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TRANSFER OF CHOLESTEROL BETWEEN LIPOSOMAL MEMBRANES

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Summary

The transfer of cholesterol between liposomal membranes was examined. On incubation of liposomes composed of egg yolk phosphatidylcholine, phosphatidic acid and cholesterol (molar percentage, 65.8 : 1.3 : 32.9 or 62.5 : 6.3 : 31.2), almost complete equilibration of the cholesterol pools was achieved within 6 to 8 h at 37°C. The rate of transfer of cholesterol from the liposomes, in which cholesterol was introduced by 'the exchange reaction', was not significantly different from that from liposomes prepared in the presence of cholesterol, in which the cholesterol was distributed homogenously. These findings indicate that half life for 'flip-flop' of cholesterol molecules in egg yolk phosphatidylcholine liposomes is less than 6 h at 37°C.

The transfer of cholesterol between liposomes was strongly dependent on temperature and was affected by the fatty acid composition of the phospholipid, suggesting that the 'fluidity' of the membranes strongly influences the transfer rate.

A preferential distribution of cholesterol molecules was observed in heterogeneous liposomes with different classes of phospholipids. The 'affinity order' of cholesterol for phospholipid deduced from the present experiments is as follows: beef brain sphingomyelin > dipalmitoylglycerophosphocholine ≒ dimyristoylglycerophosphocholine > egg yolk phosphatidylcholine.

Introduction

The mechanisms of transfer or exchange of cholesterol between membranes are unknown. It is interesting to know the mechanism governing the distribution of cholesterol in membranes, because cholesterol is important in regulating membrane fluidity. Since transfer of cholesterol was reported to occur between liposomal membranes [1,2], these membranes should be a good system for ob-

taining information about this transfer mechanism. In this work, using a different assay technique to those described by Ehnholm and Zilversmit [1] and by Haran and Shoporer [2], we examined the movement of cholesterol in sonicated liposomes of various phospholipid compositions at various temperatures.

Materials and Methods

Lipids. Egg yolk phosphatidylcholine was prepared by chromatography on Aluminum Oxide Neutral and Unisil. Phosphatidic acid was prepared from egg yolk phosphatidylcholine by treatment with cabbage phospholipase D (EC 3.1.4.4). Dilauroylglycerophosphocholine (C12:0-PC) and diundecanoylglycerophosphocholine (C11:0-PC) were synthesized by the method of Cubero et al. [4]. Other lipids were obtained from the following companies: Sigma Chemical Company, St. Louis, MO, U.S.A. (cholesterol and β,γ-dipalmitoyl-D,L-α-glycerophosphocholine); Calbiochem., San Diego, CA, U.S.A., (β,γ-dimyristoyl-L-α-glycerophosphocholine); New England Nuclear Co., Boston, MA, U.S.A. ([7-^{3}H]cholesterol and glycerol-tri[2-^{3}H]oleate); Radio Chemical Centre, Amersham, U.K., [4-^{14}C]cholesterol and glycerol-tri[1-^{14}C]-oleate).

Other chemicals. Bovine albumin (fraction V) (Nakarai Chemical Co., Kyoto, Japan), Unisil (Clarkson Chemical Co., Williamsport, PA, U.S.A.), Aluminum Oxide Neutral (Woelum, Eschwege, F.R.G.), DEAE-cellulose (DE 52, Whatman, Maidstone, Kent, U.K.) and Sepharose CL 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) were also used.

Preparation of liposomes. Liposomes composed of phosphatidylcholine, phosphatidic acid and cholesterol in various molar ratios were prepared by the method of Huang [5]. Lipids were first dispersed in 12.5 mM phosphate buffer (pH 7.4) with a Vortex mixer. The final concentration of liposomes was 10 μmol as phospholipid per ml. The preparations were used as 'non-sonicated liposomes'. Sonicated liposomes were prepared as follows: Lipids were dispersed in a vortex mixer and sonicated for 10 min with a microprobe in a Branson sonifier Model W185 (operating at 18 W) at an appropriate temperature. The preparation was centrifuged at 15 000 × *g*, for 15 min and the supernatant was used directly as 'sonicated liposomes'. To obtain homogeneous preparations in some experiment, the liposomes were 'sized' by chromatography. For this, aliquots of liposome preparations (1 ml) were applied to a column of Sepharose CL-4B (1.6 × 50 cm), and the colum was eluted with 12.5 mM phosphate buffer (pH 7.4) at flow rate of 5 ml/h. The results with sized and unsized liposomes were virtually identical. Therefore, routinely 'sonicated liposomes' were used without sizing.

Determination of transfer of cholesterol between liposomes

Cholesterol transfer was determined by the method developed for experiments on transfer of phosphatidylcholine between liposomes by van den Besselar et al. [3]. The principle of the method is that two populations of liposomes can be separated according to surface charge on a DEAE-cellulose ion-exchange column. Transfer of cholesterol between liposomes was determined by measuring the transfer of radioactive cholesterol from 'donor' to

'acceptor' liposomes. The incubation was performed at an appropriate temperature in 12.5 mM phosphate buffer (pH 7.4) with 0.5 μmol of each type of liposome preparation, in a final volume of 1.5 ml. The 'donor' liposomes (liposomes with 1.3 mole% phosphatidic acid) contained phosphatidylcholine, phosphatidic acid and cholesterol in a molar ratio of 1 : 0.02 : 0.5, a trace of [^{3}H]cholesterol and [^{14}C]glycerol-trioleate as a 'non-exchangeable marker'. The 'acceptor' liposomes (liposomes with 6.3 mol% phosphatidic acid) contained phosphatidylcholine, phosphatidic acid and cholesterol in a molar ratio of 1 : 0.1 : 0.5. A mixture of equal amounts of 'acceptor' and 'donor' liposomes (0.5 μmol each as phospholipid) was incubated at various temperatures for an appropriate period. Then the mixtures (1.5 ml) were rapidly passed through a DEAE-cellulose column (0.8 × 1 cm) equilibrated with 12.5 mM phosphate buffer at room temperature, and the column was eluted with 4.5 ml of the same buffer. Fractions of 0.6 ml were collected and their radioactivity was counted in a Packard liquid scintillation counter (Model 3320). By this procedure, the liposomes could be separated within 5 min. About 80% of the 'donor' liposomes were recovered in the eluate, while practically all the 'acceptor' liposomes were bound to the column.

The percentage transfer of [^{3}H]cholesterol from the 'donor' liposomes to the 'acceptor' liposomes was calculated as follows:

$$1 - \frac{^3\text{H}/^{14}\text{C of donor liposomes after incubation}}{^3\text{H}/^{14}\text{C of donor liposomes before incubation}} \times 100\%$$

After a long incubation (more than 12 h) recovery of the 'donor' liposomes from the column was poor (4%). This difficulty was overcome by adding serum albumin (110 μg/ml) to the incubation mixture; In the presence of albumin, recovery of 'donor' liposomes prepared with any kinds of phospholipids was about 80%. In confirmation of the observation of Bloj and Zilversmit [7] we found that the extent of cholesterol transfer was not affected by the presence of albumin in the medium.

In some experiments, the increase of the ratio of ^{14}C/^{3}H in liposomes with 1.3 mol% phosphatidic acid and [^{3}H]glyceroltrioleate was measured after incubating them with liposomes containing 6.3 mol% phosphatidic acid and [^{14}C]cholesterol.

Determination of cholesterol. Cholesterol in the eluate from the DEAE-cellulose column was determined quantitatively as follows: lipids were extracted from the eluate by the method of Bligh and Dyer [8], and then the cholesterol was isolated from the lipid extracts on a silicic acid column eluted with chloroform. The cholesterol content was determined by the method of Zlatkis et al. [9].

Results

Transfer of cholesterol between egg yolk phosphatidylcholine liposomes

The transfer of cholesterol between liposomes containing egg yolk phosphatidylcholine and cholesterol in a molar ratio of 1 : 0.5 is shown in Fig. 1. The ratio of [^{3}H]cholesterol/[^{14}C]glyceroltrioleate in liposomes with 1.3 mol%

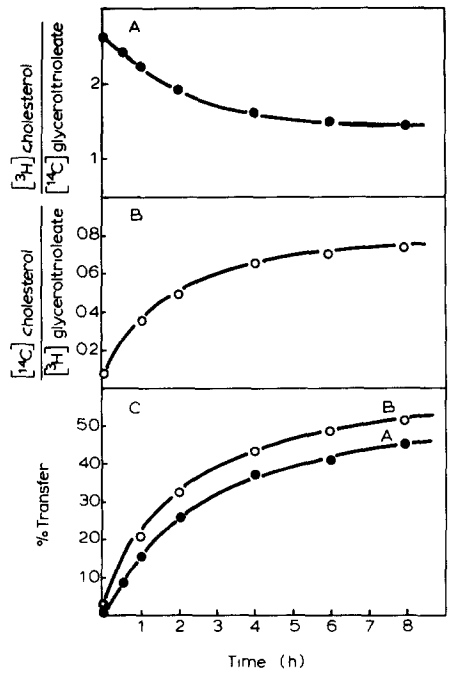

Fig. 1. Transfer of cholesterol between egg yolk phosphatidylcholine liposomes Liposomes composed of egg yolk phosphatidylcholine, phosphatidic acid, and cholesterol (molar percentage, 65.8 1.3 32.9), with trace amounts of [^{3}H]cholesterol and [^{14}C]glyceroltrioleate were incubated at 37°C with an equal amount of liposomes composed of egg yolk phosphatidylcholine, phosphatidic acid and cholesterol (molar percentage, 62.5 6.3 : 31.2). The ^{3}H/^{14}C ratio in the fraction containing liposomes with 1.3 mol% phosphatidic acid was measured (A). Liposomes composed of egg yolk phosphatidylcholine, phosphatidic acid, and cholesterol (molar percentage, 65.8 1.3 · 32.9), with a trace amount of [^{3}H]-glyceroltrioleate (6732 cpm) were incubated at 37°C with liposomes composed of egg yolk phosphatidylcholine, phosphatidic acid, and cholesterol (molar percentage, 62.5 6.3 31.2) and a trace amount of [^{14}C]cholesterol. The ^{14}C/^{3}H ratio in the fraction containing the former liposomes was measured (B). The percentage transfer shown in (C) was calculated from A (●) and B (○).

phosphatidic acid [^{3}H]cholesterol and [^{14}C]glyceroltrioleate decreased gradually (Fig. 1A) when these liposomes were incubated at 37°C with liposomes containing 6.3 mol% of phosphatidic acid. Transfer of cholesterol reached a maximum after incubation for 6 h at 37°C (Fig. 1C). The ratio of [^{14}C]cholesterol/[^{3}H]glyceroltrioleate in liposomes with 1.3 mol% phosphatidic acid and [^{3}H]glyceroltrioleate increased when these liposomes were incubated with liposomes containing 6.3 mol% phosphatidic acid and [^{14}C]-cholesterol (Fig. 1B). The transfer of cholesterol from liposomes with 1.3 mol% phosphatidic acid to liposomes with 6.3 mol% phosphatidic acid was almost the same as that observed in the reverse direction (Fig. 1C). These results show that cholesterol molecules can be exchanged between liposomes.

We next examined the possibility of asymmetric transfer of cholesterol between liposomes with 1.3 mol% phosphatidic acid and those with 6.3 mol% phosphatidic acid. Mixtures of liposomes with 1.3 mol% phosphatidic acid and with 6.3 mol% phosphatidic acid, containing equal amounts of [^{3}H]cholesterol, were incubated at 37°C for various periods. As shown in Table I, the ratio of ^{3}H/^{14}C in liposomes with 1.3 mol% phosphatidic acid increased gradually from 4.76 reaching 5.23 after 8 h.

TABLE I

UNEVEN DISTRIBUTION OF CHOLESTEROL BETWEEN LIPOSOMES WITH 1 3 MOL% PHOSPHATIDIC ACID AND THOSE WITH 6.3 MOL% PHOSPHATIDIC ACID

Liposomes with 1.3 mol% phosphatidic acid, which were composed of 0.5 μmol egg yolk phosphatidylcholine, 0.01 μmol phosphatidic acid, 0.25 μmol non-labeled cholesterol, 75 400 dpm of [^{3}H]cholesterol and 15 800 dpm of [^{14}C]glyceroltrioleate (^{3}H/^{14}C = 4.76), were incubated for various times at 37°C with an equal amount of liposomes composed of all the same components, but with 6.3 mol% phosphatidic acid instead of 1.3 mol% phosphatidic acid. After incubation, aliquots of the reaction mixture (1.5 ml) were passed through a DEAE-cellulose column to separate liposomes with 1.3 mol% phosphatidic acid. The ^{3}H/^{14}C ratio in liposomes with 1.3 mol% phosphatidic acid was determined.

Incubation time (h)	Ratio of ^{3}H/^{14}C in liposomes with 1.3 mol% phosphatidic acid	Relative increase in the ratio of ^{3}H/^{14}C
0	4.76	100
1	4.80	101
2	4.92	103
4	4.98	105
6	5.10	107
8	5.23	110

The percentage increase in the ^{3}H/^{14}C ratio after 8 h was 10%, indicating that 55.0% of the total cholesterol was present in liposomes with 1.3 mol% of phosphatidic acid, and 45.0% was present in those with 6.3 mol% of phosphatidic acid. This uneven distribution of cholesterol may explain why the percentage transfer of cholesterol from liposomes with 1.3 mol% phosphatidic acid to those with 6.3 mol% phosphatidic acid reached a plateau at about 45%, instead of 50%. Consistent with this, the percentage transfer in the reverse direction reached a plateau at 50 to 55%.

The transfer of cholesterol between non-sonicated liposomes was rather slow, and did not reach a plateau within 8 h, (data not shown). This slow transfer probably merely reflects the inaccessibility of the inner lamellae of liposomes to other liposomes. Over 20% transfer was observed between multilamellar liposomes, suggesting that cholesterol incorporated into the outermost lamellae can move into the center of the liposomes by sequential transfer between adjacent lamellae [10].

Transfer of cholesterol between homologous liposomes containing cholesterol and various phospholipids

Fig. 2 shows the transfer of cholesterol between homologous liposomes containing distearoyl-, diplamitoyl-, dimyristoyl-, dilauroyl-, and diundecanoylglycerophosphocholine, egg yolk phosphatidylcholine and beef brain sphingomyelin at 37°C. Transfer was fast with egg yolk phosphatidylcholine, and diundecanoylglycerophosphocholine liposomes, and slower with dilauroylglycerophosphocholine liposomes. Liposomes composed of dimyristoyl-, dipalmitoyl-, and distearoylglycerophosphocholine and beef sphingomyelin showed even slower transfer of cholesterol; even after incubation for 12 h, only 20—25% transfer was observed with liposomes containing dipalmitoylglycerophosphocholine. The difference in the rate of cholesterol transfer between egg phosphatidylcholine liposomes and dipalmitoylglycerophosphocholine liposomes is probably not due to a difference in vesicle size, because the two

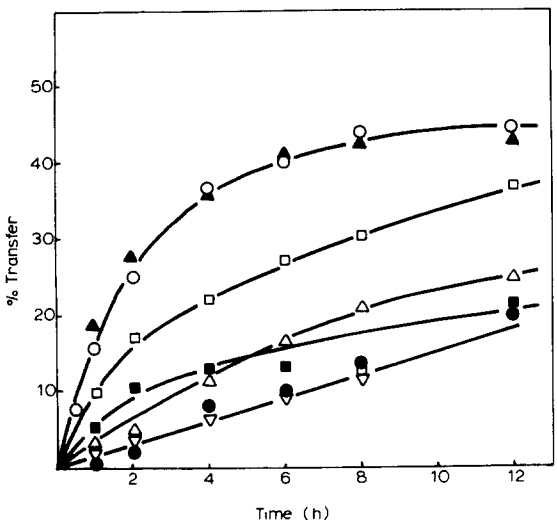

Fig. 2. Transfer of cholesterol between homologous liposomes containing cholesterol and various phospholipids, egg yolk phosphatidylcholine liposomes (○), diundecanoylglycerophosphocholine liposomes (▲), dilauroylglycerophosphocholine liposomes (□), dimyristoylglycerophosphocholine liposomes (●), dipalmitoylglycerophosphocholine liposomes (△), distearoylglycerophosphocholine liposomes (■) and beef brain sphingomyelin liposomes (▽). Liposomes composed of phosphatidylcholine or sphingomyelin, phosphatidic acid, and cholesterol (molar percentage, 65.8 1.3 : 32.9), [^{3}H]cholesterol and [^{14}C]-glyceroltrioleate were incubated at 37°C for various periods with the homologous liposomes containing phosphatidylcholine, phosphatidic acid and cholesterol (molar percentage, 62.5 : 6.3 · 31.2). The percentage transfer was obtained as described in the text.

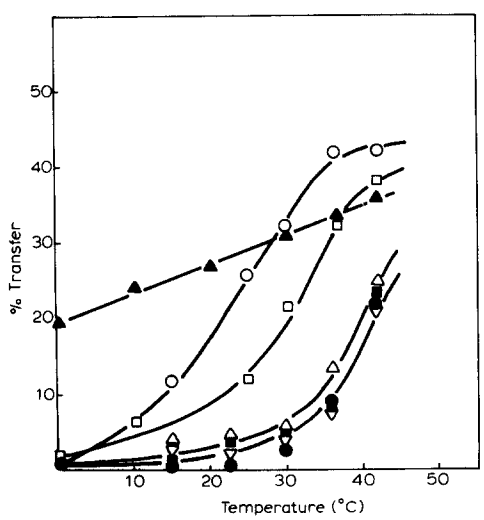

Fig. 3. Temperature-dependence of cholesterol transfer. Cholesterol transfer between homologous liposomes was determined after 6 h incubation at various temperatures. Transfer was measured between egg yolk phosphatidylcholine liposomes (○), diundecanoylglycerophosphocholine liposomes (▲), dilauroylglycerophosphocholine liposomes (□), dimyristoylglycerophosphocholine liposomes (●), dipalmitoylglycerophosphocholine liposomes (△), distearoylglycerophosphocholine liposomes (■) and sphingomyelin liposomes (▽). The assay system was as described in the legend for Fig. 1.

types of liposomes showed the same pattern of elution from a Sepharose CL 4B column.

The effect of incubation temperature on the rate of transfer was next examined (Fig. 3). The temperature required for half maximum transfer (25%) was 25°C with egg yolk phosphatidylcholine liposomes, about 30°C with dilauroylglycerophosphocholine liposomes, 45°C with both dimyristoyl- and dipalmitoylglycerophosphocholine liposomes and 50°C with distearoylglycerophosphocholine and sphingomyelin liposomes. In the system composed of diundecanoylglycerophosphocholine liposomes, the temperature-dependence of cholesterol transfer was quite peculiar, and showed no steep curve like that observed in other systems.

Effect of cholesterol content on the transfer rate

Dimyristoylglycerophosphocholine liposomes containing various amounts of cholesterol were incubated at various temperatures for 6 h. As shown in Fig. 4, the net content of cholesterol in the liposomes did not have any significant effect on the transfer rate. Liposomes containing only a trace of labeled cholesterol showed very little transfer activity below 15°C, indicating that the transfer is extremely slow when the membranes are in a gel state.

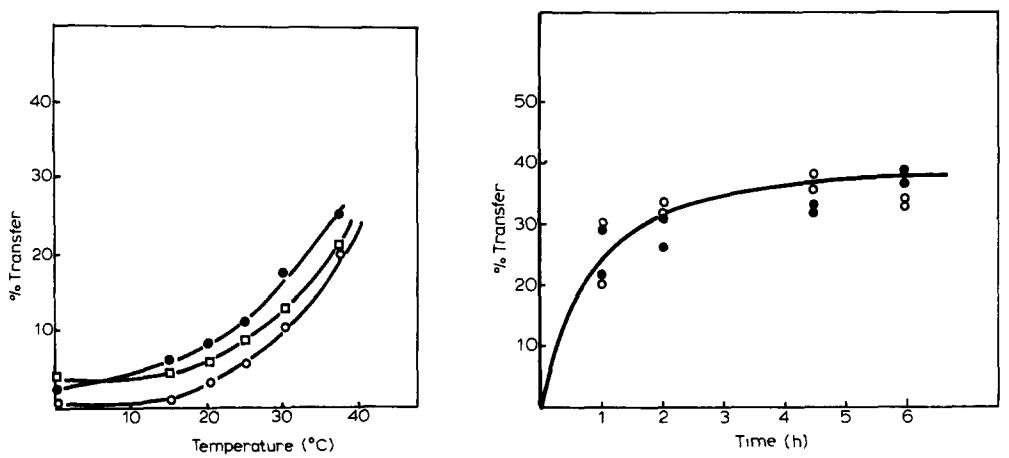

Fig. 4. Effect of cholesterol content on the transfer rate. Liposomes containing dimyristoylglycerophosphocholine and various amounts of cholesterol were incubated for 6 h at various temperatures to measure their cholesterol transfer rates. The cholesterol contents of the liposomes were as follows: •, labeled cholesterol only (35 nCi, 400 Ci/mmol); ○, 0.5; □, 1.0, as molar ratios to phospholipid.

Fig. 5. Transfer of cholesterol incorporated exogenously into liposomes. Liposomes (10 μmol as phospholipid phosphorus) containing 65.8 mol% of egg yolk phosphatidylcholine, 1.3 mol% of phosphatidic acid, and 32.9 mol% of cholesterol with a trace of [^{14}C]glyceroltrioleate were incubated for 6 h at 37°C with an equal amount of liposomes containing 62.5 mol% of egg yolk phosphatidylcholine, 6.3 mol% of phosphatidic acid, and 31.2 mol% of cholesterol with a trace of [^{3}H]cholesterol in a final volume of 4 ml. The reaction mixtures were rapidly subjected to DEAE-cellulose column chromatography (1.5 × 7 cm) to separate the former liposomes. An aliquot of the eluate (0.9 ml) containing liposomes (0.5 μmol phospholipid) with 1.3 mol% phosphatidic acid, was further incubated at 37°C with an equal amount of liposomes containing 62.5 mol% of egg yolk phosphatidylcholine, 6.3 mol% of phosphatidic acid, and 31.2 mol% of cholesterol in a final volume of 2 ml. The percentage transfer of [^{3}H]cholesterol was calculated (○). The percentage transfer of cholesterol from liposomes with 1.3 mol% phosphatidic acid and endogenous [^{3}H]-cholesterol to those with 6.3 mol% phosphatidic acid is also slown (•).

Transfer of exogenously incorporated cholesterol

If cholesterol molecules move very slowly from the outer to the inner layer of the bilayer transferred cholesterol should remain for some time in the outer layer. To test this possibility, egg yolk phosphatidylcholine liposomes with 1.3 mol% phosphatidic acid were incubated with egg yolk phosphatidylcholine liposomes with 6.3 mol% phosphatidic acid and a trace of [^{14}C]cholesterol. The former liposomes were then separated by DEAE-cellulose column chromatography, and incubated at 37° C with liposomes containing 6.3 mol% phosphatidic acid (Fig. 5). The rate of transfer of cholesterol from the liposomes was not significantly different from that from liposomes prepared in the presence of labeled cholesterol, in which the cholesterol was 'evenly' distributed between the outer and inner bilayers. This result shows that cholesterol transfered liposomes from other liposomes has the same transfer rate as that endogenously incorporated into the liposomes. Complete equilibration of labeled cholesterol in the bilayer occurred in liposomes having cholesterol in both layers of the bilayer, indicating that equilibration of transverse diffusion of cholesterol in egg yolk phosphatidylcholine liposomes was reached within 6 h at 37° C.

Asymmetric transfer of cholesterol from egg yolk phosphatidylcholine liposomes to dipalmitoylglycerophosphocholine liposomes

Egg yolk phosphatidylcholine liposomes containing labeled cholesterol were incubated at 37° C with an equimolar amount of dipalmitoylglycerophosphocholine liposomes (Fig. 6A). Transfer of labeled cholesterol from egg yolk phosphatidylcholine liposomes to dipalmitoylglycerophosphocholine liposomes reached 50% within 8 h. Although the rate of transfer was about the same as that observed between egg yolk phosphatidylcholine liposomes, the percentage transfer gradually increased even after 8 h. The reverse transfer of cholesterol from dipalmitoylglycerophosphocholine liposomes to egg yolk phosphatidylcholine liposomes was also examined. The transfer rate in this direction was about half that observed in the opposite direction, and about the same as that observed between homologous dipalmitoylglycerophosphocholine liposomes.

The net amount of cholesterol in liposomes was next examined (Fig. 6B). The amount of cholesterol in egg yolk phosphatidylcholine liposomes with 1.3 mol% phosphatidic acid ('donor liposomes') was determined after incubating them with dipalmitoylglycerophosphocholine liposomes containing 6.3 mol% phosphatidic acid ('acceptor liposomes') for various periods. The normalized cholesterol contents, calculated by dividing the observed values by the counts of [^{14}C]glycerol trioleate, changed from 2.3 to 2.0 after 2 h and finally to 1.25 after 8 h of incubation. The amount of cholesterol in dipalmitoylglycerophosphocline liposomes ('donor liposomes') was also determined after incubating these liposomes with egg yolk phosphatidylcholine liposomes ('acceptor liposomes'). The cholesterol content increased from 2.0 to 3.0 after 8 h incubation. When mixtures of egg yolk phosphatidylcholine liposomes and dipalmitoylglycerophosphocholine liposomes were incubated for 12 h, the cholesterol content of the former decreased to 30% of the original content (Fig. 7A). On the contrary, the cholesterol content of dipalmitoylglycerophosphocholine liposomes increased 45% on incubation with egg yolk phosphatidylcholine liposomes. The cholesterol contents in liposomes did not change signifi-

cantly on incubation with homologous liposomes (dipalmitoylglycerophosphocholine liposomes) (Fig. 7B). On incubation of homolgous egg yolk phosphatidylcholine liposomes, however, the cholesterol content of liposomes containing 1.3 mol% phosphatidic acid increased about 6%. This increase was quite consistent with the results obtained in the experiments described before (Table I). Cholesterol transfer between dipalmitoylglycerophosphocholine

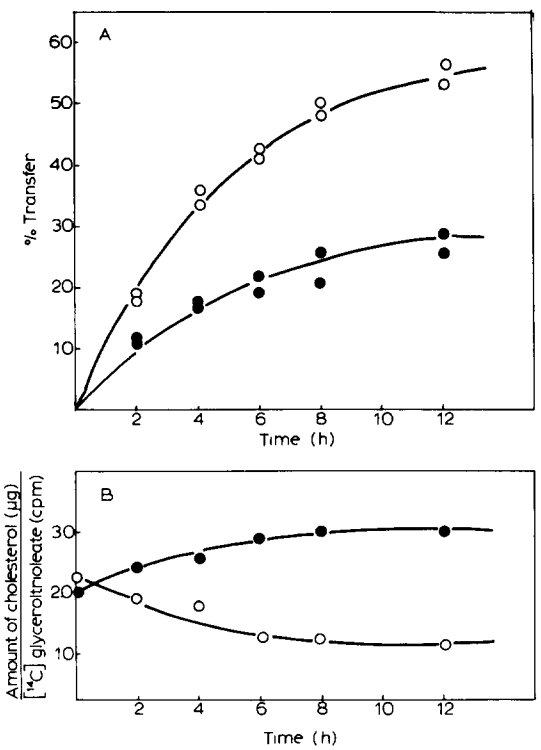

Fig. 6. Cholesterol transfer from egg phosphatidylcholine liposomes to dipalmitoylglycerophosphocholine liposomes and vice versa (A). Liposomes containing 65.8 mol% of egg phosphatidylcholine, 1.3 mol% of phosphatidic acid, and 32.9 mol% of cholesterol, with trace amounts of [^{3}H]cholesterol and [^{14}C]glyceroltrioleate were incubated at 37°C with liposomes containing 62.5 mol% of dipalmitoylglycerophosphocholine, 6.3 mol% of phosphatidic acid, and 31.2 mol% of cholesterol (○). Lipososmes containing 65.8 mol% of dipalmitoylglycerophosphocholine, 1.3 mol% of phosphatidic acid, and 32.9 mol% cholesterol, with [^{3}H]cholesterol and [^{14}C]glyceroltrioleate were also incubated with liposomes containing 62.5 mol% of egg phosphatidylcholine, 6.3 mol% of phosphatidic acid, and 31 2 mol% of cholesterol (●). Transfer of labeled cholesterol between liposomes was assayed at various times. Net content of cholesterol in liposomes during incubation of mixtures of two kinds of liposomes (B). Liposomes (1 μmol as phospholipid) containing 65.8 mol% of dipalmitoylglycerophosphocholine, 1.3 mol% of phosphatidic acid, and 32.9 mol% of cholesterol and [^{14}C]glyceroltrioleate were incubated at 37°C with an equal amount of liposomes containing 62.5 mol% of egg phosphatidylcholine, 6.3 mol% of phosphatidic acid, and 31.2 mol% of cholesterol in a final volume of 3 ml. The reaction mixtures were passed through a DEAE-cellulose column (0.6 × 2 cm) to separate the former liposomes, and the column was washed with 3 ml of phosphate buffer (pH 7.4). The radioactivity of an aliquot (0.6 ml) was counted, while the remaining eluate (4 ml) was extracted by the procedure of Bligh and Dyer. Cholesterol was determined as described in the text. The amount of cholesterol in liposomes with 1.3 mol% phosphatidic acid, which was normalized by dividing by the amount of [^{14}C]glyceroltrioleate recovered from the eluate was determined at various times (●). The cholesterol content of egg phosphatidylcholine liposomes with 1.3 mol% phosphatidic acid after incubation with dipalmitoylglycerophosphocholine liposomes with 6 3 mol% phosphatidic acid was also assayed (○).

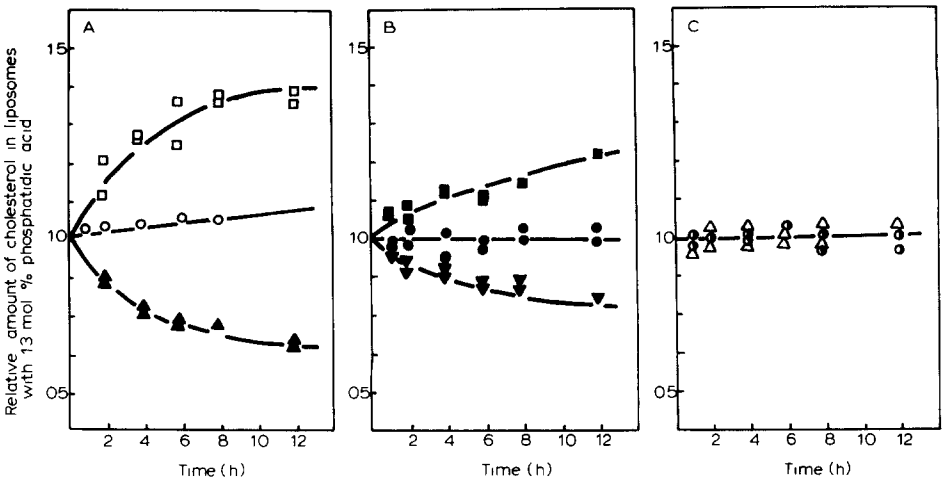

Fig 7. Asymmetric transfer of cholesterol between liposomes. Experiments were performed at 37°C by the same procedure as that described for Fig. 7B. The cholesterol content of liposomes with 1.3 mol% phosphatidic acid after incubation with liposomes with 6.3 mol% phosphatidic acid is shown relative to the original content of cholesterol (1.8 ng of cholesterol/dpm of glyceroltrioleate). The incubation systems were as follows: Dipalmitoylglycerophosphocholine liposomes with 1.3 mol% of phosphatidic acid ⇋ egg phosphatidylcholine liposomes with 6.3 mol% phosphatidic acid, □; egg phosphatidylcholine liposomes with 1.3 mol% phosphatidic acid ⇌ dipalmitoylglycerophosphocholine liposomes with 6.3 mol% phosphatidic acid, ▲, egg phosphatidylcholine liposomes with 1.3 mol% phosphatidic acid ⇌ egg phosphatidylcholine liposomes with 6.3 mol% phosphatidic acid, ○, sphingomyelin liposomes with 1.3 mol% phosphatidic acid ⇌ dipalmitoylglycerophosphocholine liposomes with 6.3 mol% phosphatidic acid, ■, dipalmitoylglycerophosphocholine liposomes with 1.3 mol% phosphatidic acid ⇌ sphingomyelin liposomes with 6 3 mol% phosphatidic acid, ▼, dipalmitoylglycerophosphocholine liposomes with 1.3 mol% phosphatidic acid ⇌ dipalmitoylglycerophosphocholine liposomes with 6.3 mol% phosphatidic acid, ●, dimyristoylglycerophosphocholine liposomes with 1.3 mol% phosphatidic acid ⇌ dipalmitoylglycerophosphocholine liposomes with 6.3 mol% phosphatidic acid, △; dipalmitoylglycerophosphocholine liposomes with 1.3 mol% phosphatidic acid ⇌ dimyristoylglycerophosphocholine liposomes with 6.3 mol% phosphatidic acid (◗)

liposomes and sphingomyelin liposomes was determined by the same procedure (Fig. 7B); the cholesterol content of sphingomyelin liposomes increased, while that of dipalmitoylglycerophosphocholine liposomes decreased. On the contrary, on incubation of heterogeneous liposomes, such as dipalmitoylglycerophosphocholine and dimyristoylglycerophosphocholine liposomes, there was no appreciable change in the cholesterol contents of the liposomes (Fig. 7C).

Effect of liposomes concentration on the transfer of cholesterol

The rate of transfer was measured as a function of the total liposome concentration (Table II). Liposomes composed of egg yolk phosphatidylcholine and cholesterol (1 : 0.5) were incubated at 37°C for 30 min. Within the concentration range of 1.33 mM to 0.083 mM phospholipid, no appreciable difference was observed in cholesterol transfer. Thus the rate of random collision between liposomes is probably not rate limiting in cholesterol transfer under the present experimental conditions. This finding is not consistent with results on cholesterol transfer between liposomes and erythrocyte ghosts [12], but we cannot explain the discrepancy at present.

TABLE II

EFFECT OF LIPOSOMAL CONCENTRATION ON THE TRANSFER RATE

Equimolar mixtures of liposomes containing 65.8 mol% of egg yolk phosphatidylcholine, 1.3 mol% of phosphatidic acid, and 32.9 mol% of cholesterol, with trace amounts of [^{3}H]cholesterol and [^{14}C]glyceroltrioleate and liposomes containing 62.5 mol% of egg yolk phosphatidylcholine, 6.3 mol% of phosphatidic acid and 31.2 mol% of cholesterol were incubated at 37°C for 2 h at various total liposome concentrations. The percentage transfer was measured as described in the Materials and Methods.

Liposomal concentration as phospholipid (mM)	Transfer of cholesterol (%)
0.08	22.5
0.17	26.5
0.33	25.0
0.67	26.5
1.33	27.5

Discussion

The transfer of cholesterol between liposomal membranes was examined. Cholesterol molecules can be exchanged between liposomal membranes in the absence of any specific protein. The present procedure for assaying cholesterol transfer between liposomes, which was originally devised for detecting phospholipid exchange by van den Besselar et al. [3], has the advantage over systems using the immune-agglutination technique [1] and the NMR technique [2], that it is rapid and so the rate of transfer can be determined easily.

On incubation of liposomes composed of egg yolk phosphatidylcholine, phosphatidic acid and cholesterol (molar ratio, 1 : 0.02 or 0.1 : 0.5) at 37°C, almost complete equilibration of the cholesterol pools was achieved within 6—9 h. Cholesterol transfered into the liposomes from the other liposomes showed the same transfer rate as that incorporated into the liposomes, exogenously. These clearly indicates that 'flip-flop' of cholesterol molecules occurs in the egg yolk phosphatidylcholine liposomes. Contradictory results have been obtained in previous studies on the rate of 'flip-flop' of cholesterol or its derivatives. The half-life for flip-flop was reported to be 70 min at 30°C for the fluorescent cholesterol analogue, sterophenol, in egg yolk phosphatidylcholine liposomes [13], 6 days for cholesterol in dipalmitoylglycerophosphocholine liposomes [6] and 13 days for cholesterol in influenza viral envelopes [14]. Quite recently, Bloj and Zilversmit [7] reported that the half-lives for transfer of cholesterol incorporated into dipalmitoyl- and egg yolk phosphatidylcholine were 4.7 and 3.1 h, respectively. In our system, the half time for cholesterol flip-flop was calculated to be shorter than 6 h in egg yolk phosphatidylcholine liposomes at 37°C. Our results agree with the observations of Bloj and Zilversmit [7]; they found that the transfer process in egg yolk phosphatidylcholine liposomes had a half-life of 2—3 h. This rate is compatible with that reported for cholesterol transfer between red blood cells and plasma [15].

Cholesterol transfers between, red blood cells and low density lipoprotein or high density lipoprotein [16], red blood cells and plasma [17], and red cell ghosts and low density lipoprotein [18] were found to be temperature-depen-

dent. In all cases, the transfer was greater at 37°C than at 10°C. In our work the temperature dependence of the transfer between liposomes was demonstrated more clearly. The temperature dependence observed in the present experiments may be related to the fluidity of the membranes. The 'fluidity' of the membranes was not, however, the only factor controlling the exchange of cholesterol, since dimyristoylglycerophosphocholine, dipalmitoylglycerophosphocholine, distearoylglycerophosphocholine and beef sphingomyelin liposomes showed almost the same temperature-dependence of cholesterol exchange, although their thermodynamic characters must be different. The activation energies for the transfer between erythrocyte ghosts and dioleoyl- and dipalmitoylphosphatidylcholine liposomes were reported to be —9.5 kcal/mol and —19.3 kcal/mol, respectively [12]. The energy for cholesterol transfer between egg phosphatidylcholine liposomes was calculated to be about —5 kcal/mol. This value corresponds fairly well with the value obtained for transfer between erythrocyte membranes and dioleoylphosphatidylcholine liposomes.

We observed asymmetric transfer of cholesterol when heterogeneous liposomes containing different classes of phospholipids were incubated together. Asymmetric transfer of cholesterol was observed from egg phosphatidylcholine liposomes, containing phospholipid with a lower melting point, to dipalmitoylglycerophosphocholine liposomes, containing phospholipid with a higher melting point. This finding suggests that cholesterol has a higher 'affinity' for phospholipid with a higher melting point. When incubated with egg yolk phosphatidylcholine liposomes, dipalmitoylglycerophosphocholine liposomes seemed to contain cholesterol in a molar ratio of 1.5 to phospholipid, if phospholipid is not transferred under the present conditions. Recently, phospholipid transfer between liposomes was reported by Martin and MacDonald [19] and be Duckwitz-Peterlein et al. [20]; they observed asymmetric transfer of phospholipid between two types of liposomes. The transfer of phospholipids in fluid membranes appears to be more rapid from more fluid to less fluid membranes than in the reverse direction. Therefore it is possible that egg phosphatidylcholine as well as cholesterol is transferred between membranes. Under the present experimental conditions, however, we did not observe any significant transfer of ^{14}C-labeled egg yolk phosphatidylcholine between egg yolk phosphatidylcholine liposomes and dipalmitoylglycerophosphocholine liposomes.

It remains to be resolved how cholesterol molecules are localized in dipalmitoylglycerophosphocholine liposomes and why the liposomes can take so much cholesterol. With a mixture of dipalmitoylglycerophosphocholine liposomes and liposomes containing a phospholipid differing by 2 carbon atoms, dimyristoylglycerophosphocholine, no increase or decrease in the cholesterol content of either type of liposomes was observed. A 'preferential affinity' of cholesterol for beef sphingomyelin was also found in mixtures of dipalmitoylglycerophosphocholine and sphingomyelin liposomes. The 'affinity order' of cholesterol for the phospholipids deduced from the present experiments is sphingomyelin > dipalmitoylglycerophosphocholine = dimyristoylglycerophosphocholine > egg yolk phosphatidylcholine. The order observed here does not agree with that obtained by differential scanning calorimetry

[21,22]. In the latter studies on the distribution of cholesterol in phospholipid bilayers, it was found that in lipid bilayers showing phase separation cholesterol interacted preferentially with the phospholipid species with the lower transition temperature, while in mixtures which differed by 2 carbon atoms and showed cocrystallization of the paraffin chains cholesterol interacted randomly with both phospholipids. The latter studies also showed a preferential affinity of cholesterol for sphingomyelin. At present, we cannot explain the difference in the affinity order obtained with the two different systems. It is interesting, however, that even with different liposomal membranes, cholesterol showed an apparent 'preferential affinity' for various phospholipid species.

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References

1 Ehnholm, C. and Zilversmit, D.B. (1973) J. Biol. Chem. 248, 1719—1724
2 Haran, N. and Shoporer, M. (1977) Biochim. Biophys. Acta 465, 11—18
3 Van den Besselar, A.M.H.P., Helmkamp, G.M. and Wirtz, K.W.A. (1975) Biochemistry 14, 1852—1858
4 Cubero-Robles, E. and van den Berg, D. (1969) Biochim. Biophys. Acta 187, 520—526
5 Huang, C. (1969) Biochemistry 8, 344—352
6 Poznansky, M. and Lange, Y. (1976) Nature 259, 420—421
7 Bloj, B and Zilversmit, D.B. (1977) Biochemistry 16, 3943—3948
8 Bligh, E.G. and Dyer, W J. (1959) Can. J. Biochem. Physiol. 37, 911—917
9 Zlatkis, A., Zak, B. and Boyle, A.J. (1953) J. Lab. Clin. Med. 41, 486—492
10 Bruckdorfer, K.R., Edwards, P.A. and Green, C. (1968) Eur. J. Biochem. 4, 506—511
11 Huang, C., Sipe, J.P., Chow, S.T. and Martin, R.B. (1974) Proc. Natl. Acad. Sci. U.S. 71, 359—362
12 Poznansky, M.J. and Lange, Y. (1978) Biochim. Biophys. Acta 506, 256—264
13 Smith, R.J.M. and Green, C. (1974) FEBS Lett. 42, 108—111
14 Lenard, J. and Rothman, J.E. (1976) Proc. Natl. Acad. Sci. U.S. 73, 391—395
15 Hagerman, J.S. and Gould, R.G. (1951) Proc. Soc. Exp. Biol. Med. 78, 329—332
16 Bell, F.P., Somer, J.B., Craig, I.H. and Schwartz, C.J. (1972) Pathology 4, 205—214
17 Murphy, J.R. (1962) J. Lab. Clin. Med. 60, 86—109
18 Bruckdorfer, K.R. and Green, C. (1967) Biochem. J. 104, 270—277
19 Martin, F.J. and MacDonald, R.C. (1976) Biochemistry 15, 321—327
20 Duckwitz-Peterlein, D., Eilenberger, G. and Overath, P. (1977) Biochim. Biophys. Acta 469, 311—325
21 De Kruyff, B., van Dijck, P.W.M., Demel, R.A., Schuijff, A., Brants, F. and van Deenen, L.L.M. (1974) Biochim. Biophys. Acta 356, 1—9
22 Demel, R A., Jansen, J.W.C.M., van Dijck, P.W.M. and van Deenen, L.L.M. (1977) Biochim. Biophys. Acta 465, 1—10