# Esterification of oxysterols in human serum: effects on distribution and cellular uptake

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**Abstract** Oxysterols, oxidized derivatives of cholesterol, may enter the circulation as contaminants of cholesterol-containing food, arise through peroxidation of lipoproteins, or be generated by enzymatic reactions. They are found in serum associated either with lipoproteins or with albumin. In these studies, 25-hydroxycholesterol (25OHC) was used as a model oxysterol to investigate the effect of esterification on the association of oxysterols with serum components and their delivery to cultured cells. 25OHC added in vitro to fresh human serum was readily esterified during incubation at 37°C, most likely by serum lecithin:cholesterol acyltransferase (LCAT) as it was blocked by known inhibitors of LCAT. The 25OHC-esters formed were identified as monoesters by comparing their elution on high performance liquid chromatography and thin-layer chromatography with that of chemically synthesized 25OHC mono- and diesters. Esterification doubled the percentage of 25OHC associated with lipoproteins, concomitantly decreasing the amount associated with albumin. Whereas unesterified 25OHC readily transferred between isolated lipoproteins, 25OHC-esters remained associated with donor lipoproteins unless human lipoprotein-deficient serum was added. That cholesteryl ester transfer protein (CETP) mediated transfer of 25OHC-esters was demonstrated by the ineffectiveness of rat lipoprotein-deficient serum as well as by the ability of IC-4, an anti-CETP monoclonal antibody, to suppress the transfer. Esterification of 25OHC in serum limited its entry into human erythrocytes and fibroblasts (GM 3468A cells) in vitro. Up-regulation of fibroblast low density lipoprotein (LDL)-receptors enhanced the uptake of esterified 25OHC from medium, but did little to enhance the total uptake of 25OHC. Thus, esterification of oxysterols in serum shifts their distribution away from albumin into LDL and high density lipoprotein (HDL) and limits their availability to cells in culture.—**Lin, C-Y., and D. W. Morel.** Esterification of oxysterols in human serum: effects on distribution and cellular uptake. *J. Lipid Res.* 1996. **37:** 168–178.

**Supplementary key words** cholesterol • lecithin:cholesterol acyltransferase • cholesteryl ester transfer protein • lipoproteins

Cholesterol undergoes oxidation in vitro and in vivo, forming biologically active derivatives known as oxysterols (1, 2). Many oxysterols, such as 27-hydroxycholesterol and 25-hydroxycholesterol (25OHC), are potent modulators of cell metabolism and, in particular, cellular cholesterol homeostasis (3, 4). Among the oxysterols, 25OHC is one of the most potent (5, 6): it inhibits cholesterol biosynthesis by suppressing 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (6, 7); it enhances cellular cholesteryl ester accumulation by stimulating the activity of acetyl CoA:cholesterol acyltransferase (ACAT) (8–10); it reduces the efflux of cholesterol to extracellular acceptors (11); and it is toxic to smooth muscle cells, endothelial cells, and fibroblasts (12–14). Because oxysterols can insert into the plasma membrane (15, 16) modifying its physicochemical properties (17, 18), oxysterols can also modulate the enzymatic activity of many membrane-bound proteins (19).

Many oxysterols, including 7-hydroxycholesterol, 7-ketocholesterol, 26-hydroxycholesterol, and 25OHC have been detected in the plasma of experimental animals (20) and humans (21, 22). Using 25OHC as a model, our previous studies demonstrated that unlike cholesterol, oxysterol distributed almost equally between lipoproteins and the lipoprotein-deficient serum (LPDS, $d \geq 1.210$ g/ml); in LPDS, the protein with which 25OHC associated was identified as albumin (23).

It is not clear what factors are important in establishing the distribution of oxysterols in serum. It is known for cholesterol that its distribution in serum among individual lipoproteins depends in part on its esterification (24, 25) and exchange between lipoproteins (26, 27). In serum, unesterified cholesterol is a

Abbreviations: VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; LPDS, lipoprotein-deficient serum; LCAT, lecithin:cholesterol acyltransferase; CETP, cholesteryl ester transfer protein; NEM, N-ethylmaleimide; DTNB, dithionitrobenzoic acid; TLC, thin-layer chromatography; HPLC, high performance liquid chromatography; 25OHC, 25-hydroxycholesterol; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; ACAT, acyl-CoA:cholesterol acyltransferase.

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surface-component of the lipoprotein particles, whereas esterified cholesterol, generated through the action of serum lecithin:cholesterol acyltransferase (LCAT) (24, 25), is essentially solubilized in the hydrophobic core (28, 29). Serum cholesteryl ester transfer protein (CETP) facilitates the exchange of cholesteryl esters and triglycerides between high density lipoprotein (HDL) and apoB-containing lipoproteins, i.e., very low density lipoprotein (VLDL) and low density lipoprotein (LDL), during lipoprotein remodeling in circulation (26, 27). In rodent serum, which lacks CETP, cholesterol associates mainly with HDL (27).

Esterified oxysterols have been detected in atherosclerotic human aortae as well as in human plasma (30, 31). Because of the structural similarity between oxysterols and cholesterol, plasma oxysteryl esters might be expected to be the products of LCAT and CETP-mediated redistribution. Given the second hydroxyl function of many oxysterols, diesters of oxysterols might also be possible. Such esterification of oxysterols in plasma might be expected to alter their distribution and association with plasma proteins and thereby influence their delivery to cells and tissues.

These studies have examined the esterification of oxysterols in human serum using 25OHC as a model. They show that 25OHC is readily esterified at 37°C in vitro, and that this esterification alters the distribution of 25OHC in serum, such that a greater proportion becomes associated with lipoproteins. In addition, esterification of 25OHC in serum, by reducing the amount of readily exchangeable 25OHC (unesterified), decreases the uptake of serum 25OHC by cultured cells.

## MATERIALS AND METHODS

### Materials

The following compounds were purchased from Sigma, St. Louis, MO: cholesterol, 25-hydroxycholesterol, 19-hydroxycholesterol acetate, oleic chloride, N-ethylmaleimide, and 5,5′-dithio-bis (2-nitrobenzoic acid). [26,27-$^{3}$H]25-hydroxycholesterol (77 Ci/mmol) and [4-$^{14}$C]cholesterol (53.0 mCi/mmol) were obtained from NEN-DuPont, Wilmington, DE. All organic solvents including ethanol, methanol, chloroform, hexanes, acetonitrile, isopropanol, benzene, ethyl acetate, and petroleum ether were from Fisher, Pittsburgh, PA. The monoclonal antibody (IC-4) against human CETP was a generous gift from Dr. M. Bamberger at Pfizer, Groton, CT.

### Labeling serum in vitro

Fresh human sera were obtained from healthy volunteers after an overnight fast. To label sera with [$^{3}$H]25OHC, radiolabeled 25OHC (20 μCi/ml) was first mixed with unlabeled 25OHC (0.7 mg/ml) in ethanol, to achieve a specific activity of 10 μCi/mmol and the solvent was evaporated under nitrogen. To study the distribution of 25OHC in human serum as a function of incubation time, the dried [$^{3}$H]25OHC was redissolved in 10–20 μl ethanol and added to 20 ml human sera in vitro to achieve a final concentration of 100 nM. [$^{14}$C]cholesterol radiolabel (10 μCi/ml in ethanol) was added in a similar fashion to serum to trace the endogenous free cholesterol. After incubation at 37°C for 0–24 h, the labeled serum was subjected to isopycnic ultracentrifugation (32) to isolate two fractions: a total lipoprotein fraction (d < 1.210 g/ml) and a lipoprotein-deficient fraction (LPDS, d ≥ 1.210 g/ml). After dialysis in phosphate-buffered saline (PBS) containing 2 mM EDTA and 0.1% sodium azide, $^{3}$H and $^{14}$C radiolabels in both fractions were quantitated by liquid scintillation counting. In some experiments, sequential density ultracentrifugation was performed to isolate the individual lipoprotein fractions including VLDL (d < 1.006 g/ml), LDL (1.006 < d < 1.063 g/ml), and HDL (1.063 < d < 1.210 g/ml). The purities of the isolated lipoproteins

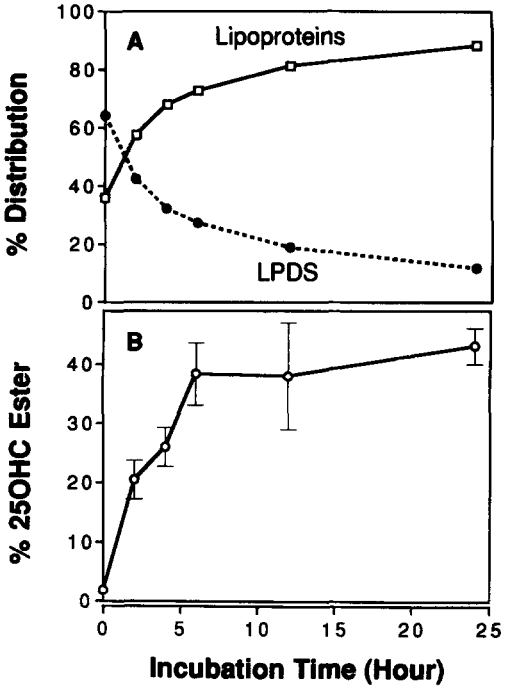


**Fig. 1.** A: Distribution of [$^{3}$H]25OHC in human serum as a function of incubation time. [$^{3}$H]25OHC (100 nM, 10 μCi/mmol), added to fresh human serum in ethanol (< 1%) in vitro, was incubated at 37°C for the indicated times, and fractionated into lipoproteins (d < 1.210 g/ml) and LPDS (d ≥ 1.210 g/ml), and the distribution of $^{3}$H radiolabel was quantitated. B: Esterification of [$^{3}$H]25OHC in human serum as a function of incubation time. Lipids extracted from an aliquot of the serum samples after incubation was analyzed by TLC as described under Methods. Data shown are means ± SD for triplicate samples.

were examined using a Beckman Lipogel electrophoresis kit, the protein content was determined according to Lowry et al. (33), and the cholesterol content was assessed enzymatically (Sigma procedure No. 352).

In experiments examining the esterification of 25OHC in vitro and the transfer of 25OHC between lipoproteins, a high concentration (0.1 mM) of [$^3$H]25OHC was used to enhance the mass of 25OHC-esters in serum and to enrich the lipoproteins with 25OHC. In some experiments, N-ethylmaleimide (NEM) or dithionitrobenzoic acid (DTNB) were added to the [$^3$H]25OHC-labeled serum at final concentrations of 5 or 2.5 mM, respectively, to inhibit LCAT. NEM was added to serum via an ethanol vehicle similar to the way 25OHC and cholesterol were added, whereas DTNB was added in PBS (pH = 7.8).

### Chemical synthesis of 25OHC-mono- and diester

25OHC-mono- and diesters were synthesized according to Lichtenstein and Brecher (34). In brief, [$^3$H]25OHC (50 μCi/5 μmol) was mixed with 40-fold oleoyl chloride and incubated at 55°C for 3 h in 0.5 ml dry pyridine. After the incubation, the lipids were extracted twice with 10 ml petroleum ether and the combined extracts were washed twice with 10 ml of 0.5 M HCl, 0.1 N NaOH in 50% ethanol and water. After evaporation under nitrogen, the lipid extract was redissolved in 100 μl of ether and mono- and diesters were separated by thin-layer chromatography (TLC) in a solvent system of benzene–ethyl acetate 75:25 (v/v). The 25OHC-mono- and dioleate migrated on the plate with calculated $R_f$ values of 0.39 and 0.75, respectively, matching well with those reported by Lichtenstein et al. (34). As the ratio of oleic chloride to 25OHC in the reaction increased, free 25OHC in the products vanished while the quantity of esterified 25OHC increased. At 20-fold molar excess of oleic chloride, about 90% of $^3$H radiolabel in the product was in a monoester form; while at 40-fold excess, 60% of the $^3$H label was in a mono ester and 30% was in a diester form. The mono- and diesters of 25OHC scraped from the TLC plate were extracted and further analyzed by HPLC (described below).

### Separation of free and esterified sterols

Lipids extracted from either the labeled sera or the isolated lipoproteins according to Radin (35) were separated by TLC on a 0.25 mm thick silica gel plate (200 × 200 mm) developed in a solvent mixture consisting of hexanes–acetone–acetic acid 70:30:2 (v/v/v). The $R_f$ values for cholesterol, 25OHC, cholesteryl esters, and 25OHC-esters were 0.54, 0.47, 0.88, and 0.80, respectively. Their identities were confirmed by comparison to pure standards of cholesterol, 25OHC, cholesteryl oleate, and 19-hydroxycholesteryl acetate as well as by elimination of the ester spots by saponification prior to TLC. Areas corresponding to free and esterified 25OHC and cholesterol were scraped from the plate into tubes and extracted twice with 3 ml of methanol–chloroform 1:1 (v/v), and quantitated by liquid scintillation counting to assess the esterification of the sterols.

In some experiments, lipid extracts from [$^3$H]25OHC- or [$^{14}$C]cholesterol-labeled sera or individual fractions from TLC were analyzed by reverse-phase HPLC (Supelcosil LC-18, 250 × 4.6 mm ID). Lipids were eluted using a gradient of acetonitrile–2-propanol (1 ml/min flow): after 5 min at the initial conditions of 75:25 (v/v), the

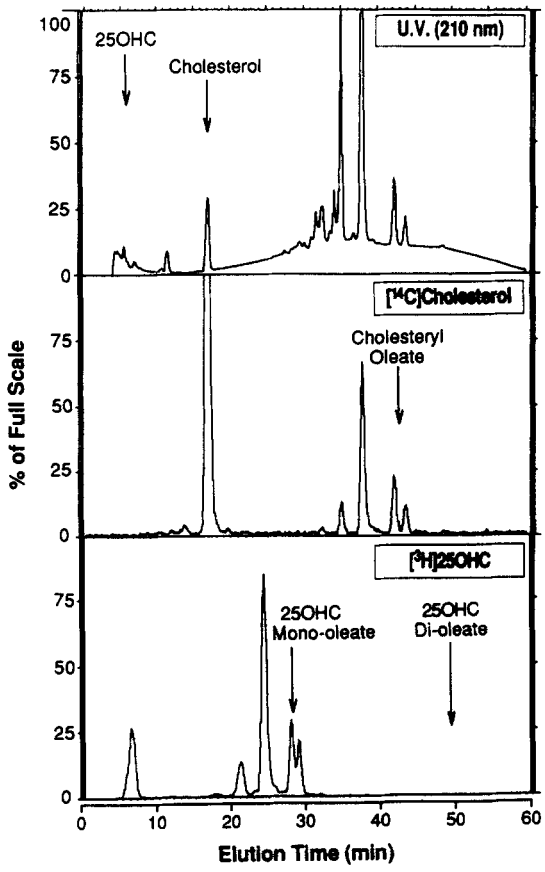


**Fig. 2.** Analysis of [$^3$H]25OHC and [$^{14}$C]cholesterol esterification by HPLC. Lipids were extracted from the [$^3$H]25OHC- and [$^{14}$C]cholesterol-labeled sera after 24 h incubation and subjected to reverse phase HPLC analysis on a LC-18 column at 1 ml/min flow rate in a gradient solvent system described under Methods. The elution profile of a typical sample is shown, with UV absorption at 210 nm (top panel), $^{14}$C (middle panel), and $^3$H (lower panel) liquid scintillation counting in a flow-through counter, displayed as percent of full scale. Full scale was 0.1 absorbance units, 300 cpm ($^{14}$C), and 2000 cpm ($^3$H), respectively. Retention times for synthesized or purchased standards are indicated by arrows. Based on the literature (46, 47), the set of four peaks between 35 and 45 min for [$^{14}$C]cholesterol and the set of four peaks between 20 and 30 min for [$^3$H]25OHC most likely represent, in order of elution, arachidonate, linoleate, oleate, and palmitate esters of the respective sterols.

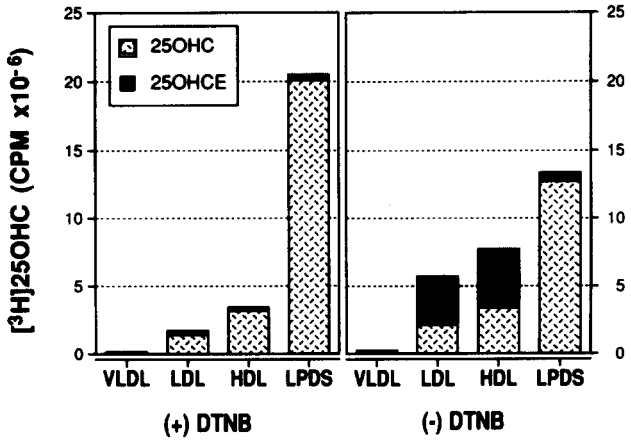


**Fig. 3.** Effect of LCAT inhibition on 25OHC distribution in human serum. Individual lipoprotein fractions isolated from [$^3$H]25OHC-labeled serum incubated with or without DTNB (2 mM) for 24 h were analyzed for total, free, and esterified 25OHC radiolabel by TLC. Data shown are means ± SD for triplicate samples.

mixture was adjusted to 40:60 (v/v) over 20 min, then maintained at 40:60 for 15 min, and returned to 75:25 over 10 min. The column effluent was monitored by UV absorbance at 210 nm, and [$^3$H]25OHC and [$^{14}$C]cholesterol radioactivities were monitored by scintillation counting using a Packard Radiomatic flow-through detector. Unesterified 25OHC and cholesterol standards eluted with retention times of 7.5 min and 17.5 min, respectively, and cholesteryl oleate eluted at 42 min; the synthesized 25OHC-mono- and dioleate standards eluted with retention times of 28 min and 50 min, respectively.

### Transfer of free and esterified 25OHC between lipoprotein particles

Donor lipoproteins for 25OHC were prepared by isolating LDL and HDL from fresh human serum incubated for 24 h with [$^3$H]25OHC in the presence or absence of 2.5 mM DTNB as described above. Acceptor-LDL and HDL were isolated from 25OHC-free sera.

Transfer experiments were conducted by mixing donor-LDL (or HDL) with an increasing volume of acceptor-HDL (or LDL) to achieve various acceptor-to-donor molar ratios. After incubation at 37°C for 18 h, the donor-lipoprotein was separated from acceptor-lipoprotein by density ultracentrifugation (d 1.063 g/ml). The donor and acceptor lipoproteins were assessed for purity, protein content, and TLC-separated free and esterified [$^3$H]25OHC radioactivity as described above.

In some experiments, human LPDS (1.0 ml, 64 mg protein/ml) was added to the donor-acceptor mixture as a source of CETP. Rat LPDS (1.0 ml, 63 mg protein/ml), which lacks CETP, was added in a separate group as a negative control. In another experiment, 8 µl of a monoclonal anti-human CETP antibody (IC-4, 2.3 mg protein/ml) was added to aliquots of human LPDS and mixed at room temperature for 2 h before the LPDS was added to the donor-acceptor mixture. The donor and acceptor lipoproteins were analyzed as described above.

In another experiment, human serum was pretreated with the anti-human CETP antibody (10 µg antibody protein/ml serum) for 2 h at room temperature, then labeled with [$^3$H]25OHC (0.1 mM) and incubated at 37°C for 24 h. After incubation, the serum was fractionated to isolate individual lipoproteins and LPDS, and analyzed as above.

### Cellular uptake of 25OHC from serum

Human erythrocytes, obtained from a local blood bank, were washed three times in 20 ml cold buffer containing PBS and 0.25% BSA, and pelleted by centrifugation (700 rpm × 15 min, 4°C). No hemolysis was observed. Cells were resuspended in the buffer at 4.75 × $10^9$ cells/ml. An increasing volume (0–1.6 ml) of serum labeled with [$^3$H]25OHC (0.1 µM) was added to a constant volume (0.25 ml) of the erythrocyte suspension. After incubation at 37°C for 18 h, the erythrocytes were pelleted by centrifugation, and washed three times

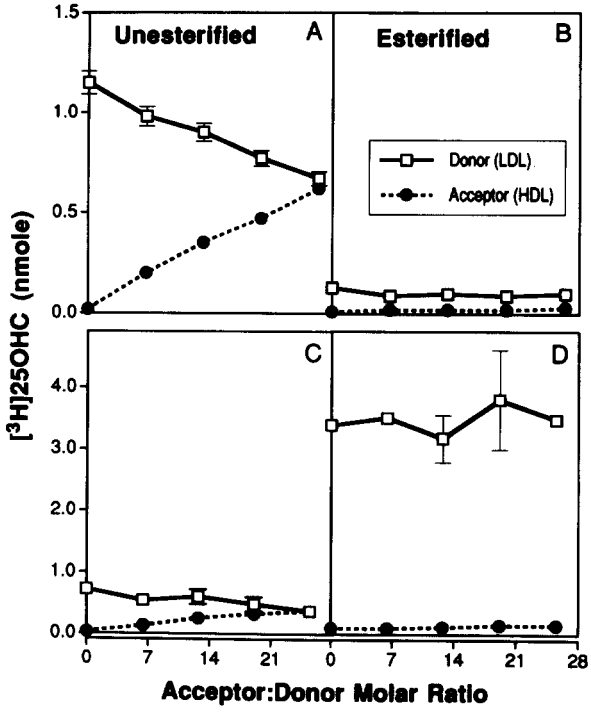


**Fig. 4.** Transfer of 25OHC from LDL to HDL. Donor-LDL isolated from the [$^3$H]25OHC-labeled serum incubated in the presence (A and B) or absence (C and D) of 2 mM DTNB, was mixed with an increasing amount of acceptor-HDL isolated from 25OHC-free serum. Donor and acceptor lipoproteins were reisolated after 18 h incubation at 37°C, and transfer was measured by quantitation of $^3$H radioactivity in each lipoproteins. Data shown are means ± range for duplicate samples.

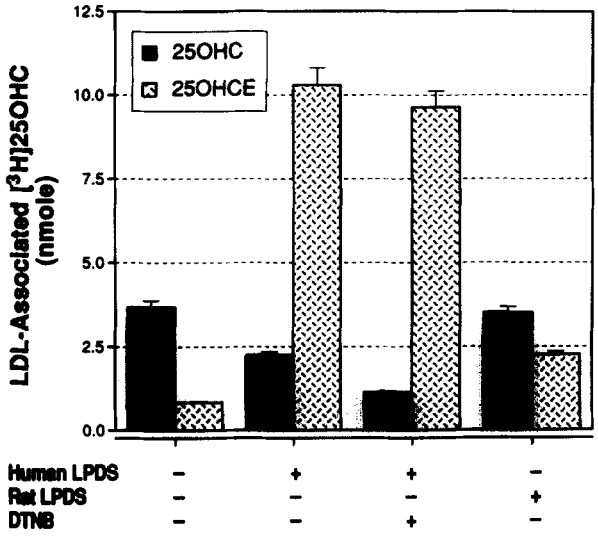


**Fig. 5.** Effect of human LPDS on the transfer of esterified 25OHC from HDL to LDL. HDL isolated from [$^3$H]25OHC-labeled sera (without DTNB treatment) was mixed with 25OHC-free LDL at 37°C in the presence or absence of 1 ml either human or rat LPDS. Transfer was assessed by reisolation of acceptor-LDL after 18 h incubation, and analysis of LDL-associated esterified and unesterified [$^3$H]25OHC (by TLC and liquid scintillation counting). Data shown are means ± range for duplicate samples.

in 20 ml cold washing buffer. Cell lipids were extracted according to Radin (35); free and unesterified [$^3$H]25OHC were separated by TLC and quantitated by liquid scintillation counting.

In other experiments, normal human foreskin fibroblasts, GM 3468A cells, obtained from the NIH Cell Repository (Coriell Institute for Medical Research, Camden, NJ) were used. These cells were maintained in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and gentamicin (45 μg/ml) at 37°C in a humidified atmosphere with 95% $O_2$ and 5% $CO_2$. For each experiment, cells were seeded in 35-mm wells and grown to confluence prior to the start of the experiment. In order to maximize the cellular uptake of 25OHC via both the LDL-receptor-dependent and -independent mechanisms, the growth medium was replaced by 2 ml MEM supplemented with 1% BSA 36 h before the experiment. In one experiment, [$^3$H]25OHC-labeled serum samples were prepared by adding [$^3$H]25OHC to final concentrations ranging from 0–1000 nM, and incubating at 37°C in the presence or absence of DTNB for 24 h. The experimental medium was made by adding these sera to MEM at 10% volume displacement (final 25OHC concentration 0–100 nM). In a parallel experiment, a single batch of labeled serum (containing 1 μM 25OHC) was added to MEM at different volume displacements (0–10%) to yield experimental media containing final concentrations of 25OHC in the same range (0–100 nM). Cells were incubated with the experimental medium (2 ml/well) for 5 h, followed by three washes in 1 ml cold PBS. Cell lipids were extracted twice in 4 ml isopropanol. Free and esterified [$^3$H]25OHC were separated by TLC, and quantitated by liquid scintillation counting. Cellular protein was dissolved in 2 ml Lowry reagent and quantitated accordingly.

To compare the uptake of serum 25OHC by fibroblasts with normal or up-regulated LDL-receptors, confluent fibroblasts were preincubated in MEM containing either 10% FBS or 1% BSA, respectively, for 36 h before the start of the experiment. The experimental media contained MEM plus 10% [$^3$H]25OHC-labeled (1 μM) human serum preincubated for 24 h at 37°C. Uptake of 25OHC by cells at times up to 24 h was determined as described above.

## RESULTS

### Esterification of 25OHC in human serum

The distribution of [$^3$H]25OHC (100 nM) and [$^{14}$C]cholesterol between serum lipoproteins and LPDS was examined after incubation at 37°C for various times up to 24 h. As shown in **Fig. 1A,** the percentage of lipoprotein-associated 25OHC increased as incubation proceeded. After 24 h, approximately 90% of 25OHC was found in the lipoprotein fraction. In contrast, cholesterol was wholly lipoprotein-associated under all con-

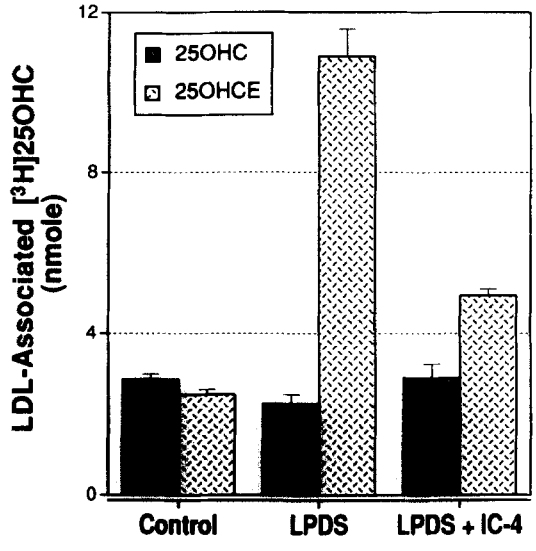


**Fig. 6.** Effect of blocking CETP on the LPDS-mediated transfer of esterified 25OHC from HDL to LDL. Human LPDS (1 ml) was incubated with a monoclonal antibody to CETP (IC-4, 8 μl, 2.3 mg protein/ml) for 2 h before addition to the HDL (donor)/LDL (acceptor) mixture. The experiment was conducted in the same fashion as described under Fig. 5. Data shown are means ± range for duplicate samples.

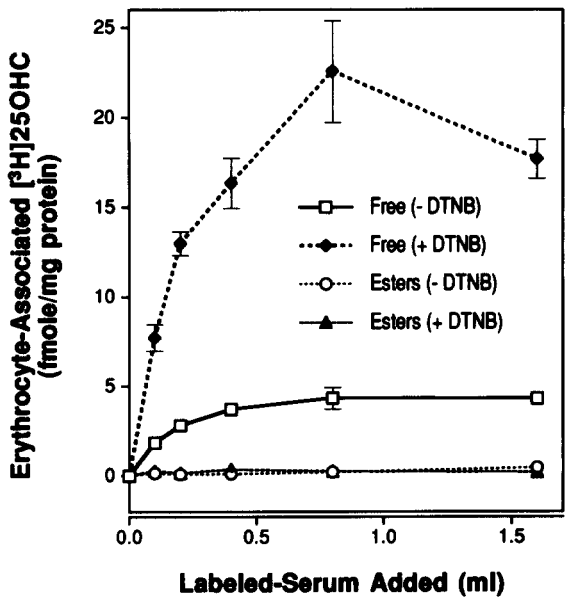


**Fig. 7.** Effect of esterification on the incorporation of serum 25OHC into human erythrocytes. Human erythrocytes ($4.75 \times 10^9$ cells/ml × 0.25 ml) were incubated with indicated volumes of [$^3$H]25OHC-labeled serum (0.1 nmol 25OHC/ml serum) at 37°C for 18 h, washed three times in 20 ml cold PBS containing 0.25% BSA, and analyzed for cell-associated free and esterified 25OHC. Data shown are means ± SD for triplicate samples.

ditions (data not shown). When the serum lipids were extracted and analyzed by TLC, both esterified and unesterified forms of [$^3$H]25OHC and [$^{14}$C]cholesterol radiolabels were detected. As shown in Fig. 1B, the percentage of esterified 25OHC increased with time of incubation up to 40% in this experiment. In other experiments, with different serum samples used with-in hours of collection, esterification over a 24-h period was as high as 90% (e.g., Fig. 4).

To explore the possibility that serum lecithin:cholesterol acyltransferase (LCAT) is involved in the esterification of 25OHC, [$^3$H]25OHC (final concentration 50 μM) and [$^{14}$C]cholesterol (0.05 μCi/ml to trace endogenous unesterified cholesterol of approximately 1.6 mM) were added to aliquots of serum and incubated for 24 h in the presence and absence of known LCAT inhibitors, NEM and DTNB (24, 36). In the absence of the inhibitors, about 76% of $^3$H and 25% of $^{14}$C radiolabels were esterified. NEM (final concentration 5.0 mM) inhibited the esterification of 25OHC and cholesterol by 80% and 72%, respectively. DTNB (final concentration 2.5 mM) inhibited esterification to an even greater extent (96% for 25OHC and 93% for cholesterol). Because DTNB inhibited the esterification of both sterols more efficiently at a lower concentration than NEM, DTNB was used in subsequent experiments.

To determine whether the 25OHC-esters formed in serum were mono- or diesters, 25OHC-mono- and dioleate were chemically synthesized according to Lichtenstein et al. (34), and used as standards to identify the oxysteryl esters formed in human serum. As shown in **Fig. 2,** HPLC analysis of the lipid extracts from [$^3$H]25OHC- and [$^{14}$C]cholesterol-labeled serum exhibited a series of $^3$H-labeled peaks with retention times between 20 and 30 min, similar to the retention time of 28 min for 25OHC-mono-oleate, and a series of $^{14}$C-labeled peaks eluting between 35 and 45 min, similar to the retention time of 42 min for cholesteryl oleate. In addition, there were peaks corresponding to unesterified 25OHC and cholesterol. No 25OHC peaks were observed with retention times in the 45–55 min range where 25OHC-dioleate eluted. The series of $^3$H and $^{14}$C peaks at 20–30 min and 35–45 min vanished with a concomitant increase in the magnitudes of the unesterified 25OHC and cholesterol peaks following saponification (data not shown), demonstrating that these peaks were indeed 25OHC and cholesteryl esters. Thus, monoesters, but not diesters, of 25OHC readily formed in human serum after incubation at 37°C. The pattern of the 25OHC-ester peaks was strikingly similar to that for the cholesteryl esters.

The effect of esterification on the distribution of [$^3$H]25OHC among individual lipoproteins and LPDS is shown in **Fig. 3.** With esterification (-DTNB), there was a net decrease in LPDS-associated 25OHC, which quantitatively accounted for the net increase of 25OHC in LDL and HDL. This increase was due to increased ester content; the content of unesterified 25OHC in these fractions remained constant. These data also show that only unesterified 25OHC associated with LPDS.

### Transfer of esterified and unesterified 25OHC between lipoproteins

To further characterize the effect of esterification on the distribution of oxysterol among lipoproteins, we examined whether unesterified and/or esterified 25OHC could transfer from one lipoprotein to another. In the experiments shown in **Fig. 4,** LDL was used as a donor of either esterified or unesterified 25OHC, and HDL was used as an acceptor. Figs. 4A and B showed that LDL isolated from [$^3$H]25OHC-labeled serum in the presence of DTNB contained mainly (90%) unesterified 25OHC. When this donor-LDL was mixed with an increasing amount of acceptor-HDL and then reisolated, the amounts of unesterified 25OHC decreased in LDL and increased in HDL, indicating a transfer of unesterified 25OHC from donor to acceptor. In contrast, LDL isolated from serum labeled without DTNB (Figs. 4C and D) contained 80% esterified 25OHC. When this LDL was mixed with the acceptor-HDL, a similar transfer of unesterified 25OHC, but no transfer of esterified 25OHC, was seen. Identical results were

observed in parallel experiments in which HDL was used as 25OHC-donor and LDL as the acceptor (data not shown).

As cholesteryl ester transfer protein (CETP), which facilitates the exchange of cholesteryl esters and triglycerides between lipoproteins, is found in LPDS after ultracentrifugation (37, 38), in another experiment human LPDS was added to a mixture of donor-HDL and acceptor-LDL as a source of CETP. Rat LPDS, which lacks CETP (26, 27), was used as a negative control. As shown in **Fig. 5,** significantly more of the esterified [$^3$H]25OHC originally associated with the donor-HDL became acceptor-associated during incubation in the presence of human LPDS. This enhanced transfer was observed even in the presence of DTNB, indicating that the appearance of 25OHC-esters in the acceptor particles was not due to hydrolysis and re-esterification of 25OHC during the incubation. In contrast to human LPDS, rat LPDS did not promote the transfer of 25OHC-esters from donor to acceptor lipoproteins.

To confirm that CETP was responsible for the lipid-transferring effect of the human LPDS, in a separate experiment, a monoclonal antibody (IC-4) against human CETP was added to the human LPDS. As illustrated in **Fig. 6,** addition of this antibody to the donor-acceptor mixture effectively decreased the amount of 25OHC-esters transferred to the acceptor (HDL) particles, consistent with CETP mediating the transfer of esterified oxysterols among lipoproteins.

That transfer of oxysteryl esters by CETP could influence the distribution of oxysterols in serum was shown in another experiment in which the distribution of [$^3$H]25OHC (0.1 mM) in serum incubated in the presence and absence of the IC-4 antibody was determined. Blocking CETP reduced the amount of LDL-associated 25OHC-esters by 40%, and increased LPDS-associated unesterified 25OHC by 30%. No substantial change was observed in HDL-associated esterified and free 25OHC. Thus, both esterification and CETP play a role in the distribution of 25OHC in serum.

### Effect of esterification on the delivery of 25OHC to cells

The influence of esterification on the delivery of oxysterols to cells was examined using human erythrocytes and human fibroblasts (GM 3468A cells) as models. Erythrocytes were chosen because of their relative simplicity with respect to intracellular organelles and plasma membrane. **Fig. 7** shows that the amount of [$^3$H]25OHC associated with the erythrocytes increased in a dose-dependent manner. This association was 5- to 6-fold greater in cells exposed to serum containing mainly unesterified 25OHC (DTNB-treated) than in cells exposed to serum containing significant amount

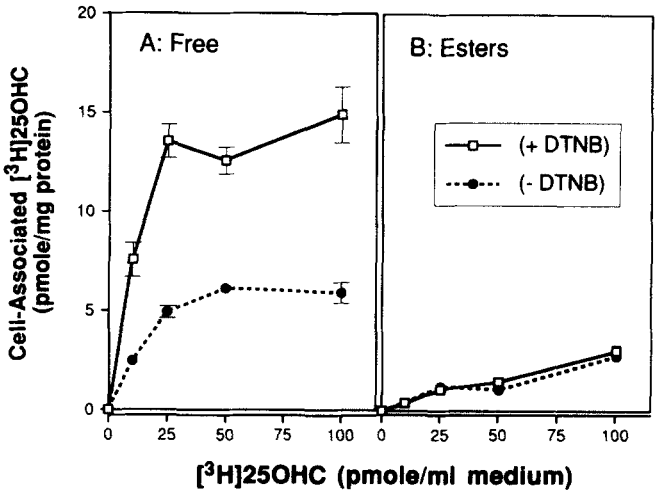


**Fig. 8.** Effect of esterification on the uptake of serum 25OHC by human fibroblasts (GM 3468A cells). The cells, grown in MEM containing 10% FBS and incubated in MEM plus 1% BSA for 36 h before the experiment, were exposed to experimental media containing [$^3$H]25OHC-labeled human serum (1 nmol 25OHC/ml serum, prepared with or without DTNB) at 0%, 1.0%, 2.5%, 5.0%, and 10% volume displacement for 5 h, then washed twice with cold PBS and analyzed for cell-associated free and esterified 25OHC. The data shown are means ± SD for triplicate wells.

(70%) of 25OHC-esters. Virtually all the cell-associated 25OHC was unesterified.

A similar dose-dependent uptake of 25OHC was seen in human fibroblasts with functional LDL-receptors, whether the dose was received by adding different amounts of serum, as shown in **Fig. 8,** or by adding a constant amount of serum containing different amounts of 25OHC (data not shown). At maximum, approximately 6% and 2% of available 25OHC from serum incubated with or without DTNB, respectively, became cell-associated. In both cases, the predominant cellular form was unesterified 25OHC. Inhibition of cellular esterification with Sandoz compound 58-035 in another experiment did not alter the relative proportions of cellular free and esterified 25OHC (data not shown).

Up-regulation of cell surface LDL-receptors might be expected to limit the protective effect of esterification by enhancing LDL uptake. In order to examine this idea, LDL receptors in fibroblasts were up-regulated by a standard protocol of pretreatment with lipoprotein-free medium (39, 40); control cells were maintained in medium containing 10% FBS. The uptake of [$^3$H]25OHC from sera preincubated with or without DTNB was compared in cells with these two levels of LDL-receptor expression. As shown in **Fig. 9,** more 25OHC became cell-associated upon incubation with serum pretreated with DTNB than with serum incubated in the absence of DTNB (A vs. B). As shown in Fig. 9B, in cells incubated with DTNB-free serum (containing mostly esteri-

fied 25OHC), up-regulation of LDL-receptors did increase the amount of cell-associated total 25OHC (as well as cell-associated 25OHC-esters, data not shown), but did not raise it to the levels achieved with DTNB-treated serum (containing mainly unesterified 25OHC). In other experiments in J774 macrophages, maximal uptakes of 25OHC from fresh human serum incubated with or without DTNB were 10% and 3%, respectively, compared to 48% from thawed fetal bovine serum, in which less than 4% of 25OHC was esterified even in the absence of DTNB (data not shown).

## DISCUSSION

These studies show that the oxysterol 25OHC is readily esterified in human serum at 37°C, and esterification leads to a redistribution of 25OHC in serum from LPDS to lipoproteins. Within the concentration range in which oxysterols are reportedly detected in human and animal plasma in vivo (21, 22), most (40–80% in various experiments) of the 25OHC added in vitro became esterified after incubation at 37°C for 24 h, and virtually all of the 25OHC-esters formed associated with the lipoproteins in serum. It is, therefore, not too surprising that under physiological conditions, serum oxysterols have been reported to associate mainly with the lipoproteins (41, 42). The variability in the extent of esterification in different serum samples is most likely related to donor variation and the degree of serum freshness.

Two lines of evidence from our studies suggest that LCAT is the enzyme catalyzing the esterification of 25OHC in human serum. *1)* The esterification of 25OHC was effectively suppressed by LCAT inhibitors, both NEM and DTNB, and *2)* the esters formed in serum were monoesters, rather than diesters. This is consistent with the finding by Piran and Nishida (43) and Kitabatake et al. (44) that many sterols with a 3β-hydroxyl group and a *trans* configuration of the A/B rings are able to serve as acyl acceptors in the LCAT-mediated transesterification reaction. Moreover, Szedlacsek et al. (45) have reported recently that oxysterols with a hydroxyl or a ketone group at the $C_7$, $C_{27}$, and $C_{25}$-positions in liposomes containing apoA-I are readily esterified in vitro by purified LCAT; 27-hydroxycholesterol could form diesters, whereas the other oxysterols formed solely monoesters at the $C_3$β-OH group.

Also consistent with LCAT as the mechanism for oxysterol esterification is the similarity between the HPLC profile of the cholesterol-derived esters and that of 25OHC-derived esters. The differential elution of various cholesteryl esters relates to the fatty acids to which they are esterified, most likely arachidonate, linoleate, oleate, and palmitate in order of elution (46, 47). The elutions of the synthesized 25OHC-monooleate and the commercial cholesteryl oleate standard are consistent with this idea (Fig. 2). The apparent similarity in fatty acyl chains for both 25OHC-esters and cholesteryl esters suggests that they are most likely derived through a common enzymatic pathway.

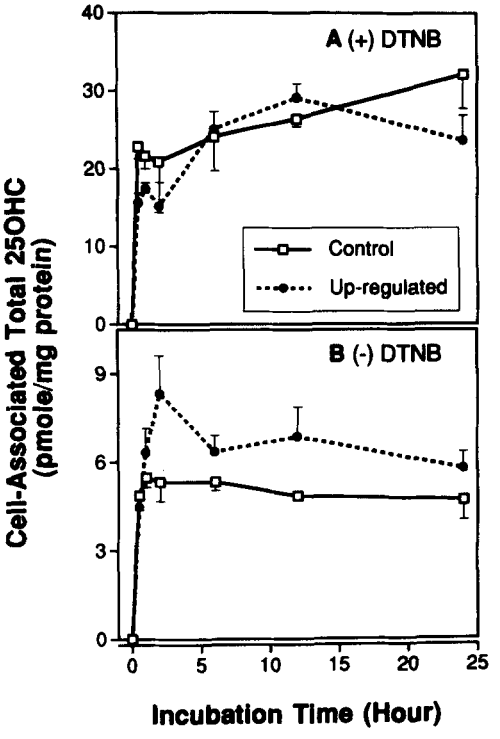


**Fig. 9.** Effect of LDL-receptor up-regulation on the uptake of serum 25OHC by GM 3468 A cells. Confluent cells were pretreated with MEM + 10% FBS (control) or MEM + 1% BSA for 36 h (to up-regulate LDL-receptors), then incubated with [$^3$H]25OHC-labeled serum (1 nmol 25OHC/ml serum) at 10% volume displacement for the indicated times. Cell lipids were extracted and quantitated by liquid scintillation counting. The data shown are means ± SD for triplicate wells.

It is not clear from these studies whether cholesterol or 25OHC is a better acyl acceptor in the LCAT reaction. The percentage of available sterol that became esterified in these studies was much higher for 25OHC (76%) than for cholesterol (25%), although the mass of cholesterol esterified, based on the specific radioactivities of [$^3$H]25OHC and [$^{14}$C]cholesterol, was 10 times greater than the mass of 25OHC esterified. These data might imply that 25OHC is a better acyl acceptor under physiological conditions, and that the greater mass of cholesteryl esters formed may reflect the greater availability of unesterified cholesterol in serum. However, according to Szedlacsek et al. (45) using discoidal bilayer particles as substrate for purified LCAT in vitro, LCAT-mediated esterification of cholesterol is comparable to that of cholesterol. In serum, the equal distribution of 25OHC-esters in serum between HDL and LDL (Fig. 3) after esterification on HDL (where apoA-I, the cofactor

for LCAT, resides) indicates a concomitant redistribution of 25OHC-esters with esterification. The removal of 25OHC-esters from HDL to LDL, presumably through the action of CETP, may, in part, explain the discrepancy between the apparent efficiency of oxysterol esterification in serum and that in vitro. In order to fully understand which sterol is preferred by LCAT in serum, kinetic experiments with equally labeled substrates in serum could be compared to those with purified enzyme.

Unesterified 25OHC is water-soluble whereas its esterified counterparts are not, suggesting that unesterified 25OHC can transfer among lipoproteins, or between lipoproteins and albumin, by aqueous diffusion (48), whereas esterified 25OHC would be expected to transfer only with the aid of plasma lipid transfer proteins. This speculation is supported by the observation that unesterified 25OHC readily transferred between HDL and LDL, whereas esterified 25OHC did not exchange unless CETP was present. It is known that in human plasma CETP mediates the exchange of cholesteryl esters and triglycerides between HDL and apoB-containing lipoproteins (26, 27). Our studies suggest that CETP facilitates the exchange of esterified oxysterols among lipoproteins as well.

Interestingly, inhibition of CETP activity by a monoclonal antibody (IC-4) not only suppressed the transfer of 25OHC-esters between the lipoproteins, but also decreased the levels of total 25OHC-esters formed in serum, suggesting that blocking CETP activity in serum may influence LCAT activity as well.

It remains unclear where esterified oxysterols are located within lipoproteins. The polar unesterified 25OHC is most likely associated with other polar lipids such as cholesterol and phospholipids on the outer surface of the lipoprotein particles. In contrast, as suggested by the requirement for CETP to transfer esterified 25OHC between lipoproteins, 25OHC-esters are probably immobilized in the lipoprotein with their fatty acyl chains anchored in the hydrophobic core. Because of the remaining free hydroxyl function, it is unlikely that the entire monoester molecules would be solubilized in the core, like cholesteryl esters. Thus, it is most likely that 25OHC-esters are aligned perpendicular to the interface, with the free hydroxyl function toward the aqueous phase and the sterol rings and long fatty acid acyl chain buried inside the hydrophobic core.

It is generally believed two means, namely diffusional transfer (48) and receptor-mediated endocytosis (29), contribute to the acquisition by cells of exogenous cholesterol and other nutrient lipids. Surface transfer is a process in which cholesterol molecules desorb from the donor particles and diffuse through the intervening aqueous layer until they collide with and are adsorbed by the acceptor cell membrane. In contrast, receptor-mediated endocytosis is not a spontaneous process, and requires metabolic energy (29). In this process, binding of LDL to the cell surface LDL-receptor triggers the internalization of LDL and hydrolysis of the LDL-associated lipids in lysosomes.

Esterification of oxysterols in serum decreases their solubility and immobilizes them in the lipoproteins, thereby limiting the diffusional transfer of serum oxysterols to cell membranes. Up-regulation of the surface LDL-receptor in fibroblasts substantially enhanced the uptake of 25OHC-esters from medium, but did not offset the limitation on the cellular access of serum 25OHC imposed by esterification. One possible explanation is that 25OHC itself is a potent inhibitor of LDL-receptor activity, as demonstrated by Brown and Goldstein (29, 49); a small amount of unesterified 25OHC incorporated into the cells quickly suppresses the receptor activity, and then inhibits the receptor-mediated further internalization of lipoprotein-associated 25OHC. Alternatively, the contribution of LDL-receptor-mediated uptake to total uptake is small. As the ability of oxysterols to modulate cholesterol homeostasis or induce cytotoxicity is dependent on the concentration of oxysterols in cells, esterification may provide a protective function against the cellular actions of oxysterols by limiting their access to cell.

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