On the rate of translocation in vitro and kinetics in vivo of the major oxysterols in human circulation: critical importance of the position of the oxygen function

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Abstract **Oxysterols possess powerful biological activities. Some of their effects on the regulation of key enzymes are similar to those of cholesterol, but are much more potent. One of the critical properties of oxysterols is their ability to pass lipophilic membranes at a high rate. Transfer of unesterified 25-hydroxycholesterol from red blood cells to plasma has been reported to occur more than 1,000 times faster than cholesterol. Here we have measured the relative rate of such translocation of the three major oxysterols in human circulation: 27-hydroxycholesterol, 24S-hydroxycholesterol, and 4β-hydroxycholesterol. The distance from the 3β-hydroxyl group to the additional hydroxyl group is the greatest possible in 27-hydroxycholesterol and the least possible in 4β-hydroxycholesterol. The rate of exchange between erythrocytes and plasma was found to be high for 27-hydroxycholesterol and 24S-hydroxycholesterol, and hardly possible to measure for 4β-hydroxycholesterol and cholesterol. When injected intravenously into humans, deuterium labeled 24- and 27-hydroxycholesterol caused an immediate high enrichment of the corresponding plasma sterols followed by a decay. After injection of labeled 4β-hydroxycholesterol, the maximum deuterium enrichment occurred after 2–3 h, when secretion of the oxysterol from the liver is likely to be the limiting factor. When radiolabeled cholesterol was injected under the same conditions, maximum appearance of label occurred after about 2 days. The results illustrate the importance of the position of the additional oxygen in oxysterols and are discussed in relation to the rate of metabolism and biological effects of these oxysterols.**—Meaney, S., K. Bodin, U. Diczfalusy, and I. Björkhem. **On the rate of translocation in vitro and kinetics in vivo of the major oxysterols in human circulation: critical importance of the position of the oxygen function.** ***J. Lipid Res.*** **2002.** 43: **2130–2135.**

Supplementary key words 27-hydroxycholesterol • 4β-hydroxycholesterol • 24*S*-hydroxycholesterol • oxysterol flux • cholesterol flux

Oxysterols are mono-oxygenated derivatives of cholesterol that have been implicated in many cellular processes (1–3). One of their most important and well-characterized roles is as intermediates or end products in cholesterol excretion pathways (1, 2, 4). Introduction of an oxygen moiety into the largely hydrophobic cholesterol designates it to a highly efficient excretion pathway with bile acids as typical end products. Oxysterols are considerably more potent than cholesterol in modulating lipid homeostasis, at least under in vitro conditions. This has lead to widespread endorsement of the homeostatic relevance of oxysterols, despite their extremely low concentrations in most physiological situations.

The rapid catabolism of oxysterols is a function of several different properties, including their ability to enter cholesterol excretion pathways at a variety of stages and their biophysical attributes vis á vis translocation between different lipophilic compartments.

The spontaneous transfer of cholesterol between different lipophilic compartments (e.g., from erythrocytes to lipoproteins) is well recognized, and several studies have investigated the kinetic parameters of this process. Lange et al. (5) characterized the transfer of cholesterol between erythrocytes and plasma, finding that it progressed in an apparently first order fashion except for an initial rapid component of ~15% when using erythrocytes as cholesterol donors. The first-order rate constant for cholesterol release from red cells to a variety of lipophilic acceptors was estimated to be $\sim 1 \times 10^{-4}$ s^{-1}, with a $t_{1/2}$ of ~2 h. In similar experiments the transfer of 25-hydroxycholesterol from erythrocytes to plasma was found to occur about 2,000 times faster than that of cholesterol, though it was not possible to estimate the kinetics of this exchange (6).

More recently, the transfer of different cholesterol hydroperoxides between erythrocyte ghosts and unilamellar

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Abbreviations: 25-hydroxycholesterol, cholest-5-ene-3β,25-diol; 7α-hydroxycholesterol, cholest-5-ene-3β,7α-diol; 7β-hydroxycholesterol, cholest-5-ene-3β,7β-diol; 4β-hydroxycholesterol, cholest-5-ene-3β,4β-diol; 7-ketocholesterol, cholest-5-ene-7-keto-3β-ol; 24*S*-hydroxycholesterol, cholest-5-ene-3β,24S-diol; 27-hydroxycholesterol, cholesten-5-ene-3β,25R(26)-diol.

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liposomes was investigated (7). Under the conditions used in these studies, the rate constant for the transfer of cholesterol hydroperoxides was estimated to be about 65 times that of cholesterol ($K_a \sim 3.27 \times 10^{-4}$ min^{-1} and $K_a \sim 2.44 \times 10^{-2}$ min^{-1} for cholesterol and cholesterol hydroperoxides, respectively).

Using a phospholipid monolayer system Theunissen et al. (8) investigated different membrane properties of the cholesterol auto-oxidation products 7-ketocholesterol, 7α-hydroxycholesterol, 7β-hydroxycholesterol, and 25-hydroxycholesterol. In this study, position dependent effects of the hydroxyl or keto group on the membrane properties (e.g., interfacial orientation, membrane surface area, and membrane condensation) of the oxysterols were observed, with a clear division between the properties of oxysterols hydroxylated in the side chain or the nucleus. In particular, the rate of transfer of radiolabeled oxysterols from a monolayer to acceptor particles (lipoproteins or liposomes) followed a clear rank order with the highest rate of transfer observed for 25-hydroxycholesterol and the lowest for 7-ketocholesterol. Under the conditions used, there was a 20-fold difference between the rates of translocation of these oxysterols, while that of cholesterol was hardly measurable.

Quantitatively, the most important oxysterols in human circulation are 24*S*-, 27-, and 4β-hydroxycholesterol (1). 24*S*-Hydroxycholesterol is predominantly formed in the brain and, as judged by animal experiments, is able to traverse the blood-brain barrier several orders of magnitude more efficiently than cholesterol. Conversely, 27-hydroxycholesterol is formed in many tissues and, based on experiments in cell culture systems, is able to rapidly cross the plasma membrane and enter the extracellular space. The ability to translocate between different physiological compartments, in addition to their obligate hepatic metabolism, permits these oxysterols to act as unidirectional transport forms of cholesterol.

The in vivo origin of 4β-hydroxycholesterol has recently been elucidated (9). This oxysterol is formed by the cytochrome P450 CYP3A4, and changes in the expression of this enzyme may have consequences for the plasma levels of 4β-hydroxycholesterol.

24*S*- and 4β-hydroxycholesterol have both been reported to be efficacious activators of the liver X receptors LXR-α (NR1H3) and -β (NR1H2), nuclear receptors believed to be important regulators of genes involved in cholesterol and lipid homeostasis (10, 11). Data on 27-hydroxycholesterol is less consistent, with one group reporting efficient activation (12) while others do not.

There are few studies detailing the properties of the quantitatively dominating oxysterols, with most studies using readily available oxysterols (often auto-oxidation products). In the present study, we investigate the relationship between the position of an oxysterol's hydroxyl group and its effect on the kinetics of the steroid when administered to human volunteers. Dramatic position dependent effects were observed on both the rate of transport of oxysterols between lipophilic compartments and the in vivo kinetics of deuterium labeled oxysterols.

MATERIALS AND METHODS

Materials

Tritium labeled cholesterol and 25-hydroxycholesterol were purchased from Perkin-Elmer, Life Sciences. Before the flux experiments they were diluted with the corresponding unlabeled compound to give a specific radioactivity of 3×10^6 cpm/μg and 0.12×10^6 cpm/μg, respectively.

In the in vivo experiment with [^{3}H]cholesterol, the material had a specific radioactivity of 0.02×10^6 cpm/μg. 7α-^{3}H-labeled 7β-hydroxycholesterol and 7β-^{3}H-labeled 7α-hydroxycholesterol were synthesized by reduction of 7-ketocholesterol with tritium labeled sodium borotritide as described previously (13), and had a specific radioactivity of 0.1×10^6 cpm/μg and 0.5×10^6 cpm/μg, respectively. ^{3}H-labeled 4β-hydroxycholesterol, 24-hydroxycholesterol, and 27-hydroxycholesterol were synthesized as described previously (14–16) and had specific radioactivities of 0.08×10^6 cpm/μg, 0.4×10^6 cpm/μg, and 0.3×10^6 cpm/μg, respectively. ^{2}H-labeled 4β-hydroxycholesterol, 24-hydroxycholesterol, and 27-hydroxycholesterol were synthesized as described previously (17, 18).

Human serum albumin, 200 mg/ml, was obtained from Amersham Biosciences (Uppsala, Sweden). All solvents and buffer salts were of analytical grade.

Preparation of labeled erythrocytes

The flux of oxysterols between erythrocytes and plasma was estimated essentially as described (6). Briefly, packed washed erythrocytes were prepared by washing freshly drawn whole blood three times in flux buffer (150 mM NaCl, 5 mM NaP_i, 5 mM glucose, pH 7.5) and carefully removing the buffy coat. One microgram of radiolabeled oxysterol was dissolved in 10 μl of ethanol and mixed with 1 ml of flux buffer and 1 ml of prepared erythrocytes. This suspension was incubated on ice for 60–90 min before the cells were washed as before. Aliquots of labeled cells (10 μl) were kept on ice until required. As judged by the absence of visible haemolysis, this treatment was not shown to affect the stability of the blood cells.

Preparation of exchange plasma

Plasma was prepared from fresh EDTA anticoagulated whole blood by centrifugation at 1,600 *g* for 5 min. Plasma was heat inactivated at 56°C for 50 min before being centrifuged at 15,000 *g* for 30 min. The clear central fraction of the plasma preparation (corresponding to about 80% of the total volume) was removed and refrigerated until required.

Oxysterol flux experiments

Ten-microliter aliquots of labeled erythrocytes were rapidly brought to 37°C. At $t = 0$, 90 μl of a 1:1 mixture of heat treated plasma and flux buffer, equilibrated to 37°C, was added to the pre-heated cells. At suitable intervals (typically $t = 5$, 10, and 30 s), the erythrocytes were pelleted at maximum speed in a chilled benchtop centrifuge. Aliquots of the supernatant were mixed with 10 ml of liquid scintillation reagent (Lumasafe, Lumac LSV, Groningen, Netherlands) and counted using a Wallac Winspectral 1414 liquid scintillation counter (Wallac LKB, Finland). To determine the sterol available for exchange, aliquots of labeled erythrocytes were lysed with deionised water and extracted with chloroform-methanol (2:1, v/v). The organic phase was dried before counting as before.

In some experiments, the direction of the flux was reversed and the labeling of the erythrocytes was followed.

In vivo experiments

Five hundred micrograms of the appropriate deuterium labeled sterols (4β-hydroxycholesterol, 24-hydroxycholesterol, or 27-hydroxycholesterol in different experiments) and, in one experiment, tritium labeled cholesterol, was dissolved in ethanol, mixed with human serum albumin and physiological sodium chloride solution (0.9%, w/v), and administered intravenously to a healthy male volunteer 60 years of age (body mass index 25) or to a healthy female volunteer 64 years of age (body mass index 24). When the same subject participated in more than one experiment, there was an interval of at least 1 month between the experiments. At suitable time intervals blood samples were collected and the deuterium enrichment was determined by GC-MS as described in detail previously (14, 17). Determination of the tracer activity of plasma was carried out by measuring the total counts in an aliquot of plasma from each time point. No quenching effect was observed in these analyses.

It should be noted that the experiments described above with the same deuterium labeled oxysterols have been performed previously (14, 17). The goal in these previous experiments was to determine the half-life of the oxysterols and the early phase of the kinetics was therefore never followed.

GC-MS analysis

Oxysterols were analyzed as trimethylsilylether derivatives as previously described (19), except that no internal standard was added. The instrument used was a Hewlett Packard Series II gas chromatograph equipped with an HP-5MS capillary column (30m × 0.25 mm, 0.25 um phase thickness) connected to an HP 5972 mass selective detector and an HP 7673A automatic sample injector. The following ions were monitored: *m/z* 579 (deuterium labeled 4β-hydroxycholesterol), *m/z* 573 (unlabeled 4β-hydroxycholesterol, *m/z* 416 (deuterium labeled 24-hydroxycholesterol, *m/z* 413 (unlabeled 24-hydroxycholesterol), *m/z* 461 (deuterium labeled 27-hydroxycholesterol), and *m/z* 456 (unlabeled 27-hydroxycholesterol).

Ethical permissions

All experiments on human volunteers were approved by the local ethical committee.

RESULTS

Transfer of oxysterol from erythrocytes to plasma

As **Fig. 1** shows, the transfer of all oxysterols tested except 4β-hydroxycholesterol, was found to occur at a much greater rate than cholesterol. The three side-chain oxidized oxysterols were translocated at a considerably higher rate than the oxysterols with an oxygen function in the C-7 position. The rate of translocation of 4β-hydroxycholesterol appeared to be even lower than that of cholesterol.

It should be emphasized that the time-course for the transfer during the first 5 s of the experiment was not possible to evaluate. During this period of time, there is a very rapid initial burst of sterol transfer. In previous work, this initial burst of cholesterol transfer has been subtracted in the calculations of the relative rates of transfer (5, 7). This background has been estimated by various studies to be between 10% and 20% (5, 7, 20, 21).

In an attempt to reduce the problems with the initial burst of transfer, we also studied a reverse transfer in the same system. In this case, we loaded the heat-inactivated plasma with the labeled sterol and measured the rate of labeling appearance of the label in erythrocytes. With this technique it was possible to reduce the initial burst of transfer of labeled cholesterol from about 10% down to 1%. The rank order between the different oxysterols did not change by this technique (results not shown).

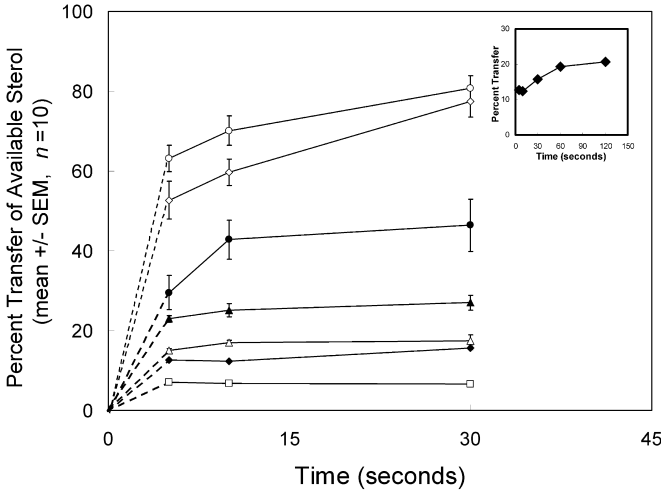

Fig. 1. Transfer of cholesterol (filled diamonds), 4β-hydroxycholesterol (open squares), 7β-hydroxycholesterol (open triangles), 7α-hydroxycholesterol (filled triangles), 24*S*-hydroxycholesterol (filled circles), 25-hydroxycholesterol (open circles), and 27-hydroxycholesterol (open diamonds) from ^{3}H-sterol labeled erythroctes to plasma. The difference between nuclear and side chain oxidized sterols is readily apparent. The inset shows the transfer of cholesterol over an extended time course.

Due to the obvious limitations of the present, as well as previous, attempts to measure rate of translocation of oxysterols, no exact figure can be given. A clear rank order can be defined, however. After the initial burst during the first 5 s of the experiment, the transfer of all side chain hydroxylated species tested occurred at a rate 30–50-fold greater than cholesterol, while transfer of the 7-oxygenated species was only about 5-fold greater than cholesterol. Using this method the transfer of cholesterol in some experiments was monitored over an extended time course. A plateau of cholesterol transfer was observed at 60 s with an apparent second phase beginning at this point (inset, Fig. 1).

Using a technique essentially identical to that previously described (5), oxysterol transfer over a time course up to 3 h was also monitored (results not shown). While a similar rank order of sterol transfer was found, the relatively large initial burst of sterol transfer precluded the use of this technique in the investigation of the physiologically dominating oxysterols; however, the similarity of the transfer of cholesterol and 4β-hydroxycholesterol was also readily apparent with this technique.

In vivo kinetics of oxysterols

To further elucidate the consequences of different rates of translocation, deuterium labeled analogues of 4β-hydroxy-

cholesterol, 24-hydroxycholesterol, and 27-hydroxycholesterol were injected as a bolus dose to healthy volunteers (at least one experiment with each oxysterols in each of the two volunteers). The elimination of deuterium labeled 27-hydroxycholesterol was so fast that deuterium enrichment of plasma 27-hydroxycholesterol was only detectable in the first sample collected. This is in accord with the previous result that the half-life of 27-hydroxycholesterol in plasma is extremely short, at least less than 0.75 h (22). No further attempts were therefore made to study the kinetics of 27-hydroxycholesterol with this technique.

The kinetics of representative experiments with deuterium labeled 24-hydroxycholesterol and 4β-hydroxycholesterol are shown in **Fig. 2**. Maximal labeling of circulating 24-hydroxycholesterol occurred within 2 min after the injection while the maximal labeling of 4β-hydroxycholesterol occurred after about 5 h. Subsequently, approximate first order kinetics were followed by both compounds with an estimated terminal half-life of ~10 h for 24*S*-hydroxycholesterol (17) and ~60 h for 4β-hydroxycholesterol (**Fig. 3**) (14). For reasons of comparison, ^{3}H-labeled cholesterol was injected in one of the volunteers under the same conditions as the above oxysterols. The time course of the plasma label is shown in the inset of Fig. 2. After an initial rapid decrease a slow increase in plasma label occurred with the tracer peak occurring after about 2 days. In accordance with previous work, elimination by first order of kinetics occurred after 2–3 days.

DISCUSSION

Erythrocytes were used as a model sterol donor system for several reasons: they lack the capacity to metabolize

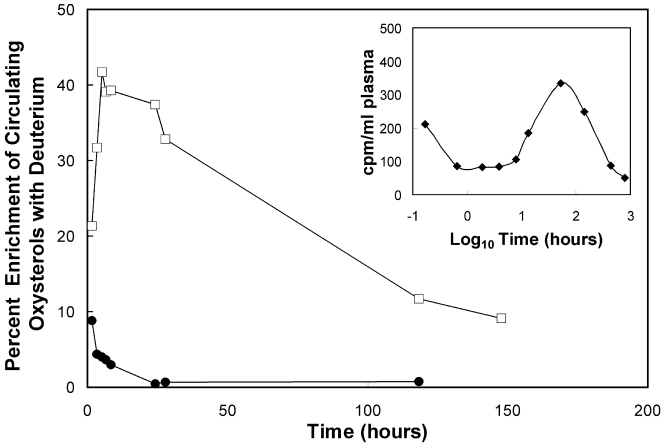

Fig. 2. Deuterium enrichment of circulating 4β-hydroxycholesterol (open squares) and 24*S*-hydroxycholesterol (filled circles) after injection of a bolus dose of deuterated sterols in a healthy volunteer. A clear secretion phase prior to an elimination phase is obvious with 4β-hydroxycholesterol, while only the secretion and elimination phases are visible for 24*S*-hydroxycholesterol. The inset shows the change in the activity of plasma after administration of ^{3}H-cholesterol (filled diamonds). As expected the redistribution, secretion, and elimination phases are clearly defined.

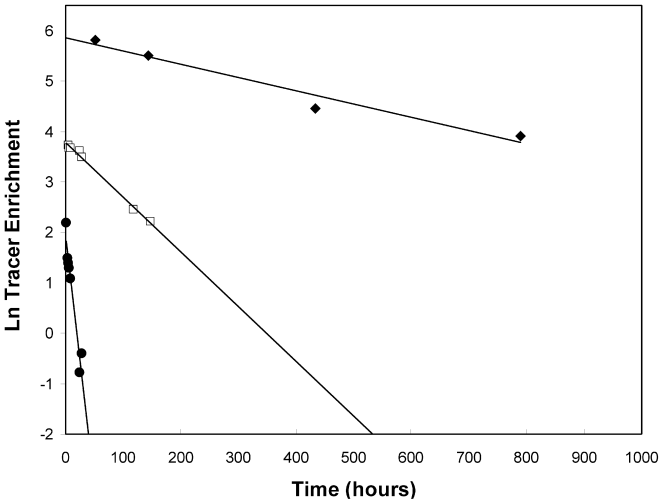

Fig. 3. Terminal elimination kinetics of injected ^{3}H-cholesterol (filled diamonds), deuterium labeled 4β-hydroxycholesterol (open squares), and 24*S*-hydroxycholesterol (filled circles). The elimination of 4β-hydroxycholesterol is between that of 24-hydroxycholesterol and cholesterol, highlighting the "cholesterol-like" character of this oxysterol.

cholesterol, are well characterized with respect to cholesterol transfer, and are readily available in a highly purified form. In previous investigations the transfer of sterols between lipophilic compartments was quenched at suitable time points by the dilution of the transfer mixture with a large excess of cold buffer followed by separation via centrifugation (5, 7, 21); however, due to the rapidity of oxysterol transfer this method was found to be unsuitable for the current studies. The early phase of the oxysterol transfer is missed, in particular in the case of the side chain oxidized sterols (unpublished observations). The rapid centrifugal separation of cells and plasma used here allowed a better evaluation of the transfer during the early phase. Despite this, the very rapid burst of transfer during the first few seconds of the experiment prevented a more precise definition of the kinetic parameters.

While there are few studies directly investigating the properties of oxysterols within lipid bilayers, cholesterol has been the subject of sustained investigation. The results of these studies are instructive in the generation of models for the behavior of oxysterols observed in the current study. When cholesterol is incorporated into a lipid bilayer its preferential orientation is such that the 3-hydroxyl group interacts with the nearby polar headgroup, with the rest of the cholesterol molecule oriented roughly perpendicular to the plane of the membrane (23) (**Fig. 4**). This configuration permits the maximum interactions between both the non-polar regions of the cholesterol molecule and the acyl chains of the bilayer lipids to occur, while minimizing the exposure of the hydroxyl group to a non-polar environment.

In the case of oxysterols, the introduction of an additional polar moiety into the hydrophobic region should lead to a redistribution of the sterol in conjunction with a local reordering of the acyl chains. This is supported by

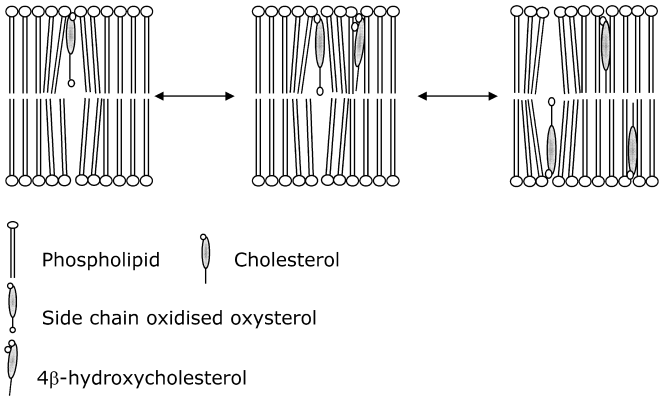

Fig. 4. Model for the orientation of oxysterols in membrane bilayers. The introduction of an hydroxyl group into the side chain leads to local reordering of the membrane phospholipids. The acyl chains are distorted by the hydrophilic hydroxyl group, and the polar phospholipid headgroups are deformed in order to maintain the membrane integrity. The net result of these changes is that it is easier for a side chain oxidized sterol to desorb from the membrane.

molecular dynamics simulations that show that the C-6 keto group of 6-ketocholestanol leads to the movement of this sterol towards the polar headgroup, with the net effect of increasing both the membrane area and the acyl chain disorder (24). As judged by pressure-area isotherms of mixtures of a phospholipid and side-chain oxidized cholesterol species, the presence of the oxysterol expanded the area of phospholipid monolayers at low molecular ratios (25). Increasing the area of the membrane is likely to facilitate desorption of an oxysterol from it. Based on biophysical studies, it has been suggested that there may be a perpendicular shuttling of 25-hydroxycholesterol between the inner and outer monolayer of the membrane, causing a local disordering of the lipids and a higher permeability of the membrane (8) (Fig. 4). Such an effect would also facilitate a desorption from the membrane and explain the high rate of translocation of side-chain oxidized sterols across biological membranes. According to this hypothesis the highest rate of transport of a side-chain oxidized oxysterol should be expected for an oxysterol in which the distance between the 3β- and the side chain hydroxyl group is the greatest possible, i.e., 27-hydroxycholesterol. As shown in Fig. 1, the rate of transfer of 25- and 27-hydroxycholesterol from erythrocytes to plasma lipids was found to be higher than that of any other of the oxysterols tested.

A consequence of the above hypothesis is that hydroxyl groups in the nucleus should have less disturbing effects on membrane lipids, and thus less effect on permeability. 7β-Hydroxycholesterol has been reported to be redistributed between a monomolecular layer and lipoproteins at a rate considerably slower than that of 25-hydroxycholesterol (8). As shown in Fig. 1, this was confirmed in our study. The rate of transport of tritium-labeled 7β-hydroxycholesterol was found to be considerably lower than the rate of transport of the three side-chain oxidized oxysterols, but greater than the rate of transport of labeled cholesterol. 4β-Hydroxycholesterol should be the most cholesterol-like oxysterol, and in accordance with this, the rate of translocation of 4β-hydroxycholesterol was similar to that of cholesterol in the system used.

The marked difference in the rate of translocation of 24*S*-hydroxycholesterol and 4β-hydroxycholesterol observed in our in vitro system is likely to be of importance for the in vivo kinetics of these oxysterols. In addition to the capacity of the systems able to metabolize the oxysterol, the rate of translocation between different compartments may be a critical factor. When cholesterol is introduced into the circulation by intravenous injection of a bolus dose, there is a rapid uptake in the liver and to some extent also in some other organs or tissues. This phase is followed by incorporation of the sterol into lipoproteins and subsequent secretion. The secretion phase is followed by a distribution phase during which cholesterol equilibrates with the exchangeable pools. In parallel with this, there is a slow elimination of cholesterol in the form of bile acids or unchanged cholesterol in bile. These different phases are well illustrated by the results of the present experiment with labeled cholesterol (inset, Fig. 2).

The fate of an administered oxysterol would be expected to be similar to that of cholesterol. In agreement with this, the distribution of oxysterols in lipoproteins is similar to that of cholesterol (9, 26). Due to the rapid translocation of a side-chain oxidized oxysterol, some of the steps may however occur too rapidly to be clearly defined. Alternatively, the efficiency of later stages may obscure one or more of the earlier steps. Among the different oxysterols administered, only 4β-hydroxycholesterol showed "cholesterol-like" kinetics with a clear secretion phase occurring prior to the elimination phase (Fig. 2). In the case of 24- and 27-hydroxycholesterol, no secretion phase could be defined, while with 24-hydroxycholesterol both an equilibration phase and an elimination phase were observed. Only part of the elimination could be followed in the case of 27-hydroxycholesterol.

The capacity of the systems involved in metabolism of the different oxysterols is likely to be the most critical factor for the elimination kinetics. The rate of translocation of the oxysterols across biological membranes must however also be of importance, and for some, oxysterols (e.g., 4β-hydroxycholesterol) this rate is likely to be a limiting factor for part of the process (e.g., the secretion phase).

There is a striking relation between the rate of translocation of steroids in biological membranes and the rate of their elimination. Side-chain oxidized oxysterols are cytotoxic and are very potent suppressors of cholesterol synthesis (1–3). For example, 25-hydroxycholesterol is a far more potent suppressor of cholesterol synthesis in cultured cells than 4β-hydroxycholesterol (27). The observed properties of side-chain oxidized oxysterols may well be linked to their membrane-disturbing effects. It may be speculated that evolution has favored metabolic systems that are able to minimize the risk for accumulation of the fast-moving membrane-disturbing oxysterols. Oxysterol 7α-hydroxylase (CYP7B1) has a wide tissue dis-

tribution and high enzymatic activity, for example towards 25- and 27-hydroxycholesterol (16, 28–32). The possibility has been discussed that one of the major biological roles of CYP7B1 may be to inactivate 25- and 27-hydroxycholesterol by 7α-hydroxylation (32). This conversion is likely to render them considerably less toxic.

To summarize, it is evident that the location of the additional oxygen function in an oxysterol is of critical importance for its rate of translocation between lipophilic compartments. As judged from the results of the investigation on the oxysterols studied here, there is a clear relation between this rate of translocation in vitro and the kinetic behavior of the oxysterol in vivo. There is also a relation between this translocation and the ability of an oxysterol to suppress cholesterol synthesis.

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