Mechanism of Cholesterol Efflux from Cells

EFFECTS OF ACCEPTOR STRUCTURE AND CONCENTRATION

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The kinetics of removal of [3H]cholesterol from Fu5AH rat hepatoma, WIRL-3C rat liver cells, and human skin fibroblasts growing in culture to phospholipid-containing acceptor particles in the extracellular medium (0.05-1.2 mg of phosphatidylcholine (PC)/ml) has been measured. The rate of release of at least one-third of the cholesterol in these cells in either monolayer or suspension culture is first order with respect to concentration of cholesterol in the cells. The rate constants or half-times (t1/2) for this process are a function of the cell type and the concentration and structure of the extracellular acceptor particles. At high acceptor concentrations, the t1/2 is independent of acceptor concentration, and the rate-limiting step is the desorption of cholesterol molecules from the cell plasma membrane into a layer of unstirred water surrounding the cell. Under these conditions, the t1/2 values are Fu5AH < WIRL-3C < fibroblasts for all acceptors used and presumably reflect differences in the structures of their plasma membranes; a common minimum t1/2 is achieved for a given cell type in a confluent monolayer or in suspension where the thickness of the unstirred water layer is reduced, indicating that collisions between acceptor particles and cholesterol molecules which have desorbed from the cell are sufficiently frequent for the absorption by the acceptor not to be rate limiting. At lower concentrations of egg PC vesicles, apo-high density lipoprotein/egg PC complexes, or sodium taurocholate/egg PC micelles, efflux decreases with decreasing PC concentration in the extracellular medium, because the lower frequency of collisions between desorbed cholesterol molecules and acceptor particles causes the diffusion barrier of the unstirred water layer to reduce the cholesterol flux. In this region, the t1/2 is a function of acceptor composition so that, at a given PC concentration in the extracellular medium, t1/2 values are in the order: PC vesicles > apo-high density lipoprotein/PC complexes > sodium taurocholate/PC micelles. These differences in cholesterol efflux with acceptor type do not simply arise from variations in acceptor particle number in the extracellular medium. Particle size is important because comparison of different acceptors on the basis of the total surface area presented to the cells normalizes their performances to a large extent.

The lipoprotein-mediated delivery and removal of unesterified cholesterol from cells plays an important part in the regulation of cellular cholesterol content (1, 2). Investigation of the removal process is essential to the understanding of the mechanisms involved in the reverse transport of cholesterol from peripheral cells to the liver. Earlier studies (3-5) have shown that the free cholesterol content of cells decreases when suitable extracellular acceptors are incubated with the cells; the quantitative aspects of the process are poorly understood. We are utilizing a system where the kinetics of the unidirectional flow of cholesterol from tissue culture cells to extracellular acceptors can be conveniently measured (6). The similarities in reaction order, rate constants, and activation energies between cholesterol transport in a model vesicle system (7) and cell systems indicate that the mechanism in both cases involves diffusion of cholesterol molecules through the aqueous phase, with the overall rate being influenced by the rate of the desorption of cholesterol molecules from the donor phospholipid-cholesterol bilayer membrane (6). An unstirred water layer around the cells presents a diffusion barrier across which the desorbed cholesterol molecules and acceptor particles must mix so that the half-time for the movement of cholesterol between cells and vesicles can be a function of extracellular vesicle concentration (6). In this paper we extend the above findings to show how variations in acceptor particle structure affect the rate of cholesterol removal from different cells.

MATERIALS AND METHODS

Cell Culture

The Fu5AH rat hepatoma cell line was originally derived from the Reuber H-35 rat hepatoma (8, 9) and has been extensively used for studies on cholesterol and cholesteryl ester metabolism (10, 11). The WIRL-3C rat liver cell line was obtained from Dr. L. Diamond, Wistar Institute, and has been maintained in culture since 1973 (12). These two cell lines do not appear to synthesize significant quantities of lipoprotein1 or to conjugate or release bile acids.2 Normal human preputial fibroblasts were initiated at the Medical College of Pennsylvania and used between the 10th to 30th passages. Stock monolayers of all cells were grown in MEM3 (Flow Labs, McLean VA) supplemented with Eagle's basal medium vitamins, 50 units/ml of penicillin, 50 µg/ml of streptomycin, and 10% calf serum (Flow Labs). Cells were grown at 36 °C in an atmosphere of 5% CO2-95% air. All cultures were free of mycoplasma as demonstrated by routine screening.

To prelabel cells with [3H]cholesterol, monolayers approximately 50-75% confluent were washed free of serum-containing medium and refed with medium supplemented with 1% fetal bovine serum, solvent-extracted delipidized calf serum protein (5 mg/ml) (13), egg PC (8 µg/ml), unesterified cholesterol (4 µg/ml), and N-[7-3H]cholesterol (0.5

1 J. B. Marsh and G. H. Rothblat, unpublished data.
2 D. Cassel and G. H. Rothblat, unpublished data.
3 The abbreviations used are: MEM, Eagle's minimal essential medium; PC, phosphatidylcholine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HDL, high density lipoprotein; LDL, low density lipoprotein.
Cholesterol Efflux from Cells

Have1 et al. (15) as modified by Marsh (16). HDL the vesicles by negative stain electron microscopy following 6-h fusion of the vesicles into multilamellar structures during the course incubation with cells demonstrated that there was no significant stain electron microscopy and gel chromatography on Sepharose ZBCL, the vesicles were homogenous unilamellar spheres with a diameter of 250 Å. For this preparation was done for the increased cholesterol synthesis which was made for the increased cholesterol synthesis which was modified by ether-ethanol extraction following the procedures described by Scamu (17). The lipid-free apo-HDL was dissolved in a solution consisting of NaCl (0.15 M), EDTA (0.001 M), and sodium azide (0.02% w/v) (pH 8.0) and stored at 4 °C. Complexes of apo-HDL with egg PC were prepared by sonicating a mixture of 1 mg/ml of PC and apo-HDL (2.5:2.1, w/w) in a Branson Sonifier 350 for 20-30 min at 4 °C. Following sonication the complexes were centrifuged at 40,000 rpm for 1 h in a Beckman type 40 rotor to remove titanium and any uncomplexed phospholipids and proteins under these conditions, combination of lipid and protein was essentially complete. Analysis of this preparation at this stage confirmed that the initial lipid/protein stoichiometry was maintained. Prior to use, the preparation was dialyzed against MEM plus HEPES and diluted to the required concentrations. Electron microscopic examination of the mostly spherical egg PC/apo-HDL complexes (cf. Ref. 18) gave the particle dimensions listed in Table I.

<table>
<thead>
<tr>
<th>Table I</th>
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<tr>
<td>Characteristics of cholesterol acceptor particles</td>
</tr>
<tr>
<td>Composition</td>
</tr>
<tr>
<td>Egg PC vesicles *</td>
</tr>
<tr>
<td>Egg PC/apo-HDL</td>
</tr>
<tr>
<td>Egg PC/Na taurocholate #</td>
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* Determined directly from electron micrographs of several representative preparations.

# Ref. 7.

* Ref. 18.

# Ref. 19.

* Thickness of disc.

Sodium Taurocholate/Egg PC Micelles—Bile salt/PC mixed micelles were obtained following the general procedure described by Mazer et al. (19). After dissolving the appropriate amounts of each lipid in methanol, the mixture was dried under N₂. An appropriate volume of MEM buffered with HEPES was then added, and the mixture was vortexed until clear. Stock solutions of the micelles were prepared at a concentration of 3.7 mg/ml of egg PC and 6.3 mg/ml of sodium taurocholate (Calbiochem-Behring). This stock was diluted to the final concentrations indicated in the text by the addition of HEPES-buffered MEM supplemented with 3 mg/ml of sodium taurocholate; this gave a solution containing sodium taurocholate at its critical micelle concentration of 5.5 mM to ensure that the integrity of the mixed micelles was preserved (see Table I for their dimensions).

Analogous preparations and Methods—Proteins were assayed by the sodium dodecyl sulfate-Lowry method (20), and lipids were extracted as described by Bligh and Dyer (21). Cholesterol was quantitated by gas liquid chromatography (22). Phospholipid phosphorus was measured by the method described by Sokoloff and Rothblat (23). ¹H and ¹³C were quantitated by liquid scintillation techniques in a Beckman LS 7500 counter using Scintiverse (Fisher).

Electron Microscopy—Lipid-containing dispersions were negatively stained with 2% w/v sodium phosphotungstate (pH 7) on Formvar-coated 200-mesh copper grids. In order to facilitate spreading, the grids were electrostatically charged in a Denton DV-602 vacuum evaporator prior to applying the sample (0.5 μl of a dispersion containing about 0.25 mg of lipid/ml) and the stain. Micrographs were then obtained by standard procedures with a Zeiss 10 transmission electron microscope operating at 80 kV.

RESULTS

Kinetics of Cholesterol Efflux—Previous studies quantitating the release of free cholesterol from cells exposed to unilamellar PC vesicles demonstrated that efflux followed first order kinetics with respect to free cholesterol concentration in the donor cells (6). In the case of FuSAH cells which exhibited rapid efflux (6) and which have been shown to have high rates of cholesterol synthesis (24), a semi-exponential plot of the fraction of the original counts remaining in the cells against time yielded a biphasic line until corrected for changing cellular cholesterol specific activity. When allowance was made for the increased cholesterol synthesis which was

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acid during our standard 2-day labeling period. The data (not previously incorporated by the cells from the medium to verified by simultaneously following the efflux of [\(^3\)H]cholesterol synthesized from [\(^4\)C]mevalonic decreases.

![Graph of Cholesterol Efflux from Cells](http://www.jbc.org/)

Fig. 1. Efflux of [\(^3\)H]cholesterol from Fu5AH rat hepatoma cell monolayers exposed to apo-HDL/egg PC complexes, 0.5 mg of PC/ml (○—○); sodium taurocholate/egg PC micelles, 250 \(\mu\)g of PC/ml (■—■); and human skin fibroblasts exposed to apo-HDL/PC, 0.5 mg of PC/ml (▲—▲). Average of duplicate cultures.

initiated after 2- to 3-h exposure to acceptors, the loss of cholesterol mass followed first order kinetics so that the rate constant \(k = \ln 2/t_{1/2}\) where \(t_{1/2}\) is the half-time for removal of cholesterol (6). Fig. 1 illustrates similar data obtained from typical experiments conducted with Fu5AH cells exposed to sodium taurocholate/PC micelles or apo-HDL/PC complexes and skin fibroblasts incubated in the presence of apo-HDL/PC acceptor particles. Due to de novo cholesterol synthesis the extent to which cholesterol specific activity decreased with time was a function of the cell type and magnitude of cellular cholesterol loss. In the examples shown in Fig. 1, the cholesterol specific activity in the fibroblasts decreased by only 8% during the 6-h incubation, whereas the decrease was 20% in Fu5AH cells exposed to the apo-HDL/PC complex and 43% in the same cells exposed to the sodium taurocholate/PC micelles. To minimize the influence of changing specific activity which occurred in the rat hepatoma cells under conditions stimulating rapid cholesterol release, using multiple time points, efflux was monitored for 3 h which is prior to the inflections apparent in Fig. 1. Since the major change in cholesterol specific activity occurred during the latter part of the incubations (6), it did not have a major effect on the \(t_{1/2}\) for cholesterol efflux calculated from the isotopic data; \(t_{1/2}\) calculated from isotopic and mass data differed by less than 0.2 h. When the \(t_{1/2}\) is very long compared to the 6-h period of the experiment, the accuracy of these values progressively decreases.

The validity of the use of the [\(^3\)H]cholesterol-labeling procedure for assay of the flux of cellular-free cholesterol was verified by simultaneously following the efflux of [\(^3\)H]cholesterol previously incorporated by the cells from the medium to the efflux of [\(^{14}\)C]cholesterol synthesized from [\(^{14}\)C]mevalonic acid during our standard 2-day labeling period. The data (not shown) demonstrated a consistent \(^3\)H/\(^{14}\)C ratio for the nonsaponifiable lipid in both the cells and in the incubation medium throughout a 6-h incubation in the presence of acceptor. In addition, thin layer analysis using solvent systems that separate cholesterol from oxidized sterol demonstrated that >99% of the \([\(^3\)H] steryl released into the culture medium migrated as cholesterol.

In addition to the changes in cholesterol-specific activity observed with the Fu5AH cells, these cells also exhibited detachment from the plastic dishes and cellular lysis when incubated in high concentrations of the bile acid/PC micelles (>250 \(\mu\)g of PC/ml). At the end of a 2-h incubation period, toxicity as measured by standard \(^5\)Cr release techniques (40, 41) was 5% at bile acid/PC concentrations of 100 \(\mu\)g of PC/ml and 250 \(\mu\)g/ml, 10% at 500 \(\mu\)g/ml, and 18% at 1 mg/ml. This toxic response increased after 2- to 3-h incubation at acceptor concentrations above 100 \(\mu\)g/ml but was not observed when cells were incubated with either sodium taurocholate (5.5 mm) or PC (2 mg/ml) added separately. Cholesterol \(t_{1/2}\) values for such cells were calculated from multiple time points taken during a 2-h incubation period which was prior to the appearance of pronounced cellular toxicity. Other cell lines did not exhibit any toxic response to the different cholesterol acceptors. Although we have not conducted detailed investigations on the cause of the cellular toxicity observed with the rat hepatoma cells, it is probable that cell lysis was caused by the massive decrease in cellular cholesterol (>70%) occurring in the presence of the bile acid/PC acceptors (Fig. 1). In support of this explanation is the observation that Fu5AH cells loaded with excess free and esterified cholesterol demonstrate no toxic response when exposed to high concentrations of the sodium taurocholate/PC acceptors for periods up to 8 h.

**Efficiency of Acceptors**—When Fu5AH cells were exposed to apo-HDL or sodium taurocholate alone, cholesterol efflux was very slow; 0.75 mg apo-HDL/ml of medium and 3 mg of sodium taurocholate/ml gave \(t_{1/2}\) values of 28 and 44 h, respectively. Significant cellular cholesterol loss occurred only when phospholipid was present in the culture medium. Fig. 2 shows the effect of acceptor concentration on cholesterol efflux with four different acceptors. The data are normalized on the basis of the phospholipid concentration in the incubation medium. At all concentrations the sodium taurocholate/egg PC micelles stimulated the fastest efflux followed by lipoprotein-deficient human serum ("1.21 bottom fraction") > apo-HDL/egg PC complex > egg PC vesicles. With all acceptors, the \(t_{1/2}\) is a function of the extracellular acceptor concentration but is not inversely proportional to the phospholipid concentration, indicating that the rate of cholesterol removal from the cells is not first order with respect to acceptor concentration. Maximum efflux was obtained with the sodium taurocholate/PC micelles when present at a concentration of 250 \(\mu\)g of PC/ml, and addition of this acceptor at higher concentrations resulted in no additional reduction in \(t_{1/2}\). The curves in Fig. 2 indicate that increasing the concentration of the other acceptors caused the rate of cholesterol loss to approach the maximum value achieved with the bile acid/PC micelles.

The dependence of \(t_{1/2}\) on acceptor concentration is not a function of the cells being in a monolayer because a similar effect was observed with the Fu5AH cells maintained in suspension (Fig. 3). Thus, increasing the concentration of apo-HDL/PC complexes over the range 25-1200 \(\mu\)g of egg PC/ml decreased the \(t_{1/2}\) by a factor of about 5, while the equivalent factor for monolayer cells was about 9. The \(t_{1/2}\) for cells in suspension was lower than that for a monolayer until a com-
minimum value was achieved.

Fig. 4 illustrates the change in cellular cholesterol concentration observed in cells exposed to the lower concentrations of acceptors (10–100 μg of PC/ml). The efficiency of the acceptors in reducing cell cholesterol content paralleled the results obtained for the $t_{1/2}$ determined by isotopic analysis.

As shown in Fig. 2, the effect of acceptor dose was most dramatic at low concentrations of acceptors. Since it was possible that the effective concentration of acceptors was significantly reduced by adsorption to, or incorporation by cells, an experiment was conducted to quantitate the loss of acceptors from the incubation medium. Table II shows the recovery of sodium taurocholate/[14C]PC acceptors after incubation with either cell monolayers or with blank plates. Maximum loss of acceptors occurred at the lowest acceptor concentration, and incubation with cell monolayers increased the loss when compared to blank plates. The results were similar at 2 h, 4 h, and 6 h, indicating that there was an initial rapid decrease in acceptor concentration, which did not change with continued incubation. These results demonstrate that although there was a small change in acceptor number at low acceptor concentrations, this change was not sufficient to explain the dose-response curves shown in Fig. 2.

The comparison of acceptor efficiencies (Fig. 2) indicated that the sodium taurocholate/PC micelle was the most effective acceptor when compared on the basis of exogenous PC concentration. Since high concentrations of sodium taurocholate were added to the incubation medium to ensure micellar integrity, it is possible that the presence of the bile acid indirectly stimulated cholesterol efflux by interacting with, and modifying, cell membranes. To study this possibility, sodium taurocholate was added to cultures exposed to apo-HDL/PC complexes. As shown in Table III, the addition of the bile acid did not influence cholesterol efflux into medium containing apo-HDL/PC as acceptors. These results indicate that the enhanced efflux obtained with the bile acid micelles was a function of the micelles themselves and not related to the presence of the sodium taurocholate.

The data presented in Fig. 2 comparing the effectiveness of acceptors clearly demonstrates that, at equivalent exogenous

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TABLE II

<table>
<thead>
<tr>
<th>Micelle concentration (μg PC/ml)</th>
<th>Fu5AH monolayer</th>
<th>Blank plate</th>
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<tr>
<td>10</td>
<td>83</td>
<td>91</td>
</tr>
<tr>
<td>50</td>
<td>95</td>
<td>99</td>
</tr>
<tr>
<td>100</td>
<td>98</td>
<td>101</td>
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</tbody>
</table>

* $% = [14C]PC in medium after 4-h incubation/[14C]PC in medium at zero time. Recovery was similar at all micelle concentrations after 2-h and 6-h incubations.

The data presented in Fig. 2 comparing the effectiveness of acceptors clearly demonstrates that, at equivalent exogenous
phospholipid concentrations, the bile acid/PC micelle stimulates the most rapid efflux of cholesterol from Fu5AH cells. Fig. 5 presents the same data replotted on the basis of the number of acceptor particles in the culture medium using the particle characteristics presented in Table I. When the results are normalized on the basis of particle number, the PC vesicles appear to be the most efficient acceptor while the bile acid/PC micelles and the apo-HDL/PC complex yield similar curves. In Fig. 6, the acceptor dose data are presented normalized on the basis of external surface area (see Table I). When the data are treated in this manner, the dose-response curves are quite similar although not identical.

Comparison of Cholesterol Efflux from Different Cell Types—Fig. 7 shows the effect of increasing concentrations of the sodium taurocholate/PC micelles on cholesterol efflux from the Fu5AH rat hepatoma, WIRL rat liver cells, and a human skin fibroblast cell line. The rat hepatoma cell consistently exhibited the fastest rate of cholesterol loss. Although there were considerable differences between cell types in the rate of cholesterol removal, all of the cell lines demonstrated the same dose-response pattern. A large effect of acceptor dose was seen at the low acceptor concentrations, but at micelle concentrations above 250 μg of PC/ml, efflux from the three cell lines became independent of acceptor concentration (cf. Refs. 4, 25, and 26).

In a previous preliminary study (6), we determined that Fu5AH hepatoma cells released cholesterol at a faster rate than WIRL or skin fibroblasts when PC vesicles were used as exogenous acceptors. The comparative data presented in Table IV demonstrate that: 1) the differences in efflux between cell types are evident when apo-HDL/PC complexes are used as acceptors, 2) that all of the cell types exhibited an acceptor dose-response to the apo-HDL/PC acceptors, and 3) the so-

### Table III

<table>
<thead>
<tr>
<th>Medium</th>
<th>Na taurocholate</th>
<th>apo-HDL/PC</th>
<th>t½</th>
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<tr>
<td></td>
<td>3.0 mg/ml</td>
<td>0.5 mg PC/ml</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>3</td>
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*Fig. 5. Dependence of the half-time for cholesterol efflux from Fu5AH rat hepatoma cell monolayers on the total number of acceptor particles present in the extracellular medium. □, egg PC vesicles; ●, apo-HDL/egg PC complexes; ×, sodium taurocholate/egg PC micelles."

*Fig. 6. Dependence of the half-time for cholesterol efflux from Fu5AH rat hepatoma cell monolayers on the total surface area of acceptor particles present in the extracellular medium. Only the external surface area of the bilayer in vesicles is included. Symbols are the same as Fig. 5.*

*Fig. 7. Dependence of the half-time for cholesterol efflux on the concentration of sodium taurocholate/egg PC micelles in the extracellular medium. Fu5AH rat hepatoma cells (ΟΟΟΟΟΟ); WIRL rat liver cells (ΟΟΟΟΟΟ); human skin fibroblasts (ΧΧΧΧΧΧΧ). Average of 2 determinations.*
from cells. This is consistent with recent measurements of the pool under our conditions for efflux. Since removal of these lipoproteins, vesicles, fraction being in the plasma membrane (4, 27) where the free constant. With fibroblasts and WIRL cells up to 35% of the in Fu5AH hepatoma cells can be described by a single rate rate of removal of more than 70% of the free cholesterol mass very little esterified. The free cholesterol is distributed among the plasma and internal membranes of the cell with a large essentially contain all the cholesterol as free cholesterol with at the start of the cholesterol removal experiment the cells

**DISCUSSION**

1. **Influence of Cell Structure on Kinetics of Cholesterol Release**—The experimental design in this study is such that at the start of the cholesterol removal experiment the cells essentially contain all the cholesterol as free cholesterol with very little esterified. The free cholesterol is distributed among the plasma and internal membranes of the cell with a large fraction being in the plasma membrane (4, 27) where the free cholesterol/phospholipid ratio is 40–45 mol % in the cell types employed here (28, 29). Obviously, the free cholesterol is in more than one physically distinct pool, but, nonetheless, the rate of removal of more than 70% of the free cholesterol mass in Fu5AH hepatoma cells can be described by a single rate constant. With fibroblasts and WIRL cells up to 35% of the initial cell-free cholesterol content behaves as a single kinetic pool under our conditions for efflux. Since removal of these levels of free cholesterol must involve removal of cholesterol from the inner monolayer of the plasma membrane, it follows that “flip-flop” of cholesterol across the plasma membranes of these cells is not rate limiting for removal of cholesterol from cells. This is consistent with recent measurements of the transmembrane movement of cholesterol across human erythrocyte membranes where \( t_{1/2} \leq 3 \) s at 37 °C (30). Cholesterol molecules in rat hepatocytes become evenly distributed around the plasma membrane in 35 min (31) so that any lateral redistribution of cholesterol between different regions of the membrane is also not rate limiting for removal from the plasma to extracellular acceptors.

Earlier studies from this laboratory (6) have established that the mechanism by which extracellular particles such as lipoproteins, vesicles, or complexes of phospholipids with apo-protein or bile acid facilitate the removal from cells involves diffusion of cholesterol molecules through the aqueous phase, with the overall rate being influenced by the desorption from the donor phospholipid-cholesterol bilayer membrane. The experimental activation energy (6) is associated with the desorption step in which a cholesterol molecule leaves the plasma membrane and partitions into the aqueous phase where it can subsequently collide with an acceptor particle and be absorbed. The packing and location of free cholesterol molecules in the donor membrane must affect the ease with which they can desorb, but these aspects of the process are poorly understood at present. Thus, a detailed interpretation of why fibroblast and WIRL cells consistently show longer \( t_{1/2} \) for efflux than Fu5AH cells is not possible and must await isolation and comparison of their plasma membranes. The only absolute measure of the rate of desorption from a membrane is the interface flux, \( J \), expressed as molecules of cholesterol desorbed per unit area in unit time. In order to derive \( J \) for cells, an accurate measure of the plasma membrane surface is required. A better estimate of this can be obtained for a suspension culture because the cells assume a spherical morphology. The data in Table V for two liver-derived cells of similar size and cholesterol content indicate that \( J \approx 46 \times 10^{-6} \) mol of cholesterol m\(^{-2} \) s\(^{-1} \) for Fu5AH cells, which is about five times the \( J \) of WIRL cells. Since this difference in \( J \) for these two cell types is maintained in monolayer and suspension cultures, it does not arise from differences in cell packing or morphology and is probably a reflection of intrinsic differences in their plasma membranes.

The dependence on lipoprotein concentration of the rate of cholesterol exchange between cells and lipoproteins has been observed in several systems (4, 25, 28) and, as shown in Fig. 7, the rate eventually assumes a maximum value (minimum \( t_{1/2} \)) which is independent of acceptor concentration. In the region where the rate of cholesterol removal is a function of acceptor concentration, raising the number of acceptor particles tends to reduce any overall rate limitation arising from the low frequency of collisions between cholesterol molecules in the aqueous phase and the acceptor particles. Also, the increase in frequency of collisions between desorbed cholesterol molecules and acceptor particles progressively eliminates the restriction on cholesterol flux due to the diffusion barrier of the unstirred water layer (32). As expected, in this acceptor concentration range \( t_{1/2} \) for suspension cultures is less than that for monolayer cultures (Fig. 3) because the suspended cells have a thinner unstirred water layer associated with them. In our experiments we observed loss of only a few micrograms of PC at most from the extracellular medium over the period of an experiment (Table II), and it has been shown for vesicles that uptake is about 1 µg of PC/mg of cell protein/h (33, 34). Since we only observe zero order kinetics at high concentrations of our most efficient acceptors, it is unlikely that \( t_{1/2} \) values become independent of acceptor concentration because of saturation binding of acceptors to cells. The occurrence of a maximum cholesterol efflux at high acceptor concentration arises because under these conditions the collision frequency of acceptor particles with desorbed cholesterol molecules in the unstirred water layer around the cells is great enough to reduce the aqueous phase concentration of free cholesterol to zero. Under these conditions the overall rate of cholesterol removal is limited by the rate of desorption of cholesterol molecules from the cell membrane. Since the minimum \( t_{1/2} \) values at high acceptor concentrations from monolayer and suspension cultures are essentially the same (Fig. 3), this value is not determined by hydrodynamic effects arising from the presence of the unstirred water layer but rather is a reflection of the properties of the plasma membrane of the donor cell.
**2. Factors Controlling Acceptor Effectiveness**—It has been established that in the presence of PC which has an apolar region to solubilize cholesterol, effective removal of cholesterol from tissue culture cells occurs (3, 35, 36). In contrast, pure apolipoproteins or bile acids are not effective (cf. Refs. 3 and 35). When the acceptor concentration is below the level to give the minimum  t_{1/2} (maximum efflux) which is a function of the donor membrane, the structure of the acceptors becomes significant, and the kinetics is a function of acceptor composition (Fig. 2). Since efflux of cholesterol involves diffusion of acceptor particles into the unstirred water layer and collision with desorbed free cholesterol molecules, the rate of cholesterol pickup by acceptors will be greatest for small particles which have higher diffusion coefficients and the maximum surface area per unit mass of PC to enhance the probability of rapid collision and absorption of cholesterol. Hence, the concentration of desorbed cholesterol molecules in the interfacial region is maintained at a minimum level, and the net rate of desorption from the cell is maximized since the reabsorption of cholesterol molecules by the cell membrane is prevented.

The recalculation of Fig. 2 efflux data, using parameters from Table I, in terms of  t_{1/2} against acceptor particle number (Fig. 5) rather than extracellular PC concentration indicates that the differences between acceptor types do not arise simply from variations in particle number. Particle size plays a role in governing the effectiveness of acceptors because comparison of different acceptors on the basis of the total external surface area presented to the cells normalizes their performances to a large extent (Fig. 6). The data in Fig. 6 show that when acceptors of different chemical compositions but the same surface areas are compared, the rates of cholesterol removal from cells are similar but not identical. Elucidation of the exact effects of variations in the surface structure of acceptors must await further study.

**3. Physiological Significance**—These model studies of unidirectional flow of cholesterol from cells suggest that in the reverse transport of free cholesterol from peripheral tissue to liver, cholesterol efflux will be enhanced when the acceptor is a small particle of high surface area per unit mass. Thus, the rates of cholesterol flow from cells bathed by plasma to HDL should be greater than to LDL because the former is smaller and in normal plasma has a greater total surface area by a factor of ~1.5 for males and 2.8 for females. Apparently, a similar situation prevails in human peripheral lymph which contains reduced concentrations of lipoproteins (37), because both LDL and HDL in this fluid can act as acceptors of tissue free cholesterol with HDL being predominant (38). Although the data presented here for the model system with homogeneous acceptor particles can be interpreted without invoking binding of acceptors to the cell surface, variation in reversible binding of acceptor (e.g. lipoproteins) occurring in vivo where the acceptor population is presumably heterogeneous might contribute to differences in the effectiveness of dissimilar lipoproteins to promote cholesterol efflux by preferentially raising the local concentration of a certain type of acceptor particle near the cell surface.

The results obtained from this study may also be relevant to the process of cholesterol transfer from liver into bile. The data in Fig. 7 show that PC/taurocholate micelles remove cholesterol from cells very effectively so that the very tight coupling of cholesterol secretion in bile to the rate of bile acid output (39) may occur just adjacent to the canalicular membrane where particles of constant bile acid/phospholipid molar ratio could pick up cholesterol molecules from the aqueous phase after they have desorbed from the membrane. Each bile acid/phospholipid micelle would pick up a constant amount of cholesterol as it flows past the canalicular membrane so that the molar ratio of cholesterol in the bile would remain within narrow limits.

The results presented here give information on the factors likely to control the rate of cholesterol movement out of cells but they do not indicate anything about the equilibrium situation. This requires determination of bidirectional flow using acceptors such as HDL and will be the subject of further study.

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