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Selective Transfer of Cholesteryl Ester over Triglyceride by Human Plasma Lipid Transfer Protein between Apolipoprotein-Activated Lipid Microemulsions†

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ABSTRACT: The substrate-specific rate of the human plasma lipid transfer protein (LTP) reaction was studied using pyrene-labeled substrate lipid analogues as probes for various lipids, by monitoring the ratio of the fluorescence intensities of their excimers to those of their monomers as an indicator of pyrene concentration in the microenvironment. Transfer of cholesteryl ester (CE) and triglyceride (TG) was demonstrated between human high-density lipoproteins, between low-density lipoproteins, and between these two lipoproteins, and the specific fractional transfer rate of CE was always higher than that of TG by a factor of 2.4–7.9. On the other hand, the transfer by LTP of CE, TG, and phosphatidylcholine (PC) was also demonstrated between lipid microemulsions having an average diameter of 25–26 nm using the same probes, but only when the emulsions were activated by apolipoproteins A-I, A-II, E, or C-III. The maximally activated rates of the transfer of CE and TG were the same when measured between the emulsions with cores composed exclusively of either lipid. The specific fractional transfer rate of pyrene-CE, however, was inversely proportional to the percentage of CE in the TG core of the emulsions, and the initial transfer of TG was almost completely inhibited by the presence of small percentages of CE in the TG core. Thus, the transfer of CE between the emulsions is highly selective over that of TG by orders of magnitude, much more selective than the reaction between any natural plasma lipoproteins, but this selectivity is not a rate-limiting step of the overall LTP reaction. The maximally activated LTP-catalyzed transfer rate of PC between the emulsions was somewhat higher than that of CE or TG and was not affected by the composition of the core lipids of the emulsion, TG or CE. When an excess amount of LTP was incubated with emulsion containing a small percentage of pyrene-CE in the TG core in the absence of the acceptor particles, excimer fluorescence rapidly decreased to the base line, and this change was suppressed when pyrene-CE was diluted with CE in the core. This result may indicate that LTP selectively disrupts pyrene-CE excimer formation on the basis of its selective interaction with the CE molecule over TG in the emulsion system as a putative background mechanism for the selective transfer of CE.

Transfer of lipids among plasma lipoproteins plays an important role in lipoprotein metabolism by redistributing lipids among lipoproteins (Tall, 1986). The reaction is catalyzed by specific plasma proteins except for the transfer of unesterified cholesterol. Two different lipid-transfer proteins have been identified in human plasma. One is known as human plasma lipid transfer protein [LTP;[1] also named as lipid transfer protein I (LTP-I) or cholesteryl ester transfer protein (CETP)] (Morton & Zilversmit, 1982; Ohnishi et al., 1990), and the other is phospholipid transfer protein [or human plasma lipid transfer protein II (LTP-II)] (Tall et al., 1983; Tollefson et al., 1988). While phospholipid transfer protein catalyzes only phospholipid transfer between plasma lipoproteins, LTP (LTP-I or CETP) catalyzes the transfer of cholesteryl ester, triglyceride, and phospholipid. This low substrate specificity seems to lead to a heteroexchange of the lipids between lipoproteins by LTP (Albers et al., 1984). Cholesteryl ester is newly synthesized mainly on high-density plasma lipoprotein (HDL), and therefore its heteroexchange with other lipids should cause a continuous net transfer of cholesteryl ester from HDL. However, the core of low-density plasma lipoprotein (LDL) is almost entirely cholesteryl ester, so that lipid transfer between LDL and HDL by LTP is mostly the exchange of cholesteryl esters and causes no net lipid transfer. Therefore, the net transfer of cholesteryl ester from HDL should occur mainly in triglyceride-rich lipoproteins such as very low density plasma lipoprotein (VLDL), chylomicrons, and their remnants as a result of the exchange with triglyceride. It is not clear, though, to what extent such a net transfer is indeed caused by the LTP reaction in plasma *in vivo*. It also should depend upon the relative selectivity of the LTP reaction for cholesteryl ester and triglyceride, if there is any. Thus, understanding the mechanism for regulation of substrate specificity of the LTP reaction is an important key to understanding the physiological role LTP plays in cholesterol transport by human plasma lipoproteins.

Many reports suggest that LTP transfers a wide variety of lipids (Morton & Zilversmit, 1982, 1983; Ohnishi et al., 1990; Tall et al., 1983; Albers et al., 1984; Ihm et al., 1982; Abbey et al., 1984, 1985; Ogawa & Fielding, 1985; Nishikawa et al.,

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[1] Abbreviations: LTP, plasma lipid transfer protein; CETP, plasma cholesteryl ester transfer protein; HDL, high-density plasma lipoprotein; VLDL and LDL, very low density and low-density plasma lipoprotein; apo, apolipoprotein; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; pyrene–cholesteryl ester, cholesteryl 1-pyrenehexanoate; pyrene–triglyceride, *rac*-1,2-dioleoyl-3-(1-pyrenedecanoyl)glycerol; pyrene–phosphatidylcholine, β-(1-pyrenedecanoyl)-γ-palmitoylphosphatidylcholine; BSA, bovine serum albumin; CE, cholesteryl ester; TG, triglyceride; PC, phosphatidylcholine.

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1988). Morton and Zilversmit (1983) reported that the lipid transfer by LTP between different classes of lipoprotein was competitive between cholesteryl ester and triglyceride, and selective for the former over the latter by a factor of 1.6–3.2. However, the transfer of phosphatidylcholine was relatively independent of these two lipids with the same rate as that for cholesteryl ester. Ihm et al. (1982) and Tall et al. (1983) also showed that the transfer rates of phosphatidylcholine and cholesteryl ester by LTP from HDL to LDL were the same. Abbey et al. (1984, 1985), however, reported that the transfer of cholesteryl ester by LTP was 5–7-fold faster than that of phosphatidylcholine or triglyceride. Thus, this previous work seems to agree that LTP reaction tends to have selectivity for cholesteryl ester over triglyceride to some extent, but is controversial for the rate of phospholipid transfer. Interestingly, LTP-catalyzed triglyceride transfer from phosphatidylcholine vesicles to LDL seemed much slower than cholesteryl ester transfer in the same system, indicating some additional regulatory factor(s) for the substrate specificity of LTP (Morton & Steinbrunner, 1990). In order to approach this problem, we applied our lipid transfer assay system with pyrene-labeled substrate lipid analogues (Milner et al., 1991; Ohnishi & Yokoyama, 1993).

Pyrene compounds form excited-state dimers (excimers) at a high local concentration giving a fluorescence emission peak around 470 nm (excimer fluorescence) and another fluorescence emission peak of the compounds below 420 nm which originates from the lowest excited single state (monomer fluorescence). The ratio of the excimer to the monomer fluorescence intensities is known to be a function of the concentration of pyrene compound in the microenvironment (Föster, 1969; Pownall & Smith, 1973; Charton et al., 1976), so that pyrene compounds have been used as probes to measure lipid-transfer reactions among lipid vesicles or lipoproteins by monitoring their local concentration (Roseman & Thompson, 1980; Massey et al., 1982; Pownall & Smith, 1989). The method has the advantages of avoiding separation of the donor and acceptor particles, a continuous monitoring of the reaction, and no involvement of apolipoproteins in the baseline condition. In previous studies, we successfully applied this method to the study of the LTP reaction and demonstrated that the presence of apolipoproteins on the surface of the microemulsion is essential for cholesteryl ester transfer reactions catalyzed by LTP (Milner et al., 1991; Ohnishi & Yokoyama, 1993).

In the present paper, we report the results of further study of the LTP reaction extending the same assay system to other lipids, with human plasma lipoproteins and with lipid microemulsions. These substrate analogues were shown to be good probes for determining lipid transfer between plasma lipoproteins, demonstrating preference for cholesteryl ester over triglyceride. Between microemulsions, LTP required activation by apolipoproteins for the transfer of all triglyceride, cholesteryl ester, and phospholipid. The reaction demonstrated almost exclusive selectivity for cholesteryl ester over triglyceride, in sharp contrast to the reaction between natural lipoproteins where the selectivity was only modest.

EXPERIMENTAL PROCEDURES

Chemicals. Cholesteryl 1-pyrenehexanoate (pyrene–cholesteryl ester), *rac*-1,2-dioleoyl-3-(1-pyrenedecanoyl)glycerol (pyrene–triglyceride), β-(1-pyrenedecanoyl)-γ-palmitoylphosphatidylcholine (pyrene–phosphatidylcholine), cholesteryl oleate, and triolein were purchased from Sigma. Egg yolk phosphatidylcholine was obtained from Avanti Polar-Lipids, Inc. Other chemicals used in the present study were of the highest quality commercially available.

Preparation of Lipoproteins, Apolipoproteins, and LTP. LDL and HDL were isolated from fresh human plasma by sequential ultracentrifugation and a subsequent washing procedure as densities of 1.006–1.063 and 1.063–1.21 g/mL, respectively, and dialyzed against 10 mM sodium phosphate buffer (pH 7.4) containing 0.15 M NaCl and 0.1 mM EDTA (PBS). The purity was verified by electrophoresis in 0.5% agarose gel. Human apolipoprotein A-I (apoA-I) and apoA-II were purified from the HDL fraction of fresh human plasma according to methods described elsewhere (Yokoyama et al., 1982; Tajima et al., 1983). ApoC-III and apoE were isolated from the human VLDL fraction as described previously (Tajima et al., 1983; Yokoyama et al., 1985). Each apolipoprotein showed a single band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and was lyophilized and stored at −75 °C before use. An aqueous solution of each apolipoprotein was prepared as previously described (Yokoyama et al., 1982, 1985; Tajima et al., 1983), and the concentration of each was determined on the basis of its absorbance at 280 nm using its respective specific molar extinction coefficient (Tajima et al., 1983; Yokoyama et al., 1985). LTP was purified from human plasma according to the method previously published by Ohnishi et al. (1990). The purified protein consisted of 69- and 66-kDa LTP, both of which had the same specific activity of cholesteryl ester transfer between LDL and HDL (Ohnishi et al., 1990). Judging from the staining density on SDS–PAGE, 90% of the particular LTP preparation used was 69-kDa protein and 10% was 66-kDa protein. The final LTP preparation, 1.4 mg/mL, was stored at −75 °C.

Preparation of Lipid Microemulsion. The previously described method (Ohnishi & Yokoyama, 1993) was slightly modified. Twenty milligrams of egg yolk phosphatidylcholine and 20 mg of core lipids (triolein and/or cholesteryl oleate) in benzene solution were placed in a sonication cup, and the solvent was completely evaporated under a stream of nitrogen. Fifteen milliliters of PBS was added to the cup, and the solution was sonicated for 30 min at 200 W in an on and off cycle of 3 and 1 min using a flat tip with a Heat Systems-Ultrasonics XL 2020 sonicator. When the cholesteryl ester-rich emulsions were prepared, sonication was prolonged to 70 min at the maximum power of the instrument (>250 W). The solution was kept exposed to a stream of nitrogen and cooled with circulated water during sonication. The emulsions formed were isolated as the top fraction after ultracentrifugation at 99 000 rpm for 30 min with a Beckman TL-100 ultracentrifuge and then fractionated by use of a Sepharose CL-4B gel permeation column (1.6 × 46 cm). Each 1-mL fraction was collected and analyzed for the lipid composition. To prepare the pyrene-donor microemulsion, 1 mg of pyrene-labeled lipid, either phosphatidylcholine, cholesteryl ester, or triglyceride, was premixed with other lipids. The microemulsions were prepared in the same manner as described above except that all procedures were conducted in darkness. In the elution profiles of various microemulsions from the column, the ratio of the sum of the core lipids to phosphatidylcholine continuously decreased as a function of the elution volume, indicating that the gel permeation mechanism functioned well for fractionation of the microemulsions on the basis of their size. The ratio of pyrene–cholesteryl ester or pyrene–triglyceride to the core lipid remained constant throughout the fractions, while the ratio of these pyrene–lipids to phosphatidylcholine or total lipid decreased as a function of the elution volume, demonstrating that both of the pyrene–lipids were exclusively distributed in the core of the microemulsion (Milner et al., 1991). The ratio of pyrene–phosphatidylcholine to the core

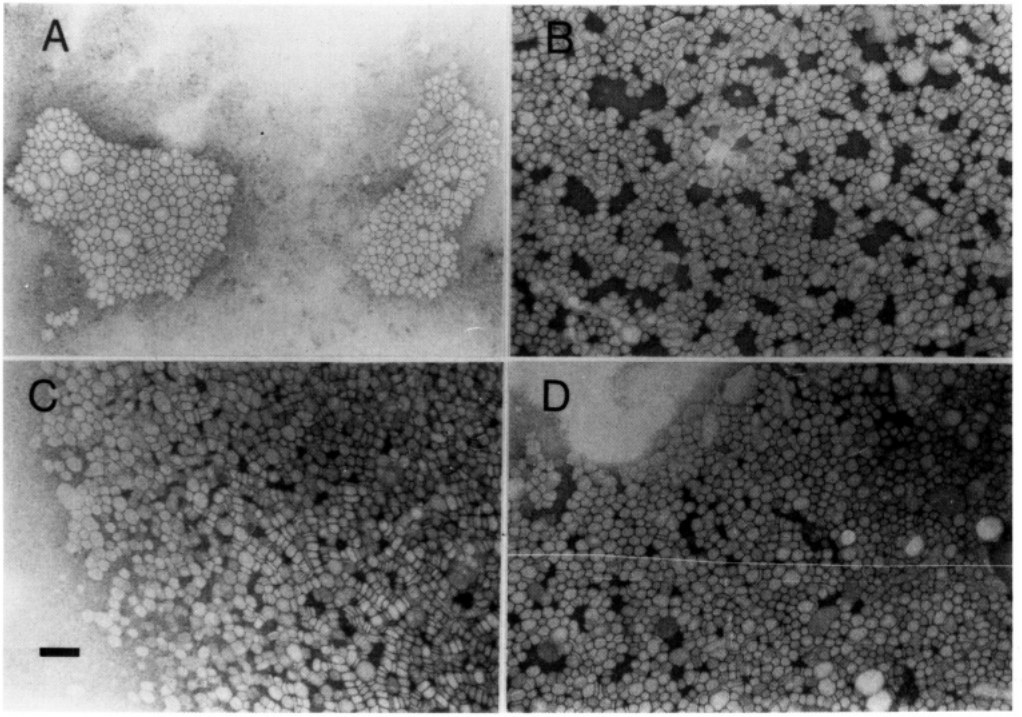

FIGURE 1: Electron micrograms of lipid microemulsions. The combined fraction 40–41 from the gel permeation column was analyzed by electron microscopy after negative staining of the samples with 1% phosphotungstate. Magnification is 63 600× (the scale bar indicates 100 nm): (A) phosphatidylcholine/triolein microemulsion; (B) phosphatidylcholine/triolein/cholesteryl oleate microemulsion (cholesteryl oleate accounted for 60.0 mol % of the total core lipids); (C) phosphatidylcholine/cholesteryl oleate; (D) phosphatidylcholine/triolein microemulsion with pyrene–cholesteryl ester, 5.3 mol % of the total core lipids.

lipid and that to the total lipid both increased as a function of the elusion volume, while the ratio of this pyrene–lipid to phosphatidylcholine remained constant throughout the fractions. Thus, pyrene–phosphatidylcholine is exclusively associated with surface phospholipid in the microemulsion. The weight ratio of the core lipid(s) (triglyceride and/or cholesteryl ester) to the surface lipid (phosphatidylcholine) was constantly around 1.2 in the inclusion peak fractions 40 and 41 regardless of the lipid composition. These fractions were combined and kept at 4 °C under argon gas and used for the experiments.

Electron micrograms of the emulsions were taken with a Hitachi H-7000 electron microscope at 75 kV after the samples were negatively stained with 1% phosphotungstate by Dr. Ming Chen, Surgical-Medical Research Institute, University of Alberta. Representative particles are shown in Figure 1. The average diameters (mean ± SD) of these particles were 24.8 ± 4.2 nm for the emulsions with 100% triolein core (Figure 1A), 26.1 ± 3.6 nm for those with triolein core containing 56.4 mol % cholesteryl ester (Figure 1B), 25.3 ± 3.5 nm for those with 100% cholesteryl oleate core (Figure 1C), and 25.3 ± 2.7 nm for those with triolein core containing 5.3 mol % pyrene–cholesteryl ester (Figure 1D). Contamination by particles with vesicular structure was minimal even in the cholesteryl ester-rich particle preparations.

Incorporation of Pyrene-Labeled Lipids into Plasma Lipoproteins. Lipoproteins labeled with pyrene–lipid were prepared according to the method described for incorporation of radiolabeled lipid into lipoproteins (Nishikawa et al., 1986; Francis et al., 1991). VLDL was removed from fresh human plasma by centrifugation at 100000*g* for 2 h in a Beckman TL-100 ultracentrifuge. The lipid microemulsion containing pyrene–cholesteryl ester was prepared by sonication of 5 mg of egg yolk phosphatidylcholine, 4 mg of triolein, and 1 mg of pyrene–cholesteryl ester in 15 mL of PBS for 36 min and subsequent ultracentrifugation at 120000*g* for 10 min to obtain the top fraction. The microemulsion containing pyrene–triglyceride was also prepared by sonication of 2 mg of egg phosphatidylcholine, 1 mg of triolein, and 1 mg of pyrene–triglyceride and subsequent ultracentrifugation under the same conditions. VLDL-free plasma samples of 1 and 2 mL were incubated with pyrene–cholesteryl ester and pyrene–triglyceride emulsions (the entire amount recovered), respectively, in the presence of 5,5′-dithiobis(2-nitrobenzoic acid), aprotinin, and gentamycin at 37 °C for 48 h (Nishikawa et al., 1986; Francis et al., 1991). LDL and HDL were isolated by ultracentrifugation as densities of 1.006–1.063 and 1.063–1.21 g/mL, respectively. Typical lipid compositions of labeled lipoproteins are listed in Table 1, showing that it is possible to incorporate pyrene–lipids into lipoproteins without introducing drastic changes in their chemical compositions.

Table 1: Lipid Weight Composition of Lipoproteins as a Percentage of Total Lipid after Incorporation of Pyrene-Labeled Lipid[a]

		PL	TG	FC	CE
LDL	unlabeled	25.7	8.4	11.9	54.0
	pyrene-CE	23.8	9.2	10.5	56.5
	pyrene-TG	24.1	10.1	10.0	55.9
HDL	unlabeled	48.4	9.8	5.7	36.1
	pyrene-CE	48.0	6.0	7.0	39.0
	pyrene-TG	48.6	6.9	7.5	37.0

[a] PL, choline–phospholipid; TG, triglyceride; FC, free cholesterol; CE, cholesteryl ester.

Measurement of Fluorescence. Fluorescence of pyrene compounds in the microemulsion solutions was measured using a Hitachi F-2000 fluorescence spectrophotometer (Ohnishi & Yokoyama, 1993) equipped with a thermostatic cuvette holder and a micromagnetic stirring unit. The pyrene-labeled lipids were excited at 320 nm, and the emission fluorescence of monomer and excimer pyrene was measured respectively at 396 and 468 nm for pyrene–cholesteryl ester, 395 and 468 nm for pyrene–triglyceride, and 396 and 472 nm for pyrene–phosphatidylcholine. The excitation light was kept off until 20 s before the measurement took place, and the fluorescence

was integrated for 5 s at each emission wavelength. The interval required for changing the emission wavelength was less than 2 s. The samples were kept under constant stirring at 37 °C during the measurement.

Measurement of Lipid-Transfer Reactions. The method was described in detail in our previous publication (Ohnishi & Yokoyama, 1993). The microemulsion or lipoprotein particles without pyrene-labeled lipids were used as acceptor particles. For plasma lipoproteins, pyrene-donor lipoproteins as the 0.96-μg core lipid were incubated with acceptor lipoproteins (8.64 μg of core lipid for LDL and 8.64 and 1.73 μg of core lipid for HDL). For microemulsions, 0.8 μg of phospholipid was used as the donor particles and 7.2 μg of phospholipid was used as the acceptor particles. The acceptor and apolipoproteins (4 μg of apolipoproteins or bovine serum albumin, when used) were first placed in the fluorescence microcuvette in a final volume of 0.36 mL, and the fluorescence of this solution was measured as a background. The pyrene-donor particles were then added to the acceptor solution in the microcuvette. After the mixture was left for 5 min at 37 °C for preincubation, the lipid-transfer reaction was initiated by adding human LTP. The final incubation volume for the reaction mixture was 0.4 mL. The fluorescence intensities of pyrene monomer and excimer were measured at various incubation times between 0 and 30 min as described above. The background fluorescence was subtracted at each wavelength from the fluorescence measured, and the ratio of the emission fluorescence of excimer pyrene to that of monomer pyrene was calculated as an indicator of the pyrene–lipid transfer from the donor to the acceptor lipid particles. The measurement was significantly improved with respect to accuracy and reproducibility by introducing a thermostatic cuvette holder with a micromagnetic stirring unit because of improved stability of the fluorescence.

Direct Interaction of Pyrene–Lipids with LTP. The microemulsion containing pyrene–cholesteryl ester (0.2 μg of phospholipid) was preincubated with 1 μg of apoA-I or bovine serum albumin in the microcuvette for 5 min. Then LTP was added to give a final volume of 0.4 mL, and the fluorescence was measured in the same manner as in the transfer-reaction assay described above in the absence of the acceptor particles. The fluorescence from the lipid microemulsion without pyrene–cholesteryl ester in the presence of apoA-I or bovine serum albumin was used as a background, which was less than 5% of the initial fluorescence of the microemulsion containing pyrene–cholesteryl ester.

Other Analytical Methods. Concentrations of lipids were determined using enzymatic assay kits for triacylglycerol, choline-containing phospholipid, and total cholesterol, purchased from Wako Pure Chemical (Richmond, Virginia). The concentration of pyrene-labeled lipids was determined on the basis of the absorbance at 342.4 nm for pyrene–cholesteryl ester, at 343 nm for pyrene–triglyceride, and at 342.6 nm for pyrene–phosphatidylcholine, using a molar extinction coefficient of 50 000 M^{-1} cm^{-1} according to an assumed analogy to pyrene (Pownall & Smith, 1973). The protein concentration of LTP was determined by a Pierce BCA protein assay kit using bovine serum albumin as a standard. SDS–PAGE was carried out according to the method of Laemmli (Laemmli, 1970), and proteins were visualized by staining the gel with Coomassie Brilliant Blue R-250.

Calculation of Lipid-Transfer Rate. We derived the empirical equation to calculate the rate of the transfer of lipids between the donor and acceptor particles from the change of the ratio of the emission fluorescence of excimer pyrene (Ex) to that of monomer pyrene (Mo) during the initial part

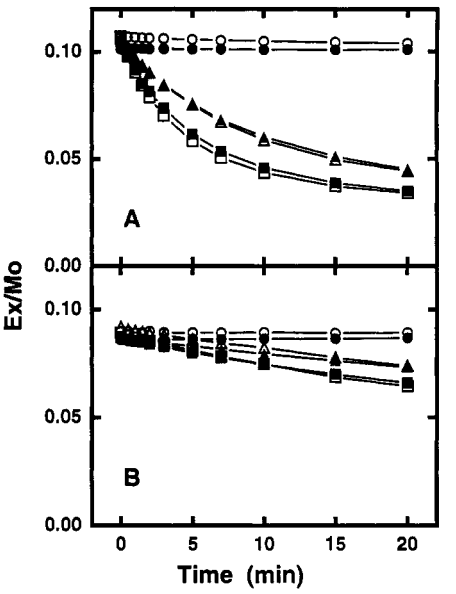

FIGURE 2: Pyrene–lipid transfer from HDL. Pyrene–lipid-labeled HDL (0.96 μg of the core lipid) was incubated with acceptor lipoproteins (8.64 μg of the core lipid) in the presence of LTP, and the Ex/Mo ratio was monitored by fluorescence measurements as described in Experimental Procedures. Panel A shows the transfer of cholesteryl ester, and panel B shows triglyceride transfer. Open symbols indicate the lipid transfer from HDL to LDL (○, △, □), and closed symbols, the transfer from HDL to HDL (●, ▲, ■), in the absence of LTP (○, ●), or with 47.1 nM LTP (△, ▲) or 94.2 nM LTP (□, ■).

(30%) of the reaction,

$$\ln[(\mathrm{Ex/Mo}) - b] = -2(k/L_1)t + \text{constant}$$

where b is the y-intercept of the linear regression of the calibration of Ex/Mo for the content of pyrene compound in the microemulsions (Milner et al., 1991; Ohnishi & Yokoyama, 1993). The value b was obtained by extrapolating Ex/Mo calibration against pyrene content in the core lipid to zero pyrene content for each experimental condition (Ohnishi & Yokoyama, 1993). The empirical rate constant k/L_1 is to be calculated as a slope of the plot of $\ln(\mathrm{Ex/Mo} - b)$ against the incubation time (t) by least-squares linear regression, where k is the average rate of the lipid transfer and L_1 is the pool size of substrate lipids in donor particles. In previous papers, we assumed that the rates of the transfer of cholesteryl ester and triglyceride by LTP differ by only a fewfold factor on the basis of the data for plasma lipoproteins published by other groups prior to our work (Tall et al., 1983; Morton & Zilversmit, 1983; Ihm et al., 1982; Abbey et al., 1984, 1985). Therefore, the data had been analyzed as if all the lipids had been in a single kinetic pool for the transfer in our previous work (Milner et al., 1991; Ohnishi & Yokoyama, 1993). However, as we are going to demonstrate in this study, this assumption is not exactly valid because LTP does not transfer various lipids equally, especially cholesteryl ester and triglyceride when both are available for the reaction in the emulsion system. Therefore, we must consider the value k/L_1 as a fractional transfer rate (an apparent first-order rate constant) of the lipid species that is specifically probed by a particular pyrene-labeled molecule.

RESULTS

Transfer of Core Lipids by LTP Between Plasma Lipoproteins. Figures 2 and 3 show the transfer of cholesteryl ester and triglyceride between lipoproteins using pyrene–lipid probes. Figure 2 represents the transfer of pyrene–cholesteryl

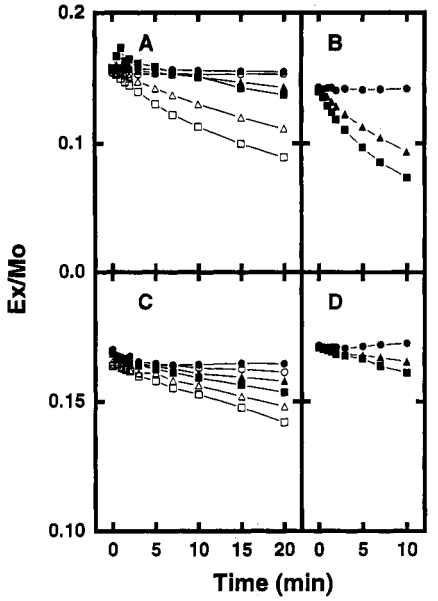

FIGURE 3: Pyrene–lipid transfer from LDL. Pyrene–lipid-labeled LDL (0.96 μg of the core lipid) was incubated with acceptor lipoproteins in the presence of LTP, and the Ex/Mo ratio was monitored by fluorescence measurements as described in Experimental Procedures. Panels A and B show cholesteryl ester transfer, and panels C and D show triglyceride transfer. Panel A demonstrates the transfer from LDL to LDL (acceptor core lipid, 8.64 μg) (○, △, □) and to HDL (acceptor core lipid, 8.64 μg) (●, ▲, ■), and panel B shows the transfer from LDL to a lesser amount of HDL (acceptor core lipid, 1.73 μg), in the absence (○, ●) or the presence of 47.1 nM LTP (△, ▲) or 94.2 nM LTP (□, ■), respectively. Conditions and symbols are the same for triglyceride transfer (panels C and D).

ester and pyrene–triglyceride from HDL to LDL and from HDL to HDL. Figure 3 demonstrates the transfer of these pyrene–lipids from LDL to LDL and from LDL to HDL. For the first time, it has been clearly demonstrated that both lipids are transferred between any combination of these lipoproteins, including between lipoproteins in the same subclass. The fractional transfer rate was very slow when LDL was the pyrene donor and HDL was the acceptor under standard assay conditions where the weight ratio of the core lipid of acceptor to donor was 9, probably because the ratio of the particle concentration is about 90 in this condition and LTP is therefore utilized for silent transfer of lipids between acceptors (Figure 3A,C). This view was indeed supported by an increase of the apparent rate when the acceptor concentration was reduced by a factor of 5 (Figure 3B,D). The fractional transfer rate (k/L_1) was calculated for a few different concentrations of LTP from the time course of the decrease in the fluorescence ratio using the equation described in Experimental Procedures. The specific fractional transfer rate was obtained as a slope of the linear dependency of k/L_1 on LTP concentration in the standard assay conditions (core lipid ratio of acceptor to donor lipoproteins was 9). As described in Table 2, the rate was always higher for cholesteryl ester than for triglyceride, by a factor of 2.43–7.86 in either direction. This difference is in good agreement with previously described rates measured between LDL and HDL or between LDL and reconstituted HDL-like lipoproteins using radioisotopes (Tall et al., 1983; Morton & Zilversmit, 1983; Ihm et al., 1982; Abbey et al., 1984, 1985). It was also demonstrated that apolipoprotein B has the same effect as other apolipoproteins with respect to activating the LTP reaction since lipid transfer was demonstrated between LDLs as well as other lipoprotein combinations.

Transfer of Core Lipids by LTP between Microemulsions. The rate of triglyceride transfer was measured between microemulsions with triglyceride core using pyrene–triglyceride as a probe. The donor particles with triolein cores containing 2.9% (mol/mol) pyrene–triglyceride were incubated with the same acceptor particles that do not contain the pyrene–lipid. Pyrene–triglyceride was transferred from the donor to the acceptor microemulsions only when the surface of the microemulsion was covered with apoA-I. The rate of this transfer was directly proportional to the concentration of LTP (Figure 4A). The LTP-catalyzed triglyceride transfer was activated by other apolipoproteins as well, and the results are summarized in Table 3 as specific fractional transfer rates [$k/(L_1$[LTP])]. The transfer was activated by all the tested apolipoproteins, apoA-I, apoA-II, apoC-III, and apoE, demonstrating the essential requirement of apolipoproteins for LTP-catalyzed triglyceride transfer. The results are consistent with our previous observation that apolipoproteins have been required for the LTP-catalyzed cholesteryl ester transfer reaction between LDL and triglyceride/phospholipid microemulsion (Nishikawa et al., 1988; Ohnishi et al., 1990) and between triglyceride/phospholipid microemulsions (Milner et al., 1991; Ohnishi & Yokoyama, 1993).

Table 2: Specific Fractional Transfer Rate [$k/(L_1$[LTP])] of Pyrene–Cholesteryl Ester (Pyrene-CE) and Pyrene–Triglyceride (Pyrene-TG) by LTP between Human Plasma Lipoproteins[a]

transfer direction, donor ⇒ acceptor	$k/(L_1$[LTP]) (1/min/μM)		
	CE	TG	CE/TG
LDL ⇒ LDL	0.204 ± 0.019	0.036 ± 0.008	5.68
LDL ⇒ HDL	0.052 ± 0.002	0.021 ± 0.003	2.43
HDL ⇒ LDL	0.791 ● 0.033	0.101 ± 0.011	7.86
HDL ⇒ HDL	0.675 ± 0.046	0.099 ± 0.012	6.85

[a] The total core lipid amount of pyrene–lipid donor lipoprotein was 0.96 μg, and that of the acceptor lipoprotein was 8.64 μg, per incubation mixture. Details for the assay conditions are described in the Experimental Procedures section. The value of $k/(L_1$[LTP]) was calculated from the slope of the plot of the fractional catabolic rate (k/L_1) against the LTP concentration (0, 47.1, and 94.2 nM) by a linear regression method (mean ± SE).

Figure 4B shows the pyrene–cholesteryl ester transfer between the microemulsions with 100% cholesteryl ester core in the presence and absence of apoA-I. The microemulsions with the core of cholesteryl oleate containing 7.9% (mol/mol) pyrene–cholesteryl ester were used as donor particles, and the same microemulsions without pyrene–cholesteryl ester were used as acceptor particles. The apolipoproteins (A-I, A-II, C-III, and E) were also required for the activation of the pyrene–cholesteryl ester transfer. The rate of the cholesteryl ester transfer was similar to that of the triglyceride transfer on the basis of the specific fractional transfer rate (Figure 4B and Table 3). Thus, the transfer of cholesteryl ester and triglyceride by LTP was essentially similar with respect to activator requirements and specific transfer rates when the cores were composed exclusively of either one of these lipids.

Interesting results were observed for pyrene–cholesteryl ester transfer between the emulsions containing both cholesteryl ester and triglyceride. Figure 4C shows the transfer of pyrene–cholesteryl ester between donor microemulsion containing 56.4% (mol/mol) cholesteryl ester including pyrene–cholesteryl ester equal to 7.0% (mol/mol) of the total core lipid and the acceptor containing 60.0% (mol/mol) cholesteryl oleate compound without pyrene. The transfer rate of pyrene–cholesteryl ester was directly proportional to the concentration of LTP in the presence of apoA-I. However, the specific fractional transfer rate [$k/(L_1$[LTP])] was about 1.6 times higher than that between microemulsions without triglyceride in the core. This apparently higher rate of the pyrene–cholesteryl ester transfer was more obvious between micro-

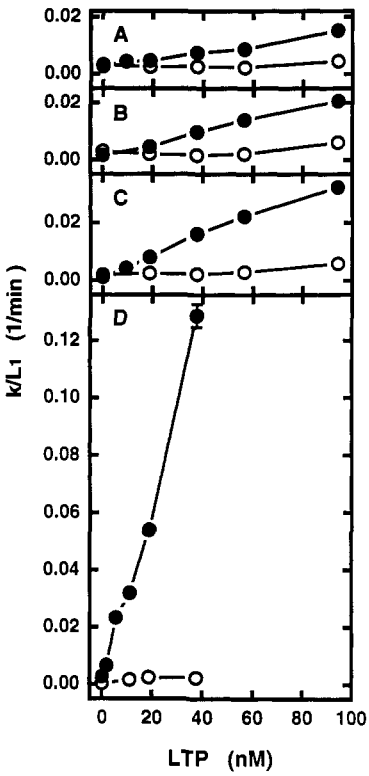

FIGURE 4: Fractional transfer rate of pyrene–triglyceride (A) and pyrene–cholesteryl ester (B, C, and D) between microemulsions with various core lipid compositions. The reactions were initiated by adding the indicated amount of LTP at 37 °C and monitored with constant stirring as a decrease in Ex/Mo as described in Experimental Procedures, in the presence of 4 μg of apoA-I (●) or bovine serum albumin (○) in a final volume of 0.4 mL. The fractional lipid transfer rate (k/L_1) was calculated as the slope of the semilogarithmic plot of [(Ex/Mo) − b] against the incubation time as described in Experimental Procedures. The standard errors of the calculated data are shown as error bars unless they are smaller than the symbols used. (A) The acceptor particle (phosphatidylcholine/triolein microemulsion, 7.2 μg of phospholipid) was mixed with the donor particle (the same microemulsion containing pyrene–triglyceride, 2.9 mol % of the core, 0.8 μg of phospholipid). (B) The acceptor particle (phosphatidylcholine/cholesteryl oleate microemulsion, 7.2 μg of phospholipid) was mixed with the donor particle (the same microemulsion containing 7.9% (mol/mol) pyrene–cholesteryl ester in the core, 0.8 μg of phospholipid). (C) The acceptor particle (phosphatidylcholine/cholesteryl oleate (60.0%) and triglyceride microemulsion, 7.2 μg of phospholipid) was mixed with the donor particle (the microemulsion containing 56.4% (mol/mol) cholesteryl ester including 7.0% (mol/mol) pyrene–cholesteryl ester, 0.8 μg of phospholipid). (D) The acceptor particle (phosphatidylcholine/triglyceride microemulsion, 7.2 μg of phospholipid) was mixed with the donor particle (the same microemulsion containing 5.3% (mol/mol) pyrene–cholesteryl ester in the core, 0.8 μg of phospholipid).

emulsions with a lower content of cholesteryl ester (Figure 4D). The microemulsions containing 5.3% pyrene–cholesteryl ester in the core triolein (mol/mol) but without additional cholesteryl oleate were used as donor particles, and the phosphatidylcholine/triolein microemulsions were used as acceptor particles. The specific fractional rate of the pyrene–cholesteryl ester transfer was more than 16 times greater than of the microemulsions with no triolein in the core. The same results were also observed when cholesteryl ester transfer was activated by other apolipoproteins (A-II, C-III, and E) (Table 3). The fractional specific transfer rate of pyrene–cholesteryl ester was almost reciprocally proportional to the content of cholesteryl ester in the core of the microemulsions with all of these activator proteins.

The fractional specific rate of cholesteryl ester transfer, $k/(L_1[\text{LTP}])$, was calculated more accurately as the slope of the plot of k/L_1 against LTP concentration for apoA-I, and the specific transfer rate of cholesteryl ester was obtained as $k/(L_1[\text{LTP}])$ times the cholesteryl ester content in the core of the emulsion (Table 4). The specific rate of the transfer became very consistent regardless of the original content of cholesteryl ester. A slight decrease in this rate was observed only when the majority of the core lipids was triglyceride. Thus, in this reaction system, cholesteryl ester transfer by LTP seemed to be competed very weakly by triglyceride.

This speculation was further consolidated when the effect of cholesteryl oleate in the core was observed on the pyrene–triglyceride transfer catalyzed by LTP (Figure 5). The core of the donor microemulsion consisted of triolein with pyrene–triglyceride and contained 15.5% (mol/mol) cholesteryl oleate, and the acceptor particle core consisted of only triolein. Both particles (0.8 and 7.2 μg of phospholipid, respectively) were incubated with 4 μg of apoA-I or bovine serum albumin, and then LTP was added. Figure 5A shows the decrease in the ratio of the excimer/monomer fluorescence peaks of the control experiment in which donor microemulsions lacked cholesteryl oleate (the same type of experiment as demonstrated in Figure 4A). The rapid decrease of the ratio in the presence of apoA-I demonstrated the transfer of pyrene–triglyceride. Figure 5B shows the experiments using donor microemulsions with cholesteryl oleate. Even in the presence of apoA-I, the fluorescence ratio hardly changed in the initial 10 min, after which it started decreasing very slowly. The fractional transfer rate for 0–5 min was plotted against the concentration of LTP for these experiments (Figure 5C). The transfer rate of triglyceride was almost negligible even with a high concentration of LTP regardless of the presence of apoA-I. Thus, the presence of a small amount of cholesteryl ester in the core almost completely inhibited the pyrene–triglyceride transfer at the initial phase. Delayed and slight initiation of the transfer of triglyceride can be interpreted as the depletion of cholesteryl oleate in the core of the donor by the initial selective transfer of cholesteryl ester by LTP. Thus, it can be concluded that cholesteryl ester undergoes highly selective transfer by LTP when both triglyceride and cholesteryl ester are present in the substrate emulsions. However, the overall rate of the lipid transfer does not seem to be a function of the lipid composition of the lipid particles.

Effect of Core Lipids on Transfer of Phospholipid. The transfer rate of phosphatidylcholine was measured utilizing the microemulsions containing pyrene-labeled phosphatidylcholine as 4.1% (mol/mol) of total phospholipid. Figure 6A shows the reaction of such emulsions with the triolein core activated by apoA-I. The phospholipid transfer was catalyzed by LTP again only in the presence of apoA-I or other apolipoproteins (Table 3). Thus, activation of LTP by apolipoproteins was required not only for core lipid transfer but also for surface lipid transfer. The specific fractional transfer rate was twice as high as the rates measured for pyrene–cholesteryl ester and pyrene–triglyceride. This transfer rate was not altered even when the transfer was measured between emulsions with cores composed of cholesteryl oleate (57.5 mol %) and triolein (Figure 6B and Table 3) or between those of 100% cholesteryl oleate (Table 3). Thus, phospholipid transfer by LTP was largely independent of the competition for transfer between cholesteryl ester and triglyceride.

Interaction of LTP with Cholesteryl Ester Molecules. A further characterization of this selective transfer was attempted in order to observe whether the selective interaction of pyrene–cholesteryl ester with LTP is involved. Small amounts of microemulsion containing pyrene–cholesteryl ester, as 0.2 μg of total phospholipid, was incubated with much greater amounts of the LTP protein than its catalytic amount in the

Table 3: Activation of LTP-Catalyzed Transfer of Cholesteryl Ester, Triglyceride, and Phosphatidylcholine between Microemulsions by Apolipoproteins[a]

pyrene–lipid	core lipid (molar ratio)	specific fractional transfer rate, $k/(L_1[LTP])$ (1/min/μM) control	BSA	apoA-I	apoA-II	apoC-III	apoE
pyrene-CE	CE (100)	0.028 ± 0.019	0.078 ± 0.047	0.256 ± 0.026	0.362 ± 0.011	0.217 ± 0.029	0.221 ± 0.013
pyrene-CE	TG/CE (43.6/56.4)	0.026 ± 0.006	0.057 ± 0.011	0.428 ± 0.008	0.565 ± 0.026	0.159 ± 0.012	0.382 ± 0.022
pyrene-CE	TG/CE (94.7/5.3)	0.046 ± 0.038	0.140 ± 0.023	2.827 ± 0.062	3.227 ± 0.067	1.314 ± 0.036	2.077 ± 0.056
pyrene-TG	TG (100)	0.069 ± 0.005	0.068 ± 0.009	0.195 ± 0.007	0.303 ± 0.021	0.248 ± 0.010	0.245 ± 0.017
pyrene-TG	TG/CE (84.5/15.5)		−0.002 ± 0.002*	−0.035 ± 0.006*			
pyrene-PC	TG (100)	−0.004 ± 0.014	0.051 ± 0.004	0.365 ± 0.012	0.610 ± 0.015	0.381 ± 0.062	0.463 ± 0.019
pyrene-PC	TG/CE (47.5/57.5)	0.038 ± 0.015	0.064 ± 0.039	0.621 ± 0.060	0.699 ± 0.020	0.515 ± 0.027	0.493 ± 0.016
pyrene-PC	CE (100)			0.537 ± 0.095*			

[a] The specific fractional transfer rate $[k/(L_1[LTP])]$ was calculated using a least-squares linear regression method as the slope of a plot of k/L_1 against LTP concentration (±SE) for the values indicated with an asterisk. The rate was calculated for other values from k/L_1 values (±SE) using the data for the reaction time course at an LTP concentration of 11.3 nM (for pyrene–cholesteryl ester transfer when the triglyceride/cholesteryl ester molar ratio in the core of the emulsion was 94.7/5.3) or 37.7 nM (for all other cases).

Table 4: Rate of Cholesteryl Ester Transfer in apoA-I-Activated LTP Reactions between Microemulsions with Various Core Lipid Compositions[a]

pyrene–lipid	core lipid	specific fractional transfer rate, $k/(L_1[LTP])$ (1/min/μM)	specific transfer rate, $[k/(L_1[LTP])]$ × [CE] (%)
pyrene-CE	CE (100%)	0.208 ± 0.010	20.80
pyrene-CE	TG and CE (56.4%)	0.338 ± 0.017	19.06
pyrene-CE	TG and CE (5.3%)	3.296 ± 0.190	17.47

[a] The specific fractional transfer rate $[k/(L_1[LTP])]$ was calculated using a least-squares linear regression method as the slope of a plot of k/L_1 against LTP concentration (±SE) from the data shown in Figure 4, and the specific transfer rate was calculated as the specific fractional transfer rate multiplied by the molar percentage of cholesteryl ester in the core of pyrene-donor particles.

absence of the acceptor particles, and the subsequent change in the ratio of the excimer/monomer peaks was monitored (Figure 7). The microemulsions contained 5.3% (mol/mol) pyrene–cholesteryl ester in the triolein core or 56.4% (mol/mol) total cholesteryl ester as cholesteryl oleate plus pyrene–cholesteryl ester (7.0 mol %) in the triolein core. In Figure 7A, the ratio of the excimer/monomer peaks was plotted against the incubation time when microemulsion containing 5.3% (mol/mol) cholesteryl ester preincubated with 1 μg of apoA-I was incubated with various amounts of LTP. The ratio rapidly decreased within 15 s after LTP was added to reach the minimum end point. In Figure 7B, this end point of the ratio was plotted against the concentration of LTP. This change in the ratio was found as a function of the LTP concentration and reached maximum above 5×10^{-8} M LTP, which is roughly equivalent to the cholesteryl ester concentration in the mixture, 4×10^{-8} M (as a component of the core). This curve was identical whether in the presence of apoA-I or bovine serum albumin. However, this reaction was strongly suppressed by the presence of an additional excessive amount of cholesteryl oleate in the core. Thus, LTP selectively disrupts the excimer of pyrene–cholesteryl ester over triglyceride, and this interaction was suppressed by dilution of the pyrene–lipid by cholesteryl oleate in the core (Figure 7B). Thus, we may speculate that the selective transfer of cholesteryl ester over triglyceride is based on the direct selective interaction of LTP with the cholesteryl ester molecule in the lipid microemulsion.

DISCUSSION

We previously reported the successful use of pyrene–cholesteryl ester for the assay of the LTP reaction and demonstrated that the lipid-bound apolipoproteins are essential for the LTP-catalyzed cholesteryl ester transfer reaction (Milner et al., 1991; Ohnishi & Yokoyama, 1993). In the present paper, we extended the study to other lipid substrates and natural lipoproteins using the same assay system for the transfer reaction. It has been shown that the pyrene–lipid method can be applied to measure the lipid transfer between plasma lipoproteins, including the reaction between lipoproteins in the same subclass, which has not been feasible to observe before. Cholesteryl ester and triglyceride were both transferred by LTP in any direction with a preferred rate for cholesteryl ester by a factor of 2.4–7.9, in good agreement with the previously reported rates measured by using radioisotope probes for hetero donor–acceptor combinations (Tall et al., 1983; Morton & Zilversmit, 1983; Ihm et al., 1982; Abbey et al., 1984, 1985). This also confirmed that pyrene–lipids are good substrate analogues for both substrates of the LTP reaction.

A more unique feature of the LTP reaction was discovered with the lipid microemulsions. It was demonstrated that the lipid emulsions could be prepared in a homogeneous size with an approximate diameter of 26 nm and with a wide range of lipid compositions including those with pure cholesteryl ester core. This was achieved only by choosing appropriate conditions for sonication unlike the extremely harsh conditions reported elsewhere (Lundberg & Saarinen, 1975; Ginsburg et al., 1982). It was indicated by the elution profiles of the emulsions from a gel permeation column that pyrene-labeled lipids were incorporated into the positions where the corresponding non-pyrene lipids were located with respect to the distribution between the core and the surface. Therefore, pyrene-labeled lipids can be considered as analogues to the corresponding lipids in terms of topological distribution in the lipid emulsions.

Pyrene–cholesteryl ester was rapidly transferred by LTP between the microemulsions containing mainly triglyceride in the core in the presence of apolipoproteins, consistent with our previous reports (Milner et al., 1991; Ohnishi & Yokoyama, 1993). However, the presence of cholesteryl oleate in the core apparently decreased the fractional rate of transfer of pyrene–cholesteryl ester, the rate being almost proportional to the total cholesteryl ester content in the core (Table 3).

The first possibility to be considered is that the physicochemical state of the core changes with the presence of cholesteryl oleate to such an extent that the reaction may be slowed down. Ginsburg et al. (1982) reported that egg yolk phosphatidylcholine/cholesteryl oleate microemulsion exhibited a phase transition of cholesteryl oleate only at 42 °C with no transition associated with phospholipid. Kroon (1981) showed that cholesteryl oleate exhibits two separate phase transitions, at 42.0 and 46.5 °C, in its bulk state. In the presence of 5% triolein the phase transition at 46.5 °C disappeared, as did the other phase transition when 22% triolein

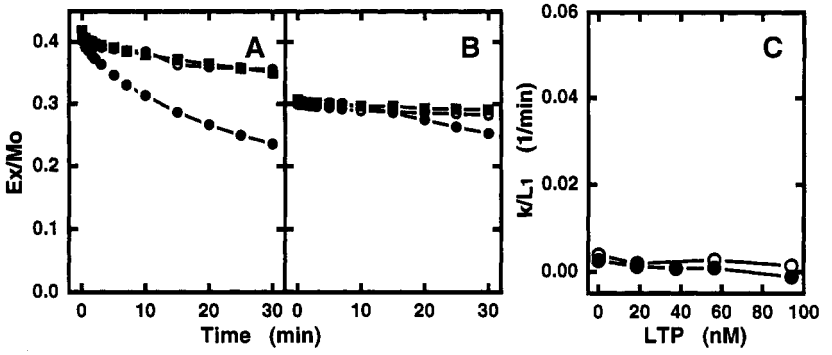

FIGURE 5: Pyrene–triglyceride transfer between microemulsions containing 15.5 % cholesteryl ester in the core. (A) The same incubations as described in the caption to Figure 4A (triglyceride transfer between emulsions without cholesteryl ester) were carried out, and the ratio of the excimer fluorescence to the monomer fluorescence was plotted against the incubation time: incubation without LTP in the presence of apoA-I (■); incubation with bovine serum albumin and 94.2 nM LTP (○); incubation with apoA-I and 94.2 nM LTP (●). (B) The acceptor particle (phosphatidylcholine/triglyceride microemulsion, 7.2 μg of phospholipid) was incubated with the donor particle (phosphatidylcholine/triolein containing cholesteryl oleate and pyrene–triglyceride (15.5 and 2.1 mol % of the core, respectively), 0.8 μg of phospholipid) in the presence of 4 μg of apoA-I or bovine serum albumin in a final volume of 0.4 mL. The reaction was initiated by adding LTP and monitored at 37 °C with constant stirring. Symbols are the same as those in panel A. (C) The fractional lipid transfer rate (k/L_1) in the reaction mixtures described in panel B was plotted against the concentration of LTP: incubation with bovine serum albumin (○); incubation with apoA-I (●).

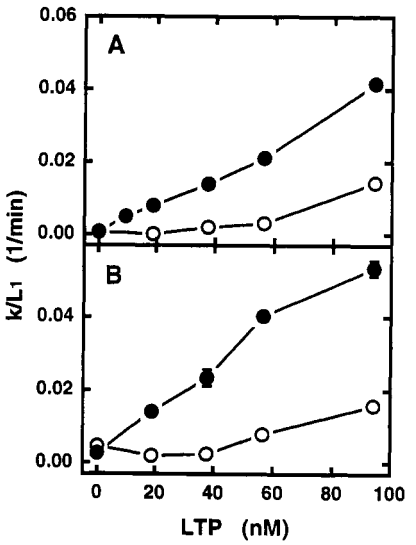

FIGURE 6: Pyrene–phosphatidylcholine transfer between microemulsions. (A) The acceptor particle (phosphatidylcholine/triolein microemulsion, 7.2 μg of phospholipid) was mixed with the donor particle (the same microemulsion containing 4.1% (mol/mol) pyrene–phosphatidylcholine, 0.8 μg of phospholipid) as phospholipid, or (B) the acceptor particle (phosphatidylcholine/triolein and cholesteryl oleate (60.0 mol % of the core) microemulsion, 7.2 μg of phospholipid), was mixed with the donor particle (containing 57.5 mol % cholesteryl oleate in the core lipids and 4.2 mol % pyrene–phosphatidylcholine in phospholipid, 0.8 μg of phospholipid), in the presence of 4 μg of apoA-I (●) or bovine serum albumin (○) in a final volume of 0.4 mL. The reaction was initiated by LTP and monitored at 37 °C with constant stirring. The fractional lipid transfer rate (k/L_1) was calculated as described in Experimental Procedures. The standard errors of the calculated data were all smaller than the symbols in panel A.

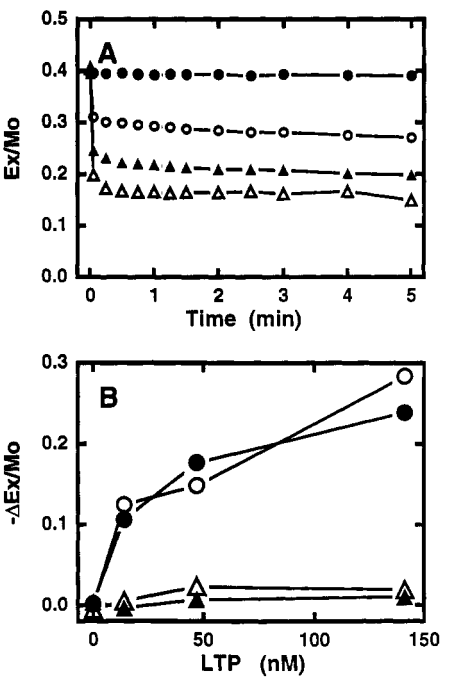

FIGURE 7: Direct interaction of LTP with pyrene–cholesteryl ester in microemulsions containing 5.3% or 56.4% cholesteryl ester in the core. Microemulsion particles (phosphatidylcholine/triolein, 0.2 μg of phospholipid) containing 5.3 mol % pyrene–cholesteryl ester in the core were incubated with 1 μg of apoA-I and various amounts of LTP (●, no LTP; ○, 14.1 nM; ▲, 47.1 nM; △, 141.2 nM) with no acceptor particle in a final volume of 0.4 mL. The ratio of the excimer fluorescence to the monomer fluorescence (Ex/Mo) was plotted against the incubation time (A). (B) The maximum decrease of the Ex/Mo ratio (–ΔEx/Mo) plotted against the concentration of LTP. The donor particle [5.3% (mol/mol) cholesteryl ester of the core] was incubated with LTP in the presence of bovine serum albumin (○) or apoA-I (●). The emulsion particle (phosphatidylcholine/triolein), containing 56.4 mol % cholesteryl ester including 7.0% (mol/mol) pyrene–cholesteryl ester in the core, was incubated with LTP in the presence of 1 μg of bovine serum albumin (△) or apoA-I (▲).

was added. Thus, at 37 °C only the pure cholesteryl ester core is below its transitional temperature among the emulsion cores used in this study. However, the change in the fractional transfer rate was continuous and linear to cholesteryl ester content in the core including this type of emulsion, so it is unlikely that the physical state of the core contributes to the transfer rate under our experimental conditions.

The second possibility is that cholesteryl ester is selectively transferred by LTP over triglyceride. Assuming that pyrene–cholesteryl ester and cholesteryl oleate were transferred by LTP with the same rate, the apparent fractional rate of the transfer (k/L_1) could be converted to the rate of cholesteryl ester transfer (k) by multiplying the k/L_1 value with the amount of total cholesteryl ester in the donor emulsion. The transfer rate calculated in this manner was almost constant with various contents of cholesteryl ester in the core (Table 4). Therefore, it was fair to conclude that cholesteryl ester is almost exclusively transferred by LTP when both cholesteryl ester and triglyceride are present in the core. This hypothesis was also supported by the almost complete inhibition of triglyceride transfer by the presence of a small amount of cholesteryl ester in the core (Figure 5). In addition, it was

shown that the triglyceride transfer was slowly initiated only after a substantial amount of cholesteryl ester was selectively removed from the donor particles in the initial phase of the reaction. These results also almost excluded the possibility that this specificity is caused by an artifact of the pyrene–lipids. Thus, we concluded that both cholesteryl ester and triglyceride fully compete for LTP transfer and that LTP transfers the former substrate with much higher selectivity between the apolipoprotein-activated lipid microemulsions.

Interestingly, the rates of the transfer of cholesteryl ester and triglyceride are similar when no competitive lipids are present in the core of the emulsions. This means that the selective interaction of substrate with LTP is not a rate-limiting step of the reaction. Thus, the rate-limiting step should be either association of LTP with the lipid surface or dissociation of the LTP–substrate complex from the surface if a lipid-carrying model is adopted, or it should be flip-flop exchange of lipids in a ternary complex model. In the former case, the dissociation of LTP from lipid particles is more likely to be the rate-limiting reaction than its association because of the hydrophobic nature of the protein. Accordingly, the slightly lower rate of cholesteryl ester transfer in the presence of triglyceride could still be caused by weak competitive inhibition by triglyceride. The difference of fractional specific transfer rate thereby can be estimated to be as much as 90-fold or even higher for cholesteryl ester assuming that the sum of the cholesteryl ester and triglyceride transfer rates is always constant.

Excimers of pyrene–cholesteryl ester were apparently disrupted by LTP, and this association between LTP and cholesteryl ester molecules may be stoichiometric. The interaction was strongly inhibited by the presence of cholesteryl oleate presumably due to the dilution of the pyrene–lipid by cholesteryl oleate. Thus, LTP may interact with one or more cholesteryl ester molecules more selectively than with triglyceride in the emulsion. However, apoA-I did not show the specific effect on this direct interaction, unlike its effect on the transfer reaction. When an excess amount of LTP was given, LTP seemed to catalyze the lipid transfer even without apolipoprotein activation in a nonlinear dose-dependent manner (Ohnishi & Yokoyama, 1993). This may indicate that the binding of LTP itself to the lipid surface provides a condition similar to that created by apolipoproteins and leads to its "self-activation". The relatively large amount of LTP used for the experiments on the direct LTP–lipid interaction was in such a range that apolipoproteins may not be absolutely required for the interaction. We do not know yet the nature of this interaction, whether it is due to selective removal by LTP of cholesteryl ester from the emulsion or to formation of LTP–cholesteryl ester complex on the lipid surface, or whether it is limited to disruption of pyrene–excimer formation in the emulsion. However, the saturation of the reaction seems to occur when the molar ratio of LTP to emulsion particles is more than 200:1, assuming 2600 phospholipids per emulsion particle (Figure 7B). It is very unlikely that the surface of the emulsion is reaching the saturation of LTP binding at this level, considering LTP's strong binding to this type of emulsion (Ko et al., 1993; T. Ohnishi and S. Yokoyama, unpublished data).

The criticism for the interpretation and discussion above may be that the finding is limited to the pyrene–lipids. This criticism can be addressed in several ways. The substrate specific transfer rate based on the pyrene–lipid transfer was similar to that measured with radioisotope-labeled lipid among natural plasma lipoproteins in terms of the ratio between the transfer rates of cholesteryl ester and triglyceride (Table 2). In the emulsion system, dilution of pyrene–cholesteryl ester with cholesteryl oleate for the transfer indicates that both lipids are almost identical in the competition for LTP (Figure 4 and Tables 3 and 4), although the dilution may be a little overeffective on the selective disruption of pyrene excimer, suggesting a slightly higher affinity for the natural lipid than for the pyrene–lipid (Figure 7). Pyrene–cholesteryl ester undergoes the transfer far more selectively than triolein, but the latter may compete somewhat against the former (Figure 4 and Tables 3 and 4). Finally, pyrene–triglyceride transfer may be completely blocked by a small amount of cholesteryl oleate (Figure 5). Thus, the order of the transfer selectivity among the substrates can be speculated as pyrene–triglyceride $\leq$ triolein $\ll$ pyrene–cholesteryl ester $\leq$ cholesteryl oleate, but the differences between the respective pyrene-labeled and unlabeled lipids seem very small. It is not possible to measure the transfer of either lipid mass or radiolabeled lipid between identical lipid particles, so that we believe the data presented here using the pyrene–lipids is the best probe of the reaction in such conditions.

The mechanism for selective interaction of LTP with cholesteryl ester is unknown. A higher affinity of LTP for cholesteryl ester molecules seems to be one of the rational interpretations of the substrate selectivity. This discussion may not be inconsistent with the selective suppression of the triglyceride transfer by chemical modification of LTP with *p*-chloromercuriphenylsulfate (Morton & Zilversmit, 1983) or by monoclonal antibodies against human (Fukasawa et al., 1992) and rabbit LTP (K. W. Ko and S. Yokoyama, unpublished data), since these data can be interpreted by the hypothesis that the treatments interfere with the LTP–substrate interaction to the extent that only the transfer based on the weak interaction is influenced.

On the other hand, it was proposed (Morton & Steinbrunner, 1990) that LTP transfers lipids present in the surface membrane. This may lead to a hypothesis that in emulsions cholesteryl ester occupies a specific surface position accessible by LTP, displacing triglyceride. However, this view argues against the reports by Miller and Small (1982, 1983) that triglyceride is dissolved in the surface membrane of the emulsion to a much greater extent than cholesteryl ester.

The excimer/monomer ratios in pyrene fluorescence were approximately the same for triglyceride and cholesteryl ester for similar concentrations in the core regardless of the core lipid composition (0.3–0.4 for 5–7 mol % of the core lipid). Thus, most of these pyrene–lipids seem to be homogeneously distributed in the core. On the other hand, rapid and complete disruption of pyrene–cholesteryl ester excimer in the emulsion by a stoichiometric amount of LTP (Figure 7) suggests that the selective interaction between LTP and cholesteryl ester eventually takes place with all of the pyrene–lipid, including the molecules buried in the core. Therefore, granted that cholesteryl ester molecules may undergo the interaction with LTP for the transfer only at the surface, they are replaced rapidly by the molecules in the core. Thus, the surface concentration of the substrate at a certain moment may not absolutely be a rate-limiting factor for the transfer reaction.

Nevertheless, we cannot completely exclude the possibility that cholesteryl ester molecules are selectively accessible by LTP due to the structure of certain lipid particles until we are able to determine whether or not there is such an accessible pool of cholesteryl ester in the emulsions.

The significantly lower substrate selectivity in the lipoproteins also remains to be explained. Lipids may be randomly incorporated into the emulsion during sonication, and the structure becomes stable only on the basis of their thermo-

dynamic interaction, so that LTP may see cholesteryl ester preferably according to its primitive molecular nature on the basis of the molecular affinity for the substrates or due to a specific accessible pool of cholesteryl ester for LTP. Natural lipoproteins, on the other hand, are assembled by many sequential reactions including pyrene–lipid incorporation leading to a highly organized lipid structure that may expose triglyceride to LTP in a more accessible manner or cholesteryl ester in a less susceptible way, for example, by their conformation or by an accessible pool in the surface layer. Apolipoproteins bound to emulsions may penetrate into the phospholipid surface monolayer, but only to a limited extent, while they may be more deeply integrated in the surface layer of natural lipoproteins in a more highly organized manner providing a microenvironment for decreased substrate selectivity of LTP. Some other unknown factor(s) in lipoproteins may specifically alter LTP's substrate selectivity, such as free cholesterol on the surface or unknown protein factors, by interacting with LTP or by modifying the properties of the surface.

The transfer rate of pyrene–phosphatidylcholine between the microemulsions was on the same order as those of triglyceride and cholesteryl ester, and it was not affected by the composition of the core lipids. This implicates the possibility that the site and nature of binding of LTP to phosphatidylcholine may not be exactly the same as those of binding of LTP to triglyceride or cholesteryl ester (Morton & Zilversmit, 1983; Morton & Steinbrunner, 1990). Also, apolipoproteins were shown to be required for phospholipid transfer, demonstrating that this effect is not only seen for the core lipids. Thus, apolipoproteins seem to reduce the energy barrier between the aqueous phase and the hydrophobic phase including acyl chains of phospholipid (Ohnishi & Yokoyama, 1993).

The overall picture derived from the present experiments leads us to the hypothesis that cholesteryl ester and triglyceride compete for transfer by LTP but that the former substrate is much more preferred. This substrate specificity is significantly reduced in natural plasma lipoproteins. Phospholipid transfer by LTP is carried out by a somewhat independent mechanism.

LTP is thought to play an important role in the transport of cholesteryl ester by plasma lipoproteins. However, it is not exactly clear what the physiological role of this protein is. A remarkable accumulation of cholesteryl ester in HDL in patients with a genetic defect of LTP suggests that this protein moves cholesteryl ester generated on HDL to other lipoproteins (Inazu et al., 1990). Since LTP catalyzes nondirectional transfer of cholesteryl ester and other lipids, the net transfer of cholesteryl ester should occur by the heteroexchange with other lipids, mainly triglyceride. The present data indicate that the heteroexchange may indeed be catalyzed widely by LTP among any plasma lipoproteins. However, it was also demonstrated that highly selective cholesteryl ester transfer occurs under certain conditions, suggesting that some specific factor or factors contribute to the regulation of cholesterol transport by plasma lipoproteins by altering substrate selectivity of the LTP reaction. We will continue extensive investigations to identify this factor (these factors).

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