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Molecular species composition of membrane phosphatidylcholine influences the rate of cholesterol efflux from human erythrocytes and vesicles of erythrocyte lipid

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The efflux of [^{3}H]cholesterol from prelabelled human erythrocytes having modified phosphatidylcholine compositions was measured during 24-h incubations in the presence of unlabelled acceptor liposomes composed of equimolar amounts of egg phosphatidylcholine and cholesterol. The cells were modified by replacement of part of the native phosphatidylcholine with either dipalmitoylphosphatidylcholine, palmitoyloleoylphosphatidylcholine or dilinoleoylphosphatidylcholine catalyzed by phosphatidylcholine-specific transfer protein from bovine liver. The results indicated that the efflux of [^{3}H]cholesterol was faster from erythrocytes in which the dipalmitoylphosphatidylcholine content was increased from 7 to 25% of the total, than from cells enriched in palmitoyloleoylphosphatidylcholine or dioleoylphosphatidylcholine. Incorporation of dilinoleoylphosphatidylcholine to a level of 13% of the total phosphatidylcholine slowed the rate of efflux of [^{3}H]sterol. The phosphatidylcholine replacements produced no significant differences in cholesterol/phospholipid ratio before or after 24 h of incubation with the acceptor egg phosphatidylcholine-cholesterol vesicles. Using vesicles prepared from erythrocyte lipid, modified to reflect the changes in the phosphatidylcholine composition induced in the whole cells, the same influence of composition on the rate of cholesterol exchange was evident. Enhancement of the dipalmitoylphosphatidylcholine content from 7 to 25% of the total phosphatidylcholine pool increased the rate of [^{3}H]cholesterol efflux, while the addition of the same amount of dilinoleoylphosphatidylcholine slowed it compared to controls. The magnitude of the effect was comparable in intact cells and erythrocyte lipid vesicles enriched in dipalmitoylphosphatidylcholine, while the influence of dilinoleoylphosphatidylcholine was more marked in the intact cells. These results demonstrate that changes in the molecular species composition of the phosphatidylcholine pool can influence the rate of exchange of cholesterol but not necessarily the cellular content of sterol in the human erythrocyte. The influence of this phospholipid appears to be expressed independently of the presence of membrane protein or an underlying cytoskeleton.

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Abbreviations: GLC, gas-liquid chromatography; palmitoyloleoylphosphatidylcholine, 1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphocholine. All other diacylphosphatidylcholines were 1,2-diacyl-*sn*-glycero-3-phosphocholines.

Introduction

Much progress has been made over the past 5 years toward an understanding of the mechanism of the exchange of cholesterol between membranes. An important step forward has been the

identification of rate-limiting steps in the process. Several laboratories have provided evidence that the desorption of sterol from the membrane is rate-limiting, rather than the uptake stage. This has been found to be consistent for a wide variety of systems including erythrocytes [1–3], fibroblasts in culture [4], plasma lipoproteins [1] and cholesterol-phosphatidylcholine liposomes [1,4]. Furthermore, although differences in the rate constants of cholesterol efflux between cell types have been reported [3], they appear to be remarkably similar to those describing the efflux from cholesterol-phosphatidylcholine liposomes [1,4].

Clearly, an elucidation of the factors that influence the desorption of cholesterol from membranes would facilitate a further understanding of sterol exchange. The reported similarity in the rate constants derived from whole cells and liposomes suggests that one of the determinants may be interactions occurring between cholesterol and the phospholipid component of the membrane. A clear relationship between the rate of cholesterol exchange and the structure of phosphatidylcholine has been established with sonicated liposomes composed of cholesterol and a single, pure phosphatidylcholine species [5,6]. In these experiments, cholesterol effused more slowly from vesicles composed of saturated phosphatidylcholine species than from those of unsaturated species. A relationship between fatty acyl composition and cholesterol exchangeability is also implied in experiments using *Acholeplasmas* [7]. Gershfeld and co-workers [7] demonstrated that cholesterol accumulated to a greater extent in cells grown on palmitic acid compared to those grown on oleic acid. Although the influence of the fatty acyl substrates on cellular structures is likely to be complex under the latter conditions, the implication is that cholesterol was retained to a greater extent by glycerolipid matrices enriched in saturated fatty acids.

Despite the results from liposomal model systems and the indications from whole cell experiments, however, it remains to be demonstrated that the structure of phosphatidylcholine has any influence on cholesterol exchange in a biological membrane. In this report, we describe experiments designed to investigate the role of the cellular phosphatidylcholine composition in the exchange of cholesterol from human erythrocytes, by taking advantage of the ability of phosphatidylcholine-specific transfer protein from bovine liver to replace the native erythrocyte phosphatidylcholine in the outer monolayer with species of our own chosing [8,9]. The results demonstrate a relationship between cholesterol exchange and the structure of the membrane phosphatidylcholine in erythrocytes modified by the above technique and also in vesicles composed of human erythrocyte lipid.

Materials and Methods

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine and 1,2-dioleoyl-*sn*-3-phosphocholine were generously provided by W.S.M. Geurts van Kessel (Biochemistry Department, Utrecht, The Netherlands). Egg phosphatidylcholine, egg phosphatidic acid and 1,2-dilinoleoyl-*sn*-glycero-3-phosphocholine were purchased in the highest available purity from Sigma Corporation (St. Louis, MO, U.S.A.) and were used without further purification.

Cholesterol [^{14}C]stearate, 1,2-di[1-^{14}C]-palmitoyl-*sn*-glycero-3-phosphocholine, 1-palmitoyl-2-[^{14}C]oleoyl-*sn*-glycero-3-phosphocholine and [1,2-^{3}H]cholesterol were purchased from Amersham International (Amersham, Bucks, U.K.). Soy phosphatidyl[*N-methyl*-^{14}C]choline was available in the laboratory from previous studies. The radioactive cholesterol and phosphatidylcholines each gave one band following thin-layer chromatography on silica gel H plates developed with ether/hexane (85 : 15, v/v) and chloroform/methanol/acetic acid/water (75 : 45 : 12 : 6, v/v) [10], respectively. Scans of the resulting TLC plates showed that more than 95% of the radioactivity migrated with the carrier compound indicated by exposure to iodine.

Phosphatidylcholine-specific transfer protein from bovine liver was purified and prepared for experimental use as previously described [9,11].

Fresh human erythrocytes were obtained by venipuncture using acid/citrate/dextrose as anticoagulant. The cells were centrifuged for 10 min at $900 \times g$ and the plasma and buffy coat carefully removed. The pellet was dispersed in 5 vol. of buffer comprising 150 mM NaCl/25 mM glucose/10 mM Tris-HCl/1 mM EDTA (pH 7.4) (referred to as 'buffer' throughout) and recentri-

fuged. This washing procedure was repeated three times. The erythrocytes were used within 2 h of their preparation.

Preparation of vesicles. Vesicles used in the modification of phosphatidylcholine composition of the erythrocytes contained 47 mol% phosphatidylcholine, 47 mol% cholesterol, 6% egg phosphatidic acid. Trace amounts of [^{14}C]phosphatidylcholine were added giving a final specific radioactivity of $1.5 \cdot 10^5$ ^{14}C dpm/nmol phosphatidylcholine. The various components were dispensed from stock solutions in organic solvent into a rotary flask and dried by rotary evaporation. For vesicles prepared from dipalmitoylphosphatidylcholine, dioleoylphosphatidylcholine or palmitoyloleoylphosphatidylcholine, the dried mixtures were dispersed in buffer (37°C) to give a final concentration of 4.9 μmol phosphatidylcholine/ml. The dispersions were then sonicated under nitrogen with a Branson Sonifier fitted with a standard probe (2 × 5 min at 0°C, 50 W), and subsequently centrifuged at 100 000 × *g* for 30 min. The pellet was discarded and the supernatant used in the experiments described.

A modified procedure was used for the preparation of dilinoleoylphosphatidylcholine vesicles because of the risk of peroxidation of this species during sonication. In this case, the dried lipid mixture was dissolved in 100–200 μl ether and released, by syringe, into buffer at 65°C under nitrogen. The suspension was then centrifuged as above. The yield of vesicles after this procedure was typically 10–20% compared to more than 50% with the sonication method.

Nonlabelled cholesterol acceptor vesicles used in the efflux experiments were composed of cholesterol, egg phosphatidylcholine and egg phosphatidic acid (0.8 : 1.0 : 0.10, mole ratio) and were prepared in the same manner as the phosphatidylcholine donor vesicles. To the dried components, buffer was added to give a final concentration of 2 μmol phosphatidylcholine/ml. The remainder of the preparation was the same as that of the phosphatidylcholine donor liposomes.

For the preparation of vesicles of erythrocyte lipids, a total lipid extract was made (from 15 ml packed cells) according to the method of Rose and Öklander [12]. The organic extract was evaporated under vacuum and the residue taken up in 12 ml chloroform/methanol (2 : 1, v/v). The extract was washed with 3 ml physiological saline [13] and the organic phase removed and evaporated to a small volume.

[^{3}H]Cholesterol and cholesterol [^{14}C]stearate were added to aliquots of the extract followed by enough dipalmitoyl phosphatidylcholine, dilinoleoylphosphatidylcholine or egg phosphatidylcholine to increase the phosphatidylcholine content by 20%. Subsequently, the extract was evaporated and buffer was added giving a final concentration of 0.5 μmol phosphatidylcholine/ml. The samples were sonicated at 0°C under nitrogen for the same times as before. The level of buffer was restored to the original and the mixtures centrifuged at 10 000 × *g* for 10 min. The yield of vesicles was greater than 90% by the procedure as judged by the recovery of radioactivity in the supernatant. There was no dependence of the recovery on the phosphatidylcholine species used to enrich the erythrocyte lipid.

Labelling of the cholesterol pool in erythrocytes. The erythrocytes were labelled by exchange in the presence of [^{3}H]cholesterol-containing plasma by a method based on that of Murphy [14]. Freshly isolated plasma, containing acid/citrate/dextrose anticoagulant, was heated at 56°C for 30 min to inactivate phosphatidylcholine-cholesterol acyltransferase. After cooling and centrifugation for 10 min at 3000 × *g*, the supernatant (8 ml) was incubated in a 50 ml round-bottom flask into which [^{3}H]cholesterol (4 μCi) had been previously introduced and dried under a nitrogen stream.

Glucose (8 mg) and a streptomycin/penicillin cocktail were added and the mixture shaken in a 37°C water-bath. After 24 h, the plasma was centrifuged at 2000 × *g* for 30 min and the supernatant used for labelling the erythrocytes. Washed erythrocytes (2.5 ml packed cells) were incubated with 8 ml of the plasma supernatant at 37°C for 16 h on a clinical blood rotator. The cells were subsequently washed twice with 5 vol. of buffer before treatment with phosphatidylcholine transfer protein to modify the phosphatidylcholine compositions.

Modification of the cellular phosphatidylcholine composition. The replacement of erythrocyte phosphatidylcholine with species of defined composition was carried out with phosphatidylcholine

transfer protein essentially as described by Kuypers et al. [9]. The incubations were performed in plastic liquid scintillation vials at 37°C in a shaking water-bath and typically contained washed prelabelled erythrocytes (1 ml, 1200 nmol phosphatidylcholine), vesicles (1230 nmol phosphatidylcholine) and phosphatidylcholine transfer protein (240 μg protein) in a final volume of 3.6 ml buffer. After 3 h, the mixtures were centrifuged 10 min at $2500 \times g$ and the supernatants (donor vesicles) were removed. The erythrocytes were washed a further three times with 5 vol. of buffer prior to subsequent analysis. The percentage replacement was determined either by the radioactivity appearing in the phosphatidylcholine fraction of the cells [9] or by the weight percentage of erythrocyte phosphatidylcholine species appearing in the donor vesicles measured by GLC [15].

The efflux of [^{3}H]cholesterol from prelabelled erythrocytes with modified phosphatidylcholine compositions. The rate of efflux of [^{3}H]cholesterol from prelabelled cells during incubation with unlabelled cholesterol-egg phosphatidylcholine vesicles was measured using a modification of the technique described by Lange et al. [1]. The prelabelled cells were incubated at 37°C in solutions prepared in triplicate, containing the cells (125 μl, 0.50 μmol phospholipid), vesicles (0.5–1.5 μmol phosphatidylcholine) and buffer, to give a final hematocrit of 10%. Samples (100 μl) were withdrawn from each solution at various times, dispersed in 0.5 ml buffer and centrifuged for 0.5 min at $8000 \times g$. The supernatant was removed and placed directly in a liquid scintillation vial, followed by 4.5 ml Instagel liquid scintillation emulsifier cocktail (Packard Becker B.V., Groningen, The Netherlands). The cell pellet (typically 10 μl) was washed twice with 0.5 ml buffer and lysed with 20 μl distilled water. An extract of the cell lysate was made with 0.5 ml isopropanol and following extensive vortexing and centrifugation, was transferred to a scintillation vial followed by 4.5 ml Instagel. The recovery of the labelled cholesterol from the cells by this technique was not significantly different from that of control cells extracted with chloroform/methanol using standard procedures [13]. The incubation supernatant and the erythrocyte extracts (in triplicate) were analyzed in a Prias PLD liquid scintillation counter (Packard). The data was expressed as the ratio of the dpm remaining in the cells or supernatant, divided by the total dpm in the sample at each time point.

Efflux of [^{3}H]cholesterol from vesicles of erythrocyte lipid. These experiments were carried out essentially as the cellular efflux experiments, with the exception that the [^{3}H]cholesterol was initially present in the vesicles and the acceptor membranes were those of untreated red cells. The incubation mixtures contained erythrocytes (0.3 ml, 0.68 μmol phospholipid), vesicles (50 μl, 0.025 μmol phosphatidylcholine) and buffer to give a hematocrit of 30%.

Gas liquid chromatography. The molecular species composition of erythrocyte phosphatidylcholine was determined by capillary and packed column GLC of the trimethylsilyl ethers of diacylglycerols liberated by phospholipase C, as described in detail in previous reports [15,16].

Cholesterol was analyzed chromatographically as the trimethylsilyl ether using cholesterol acetate (Sigma Corp., St. Louis, U.S.A.) as the internal standard.

Results and Discussion

In the preparation of modified erythrocytes for use in cholesterol efflux studies, the cells were first labelled with [^{3}H]cholesterol by an incubation in the presence of radioactive plasma. Subsequently, these cells were incubated with phosphatidylcholine transfer protein and vesicles containing cholesterol and the pure phosphatidylcholine species of choice, to bring about the modification of the cellular phosphatidylcholine pool. These erythrocytes, possessing cholesterol marked with ^{3}H and having modified phosphatidylcholine compositions, were then incubated with nonradioactive vesicles of egg phosphatidylcholine-cholesterol and the release of ^{3}H from the cells was monitored. The status of the cells after each of these treatments is described below under appropriate subheadings.

Labelling of erythrocytes with [^{3}H]cholesterol

Under the labelling conditions used, the cells attained a specific radioactivity of about 1000 dpm/nmol erythrocyte cholesterol or 1 μCi/ml packed cells. In accordance with previous reports

[1,14], this treatment caused no change in the cholesterol/phospholipid ratio of the cells and their shape remained discoidal.

Modification of cellular phosphatidylcholine composition

In accordance with previous reports [9,17], the phospholipid headgroup composition of the erythrocytes remained unchanged and there was no significant change in the cellular cholesterol/phospholipid ratio as a result of the incubation of the cells with vesicles of cholesterol and phosphatidylcholine in the presence of the transfer protein (Table I). The exchange of phosphatidylcholine was indicated by the appearance of radioactive label in the phosphatidylcholine fraction of the erythrocytes. From these data, the extent of replacement was calculated after correcting for adherent vesicle material estimated in control experiments using [^{3}H]trioleoylglycerol as a nonexchangeable marker (Table I). The percentage replacement was not allowed to exceed 30% of the total phosphatidylcholine pool to minimize the influence of the substitutions on cell shape, osmotic fragility, potassium leakage and hemolysis [8,9]. At the levels of exchange indicated in Table I, the cells generally retained a discoidal shape, but the dipalmitoylphosphatidylcholine-enriched cells contained a greater percentage of echinocytes while dioleoylphosphatidylcholine and dilinoleoylphosphatidylcholine-treated preparations contained greater proportions of cup-shaped cells than palmitoyloleoylphosphatidylcholine-treated erythrocytes. The exchange of cellular phosphatidylcholine is reflected in the mean number of double bonds per phosphatidylcholine molecule in the modified erythrocytes, but the mean acyl chain-length was not greatly affected by the modifications (Table I).

Characterization of cellular cholesterol content and labelling after phosphatidylcholine modification

The experimental sequence of prelabelling the cholesterol pool before carrying out the modifications to the cellular phospholipid allows adherent labelled plasma to be washed from the cells before the performance of the efflux experiments but also allows some sterol exchange to occur because the phosphatidylcholine exchange vesicles necessarily contain cholesterol. The exchange of cholesterol during this period was, therefore, examined by two methods and the results shown in Table I. The [^{3}H]cholesterol specific radioactivities did not differ significantly following the various treatments with phosphatidylcholine transfer protein. The fraction of radioactivity remaining in the cells after 3 h in the presence of phosphatidylcholine transfer protein was not significantly different between dipalmitoyl-, dioleoyl- and palmitoyloleoylphosphatidylcholine-treated cells, but there

TABLE I

CHARACTERISTICS OF ERYTHROCYTES PRELABELLED WITH [^{3}H]CHOLESTEROL AFTER TREATMENT WITH PHOSPHATIDYLCHOLINE-CHOLESTEROL VESICLES PLUS TRANSFER PROTEIN

Human erythrocytes were prelabelled with [^{3}H]cholesterol and subsequently treated with transfer protein as described in Materials and Methods. The double bond index represents the mean number of double bonds per phosphatidylcholine molecule and was derived from GLC analysis of the molecular species of each cell type as outlined in Materials and Methods. RBC, red blood cell; PC, phosphatidylcholine.

PC species in donor vesicles	Erythrocyte phosphatidylcholines			Erythrocyte cholesterol		
	percentage replacement	double bond index	mean acyl chain-length	[cholesterol/phospholipid] (mol/mol)	[^{3}H dpm/nmol cholesterol]	[^{3}H dpm$_{RBC}$/(^{3}H dpm$_{RBC}$ + ^{3}H dpm$_{vesicles}$)]
Dipalmitoyl PC	15	1.39	33.2	0.86 ± 0.03	967 ± 17	0.92 ± 0.01
Palmitoyloleoyl PC	25	1.48	34.4	0.85 ± 0.04	1036 ± 104	0.91 ± 0.01
Dioleoyl PC	30	1.80	35.1	0.86 ± 0.06	940 ± 119	0.92 ± 0.01
Dilinoleoyl PC	13	1.95	34.4	0.88 ± 0.09	959 ± 97	0.99 ± 0.01
Untreated	0	1.64	34.4	0.89 ± 0.03	1038 ± 245	–

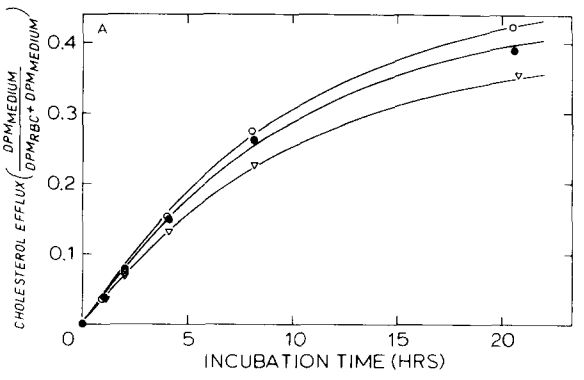
was significantly less exchange of labelled cholesterol in the dilinoleoylphosphatidylcholine-treated cells. This is consistent with the slightly lower concentration of vesicle phospholipid in the dilinoleoylphosphatidylcholine incubations compared to the others. No explanation for the discrepancy between the two data sets was found. With both methods of determination, the loss of radioactivity from the cholesterol pool during the preincubation represented less than 10% of the total in each case.

Cholesterol efflux from modified erythrocytes in the presence of unlabelled vesicles

Incubation of [^{3}H]cholesterol-labelled cells having modified phosphatidylcholine compositions with vesicles composed of cholesterol and egg phosphatidylcholine promotes the exchange of cholesterol that can be followed as a loss of ^{3}H from the cells and its appearance in the vesicle fraction following their separation by rapid centrifugation. The efflux of [^{3}H]cholesterol from erythrocytes enriched in dipalmitoylphosphatidylcholine, palmitoyloleoylphosphatidylcholine or dilinoleoylphosphatidylcholine, expressed as the fraction of the total sample ^{3}H dpm appearing in the vesicles at each time point, is shown in Fig. 1A. The same data are plotted semilogarithmically in Fig. 1B.

The curves show the same general shape as that reported by previous authors [1,18], but it is evident that the approach to equilibrium proceeded most rapidly with the dipalmitoylphosphatidylcholine-enriched cells and least rapidly with dilinoleoylphosphatidylcholine-enriched cells. The efflux of [^{3}H]cholesterol from palmitoyloleoylphosphatidylcholine-treated cells was not significantly different from nontreated cells or those enriched in dioleoylphosphatidylcholine (data not shown).

The level of cell hemolysis did not exceed 8% during 20 h of incubation but longer incubations were prohibited by the occurrence of greater than 10% lysis. There was no significant difference in the amount of lysis occurring in the three types of erythrocytes in this experiment up to 12 h, but thereafter the dipalmitoylphosphatidylcholine-treated cells tended to become more lytic. After 20 h of incubation, this difference did not exceed 2%



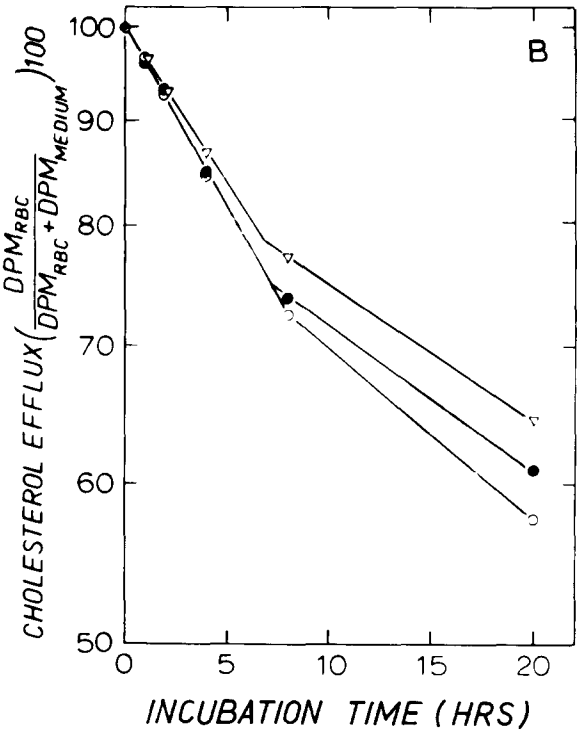


Fig. 1. The efflux of [^{3}H]cholesterol from erythrocytes with modified phosphatidylcholine compositions. Erythrocytes were prelabelled with [^{3}H]cholesterol and enriched in either dipalmitoylphosphatidylcholine (○), palmitoyloleoylphosphatidylcholine (●) or dilinoleoylphosphatidylcholine (▽) to the levels of replacement indicated in Table I. The cells (125 μl) were incubated with cholesterol-egg phosphatidylcholine vesicles (1.5 μmol phosphatidylcholine) and buffer in a final volume of 1.25 ml. (A) The data plotted are expressed as the fraction of radioactivity in each sample that is present in the vesicle fraction after centrifugation, as described in Materials and Methods. Each data point is the mean of three samples. The same data are plotted semilogarithmically in (B).

(8% vs. 6%). Small amounts of hemoglobin present in the supernatants (90 μl of supernatant diluted to 590 μl) did not interfere with the conversion of the ^3H cpm to dpm, as determined with standard mixtures containing increasing amounts of cell lysate.

We were unable to detect any significant change in the cholesterol/phospholipid ratio of the cells following 20 h of incubation with cholesterol-egg phosphatidylcholine liposomes. This was of interest in view of the speculation, based on the behavior of vesicles of dipalmitoylphosphatidylcholine, that the structure of membrane phosphatidylcholine may dictate the cholesterol level [19]. From our experiments, it is clear that increasing the dipalmitoylphosphatidylcholine content of the cellular phosphatidylcholine from 7% of the total to 25% did not lead to gross changes in the cholesterol content of the cell before or after subsequent exchange incubations. It is possible, however, that small changes beneath the level of our detection did occur and it should be noted that in Fig. 1, for example, the difference between the two extremes in rate give rise to only an 8% difference in the [^{3}H]cholesterol remaining in the cell after 20 h of incubation. Kuypers et al. [9] have, however, reported that there is no change in the cholesterol content of erythrocytes treated with dipalmitoylphosphatidylcholine or dilinoleoylphosphatidylcholine to much higher levels of replacement than those described here.

To confirm that the exchange of cellular phosphatidylcholine was required for the observed differences in cholesterol efflux, two populations of [^{3}H]cholesterol-labelled erythrocytes were incubated for 3 h in the presence of dipalmitoylphosphatidylcholine-cholesterol vesicles, one with and one without the transfer protein. The efflux of [^{3}H]cholesterol during subsequent incubation of the red cells with unlabelled acceptor vesicles was measured and the results are shown in Fig. 2. Clearly, the rate of efflux is greater from the cells in which the dipalmitoylphosphatidylcholine content had been enriched by the transfer protein. In this experiment, the differences were evident at all time points. No significant difference in hemolysis was observed between the two cell types at the earlier time points shown in the graph, but there was a 2% greater degree of lysis in the dipalmitoylphosphatidylcholine-enriched preparation after 20 h (9% vs. 7%).

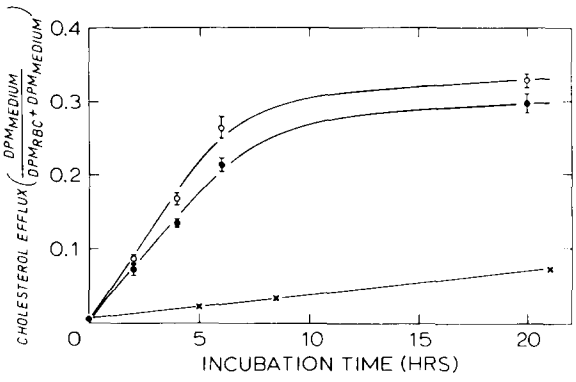


Fig. 2. The efflux of [^{3}H]cholesterol from dipalmitoylphosphatidylcholine-enriched and dipalmitoylphosphatidylcholine-incubated erythrocytes. Modified erythrocytes for this experiment were prepared by exposing [^{3}H]cholesterol-prelabelled cells to vesicles composed of dipalmitoylphosphatidylcholine/cholesterol in the presence (○) or absence (●) of phosphatidylcholine transfer protein. The cells (125 μl) were subsequently incubated with cholesterol-egg phosphatidylcholine vesicles (0.6 μmol phosphatidylcholine) and the above data describe the efflux of [^{3}H]cholesterol occurring during this period. The data are the mean of triplicate incubations and are expressed as in Fig. 1. The degree of cell cysis occurring during the incubation is plotted (×) in the lower portion of the graph.

The results of this experiment are consistent with a requirement for phosphatidylcholine replacement for an inducement of the observed change in sterol exchange rate. They are not consistent with an influence of the dipalmitoylphosphatidylcholine vesicles mediated through impurities in the preparation (e.g., lysophosphatidylcholine), by interference with other membrane components, or by vesicle-specific alterations in the specific radioactivity of the labelled sterol pool during the initial treatment of the cells.

Efflux of [^{3}H]cholesterol from vesicles of erythrocyte lipid

To determine the influence of phosphatidylcholine composition independent of membrane protein and an underlying cytoskeleton, we investigated the exchange of [^{3}H]cholesterol from vesicles prepared from purified erythrocyte lipids. The release of cholesterol from these vesicles, occurring during exchange in the presence of unlabelled

erythrocytes, is shown in Fig. 3. The curves show the same general shape as with the [^{3}H]-cholesterol-labelled erythrocytes, tending towards equilibrium after 24 h in the presence of a large excess of the acceptor species. This is in accordance with a previous study of cholesterol exchange from vesicles of erythrocyte lipid [20]. In panel A of this figure which compares dipalmitoylphosphatidylcholine- and egg phosphatidylcholine-enriched vesicles, it is clear that at all time points, the release of ^{3}H label is faster from the vesicles containing the more saturated species. The similarity between this figure and Fig. 2, expressing data from erythrocytes, is quite striking considering that the phosphatidylcholine content of the vesicles was increased by 20% compared to the intact cells and that the vesicles contain no membrane protein.

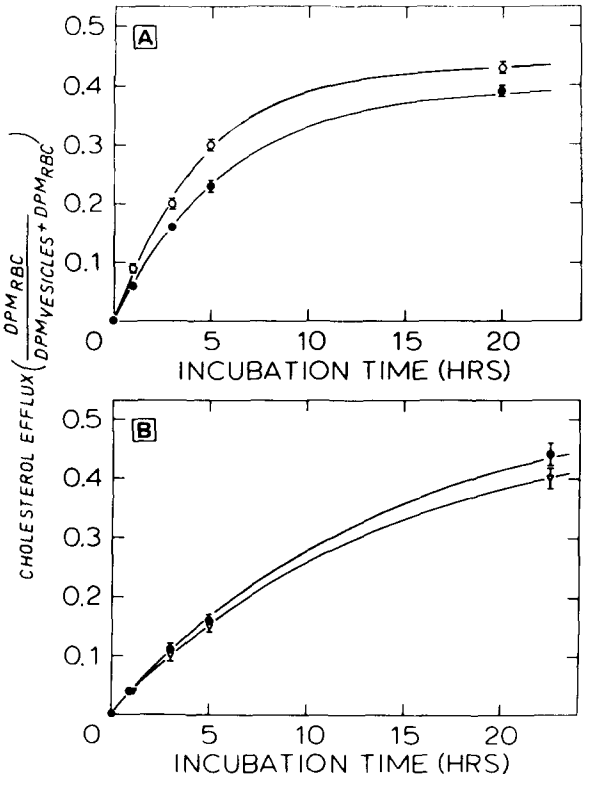


Fig. 3. Efflux of [^{3}H]cholesterol from vesicles of erythrocyte lipid having modified phosphatidylcholine compositions. Vesicles were prepared from erythrocyte total lipids enriched in dipalmitoylphosphatidylcholine, egg phosphatidylcholine or dilinoleoylphosphatidylcholine to a level of 20% of the total phosphatidylcholine content as described in Materials and Methods. The vesicles (0.025 μmol phosphatidylcholine) were incubated with unlabelled erythrocytes (300 μl) in a final volume of 0.95 ml. The data represent the [^{3}H]cholesterol appearing in the erythrocytes and are the means of values derived from triplicate incubation mixtures. Panel A: comparison of dipalmitoylphosphatidylcholine- (○) and egg phosphatidylcholine- (●) enriched vesicles. Panel B: comparison of dilinoleoylphosphatidylcholine- (▽) and egg phosphatidylcholine- (●) enriched vesicles.

The vesicles adhering to the cells were measured by the inclusion of the nontransferable marker, cholesterol [^{14}C]stearate, into the vesicles and the data given in Fig. 3 are corrected for this factor. The ^{14}C dpm indicated that the amount of vesicles isolated with the red cell pellet during the centrifugations did not exceed 10% of the total at any time (6% at $t = 20$ h with egg phosphatidylcholine-enriched and 10% at $t = 20$ h with dipalmitoylphosphatidylcholine-enriched vesicles).

Panel B of Fig. 3 represents similar experiments using dilinoleoylphosphatidylcholine- and egg phosphatidylcholine-enriched vesicles. In this case, the difference between the efflux rates was less marked than in the previous experiment, but the rate is nonetheless significantly slower from the dilinoleoylphosphatidylcholine-enriched vesicles compared to the egg phosphatidylcholine-enriched controls. The data are corrected for vesicles adherent to the erythrocyte pellet, which represented 4.5 and 6.5% of the total for dilinoleoylphosphatidylcholine- and egg phosphatidylcholine-enriched, respectively, after 21.5 h of incubation.

Influence of membrane components on cholesterol exchangeability

The results of these experiments provide evidence that the molecular species composition of phosphatidylcholine in erythrocytes or vesicles of erythrocyte lipid can modify the rate of efflux of cholesterol from the membrane. In each case, the efflux of cholesterol decreased as the membrane phosphatidylcholine was made more unsaturated with dilinoleoylphosphatidylcholine and increased when the dipalmitoylphosphatidylcholine content was enhanced. Thus, qualitatively, the rate of cholesterol efflux varied inversely with membrane phosphatidylcholine unsaturation in this experimental system. This is the opposite trend to that previously observed with vesicles of pure phos-

phatidylcholine species and cholesterol [5,6]. This discrepancy strongly suggests that other lipid components of the cellular membrane, or the mixture of phosphatidylcholines, play a role in determining the nature of the influence that a given species of phosphatidylcholine will have over the exchange of cholesterol.

The trends observed in the present experiments suggest that specific cholesterol-phosphatidylcholine interactions do not determine the rate of sterol desorption from the membrane. Previous experiments [21] have demonstrated that cholesterol interacts strongly with stearoyloleoylphosphatidylcholine to reduce the permeability of liposomes of that species. In the same experiments, the permeability of liposomes of dipalmitoylphosphatidylcholine below the transition temperature (41°C) or dilinoleoylphosphatidylcholine was not reduced by the addition of cholesterol [21], implying that the interaction between the sterol and these phosphatidylcholine species is not as strong. Clearly, if the desorption of cholesterol from the bilayer were governed by similar interactions with phosphatidylcholine, then the efflux from palmitoyloleoylphosphatidylcholine- or egg phosphatidylcholine-enriched cells would be expected to be slower than from dipalmitoylphosphatidylcholine- or dilinoleoylphosphatidylcholine-enriched membranes.

Interactions of cholesterol with membrane components other than phosphatidylcholine may explain this observation as well as the apparent differences in the influence of phosphatidylcholine structure observed in the present experiments and those of previous workers [5,6,21]. Wattenberg and Silbert [22] have provided evidence that the cholesterol content of phospholipid bilayer membranes is influenced by the sphingomyelin content, a concept that was previously suggested by Patton [23]. Strong cholesterol-sphingomyelin interactions are implied in the work of Cullis and Hope [24] and have been directly measured by differential scanning calorimetry by Demel et al. [25]. Sphingomyelin is known to be present in a roughly equimolar proportion with phosphatidylcholine in the outer monolayer of the human erythrocyte membrane [26] and since cholesterol desorption necessarily occurs from this monolayer, any interaction bteween cholesterol and sphingomyelin would be expected to influence the exchange process as well as the level of sterol in the membrane.

A model consistent with the current data and not dependent upon specific cholesterol-phosphatidylcholine interactions postulates a strong association between cholesterol and sphingomyelin that may be competitively displaced by phosphatidylcholine-sphingomyelin interactions. According to this model, dipalmitoylphosphatidylcholine may be expected to hydrophobically interact strongly with sphingomyelin by virtue of its fully saturated acyl chains, thus displacing cholesterol and increasing the rate of exchange.

We have previously suggested a strong hydrophobic interaction between dipalmitoylphosphatidylcholine and sphingomyelin to explain the slow rate of exchange of the saturated phosphatidylcholine from the erythrocyte membrane [15]. Evidence has previously been presented [22] that such an interaction also occurs in other cell types. We cannot, however, rule out that the influence of membrane phosphatidylcholine composition on cholesterol exchange is not mediated through changes in acyl chain packing or mobility within the membrane, arising as a result of the specific molecular shape of the phosphatidylcholine species introduced in these experiments.

We conclude that the rate of cholesterol efflux from human erythrocytes can be influenced by modest changes to the membrane phosphatidylcholine composition. The close correspondence between results obtained with whole cells and protein-free vesicles of erythrocyte lipid show that the effect is mediated by the lipid component of the membrane. The results suggest, however, that phosphatidylcholine does not exert the only influence over cholesterol desorption, and the possibility that this phospholipid does act in concert with others within the membrane to govern cholesterol exchange must be considered.

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