The Bidirectional Flux of Cholesterol between Cells and Lipoproteins

EFFECTS OF PHOSPHOLIPID DEPLETION OF HIGH DENSITY LIPOPROTEIN*

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The bidirectional surface transfer of free cholesterol (FC) between FuSAH rat hepatoma cells and human high density lipoprotein (HDL) was studied. Cells and HDL were prelabeled with [4-14C]FC and [7-3H]FC, respectively. Influx and efflux of FC were measured simultaneously from the appearance of 3H counts in cells and 14C counts in medium. Results were analyzed by a computerized procedure which fitted sets of kinetic data to a model assuming that cell and HDL FC populations each formed a single homogeneous pool and that together the pools formed a closed system. This analysis yielded values for the first-order rate constants of FC influx and efflux (k1 and k1), from which influx and efflux of FC mass (F1 and F1) could be calculated. With normal HDL, the uptake and release of FC tracers conformed well to the above-described model; F1 and F1 were approximately equal, suggesting an exchange of FC between cells and HDL. HDL was depleted of phospholipid (PL) by treatment with either phospholipase A2 or heparin-releasable rat hepatic lipase, followed by incubation with bovine serum albumin. PL depletion of HDL had little or no effect on k1, but reduced k1, indicating that PL-deficient HDL is a relatively poor acceptor of cell cholesterol. The reduction in k1 resulted in initial F1 > F1 and, thus, in net uptake of FC by the cells. This result explained previous results demonstrating net uptake of FC from PL-depleted HDL. In the presence of an inhibitor of acyl coenzyme A:cholesterol acyltransferase, the steady state distribution of FC mass between cells and HDL was accurately predicted by the ratio of rate constants for FC flux. This result provided additional validation for describing FC flux in terms of first-order rate constants and homogeneous cell and HDL FC pools.

High density lipoprotein (HDL) is thought to mediate reverse cholesterol transport, the process by which excess cholesterol from peripheral tissues is transported in blood and lymph to the liver for excretion (1). One of the ways in which HDL may contribute to reverse cholesterol transport is by the delivery of free (unesterified) cholesterol (FC) to hepatocyte plasma membranes by a surface transfer process which occurs independently of the uptake and degradation of the lipoprotein (2).

The surface transfer of FC between HDL and most cells is bidirectional (i.e. FC is taken up and released simultaneously), and the transfer occurs passively and without the direct input of metabolic energy (reviewed in Ref. 3). The phospholipid (PL) content of HDL and cell membranes is largely responsible for the affinity of HDL and cells for FC (4-6). Owing to the above characteristics, FC probably tends to distribute between HDL and cell surface membranes in a simple equilibrium determined largely by the relative PL content of these structures (6). Hepatic endothelium contains an extracellular, heparin-releasable lipase (hepatic lipase) which acts on the PL of HDL and thereby reduces the PL content of lipoprotein (7-9). Jansen and Hülsmann (10) suggested that this reduction may favor the passive redistribution of FC from HDL to hepatocyte plasma membranes, thereby promoting the component of reverse cholesterol transport which depends on surface transfer.

An established in vitro model for studying the surface transfer of FC between HDL and cells is the rat hepatoma cell line FuSAH (4, 9, 11, 12). In this cell, the uptake and degradation of intact HDL are small relative to the uptake of FC from the lipoprotein (9, 12). Likewise, the secretion of cholesterol and cholesterol-derived products (e.g. bile salts) in the absence of lipoproteins is small relative to the release of cellular FC in the presence of HDL (11). Thus, by employing this cell line, it is possible to study FC surface transfer mechanisms without significant interference from holo-HDL uptake and the secretion of lipoproteins and steroidal metabolic products. During incubation with normal human HDL (9, 12), FuSAH cells incorporated [3H]FC from the lipoprotein, but their content of free and esterified cholesterol mass increased only modestly, suggesting that an approximate exchange of FC mass occurred. Depletion of PL from HDL (by treatment with hepatic lipase or Crotalus venom phospholipase A2) stimulated the net uptake of [3H]FC from the lipoprotein and significantly enhanced the cellular accumulation of FC and cholesteryl ester (CE) mass. The effects of PL depletion could not be attributed to increased uptake and degradation of HDL or to increased cellular cholesterol synthesis. Thus, in accord with the hypothesis of Jansen and Hülsmann (10), the lipase-induced accumulation of cholesterol appeared to have resulted from the stimulated net surface transfer of FC from HDL into the cells.

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1 The abbreviations used are: HDL, high density lipoprotein; BSA, bovine serum albumin; CE, cholesteryl ester; ECR, esterified cholesterol; TC, total cholesterol; F1 and F1, influx and efflux, respectively, of free cholesterol mass; FC, free cholesterol; FC, mass of cellular and medium free cholesterol, respectively; GLC, gas-liquid chromatography; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; k1 and k1, rate constants for free cholesterol efflux and influx, respectively; MEM, Eagle’s minimum essential medium; PL, phospholipid; SDS, sodium dodecyl sulfate.
The transfer of FC between Fu5AH cells and HDL is demonstrably bidirectional, with the uptake and release of FC occurring simultaneously (11). Consequently, any modification of the surface transfer properties which makes the difference of influx minus efflux more positive will stimulate the net cellular uptake of FC from HDL. The present studies investigated the mechanism by which depletion of PL from HDL stimulates Fu5AH cellular cholesterol accumulation in terms of independent effects on influx and efflux of FC. Methods for the simultaneous measurement of the uptake and release of radiolabeled FC between HDL and Fu5AH cells were developed. Kinetic data on the transfer of FC tracers were analyzed using a computerized curve-fitting procedure to yield, for the first time, the rate constants characterizing the bidirectional surface transfer of FC between HDL and cells. These methods were used to investigate the effects of PL depletion of HDL on FC influx and efflux.

EXPERIMENTAL PROCEDURES

The experimental system was designed specifically for analysis of cholesterol flux between cells and extracellular vehicles. The model system was the rat hepatoma cell line Fu5AH, as already discussed in the Introduction. The external vehicle was total HDL, prepared from fresh plasma treated with N-ethylmaleimide and then purified by column chromatography on heparin-Sepharose. N-Ethylmaleimide treatment of plasma irreversibly inhibited the plasma enzyme lecithin:cholesterol acyltransferase and thus prevented esterification of cholesterol in the incubation media. Heparin-Sepharose chromatography eliminated lipoproteins containing apolipoprotein B and thus prevented internalization of lipoproteins mediated by cellular receptors for these apolipoproteins to that occurring by surface transfer processes. Cells and HDL were prelabeled with [3H]FC and [14C]FC, respectively. During incubations, simultaneous measurements of influx and efflux were accomplished by monitoring the accumulation of [3H] in cells and the release of [14C] to the medium, respectively.

Materials

Sources of chemical supplies were as follows: [4-14C]cholesterol, Research Products International Corp., Mount Prospect, IL; [7-3H]cholesterol, ICN, Irvine, CA; unlabeled cholesterol (99% grade), egg yolk phosphatidylcholine (type I11-E), N-ethylmaleimide, and bovine serum albumin (BSA; Fraction V, fatty acid-free), Sigma; phospholipase A2 (derived from venom of Crotalus adamanteus), Boehringer Mannheim; thin layer chromatography plates, Gelman Sciences, Inc., Ann Arbor, MI, and J. T. Baker Chemical Co., Phillipsburg, NJ; media and antibiotics for tissue culture, GIBCO, Grand Island, NY, for tissue culture, Flow Laboratories, Inc., McLean VA; and compound 58-035 ([decyldimethylsilyl]-N-[2-(4-methylphenyl)-1-phenylethyl]propanol) as a gift from Dr. John Heider, Sandzio, Inc., East Hanover, NJ.

Solutions used routinely in these studies were phosphate-buffered saline (0.137 M NaCl, 8.3 mM sodium phosphate, 1.5 mM potassium phosphate, 2.7 mM KCl, 0.9 mM CaCl2, 0.5 mM MgSO4, pH 7.4), phosphate-buffered NaCl with antibiotics (0.15 M NaCl, 2 mM sodium phosphate, 50 IU of penicillin/ml, 50 µg of streptomycin/ml, pH 7.4), and Eagle’s minimum essential medium buffered to pH 7.4 with either 24 mM NaHCO3 (MEM bicarb) or 14 mM HEPES (MEM-HEPES). All culture and incubation media were supplemented with 50 µg of gentamicin/ml to prevent bacterial contamination. Solutions buffered with bicarbonate were used under an atmosphere of 5% CO2 in air, Solutions buffered with HEPES or phosphate were used under an atmosphere of air.

Methods

Preparation of HDL—Blood was collected from nonfasted to normal human donors and then made 2 mM in disodium EDTA prevent clotting and centrifuged to obtain plasma. Plasma was made 5 mM in N-ethylmaleimide to inhibit lecithin:cholesterol acyltransferase, and then total HDL (d 1.063–1.21 g/ml fraction) was isolated by sequential ultracentrifugation, as adapted from Hatch and Lees (13). HDL was dialyzed to remove KBr and chromatographed on heparin-Sepharose to remove particles containing apolipoproteins B and E, as described by Bamberger et al. (9).

Analytical Procedures—Lipids in media and HDL samples were extracted according to Bligh and Dyer (14). Cellular lipids in tissue culture dishes were extracted with isopropanol alcohol. Free and total cholesterol were quantitated in lipid extracts by GLC, using capped cholesterol as an internal standard (15). Lipid phosphorus was determined by the method of Sokoloff and Rothblat (16). Total protein was determined by the Lowry procedure, as modified by Markwell et al. (17). Cellular protein (delipidated and fixed to tissue culture dishes by extraction with isopropanol alcohol) was prepared for the protein assay by solubilization in 1 N NaOH. The distribution of cholesterol radioactivity into polar-impurity, free and esterified fractions was assayed in total lipid extracts following separation by TLC, using Silica Gel IB2 or G as the stationary phase and benzene or diethyl ether as the mobile phase. Phospholipids and fatty acids in HDL were identified using a TLC system of silica gel and diethyl ether (9). The methods for modification with cholesterol or lipid phosphorus. However, on SDS gel electrophoresis, it contained trace amounts of material co-migrating with apolipoproteins A-I, A-II, and C.

Preparation of Incubation Media—The resulting HDL was diluted into MEM-HEPES to prepare experimental incubation media. It is a given experiment, media containing different types of HDL to be compared to one another were prepared to have equal initial concentrations of HDL protein and FC. Concentrations of HDL in media are routinely expressed in terms of protein, because this parameter of HDL composition was least variable. Media also contained 0.2 or 2% (w/v) BSA. Analysis of the BSA indicated no significant contamination with cholesterol or phospholipids. However, on SDS gel electrophoresis, it contained trace amounts of material co-migrating with apolipoproteins A-I, A-II, and C.

Preparation of Cells—Cells were grown to near confluency in T-75 flasks containing 15 ml of medium consisting of 95% (v/v) MEM-bicarb and 5% (v/v) calf serum. Monolayers in 7-76 flasks were prepared by incubation for 24 hr in 15 ml of MEM medium, 5% (v/v) fetal bovine serum, 7 µg/ml egg yolk phosphatidylcholine or dipalmitoylphosphatidylcholine, and 6 µg/ml [4-14C]FC (21 mCi/mmol). The lipids were added to the labeling medium dissolved in 0.1 ml of less than 0.1 ml of ethanol (final concentration of ethanol, less than 0.5%). After 2 days of labeling, cells were rinsed three times with 15 ml of MEM-
bicarbonate, detached from the flasks with trypsin (2.5 mg/ml in MEM-HEPES), suspended in medium consisting of MEM-bicarb supplemented with 5 mg of delipidized serum protein/ml, and replated in 22-mm wells of 12-well tissue culture plates or 24-well plastic culture dishes. Cells were firmly attached and 70–100% confluent after 18–24 h of incubation, at which time cholesterol flux experiments were initiated. Analysis of cellular lipids at this time showed 90–98% of 14C label in FC and 2–10% in CE.

Incubation Procedures—To begin incubations, cells were rinsed three times with 2 ml of phosphate-buffered saline and then 0.5 ml (to 22-mm wells) or 1.0 ml (to 35-mm plates) of prewarmed medium was added. Plates were covered and incubated at 37 °C without shaking in an atmosphere of air for periods usually no longer than 100 min.

Incubations were ended by removing and cooling the medium on ice. The cells were rinsed once with 3 ml of cold phosphate-buffered saline supplemented with 0.2% (w/v) BSA and twice with 2 ml of cold phosphate-buffered saline. After the final rinse, cellular lipids were extracted by adding 2 ml of isopropyl alcohol to each well, covering the plates, and leaving them undisturbed overnight at room temperature in a chamber saturated with isopropyl alcohol vapor.

Samples of medium were centrifuged briefly, and aliquots were taken for isotopic and lipid analysis. Isopropyl alcohol extracts of cellular lipids were sampled for isotopic and lipid analysis. Control incubations performed in each experiment demonstrated that both the association of HDL cholesterol with the plastic of tissue culture dishes and the release of cellular cholesterol to media lacking HDL were negligible. Thus, our sampling procedures appeared to be valid for assaying the transfer of cholesterol between cells and HDL.

Analysis of Data—As described under “Results,” the movement of isotopic tracers between cells and media was representative of the movement of FC. Therefore, the uptake and release of FC were determined, respectively, from the accumulation of ³H in cells and the release of ¹⁴C to media.

The kinetic analysis of tracer movement was accomplished by a computer program which fitted measurements of ³H uptake and ¹⁴C release to a compartmental model which assumed that FC in this system formed only two kinetic pools, one comprised of all cellular FC and the other of all HDL FC, and that together the two pools formed a closed system. We felt that the first of these assumptions was justified because of several previous experimental findings. 1) Results of Lund-Katz and Phillips (21) suggested very rapid equilibration of the two identifiable pools of FC within HDL (tₘ of equilibration falling in the range 10 ms to 300 s). 2) Other results of Lund-Katz et al. (22) showed that the FC in HDL could equilibrate completely with the FC in low density lipoprotein with a very low tₘ (180 s). 3) Results of Rothblat and Phillips (4) had shown that, during rapid release of FC, at least 70% of Fu5AH cellular FC was lost in a single polynuclear fashion. The second assumption of the modeling (that of a closed system) was met in most experiments by limiting incubations to periods less than 100 min, thus preventing cholesterol synthesis and the cycle of cholesterol ester formation and hydrolysis from seriously affecting the total labeled and unlabeled FC within the system. As described in the “Appendix,” the fitting procedure produced mathematical relationships describing the time course of tracer uptake and release. These were used to estimate the rate constants of FC influx and efflux (kᵢ and kₑ) and the unidirectional influx and efflux of FC mass (Fᵢ and Fₑ). In making predictions of FC mass redistribution from determinations of kᵢ and kₑ, the rate constants characteristic of a particular concentration of an HDL type were assumed not to vary as the content of FC within cells and HDL particles varied.

Statistical comparisons between sets of measurements (n ≥ 3) were performed by Student’s t test for unpaired observations (29). Linear regression analysis was performed on a Franklin 1000 computer, using the Osborne mathematic software package.

RESULTS

General Features of the Bidirectional Surface Transfer of FC

As a preliminary experiment, Fu5AH cells labeled with [¹⁴C]-cholesterol and HDL labeled with [³H]-cholesterol were combined, and the distribution of the isotopic tracers between cells and HDL was allowed to proceed to steady state (Fig. 1a). The results show that the redistribution of tracers was rapid and essentially complete after 400 min of incubation.

![Fig. 1](image_url)
The smooth curves drawn through the points in Fig. 1a were obtained by a computerized procedure, which fitted a set of measurements for tracer uptake or release to a compartmental model which assumed that the cell and HDL populations of FC molecules each formed a kinetically homogeneous pool and that together the two pools formed a closed system (see “Appendix”). The equations for the curves and the rate constants for FC flux \((k_i\) and \(k_e\)) calculated from the equations are given in columns 1 and 2 of Table I. This analysis produced equations which accurately represented tracer movement over the entire 480-min period of the experiment, suggesting that FC interchange between HDL and Fu5AH cells conformed well to the above-described model.

In most experiments, we planned to limit the kinetic analysis to results obtained in the first 60-100 min of incubation. In this way, we hoped to study the flux of FC without significant interference from the processes of cell growth, cholesterol synthesis, and the cycle of cholesteryl ester formation and hydrolysis. We wished to verify that this approach would reliably characterize FC movement between HDL and Fu5AH cells. For this verification, results from Fig. 1a were again analyzed, in this case using only measurements taken in the first 90 min of the experiment for the computerized curve-fitting. The equilibration curves and rate constants in this way were compared to those obtained from analysis of the complete experimental time course, as described above. If the assumptions of the modeling were valid, both methods of analysis were expected to yield similar equations for tracer equilibration and similar values for the rate constants of FC flux. As shown in Fig. 1, \(a\) and \(b\), analysis of the early measurements (0-90 min) yielded curves which accurately represented tracer movement in the initial 90-min interval and which did not deviate from later measurements or from the curves in Fig. 1a by more than 10%. Additionally, as shown in Table I (columns 2 and 4), the rate constants derived from the early measurements differed by no more than 10% from those derived from the complete experimental time course. The analysis of results from the first 90 min of incubation thus appeared to reliably characterize the transfer of FC between HDL and Fu5AH cells. In all subsequent experiments, the kinetic analysis of FC flux was confined to results obtained in such short-term incubations.

**Effect of Phospholipid Depletion of HDL on Bidirectional Flux of FC**

Phospholipid Depletion Mediated by Phospholipase A\(_2\)–HDL, which had been prelabeled with \([^{3}H]\)FC, was incubated with phospholipase \(A_2\) in the presence (control HDL) or absence (modified HDL) of EDTA, post-incubated with BSA to remove the products of lipolysis, and then resolated by ultracentrifugation. The compositions of the resulting HDL are summarized in Table II. It is shown that exposure to active lipase selectively reduced the PL content of HDL relative to protein, with no significant effect on FC or TC. Regarding the reduction in PL, TLC analysis of the PL and free fatty acid of the HDL showed that the modification caused selective reduction of phosphatidylcholine, with no significant increase in lysophosphatidylcholine or free fatty acid. In preliminary experiments (data not given), we found that HDL which had undergone the control exposure to phospholipase produced influx and efflux of FC which were identical to that produced by normal untreated HDL, showing that the control treatment and resolation of HDL did not affect its ability to interchange FC with Fu5AH cells.

The effect of PL depletion of HDL on the time course of FC tracer uptake and release at an HDL concentration of 1.2 mg of protein/ml (initial [FC] = 56 \(\mu\)g/ml) is given in Fig. 2. These results demonstrated two major points. 1) The modification clearly reduced the initial influx of cell-derived \([^{14}C]\) FC and over the entire time course of the experiment caused greater cellular retention of \([^{14}C]FC\). 2) Although the modification eventually resulted in greater accumulation of HDL-derived \([^{3}H]\)cholesterol in cells, it had very little effect on the initial influx of \([^{3}H]\)FC. The initial (\(t = 0\)) slopes of the plots in Fig. 2 are directly convertible to the rate constants of FC influx and efflux, \(k_i\) and \(k_e\) (Ref. 24; also see “Appendix”). The results thus indicate that the primary effect of PL depletion of HDL was to reduce \(k_e\), the rate constant for efflux of FC.

Under the same conditions described for Fig. 2, \(k_i\) and \(k_e\) for FC over a range of concentrations of control and phospholipase-modified HDL were measured. The rate constants were converted to values for the initial flux of FC mass (\(F_i\) and \(F_e\)), as described in the “Appendix.” The dependence of these fluxes on HDL concentration is plotted in Fig. 3. The results show that, over the entire concentration range (0.12–3.5 mg of protein/ml, initial [FC] = 5–145 \(\mu\)g/ml), initial influx of FC was not significantly affected by the modification, whereas initial efflux was reduced by approximately 25%. It is also apparent that, with control HDL, initial influx and efflux were very nearly equal at all HDL concentrations, whereas with the modified HDL, influx was consistently greater than efflux. These results again illustrate that the

### Table I

**Equations and rate constants to describe the bidirectional flux of cholesterol between HDL (0.4 mg of protein/ml) and Fu5AH cells**

Incubation conditions were as described for Fig. 1. Equations and rate constants were obtained by the computerized fitting of results to a model for the interchange of FC between two pools comprising a closed system, as described in the “Appendix.”

<table>
<thead>
<tr>
<th>Incubation interval providing measurements analyzed by curve-fitting program</th>
<th>0.480 min</th>
<th>0.900 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equations describing tracer uptake ((^{3}H)) and retention ((^{14}C))</td>
<td>(10^{9} k_i)</td>
<td>(10^{9} k_e)</td>
</tr>
<tr>
<td>Cellular uptake of ([^{3}H])FC) (^{3}H_o/^{3}H_{eo} = -0.30 e^{-(0.60 \times 10^{-6} min^{-1}) t} + 0.31)</td>
<td>3.1</td>
<td>(^{3}H_o/^{3}H_{eo} = -0.28 e^{-(0.60 \times 10^{-6} min^{-1}) t} + 0.28)</td>
</tr>
<tr>
<td>Cellular retention of ([^{14}C])FC) ([^{14}C_o]/^{14}C_{eo} = 0.70 e^{-(0.012 \times 10^{-6} min^{-1}) t} + 0.31)</td>
<td>8.4</td>
<td>([^{14}C_o]/^{14}C_{eo} = 0.65 e^{-(0.014 \times 10^{-6} min^{-1}) t} + 0.35)</td>
</tr>
</tbody>
</table>

*Values of \(k_i\) are dependent on medium volume and cell number/tissue culture plate and should be converted to clearance (Cl) to provide an indication of the intrinsic tendency of FC to move from HDL to cells (see “Appendix”).*
procedure, except that 12 mM EDTA was present during exposure to lipase. Compositional data on control and modified HDL are summarized in Table II. Incubation media contained HDL at a concentration of 1.2 mg of protein/ml (initial [FC] = 50 μg/ml) and 0.2% (w/v) BSA in MEM-HEPES. 0.5 ml aliquots of the medium were incubated with monolayers of Fu5AH cells plated in 22-mm wells of 12-well tissue culture dishes (0.29 mg of cellular protein and 5.4 μg of cellular FC/well), and then cells and medium were separated and analyzed. Incubation duration was 0 and approximately 15, 30, 45, and 60 min. The initial specific activities of FC were 4601 14C cpm and 7835 3H cpm/μg of FC in cells and medium, respectively. Results are plotted as the fractional retention (a) or the fractional uptake (b) of isotopic FC in the presence of control (○) or modified (×) HDL. Each point is a single experimental determination. Curves were obtained by the computerized fitting procedure, as described for Fig. 1.

TABLE II

<table>
<thead>
<tr>
<th>Compositional parameter</th>
<th>HDL</th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Modified</td>
<td></td>
</tr>
<tr>
<td>FC/protein (w/w)</td>
<td>0.041</td>
<td>0.042</td>
<td></td>
</tr>
<tr>
<td>TC/protein (w/w)</td>
<td>3.26</td>
<td>3.26</td>
<td></td>
</tr>
<tr>
<td>PL/protein (w/w)</td>
<td>0.37</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td>PL/FC (mol/mol)</td>
<td>4.6 ± 0.5</td>
<td>2.3 ± 0.4</td>
<td></td>
</tr>
</tbody>
</table>

The effect of modifying HDL with phospholipase A2 on the bidirectional flux of FC with Fu5AH cells. HDL, prelabeled with [3H]FC, was treated with phospholipase A2 (C. adamanteus) post-incubated with BSA to remove hydrolysis products, and then reisolated by ultracentrifugation, as described by Pattnaik and Bamberger.

lipase-mediated modification selectively reduced $k_i$, with little or no effect on $k_e$. Additionally, they suggest that, with control HDL, there was an approximate steady state of FC mass distribution between the lipoprotein and Fu5AH cells and that the modification of HDL, by the selective reduction of $k_i$, perturbed this steady state toward net uptake of FC from the lipoprotein.

It can also be seen in Fig. 3 that, with a given type of HDL, both initial influx and efflux of FC increased as HDL concentration was raised but that the rate of increase was greatest at low HDL concentrations. From the relationship

$$\frac{F_i}{F_m} = k_i$$

It can be seen in Fig. 2 that, after approximately 30 min of incubation, PL depletion of HDL stimulated the cellular retention of both the [14C]cholesterol initially present in cells and the [3H]cholesterol derived from HDL. Both effects were consistent observations from experiment to experiment. Two processes may have contributed to the enhanced retention of cholesterol tracers: 1) the net redistribu-
tion of FC mass from HDL into cells which resulted from the selective reduction of efflux and 2) enhanced cellular esterification of cholesterol, which occurs in Fu5AH cells in response to FC enrichment (9, 25). To determine the relative importance of these processes, we employed the Sandoz compound 58-035, a specific inhibitor of acyl coenzyme A:cholesterol acyltransferase in Fu5AH cells (25). The effect of the inhibitor on the enhanced cell association of cholesterol tracers during incubation with PL-depleted HDL is shown in Fig. 4. It can be seen that, using either control or PL-depleted HDL, the inhibitor had no effect on the initial influx or efflux of FC or on the cell association of cholesterol tracers during the first 90 min of incubation. TLC analysis of isotopic cholesterol and GLC analysis of cholesterol mass confirmed that the inhibitor prevented essentially all cellular esterification of cholesterol. The results thus suggest that the enhanced cell association of cholesterol tracers during incubation with PL-depleted HDL was not due to increased cellular esterification of cholesterol.

It may be noticed in Fig. 4 that depletion of HDL PL had the expected large negative effect on initial FC efflux (35% reduction) but also appeared to enhance FC influx to a small degree (10% increase). The level of deletion of HDL PL was 63% in this experiment (see legend of Fig. 4). Typical depletion levels, as described for Figs. 3 and 5, were only 50%, in which case efflux was depressed by 30 ± 6% (n = 6, from data in Figs. 3 and 5), whereas influx was not significantly affected (average change of -7 ± 6%, n = 6, from data in Figs. 3 and 5). Thus, the stimulation of influx discernible in Fig. 4 was somewhat unusual and may have arisen from a higher than normal level of depletion of HDL PL. In all other respects, the results of this experiment were quite typical.

In the same experiment, some sets of cells were incubated for an extended period (415 min) under the four conditions described for Fig. 4 (control or PL-depleted HDL ± compound 58-0355), and then changes in cellular free and esterified cholesterol mass (as quantitated by GLC) were compared in the four sets (Table III). Initially (t = 0), cells contained 17.5 μg of FC/mg of protein and no detectable esterified cholesterol (EC). As expected from previous results (9), in the absence of compound 58-035 (line 1 of Table III), there was a moderate increase in cellular FC and a significant accumulation of EC during the 7-h incubation with control HDL, suggesting a slow net increase in cellular cholesterol. The increases in both of these quantities were significantly greater when the medium contained modified HDL. In the presence of compound 58-035 (line 2 of Table III), there was no significant accumulation of EC during incubation with either HDL. There were, however, significant net increases in FC, with that caused by modified HDL being much greater than that caused by control HDL. The recovery of isotope from these cells indicated that the differences in FC and EC apparent in Table III were due neither to the de novo synthesis of cholesterol nor to the uptake of unlabeled CE from HDL. The results provide additional evidence that the depletion of PL from HDL stimulated the net transfer of FC from HDL to cells independently of acyl coenzyme A:cholesterol acyltransferase-mediated cholesterol esterification. It is also clear from the results, however, that, when acyl coenzyme A:cholesterol acyltransferase was allowed to function, it effectively limited the accumulation of cellular FC to a low level.

In the absence of interfering metabolic processes (cell growth, cholesterol synthesis, and cholesterol esterification), the ratio of rate constants for FC flux should predict the steady state distribution of FC mass between cells and HDL (i.e. k₁/k₅ = FC₅/FC₇ at steady state) (24). Additionally, as

![Fig. 4. Effect of inhibition of cellular acyl coenzyme A:cholesterol acyltransferase on the bidirectional flux of FC between HDL and Fu5AH cells.](image-url)
TABLE III

Effect of PL depletion of HDL and inhibition of acyl coenzyme A-cholesterol acyltransferase on cellular cholesterol content

<table>
<thead>
<tr>
<th></th>
<th>Control HDL</th>
<th>Modified HDL</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>FC</td>
<td>EC</td>
</tr>
<tr>
<td>[58-035]</td>
<td>21.2 ± 20.5</td>
<td>11.8 ± 11.3</td>
</tr>
<tr>
<td>[58-035]</td>
<td>25.7 ± 23.5</td>
<td>0.7</td>
</tr>
</tbody>
</table>

* Media lacked compound 58-035.

* Media contained 1 µg of compound 58-035/mL.

TABLE IV

Comparison between the steady state distribution of FC mass between cells and HDL and the ratio of rate constants for FC flux

<table>
<thead>
<tr>
<th>HDL type</th>
<th>FC mass*</th>
<th>Rate constants for FC flux</th>
<th>10^9 k</th>
<th>10^9 k</th>
<th>kF/kC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells</td>
<td>HDL</td>
<td>Cells/HDL</td>
<td>min⁻¹</td>
<td>min⁻¹</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5.4</td>
<td>36</td>
<td>0.15</td>
<td>1.8</td>
<td>14.2</td>
</tr>
<tr>
<td>Modified</td>
<td>8.0</td>
<td>34</td>
<td>0.24</td>
<td>2.0</td>
<td>9.3</td>
</tr>
</tbody>
</table>

* Mass quantitation of FC in cells and medium/22-mm tissue culture well, containing 0.22 mg of cell protein and 0.5 mL of medium.

The table shows the ratio of rate constants for FC flux between cells and HDL, calculated as the ratio of the initial (t = 0) mass of cellular protein. Each value is a single experimental determination. Initially, cells contained 17.5 ± 0.5 µg of FC/mg of cell protein (n = 4) and no detectable EC.

Predictions provide additional validation for the modeling of FC movement between HDL and Fu5AH cells in terms of first-order rate constants and homogeneous FC pools.

In Fig. 5, the relationship between degree of modification of HDL with phospholipase A2 and cholesterol flux is shown. The results are plotted as kF or kC for FC flux versus the ratio of PL to protein (w/w) at constant HDL protein (and initial FC) concentration. Linear regression analysis of the results demonstrated that there was a significant negative relationship between kF and PL/protein ratio (coefficients of determination, r², equal to 0.96 and 0.93 at HDL concentrations of 0.09 and 0.53 mg of protein/mL, respectively) but no significant relationship between kC and PL/protein ratio (r² = 0.52 and 0.05 at 0.09 and 0.53 mg of protein/mL, respectively).

The table shows the ratio of rate constants for FC flux between cells and HDL, calculated as the ratio of the initial (t = 0) mass of cellular protein. Each value is a single experimental determination. Initially, cells contained 17.5 ± 0.5 µg of FC/mg of cell protein (n = 4) and no detectable EC.

Depletion of Phospholipids from HDL Mediated by Hepatic Lipase—HDL was modified with hepatic lipase and reisolated, as described under "Experimental Procedures." Heat-inactivated hepatic lipase was used in the preparation of control HDL. Compositional data for HDL prepared in this way were summarized by Bamberger et al. (9). For the experiment described here, the PL/protein ratio of the modified and control HDL were 0.44 and 0.54 (w/w), respectively (initial PL/FC = 3.8 and 4.7 mol/mol, respectively). At an HDL concentration of 0.54 mg of protein/ml (initial [FC] = 32 µg/mL), the modification reduced kF for FC by 14% and increased kC by 7%. Additionally, it caused obvious enhancement of the cellular accumulation of HDL-derived [3H]FC after 60 min of incubation and doubled the extent of esterification of cell-associated [3H]FC, after 240 min of incubation (10.0 and 5.2% in cells exposed to modified and control HDL, respectively). These effects were qualitatively similar to those produced by a low level of modification of HDL with phospholipase A2 as described above. The results suggest that the two forms of HDL modification (by hepatic lipase and by phospholipase A2) stimulate cholesterol uptake by the same mechanism(s).

**DISCUSSION**

Data presented in this paper show that the surface transfer of FC between HDL and Fu5AH cells was bidirectional and that essentially all FC in both cells and HDL was available for movement across the cell surface boundary. The kinetics of movement of FC tracers between cells and HDL and HDL formed well to a model which assumed that the FC populations in cells and HDL, formed a closed system of only two kinetic pools. The computerized analysis of kinetic data in terms of this model yielded estimates of the rate constants of cellular FC influx and efflux under a variety of incubation conditions. These, in turn, were used to calculate the influx and efflux of FC mass under various conditions and to predict the steady state distribution of FC mass between cells and HDL.

The bidirectional nature of FC transfer in this system was similar to FC interchange between HDL and a variety of other cells and micellar complexes, for example, erythrocytes (28), rabbit primary hepatocytes (29), transformed lung fibroblasts (27), small unilamellar vesicles (28), and low density lipoprotein (22). The present results demonstrate that the Fu5AH cell is a convenient model for studying the bidirectional transfer of FC between HDL and intact cultured cells.

There are two possible reasons for the FC of Fu5AH cells forming a single kinetic pool for eflux. 1) Negligibly small amounts of FC may normally reside in non-surface membranes of the cells. 2) The surface and internal pools of cell FC may be in very rapid equilibrium compared to the rate of equilibration of HDL and cell plasma membrane pools. Existing data are not sufficient to distinguish between these possibilities. It is clear, however, that the intracellular disposition of FC in Fu5AH cells is different from that in certain lines of mammalian macrophage (29, 30) and in human fibroblasts (31), in which kinetically distinct surface and internal pools of FC seem to exist.

Several mechanisms operate in the delivery of cholesterol to the liver and therefore contribute to reverse cholesterol transport. These include endocytic uptake and degradation of lipoproteins, including HDL (2, 32–34), and the surface transfer of CE (35) and FC (2) from non-endocytosed HDL. Jansen and Hulsman (10) suggested that heparin-releasable hepatic lipase may promote the surface transfer of FC mediated by the hydrolysis of HDL PL within the liver, thereby reducing the capacity of the lipoprotein to hold FC. In the experiments which served as the basis for the present studies (9, 12), this hypothesis was tested by studying the interactions between HDL and Fu5AH rat hepatoma cells in culture. It was found that depletion of PL from HDL, by treatment with hepatic lipase or snake venom phospholipase A2, enhanced the accumulation of FC and CE mass in cells during long-term incubations and stimulated the net delivery of [3H]FC from HDL to the cells. These effects could not be attributed to uptake and degradation of the intact HDL, enhanced uptake and degradation of CE from HDL, or increased cellular synthesis of cholesterol, suggesting, in accord with the hy-
The preceding comment is not intended to deny a role for the depletion of PL from HDL stimulated the accumulation of cholesterol in cells exposed to PL-depleted HDL. The functioning of the enzyme, acyl coenzyme A:cholesterol acyltransferase, in its mediation of CE formation, functioned to limit the accumulation of FC in Fu5AH cells. When cells experienced net inward flux of FC, as in the presence of PL-depleted HDL, acyl coenzyme A:cholesterol acyltransferase apparently reacted to the accumulation by increasing the rate at which the cellular FC was esterified. This prevented unlimited increase in the size of FCC, the cellular FC pool. By this mechanism, the unidirectional outward flux of FC \((F_i = FC_p \times k_c)\) would also have been limited, and the net inward flux of FC \((F_i - F_o)\) would have been sustained. Thus, acyl coenzyme A:cholesterol acyltransferase, even though it functioned to limit the size of the cellular FC pool, would actually have promoted the net uptake of FC from PL-depleted HDL. The functioning of the enzyme, however, would not have been the primary cause of the uptake, as its contribution would have arisen as it reacted to the accumulation of FC, which would have occurred initially.

**Fig. 5.** Bidirectional flux of FC between Fu5AH and HDL, as influenced by extent of modification of HDL with phospholipase A₂. HDL was modified as described for Fig. 2. Two levels of modification were achieved by use of two concentrations of lipase (50 and 160 μg/ml). Control HDL was exposed to the higher concentration of enzyme in the presence of 12 mM EDTA. Media contained 0.2% (v/v) BSA and 0.09 or 0.53 mg of HDL protein/ml (initial [FC] = 5.2 or 31 μg/ml, 3832 3H cpm/μg of FC) in MEM-HEPES. Other incubation conditions were as for Fig. 2. Cells in each 22-mm well contained 0.20 mg of protein and 2.7 pg of FC (3764 14C cpm/μg of FC) at the initiation of incubations. With each medium, triplicate series of incubations were performed, samples being taken after 0 and approximately 15, 25, 40, 60, and 90 min of incubation. The measurements from a series were used to estimate the rate constants for FC flux \(k_i, k_e, k_c\) by the computerized fitting procedure, as described for Fig. 1. Results are plotted as \(k_i\) or \(k_e\) versus weight ratio of PL to protein in HDL at a constant concentration of HDL protein. Each point is a single experimental determination. Lines were obtained by linear regression analysis of a set of points. Values of \(k_i\) are dependent on the medium volume and cell number/tissue culture well and should be converted to clearance \(Cl_i\) to provide an indication of the intrinsic tendency of FC to move from HDL to cells (see "Appendix").
through FC surface transfer mechanisms alone.

Arguments presented under “Results” assume that, at a given concentration of a specific type of HDL, the only mechanism for equalizing $F_F$ and $F_C$ for FC would have been readjustment of pool sizes. This is equivalent to saying that, at a specific concentration of an HDL type, $k_i$ and $k_e$ for FC were simple first-order rate constants and thus independent of the sizes of FC, and FC$_m$. Results in Table IV suggest that this assumption probably was valid with respect to FC$_m$. Here, it is demonstrated that rate constants, which were determined from the initial fluxes of FC, correctly predicted the steady state distribution of FC mass between cells and modified HDL when the size of FC$_m$ more than doubled during the approach to the steady state. That the assumption was valid with respect to the size of FC$_m$ was not adequately tested, since, even in cases of considerable net cellular accumulation of cholesterol, medium FC was seldom seriously depleted.

The present results show that reducing the PL content of HDL rendered the lipoprotein a relatively poor acceptor of cellular FC. Two factors may have contributed to the reduced efficiency of this HDL as an acceptor of FC. One was the simple reduction of PL$_a$, a class of molecules which can readily solubilize FC (36, 37) and which are capable of independently causing the release of cellular FC (4). The second factor has to do with the reorganization of lipid molecules within HDL known to result from PL depletion. Bamberger et al. (12) showed that reducing the PL content of HDL causes FC to redistribute within the lipoprotein particle so that a greater fraction of its FC resides in the surface lipid monolayer. This redistribution would put a larger fraction of the FC of HDL in immediate contact with the lipoproteins’ PL, and thus may further reduce the effective extracellular PL available for interaction with FC transferring from the cell surface.

Free cholesterol in a human HDL particle has an average residence time of 5 min (22). The FC molecules desorb from the HDL surface into the aqueous phase and exchange rapidly with FC molecules in other lipoprotein particles or in cell membranes. Similarly, FC in cell plasma membranes can desorb into the extracellular aqueous phase and be absorbed by a PL-containing acceptor particle (4). Thus, the aqueous diffusion mechanism, in all probability, underlies the equilibration of FC between HDL and Fu5AH cells observed in this study. Consistent with this suggestion the rates of FC release to HDL observed in the present studies were such that the release would have occurred by an aqueous diffusion mechanism. The maximum observed $k_e$ for FC was 0.022 min$^{-1}$. (This value can be obtained from the results for efflux of FC in Fig. 3 by dividing the initial cellular FC mass, 5.4 µg, into the maximum observed initial efflux of FC in the presence of control HDL, 410 ng of FC min$^{-1}$ (mg of cell protein)$^{-1}$, and then multiplying the result by the initial mass of cellular protein, 0.29 mg.) This $k_e$ corresponds to a $t_\text{a}$ for efflux of 31 min, which is similar in magnitude to minimum $t_\text{a}$ values for FC efflux from Fu5AH cells to various artificial acceptors known to promote FC release by an aqueous diffusion mechanism (4).

Under the conditions of Fig. 3, the efflux of FC was never zero order with respect to HDL concentration, indicating that desorption of FC molecules from the plasma membrane is not rate-limiting for FC transfer from cells to HDL (cf. Ref. 4). Consequently, the absorption process, whereby cholesterol molecules in the water are incorporated into HDL, affects the overall rate of transfer. Since removal of PL from HDL reduces $k_e$ (Table IV), it seems that the incorporation of FC molecules into a PL-depleted HDL is relatively slow. The observation that $k_e$ is not sensitive to PL depletion of HDL implies that the rate of desorption of FC molecules from HDL is independent of the FC/PL ratio of the particle; this observation is consistent with prior observations on the equilibration of FC between PL/FC vesicles (38).

Since HDL can bind specifically to receptor sites on the plasma membrane of several cell types (39–43), an intriguing question is whether this interaction affects the values of $k_i$ and $k_e$ measured here. It should be noted that a receptor-mediated process is not inconsistent with the aqueous diffusion mechanism; the FC molecules may transfer between the bound HDL and the plasma membrane by desorbing and diffusing through the intervening water layer. The kinetics of surface transfer of FC mediated by specific HDL binding should exhibit a hyperbolic dependence on HDL concentration, which mimics the concentration dependence of specific binding. The specific binding of HDL to mammalian cells typically exhibits a $K_m$ in the range of 2–30 µg of HDL protein/ml (39, 41–44). In the present study (Fig. 3, control curves), influx and efflux resembled one another in their dependence on HDL concentration, rising rapidly in the range 0.1–1 mg of HDL protein/ml, and then showing a slower linear rate of increase in the range 1–5.5 mg of protein/ml. These results show clearly that influx and efflux do not approach maximal values within HDL concentrations which normally allow maximal specific binding. This suggests that specific HDL binding, although it may mediate FC transfer at low HDL concentrations, cannot account for the bulk of FC transfer between HDL and the Fu5AH cells. The point might also be made that reduced binding is an unlikely explanation for reduced FC efflux in the presence of PL-depleted HDL. Apart from the concentration dependence of flux, two facts argue against this explanation. First, when Bamberger et al. (9) measured the binding and degradation of control and hepatic lipase-modified HDL, they found no difference between the two forms of the lipoprotein. Second, if important to FC transfer, binding is likely to enhance both influx and efflux to similar degrees. If reduced binding were an adequate explanation for the reduction in efflux, then influx should have been reduced in a parallel fashion. However, in the present experiments, the effects of PL depletion of HDL on influx and efflux were distinctly different. Studies to further evaluate any possible relationship between HDL binding and FC surface transfer are in progress in this laboratory.

HDL concentration in normal human blood plasma is 1.6–1.8 mg of protein/ml (45), and the concentration in peripheral lymph is probably about 10% that in plasma (46) or approximately 0.2 mg/ml. These values probably are near the extremes of normal human physiological HDL concentrations. They are included within the range of HDL concentrations employed in the present experiments, indicating that conclusions drawn from the present experiments may be applied to situations where physiological concentrations of HDL are encountered.

The present results and those of Bamberger et al. (9, 12) show that the ratio of FC to PL in HDL is important in determining the net direction of FC transfer between HDL and cells. These results are similar to the hypothesis of Fielding (47) that FC concentrations in plasma lipoproteins are important in determining the direction of net transfer of FC between the different lipoprotein classes. Taken together, these observations suggest that an important component of FC transfer among the biochemical and morphological compartments in intact animals is non-active (i.e. occurs without the direct expenditure of metabolic energy) and thus is directed by the creation of negative free-energy gradients. The
present results show that hepatic lipase and cellular acyl coenzyme A:cholesterol acyltransferase can influence such gradients and consequent FC flux. Other physiological processes likely to have a significant influence on free-energy gradients for FC are the action of lecithin:cholesterol acyltransferase in plasma, the synthesis of bile salts and steroid hormones in steroidogenic tissues, the secretion of FC in bile and lipoproteins, and the activity of cholesteryl-ester hydrolases in cells. How these processes affect the flux of FC between cells and lipoproteins will be the subject of future investigations.

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APPENDIX

The following definitions of terms apply to the kinetic analysis of tracer flux: $t$, incubation time, in minutes; $q_s$ either the $^3$H counts/minute taken up by cells during incubation time ($t$) or the difference of the initial $^3$C counts/minute in cellular FC minus the $^3$C counts/minute released to medium during incubation time ($t$); $q_{oa}$, initial total of either $^3$H counts/minute in medium during incubation time or $^3$C counts/minute in cellular FC; $q_{ao}$, the fractional uptake of $[^3H]$ FC or the fractional retention of $[^3C]$ FC by cells; $e$, the base of natural logarithms (2.7183); $k_i$ and $k_e$, the rate constants of FC influx and efflux, respectively, in minute$^{-1}$; FC, cellular FC pool, in nanograms or micrograms; $F_{cm}$, extracellular (medium) FC pool, in nanograms or micrograms; $F_{cm}$, unidirectional influx and efflux of FC, in nanograms or FC minute$^{-1}$ or nanograms of FC minute$^{-1}$ (mg of cell protein)$^{-1}$.

Analysis of tracer flux was performed on a Franklin 1000 computer programme to determine relationships of the form

$$q_{oa}/q_{ao} = H e^{-eq_0} + H_2$$

(1)

which best described the time course of $^3$H uptake or $^3C$ retention by cells. ($H_2$, $g_1$, and $H_2$ were constants adjusted by the program to provide the most accurate representation of data.) Relationships in the form of Equation 1 are expected to describe tracer uptake and retention if the cell-associated and HDL-associated FC populations each forms a single kinetic pool in a closed system. The program for the fitting procedure was based on the ACM algorithm (48) for approximating the solution by the Taylor series modification of the classical least squares method. The only significant change from the ACM algorithm was the convergence criterion. The algorithm used in the present study continued to iterate until the "fractional squared error" ($|E^2(n) - E^2(n-1)|/E^2(n)$, where $E^2$ is the sum of the residuals and $n$ is the number of iterations) was less than 0.01.

Under the assumptions of this analysis, $k_i = -H_i g_1$, from measurements of cellular uptake of $[^3H]$ FC, and $k_e = H_e g_2$, from measurements of retention of cellular $[^3C]$ FC (24). The values of $k_i$ and $k_e$ are the initial ($t = 0$) slopes of the curves describing $[^3H]$ FC uptake and $[^3C]$ FC retention, respectively. $F_i = k_i \times F_{cm}$ and $F_e = k_e \times F_C$ (24). In making predictions of FC mass redistribution from determinations of $k_i$ and $k_e$, the rate constants for a specific concentration of a specific type of HDL were assumed to be constant, whereas $F_{cm}$ and FC varied.

The initial flux of FC between cells and HDL would be limited by HDL concentration and by the number of cells exposed to the HDL-containing medium, but probably not by volume of medium applied to cells. The rate constant of influx, $k_i$, (in minutes$^{-1}$), equals $F_i/F_{cm}$ (in nanograms minute$^{-1}$/nanograms). Thus, as the volume of medium increased at constant cell number, initial FC$_{cm}$ would increase, whereas initial $F_i$ would remain constant, and the measured value of $k_i$ would be inversely proportional to medium volume. Similarly, as cell number increased at constant medium volume, initial $F_i$ would increase, whereas initial FC$_{cm}$ would remain constant, and the measured value of $k_i$ would be directly proportional to cell number. These considerations indicate that the values of $k_i$, as presented here is simple terms of minute$^{-1}$, are dependent on the volume of medium applied to cells and the number of cells exposed to the HDL-containing medium and thus are not immediately representative of the intrinsic tendency of FC to move from HDL to cells. To remove the dependence on medium volume and cell number, values of $k_i$ may be re-expressed in terms of clearance of HDL FC by cells, $C_l$, which is equal to $k_i \times$ volume of HDL solution applied to cells/mass cell protein (24). The values necessary for calculating clearance from $k_i$ are supplied in the figure legends. In a given experiment, the volume of medium and cell mass in each tissue culture well were constant; this allowed valid comparisons of $k_i$ values when expressed simply in terms of minute$^{-1}$. Similar problems do not arise in the expression of $k_e$ because initial mass of cell FC (initial FC$_{cm}$) would be directly proportional to cell number and completely independent of medium volume. Thus, at a given HDL concentration, initial $F_e$ would simply increase in direct proportion to initial FC$_{cm}$, and measured $k_e$ ($=F_e/F_{cm}$, in nanograms minute$^{-1}$/nanograms or minute$^{-1}$) would be independent of cell number and medium volume.

REFERENCES


Bidirectional Cholesterol Flux

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