Concentration of neutral lipids in the phospholipid surface of substrate particles determines lipid transfer protein activity

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Abstract To better understand the mechanism of lipid transfer protein (LTP) action and the effects of altered lipoprotein composition on its activity, we evaluated the dependence of LTP activity on the concentrations of cholesteryl ester (CE) and/or triglyceride (TG) in the phospholipid bilayer of substrate particles. Phosphatidylcholine (PC)-cholesterol liposomes containing up to 2 mole% TG and/or CE were prepared by cholate dialysis and used as either the donor of lipids to, or the acceptor of lipids from, low density lipoproteins (LDL). CE or TG transfer from liposomes of varying neutral lipid content to LDL showed saturation kinetics with an apparent K_m of ≤ 0.2 mole%. Throughout this concentration-dependent response, PC transfer, which depended on the same LTP-donor particle binding interactions as those required for neutral lipid transfer, was essentially unchanged. Lipid transfer in the reverse direction (from LDL to liposomes of varying neutral lipid content) followed the same kinetics showing that transfer between the two particles is tightly coupled and bidirectional. When liposomes contained both TG and CE, these lipids competed for transfer in a manner analogous to that previously noted with lipoprotein substrates. In conclusion, CE and TG transfer activities are determined by the concentration of these lipids in the phospholipid surface of donor and acceptor particles. At low TG and CE concentrations, LTP bound to the liposome surface as indicated by PC transfer, but only a portion of these interactions actually facilitated a neutral lipid transfer event. Thus, the overall rate of neutral lipid transfer, and the competition between TG and CE for transfer, depend on the concentrations of these lipids in the phospholipid layer.—**Morton, R. E., and J. V. Steinbrunner.** Concentration of neutral lipids in the phospholipid surface of substrate particles determines lipid transfer protein activity. *J. Lipid Res.* 1990. **31:** 1559–1567.

Supplementary key words lipid transfer protein • liposomes • cholesteryl ester • triglyceride • phosphatidylcholine • coupled transfer • reciprocal transfer

The interlipoprotein transfer of triglyceride and cholesteryl ester is facilitated by a single plasma protein designated by various investigators as LTP (1), cholesteryl ester transfer protein (2, 3), neutral or core lipid transfer protein (4), or lipid transfer protein-I (5). LTP facilitates TG and CE transfer by an exchange mechanism whereby lipid transfer between two lipoproteins involves either the exchange of CE for CE or TG for TG, or the heteroexchange of CE for TG (6). Thus, LTP can equilibrate the molecular species of TG or CE between lipoproteins and promote the remodeling of lipoprotein lipid composition (4, 7, 8). Although LTP has a marked preference for transferring CE (1, 3), it is apparent that CE and TG compete for transfer (6), perhaps due to shared binding sites on LTP (9). The selection of TG versus CE for transfer is driven, in large part, by the ratio of these two lipids in the lipoprotein substrate; TG-rich lipoproteins are better TG donors than CE-rich lipoproteins, and conversely for CE transfer (6). Although the mechanism of transfer is yet to be clearly defined, it is apparent that the physical association or binding of LTP to the donor lipoprotein is an integral part of the transfer process (10).

In recent study we observed that the typical competition of CE and TG for transfer by LTP is perturbed by changes in the free cholesterol content of substrate lipoproteins (11). For LDL and VLDL, the effect of free cholesterol enrichment was to make these lipoproteins progressively poorer donors of CE without affecting (increasing or decreasing) their ability to donate TG. We proposed, based on the lipid solubility studies of others (12–15), that the effects of free cholesterol on lipid transfer were due to a selective modification in the availability of CE relative to TG at the phospholipid surface of lipoproteins where it can interact with LTP. For such a mechanism to explain these data, however, requires LTP activity to be dependent on the concentration of neutral lipids in the phospholipid coat of lipoproteins. Furthermore, the operative mechanism would have to be able to

Abbreviations: LTP, lipid transfer protein; PC, phosphatidylcholine; LDL, low density lipoprotein(s); HDL, high density lipoprotein(s); BSA, bovine serum albumin; TG, triglyceride; CE, cholesteryl ester.

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explain how TG and CE can compete for transfer, and yet how TG transfer can remain unchanged while CE transfer is decreased by free cholesterol. None of these issues has been addressed to date.

To better understand how free cholesterol and other agents that may alter the molecular organization of lipoproteins affect LTP activity, we have studied the dependence of LTP activity on the amounts of CE and/or TG present in the phospholipid surface of donor particles. Phosphatidylcholine liposomes of varying TG and/or CE content were prepared, characterized, and used as donors of lipid to, or acceptors of lipid from, LDL.

EXPERIMENTAL PROCEDURES

Materials

Tri[9,10-^{3}H]oleoylglycerol (26.8 Ci/mmol) was obtained from New England Nuclear (Boston, MA), and 1-palmitoyl-2-[1-^{14}C]oleoly-*sn*-3-phosphocholine (52 mCi/mmol), [4-^{14}C]cholesterol (55 mCi/mmol), and [1α,2α(n)-^{3}H]cholesterol (45.6 Ci/mmol) were purchased from Amersham Corp. (Arlington Heights, IL). [^{3}H]- and [^{14}C]cholesteryl oleate were synthesized from their respective radiolabeled cholesterol moiety an oleoyl chloride (Nu-Chek, Elysian, MN) as described by Pinter, Hamilton, and Muldrey (16). Radiolabeled triglyceride and cholesteryl esters with purities of $<98\%$ were repurified by thin-layer chromatography on silica gel 60A plates (Whatman Chemical Separations, Inc., Clifton, NJ) in a developing system of hexanes–diethyl ether 80:20 (v/v) prior to their use.

BSA (fraction V), diethyl *p*-nitrophenyl phosphate, egg PC, butylated hydroxytoluene, and all reagents for salt and buffer solutions and for enzymatic assays were obtained from Sigma Chemical Co. (St. Louis, MO). Cholesterol, cholesteryl oleate, and triolein were purchased from Nu-Chek. Lipid solutions were prepared in chloroform containing 10 μg/ml butylated hydroxytoluene and stored under N_2 at -20°C. Phenyl Sepharose CL-4B, Con A Sepharose 4B, and dextran sulfate (M_r = 500,000) were from Pharmacia Fine Chemicals (Piscataway, NJ), and Bio-Gel A-15m was from Bio-Rad Laboratories (Richmond, CA).

Isolation of LTP

Partially purified LTP was isolated from human plasma as previously described (1). Briefly, citrated human plasma was made lipoprotein-deficient by the dextran sulfate–$MnCl_2$ precipitation procedure of Burstein, Scholnick, and Morfin (17). LTP was then partially purified by sequential hydrophobic and ion exchange chromatographies (1). Partially purified LTP, which was routinely enriched 700 to 900-fold in both triglyceride and cholesteryl ester transfer specific activities, was stored at 4°C in 0.27 mM disodium EDTA, pH 7.4. This fraction of LTP was used in all experiments. Partially purified LTP did not contain detectable lecithin:cholesterol acyltransferase (18) or the LTP inhibitor protein (19), and was devoid of the phospholipid-specific transfer protein activity (LTP-II, (5)) based on the absence of significant heat-labile phospholipid transfer activity in this fraction (88% of the PC transfer activity remained after 60 min at 58°C). During the purification protocol, LTP activity was routinely assayed by determining the extent of radiolabel transferred from [^{3}H]TG, [^{14}C]CE-labeled LDL to unlabeled HDL (18, 20). The plasma-derived inhibitor protein of LTP was isolated by the same chromatographic steps as for LTP, except that the inhibitor was eluted from the hydrophobic column with 15% ethanol after LTP removal, and then further purified on the ion exchange column where it eluted at a lower NaCl concentration than did LTP (19).

Isolation and radiolabeling of lipoproteins

Human lipoproteins were labeled by the lipid dispersion technique of Morton and Zilversmit (19). For this procedure, fresh human plasma was incubated with a lipid dispersion containing egg PC, [^{3}H]triolein (0.2 mol%) and [^{14}C]cholesteryl oleate (19.8 mol%) at 37°C for 24 h in the presence of diethyl *p*-nitrophenyl phosphate. Labeled and unlabeled lipoproteins were isolated at 4°C by sequential ultracentrifugation (21) at solvent densities of 1.019, 1.063, and 1.21 g/ml to yield very low density lipoproteins, LDL, and HDL, respectively. Under these labeling conditions, radiolabeled lipoproteins contained TG and CE specific activities of approximately 1.6×10^3 and 4.9×10^2 dpm/μg lipoprotein cholesterol, respectively. Alternatively, for use in some liposome transfer experiments, LDL was labeled by radiolabel exchange from reconstituted HDL (22). LDL (500 μg cholesterol), reconstituted HDL (4.3×10^6 cpm [^{3}H]TG and 1×10^6 cpm [^{14}C]CE, LTP (380 μg), and 250 μl of 3.5% BSA in 50 mM Tris-HCl, pH 7.4 (total volume 2.5 ml) were co-incubated for 7 h at 37°C, followed by re-isolation of the labeled LDL by ultracentrifugation as described above. This LDL had ^{3}H and ^{14}C specific activities that were 3- to 5-fold higher than that labeled by the dispersion method. All lipoproteins were extensively dialyzed against 0.9% NaCl, 0.01% EDTA, 0.02% NaN_3, pH 7.4, and stored at 4°C. Lipoproteins were quantitated based on their total cholesterol content.

Preparation of liposome substrates

PC-cholesterol liposomes were prepared by cholate dialysis using a modification of the method of Brunner, Skrabal, and Hauser (23). Typically, egg PC (10 μmol), cholesterol (2.5 μmol), up to 300 nmol of CE and/or TG, and trace quantities of radiolabeled lipids (2.7×10^5 cpm [^{14}C]PC and 5.0×10^5 cpm of either [^{3}H]TG or [^{3}H]CE) were combined, the solvent was removed under N_2, and

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the residue was washed twice with alumina-treated diethyl ether followed by evaporation under N_2. Dried lipids were suspended in 50 μl of 1 M sodium cholate by vigorous vortexing, then combined with 1.95 ml of 10 mM Tris-HCl, 100 mM NaCl, 0.02% EDTA, 0.02% NaN_3, pH 7.4, at 25°C. Samples were dialyzed versus the same Tris/NaCl buffer (twice vs 2 l for 2 h each, then against 2 l overnight) at room temperature. Dialyzed samples were centrifuged at 41,700 *g* for 30 min, the supernatant was removed and adjusted to 0.35% BSA, and then filtered through a glass-wool plug before use. Liposomes were routinely characterized with respect to radiolabel and phospholipid phosphorus content.

Assay of LTP activity

Lipid transfer assays were carried out as previously described (18, 20). In most instances, radiolabeled donor particles (lipoprotein or liposome) and unlabeled acceptor particles were incubated with or without LTP at 37°C for up to 2 h. In assays where lipoproteins were used as the donor and acceptor particles, lipid transfer activity was terminated by selectively precipitating one of the two lipoproteins by the addition of PO_4^{3-} and Mn^{2+} (20). When the assay involved liposomes and LDL as the donor/acceptor particles, the transfer assay was terminated by affinity adsorption of the LDL by Con A Sepharose (1). The radioactivity in an aliquot of the acceptor (lipoprotein or liposome) fraction was determined by liquid scintillation counting. The fraction of radiolabeled, donor lipid that was transferred (kt) to the acceptor particle was calculated as described before (18), and is reported either as the percent lipid transferred (kt × 100), or as the mass of lipid transferred, which was calculated by multiplying the %kt value by the mass of the lipid in the donor particle. Radiolabeled lipid "transfer" in the absence of LTP was subtracted before these calculations; for TG and CE these blank values (generally ~1.5–2.5%) mostly reflected the incomplete separation of donor and acceptor at the end of the assay. The spontaneous, non-LTP-facilitated transfer of PC from liposomes to LDL was ≤0.5% in 2 h (after correcting for incomplete donor/acceptor separation), consistent with the reported long half-time for spontaneous PC transfer from liposomes of similar composition (24). For the various liposome donor particles, lipid transfer was near-linear up to 20% kt, with a doubling of LTP in the assay yielding a ~1.8-fold increase in transfer activity. Unless indicated otherwise, the transfer values presented are the average of values determined for two to three samples; replicate determinations generally differed <10% from the mean.

Analytical procedures

Protein was quantitated by the method of Lowry et al. (25) as modified by Peterson (26), with BSA as standard. Total cholesterol in aqueous samples was assayed by a colorimetric, enzymatic method using Reagent-Set (Boehringer-Mannheim). Alternatively, free and total cholesterol levels were quantitated by the fluorometric, enzymatic method of Gamble et al. (27); cholesteryl ester was determined by the difference between these two values, times a conversion factor of 1.69 to correct for the fatty acid content of the sterol ester. The cholesterol content of organic solutions of lipids was assayed by the method of Zak et al. (28). Triglyceride was measured either by a colorimetric, chemical assay (29) or by the fluorometric, enzymatic method Mendez, Cabeza, and Hsia (30). Lipid phosphorus was assayed by the method of Bartlett (31), and a conversion factor of 25 was used to determine phospholipid mass. For liposomes, the mole% values for TG and/or CE were based on the mole ratio of the neutral lipid relative to the liposome PC content.

RESULTS

Characterization of liposome substrates

The complete chemical compositions of representative preparations of liposomes are shown in **Table 1**. Liposome preparations varying up to 80-fold in TG or CE content were prepared. Over this range, the recovery of each component in the final liposome preparation was similar except at the higher levels of CE or TG; as a result, liposomes were very similar with respect to their polar lipid content and differed only by their neutral lipid content. For a given group of liposomes containing CE or TG, the free cholesterol to phospholipid ratio was essentially constant. By gel filtration, all liposome preparations were more heterogeneous in size than plasma lipoproteins, but the extent of this heterogeneity was similar between liposomes of differing CE or TG content; data for CE liposomes are shown in **Fig. 1**. Throughout the elution profile, the CE content of liposomes was remarkably constant relative to phospholipid; the [^{3}H]CE/[^{14}C]PC ratios of the 10 major fractions constituting the elution peaks for liposomes containing 0.02, 0.09, and 0.36 mole% CE (Fig. 1) were 2.3 ± 0.1, 2.3 ± 0.1, and 2.1 ± 0.1 (mean ± SD), respectively. Data similar to that described in Table 1 and Fig. 1 were observed for TG and TG-CE containing liposomes (data not shown).

Properties of PC transfer

In the present report, a principal objective was to investigate how the rate of lipid transfer is affected by the amount of TG and CE incorporated into the surface lipids of the donor particle. Since lipid transfer activity is known to depend on the binding of LTP to the surface of the donor particle (10), it was important to distinguish between altered transfer rates due to variations in TG or CE

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TABLE 1. Incorporation of lipids into phospholipid liposomes prepared by cholate dialysis

No.	CE-Containing Liposomes						TG-Containing Liposomes					
	Initial Composition			Final Composition			Initial Composition			Final Composition		
	PC	FC	CE	PC	FC	CE	PC	FC	TG	PC	FC	TG
	μmol		*nmol*	*μmol*		*nmol*	*μmol*		*nmol*	*μmol*		*nmol*
1	10	2.5	2.2	7.8	1.9	1.8	10	2.5	3.8	9.2	1.7	3.4
2	10	2.5	4.4	7.2	1.8	3.7	10	2.5	7.5	9.2	1.7	6.8
3	10	2.5	8.8	8.0	1.9	7.8	10	2.5	15	9.3	1.9	13.5
4	10	2.5	17.7	8.0	1.9	15.0	10	2.5	30	9.3	1.7	27.2
5	10	2.5	35.5	7.6	2.0	30.0	10	2.5	60	9.1	1.7	52.3
6	10	2.5	66.0	7.9	2.0	61.1	10	2.5	111	9.3	2.0	102
7	10	2.5	111	8.0	2.0	80.6	10	2.5	188	9.6	1.9	170
8	10	2.5	178	7.6	1.9	117	10	2.5	300	8.5	1.9	228

The indicated quantities of phosphatidylcholine (PC), free cholesterol (FC), and either cholesteryl oleate (CE) or triolein (TG) were combined and incorporated into liposomes as described in Experimental Procedures. Recoveries were based on chemical determination for PC and FC, and on radiolabel recovery for CE and TG.

concentration and altered rates due to perturbed binding of LTP to liposomes of differing composition. One potential means of monitoring the extent of interactions between LTP and liposomes is the transfer of PC by LTP, since previous studies have shown that, unlike TG and CE which compete for transfer, PC transfer by LTP occurs independently of TG and/or CE transfers (6). However, it is yet to be established whether the transfer of PC by LTP is dependent on the same LTP-donor particle binding interactions as previously shown to be required for TG and CE transfer (10). To address this, we have used two approaches to perturb LTP-donor particle interactions and then compared the effects of the perturbation on PC and TG transfers. In the first, the LTP transfer reaction was titrated with divalent cations, which have been shown to prevent the binding of LTP to lipoproteins (32). As shown in **Fig. 2**, the LTP-mediated transfer of TG and PC from liposomes to LDL was progressively inhibited by increasing Mn^{2+} in the assay; essentially complete inhibition of both lipid transfers occurred at 0.3 M. This inhibition was not due to LDL precipitation, since this requires the presence of PO_4^{3-} or another polyvalent anion. Likewise, the plasma-derived inhibitor of LTP (19, 33), which inhibits LTP activity by disrupting and/or preventing the formation of LTP-lipoprotein complexes (10), identically suppressed the transfers of both PC and TG from liposomes to LDL (**Fig. 3**). Similar inhibitor results have been recently reported by Nishide, Tollefson, and Albers (33). Collectively, these results indicate that PC transfer depends on the same LTP binding interactions as neutral lipid transfer, and that LTP-facilitated PC transfer provides a reliable measure of the interaction of LTP with the donor particle.

Effects of TG or CE concentration on lipid transfer

The transfer of CE by LTP was markedly affected by the concentration of CE incorporated into the donor liposome (**Fig. 4**). The transfer rate for CE showed a concentration-dependent, saturable response with respect to CE, with half-maximum activity occurring at ~0.1 mole%. Over this CE concentration range, LTP-mediated PC transfer was essentially constant, varying less than 15% from the mean. Similar results were obtained for TG transfer from liposomes containing variable TG concentrations (**Fig. 5**), although the slope of the initial portion of the dose–response curve was less than that observed for CE liposomes, resulting in a half-maximum response at 0.2 mole% TG. Again, PC transfer was essentially constant over the TG range where TG transfer was concentration-dependent. However, when TG and CE were both incorporated into the same liposome and their concentrations were increased in parallel (TG/CE mole ratio = 1), identical saturation curves were observed for TG and CE,

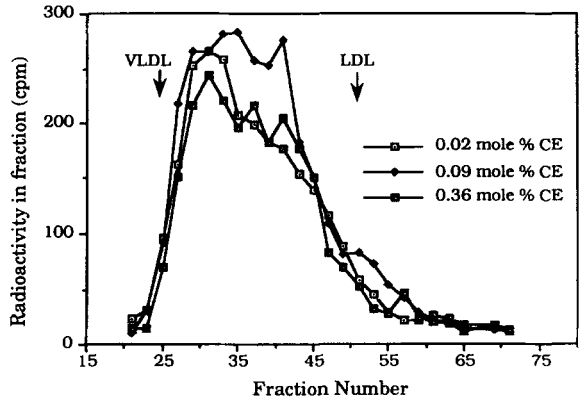

Fig. 1. Gel filtration profiles of cholesteryl ester-containing liposomes. [^{14}C]Phosphatidylcholine liposomes containing the indicated mole% of [^{3}H]cholesteryl oleate were applied to a Bio-Gel A-15m column (1 × 46 cm) equilibrated with Tris/NaCl buffer containing 0.1% BSA at 4°C. Samples were eluted at 7 ml/h; 0.5-ml fractions were collected, and the distribution of radiolabeled lipids was determined by liquid scintillation counting. Data are shown for [^{3}H]cholesteryl oleate only. Arrows mark the elution peaks of VLDL and LDL; LDL eluted in fractions #45–60.

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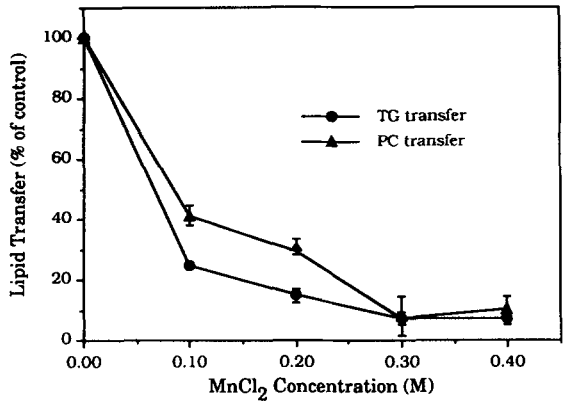

Fig. 2. Effect of Mn^{2+} on triglyceride and phosphatidylcholine transfer. The effect of Mn^{2+} on LTP-mediated transfer of [^{3}H]TG and [^{14}C]PC from radiolabeled donor liposomes (200 nmol PC) containing 0.3 mole% TG to unlabeled LDL (10 μg cholesterol) was measured as described in the Experimental Procedures. LTP-mediated transfer of TG was determined at each $MnCl_2$ concentration as the difference between samples containing partially purified LTP (13 μg protein) and incubated for 0 or 1.5 h. PC-facilitated transfer was assayed in the same manner except that 65 μg LTP was used. The data shown are the means ± SE of duplicate determinations at each $MnCl_2$ concentration. In the absence of $MnCl_2$, LTP-mediated transfers of TG and PC were 28.7 and 4.0% kt, respectively. The results are representative of two similar experiments.

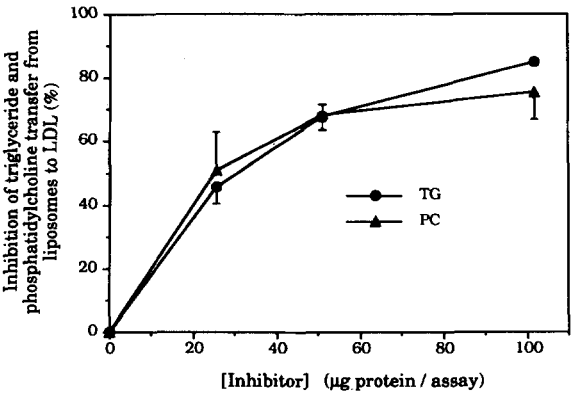

Fig. 3. Suppression of TG and PC transfer by LTP inhibitor protein. The effect of the plasma-derived inhibitor of LTP on the facilitated transfer of [^{3}H]TG and [^{14}C]PC from radiolabeled liposomes (120 nmol PC) containing 0.3 mole% TG to unlabeled LDL (6 μg cholesterol) was determined as described in the Experimental Procedures except that the donor and acceptor particles were separated by $MnCl_2$ precipitation. The transfer mediated by partially purified LTP (13 μg protein) in the absence or presence of the indicated amount of inhibitor fraction was calculated from the difference between blank samples (t = 0 h) and those incubated for 1 h (TG transfer) or for 2 h (PC transfer). Facilitated transfer in the absence of inhibitor was 23.5 and 1.9% kt for TG and PC, respectively. The data points shown are the means ± SE of four to six samples at each inhibitor concentration.

with an apparent K_m intermediate to those observed above with either lipid alone (**Fig. 6**). Thus, these results demonstrate that in the face of a constant number of LTP-liposome interactions, as determined by PC transfer, the rate of CE or TG transfer was directly dependent on the concentration of these lipids in the phospholipid bilayer of the liposome.

Previous studies have shown that the TG/CE composition of a lipoprotein is a strong determinant of whether that particle is a better donor of TG or CE to LTP, indicating that TG and CE compete for transfer (6). In the present study, the liposome assay was used to determine whether this relationship could be modeled by altering the TG/CE composition of the surface lipids. As shown in **Fig. 7**, as the liposome concentration of CE was increased and its content of TG was maintained constant (0.1 mole%), CE transfer increased in a fashion similar to that shown in Fig. 4, but TG transfer was progressively inhibited. However, consistent with the nearly linear kinetics observed at low neutral lipid concentrations (Fig. 6), TG transfer did not appear to be significantly affected by the level of CE in the liposome until the CE concentration exceeded ~0.1 mole%. These data suggest that the competition between CE and TG observed with lipoprotein substrates can be explained by compositional changes in the surface lipids, and that physiologically, since the competition does occur, the concentration of CE and TG in the phospholipid surface of lipoproteins must be sufficient to support mixed order LTP kinetics.

Coupling of transfer

In the above experiments, lipid transfer was measured from a donor (liposomes) containing small amounts of CE and/or TG to an acceptor (LDL) which, by comparison, contained large amounts of these lipids. To determine how the limited availability of TG or CE in one particle affects

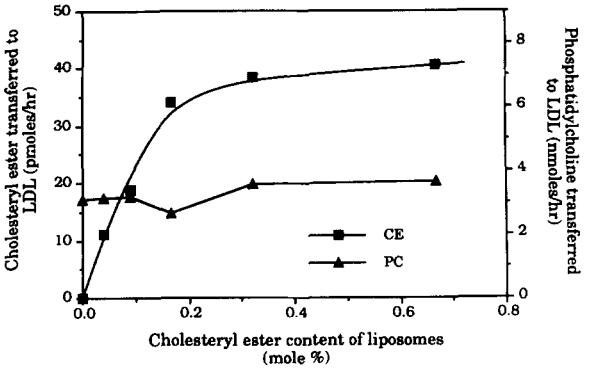

Fig. 4. Effect of liposome CE concentration on CE and PC transfer. The effect of varying the CE content of donor liposomes on the rate of [^{3}H]CE and [^{14}C]PC transfer from radiolabeled liposomes (190 nmol PC) to unlabeled LDL (10 μg cholesterol) was determined as described in the Experimental Procedures. Partially purified LTP (0.9 or 11 μg protein for CE or PC transfer, respectively), if present, and all other assay components except LDL were pre-incubated for 30 min at 37°C, then LDL was added and the incubation was continued for an additional 60 min. Data points are the mean of duplicate determinations. Transfers did not exceed 15% kt for CE with any liposome substrate. Values on the abscissa are expressed as the mole% of CE relative to the liposome PC content. These results are representative of three similar experiments.

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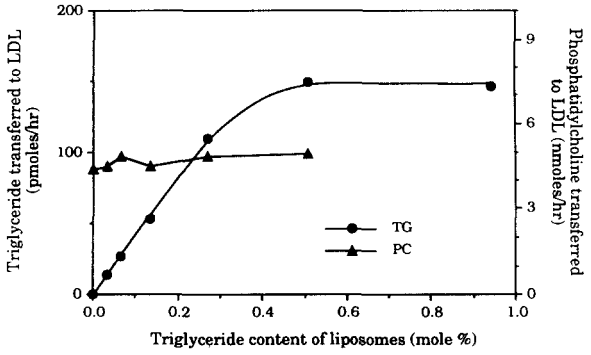

Fig. 5. Effect of liposome TG concentration on TG and PC transfer. The effect of varying the TG content of donor liposomes on the rate of [^{3}H]TG and [^{14}C]PC transfer from radiolabeled liposomes (308 nmol PC) to unlabeled LDL (10 μg cholesterol) was determined as described in the Experimental Procedures. Partially purified LTP (8.4 μg protein), if present, and all other assay components except LDL were pre-incubated for 30 min at 37°C, then LDL was added and the incubation was continued for an additional 60 min. Data points are the mean of duplicate determinations. Transfers did not exceed 20% kt for TG with any liposome substrate. PC data for liposomes containing >0.75 mole% TG were highly variable between preparations of liposomes, thus the PC transfer data for liposomes containing the highest TG level have been omitted. These results are representative of three similar experiments.

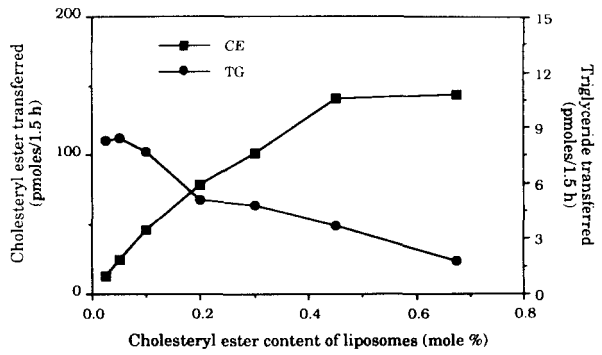

Fig. 7. Competition of CE and TG for transfer. The effect of varying the CE content of TG-containing liposomes on the competition of [^{3}H]TG and [^{14}C]CE for transfer was measured as described in the Experimental Procedures. Labeled liposomes (290 nmol PC) with the indicated [^{14}C]CE content but also containing 0.1 mole% [^{3}H]TG were incubated with unlabeled LDL (10 μg cholesterol), and ± partially purified LTP (27 μg protein) for 1.5 h. The extent of TG and CE transfer was determined by the LTP-facilitated increase in LDL radioactivity compared to blank samples incubated without LTP. The data points are the means of four values. These results are representative of two similar experiments.

the bidirectional movement of lipids between liposomes and LDL, we determined the dependence of lipid transfer in the reverse direction, i.e., from LDL to liposomes, on the amount of TG or CE incorporated into the acceptor liposomes. As seen in **Fig. 8**, CE transfer from LDL to liposomes followed saturation kinetics that was dependent on the CE content of the liposomes despite the fact that this lipid was present in "high" constant levels in the LDL donor. Very similar results were obtained for TG transfer from LDL to TG liposomes (data not shown). The tight coupling of lipid transfer between LDL and liposomes is clearly illustrated in **Fig. 9**; the transfer of CE from liposomes to LDL, and in the reverse direction, showed nearly identical dependence of the concentration of CE in the liposome particle.

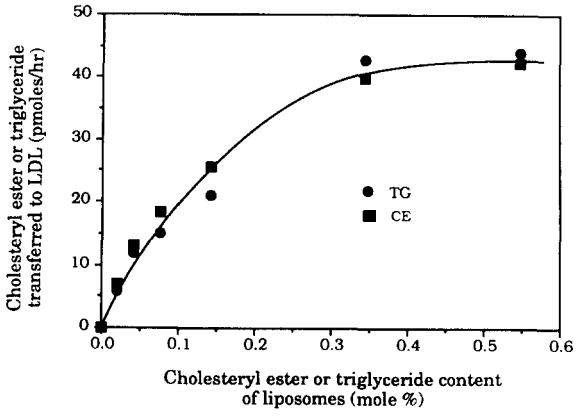

Fig. 6. Effect of TG and CE concentrations on their transfer from liposomes. The effect of simultaneously varying the CE and TG content of liposomes on the transfer of [^{3}H]TG and [^{14}C]CE from liposomes to LDL was measured as described in the Experimental Procedures. Radiolabeled liposomes (183 nmol PC) containing the indicated amount of CE or TG (CE/TG mole ratio held constant at 1) were combined with or without LTP (3.2 μg protein for TG transfers and 0.6 μg for CE transfers) and other usual assay reagents and incubated at 37°C for 30 min. Following the addition of LDL (10 μg cholesterol), the incubation was continued for 60 min. The results shown are the average of duplicate samples; these results are representative of three similar experiments.

DISCUSSION

Several lines of evidence suggest that the actual substrates for LTP are the small amounts of TG and CE that are solubilized by the phospholipid coat of the lipoprotein, and thus are more accessible to the surface (34, 35), rather than the bulk of CE and TG that reside in the lipoprotein core. First, TG and CE compete for transfer by LTP; this competition is driven by the ratio of TG and CE in the lipoprotein, and the sum of TG and CE transferred is essentially constant over a range of TG/CE compositions that encompass the TG/CE ratios in normolipemic lipoproteins (6). This competition for transfer is paralleled by the observation that in liposomes containing TG/CE ratios similar to those in plasma lipoproteins, TG and CE compete for solubility in the phospholipid bilayer and the sum of TG and CE dissolved in the phospholipids is almost constant (36). And secondly, recent in vitro studies have demonstrated that the free cholesterol in lipoproteins, which resides principally in the phospholipid monolayer of lipoproteins (34, 37), has marked effects on CE transfer but minimal effects on TG transfer (11)—a pattern of activity that is consistent with the effects that free

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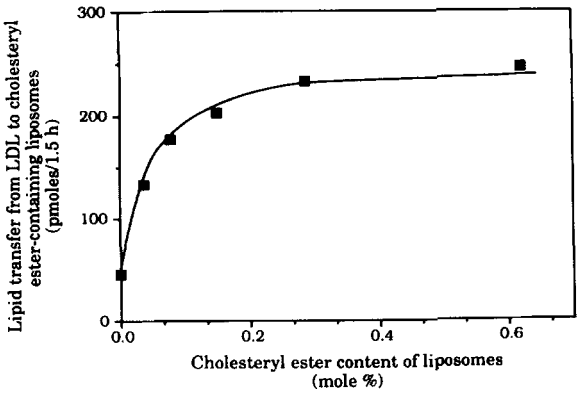

Fig. 8. Effect of the CE content of acceptor liposomes on lipid transfer from LDL. The effect of varying the CE content of the acceptor particle on the transfer of CE from LDL to that particle was studied by incubating [^{14}C]CE-labeled LDL (3 μg cholesterol, 5.2 nmol CE) with unlabeled liposomes (1435 nmol PC) containing 0–0.6 mole% CE, in the presence or absence of partially purified LTP (29 μg protein) for 1.5 h as described in the Experimental Procedures. Due to the small pool of CE in the acceptor particle, assay conditions were chosen so that the extent of transfer of CE from LDL was relatively small (<26%) compared to the pool of CE available in the acceptor liposome (except for the liposome containing no CE). Calculation of transfer values assuming bidirectional exchange of label, instead of the usual calculation which assumes unidirectional radiolabel transfer, did not result in a curve markedly different than that shown above. The data points are the means of duplicate samples; these results are representative of three similar experiments.

cholesterol has on the solubility of these lipids in the surface phospholipids of lipoproteins model systems (12–15).

Consistent with the forgoing data, in this study we have demonstrated that the transfer rates of CE and TG are highly dependent on the concentration of these lipids incorporated into the PC of substrate liposomes. Saturation kinetics of LTP activity was observed at TG or CE concentrations that were well below the solubility of these lipids in the phospholipid layer (14, 15), indicating that the saturability of the response was not due to physical solubility limits. The steepness of the first-order portion of the dose–response curve illustrates that small changes in TG or CE concentration in the phospholipid surface have marked effects on LTP activity; transfer activity was stimulated twofold by a 0.1 mole% increase in TG or CE concentration. Importantly, throughout the titration of CE and TG transfer activities, phospholipid transfer, an indicator of LTP-donor particle binding interactions, was essentially constant. Thus, not only are transfer rates dependent on the concentration of CE and TG that are present in the phospholipid bilayer, but the efficiency of LTP in transferring CE or TG increased with the concentration of these lipids. At low concentrations of neutral lipid, LTP binds to the liposome but a portion of these complexes dissociate without facilitating neutral lipid transfer, and with increasing concentrations each binding event is more likely to be productive. It should be noted that the interaction of LTP with the liposome substrate did not require the presence of CE or TG in this particle since LTP-mediated phospholipid transfer was the same for liposomes containing or lacking these lipids.

In reverse experiments in which the CE or TG content of the acceptor liposome was varied and the neutral lipid content of the donor remained constant, lipid transfer again proceeded at a rate that was defined by the concentration of neutral lipids in the liposome despite the fact that the donor TG and CE content was much higher. In fact, lipid transfer from liposomes to LDL (standard assay) and from LDL to liposomes (reverse assay) were identically dependent on the concentration of CE or TG in the liposome phospholipid bilayer. This result is consistent with the lipid exchange mechanism previously described for LTP (6) and further demonstrates that LTP-mediated lipid transfer is a tightly coupled, bidirectional process.

It should be pointed out that the saturable dependence of lipid transfer on increasing concentrations of neutral lipid in liposomes may not reflect saturation of the transfer protein with substrate per se, but may rather reflect the point at which the effective concentration of TG and/or CE in the other particle (LDL) becomes rate-limiting. This possibility is supported by preliminary studies in which the dose–response curves observed herein could be shifted by alterations (11) in the lipid composition of LDL (Morton, R. E., and J. V. Steinbrunner, unpublished observations). If true, this suggests that LTP may be a useful tool to determine the effective concentration of substrate lipids in the surface of native lipoproteins. Nonetheless, the data from Fig. 7, in which the TG/CE ratio of liposomes was varied, suggest that the surface concentration of TG and CE in all plasma lipoproteins must exceed an effective concentration of ~0.1 mole%, since it was only above this level that TG and CE appeared to compete for transfer by LTP—a characteristic of lipid transfer from lipoproteins (6).

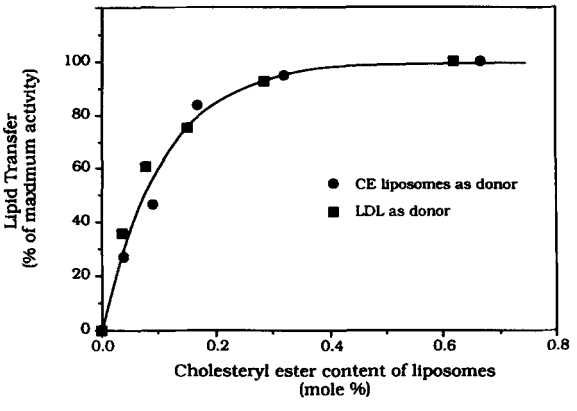

Fig. 9. Coupled bidirectional lipid transfer. The transfer data from Figs. 5 and 7 are plotted as % of maximum activity; transfer that occurred with liposomes containing no CE was set to zero.

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Numerous studies have shown that the chemical composition of lipoproteins affects their ability to function as LTP substrates. For example, modification of lipoprotein surface properties by prostaglandin E_1 (38) or by apoproteins (39) has been shown to markedly increase LTP-mediated neutral lipid transfer. Additionally, lipoproteins from subjects with hyperbetalipoproteinemia or dysbetalipoproteinemia (40), or from patients with end-stage renal disease undergoing hemodialysis (41), exhibit reduced rates of CE transfer compared to lipoproteins from normolipemic individuals. In contrast, lipoproteins from insulin-dependent diabetics (42) or those collected from plasma during alimentary lipemia (43) facilitate increased CE transfer activity when compared to control lipoproteins assayed under standardized conditions. The basis for these changes in LTP reactivity, for the most part, remains unclear, but may relate to the availability of TG and CE for transfer by LTP.

The data presented herein are consistent with the following proposed mechanism of action for LTP. LTP binds to the surface of donor lipoproteins either as a donor-LTP complex, from which it will subsequently facilitate lipid transfer by a carrier mechanism after dissociation of the complex (44), or as a donor-acceptor-LTP complex, from which it will mediate lipid transfer between those lipoprotein particles within the complex (45). These complexes are short-lived relative to the rate at which CE or TG can diffuse within the phospholipid layer to LTP, thus the rate of transfer increases with increasing CE or TG concentration. In the absence of saturating TG or CE concentrations, LTP complexes may dissociate without facilitating lipid transfer, or without initiating those events required for neutral lipid transfer to occur. At a given CE + TG concentration in the lipoprotein surface, the "percent successful transfers" would be constant; thus, TG and CE transfers would be determined by their relative concentrations in the donor particle surface. However, if the concentrations of neutral lipids in the surface were to vary, then the overall rate of lipid transfer could change. If only the concentration of CE or TG is changed and the other remained unchanged, such as the proposed to occur with free cholesterol modification (11), then only the transfer rate of the changed lipid would be altered while the other would remain the same since its chance of interacting with LTP before the complex dissociates would not have changed. Such a mechanism, however, requires that the concentration of neutral lipids in the surface of lipoproteins is below saturation of the LTP reaction, and this has yet to be determined.

In conclusion, we have demonstrated in this study that the rate of lipid transfer is closely dependent on the concentration of neutral lipids dissolved in the phospholipid surface of donor and acceptor particles. Since the binding interaction of LTP with liposomes was independent of their neutral lipid content, the data indicate that an unfruitful LTP-donor particle complex can form, resulting in LTP dissociating "empty-handed" with respect to CE or TG. By titrating the neutral lipid content of the donor and acceptor particles, we have further demonstrated that lipid transfer between two particles is tightly coupled and bidirectional. We propose that LTP activity can be regulated by any lipoprotein modification that alters the surface availability of TG and CE to the transfer protein; the effect this has on lipoprotein metabolism would be more profound if the modification preferentially affected certain lipoprotein classes or one lipid species more than another.

The technical assistance of Diane Greene and Tracy Evans is gratefully acknowledged. This research was supported, in part, by grants HL 36595 and HL 29582 from the National Heart, Lung, and Blood Institute of the National Institutes of Health.

Manuscript received 10 January 1990 and in revised form 30 April 1990.

REFERENCES

1. Morton, R. E., and D. B. Zilversmit. 1982. Purification and characterization of lipid transfer protein(s) from human lipoprotein-deficient plasma. *J. Lipid Res.* **23:** 1058–1067.
2. Hesler, C. B., A. R. Tall, T. L. Swenson, P. K. Weech, Y. L. Marcel, and R. W. Milne. 1988. Monoclonal antibodies to the M_r 74,000 cholesteryl ester transfer protein neutralize all of the cholesteryl ester and triglyceride transfer activities in human plasma. *J. Biol. Chem.* **263:** 5020–5023.
3. Hesler, C. B., T. L. Swenson, and A. R. Tall. 1987. Purification and characterization of a human plasma cholesteryl ester transfer protein. *J. Biol. Chem.* **262:** 2275–2282.
4. Jarnagin, A. S., W. Kohr, and C. Fielding. 1987. Isolation and specificity of a M_r 74,000 cholesteryl ester transfer protein from human plasma. *Proc. Natl. Acad. Sci. USA.* **84:** 1854–1857.
5. Albers, J. J., J. H. Tollefson, C-H. Chen, and A. Steinmetz. 1984. Isolation and characterization of human lipid transfer proteins. *Arteriosclerosis.* **4:** 49–58.
6. Morton, R. E., and D. B. Zilversmit. 1983. Inter-relationship of lipids transferred by the lipid-transfer protein isolated from human lipoprotein-deficient plasma. *J. Biol. Chem.* **258:** 11751–11757.
7. Morton, R. E. 1986. Specificity of lipid transfer protein from molecular species of cholesteryl ester. *J. Lipid Res.* **27:** 523–529.
8. Deckelbaum, R. J., S. Eisenberg, Y. Oschry, E. Butbul, I. Sharon, and T. Olivecrona. 1982. Reversible modification of human plasma low density lipoproteins toward triglyceride-rich particles: a mechanism for losing excess cholesterol esters. *J. Biol. Chem.* **257:** 6509–6517.
9. Swenson, T. L., R. W. Brocia, and A. R. Tall. 1988. Plasma cholesteryl ester transfer protein has binding sites for neutral lipids and phospholipids. *J. Biol. Chem.* **263:** 5150–5157.
10. Morton, R. E. 1985. Binding of plasma-derived lipid transfer protein to lipoprotein substrates. *J. Biol. Chem.* **260:** 12593–12599.
11. Morton, R. E. 1988. Free cholesterol is a potent regulator

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of lipid transfer protein function. *J. Biol. Chem.* **263:** 12235–12241.
12. Miller, K. W., and D. M. Small. 1983. Triolein-cholesteryl oleate-cholesterol-lecithin emulsions: structural models of triglyceride-rich lipoproteins. *Biochemistry.* **22:** 443–451.
13. Miller, K. W., and D. M. Small. 1982. The phase behavior of triolein, cholesterol, and lecithin emulsions. *J. Colloid Interface Sci.* **89:** 466–478.
14. Spooner, P. J. R., J. A. Hamilton, D. L. Gantz, and D. M. Small. 1986. The effect of free cholesterol on the solubilization of cholesteryl oleate in phosphatidylcholine bilayers: a ^{13}C-NMR study. *Biochim. Biophys. Acta.* **860:** 345–353.
15. Spooner, P. J. R., and D. M. Small. 1987. Effect of free cholesterol on incorporation of triolein in phospholipid bilayers. *Biochemistry.* **26:** 5820–5825.
16. Pinter, K. G., J. G. Hamilton, and J. E. Muldrey. 1964. A method for rapid microsynthesis of radioactive cholesterol esters. *J. Lipid Res.* **5:** 273–274.
17. Burstein, M., H. R. Scholnick, and R. Morfin. 1970. Rapid method for the isolation of lipoproteins from human serum by precipitation with polyanions. *J. Lipid Res.* **11:** 583–595.
18. Pattnaik, N. M., A. 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.
19. Morton, R. E., and D. B. Zilversmit. 1981. A plasma inhibitor of triglyceride and cholesteryl ester transfer activities. *J. Biol. Chem.* **256:** 11992–11995.
20. Morton, R. E., and D. B. Zilversmit. 1981. The separation of apolipoprotein D from cholesteryl ester transfer protein. *Biochim. Biophys. Acta.* **663:** 350–355.
21. Havel, R. J., H. A. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34:** 1345–1353.
22. Ritter, M. C., and A. M. Scanu. 1977. Role of apolipoprotein A-I in the structure of human serum high density lipoproteins. *J. Biol. Chem.* **252:** 1208–1216.
23. Brunner, J., P. Skrabal, and H. Hauser. 1976. Single bilayer vesicles prepared without sonication: physico-chemical properties. *Biochim. Biophys. Acta.* **455:** 322–331.
24. Phillips, M. C., W. J. Johnson, and G. H. Rothblat. 1987. Mechanisms and consequences of cellular cholesterol exchange and transfer. *Biochim. Biophys. Acta.* **906:** 223–276.
25. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
26. Peterson, G. L. 1977. A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Anal. Biochem.* **83:** 346–356.
27. Gamble, W., M. Vaughan, H. S. Kruth, and J. Avigan. 1978. Procedure for determination of free and total cholesterol in micro- or nanogram amounts suitable for studies with cultured cells. *J. Lipid Res.* **19:** 1068–1070.
28. Zak, B., N. Moss, A. J. Boyle, and A. Zlatkis. 1954. Reactions of certain unsaturated steroids with acid iron reagent. *Anal. Chem.* **26:** 776–777.
29. Sardesai, V. M., and J. A. Manning. 1968. The determination of triglycerides in plasma and tissues. *Clin. Chem.* **14:** 156–161.
30. Mendez, A. J., C. Cabeza, and S. L. Hsia. 1986. A fluorometric method for the determination of triglycerides in nanomolar quantities. *Anal. Biochem.* **156:** 386–389.
31. Bartlett, G. R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* **234:** 466–468.
32. Pattnaik, N. M., and D. B. Zilversmit. 1979. Interaction of cholesterol ester exchange protein with human plasma lipoproteins and phospholipid vesicles. *J. Biol. Chem.* **254:** 2782–2786.
33. Nishide, T., J. H. Tollefson, and J. J. Albers. 1989. Inhibition of lipid transfer by a unique high density lipoprotein subclass containing an inhibitor protein. *J. Lipid Res.* **30:** 149–158.
34. Miller, K. W., and D. M. Small. 1983. Surface-to-core and interparticle equilibrium distributions of triglyceride-rich lipoprotein lipids. *J. Biol. Chem.* **258:** 13772–13784.
35. Schneider, H., R. S. Morrod, J. R. Colvin, and N. H. Tattrie. 1973. The lipid core model of lipoproteins. *Chem. Phys. Lipids.* **10:** 328–353.
36. Hamilton, J. A., K. W. Miller, and D. M. Small. 1983. Solubilization of triolein and cholesteryl oleate in egg phosphatidylcholine vesicles. *J. Biol. Chem.* **258:** 12821–12826.
37. Deckelbaum, R. J., G. G. Shipley, and D. M. Small. 1977. Structure and interactions of lipids in human plasma low density lipoproteins. *J. Biol. Chem.* **252:** 744–754.
38. Muzya, G. I., E. N. Korobkova, N. K. Golovanova, and L. D. Bergelson. 1987. Influence of prostaglandins on the lipid transfer between human high density and low density lipoproteins. *FEBS Lett.* **220:** 371–375.
39. Nishikawa, O., S. Yokoyama, H. Okabe, and A. Yamamoto. 1988. Enhancement of non-polar lipid transfer reaction through stabilization of substrate lipid particles with apolipoproteins. *J. Biochem.* **103:** 188–194.
40. Fielding, P. E., C. J. Fielding, R. J. Havel, J. P. Kane, and P. Tun. 1983. Cholesterol net transport, esterification, and transfer in human hyperlipidemic plasma. *J. Clin. Invest.* **71:** 449–460.
41. Dieplinger, H., P. Y. Schoenfeld, and C. J. Fielding. 1986. Plasma cholesterol metabolism in end-stage renal disease: differences between treatment by hemodialysis or peritoneal dialysis. *J. Clin. Invest.* **77:** 1071–1083.
42. Bagdade, J. D., M. Ritter, R. Levy, and P. V. Subbaiah. 1987. Accelerated cholesterol ester transfer and abnormal surface lipoprotein composition in type 1 diabetics. *Circulation.* **76, Suppl. IV:** 416.
43. Tall, A., D. Sammett, and E. Granot. 1986. Mechanisms of enhanced cholesteryl ester transfer from high density lipoproteins to apolipoprotein B-containing lipoproteins during alimentary lipemia. *J. Clin. Invest.* **77:** 1163–1172.
44. Barter, P. J., G. J. Hopkins, L. Gorjatschko, and M. E. Jones. 1982. A unified model of esterified cholesterol exchanges between human plasma lipoproteins. *Atherosclerosis.* **44:** 27–40.
45. Ihm, J., D. M. Quinn, S. J. Busch, B. Chataing, and J. A. K. Harmony. 1982. Kinetics of plasma protein-catalyzed exchange of phosphatidylcholine and cholesteryl ester between plasma lipoproteins. *J. Lipid Res.* **23:** 1328–1341.

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