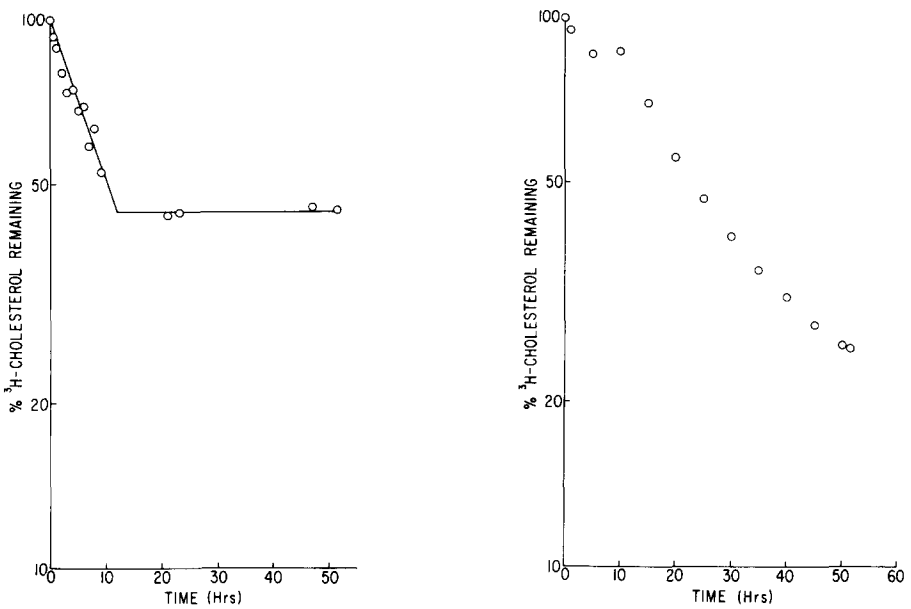

Fig. 5. Exchange of [^{3}H]cholesterol between unfractionated glycolipid-containing vesicles and erythrocyte ghosts. Unfractionated donor vesicles (0.7 μmol phospholipid) were incubated at 37°C with erythrocyte ghosts (21 μmol phospholipid) in a final volume of 5 ml containing serum albumin (5 mg/ml) and 0.5 mM NaN_3. The exchange of [^{3}H]cholesterol was then determined.

Fig. 6. Exchange of [^{3}H]cholesterol between multilamellar vesicles containing glycolipid and erythrocyte ghosts. Multilamellar vesicles were collected from the first fraction obtained after chromatography of a sonicated lipid dispersion (lecithin-cholesterol-glycolipid) on Sepharose 4B. These vesicles (0.25 μmol phospholipid) were then incubated at 37°C with erythrocyte ghosts (7.5 μmol phospholipid) in a final volume of 5 ml, which included 5 mg/ml bovine serum albumin and 0.5 mM NaN_3. The extent of exchange of [^{3}H]cholesterol was then determined in the same manner as that described for the small unilamellar vesicles.

We had demonstrated that all of the [^{3}H]cholesterol in fractionated small vesicles was available for exchange. It was therefore thought that the non-exchangeable pool of cholesterol observed in unfractionated vesicles could be due to the presence of multilayers which were normally separated from the small vesicles by molecular-sieve chromatography. We prepared multilamellar vesicles by chromatography on Sepharose 4B of a sonicated dispersion of egg lecithin, cholesterol, glycolipid, [^{3}H]cholesterol and a trace of [^{14}C]cholesteryl oleate. However, when exchange experiments were conducted between these multilayers and an excess of red cell ghosts, 75% of the[^{3}H]cholesterol in the multilayer was exchangeable with no appearance of a non-exchangeable pool (Fig. 6). Similar findings have been reported by Bloj and Zilversmit [18]. Thus, it appears unlikely that the non-exchangeable pool of cholesterol found in exchange incubations involving unfractionated donor vesicles is due to the presence of multilayers.

It is unclear what causes the different exchange behavior of cholesterol between fractionated and unfractionated vesicles. Nevertheless, our findings indicate that all precautions must be taken to insure that vesicles used in these type of experiments are produced in a well-defined and reproducible manner.

In their most recent publication, Poznansky and Lange [17] have argued that transmembrane movement of cholesterol can be induced in lecithin-cholesterol vesicles under the non-equilibrium conditions which are produced when vesicle cholesterol is depleted. To achieve depletion they used cholesterol-depleted ghosts as acceptors which had a significantly lower cholesterol content than the donors. However, neither Poznansky and Lange [17] nor Bruckdorfer et al. [31] have been able to detect any depletion of cholesterol from donor vesicles when the cholesterol contents of the donor and acceptor are comparable. Both in our experiments and those of Bloj and Zilversmit [18], this latter situation is the case. Thus, both our observations and those of Bloj and Zilversmit of a rapid flip-flop of cholesterol in small unilamellar vesicles are not artifacts of inadvertant cholesterol depletion.

In the light of our results it is not surprising that the transmembrane movement of cholesterol has been detected in erythrocytes [14,15] and vesicular stomatitis virus [32]. On the other hand, it is somewhat puzzling that cholesterol flip-flop has not been detected in influenza virus [33] or *Mycoplasma gallisepticum* [34]. It is possible that the presence of certain proteins in these membranes might inhibit the transmembrane movement of cholesterol. Further studies will be necessary to clarify this point.

Appendix

The amount of [^{3}H]cholesterol exchanged from the donor to acceptor vesicle must be corrected for the incomplete precipitation of the donor. The ^{3}H/^{14}C ratio of the precipitated donors R_t is given by:

$$R_t = \frac{H_t - H_e}{C_t}$$

where: H_t, total ^{3}H in the aliquot; C_t, total ^{14}C in the aliquot; and H_e, ^{3}H

exchanged from donor. This can be rewritten as:

$$R_t = \frac{H_t - (H_s - H_c)}{C_t}$$

where: H_s, ^3H in supernatant after removal of donors, and H_c, ^3H in unprecipitated donors.

The appearance of ^{14}C counts in the supernatant is due to the presence of unprecipitated donors. We can therefore substitute using:

$$H_c = R_t C_s$$

This leads to:

$$R_t = \frac{H_t - H_s}{C_t - C_s} \tag{1}$$

The ratio R_t coupled with the recovery of the non-exchangeable ^{14}C counts recovered in the supernatant gives:

$$H_s = H_e + R_t C_s$$

from which we can write:

$$H_e = H_s - R_t C_s \tag{2}$$

Finally, we use the expression:

$$R_v \equiv H_t / C_t \tag{3}$$

Solution of the above equations yields the relationship that:

$$\% \, [^3H]\text{cholesterol exchanged} \equiv \frac{H_e}{H_t} \cdot 100 = \frac{H_s - C_s R_v}{H_t - C_s R_v} \cdot 100 \, .$$

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References

1 Bretscher, M.S. (1972) Nat. New Biol. 236, 11—12
2 Bretscher, M.S. (1972) J. Mol. Biol. 71, 523—528
3 Rothman, J.E. and Lenard, J. (1977) Science 195, 743—753
4 Rothman, J.E. and Dawidowicz, E.A. (1975) Biochemistry 14, 2809—2816
5 Johnson, L.W., Hughes, M.E. and Zilversmit, D.B. (1975) Biochim. Biophys. Acta 375, 176—185
6 Shaw, J.M., Hutton, W.C., Lentz, B.R. and Thompson, T.E. (1977) Biochemistry 16, 4156—4163

7 Roseman, M., Litman, B.J. and Thompson, T.E. (1975) Biochemistry 14, 4826—4830
8 Renooij, W., van Golde, L.M.G., Zwaal, R.F.A. and van Deenen, L.L.M. (1976) Eur. J. Biochem. 61, 53—58
9 Bloj, B. and Zilversmit, D.B. (1976) Biochemistry 15, 1277—1283
10 Rothman, J.E. and Kennedy, E.P. (1977) Proc. Natl. Acad. Sci. U.S. 74, 1821—1825
11 Zilversmit, D.B. and Hughes, M.E. (1977) Biochim. Biophys. Acta 469, 99—110
12 Van den Besselaar, A.M.H.P., de Kruijff, B., van den Bosch, H. and van Deenen, L.L.M. (1978) Biochim. Biophys. Acta 510, 242—255
13 Bruckdorfer, K.R. and Green, C. (1967) Biochem. J. 104, 270—277
14 Lange, Y., Cohen, C.M. and Poznansky, M.J. (1977) Proc. Natl. Acad. Sci. U.S. 74, 1538—1542
15 Bloj, B. and Zilversmit, D.B. (1977) Proc. Soc. Exp. Biol. Med. 156, 539—543
16 Poznansky, M.J. and Lange, Y. (1976) Nature 259, 420—421
17 Poznansky, M.J. and Lange, Y. (1978) Biochim. Biophys. Acta 506, 256—264
18 Bloj, B. and Zilversmit, D.B. (1977) Biochemistry 16, 3943—3948
19 Tanford, C. (1973) in The Hydrophobic Effect, p. 187, Wiley-Interscience, New York
20 Litman, B.J. (1973) Biochemistry 12, 2545—2554
21 Klein, R.A. (1970) Biochim. Biophys. Acta 210, 486—489
22 Patterson, M.S. and Greene, R.C. (1965) Anal. Chem. 37, 854—857
23 Hanahan, D.J. and Ekholm, J.E. (1974) Methods Enzymol. 31A, 168—172
24 Podder, S.K., Surolia, A. and Backhawat, B.K. (1974) Eur. J. Biochem. 44, 151—160
25 Huang, C. and Thompson, T.E. (1974) Methods Enzymol. 32, 485—489
26 Huang, C. (1969) Biochemistry 8, 344—352
27 Gomori, G. (1942) J. Lab. Clin. Med. 27, 955—960
28 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
29 Curatolo, C., Shipley, G.G., Small, D.M., Sears, B. and Neuringer, L. (1977) J. Am. Chem. Soc. 99, 6771—6772
30 Huang, C., Sipe, J.P., Chow, S.T. and Martin, R.B. (1974) Proc. Natl. Acad. Sci. U.S. 71, 359—362
31 Bruckdorfer, K.R., Edwards, P.A. and Green, C. (1968) Eur. J. Biochem. 4, 506—511
32 Patzer, E.J., Shaw, J.M., Moore, N.F., Thompson, T.E. and Wagner, R.R. (1978) Biochemistry 17, 4192—4200
33 Lenard, J. and Rothman, J.E. (1976) Proc. Natl. Acad. Sci. U.S. 73, 391—395
34 Rottem, S., Slutzky, G.M. and Bittman, R. (1978) Biochemistry 17, 2723—2726