The surface cholesteryl ester content of donor and acceptor particles regulates CETP: a liposome-based approach to assess the substrate properties of lipoproteins

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Abstract **Cholesteryl ester transfer protein (CETP) activity is regulated, in part, by lipoprotein composition. We previously demonstrated that CETP activity follows saturation kinetics as cholesteryl ester (CE) levels in the phospholipid surface of donor particles are increased. We propose here that the plateau of CETP activity occurs because the surface concentration of CE in the acceptor becomes rate limiting. This hypothesis was tested in CETP assays between synthetic liposomes whose CE content was varied independently. As donor CE increased, CETP activity followed saturable kinetics, but the slope of the first-order portion of the curve and the maximum achievable CE transfer rate were linearly related to the acceptor's surface CE concentration. These findings, plus studies with free cholesterol-modified LDL, strongly suggest that CE-rich donor liposomes can measure the CETP-accessible CE in acceptor lipoproteins. CETP activity from CE-rich liposomes to multiple control LDLs ranged 1.8-fold despite equivalent CETP binding capacity, suggesting that LDLs vary widely in their capacity to present CE to CETP. Thus, CETP activity depends on the surface availability of substrate lipids in the donor and acceptor. Donor liposomes with high CE content can be used to assess how subtle changes in composition alter the substrate potential of plasma lipoproteins.**—Morton, R. E., and D. J. Greene. **The surface cholesteryl ester content of donor and acceptor particles regulates CETP: a liposome-based approach to assess the substrate properties of lipoproteins. *J. Lipid Res.* 2003.** 44: **1364–1372.**

Supplementary key words liposomes • low density lipoprotein • free cholesterol • kinetics • cholesteryl ester transfer protein binding

Cholesteryl ester transfer protein (CETP) mediates the exchange and net transfer of cholesteryl ester (CE) and triglyceride (TG) molecules between plasma lipoproteins (1, 2). The ability of CETP to facilitate the remodeling of lipoprotein composition endows an important role to this protein in the intravascular metabolism of lipids. This remodeling influences the inter-conversion of HDL subfractions (3–5), facilitates aspects of reverse cholesterol transport (2, 6–8), affects the level of LDL subfractions and their interaction with the LDL receptor (8–13), and facilitates the conversion of VLDL to LDL (1, 14). Overall, CETP activity significantly influences the structure and function of the lipoproteins with which it interacts.

Two mechanisms have been proposed for the CETP-mediated transfer of lipids. The carrier model suggests that CETP binds to the lipoprotein surface, where it picks up CE or TG and diffuses through the aqueous media to dock on an acceptor lipoprotein, where it releases its bound lipids (15). Alternatively, the ternary complex model (16) suggests that CETP transfer occurs within a temporary complex between itself and two lipoproteins. It has been argued that both mechanisms may occur physiologically depending on the levels of other factors, such as free fatty acids (17). However, several detailed kinetic studies (18–20), the demonstration that CETP has a specific binding site for CE and/or TG (21), that purified CETP contains neutral lipid bound to this site (21, 22), and that lipids bound to this site can be subsequently transferred to a lipoprotein (20, 22) strongly implicate the carrier model as the dominant mechanism of transfer. Regardless of the functional mechanism, it is apparent that the initial obligatory step for transfer is the binding of CETP to a lipoprotein (21, 23–26) through interaction with its surface phospholipids (PLs) (18, 23, 24, 26).

Most data suggest that the transfer of CE and TG between lipoproteins is a coupled process; i.e., CETP mediates the exchange of lipids between lipoproteins rather than unidirectional flux. This is supported by some long-term mass studies where CE loss and TG gain among lipoprotein fractions are nearly equimolar (1, 27–29), and by detailed radioisotope transfer studies (30), although this coupling has not always been observed (31–33). More-

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Abbreviations: CE, cholesteryl ester; CETP, cholesteryl ester transfer protein; FC, free cholesterol; PC, phosphatidylcholine; PL, phospholipid; TG, triglyceride.

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over, CE transfer to liposomes lacking CE or TG does not occur at significant rates, even though the donor particles are competent substrates (27, 34) and the acceptor liposomes lacking neutral lipids bind CETP normally (35). This also does not reflect an aberrant structure of the neutral lipid-lacking particle since the same results are observed for CE transfer when the acceptor contains only TG (no CE) but CETP has been chemically modified such that its capacity to bind TG has been blocked (27). Together, these data suggest that CETP-mediated transfer between two particles is predominately a coupled, i.e., true exchange, process.

We have previously shown that the rate of CE transfer from liposomes to LDL is dependent on the concentration of CE dissolved in the surface PLs of the donor particle (34). CETP activity was observed to increase and then plateau as the donor CE concentration increases. Since CETP-mediated PL transfer from these particles was unaffected by variations in the donor CE content, we concluded that binding of CETP to the lipoprotein surface, which is also required for PL transfer, is unaffected by the CE content of the acceptor (23, 34). These observations were interpreted to mean that CETP binds to a particle and may leave the surface without acquiring (or depositing) neutral lipid; the frequency at which CETP mediates a neutral lipid transfer event increases as the surface CE concentration in the donor particle increases.

As noted above, CETP-mediated transfer of CE between liposomes and LDL demonstrates saturation-type kinetics as the CE content of the liposome particle is increased. Although this saturation event could be interpreted as reflecting the saturation of CETP's lipid binding site with CE, the existing evidence suggesting that lipid transfer between particles is an exchange process led us to propose that these kinetics reflect conditions where the surface-available neutral lipid on the acceptor LDL particle becomes rate limiting. Here we test the hypothesis that CETP transfer activity is dependent on the concentration of CETP-accessible neutral lipid in both the donor and acceptor particle. This hypothesis is tested in assays between synthetic liposomes of defined CE content. These studies also led to an experimental approach that has been used to assess the functional surface CE concentration of native and modified lipoproteins.

EXPERIMENTAL PROCEDURES

Materials

Glycerol tri[9,10-^{3}H]oleate (26.8 Ci/mmol) was obtained from New England Nuclear (Boston, MA). [1α,2α(n)-^{3}H]cholesteryl oleate (48 Ci/mmol), cholesteryl [1-^{14}C]oleate (56 mCi/mmol), and 1-palmitoyl, 2-[^{14}C]oleoyl phosphatidylcholine (PC) (50.8 mCi/mmol) were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). BSA (fraction V), diethyl *p*-nitrophenyl phosphate, egg PC, butylated hydroxytoluene, dithiothreitol, biotin, avidin, CNBr-Sepharose, and all reagents for salt and buffer solutions were obtained from Sigma Chemical Co. (St. Louis, MO). Cholesterol and cholesteryl oleate were purchased from NuChek, and cardiolipin (beef heart) was from Serdary Research Labs, Inc. (Port Huron, MI). Lipid solutions were prepared in chloroform containing 10 μg/ml butylated hydroxytoluene and stored at −20°C. Phenyl Sepharose CL-4B, Con A-Sepharose, and dextran sulfate (M_r = 500,000) were from Amersham Pharmacia Biotech, and CM52-cellulose was from Whatman Chemical Separations, Inc. (Clifton, NJ).

Isolation of CETP

Partially purified CETP was isolated from lipoprotein-deficient human plasma by hydrophobic and ion exchange chromatography, as previously described (36). During the purification of CETP, lipid transfer activity was routinely assayed by determining the extent of radiolabel transferred from [^{3}H]CE-labeled LDL to unlabeled HDL (10 μg cholesterol each) (37, 38). These preparations of CETP are deficient in PL transfer protein (34), lipid transfer inhibitor protein (39), and lecithin:cholesterol acyltransferase activities (37), and do not differ from highly purified CETP with respect to TG-CE preference (36, 40) or binding affinity for lipoproteins (23, 41).

Lipoprotein isolation, radiolabeling, and modification

Fresh human plasma from the Blood Bank of the Cleveland Clinic Foundation was the source of LDL and HDL. Lipoproteins were isolated at 4°C by sequential ultracentrifugation (42), extensively dialyzed against 0.9% NaCl and 0.02% EDTA, pH 7.4, and stored at 4°C. In some instances, before isolation from plasma, lipoproteins were radiolabeled by a lipid dispersion technique (43). Under these labeling conditions, lipoproteins typically contained $\geq 1.6 \times 10^3$ dpm [^{3}H]CE/μg cholesterol. LDL free cholesterol (FC) content was altered by incubation with dipalmitoyl PC-FC dispersions (44). Modified lipoproteins were reisolated by heparin affinity chromatography; LDL was applied to the affinity matrix in 50 mM Tris-HCl, 150 mM NaCl, 0.02% EDTA, and 0.02% NaN_3, pH 7.4, then eluted with 3 M NaCl and dialyzed against the starting buffer.

Liposome preparation

PC-FC-CE liposomes with or without [^{3}H]CE (±[^{14}C]PC), and PC-FC-CE liposomes containing [^{3}H]CE plus 10 mol% cardiolipin were prepared by cholate dialysis using a modification (34) of the method of Brunner et al. (45). Egg PC was used in all liposomes. The content and the specific activity of CE in liposome preparations varied, depending on the experimental design. Generally, cardiolipin-PC liposome preparations contained 6.7×10^5 cpm/μmol PL and PC liposomes contained 1×10^5 cpm/μmol PL. Liposomes were routinely characterized with respect to radiolabel and PL phosphorus content.

CETP assays

Liposome-to-liposome assays. To measure CE transfer from [^{3}H]CE-cardiolipin-PC liposomes to unlabeled PC liposomes, donor and acceptor liposomes (at levels as indicated in the figure legends) were added to 3.5% BSA (20 μl), ±CETP and buffer, to a final volume of 120–145 μl. After incubation for 1.5 h at 37°C, samples were placed on ice until further processing. Donor and acceptor liposomes were separated by electrophoresis on premade 1% agarose gels (Ciba-Corning Diagnostics Inc., Palo Alto, CA). Two 2 μl aliquots of the assay mixture were applied, and the gels electrophoresed at 70 V for 30 min. Each lane was cut into two 4 cm strips, and their radioactivity content determined by scintillation counting in the presence of 0.1% SDS. In control experiments, >95% of cardiolipin-PC liposomes and PC liposomes were recovered in the upper (faster migrating) and lower portions of the gel, respectively. Variable CE content did not alter the electrophoretic mobility of liposomes to any significant extent. When calculating CETP-mediated CE transfer rates (see

below), the transfer from a given donor to an acceptor liposome lacking CE was subtracted from the observed transfers. This value does not reflect true CETP-mediated neutral lipid transfer, as evidenced by the lack of a normal preference of CETP for CE over TG (27), and probably reflects either changes in the electrophoretic mobility of a small fraction of liposomes due to the binding of CETP or minor exchange of cardiolipin between liposomes due to the PL transfer activity of CETP (46).

Liposome-to-LDL assays. CE transfer from [^{3}H]CE-PC liposomes to LDL was performed as previously described for lipoprotein-to-lipoprotein assays (37, 38). Liposome, LDL, and CETP concentrations are specified in the figure legends. After incubation, LDL was absorbed with Con A-Sepharose (34), which gave near-complete recovery of lipoprotein with low, reproducible contamination with donor particles.

Lipoprotein-to-lipoprotein assays. CE and TG transfers between radiolabeled HDL and LDL acceptors were determined as previously described (47).

All assays were carried out in a shaking water bath (Bellco Glass Inc., Vineland, NJ) at 37°C. In all instances, the radiolabel content of the acceptor fraction was determined after separation of the donor and acceptor, as referenced above. The fraction of radiolabeled donor lipid that was transferred to the acceptor particle was calculated as described before (37). In most instances, the fraction of CE transferred was small (<15%). The mass of CE transferred was therefore calculated from the fraction of radiolabel transferred times the specificity activity of CE in the donor at time zero. Radiolabel transfer in the absence of CETP was subtracted from the values reported. Duplicate values generally differed by <10%. The extent of transfer was linear over the assay time and the CETP concentrations used.

CETP binding assay. To measure the steady-state binding of CETP to different LDL preparations, LDLs were coupled to CNBr-Sepharose (1 mg LDL protein/ml gel) as previously described (23). Aliquots of LDL-Sepharose (150 μg protein) were incubated at 25°C in 50 mM Tris-HCl buffer, pH 7.4, containing 150 mM NaCl, 0.02% EDTA, 0.02% BSA, and 37–150 μg partially purified CETP. Samples were mixed continuously for 3 h, followed by brief low-speed centrifugation. Aliquots of the supernatant were assayed for CETP content in a [^{3}H]CE-LDL-to-HDL transfer assay (37, 38). Bound CETP was calculated as the difference between the unbound CETP in the supernatant and the total CETP added to the LDL-Sepharose.

Analytical procedures. Protein was quantitated by the method of Lowry et al. (48) as modified by Peterson (49), with BSA as standard. Total cholesterol of lipoproteins was assayed by a colorimetric, enzymatic kit (Sigma). FC was determined by a kit from Wako Diagnostics (Richmond, VA), and CE was calculated from the difference between total and FC values times 1.69 to correct for the fatty acid content of CE. The FC and CE content of organic solutions of lipids was assayed by the method of Zak et al. (50). TG was measured by the glycerol phosphate oxidase-Trinder enzymatic method (Sigma). Lipid phosphorus was quantitated by the method of Bartlett (51). For mole calculations, 653, 885, and 800 were used as average molecular weights of CE, TG, and PL, respectively.

RESULTS

CETP-mediated CE transfer between liposomes

Our earlier studies demonstrated that the transfer of CE from liposomes to LDL is characterized by a saturable response as the CE content of the donor liposome increased (34). To determine if these kinetics reflected saturation of CETP with CE substrate, or whether this reflected the point where the acceptor LDL became rate limiting, a series of studies were performed with liposomes as the donor and acceptor. The composition of a typical series of liposomes is shown in **Table 1**. Donor and acceptor liposome CE content was varied independently to determine transfer kinetics as a function of CE availability in each particle. As shown in **Fig. 1A**, the transfer of CE between liposomes was strongly influenced by the CE content of both the donor and acceptor. These transfer data were well described by the theoretical curve derived from a hyperbolic fit, indicating that under these conditions, CE transfer mimicked Michaelis-Menten kinetics. At low donor CE levels where the response was first-order, the slope of the curves was linearly related to the CE content of the acceptor ($r = 0.97$) (Fig. 1B). This indicates that even when donor CE is low, increased acceptor CE content can augment the rate of transfer. Similarly, maximum achievable transfer rates were directly related to CE availability in the acceptor liposome (Fig. 1C). By analogy to Michaelis-Menten kinetics, relative substrate specificity [maximum transfer activity (V_{max})/apparent K_m] increased 3-fold from the lowest to the highest acceptor CE content. This suggests that the frequency of successful transfers, i.e., where CETP binding leads to CE transfer instead of dissociation without affecting CE transfer, increases as CE content rises. Overall, the dependence of CE transfer activity on the availability of CE in both participating particles suggests that under conditions where CE in the donor is high, the rate of CE transfer is limited by the concentration of CE available on the surface of the acceptor particle.

The capacity of the CETP-mediated lipid transfer process to be used to assess the functional availability of CE in the acceptor was tested by measuring CE transfers from donor liposomes containing high CE levels to liposomes containing varying, lower levels of CE. Under these conditions, CETP transfer activity was highly correlated with ac-

TABLE 1. Phosphatidylcholine-cardiolipin liposomes

	Initial Composition		Final Composition		
Prep #	CE	CE/PL	CE	PL	CE/PL
	nmol	*mol%*	*% recovery*		*mol%*
1	2.1	0.021	73.7	72.4	0.021
2	4.0	0.040	71.6	69.3	0.042
3	8.2	0.082	69.7	72.1	0.079
4	16.3	0.163	66.8	72.9	0.150
5	24.7	0.247	70.2	75.2	0.230
6	41.0	0.410	74.9	76.2	0.403
7	61.5	0.615	75.0	75.5	0.614
8	86.0	0.860	68.6	72.1	0.818

CE, cholesteryl ester; FC, free cholesterol; PL, phospholipid. Liposomes were prepared by the cholate dialysis procedure described in the Experimental Procedures section. Initially, solutions contained 9 μmol phosphatidylcholine (PC), 1 μmol cardiolipin, 2.5 μmol FC, and the indicated amount of cholesteryl oleate. All preparations received 6.74×10^6 cpm [^{3}H]cholesteryl oleate. The recovery of PL was determined by phosphorus analysis of the final preparations after dialysis, centrifugation, and ultrafiltration (0.45 μm). Cholesteryl oleate recovery was based on its radioactivity.

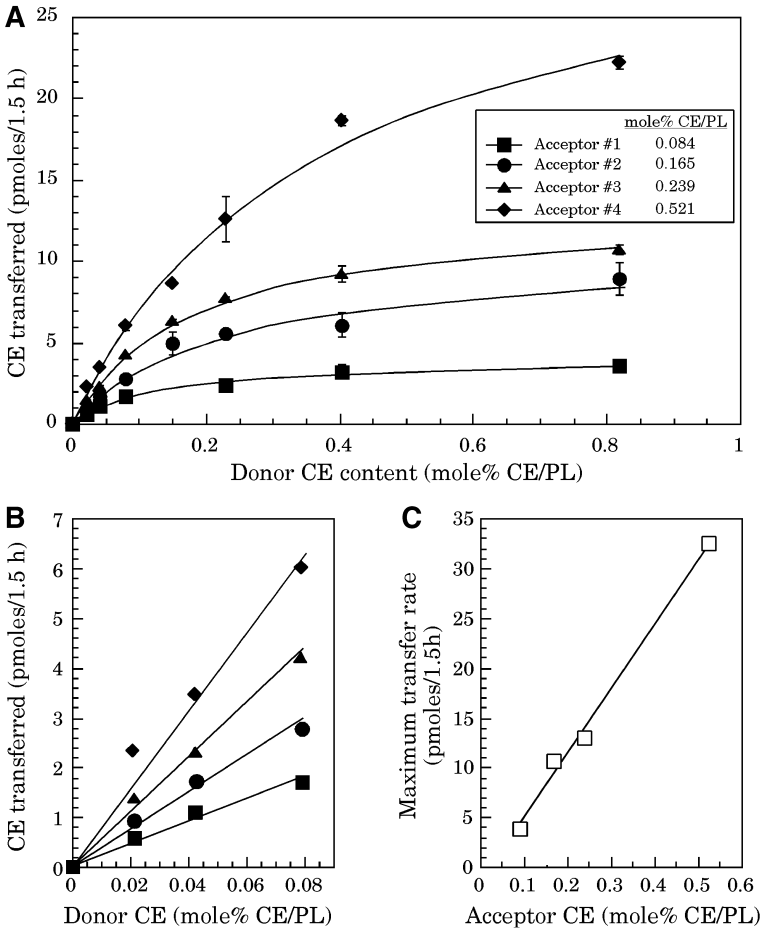

Fig. 1. Dependence of cholesteryl ester transfer protein (CETP) activity on the concentration of cholesteryl ester (CE) in donor and acceptor liposomes. A: [^{3}H]CE-labeled cardiolipin-phosphatidylcholine (PC) liposomes [53 nmol phospholipid (PL), 4.6×10^4 cpm [^{3}H]CE] and unlabeled PC liposomes (265 nmol PL) containing the indicated mol% CE (relative to PL) were incubated with CETP (0.39 μg) as described in the Experimental Procedures. Data points are mean ± SD. The lines are computer-generated fits to a hyperbolic function [y = mx/(n+x), where y is the transfer rate, x is the substrate concentration, and m and n are equivalent to V_{max} and K_m parameters, respectively). B: Plot of transfer rates at low donor CE content. Data are the same as those shown in A except the scale is expanded to show the response of CETP activity to the three liposome donors containing the lowest CE levels. C: Variations in maximum transfer values as a function of the CE content of the acceptor. Maximum transfer rates were determined from the curve fit parameters obtained in A. Results are the average of duplicate determinations and are representative of at least three similar experiments.

ceptor CE levels (**Fig. 2**). By extrapolation, this curvilinear response appeared to approach a maximum rate where the acceptor CE content was equivalent to the donor CE level.

CE surface-accessibility in LDL

The foregoing data indicate that CETP activity can be used to determine the functionally accessible CE on the surface of substrate particles, provided that the corresponding particle used in the transfer assay contains high levels of CE. In a previous study, an increased FC content of LDL resulted in decreased CE transfer rates (44). It was proposed that this decrease was due to a reduction in the CE available at the lipoprotein surface since FC and CE may compete for residency in the PL monolayer (52, 53). To test this hypothesis directly, the kinetics of lipid transfer from liposomes with progressively increasing CE content to FC-modified LDL (**Table 2**) were measured. Elevated LDL FC/PL ratios reflected increased FC content without significant change in PL (44). FC enrichment resulted in LDL particles that were progressively poorer substrates for CE transfer (**Fig. 3A**). FC enrichment led to a dose-dependent linear decrease in the maximum achievable transfer rate and the apparent K_m for CE (Fig. 3B and inset). Maximum achievable transfer activity declined with a slope of −152% (r = 0.994) for each unit increase in LDL FC/PC. A doubling of the FC/PL ratio, a variation commonly observed in vivo (54–56), resulted in a >2-fold decrease in maximum CETP activity. Similarly, the apparent K_m for CE transfer decreased with a slope of −0.35 mol% CE per unit increase in FC/PC (R = 0.955). These kinetics suggest that FC functions as an uncompetitive inhibitor of CE transfer, which is consistent with a mechanism whereby FC displaces CE from the PL surface, resulting in the formation of unproductive CETP-lipoprotein complexes that dissociate without affecting a transfer event. The magnitude of the FC effect on CE transfer, compared with the rather small increase in total surface lipid (FC+PL) upon FC enrichment, makes it unlikely that the suppression of CE transfer is mediated simply by dilution of surface CE by FC.

We have previously observed that the capacity of native LDL preparations to serve as acceptors of CE transferred from HDL is variable. For example, among LDL isolated from six random blood bank donors and subsequently assayed with the same [^{3}H-CE]HDL donor and CETP source, CE transfer activity ranged from 8.7% to 16.1% CE transfer/1.5 h. To better understand the cause of this variability in the substrate properties of native LDL, we subsequently isolated LDLs from six normolipidemic individuals and determined their capacity to bind CETP and to support CE transfer. These LDLs were similar in FC/PL content (range 0.93–1.01 mol/mol) and PL-protein (range 0.71–0.75 μg/μg). Isolated LDLs were used as acceptors of

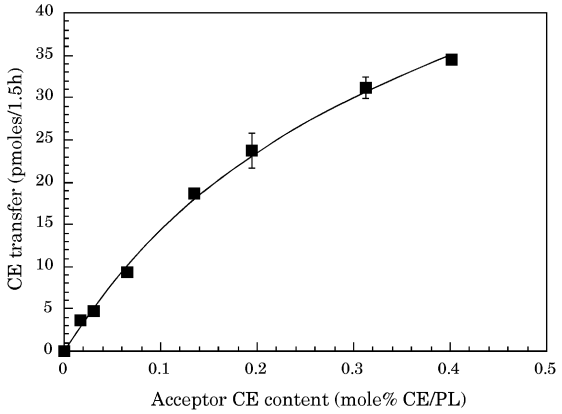

Fig. 2. CETP activity dependence on the CE content of acceptor particles. CE transfer from a radiolabeled donor containing high levels of CE [0.619 mol% (mol% CE/PL), 300 pmol/assay] to acceptor liposomes containing varying lower levels of CE was determined as described in Fig. 1. The line is a computer-generated fit to a hyperbolic function [y = mx/(n+x)] (see Fig. 1). Data points are mean ± SD.

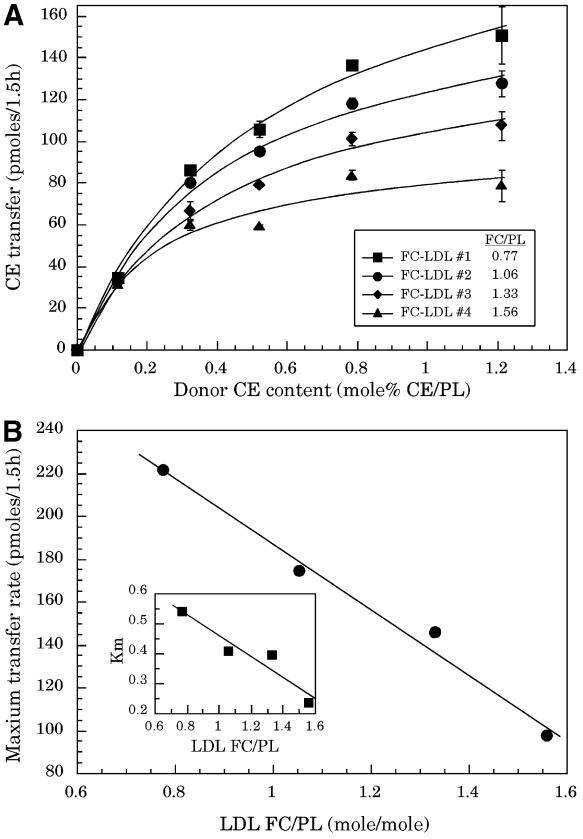

Fig. 3. Kinetics of CE transfer from liposomes to free cholesterol (FC)-modified LDL. The FC content of LDL was modified by incubation with FC-PL vesicles as described in the Experimental Procedures. A: CE transfer from PC liposomes (190 nmol PL, ~1 × 10^4 cpm [^{3}H]CE) containing the indicated level of CE to FC-modified LDL (10 μg protein) by CETP (7.7 μg) was determined under standardized assay conditions as described in the Experimental Procedures. Data points are mean ± SD. Lines are computer-generated fits to a hyperbolic function [y = mx/(n+x)] (see Fig. 1). B: Kinetic parameters derived from the experiment shown in A are shown. The line of best fit is shown (slope = −151.7, r = 0.994). Inset: Apparent K_m values derived from data in A. The line of best fit is shown (slope = −0.35, r = 0.955). Results are the average of duplicate determinations and are representative of at least three similar experiments.

CE from donor liposomes with high CE content, such that the rate of transfer would be dependent on the CE availability in the LDL donor, as demonstrated above. Among these LDLs added to assays on an equal protein basis, CE transfer varied up to 1.5-fold (**Fig. 4A**). To determine if variations in CE transfer activity reflected differences in CETP binding capacity, LDLs were coupled to CNBr-Sepharose then incubated with CETP at CETP/LDL ratios similar to those used in the CE transfer assays. Steady-state binding values are shown in Fig. 4B. Excluding LDL "B," CETP binding capacities were not statistically different except for a mildly elevated binding to LDL "C" at the higher CETP level. Compared with the mean, the 29% lower CETP activity of LDL "B" (Fig. 4A) was mirrored by a 27% lower CETP binding capacity (Fig. 4B), indicating that for this particular LDL, diminished CETP binding could explain the lower CE transfer activity. For the remaining five samples in which CETP binding could not explain differences in their ability to support CE transfer, there was no correlation between CE transfer activity or CETP binding with the ratio of CE/TG, CE/protein, or the ratio of core to surface components [(CE+TG)/(protein+PL+FC), i.e., lipoprotein size] of LDL. Consistent with their similar CETP binding capacities, by immunoblot the content of lipid transfer inhibitor protein (39), which inhibits the binding of CETP (23), was low (<20% of plasma inhibitor levels) and the same (on a protein basis) for all LDL preparations except for LDL "E", which was ~50% lower (data not shown). As seen with these five LDL preparations, in an additional group of seven LDL samples, CE transfer ranged 1.8-fold, but the variation in CETP binding, measured by PL transfer activity (23, 34), was small (data not shown). Finally, the variable CE transfer activities observed in assays using artificial donor particles (CE-enriched liposomes) and different LDLs as acceptors were also observed in assays employing HDL as the donor. With HDL as donor, both CE and TG transfer activities to representative LDL preparations were linearly

TABLE 2. Modification of LDL FC content

Prep #	Dispersion (FC/PL)	Dispersion/LDL (FC/FC)	Modified LDL (FC/PL)
1	0.43	4.0	0.771
2	1.07	5.0	1.055
3	2.56	2.0	1.329
4	2.56	5.0	1.557
Native LDL	—	—	0.822

FC, free cholesterol. Dispersions of FC and dipalmitoyl PC were prepared by sonication as described in the Experimental Procedures. Dispersions of the final composition shown were incubated with human LDL at the final dispersion to LDL/FC ratio indicated. Modified LDL was isolated by heparin Sepharose affinity chromatography. Procedures for LDL modification and characterization are described in the Experimental Procedures. All values are mol/mol ratios.

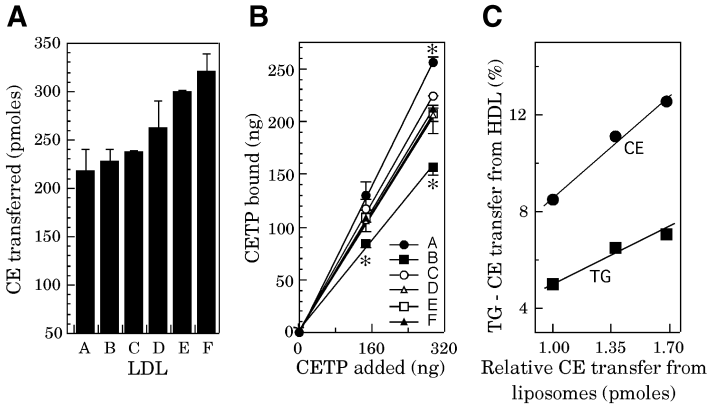

Fig. 4. Determination of the substrate capacity of LDL isolated from different donors. A: CE transfer, mediated by 3 μg partially purified CETP, from labeled liposomes [190 nmol PL, 1.2 mol% CE; [^{3}H]CE (9.8×10^3 cpm)] to LDL isolated from six normolipidemic volunteers (20 μg protein) was determined as described in the Experimental Procedures. Results are the average ± SD of duplicate determinations and are representative of two assays. B: CETP binding to LDL was determined by coupling individual LDL preparations to CNBr-Sepharose and incubating the solid-phase lipoprotein (150 μg protein) with 37 μg or 74 μg partially purified CETP (containing 150 ng or 300 ng CETP, respectively) as described in the Experimental Procedures. Values are mean ± SD (n = 3). * Indicates data points statistically different from sample F, which typified the average binding response. C: Comparison of lipid transfer from radiolabeled HDL- or CE-enriched liposomes to different LDL acceptors. Values of the *y* axis, showing CE and triglyceride transfer from doubly labeled HDL (10 μg cholesterol) to different LDL acceptors (20 μg protein) mediated by 8.6 μg CETP, were determined as described in the Experimental Procedures. The *x* axis shows CE transfer from liposomes to the same LDL acceptors determined as described in A. Data points are mean ± SD.

related to the transfer rates observed with liposome donors (Fig. 4C). Together, these data suggest that the observed variability in transfer rates between liposomes or HDL and native LDL can be explained by a reduced capacity of these LDLs to present lipid to CETP once the transfer protein has docked on the lipoprotein surface. The physical/chemical properties of LDL that lower the CETP-accessible pool of CE remain to be determined.

DISCUSSION

Plasma CETP activity is regulated at multiple points. In normolipidemic individuals, since CETP mass is not rate limiting for net mass transfer (57, 58), other factors are probably more important in defining the extent to which CETP mediates lipoprotein remodeling in these individuals. For example, the levels and composition of lipoprotein substrates influence CETP activity. Numerous studies have illustrated the variability in substrate capacities of lipoprotein fractions isolated from normal and hyperlipidemic individuals (25, 47, 59–61). While some of these variations can be attributed to altered capacities of aberrant lipoproteins to bind CETP (25), in other instances the mechanism underlying altered substrate capacity has not been determined. In previous studies, we illustrated the importance of the lipoprotein surface in facilitating lipid transfer. These studies demonstrated that lipid transfer is strongly influenced by the mol% of neutral lipid dissolved in the PL surface of donor particles (34). However, as the concentration of CE in the donor particle was increased, lipid transfer to the acceptor (LDL) demonstrated saturable kinetics, although CETP binding was not measurably altered. From these studies, the concept arose that CETP can bind to a lipoprotein particle but may dissociate from the surface without facilitating neutral lipid transfer. The frequency of unfruitful binding events decreases as the surface concentration of neutral lipids increases. However, from these kinetic studies, it was not possible to determine the mechanism underlying the "saturation" or plateau of lipid transfer at high donor CE levels.

The present study investigated whether the above-described kinetic response reflects conditions where neutral lipid availability on the surface of the acceptor particle becomes rate limiting. Our data demonstrate that the rate of radiolabeled lipid transfer from a donor to an acceptor particle is determined by the surface availability of substrate lipids in both the donor and acceptor particles. These results further emphasize that lipid transfer is predominately an exchange reaction, in that the rate of unidirectional radiolabeled lipid flux is mechanistically coupled to the successful "on-loading" of an exchangeable lipid substrate on the surface of the acceptor particle. The observation that increased acceptor CE content stimulates CE transfer, even when the donor CE content is comparatively low (Fig. 1), implies that the frequency at which CETP successfully acquires neutral lipid, instead of dissociating without facilitating transfer, must be relatively low. That is, enhancing the probability of successful interactions on either particle will increase the overall rate of transfer. Overall, these results emphasize that CE transfer between two particles is a tightly coupled process with both the "forward" and "reverse" reactions kinetically linked.

CETP-mediated lipid transfer with a lipoprotein can be broadly divided into three steps: the binding of CETP to the lipoprotein surface, the successful interaction of CETP with neutral lipids in the PL monolayer, and the subsequent dissociation of CETP from the surface where it is available to interact with another lipoprotein substrate. Although steady-state binding kinetics can assess the balance of on/off rates for CETP binding to lipoproteins, to our knowledge there are no methods available to determine the concentration of neutral lipids solubilized in the surface of lipoproteins such as LDL. However, the kinetic coupling of CETP's forward and reverse reactions provides a means of assessing the levels of functional, CETP-accessible neutral lipid in lipoproteins when assayed in the presence of liposome donors containing high levels of CE. As a test of this approach, we show that increasing the FC content of LDL over a physiologically relevant range (54–56) results in lipoproteins that are markedly deficient in their capacity to participate in CE transfer with liposome donors. This difference was not due to the altered capacity of these modified lipoproteins to bind CETP since CETP-mediated TG and PL transfers are unaffected by FC enrichment (44). Increasing FC would be expected to alter the physical status of the lipoprotein surface and potentially influence the conformation of associated proteins. However, since CE and TG transfers occur through a common site on CETP (21) and TG transfer is not altered, the decrease in CE transfer by FC enrichment is unlikely to result from an altered CETP conformation when bound to the modified lipoprotein surface. The data presented here support our previous hypothesis that FC reduces CE transfer from LDL by displacing CE from the surface of the lipoprotein (44), as suggested by the lipid solubility studies of Miller and Small (52, 53). This mechanism is consistent with the uncompetitive inhibition kinetics noted for FC. These studies further illustrate how variations in lipoprotein composition can lead to an altered ability of lipoproteins to participate in CETP reactions even when CETP binding is unaffected.

Not only could the CETP-accessible CE be altered by modification of FC content, analysis of LDL isolated from a number of individuals indicates that there is marked variability in the substrate capacity of LDL derived from normolipidemic individuals whether assayed with liposomes or HDL. This nearly 2-fold variability was observed among LDLs that were similar in total lipid composition. These differences were not due to variations in the capacity of these LDLs to bind CETP, suggesting that true differences in substrate presentation exist among control LDLs. The compositional differences that contribute to these results are of obvious interest, and likely relate to the capacity of the surface lipids to solubilize CE (and TG) where it is available for interaction with CETP (44). Alterations in the molecular packing of the PL surface due to variations in PL fatty acid composition, the sphingomyelin-to-PL ratio, or the presence of minor PL classes would appear to be reasonable candidates to investigate initially.

In summary, these data demonstrate that CE transfer between substrate particles is a tightly-coupled process in that unidirectional transfer of radiolabeled CE from a donor to an acceptor is defined by the capacity of both particles to present neutral lipids to the transfer protein. The obligatory coupling of the transfer process under these assay conditions provides a means of assaying the relative levels of functionally available neutral lipids in the surface of test lipoproteins when CETP binding is constant. This assay approach permitted us to observe that the level of CETP-accessible lipids varies significantly among native LDLs and that this pool can be altered by changes in lipoprotein FC content, as commonly occurs in hyperlipidemia and diabetes (54–56). These results suggest that variations in surface availability of neutral lipids may be major regulators of CETP activity on lipoproteins. The use of synthetic liposomes of high CE content as donors of lipid to lipoproteins will provide a useful tool to interrogate the functional properties of lipoproteins and to carefully analyze and define the lipid and protein components of lipoproteins that influence the substrate capacity of lipoproteins through their ability to alter the presentation of CE and TG to CETP.

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REFERENCES

1. Morton, R. E. 1990. Interaction of lipid transfer protein with plasma lipoproteins and cell membranes. *Experientia.* **46:** 552–560.
2. Tall, A. 1995. Plasma lipid transfer proteins. *Annu. Rev. Biochem.* **64:** 235–257.
3. Zechner, R., H. Dieplinger, E. Steyrer, J. Groener, D. Calvert, and G. M. Kostner. 1987. In vitro formation of HDL-2 from HDL-3 and triacylglycerol-rich lipoproteins by the action of lecithin-cholesterol acyltransferase and cholesterol ester transfer protein. *Biochim. Biophys. Acta.* **918:** 27–35.
4. Ellsworth, J. L., M. L. Kashyap, R. L. Jackson, and J. A. K. Harmony. 1987. Human plasma lipid transfer protein catalyzes the speciation of high density lipoproteins. *Biochim. Biophys. Acta.* **918:** 260–266.
5. Deckelbaum, R. J., S. Eisenberg, Y. Oschry, E. Granot, I. Sharon, and G. Bengtsson-Olivecrona. 1986. Conversion of human plasma high density lipoprotein-2 to high density lipoprotein-3: roles of neutral lipid exchange and triglyceride lipases. *J. Biol. Chem.* **261:** 5201–5208.
6. Tall, A. R. 1993. Plasma cholesteryl ester transfer protein. *J. Lipid Res.* **34:** 1255–1274.
7. Morton, R. E. 1988. Interaction of plasma-derived lipid transfer protein with macrophages in culture. *J. Lipid Res.* **29:** 1367–1377.
8. Skeggs, J. W., and R. E. Morton. 2002. LDL and HDL enriched in triglyceride promote abnormal cholesterol transport. *J. Lipid Res.* **43:** 1264–1274.
9. Chait, A., S. Eisenberg, A. Steinmetz, J. J. Albers, and E. L. Bierman. 1984. Low-density lipoproteins modified by lipid transfer protein have altered biological activity. *Biochim. Biophys. Acta.* **795:** 314–325.
10. Kinoshita, M., E. S. Krul, and G. Schonfeld. 1990. Modification of the core lipids of low density lipoproteins produces selective alterations in the expression of apoB-100 epitopes. *J. Lipid Res.* **31:** 701–708.
11. Deckelbaum, R. J., S. Eisenberg, Y. Oschry, and L. L. Rudel. 1988. Neutral lipid transfer and lipolysis convert high molecular weight LDL from cholesterol-fed nonhuman primates towards

normal: a molecular analysis. *Biochim. Biophys. Acta.* **961:** 223–233.
12. Lagrost, L., P. Gambert, and C. Lallemant. 1994. Combined effects of lipid transfers and lipolysis on gradient gel patterns of human plasma LDL. *Arterioscler. Thromb.* **14:** 1327–1336.
13. Viens, L., and L. Lagrost. 1997. Effect of lipid transfer activity and triglyceride hydrolysis on apolipoprotein B immunoreactivity in modified low density lipoproteins. *J. Lipid Res.* **38:** 1129–1138.
14. Deckelbaum, R. J., S. Eisenberg, M. Fainaru, Y. Barenholz, and T. Olivecrona. 1979. In vitro production of human plasma low density lipoprotein-like particles: a model for very low density lipoprotein catabolism. *J. Biol. Chem.* **254:** 6079–6087.
15. 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.
16. 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.
17. Barter, P. J. 1991. Role of non-esterified fatty acids in regulating plasma cholesterol transport. *Clin. Exp. Pharmacol. Physiol.* **18:** 77–79.
18. Weinberg, R. B., V. R. Cook, J. B. Jones, P. Kussie, and A. R. Tall. 1994. Interfacial properties of recombinant human cholesterol ester transfer protein. *J. Biol. Chem.* **269:** 29588–29591.
19. Epps, D. E., K. A. Greenlee, J. S. Harris, E. W. Thomas, C. K. Castle, J. F. Fisher, R. R. Hozak, C. K. Marschke, G. W. Melchior, and F. J. Kézdy. 1995. Kinetics and inhibition of lipid exchange catalyzed by plasma cholesteryl ester transfer protein (lipid transfer protein). *Biochemistry.* **34:** 12560–12569.
20. Connolly, D. T., J. Mclntyre, D. Heuvelman, E. E. Remsen, R. E. McKinnie, L. Vu, M. Melton, R. Monsell, E. S. Krul, and K. Glenn. 1996. Physical and kinetic characterization of recombinant human cholesteryl ester transfer protein. *Biochem. J.* **320:** 39–47.
21. Wang, S., P. Kussie, L. Deng, and A. Tall. 1995. Defective binding of neutral lipids by a carboxyl-terminal deletion mutant of cholesteryl ester transfer protein. Evidence for a carboxyl-terminal cholesteryl ester binding site essential for neutral lipid transfer activity. *J. Biol. Chem.* **270:** 612–618.
22. 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.
23. Morton, R. E. 1985. Binding of plasma-derived lipid transfer protein to lipoprotein substrates: the role of binding in the lipid transfer process. *J. Biol. Chem.* **260:** 12593–12599.
24. Pattnaik, N. M., and D. B. Zilversmit. 1979. Interaction of cholesteryl ester exchange protein with human plasma lipoproteins and phospholipid vesicles. *J. Biol. Chem.* **254:** 2782–2786.
25. Sammett, D., and A. R. Tall. 1985. Mechanisms of enhancement of cholesteryl ester transfer protein activity by lipolysis. *J. Biol. Chem.* **260:** 6687–6697.
26. Wang, S., L. Deng, M. L. Brown, L. B. Agellon, and A. R. Tall. 1991. Structure-function studies of human cholesteryl ester transfer protein by linker insertion scanning mutagenesis. *Biochemistry.* **30:** 3484–3490.
27. 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.
28. Ko, K. W. S., T. Ohnishi, and S. Yokoyama. 1994. Triglyceride transfer is required for net cholesteryl ester transfer between lipoproteins in plasma by lipid transfer protein. Evidence for a hetero-exchange transfer mechanism demonstrated by using novel monoclonal antibodies. *J. Biol. Chem.* **269:** 28206–28213.
29. Serdyuk, A. P., and R. E. Morton. 1997. Lipid transfer inhibitor protein activity deficiency in normolipidemic uremic patients on continuous ambulatory peritoneal dialysis. *Arterioscler. Thromb. Vasc. Biol.* **17:** 1716–1724.
30. Serdyuk, A. P., and R. E. Morton. 1999. Lipid transfer inhibitor protein defines the participation of lipoproteins in lipid transfer reactions: CETP has no preference for cholesteryl esters in HDL versus LDL. *Arterioscler. Thromb. Vasc. Biol.* **19:** 718–726.
31. Van Tol, A., L. M. Scheek, and J. E. M. Groener. 1991. Net mass transfer of cholesteryl esters from low density lipoproteins to high density lipoproteins in plasma from normolipidemic subjects. *Arterioscler. Thromb.* **11:** 55–63.
32. Yen, F. T., R. J. Deckelbaum, C. J. Mann, Y. L. Marcel, R. W. Milne, and A. R. Tall. 1989. Inhibition of cholesteryl ester transfer protein activity by monoclonal antibody. Effects on cholesteryl ester formation and neutral lipid mass transfer in human plasma. *J. Clin. Invest.* **83:** 2018–2024.
33. Liu, X. Q., and J. D. Bagdade. 1995. Neutral lipid mass transfer among lipoproteins in plasma from normolipidemic subjects is not an equimolar heteroexchange. *J. Lipid Res.* **36:** 2574–2579.
34. Morton, R. E., and J. V. Steinbrunner. 1990. Concentration of neutral lipids in the phospholipid surface of substrate particles determines lipid transfer protein activity. *J. Lipid Res.* **31:** 1559–1567.
35. Bruce, C., W. S. Davidson, P. Kussie, S. Lund-Katz, M. C. Phillips, R. Ghosh, and A. R. Tall. 1995. Molecular determinants of plasma cholesteryl ester transfer protein binding to high density lipoproteins. *J. Biol. Chem.* **270:** 11532–11542.
36. 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.
37. 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.
38. Morton, R. E., and D. B. Zilversmit. 1981. The separation of apolipoprotein D from cholesteryl ester transfer protein. *Biochim. Biophys. Acta.* **663:** 350–355.
39. Austin, M. A., J. L. Breslow, C. H. Hennekens, J. E. Buring, W. C. Willet, and R. M. Krauss. 1988. Low density lipoprotein subclass patterns and risk of myocardial infarction. *JAMA.* **260:** 1917–1921.
40. Jarnagin, A. S., W. Kohr, and C. Fielding. 1987. Isolation and specificity of a Mr 74,000 cholesteryl ester transfer protein from human plasma. *Proc. Natl. Acad. Sci. USA.* **84:** 1854–1857.
41. Nishida, H. I., H. Arai, and T. Nishida. 1993. Cholesterol ester transfer mediated by lipid transfer protein as influenced by changes in the charge characteristics of plasma lipoproteins. *J. Biol. Chem.* **268:** 16352–16360.
42. 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.
43. Morton, R. E., and D. B. Zilversmit. 1981. A plasma inhibitor of triglyceride and cholesteryl ester transfer activities. *J. Biol. Chem.* **256:** 11992–11995.
44. Morton, R. E. 1988. Free cholesterol is a potent regulator of lipid transfer protein function. *J. Biol. Chem.* **263:** 12235–12241.
45. Brunner, J., P. Skrabal, and H. Hauser. 1976. Single bilayer vesicles prepared without sonication: physico-chemical properties. *Biochim. Biophys. Acta.* **455:** 322–331.
46. Lagrost, L., A. Athias, P. Gambert, and C. Lallemant. 1994. Comparative study of phospholipid transfer activities mediated by cholesteryl ester transfer protein and phospholipid transfer protein. *J. Lipid Res.* **35:** 825–835.
47. Morton, R. E., and J. S. Parks. 1996. Plasma cholesteryl ester transfer activity is modulated by the phase transition of the lipoprotein core. *J. Lipid Res.* **37:** 1915–1923.
48. 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.
49. 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.
50. 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.
51. Bartlett, G. R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* **234:** 466–468.
52. Miller, K. W., and D. M. Small. 1983. Triolein-cholesteryl oleate-cholesterol-lecithin emulsions: structural models of triglyceride-rich lipoproteins. *Biochemistry.* **22:** 443–451.
53. Miller, K. W., and D. M. Small. 1982. The phase behavior of triolein, cholesterol, and lecithin emulsions. *J. Colloid Interface. Sci.* **89:** 466–478.
54. Fielding, C. J. 1984. The origin and properties of free cholesterol potential gradients in plasma, and their relation to atherogenesis. *J. Lipid Res.* **25:** 1624–1628.
55. Fielding, C. J., G. M. Reaven, G. Liu, and P. E. Fielding. 1984. Increased free cholesterol in plasma low and very low density lipoproteins in non-insulin-dependent diabetes mellitus: its role in the

inhibition of cholesteryl ester transfer. *Proc. Natl. Acad. Sci. USA.* **81:** 2512–2516.
56. Bagdade, J. D., E. Helve, and M. R. Taskinen. 1991. Effects of continuous insulin infusion therapy on lipoprotein surface and core lipid composition in insulin-dependent diabetes mellitus. *Metabolism.* **40:** 445–449.
57. Mann, C. J., F. T. Yen, A. M. Grant, and B. E. Bihain. 1991. Mechanism of plasma cholesteryl ester transfer in hypertriglyceridemia. *J. Clin. Invest.* **88:** 2059–2066.
58. Murakami, T., S. Michelagnoli, R. Longhi, G. Gianfranceschi, F. Pazzucconi, L. Calabresi, C. R. Sirtori, and G. Franceschini. 1995. Triglycerides are major determinants of cholesterol esterification/transfer and HDL remodeling in human plasma. *Arterioscler. Thromb. Vasc. Biol.* **15:** 1819–1828.
59. Bagdade, J. D., D. Kaufman, M. C. Ritter, and P. V. Subbaiah. 1990. Probucol treatment in hypercholesterolemic patients: effects on lipoprotein composition, HDL particle size, and cholesteryl ester transfer. *Atherosclerosis.* **84:** 145–154.
60. Bagdade, J. D., J. T. Lane, P. V. Subbaiah, M. E. Otto, and M. C. Ritter. 1993. Accelerated cholesteryl ester transfer in noninsulin-dependent diabetes mellitus. *Atherosclerosis.* **104:** 69–77.
61. Bagdade, J. D., M. C. Ritter, and P. V. Subbaiah. 1991. Accelerated cholesteryl ester transfer in patients with insulin-dependent diabetes mellitus. *Eur. J. Clin. Invest.* **21:** 161–167.