Cholesteryl ester transfer from phospholipid vesicles to human high density lipoproteins

Patricia M. Young and Peter Brecher

Departments of Biochemistry and Medicine, Boston University School of Medicine, Boston, MA 02118

Abstract The exchange of cholesteryl esters between different lipoproteins was reported to be mediated by a protein present in human plasma. In this study we have examined the movement of cholesteryl ester from unilamellar phospholipid vesicles to high density lipoprotein (HDL). Experimental conditions were established so that vesicles containing egg yolk lecithin and cholesteryl oleate (molar ratio of 86:1) could be incubated with human HDL so that neither disruption of particles nor transfer of lipid occurred. Addition of human lipoprotein-deficient plasma to the system promoted the transfer of cholesteryl oleate, but not phospholipid, from vesicles to HDL. Cholesteryl oleate transfer was dependent upon amount of HDL or lipoprotein-deficient plasma added and occurred when either HDL_2 or HDL_3 were present. Addition of unesterified cholesterol to the vesicles did not influence cholesteryl ester transfer to HDL. When phospholipid vesicles containing both cholesteryl oleate and triolein (molar ratio 86:1:1) were incubated with HDL and lipoprotein-deficient plasma, only cholesteryl oleate was transferred from the vesicles to HDL. Lipoprotein-deficient plasma derived from rabbits promoted the selective transfer of cholesteryl oleate from these vesicles, but rat plasma did not cause any movement of cholesteryl oleate or triolein from vesicles to HDL. HDL containing labeled cholesteryl esters was prepared and incubated with vesicles containing unlabeled cholesteryl esters or phospholipid alone. Addition of lipoprotein-deficient plasma did not promote transfer of cholesteryl esters from HDL to vesicles, whereas transfer from HDL to low density lipoprotein was readily observed. The results indicated that a protein present in rabbit and human plasma is effective in the selective, unidirectional transport of cholesteryl esters from a phospholipid bilayer to a plasma lipoprotein.—**Young, P. M., and P. Brecher.** Cholesteryl ester transfer from phospholipid vesicles to human high density lipoproteins. *J. Lipid Res.* 1981. **22:** 944–954.

Supplementary key words cholesteryl oleate · lipoprotein-deficient plasma · cholesteryl ester transfer protein

Cholesteryl ester is a component of all the major lipoprotein classes and is localized predominantly within the hydrophobic core of these particles (1). It has been recognized for some time that, in man, cholesteryl ester can move between different lipoproteins (2, 3), but only recently has it been established that specific substances, present in human plasma, can mediate either the exchange or net transfer of lipoprotein-associated cholesteryl ester. A cholesteryl ester exchange protein has been described both in rabbit and human plasma that was not associated with lipoproteins following ultracentrifugation of plasma at d 1.25 g/ml, and the activity in this lipoprotein-free plasma was shown to catalyze cholesteryl ester exchange between different lipoprotein classes (4–11). On the basis of kinetic studies on cholesteryl ester exchange between LDL and HDL using lipoprotein-deficient plasma as the source of exchange activity, it was proposed that an exchange protein had a greater affinity for HDL than for LDL (8). An exchange protein from human plasma was purified and found to be a glycoprotein that facilitated exchange between all combinations of high density, low density, and very low density lipoproteins (12). This purified protein did associate specifically with HDL, but not LDL or VLDL, to form an isolable complex and also associated with unilamellar lecithin vesicles (13). Isolation of a cholesteryl ester transfer protein from human serum, called apoprotein D, also was reported (14) and was shown to catalyze the net transfer of cholesteryl ester from HDL to VLDL or LDL. This transfer was accompanied by a reciprocal back-transport of triglyceride from VLDL or LDL to HDL. In a subsequent study (15), this transfer protein was shown to exist in plasma in complexes with other apoproteins and the enzyme lecithin:cholesterol acyltransferase, (LCAT). Most recently the isolated transfer protein was shown to facilitate the transfer of cholesteryl ester between phospholipid vesicles of different chemical composition (16).

The precise mechanism involved in either cholesteryl ester transfer or exchange remain unre-

Abbreviations: HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein; DTNB, 5,5′-dithiobis (2-nitrobenzoic acid); NaN_3, sodium azide; LCAT, lecithin: cholesterol acyl transferase.

solved. In the present study we have described the properties of an in vitro system containing phospholipid vesicles, HDL, and human lipoprotein-deficient plasma where cholesteryl ester transfer from unilamellar lecithin vesicles to HDL can be demonstrated.

EXPERIMENTAL PROCEDURES

Materials

Cholesteryl [1-^{14}C]oleate (50 mCi/mmol), [7-^{3}H]-cholesterol (11 Ci/mmol), cholesteryl [9,10-^{3}H]oleate (8.1 Ci/mmol), tri [1-^{14}C]oleoylglycerol (52 mCi/mmol), tri [9,10-^{3}H]oleoylglycerol (150 Ci/mmole), and [1-^{14}C]dipalmitoyl phosphatidylcholine (98 mCi/mmol) were obtained from New England Nuclear Corp. The [9,10-^{3}H]dipalmitoyl phosphatidylcholine was purchased from Applied Science, Inc. Egg phosphatidylcholine (Grade I) was obtained from Lipid Products (Surrey, United Kingdom). Cholesterol, cholesteryl oleate, trioleoylglycerol, and oleic acid were purchased from Applied Science, Inc. Sepharose 4B was obtained from Pharmacia, Inc., and 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) was from Aldrich Chemical Corp.

Preparation of phospholipid vesicles

Most vesicle preparations were prepared essentially as described previously (17). The designated amounts of labeled and unlabeled lipids were dissolved in chloroform–methanol (2:1), evaporated under a stream of N_2, and then lyophilized. The dried lipids were resuspended in 6 ml of a solution containing 0.1 M NaCl, 0.01 M Tris, pH 7.4, and 0.02% NaN_3. The suspension was sonicated for 10–12 min in a N_2 atmosphere at 22°–25°C using Heat Systems Sonifier (W-350) equipped with a standard 0.5 inch horn at a power setting of 3. The resulting solution was centrifuged at 191,000 *g* for 1 hr in Beckman 50 Ti rotor at 4°C and the upper 75% of the solution was removed and used for subsequent studies. All vesicle preparations were prepared using egg yolk lecithin (assumed mol wt of 787) such that the final phospholipid concentration was 12.7 mM, and the double-labeled preparations containing egg lecithin and cholesteryl oleate had a molar ratio of lecithin to cholesteryl oleate of 86:1. For these preparations, double-labeled vesicles contained either 0.33 μCi/ml of [^{14}C]lecithin and 1.67 μCi/ml of [^{3}H]cholesteryl oleate as markers, or 1.67 μCi/ml of [^{3}H]lecithin and 0.33 μCi/ml of cholesteryl[1-^{14}C]oleate. For the preparation of vesicles containing egg lecithin–cholesterol–cholesteryl oleate (molar ratio 100:50:1.2) sonication was performed for 3 hr using a 50% duty cycle under a stream of humidified N_2. These vesicles contained 0.33 μCi/ml of [^{14}C]cholesteryl oleate and 0.97 μCi/ml of [^{3}H]cholesterol.

Isolation of lipoproteins and lipoprotein-deficient plasma

HDL (d 1.063–1.21 g/ml) was isolated by sequential preparative ultracentrifugation as described previously (17). Following centrifugation of plasma at d 1.063 g/ml for 24 hr at 290,000 *g* in a Beckman 60 Ti rotor at 13°C, the infranatant was adjusted to d 1.21 g/ml and recentrifuged for 48 hr at 290,000 *g*. The HDL and the lower half of the infranatant (d > 1.21 g/ml) were collected separately and dialyzed against a solution containing 0.195 M NaCl, 1 mM EDTA, pH 7.5. The dialyzed infranatant is referred to as lipoprotein-deficient plasma. The protein concentration of the fraction ranged between 58–63 mg/ml. The ratio of total cholesterol to protein for the HDL used in these studies averaged 0.65 for three separate preparations. In selected experiments plasma was ultracentrifuged sequentially to obtain HDL_2 (d 1.063–1.125 g/ml) and HDL_3 (d 1.125–1.21 g/ml). The total cholesterol:protein ratio for HDL_2 and HDL_3 was 0.7 and 0.42, respectively.

Incubation conditions

Aliquots of the HDL and lipoprotein-deficient plasma samples were incubated with the phospholipid vesicle preparation in stoppered 13 × 100 mm test tubes. The total incubation volume was adjusted to 0.6 ml using a solution containing 0.1 M sodium phosphate, pH 7.4, 0.01% EDTA, and 0.02% NaN_3. Following incubation, samples were applied immediately to Sepharose 4B columns. Under standardized conditions, the reaction mixtures contained HDL (100–110 μg of protein), lipoprotein-deficient plasma (4.5 mg of protein), and double-labeled vesicles (2 mg of lecithin and 20 μg of cholesteryl oleate) in 0.6 ml, and incubation was performed at 37°C for 16 hr in a metabolic shaker.

Sepharose gel filtration

Sepharose 4B columns pre-equilibrated with 0.1 M NaCl, 0.01 M Tris, pH 7.4, and 0.02% NaN_3 were used to characterize the reaction mixtures. Samples were applied to the columns and eluted by ascending chromatography with the pre-equilibration buffer at flow rates of 20–25 ml/hr. Several columns were used in these studies, with dimensions of 2.6 × 26–30 cm. All column chromatography was performed at ambient temperatures (22°–25°C). Eluted fractions were analyzed for radioactivity using Liquiscint (Na-

tional Diagnostics) as the scintillation cocktail. Double isotope counting conditions were established such that ^{14}C was counted at 65% efficiency with no crossover of ^{3}H into the ^{14}C channel. Tritium was counted at 27% efficiency with a 14% crossover of ^{14}C into the ^{3}H channel. Data were expressed as dpm per ml of the eluted fraction after correcting for crossover.

Analytical procedures

Protein was determined by the method of Lowry et al. (18) using bovine serum albumin as a standard. Lipid phosphorus was measured by the method of Bartlett (19), using a factor of 25 to estimate phospholipid. Cholesterol was determined either by the method of Rudel and Morris (20) or by our modification (21) of the densitometric procedure of Katz, Shipley, and Small (22).

Lipid extracts were obtained by the method of Folch, Lees, and Sloane Stanley (23). Thin-layer chromatography was performed on silica gel G 20 × 20 cm plates (Applied Science, Inc.). Individual phospholipids were separated using chloroform–methanol–water 65:25:4 (v/v) as a developing solvent. Neutral lipid classes were resolved using hexane–diethyl ether–acetic acid 70:30:1 (v/v) as a developing solvent.

In certain experiments where it was necessary to distinguish between labeled unesterified and esterified cholesterol in the column eluates (Fig. 5), 3-ml aliquots of the fractions were lyophilized, then resuspended in 5 ml of chloroform–methanol 2:1 and lipid extracts obtained by the procedure of Folch et al. (23). These lipid extracts were subjected to thin-layer chromatography (hexane–diethyl ether–acetic acid 70:30:1) and the regions corresponding to standard cholesterol and cholesteryl oleate were scraped directly into scintillation vials for determination of radioactivity.

Preparation of ^{3}H-labeled HDL

Human HDL was labeled with [7-^{3}H]cholesterol using a modification of the procedures of Nilsson and Zilversmit (24). Human plasma was collected in 0.1% EDTA and 0.02% NaN_3, and 5 ml was incubated with 75 μCi of [7-^{3}H]cholesterol which was absorbed onto a 1-cm^2 piece of Whatman #1 filter paper. Following incubation at 37°C for 48 hr, the plasma was treated by sequential preparative ultracentrifugation as described above, but using a Beckman 50 Ti rotor at 190,000 *g*. The HDL fraction obtained was dialyzed against 0.195 M NaCl, 1 mM EDTA, and 0.02% NaN_3. Chemical and isotopic analyses of three such preparations indicated 65–75% of the total radioactivity was associated with cholesteryl ester, and the specific activity for unesterified and esterified cholesterol ranged between 4–5.5 × 10^4 dpm/μg of cholesterol and 2.8–3.6 × 10^3 dpm/μg of esterified cholesterol.

RESULTS

Experimental conditions were established where phospholipid vesicles containing cholesteryl oleate

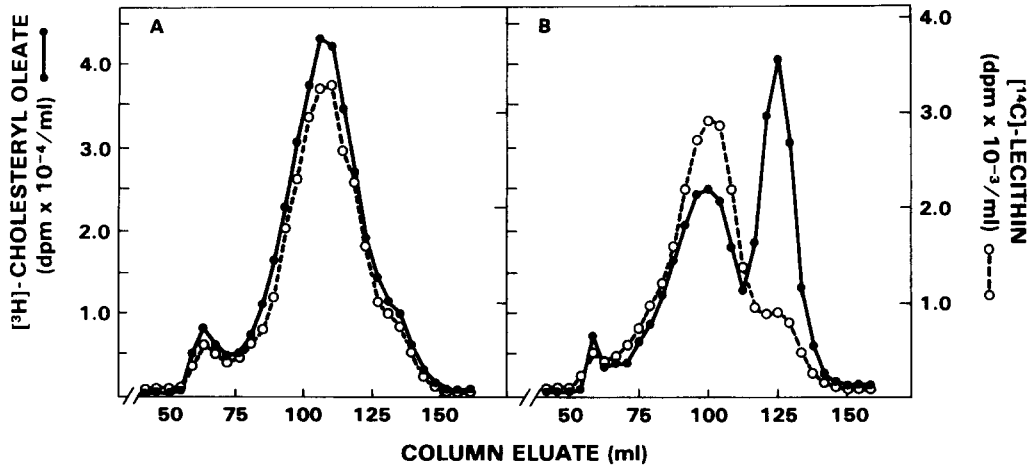

Fig. 1. Effect of lipoprotein-deficient plasma on the transfer of cholesterol [9,10-^{3}H]oleate from [^{14}C]lecithin vesicles to HDL. Double-labeled egg lecithin vesicles (2 mg phospholipid) containing [^{14}C]-lecithin and [^{3}H]cholesteryl oleate (molar ratio 86:1) were incubated with human HDL (105 μg protein, 30–40 μg cholesteryl ester) in a total volume of 0.6 ml at 37°C for 16 hr in the absence (Fig. 1A) or presence (Fig. 1B) of lipoprotein-deficient plasma (4.5 mg protein). Following incubation, a 550-μl aliquot of the reaction mixtures was applied to a Sepharose 4B column (2.6 × 30 cm) and the eluted fractions were analyzed for radioactivity (see Experimental Procedures).

could be incubated with a small amount of human HDL so that neither disruption of vesicles nor transfer of cholesteryl oleate occurred following prolonged incubation. **Fig. 1A** shows the Sepharose 4B elution profile of a reaction mixture containing double-labeled phospholipid vesicles (3.33 mg phospholipid/ml, ^{14}C-labeled phospholipid 0.3 μCi/ml, cholesteryl [^{3}H]oleate 1.5 μCi/ml) after incubation at 37°C for 16 hr with HDL (175 μg protein/ml). Over 95% of the labeled phospholipid and cholesteryl oleate eluted as a single peak corresponding in size to unilamellar vesicles. A smaller peak appeared at the void volume, and a shoulder in the region where HDL normally eluted also was observed in most experiments, but in all fractions both labeled lipids coeluted proportionately. In contrast, when lipoprotein-deficient plasma (7.5 mg protein/ml) was included in the incubation solution, about 40% of the labeled cholesteryl oleate appeared in the region where HDL eluted whereas only a slight change in the phospholipid elution pattern was observed, (Fig. 1B).

Cholesteryl oleate transfer from the phospholipid vesicles to HDL was dependent upon the amount of HDL added. **Fig. 2** shows results of experiments where increasing amounts of HDL were incubated in a reaction mixture containing a fixed amount of double-labeled vesicles and lipoprotein-deficient plasma. The elution patterns of labeled cholesteryl oleate (Fig. 2A) show that without HDL, no transfer occurred and increasing amounts of HDL produced increasing transfer of cholesteryl oleate to the HDL region. As shown in Fig. 2B, most of the labeled phospholipid eluted as vesicles when the amount of HDL added was 175 μg/ml or less, but if an excess of HDL was added (1.3 mg protein/ml), disruption of the vesicles occurred as indicated by appearance of the labeled phospholipid in the HDL fraction. This disruption of phospholipid vesicles by excess HDL also occurred in the absence of lipoprotein-deficient plasma, and chemical analysis of phospholipid in the eluted fractions showed the labeled phospholipid was an accurate measure of total mass (data not shown), consistent with our previous studies (17). Thus, the data show that in the presence of an appropriate amount of HDL, (175 μg protein/ml), a substance in lipoprotein-deficient plasma promoted the selective transfer of cholesteryl oleate from phospholipid vesicles to HDL.

Using standardized incubation conditions (lecithin vesicles, 3.3 mg phospholipid/ml), HDL (175 μg protein/ml) and lipoprotein-deficient plasma (7.5 mg protein/ml), 9 ml of such a reaction mixture was incubated at 37°C for 16 hr. Following incubation the reaction mixture was adjusted to d 1.063 g/ml and

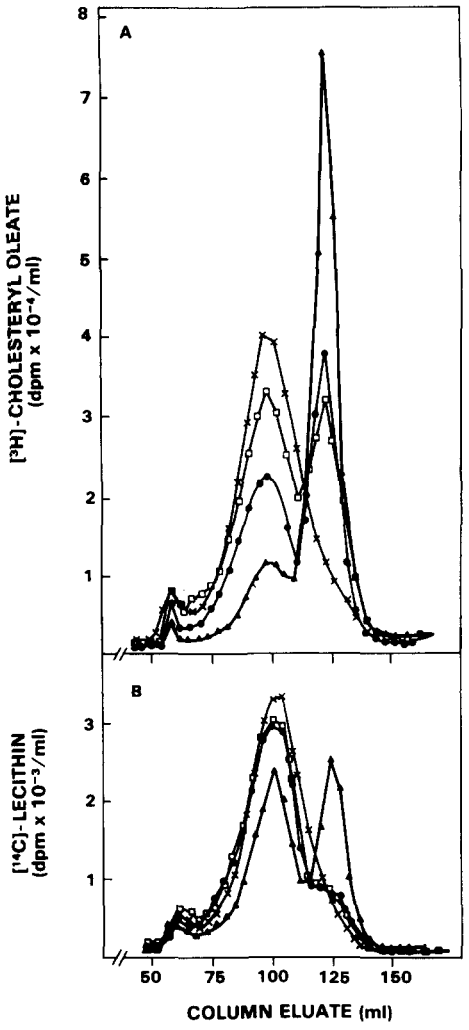

Fig. 2. Sepharose 4B elution profiles of double-labeled [^{14}C]-lecithin vesicles containing cholesteryl[9,10-^{3}H]oleate following incubation with lipoprotein-deficient plasma and varying amounts of HDL. Reactions were performed under standardized conditions (see Experimental Procedures) and contained double-labeled vesicles (3.33 mg phospholipid/ml), lipoprotein-deficient plasma (7.5 mg protein/ml), and the designated amount of HDL. Following incubation at 37°C for 16 hr, a 550-μl aliquot of the reaction mixture was analyzed by Sepharose 4B chromatography (see Experimental Procedures). Fig. 2A, Elution profiles of cholesteryl-[9,10-^{3}H]oleate. Fig. 2B, Elution profiles of [^{14}C]lecithin. HDL added (protein/ml): none (x —— x), 83 μg/ml (□ —— □), 175 μg/ml (● —— ●), 1300 μg/ml (▲ —— ▲).

ultracentrifuged at 48,000 rpm for 24 hr at 5°C in a Beckman 50 Ti rotor. The resulting supernatant and infranatant were collected and aliquots were analyzed by Sepharose 4B chromatography. **Fig. 3** shows the elution profiles of the samples and confirms that the radioactivity associated with the vesicle region had a density less than 1.063 g/ml, whereas the labeled cholesteryl oleate transferred to the HDL region had a density greater than 1.063 g/ml. Separate experiments established that the radioactivity in the d > 1.063 g/ml fraction floated at d

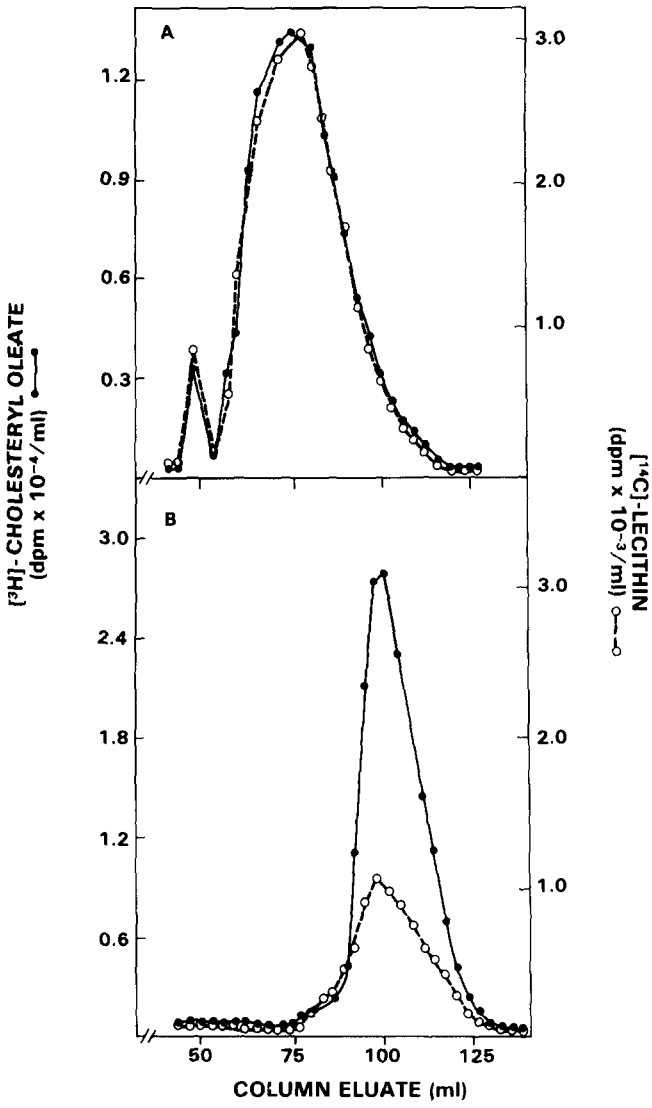

Fig. 3. Sepharose 4B elution profiles of the d < 1.063 g/ml and d > 1.063 g/ml fractions obtained from a reaction mixture containing double-labeled vesicles, HDL, and lipoprotein-deficient plasma. Nine ml of a reaction mixture including double-labeled [^{14}C]lecithin vesicles (3.33 mg phospholipid/ml) containing cholesteryl[9, 10-^{3}H]oleate, HDL (175 μg protein/ml), and lipoprotein-deficient plasma (7.5 mg/ml) were incubated for 16 hr at 37°C and an 8.4-ml aliquot was adjusted to d 1.063 g/ml using solid KBr. The reaction mixture was centrifuged at 45,000 rpm for 18 hr at 4°C using a Beckman 50 Ti rotor. Following ultracentrifugation, an aliquot of the opalescent material at the top of the centrifuge tube (d < 1.063 g/ml) was analyzed by Sepharose 4B chromatography (Fig. 3A). Fig. 3B is the Sepharose elution profile of a 0.5-ml aliquot of the fraction obtained from the lower half of the centrifuge tube (d > 1.063 g/ml).

1.21 g/ml and again chromatographed as HDL on Sepharose 4B (data not shown). If the original incubation was performed in the absence of lipoprotein-deficient plasma, the elution profile of the d < 1.063 g/ml fraction was similar to that of the original vesicle preparation (see Fig. 1A), whereas in the d > 1.063 g/ml fraction, no labeled cholesteryl oleate was associated with the HDL region.

Table 1 lists the percentage of vesicle-associated, labeled cholesteryl oleate transferred to HDL under various experimental conditions. The elution pattern shown in Fig. 1A, showing essentially no transfer, was representative of results obtained when vesicles were incubated at 37°C for 16 hr with buffer only; lipoprotein-deficient plasma (7.5 mg/ml) or HDL (165 μg/ml) only; or HDL plus bovine serum albumin (7.5 mg/ml). Selective transfer of labeled cholesteryl oleate, similar to that shown in Fig. 1B, was observed routinely under standardized incubation conditions, averaging 45% transfer of the labeled cholesteryl ester present originally in the vesicles. Decreasing the amount of lipoprotein-deficient plasma added caused a proportional decrease in the transfer of cholesteryl oleate, and a 5-fold increase in the amount of plasma normally added produced a small but definite increase in transfer (45% to 60%). Transfer was dependent upon incubation time since less transfer was observed at 2 hr than at 8 hr, but prolonged incubation for up to 43 hr had little additional ef-

TABLE 1. Effect of different conditions on the transfer of cholesteryl oleate from vesicles to HDL

Substances Added	Labeled Cholesteryl Oleate Transferred
	% of total
Vesicles	
No addition	<2
+ Lipoprotein-deficient plasma (7.5 mg/ml)	<2
Vesicles + HDL (165 μg/ml)	<2
+ Bovine serum albumin[a] (7.5 mg/ml)	<2
+ Lipoprotein-deficient plasma (7.5 mg/ml)	45 ± 3.4[b]
+ Lipoprotein-deficient plasma (3.75 mg/ml)	28
+ Lipoprotein-deficient plasma (37.5 mg/ml)	60
Vesicles + HDL (165 μg/ml) + lipoprotein-deficient plasma (7.5 mg/ml)	
37°C/2 hr	18
37°C/8 hr	32
37°C/43 hr	52
+ 1.6 mM DTNB	45
+ 0.25 M $CaCl_2$	40
+ Heat inactivated plasma (56°C/1 hr, 7.5 mg/ml	42
Vesicles + lipoprotein-deficient plasma (7.5 mg/ml)	
+ HDL_2 (193 μg/ml)	40
+ HDL_3 (175 μg/ml)	44

[a] Essentially fatty acid-free, fraction V, purchased from Sigma Chemical Co.

[b] Mean ± standard deviation for eight separate determinations.

All incubations included double-labeled lecithin vesicles (3.3 mg phospholipid/ml) containing both egg lecithin and cholesteryl oleate. Unless specified in the table, incubations were for 16 hr at 37°C and aliquots were then applied to a Sepharose 4B column for estimation of cholesteryl oleate transfer to HDL. The values shown are the average of at least two separate experiments.

fect. Cholesteryl oleate transfer was not affected by treatments known to inhibit lecithin:cholesterol acyltransferase (addition of DTNB or heat-inactivation of the plasma) nor was transfer affected by addition of 0.25 M $CaCl_2$, which was reported to reduce the interaction between purified cholesteryl ester transfer protein and egg yolk lecithin vesicles (13). $CaCl_2$ did cause about 3-fold more labeled phospholipid and cholesteryl ester to elute in the void volume. Cholesteryl ester transfer also occurred in the presence of the major subfractions of HDL, HDL_2 (d 1.063–1.125 g/ml) and HDL_3 (d 1.125–1.21 g/ml). If the amount of HDL_3 added was increased over the concentration listed in Table 1, disruption of the vesicles accompanied the transfer at higher concentrations. As was the case with total HDL, the disruption of vesicles could be shown by chemical or isotopic analysis of the phospholipid and lipoprotein-deficient plasma was not required to effect the disruption (data not shown).

Chemical and isotopic techniques were employed to determine if hydrolysis of either cholesteryl oleate or lecithin occurred during incubation of vesicles with HDL or lipoprotein-deficient plasma. Following incubation under standardized conditions, aliquots of the reaction mixture were extracted (23) and the lipid extract was subjected to thin-layer chromatography using either hexane–diethyl ether–acetic acid (70:30:1 (v/v) or chloroform–methanol–water 65:25:4 as developing solvents. Analysis of the chromatograms (see methods) showed no evidence for the presence of labeled free fatty acid or lysolecithin, and virtually all the radioactivity present on the plate co-migrated with standard cholesteryl oleate or egg yolk lecithin. In additional experiments, aliquots of incubation mixtures or fractions obtained from Sepharose 4B columns were treated in a solvent partition system (25) designed to separate fatty acid from cholesteryl ester or phospholipid, and no labeled free fatty acid was detected. These experiments showed that cholesterol esterase or phospholipase activity towards the vesicles was not present under standardized conditions.

Lecithin vesicles were prepared containing both labeled cholesterol and cholesteryl oleate at molar ratios of 100:50:1.2, lecithin–cholesterol–cholesteryl oleate. The elution profile of this preparation of Sepharose 4B is shown in **Fig. 4A.** The vesicles eluted as larger particles than egg lecithin vesicles lacking cholesterol and more material appeared at the void volume, yet the relative amounts of phospholipid, cholesterol, and cholesteryl oleate were similar in all fractions.

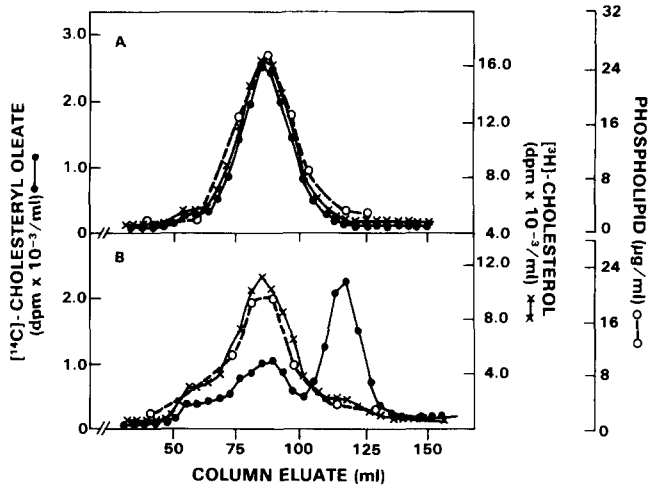

Fig. 4. Effect of lipoprotein-deficient plasma on the transfer of cholesteryl oleate from lecithin vesicles containing unesterified cholesterol (molar ratio 100:50:1.2, lecithin–cholesterol–cholesteryl oleate). Lecithin vesicles (3.33 mg phospholipid ml) containing cholesteryl[1-^{14}C]oleate and [7-^{3}H]cholesterol (see Experimental Procedures) were incubated with HDL (175 µg/ml) at 37°C for 16 hr in the absence (Fig. 4A) or presence (Fig. 4B) of lipoprotein-deficient plasma (7.5 mg). Aliquots (550 µl) of each reaction mixture were applied to Sepharose 4B (2.6 × 30 cm) columns.

When these vesicles were incubated at 37°C for 16 hr in the presence of either bovine serum albumin (7.5 mg/ml) or albumin plus HDL (175 µg/ml), no change in the elution profile shown in Fig. 4A was observed; however, incubation with HDL plus lipoprotein-deficient plasma caused selective transfer of cholesteryl oleate into the HDL region (Fig. 4B). Although most of the cholesterol remained associated with phospholipid vesicles, a small amount was present in the HDL region both with or without addition of plasma. Since there was a large difference in the amount of cholesterol originally associated with vesicles (800 µg/reaction tube) and that in the HDL (4.5 µg/reaction tube), no attempt was made to determine if transfer or exchange processes for cholesterol were involved. Results identical to those shown in Fig. 4A and 4B were obtained when the reaction mixture contained 1.4 mM DTNB.

To determine if a substance present in the lipoprotein-deficient plasma could promote the transfer of cholesteryl ester from HDL to vesicles, HDL was prepared from plasma pre-equilibrated with [7-^{3}H]cholesterol (see Experimental Procedures), so that 7-^{3}H-labeled cholesteryl esters would be present in HDL. The tritiated HDL, containing label both in free and esterified cholesterol, was then incubated with vesicles containing cholesteryl oleate, both without (**Fig. 5A** and 5C) or with (Fig. 5B and 5D) the addition of lipoprotein-deficient plasma. All incubations contained

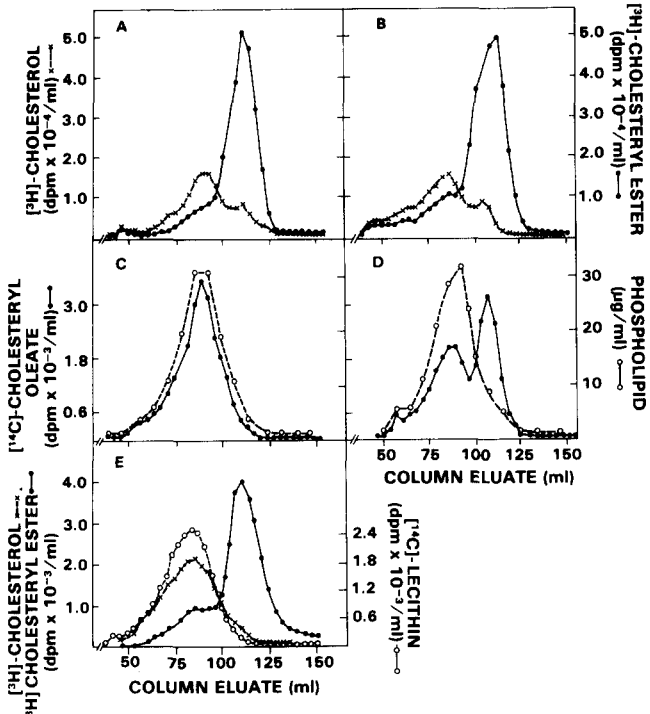

Fig. 5. Sepharose 4B elution profile of reaction mixtures containing ^{3}H-labeled HDL and phospholipid vesicles. ^{3}H-labeled HDL was prepared as described in Experimental Procedures and incubated at 37°C for 16 hr with phospholipid vesicles as follows: Fig. 5A, Reaction mixtures contained: ^{3}H-labeled HDL (175 μg protein/ml), unlabeled lecithin vesicles containing unlabeled cholesteryl oleate (molar ratio 86.1, 3.3 mg phospholipid/ml), and 1.4 mM DTNB. A 550-μl aliquot was applied to a Sepharose 4B column (2.6 × 26 cm); Fig. 5B, Experimental conditions were similar to those of Fig. 5A except lipoprotein-deficient plasma (7.5 mg protein/ml) was included in the reaction mixture. Fig. 5C, ^{3}H-labeled HDL (175 μg protein/ml) was incubated with phospholipid vesicles containing cholesteryl-[1-^{14}C]oleate (molar ratio 86:1, 3.3 mg phospholipid/ml) and 1.4 mM DTNB. Fig. 5D, Conditions similar to that of Fig. 5C except lipoprotein-deficient plasma (7.5 mg protein/ml) was included in the reaction mixture. Fig. 5E. ^{3}H-labeled HDL (65 μg/ml) was incubated with [^{14}C]lecithin vesicles (3.3 mg phospholipid/ml) but containing no cholesteryl oleate in the presence of 1.4 mM DTNB.

1.4 mM DTNB to minimize LCAT activity. The Sepharose elution profiles of these incubations are shown in Fig. 5A–5D, expressing the distribution of [^{3}H]cholesterol, and ^{3}H-labeled cholesteryl ester derived from HDL in Fig. 5A and 5B and the [^{14}C]-cholesteryl oleate derived from the vesicles in Fig. 5C and 5D. Phospholipid, which was determined chemically also, is plotted in Fig. 5C and 5D.

As seen in Fig. 5A, following incubation with vesicles, more than 90% of the [7-^{3}H]cholesterol (unesterified) was associated with the vesicle region, whereas approximately 80% of the [7-^{3}H]cholesteryl ester remained in the HDL region with only about 15% eluting into the vesicle region. Lipoprotein-deficient plasma did not alter the distribution of either [7-^{3}H]cholesterol or [7-^{3}H]cholesteryl ester between HDL and vesicles (Fig. 5B). In contrast, cholesteryl [1-^{14}C]oleate originating in vesicles was selectively transferred to HDL only when lipoprotein-deficient plasma was contained in the reaction mixture (Fig. 5C versus 5D). In additional experiments, an aliquot of a reaction mixture containing cholesteryl [1-^{14}C]-oleate-labeled lecithin vesicles, ^{3}H-labeled HDL, and lipoprotein-deficient plasma was subjected to differential density ultracentrifugation and the HDL fraction (d 1.063–1.21 g/ml) contained both cholesteryl [1-^{14}C]oleate and [7-^{3}H]cholesteryl ester which co-chromatographed on Sepharose 4B columns in the HDL region (data not shown).

Although the movement of unesterified [^{3}H]cholesterol from HDL to vesicles clearly did not represent true exchange, (since cholesterol was not present in the original vesicles), it was possible that the shift of approximately 15% of [^{3}H]cholesteryl ester into the vesicle region was mediated by an exchange process, perhaps by a protein associated with HDL. To determine if the movement of [7-^{3}H]cholesteryl ester from HDL to vesicles was due to an exchange process, the ^{3}H-labeled HDL was incubated with vesicles containing no cholesteryl oleate, but containing [^{14}C]lecithin as a marker. Following incubation at 37°C for 16 hr in the absence of lipoprotein-deficient plasma, the reaction was chromatographed on Sepharose 4B (Fig. 5E). Again, most of the [7-^{3}H]-cholesterol was associated with the vesicles and about 15% of the [7-^{3}H]cholesteryl ester also moved from HDL to vesicles. Results similar to those shown in Fig. 5E were obtained if lipoprotein-deficient plasma was included in the original reaction mixture (data not shown).

To determine if a substance in lipoprotein-deficient plasma could influence the movement of ^{3}H-labeled cholesteryl esters from HDL to another lipoprotein particle, ^{3}H-labeled HDL was incubated with low density lipoprotein (LDL) in the absence or presence of lipoprotein-deficient plasma. As shown in **Fig. 6,** in the absence of the plasma virtually all the [^{3}H]-cholesterol (free and esterified) eluted as a single peak in the HDL region. In contrast, if lipoprotein-deficient plasma was included in the reaction mixture, almost 75% of the total labeled cholesterol was associated with the region corresponding to where LDL normally eluted.

To determine if triacylglycerol could be transferred from phospholipid vesicles to HDL, vesicles containing egg yolk lecithin, cholesteryl [1-^{14}C]oleate, and tri[9,10-^{3}H]oleoylglycerol in a molar ratio of 86:1:1, respectively, were incubated with HDL under standardized conditions. **Fig. 7** shows the Sepharose

TABLE 2. Species specificity for the transfer of both cholesteryl oleate and triolein from vesicles to human HDL by lipoprotein-deficient plasma

Plasma Source	Cholesteryl Ester Transfer	Triacylglycerol Transfer
	% of total	
Human	45	0
Rabbit	56	0
Rat	0	0

Human HDL (175 μg protein/ml) was incubated with lecithin vesicles containing both cholesteryl [1-^{14}C]oleate and [^{3}H]triolein (molar ratio 86:1:1, 3.3 mg phospholipid/ml) in the presence of lipoprotein-deficient plasma (7.5 mg/ml) derived from human, rabbit, and rat. Following incubation at 37°C for 16 hr, 550-μl aliquots were applied to a Sepharose 4B column (2.6 × 26 cm). The lipoprotein-deficient plasma samples were obtained from freshly collected plasma of male Sprague-Dawley rats (300–350 g), New Zealand white rabbits (2.5 kg), or human volunteers. The plasma was adjusted to d 1.21 g/ml and centrifuged at 190,000 *g* for 48 hr in a Beckman 50 Ti rotor. The resulting infranatant was dialyzed extensively against 0.195 M NaCl, 1 mM EDTA, and 0.02% NaN_3, pH 7.4. Data are the average of three separate determinations using different plasma preparations.

4B elution profiles of the reaction mixtures following incubation in the absence (Fig. 7A) or presence (Fig. 7B) of lipoprotein-deficient plasma. Both labeled neutral lipids co-eluted in the region corresponding to that of unilamellar vesicles when plasma was omitted from the reaction mixture (Fig. 7A), indicating uniform incorporation into the bilayer. In the presence of lipoprotein-deficient plasma, cholesteryl oleate transfer to HDL occurred whereas the labeled triolein remained associated with the vesicles. The inability of a factor in lipoprotein-deficient plasma to mediate transfer of triolein from vesicles to HDL was confirmed in studies employing vesicles containing ^{14}C-labeled lecithin and [^{3}H]triolein (molar ratio 86:1) in which both labeled moieties co-eluted in the vesicle region following incubation with lipoprotein-deficient plasma (data not shown). In all the experiments with labeled triolein no evidence for hydrolysis was obtained using the thin-layer chromatographic techniques or the solvent partition procedure described previously.

The species specificity for cholesteryl ester transfer from vesicles to HDL was tested by incubating vesicles containing both labeled cholesteryl oleate and triolein with human HDL in the presence of lipoprotein-deficient plasma from the rat, rabbit, and human. As shown in **Table 2,** rabbit plasma contained transfer activity comparable to that of the human since significant transfer of cholesteryl oleate but not triolein occurred, whereas the lipoprotein-deficient plasma from rat contained no activity toward either cholesteryl oleate or triolein.

DISCUSSION

These studies have established the utility of lecithin vesicles as an artificial substrate for in vitro studies related to cholesteryl ester transfer or exchange. We have characterized an experimental system where cholesteryl ester is transferred from a lecithin bilayer to a particle with the size and density normally associated with the HDL particle population. Cholesteryl ester contained in lecithin vesicles obviously is accessible to a transfer protein, as demonstrated by our studies and in a recent report by Chajek, Aron, and Fielding (16) which showed that cholesteryl ester formed in cholesterol-containing dioleoyllecithin vesicles by the action of LCAT, in the presence of apoA-1, could be subsequently transferred to sphingomyelin-cholesterol vesicles, which were immobilized on Sepharose beads, provided apoD was added to the reaction mixture. In an earlier study, Rose (26) had also shown that lecithin-cholesterol vesicles, if pretreated with partially purified LCAT to form esterified cholesterol and subsequently added to human plasma, would result in the distribution of labeled cholesteryl esters among different lipoprotein classes.

Our studies have focused on the movement of cholesteryl ester from vesicles to HDL and included careful characterization of the vesicle preparations

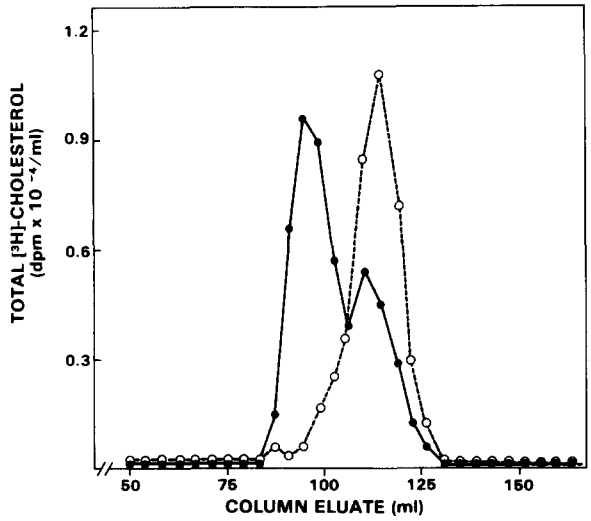

Fig. 6. Effect of lipoprotein-deficient plasma on the interaction between ^{3}H-labeled HDL and unlabeled LDL. Reaction mixture containing ^{3}H-labeled HDL (175 μg protein/ml; 58 μg total cholesterol/ml), unlabeled LDL (180 μg protein/ml, 110 μg total cholesterol/ml), and 1.4 mM DTNB were incubated at 37°C for 16 hr in the absence (○ —— ○) and the presence (● —— ●) of lipoprotein-deficient plasma (7.5 mg protein/ml). Aliquots (550 μl) of the reaction mixture were applied to Sepharose 4B columns (2.6 × 26 cm).

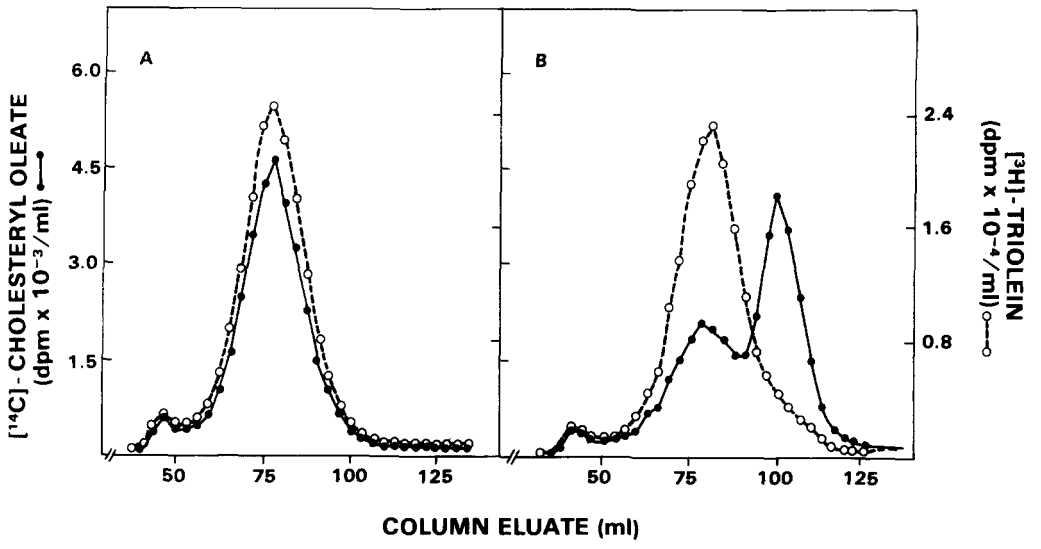

Fig. 7. Selective transfer to HDL of cholesteryl oleate from vesicles containing both labeled cholesteryl oleate and triolein. HDL (175 μg protein/ml) was incubated with lecithin vesicles containing both cholesteryl[1-^{14}C]oleate and [^{3}H]triolein (molar ratio 86:1:1, 3.3 mg/phospholipid/ml) in the absence (Fig. 7A) or presence (Fig. 7B) of lipoprotein-deficient plasma (7.5 mg protein/ml). Following incubation at 37°C for 16 hr, 550-μl aliquots were applied to a Sepharose 4B column (2.6 × 26 cm).

after incubations were performed. We were able to demonstrate the importance of the relative amounts of vesicles in the reaction mixture so that transfer could be demonstrated and vesicle disruption avoided. In our standard assay system the concentrations and relative amount of HDL and lipoprotein-deficient plasma were similar to that expected in normal human plasma diluted about 8-fold.

The experiments where ^{3}H-labeled HDL was used, summarized in Fig. 5, indicated that movement of cholesteryl ester from HDL to vesicles occurred to a limited extent, but was not dependent on a substance present in lipoprotein-deficient plasma, nor did the process require cholesteryl ester to be originally incorporated into vesicles. Although our data do not provide an explanation for the movement of cholesteryl ester from HDL to the region where vesicles normally elute, this process occurred even in the absence of plasma and may result from the formation of larger, less dense, cholesteryl ester-rich particles from a subpopulation of HDL rendered unstable during the incubation with a relative excess of phospholipid vesicles. Fusion of HDL particles has been described to occur under analogous, but not identical, conditions (27). In contrast, the transfer of cholesteryl ester from vesicles to HDL was dependent completely on the presence of plasma; it occurred using the major subfractions of HDL, HDL_2, or HDL_3, and most likely can occur independent of any apparent back-transfer. Since it is possible that the labeled HDL preparation was altered during the in vitro preincubation required to form labeled cholesteryl esters, we demonstrated that the labeled HDL preparation still retained the ability to accept labeled cholesteryl oleate from vesicles, provided that lipoprotein-deficient plasma was included in the reaction mixture. Furthermore, the ability of the tritiated cholesteryl ester in HDL to undergo transfer or exchange to LDL was established by the experiment shown in Fig. 6. Thus, it would appear that transfer proteins in plasma have specificity toward the acceptor particle as well as for the donor. Simultaneous transfer of cholesteryl ester and phospholipid from HDL to LDL in the presence of a purified protein from human plasma was described recently (28). In the present study, movement of phospholipid from vesicles to HDL occurred, but this effect was seen both in the absence and presence of lipoprotein-deficient plasma, in marked contrast to the selective movement of cholesteryl oleate, which clearly required the presence of lipoprotein-deficient plasma.

When triolein was incorporated into lecithin vesicles, no evidence for triglyceride transfer was obtained. This was shown clearly in the experiments where both labeled triolein and cholesteryl oleate were incorporated into vesicles and only the cholesteryl ester was transferred to HDL. Other studies have shown that a substance in rabbit or human lipoprotein-free plasma could promote the exchange of triolein between rabbit HDL and LDL or VLDL (29), and earlier work demonstrated a reciprocal transfer of cholesteryl esters for glycerides in human serum (3).

Using a partially purified cholesteryl ester transfer protein (apoD) from human serum, Chajek et al. (14) showed a reciprocal and equimolar back-transport of triglyceride to HDL when cholesteryl ester was transferred from HDL to VLDL or LDL. The inability of lipoprotein-free human plasma to catalyze transfer of triolein in vesicles to HDL may be related to the physical state of triolein in vesicles, where it could be distributed within the bilayer in a position not readily accessible to specific transfer or exchange proteins. Alternatively, the data may reflect the specificity of a transfer protein in a unidirectional transfer process.

The position of either cholesteryl oleate or triolein within a phospholipid bilayer has not been established. Valie et al. (30) have used deuterium magnetic resonance to determine the conformational states of cholesteryl palmitate in egg lecithin liposomes and have suggested that two forms of "dissolved" cholesteryl palmitate exist: a homogeneous fraction at relative concentrations below 0.2 mol % and crystalline patches at higher concentrations. This is in contrast to studies by Janiak et al. (31) showing that a homogeneous phase of cholesteryl linoleate in egg lecithin is present at relative concentrations up to 3.6 mol %. Chajek et al. (16) have shown that cholesterol:lecithin vesicles containing greater than 3.0 weight % cholesteryl ester were no longer an effective substrate for LCAT, and that vesicle-associated cholesteryl ester, originating from the preparation procedure or incorporated by enzymatic action, was equally available for transfer to another vesicle population. None of the above studies provides definitive data on the conformation of cholesteryl ester within a unilamellar vesicle, thus it is not clear what the required physical properties of the vesicle substrate are to permit interaction with a plasma transfer or exchange protein and to effect transfer to a suitable acceptor.

Several reports have shown that cholesterol:lecithin vesicles prepared at molar ratios exceeding 0.4 cause changes in several properties of the lipid bilayer including hydrodynamic properties (32), accessibility to cholesterol oxidase (33), and stability in the presence of serum (34). Our studies show that cholesterol-containing vesicles (0.5 molar ratio) did not influence cholesteryl ester transfer to HDL, yet the vesicles were larger than unilamellar vesicles lacking cholesterol, and the small amount of cholesteryl oleate incorporated was evenly distributed throughout the fairly homogeneous vesicle population. Interestingly, we were unable to find a previous report illustrating the gel elution profile of cholesterol: phospholipid vesicles at high molar ratios.

Cholesteryl esters are known to have a limited solubility within phospholipid bilayers (31), but it is likely that cholesteryl ester formation occurs within a bilayer, both in plasma through the action of LCAT, or intracellularly by acyl CoA:cholesterol acyltransferase that is associated with microsomal fractions (35). It is possible that newly formed cholesteryl esters exist transiently within phospholipid bilayers of discoidal particles in plasma or intracellular membranes, and that transfer proteins remove cholesteryl esters from these bilayers in a unidirectional manner analogous to the process described in these studies.

This work was supported by United States Public Health Service Grants HL 12869 and HL 25157 and the U. A. Whitaker Fund.

Manuscript received 20 January 1981 and in revised form 24 April 1981.

REFERENCES

1. Havel, R. J., J. L. Goldstein, and M. J. Brown. 1980. Lipoproteins and lipid transport. *In* Metabolic Control and Disease. P. K. Bondy and L. E. Rosenberg, editors. W. B. Saunders, Philadelphia. 393–494.
2. Rehnborg, C. S., and A. V. Nichols. 1964. The fate of cholesteryl esters in human serum incubated in vitro at 38°. *Biochim. Biophys. Acta.* **84:** 596–603.
3. Nichols, A. V., and L. Smith. 1965. Effect of very low density lipoproteins on lipid transfer in incubated serum. *J. Lipid Res.* **6:** 206–210.
4. Zilversmit, D. B., L. B. Hughes, and J. Baker. 1975. Stimulation of cholesteryl ester exchange by lipoprotein-free rabbit plasma. *Biochim. Biophys. Acta.* **409:** 393–398.
5. Barter, P. J., and J. I. Lally. 1978. The activity of an esterified cholesterol transferring factor in human and rat serum. *Biochim. Biophys. Acta.* **531:** 233–236.
6. Barter, P. J., and J. I. Lally. 1978. Metabolism of esterified cholesterol in the plasma very low density lipoproteins of the rabbit. *Atherosclerosis.* **31:** 355–364.
7. Barter, P. J., and J. I. Lally. 1979. The metabolism of esterified cholesterol in rabbit plasma low density lipoproteins. *Biochim. Biophys. Acta.* **572:** 510–518.
8. Barter, P. J., and M. E. Jones. 1980. Kinetic studies of the transfer of esterified cholesterol between human low and high density lipoproteins. *J. Lipid Res.* **21:** 238–249.
9. Barter, P. J., and J. I. Lally. 1979. In vitro exchanges of esterified cholesterol between serum lipoprotein fractions: studies of humans and rabbits. *Metabolism.* **28:** 230–236.
10. Marcel, Y. L., C. Vezina, B. Teng, and A. Sniderman. 1980. Transfer of cholesteryl esters between human high density lipoproteins and triglyceride-rich lipoproteins controlled by a plasma protein factor. *Atherosclerosis.* **35:** 127–133.
11. Sniderman, A., B. Teng, C. Vezina, and Y. L. Marcel. 1978. Cholesteryl ester exchange between human plasma high and low density lipoproteins mediated by a plasma protein factor. *Atherosclerosis.* **31:** 327–333.

12. Pattnaik, N. M., A. M. Montes, L. B. Hughes, and D. B. Zilversmit. 1978. Cholesteryl ester exchange protein in human plasma isolation and characterization. *Biochim. Biophys. Acta.* **530:** 428–438.
13. Pattnaik, N. M., and D. B. Zilversmit. 1979. Interaction of cholesteryl ester exchange protein with human plasma lipoproteins and phospholipid vesicles. *J. Biol. Chem.* **254:** 2782–2786.
14. Chajek, J., and C. J. Fielding. 1978. Isolation and characterization of a human serum cholesteryl ester transfer protein. *Proc. Natl. Acad. Sci. USA.* **75:** 3445–3449.
15. Fielding, P. E., and C. J. Fielding. 1980. A cholesteryl ester transfer complex in human plasma. *Proc. Natl. Acad. Sci. USA.* **77:** 3327–3330.
16. Chajek, T., L. Aron, and C. J. Fielding. 1980. Interaction of lecithin:cholesterol acyltransferase and cholesteryl ester transfer protein in the transport of cholesteryl ester into sphingomyelin liposomes. *Biochemistry.* **19:** 3673–3677.
17. Chobanian, A. V., A. R. Tall, P. I. Brecher. 1979. Interaction between unilamellar egg yolk lecithin vesicles and human high density lipoproteins. *Biochemistry.* **18:** 180–187.
18. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein determination with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
19. Bartlett, G. R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* **234:** 466–468.
20. Rudel, L. L., and M. D. Morris. 1973. Determination of cholesterol using o-phthalaldehyde. *J. Lipid Res.* **14:** 364–366.
21. Chan, C. T., P. Brecher, C. Haudenschild, and A. V. Chobanian. 1979. The effect of cholesterol feeding on the metabolism of rabbit cerebral microvessels. *Microvasc. Res.* **18:** 353–369.
22. Katz, S. S., G. G. Shipley, and D. M. Small. 1976. Physical chemistry of the lipids of human atherosclerotic lesions. *J. Clin. Invest.* **58:** 200–211.
23. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226:** 497–509.
24. Nilsson, A., and D. B. Zilversmit. 1972. Fate of intravenously administered particulate and lipoprotein cholesterol in the rat. *J. Lipid Res.* **13:** 32–37.
25. Pittman, R. C., J. C. Khow, and D. Steinberg. 1975. Cholesterol esterase in rat adipose tissue and its activation by cyclic adenosine 3′:5′-monophosphate-dependent protein kinase. *J. Biol. Chem.* **260:** 4505–4511.
26. Rose, H. G. 1978. Transfer and equilibration of LCAT-generated cholesteryl esters in human lipoproteins. *Scand J. Clin. Lab. Invest.* **38:** 91–97.
27. Tall, A. R., V. Hogan, L. Askinazi, and D. M. Small. 1978. Interaction of plasma high density lipoproteins with dimyristoyllecithin multilamellar liposomes. *Biochemistry.* **17:** 322–326.
28. Ihm, J., J. A. K. Harmony, J. Ellsworth, and R. L. Jackson. 1980. Simultaneous transfer of cholesteryl ester and phospholipid by protein(s) isolated from lipoprotein-free plasma. *Biochem. Biophys. Res. Commun.* **93:** 1114–1120.
29. Barter, P. J., J. M. Gooden, and O. V. Rajaran. 1979. Species differences in the activity of a serum triglyceride transferring factor. *Atherosclerosis.* **33:** 165–169.
30. Valie, M. I., H. Gorrissen, R. J. Cushley, and M. Bloom. 1979. Deuterium magnetic resonance study of cholesteryl esters in membranes. *Biochemistry.* **18:** 654–659.
31. Janiak, M. J., C. R. Loomis, G. G. Shipley, and D. M. Small. 1974. The ternary phase diagram of lecithin cholesteryl linoleate and water: phase behavior and structure. *J. Mol. Biol.* **86:** 325–339.
32. Huang, C., J. Sipe, S. T. Chow, and R. B. Martin. 1974. Differential interactions of cholesterol with phosphatidylcholine on the inner and outer surfaces of lipid bilayer vesicles. *Proc. Natl. Acad. Sci. USA.* **71:** 359–362.
33. Pal, R., V. Barenholtz, and R. R. Wagner. 1980. Effect of cholesterol concentration on organization of viral and vesicle membranes. *J. Biol. Chem.* **255:** 5802–5806.
34. Kirby, C., J. Clarke, and G. Gregoriadis. 1980. Cholesterol content of small unilamellar liposomes controls phospholipid loss to high density lipoproteins in the presence of serum. *Biochem. J.* **186:** 591–598.
35. Spector, A. A., S. N. Mathur, and T. L. Kaduce. 1979. Role of acyl coenzyme A: cholesterol O-acyltransferase in cholesterol metabolism. *Prog. Lipid Res.* **18:** 31–53.