Comparison of the intracellular metabolism and trafficking of 25-hydroxycholesterol and cholesterol in macrophages

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Abstract Oxysterols arising from the diet or through lipid peroxidation may be important in the modulation of cellular cholesterol metabolism. In this study, the metabolism of one of the oxysterols, 25-hydroxycholesterol (25OHC), was examined in J774 and mouse peritoneal macrophages. Uptake of 25OHC from serum was rapid and substantial. Esterification of the cellular 25OHC was also rapid as was hydrolysis of pre-formed esters. Like cholesterol, 25OHC was removed from cells by an extracellular acceptor such as high density lipoprotein. Unlike cholesterol, 25OHC was also rapidly and extensively removed from cells by serum albumin, but not by ovalbumin. The differential removal of oxysterols and cholesterol from cells by albumin allows separation of cellular effects due to oxysterols and cholesterol. In order to understand more about this differential efflux of sterols, a computer model for sterol mass transport in cells was used to compare intracellular trafficking of cholesterol and 25OHC. The rate constants determined by this model for movement of sterols between cytoplasm and plasma membrane were similar for both cholesterol and 25OHC, whereas those for esterification and ester hydrolysis as well as those for bidirectional movement between plasma membrane and extracellular medium were greater for 25OHC than for cholesterol. For both sterols, the rate-limiting step for removal of cellular esters appeared to be the rate of cytoplasmic ester hydrolysis. As 25OHC and cholesterol differ significantly in aqueous solubility, the similarity in their rate constants for movement between cytoplasm and plasma membrane is consistent with facilitation of transport between these two loci.–**Morel, D. W., M. E. Edgerton, G. E. Warner, W. J. Johnson, M. C. Phillips, and G. H. Rothblat.** Comparison of the intracellular metabolism and trafficking of 25-hydroxycholesterol and cholesterol in macrophages. *J. Lipid Res.* 1996. **37:** 2041–2051.

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Oxysterols are derivatives of cholesterol that contain an additional oxygen function. They may arise through cholesterol autoxidation (1, 2) or by enzymatic oxidation (3, 4). They are of interest because of their ability to modify membrane packing and the activity of membrane-associated enzymes (5, 6), their ability to inhibit de novo cholesterol synthesis via HMG-CoA reductase (7, 8), their modulatory effects on cholesterol esterification (8–10), and their cytotoxic effects in vitro (11, 12) and in vivo (2, 13).

Of the known oxysterols, 25-hydroxycholesterol (25OHC) is one of the most potent inhibitors of HMG-CoA reductase (6) and stimulators of cholesterol esterification (8). It is found in prepared foodstuffs that contain cholesterol (14–16) and presumably can enter circulation by ingestion. Different types of oxysterols at various levels have been detected in serum. Sera from normal humans as well as fetal calf serum have been reported to contain low levels of oxysterols such as 7α-hydroxycholesterol, cholestanetriol, and 25- and 26-hydroxycholesterol, with 7α-hydroxycholesterol being the most abundant (17). After ingestion of a meal rich in oxysterols, 25OHC as well as other oxysterols are found in postprandial human plasma (18); 25OHC is also detected in serum after a dietary cholesterol challenge (19). The levels of oxysterols measured in hypercholesterolemic serum are higher than those found in normocholesterolemic serum (20, 21). In addition, 25OHC has been detected in normal fibroblasts grown in culture (22).

Recent studies by Björkhem et al. (23) have suggested that excess cellular cholesterol can be converted readily to oxysterols for rapid removal by lipid carriers in serum. Whereas cholesterol is required by cells for their viability, in excess it may be detrimental and may be involved

Abbreviations: 25OHC, 25-hydroxycholesterol; CHOL, cholesterol; ACAT, acyl CoA:cholesterol acyltransferase; BSA, bovine serum albumin; OVA, ovalbumin; HDL, high density lipoprotein.

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in the pathogenesis of atherosclerotic heart disease (24). Based on our previous studies showing that 25OHC associates with both lipoproteins and albumin in human serum (25), we hypothesize that high density lipoprotein, an acceptor for cellular cholesterol, as well as serum albumin act as acceptors for oxysterols such as 25OHC. In these studies we have looked more closely at the intracellular metabolism of 25OHC by two types of macrophages, the J774 cell line and mouse peritoneal macrophages, showing that 25OHC is in many ways metabolized similarly to cholesterol but more rapidly. Unlike cholesterol, 25OHC efflux from cells is enhanced by both HDL and albumin. However, despite its rapid removal from cells, preincubation with 25OHC reduces the efflux of cholesterol from cells to a number of acceptors (26). In order to understand more about the possible differences in metabolism between these two sterols, we have in these studies also explored the kinetics of intracellular movement of 25OHC as compared to that of cholesterol using a computer model of intracellular sterol trafficking.

MATERIALS AND METHODS

Materials

Radiolabeled [26, 27-^{3}H]25OHC and [4-^{14}C]cholesterol were purchased from New England Nuclear/DuPont (Wilmington, DE); 25OHC was purchased from Sigma (St. Louis, MO). Sterols were checked for purity by thin-layer chromatography and HPLC prior to use. Sandoz compound 58-035 and compound Q7 were kind gifts from Dr. John Heider and Dr. Daniel Quinn, respectively. Tissue culture supplies were obtained from GIBCO (Grand Island, NY). J774 macrophages were obtained from frozen stocks through a tissue culture core facility and routinely screened for mycoplasma contamination. Mouse peritoneal macrophages were freshly obtained from peritoneal lavage from $B_6C_3F_1$ mice (Taconic Farms, Germantown, NY) that were previously injected with 0.5 ml of sterile 10% thioglycollate as has been previously described (27). This procedure is a protocol approved by the Institutional Animal Care and Use Committee. The macrophages were pelleted by centrifugation, resuspended, and plated in 22-mm wells at a concentration of 1.5×10^6 cells/ml.

Cellular uptake and metabolism

J774 cells were maintained in RPMI medium containing 10% fetal bovine serum (FBS) and gentamycin. Prior to an experiment, cells were plated in 35-mm, 6-well plates and allowed to grow 6–10 days with medium changes every 3 days. Labeling medium was prepared by adding [^{3}H]25OHC (0.5 μCi/ml, final concentration) in the presence of various concentrations of unlabeled 25OHC (0.1–6 μg/ml) in an ethanol suspension (0.1%, final volume displacement) to RPMI medium containing 5% FBS. This labeling medium was added to cells for various periods of time. Cells were then washed with phosphate-buffered saline and the cell lipids were extracted. In some experiments, 58-035 or Q7 was added to tissue culture medium as a concentrate in dimethyl sulfoxide (0.25%, final volume displacement); the final concentrations of these compounds, selected because of their known ability (28, 29) to block esterification of cholesterol (58-035) and hydrolysis of cholesterol esters (Q7), were 1 μg/ml and 0.1 mM, respectively, unless otherwise indicated. For all additions, control cultures received the same amount of vehicle without the active compound.

Cell lipids were extracted with isopropanol. Protein in the precipitated cell monolayer was measured by the Markwell modification of the Lowry procedure (30). Cellular uptake of 25OHC was determined from the cell-associated radioactivity, using the specific activity of the labeling medium to determine mass uptake. Free and esterified 25OHC (and cholesterol where specified) in the cell lipid extract were separated by thin-layer chromatography on Silica gel G eluted with hexane–acetone–acetic acid 70:30:2. The R_f values for free and esterified 25OHC were 0.3 and 0.5, respectively, and the equivalent numbers for free and esterified cholesterol were 0.4 and 0.7, respectively; recoveries were greater than 90%. Quantitation was achieved by scraping areas of the plates corresponding to each fraction, extracting the lipids into chloroform–methanol 2:1, drying, and liquid scintillation counting. Cell total and free cholesterol mass, where described, were measured enzymatically as described by Gamble et al. (31) with esterified cholesterol calculated by difference.

Efflux from cells

Cells were prelabeled for 24 h with [^{3}H]25OHC (0.5 μCi/ml) in the presence of unlabeled 25OHC (4 μg/ml). In some experiments cellular cholesterol was radiolabeled by preincubation with [^{14}C]cholesterol (0.5 μCi/ml). Cells were washed thrice with phosphate-buffered saline containing 0.1% bovine serum albumin, then exposed to serum-free medium containing various acceptors for periods up to 12 h. To determine the efflux of 25OHC or cholesterol from cells, aliquots of medium were removed at sequential times, centrifuged to remove any cellular debris, and the radioactivity was quantitated by liquid scintillation counting. Efflux is expressed as the percent of cell-associated radioactivity appearing in the medium at each time point. For some experiments, the apparent rate constants (k) for efflux were estimated from the slope of the initial disappear-

ance of label from the cells (i.e., time vs. the natural logarithm of the fraction of label remaining in the cells) and used to calculate the half time for efflux ($t_{1/2} = \ln 2/k$).

In some experiments, mouse peritoneal macrophages were radiolabeled by incubation for 48 h with 0.5 μCi/ml [^{14}C]cholesterol or 1.0 μCi/ml [^{3}H]25OHC, in combination with acetylated LDL (100 μg protein/ml) and 1% fetal bovine serum. After the labeling period, the cells were equilibrated overnight before the addition of test media. Liquid scintillation counting was used to assess total cellular and medium contents of radiolabeled sterols as a function of time of incubation with HDL, bovine serum albumin or ovalbumin; cellular free and esterified forms of the two sterols were separated by TLC and quantitated by liquid scintillation counting.

Computer modelling

For some experiments, a prototype model for intracellular trafficking of sterols was used to examine possible differences in intracellular trafficking between 25OHC and cholesterol. In this model, as shown in **Fig. 1,** the cell has been divided into two compartments, the cytoplasm and the plasma membrane. The external medium represents a third compartment in the system. This model represents a cell with plentiful sterol in which de novo synthesis of cholesterol via HMG-CoA reductase is down-regulated (32); thus, the contribution of de novo synthesis of cholesterol is assumed to be negligible and is therefore ignored. A third cellular compartment, the lysosome, can be added for a more complete version of this model but is not included here because the contribution of lipoprotein-derived cholesterol is nil under the experimental conditions used.

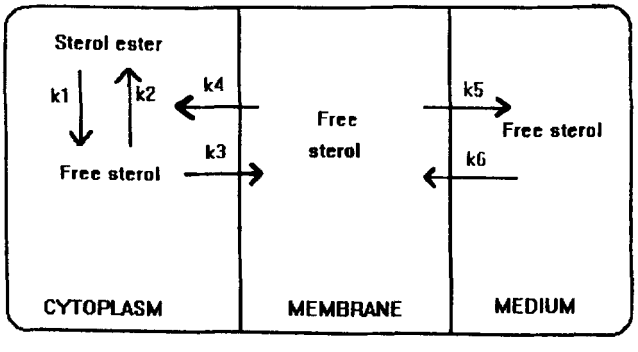

Fig. 1. Schematic representation of a three-compartment model (cytoplasm, plasma membrane, and tissue culture medium) used to analyze intracellular sterol trafficking; both cholesterol and 25-hydroxycholesterol species are examined. Newly synthesized sterols and lysosomally derived sterols are negligible contributors to the cellular sterol pool under the experimental conditions used and are ignored in the model. In addition, all the cellular free sterol is assumed to be in the plasma membrane at time zero.

A set of pseudo first order, time-dependent mass transfer equations is used to describe the kinetics of cholesterol (and 25OHC) movement through the cell. The concentrations used in the model can be expressed as fractions or percentages of the total and the rate constants are expressed in fractions per unit time. An example of the mathematical equations representing the kinetics of the cytoplasmic compartment are as follows:

$$d[FS_c]/dt = k_1[SE_c] - (k_2 + k_3)[FS_c] + k_4[FS_{mem}]$$
$$d[SE_c]/dt = k_2[FS_c] - k_1[SE_c]$$

where FS represents free sterol (cholesterol or 25OHC), SE represents sterol ester, the subscript c represents the cytoplasmic compartment and the subscript mem represents the plasma membrane compartment (Fig. 1). The concentration of free sterol (cholesterol or 25OHC) in the plasma membrane pool is described by the following equation:

$$d[FS_{mem}]/dt = k_3[FS_c] + k_6[FS_x] - (k_5 + k_4)[FS_{mem}]$$

where free sterol in the external medium is denoted by the subscript x. It is further assumed that the fraction of cellular free sterol in the membrane at time zero is one. A similar equation is used to describe the time-dependent mass transfer of sterols into the medium:

$$d[FS_x]/dt = k_5[FS_{mem}] - k_6[FS_x]$$

A variable step Runge-Kutta method, stable and efficient for integration of equations with a wide variation of rate constants such as can be found in biological systems (33), is used in the model to integrate the equations and simulate cellular mass transfer of sterols. Optimization is achieved by minimization of an error term which is shown below.

$$ERR = \sum_{t=t_1}^{t_n} (\Sigma_i(x_{exp}(i) - x_{sim}(i))^2 / \Sigma_i x_{exp}(i)^2)$$

where $x_{exp}(i)$ = experimental value of the "ith" species, $x_{sim}(i)$ = fitted value of "ith" species, t_1 = first time point and t_n is the final time point. Thus, at each time point, the difference between the experimental and simulated value for each species group is squared and this value is summed over all the species groups. It is normalized against the sum of the squared experimental values for each species group. This value is then summed over the time points of the experiment. Each time point has equal weight in the fit. As can be seen from this error function, the species that have the larger error will dominate the error term at any one time point. This

gives greater weight to the larger data values over the smaller data values which are prone to greater error in quantification. This error function was superior to others examined in preliminary experiments. The optimization method is a "complex" method that uses a sequential search technique proven effective in solving problems with non-linear objective functions (34). The fitted values may be constrained and in this case are given physiologic minimum and maximum values.

A second tool has been developed in which the sensitivity of the error function at each time point to each fitted constant is determined and normalized. This sensitivity function is:

$$S = (dERR/dk)/(ERR/k)$$

where ERR is the error function described above and k is the rate constant being evaluated. Given the complexity of the system being solved here, care must be taken to determine whether or not the experimental data are adequate for assigning or fitting a rate constant. The normalized sensitivity provides the tool for making this judgment. Values for S of 10 or more mean that the fitted constants have been adequately assessed from the experimental data (as a doubling in the rate constant would alter the error function by a factor of 10 or more yielding unsatisfactory fits). Values less than 1 signify that the data do not reflect the rate constant in question and therefore an accurate value cannot be assigned from this experiment. A value for S greater than 1 but less than 10 indicates that the data are relatively sensitive to the parameter being fit, depending on the maximum normalized sensitivity. Thus, the maximum sensitivity reached during the time course of the experiment can be used to compare the relative sensitivities of the rate constants.

Statistical considerations for experiments

All experiments were performed in triplicate wells and repeated at least twice; representative data are presented. Differences between treatment groups were tested for statistical significance using Student's unpaired *t* test.

RESULTS

Cell metabolism

The effect of 25OHC dose and incubation time on the uptake and esterification of 25OHC in J774 cells is shown in **Fig. 2.** Approximately 50% of the added 25OHC became cell-associated, with approximately 30% of cell-associated 25OHC becoming esterified. At the higher doses (10 and 12 μg 25OHC/well) in this and other experiments, cell protein after 24 h was decreased by 10–15%, suggesting toxicity of 25OHC as has been described in other cell systems (2, 12). In subsequent experiments, the maximal amount of 25OHC to which cells were exposed was 8 μg/well (4 μg/ml). As shown in Fig. 2B, the time course of uptake of 25OHC was rapid, with the majority of the accumulation complete in 4 h. Esterification was also rapid, with approximately 25% of the cell-associated 25OHC found as ester at all time points.

An inhibitor of cholesterol esterification, Sandoz compound 58-035 (28), had no effect on the uptake of 25OHC but dramatically reduced esterification of 25OHC. The extent of reduction was dependent on the

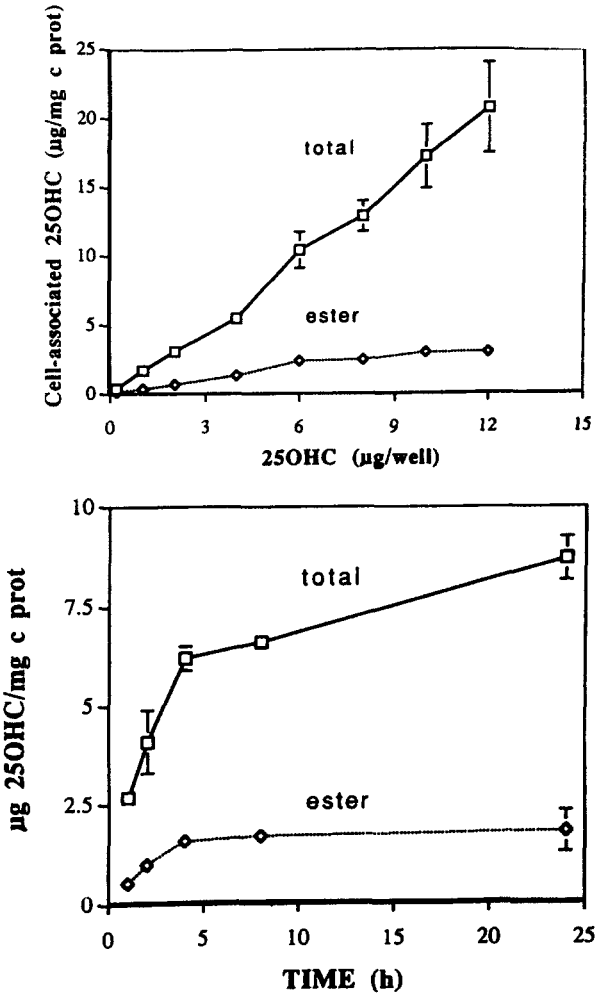

Fig. 2. Dose response (after 24 h incubation, upper panel) and time course (4 μg/ml 25OHC, lower panel) for uptake and esterification of 25-hydroxycholesterol (25OHC) added to J774 cells in medium containing 5% fetal bovine serum. Cell-associated 25OHC was determined by liquid scintillation counting of lipid extracts before (total) and after TLC separation (free and ester) using the specific activity of [^{3}H]25OHC in the labeling medium to determine mass uptake. Data are means ± SD for triplicate wells and are representative of multiple experiments.

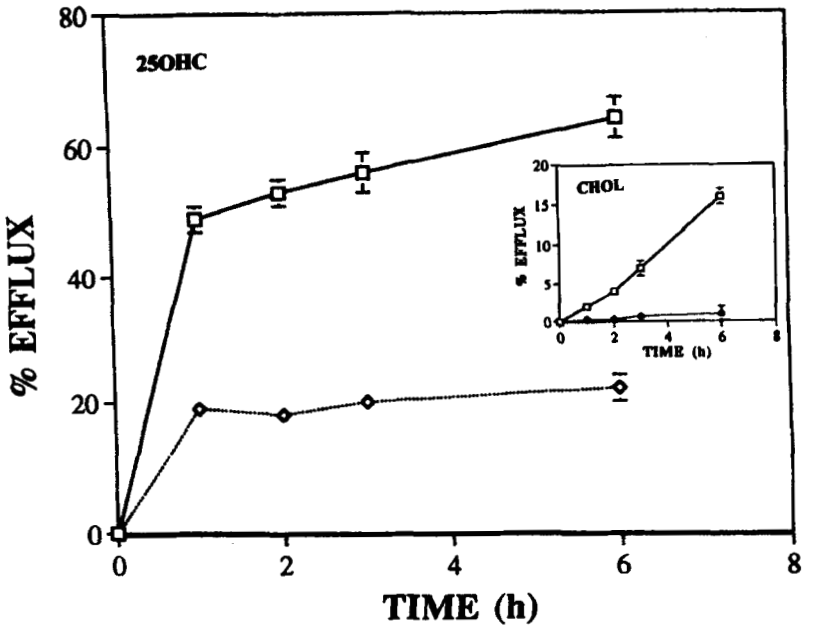

Fig. 3. Efflux of 25OHC from J774 cells labeled with [^{3}H]25OHC for 24 h to medium containing bovine serum albumin (BSA, 0.2%, ◇) or BSA plus HDL (300 μg protein/ml, □). Inset shows efflux of radiolabeled cholesterol to the same acceptors in a parallel set of wells. Data shown are means ± SD for triplicate wells and are representative of multiple experiments.

concentration of 58-035 and the time of exposure. Incubation with 1 μg/ml 58-035 for 24 h or 2 μg/ml for shorter times virtually abolished esterification of 25OHC (data not shown). Q7, a diethyl umberiferol phosphate known to inhibit cholesteryl ester hydrolysis (29), also had no effect on the uptake of 25OHC after 24 h but almost doubled the accumulation of 25OHC esters over the 24-h incubation (data not shown).

Efflux from cells

To determine how readily 25OHC could be removed from cells, J774 cells were labeled for 24 h with [^{3}H]25OHC in the presence of 58-035 to inhibit any esterification, then exposed to medium containing bovine serum albumin (BSA, 0.2%) or BSA plus HDL for time periods up to 6 h. For comparison, parallel cultures were labeled with [^{14}C]cholesterol in an analogous fashion. As shown in **Fig. 3,** efflux of 25OHC to HDL was rapid and extensive, with 50% of the 25OHC being removed from cells within the first hour. The inset to Fig. 3 shows the efflux of cholesterol in parallel wells. Cholesterol efflux to HDL was less rapid and less extensive, with only 15% efflux over the 6-h time course. The half times for cholesterol efflux and for 25OHC efflux estimated from the initial rate of decline in the fraction of label remaining in the cell were approximately 9 h and 1 h, respectively. Moreover, a significant amount of 25OHC was rapidly removed from the cell in the presence of BSA alone whereas virtually no efflux of cholesterol was seen under these conditions. Thus, both HDL and BSA stimulated removal of 25OHC from cells.

In contrast, in a similar experiment shown in **Fig. 4A,** there was little efflux of 25OHC to serum-free medium (0 mg/ml BSA). Figure 4A also shows the dose-dependent increase in the rate and extent of 25OHC efflux to BSA. Based on the apparent initial rates of efflux, the half time for efflux of 25OHC to BSA at 1 mg/ml was approximately 17 h, close to that for cholesterol efflux to HDL (300 μg protein/ml, Fig. 3, 23 h), and decreased to approximately 1 h for BSA at 20 mg/ml. To determine whether any media containing protein could promote the efflux of 25OHC, ovalbumin (OVA) and casein were tested as possible acceptors of cellular 25OHC. Very little 25OHC efflux to media containing OVA, as shown in Fig. 4B, or casein (data not shown), was observed, even after 6 h. However, as shown in Fig. 4B, when cells exposed to OVA (10 mg/ml) for 3 h were switched to medium containing BSA (10 mg/ml), the total efflux at the end of 6 h was comparable to that from cells exposed to 10 mg/ml BSA for the entire 6 h. Moreover, a further increase in the 25OHC removed from cells was seen when cells exposed to BSA for 3 h were exposed to fresh BSA-containing medium. That this was not due to medium components other than BSA was indicated by the lack of further 25OHC efflux when cells were switched at 3 h to fresh medium containing OVA rather than BSA (data not shown).

To further examine the cellular metabolism of 25OHC, J774 cells were prelabeled as described in Methods (-58-035) and the contents of free and esterified 25OHC were measured before and after incubation with OVA and BSA under conditions in which ester hydrolysis (+0.1 mM Q7) or esterification (+1 μg/ml 58-035) were blocked. As might be expected, incubation with 10 mg/ml BSA removed most of the cellular 25OHC with dramatic decreases in both free and esteri-

fied 25OHC whereas very little 25OHC was removed from cells by incubation with 10 mg/ml OVA. In addition, inhibition of ester hydrolysis with Q7 limited the ability of BSA to clear 25OHC esters although the free 25OHC content in the cells was reduced to the same extent as in cells exposed to BSA alone (data not shown).

Modelling of cellular sterol trafficking

In order to understand more about the intracellular trafficking of sterols, the dynamics of clearance of 25OHC and cholesterol esters from mouse peritoneal macrophages were examined and simulated using the model of sterol mass transport shown in Fig. 1. Murine macrophages were used for the modelling because they do not multiply in culture, thus simplifying the analysis. In addition, a significant amount of both 25OHC and cholesterol became esterified during incubation in the absence of 58-035, making measurement of changes in cellular concentrations of ester more reliable. As shown in **Fig. 5,** the efflux of cellular cholesterol and 25OHC into medium containing OVA, BSA, or HDL plus or minus 58-035 is similar in profile to that observed earlier with J774 cells. Based on the initial rate of decline in the fraction of label remaining in the cells, the half times for efflux of cholesterol from murine macrophages to HDL, HDL plus 58-035, and BSA were estimated to be 14 h, 12 h and 137 h, respectively; the half times for efflux of 25OHC to HDL, HDL plus 58-035 and BSA were estimated to be 18 min, 21 min, and 48 min, respectively.

In this experiment, cellular sterols were further analyzed by TLC to determine their chemical form (free or ester). These data were then used in the computer model for optimization of rate constants for esterification, ester hydrolysis, transfer from cytoplasm to plasma membrane, and movement between plasma membrane and the extracellular medium. The theoretical and experimental curves for the measured parameters, i.e.,

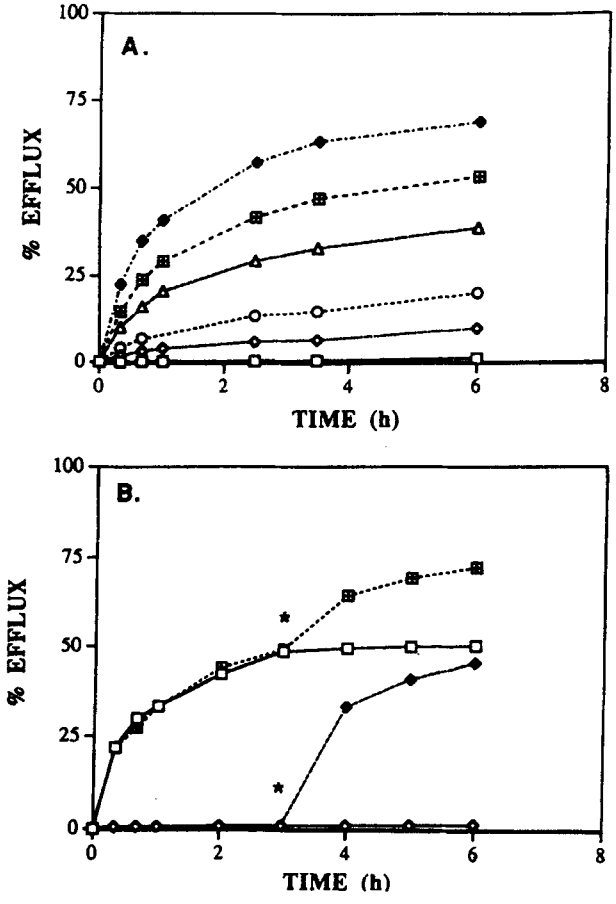

Fig. 4. Efflux of 25OHC from prelabeled J774 cells to medium containing bovine serum albumin (BSA) or ovalbumin (OVA). Panel A shows efflux to 0 (□), 1 (◇), 2 (○), 5 (△), 10 (⊞) or 20 (◆) mg/ml BSA. Panel B shows efflux to medium containing 10 mg/ml BSA (squares) or OVA (diamonds) over the full 6 h (open) or switched to fresh medium (filled, denoted by *) containing 10 mg/ml BSA at 3 h. Data are means ± SD for triplicate wells.

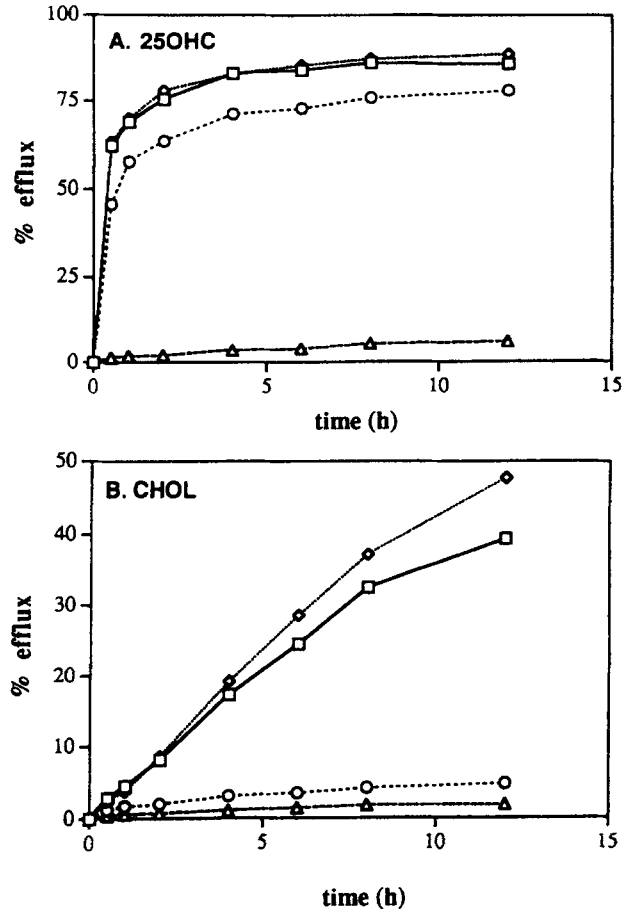

Fig. 5. Efflux of 25OHC and cholesterol from prelabeled mouse peritoneal macrophages to medium containing HDL (◇, 400 μg prot/ml), HDL plus ACAT inhibitor (□, 58-035, 2 μg/ml), bovine serum albumin (○, BSA, 0.5%) or ovalbumin (△, OVA, 0.5%). Data are means ± SD for triplicate wells.

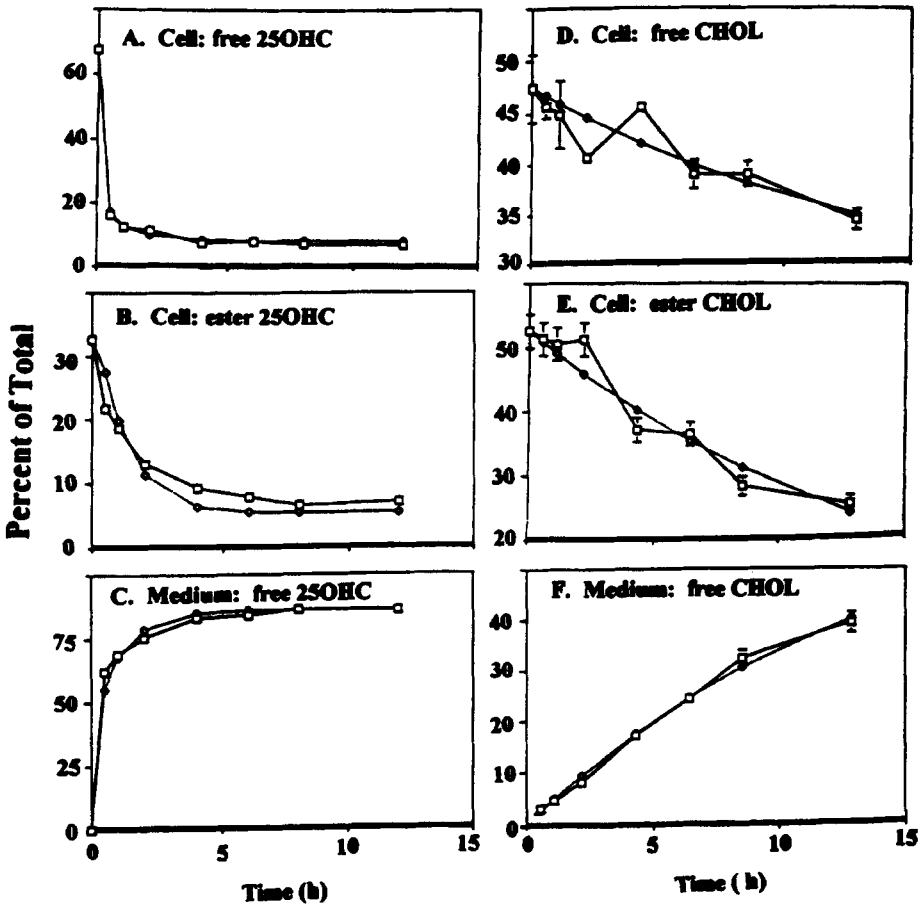

Fig. 6. Theoretical data simulated by the model (circles) shown schematically in Fig. 1 versus the experimentally derived data (squares) for cellular free, cellular ester, and extracellular 25OHC (panels A, B, C, respectively) and cholesterol (panels D, E, F, respectively). Experimental data are means ± SD for triplicate wells.

cellular free, cellular esterified and extracellular cholesterol, and 25OHC, when HDL (400 μg protein/ml) was used as a sterol acceptor are shown in **Fig. 6.** Despite marked differences in the time courses of the cellular and extracellular concentrations of the two sterols, the theoretical values for sterol concentrations calculated by optimization of the model approximated the experimental data reasonably well. The rate constant for cholesterol movement from the plasma membrane to HDL-containing medium (k_5) using this model was 0.12, corresponding to a half time of 5.8 h; when bidirectional flux at the membrane was taken into consideration (k_5 and k_6), the apparent half time for efflux was 9 h.

The rate constants derived from the model for intracellular movement of 25OHC and cholesterol in response to various acceptors in the medium are shown in **Table 1.** Again the correspondence between the simulated and experimentally measured concentrations of sterols (shown in Fig. 6) is illustrated by the small final error associated with optimization of the model parameters. The rate constants for mass transfer from cytoplasm to plasma membrane were similar for both 25OHC and cholesterol while those for mass transfer from medium to plasma membrane, and especially from plasma membrane to medium were many fold greater for 25OHC than for cholesterol, consistent with the greater polarity and aqueous solubility of 25OHC. In addition, it is interesting to note that for both sterols, except when esterification was blocked by the presence of 58-035, the rate constant for ester hydrolysis (k_1) of each sterol was much less than that for movement of that sterol into the membrane (k_3) and out of the cell (k_5). When compared to the rate constants for other processes involved in the removal of sterol, the hydrolysis of the ester form of the sterol appeared to be rate-limiting for removal of pre-formed esters.

The value of 10 for the rate constant of mass transfer from cytoplasm to plasma membrane (k_3) is maximal under the constraints assigned to the optimization procedure suggesting that this process is effectively instantaneous within the ability of the experiment to measure it. That maximization of this rate constant is appropriate

TABLE 1. Rate constants for mass transfer of cholesterol and 25-hydroxycholesterol between intracellular compartments and the extracellular medium

	k_1	k_2	k_3	k_4	k_5	k_6	Error
I. Cholesterol							
HDL	0.09	0.25	10	1.0	0.12	0.04	0.0085
HDL + 58035[a]	0.08	"0"	10	1.0	0.13	0.02	0.0047
BSA	0.11	0.46	10	1.0	0.04	0.49	0.011
II. 25-Hydroxycholesterol							
HDL	1.6	5.0	10	2.6	5.0	0.39	0.058
HDL + 58035[a]	0.5	"0"	10	0.0[b]	5.0	0.34	0.110
BSA	1.6	5.0	10	2.1	2.8	0.52	0.044

Rate constants are given for reactions illustrated in Fig. 1 and described in Methods; units are fractions/h. An error term, as described in Methods, was calculated as $ERR = \Sigma_{t=t_1}^{t_n} (\Sigma_i (x_{exp}(i) - x_{sim}(i))^2 / \Sigma_i x_{exp}(i)^2)$. The sensitivity of the data to changes in the rate constants is shown in Table 2. In addition, all optimizations were run from more than one set of initial values in order to determine that a global minimization of error was obtained.

[a]Rate constant k_2 was set to zero, then others were fit by model.

[b]Effectively nil over the time course of the experiment.

and necessary for the description of the system is demonstrated by the relatively high sensitivity of the data to fitting k_3 shown in **Table 2.** In addition, the data in Table 2 demonstrate that the experimental data were adequately sensitive for estimation of almost all the rate constants, with k_4 and k_6 for 25OHC in the presence of HDL and HDL/58035, respectively, being the only exceptions.

DISCUSSION

The metabolism of 25OHC in macrophages is in many ways analogous to that of cholesterol, with ester and free alcohol being the predominant species present in the cell. These esters are most likely formed by the same enzymes that catalyze the formation and hydrolysis of cholesteryl esters as their formation is blocked by the same inhibitors. This is consistent with the recent work of Cheng et al. (35) showing that recombinant human ACAT expressed in insect cells can use 25OHC as a substrate. However, as might be expected from the concentration gradient (i.e., from low in the cell to μg/ml outside) and the greater polarity (and increased aqueous solubility) of 25OHC, the metabolism of 25OHC occurs very rapidly when compared to the metabolism of cholesterol. Despite the rapidity of uptake and esterification, little accumulation of 25OHC esters occurs, even at high 25OHC doses or after extended periods of time. This is most likely due to rapid hydrolysis of 25OHC esters rather than saturation of ACAT as the concentration of 25OHC is low compared to that of cholesterol. The values for ester hydrolysis in murine macrophages derived from the model (k_1 in Table 1) are consistent with this supposition.

The most dramatic distinction in the metabolism of 25OHC in macrophages is its rapid and extensive efflux from cells to medium containing BSA. This observation is consistent with other studies showing that 25OHC associates with albumin in serum as well as with lipoproteins (25). This differential 25OHC efflux, rapid and extensive to serum albumin and minimal to other proteins such as ovalbumin, may be useful as a tool to distinguish the direct and indirect effects of 25OHC on cellular processes; in particular, studies on the regulation of cholesterol metabolism may benefit because cellular oxysterol but not cholesterol levels can be modulated by albumin. The time course for efflux of either cholesterol or 25OHC to various acceptors was similar in peritoneal macrophages and in J774 cells. In all cases

TABLE 2. Maximum relative sensitivity for sterol transport parameters

Sterol	Acceptor	k_1	k_2	k_3	k_4	k_5	k_6
CHOL	HDL	23.0	7.0	17.0	8.0	70.0	7.0
CHOL	HDL/58035	5.8	–	5.0	1.5	17.0	1.3
CHOL	BSA	2.0	1.1	2.2	1.5	10.0	10.0
25OHC	HDL	3.5	3.5	3.6	1.7	2.6	0.8
25OHC	HDL/58035	10.0	–	2.1	0.17	18.0	16.0
25OHC	BSA	4.3	4.0	4.0	2.1	3.0	4.5

Relative sensitivity values for each of the model parameters were determined over the time course as the absolute value of $[d\,ERR(t)/d\,k)/(ERR(t)/k)]$ where ; $ERR = \Sigma_{t=t_1}^{t_n} (\Sigma_i (x_{exp}(i) - x_{sim}(i))^2 / \Sigma_i x_{exp}(i)^2)$, x = measured variable, k = rate constant, and t = time. These values provide a tool to judge whether or not the experimental data are adequate for fitting of rate constants (shown in Table 1). The maximum values of sensitivity for each parameter over the time course are shown; values less than one signify that the data do not reflect the rate constant in question; values greater than 1 indicate that the data are relatively sensitive to the parameter being fit; values greater than 10 indicate that the data are highly sensitive to the parameter being fit.

efflux of 25OHC was many fold faster than that of cholesterol and 25OHC, but not cholesterol, could be removed effectively by serum albumin. For both 25OHC and cholesterol, efflux from murine macrophages was more rapid than from J774, despite the greater proportion of sterol as ester.

These observations complement the recent studies by Björkhem et al. (23) in which excess cellular cholesterol was converted by cells to oxysterols for excretion to components found in serum. Our findings suggest that the components are most likely HDL and albumin. The relative distribution of oxysterol among albumin, HDL and other lipoproteins in serum has been shown to depend on lipoprotein concentrations and the activities of lecithin:cholesterol acyltransferase and cholesteryl ester transfer protein (36).

A generalized model of sterol flux between cytoplasm and membrane and membrane and extracellular medium was used to estimate how various transfer processes might combine to produce the measureable, time-dependent changes in cellular free and ester contents and medium contents. Despite markedly different time courses of movement to various acceptors, both cholesterol and 25OHC data were able to be fit by this approach.

The rate constants for mass transport of oxysterols give some insight into the differential handling of oxysterol and cholesterol. The observation that many of the rate constants for oxysterol movement are many fold greater than that of cholesterol is consistent with the greater polarity of oxysterols. In general, diffusion of these polar compounds occurs much more rapidly because of their increased aqueous solubility. The rates of transfer of cholesterol and 25OHC from phospholipid bilayer vesicles by aqueous diffusion have been estimated to be 2 h and 2 min, respectively (37). These estimates were based on the critical micelle concentrations for each sterol and, at least for cholesterol, these estimates agree with experimental values (38). Furthermore, the ratio of k_8 values shown in Table 1 for cholesterol and 25OHC is in good agreement with these estimates (i.e., factor of 50 vs. factor of 60 in diffusion rates). Enhanced diffusion may contribute to the greater rate constant for esterification of 25OHC compared to that for cholesterol because the more polar 25OHC may have better access to ACAT.

It is striking that, despite the differences in polarity of the two sterols, the rate constants (k_3) for cytoplasmic transport of both cholesterol and 25OHC can be fit with the same rate constants (Table 1) which are very rapid relative to the rate constants for other cell transport processes. The analysis of sensitivity (Table 2) suggests that the data are relatively sensitive to the fitting of k_3. Consequently, it is most likely the movement of both sterols from cytoplasm to membrane is indeed fast. The high values for k_3 for both sterols are consistent with the idea that movement from cytoplasm to membrane occurs via facilitated transport as has been previously suggested for cholesterol (39). This is also consistent with the recent work of Lange, Ye, and Strebel (40) suggesting that both 25OHC and cholesterol move between the plasma membrane and endoplasmic reticulum by a common transport mechanism. In a hepatoma cell system, the fractional rate of 25OHC esterification exceeded that of cholesterol by more than 30-fold whereas at least a 20-fold difference was seen here (k_2 in Table 1) in macrophages. Vesicular transport has been suggested as a means of transport for newly synthesized cholesterol (41, 42) and could possibly play a role in the cytoplasm-to-membrane transport of cholesterol and 25OHC. Alternatively, sterol carrier proteins (43) might be involved.

It is of interest to note that despite the differences in polarity of 25OHC and cholesterol, their rates of influx into cells (k_6) from medium containing BSA, but not HDL, are similar. Thus, the net accumulation of 25OHC but not cholesterol in BSA-containing medium (26) is related wholly to its greater efflux rate rather than a decreased influx rate.

Whether or not oxysterols other than 25OHC behave similarly remains to be determined. Studies in model membrane systems suggest that 25OHC interacts with cholesterol in a manner similar to other oxysterols such as 7-ketocholesterol (44) and exhibits similar rapid exchange rates between lipid vesicles (45). However, other studies suggest that, in contrast to the studies of Björkhem et al. (23) suggesting conversion of excess cellular cholesterol to oxysterols for rapid efflux, oxidized LDL-derived 7-ketocholesterol is selectively retained in macrophages and exhibits decreased efflux to HDL relative to cholesterol (46, 47). Further work will be needed to clarify differences in oxysterol metabolism among individual oxysterols derived from different sources (i.e., oxidized LDL or enzymatic conversion of cholesterol) and to understand the interplay between cholesterol and oxysterols in the etiology of atherosclerosis.

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