Evidence that the major oxysterols in human circulation originate from distinct pools of cholesterol: a stable isotope study

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Abstract **The major oxysterols in human circulation are 7α-, 27-, and (24*S*)-hydroxycholesterol. Two unique experiments were performed to elucidate their origin and kinetics. A volunteer was exposed to $^{18}O_2$-enriched air. A rapid incorporation of ^{18}O in both 7α- and 27-hydroxycholesterol and disappearance of label after exposure were observed. The half-life was estimated to be less than 1 h. Incorporation of ^{18}O in (24*S*)-hydroxycholesterol was not significant. In the second experiment a volunteer was infused with liposomes containing 10 g of [$^2H_6$]cholesterol. This resulted in an enrichment of plasma cholesterol with ^2H of up to 13%, and less than 0.5% in cerebrospinal fluid cholesterol. The content of ^2H in circulating 7α-hydroxycholesterol remained approximately equal to that of plasma cholesterol and decreased with a half-life of about 13 days. The ^2H content of circulating 27-hydroxycholesterol was initially lower than that of cholesterol but in the last phase of the experiment it exceeded that of cholesterol. No significant incorporation of ^2H in (24*S*)-hydroxycholesterol was observed. It is evident that 7α-hydroxycholesterol must originate from a rapidly miscible pool, about 80% of 27-hydroxycholesterol from a more slowly exchangeable pool, and more than 90% of (24*S*)-hydroxycholesterol from a nonexchangeable pool, presumably the brain. The results are discussed in relation to the role of oxysterols in cholesterol homeostasis and their use as markers for pathological conditions.**—Meaney, S., M. Hassan, A. Sakinis, D. Lütjohann, K. von Bergmann, Å. Wennmalm, U. Diczfalusy, and I. Björkhem. **Evidence that the major oxysterols in human circulation originate from distinct pools of cholesterol: a stable isotope study.** ***J. Lipid Res.*** **2001.** 42: **70–78.**

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Mono-oxygenated derivatives of cholesterol, also called oxysterols, are formed from cholesterol by autoxidation or by action of specific cytochrome P450s such as cholesterol 7α-hydroxylase (CYP7A), sterol 27-hydroxylase (CYP27), and cholesterol 24-hydroxylase (CYP46) (**Fig. 1**). Introduction of an oxygen function increases the rate of degradation of cholesterol to more polar compounds, including bile acids. Oxysterols, in particular those with additional oxygen functions in the steroid side chain, can easily be transported out of cells and thus facilitate elimination of the cholesterol from extrahepatic sources (1). In addition to being intermediates in cholesterol degradation, oxysterols have a broad spectrum of biological effects, including modulation of the activity of key proteins involved in cholesterol homeostasis [for general reviews, see (2–6)]. Despite this and the description of nuclear receptors with high affinity toward selected oxysterols (7–9), no definitive evidence has yet been presented that these compounds are of regulatory importance under in vivo conditions.

The concentration of oxysterols in biological fluids is determined by their rate of formation and metabolism, and in general the rate of metabolism of these compounds is considerably higher than that of cholesterol. The major oxysterols present in human circulation are 7α-hydroxycholesterol, 27-hydroxycholesterol, and (24*S*)-hydroxycholesterol.

7α-Hydroxycholesterol is formed by the action of cholesterol 7α-hydroxylase in the liver, which is the rate-limiting enzyme in the major pathway from cholesterol to bile acids (10). Small amounts of 7α-hydroxycholesterol may also be formed as a side product of lipid peroxidation or directly by radical attack (11). It has been shown that the concentration of 7α-hydroxylated sterols in human circulation changes in parallel with the activity of cholesterol 7α-hydroxylase (12, 13). Thus it is likely that most of the

Abbreviations: CYP7A, cholesterol 7α-hydroxylase; CYP27, sterol 27-hydroxylase; CYP46, cholesterol 24-hydroxylase; GC-MS, gas chromatography-mass spectrometry; LDL, low density lipoprotein; VLDL, very low density lipoprotein.

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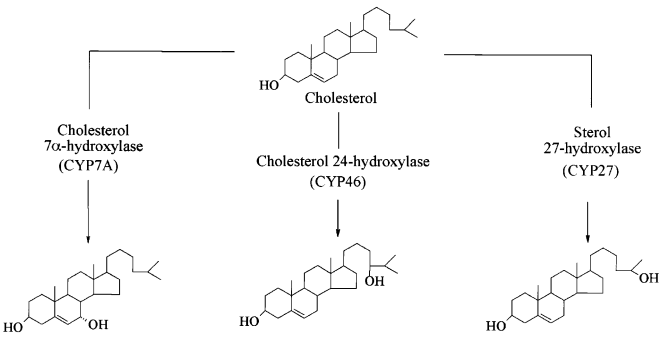

Fig. 1. P450-mediated conversion of cholesterol into the three major oxysterols present in human circulation.

circulating 7α-hydroxycholesterol originates from the liver.

The CYP27-mediated production of 27-hydroxycholesterol in extrahepatic tissues and its subsequent transport to the liver may be viewed as an alternative to classic reverse cholesterol transport and a potential antiatherogenic mechanism (1, 14). In preliminary work from this laboratory, some patients with advanced atherosclerosis were found to have high levels of 27-hydroxycholesterol, and its metabolites, in the circulation (A. Babiker et al., unpublished observation). Evaluation of such findings is facilitated by knowing the relative contributions of hepatic and extrahepatic sources to circulating 27-hydroxycholesterol.

It has been shown that there is a constant flux of (24*S*)-hydroxycholesterol from the brain into the circulation which is similar to the uptake of this oxysterol in the liver (15, 16). This is consistent with a cerebral origin of most of the (24*S*)-hydroxycholesterol in human circulation, although an extracerebral origin of a considerable part of the (24*S*)-hydroxycholesterol in the circulation cannot be excluded. It should be mentioned that purified sterol 27-hydroxylase from pig liver has a small but significant capacity to synthesize (24*S*)-hydroxycholesterol from cholesterol (17). Further, disruption of the gene coding for the cholesterol (24*S*)-hydroxylase in mice does not result in complete disappearance of (24*S*)-hydroxycholesterol from the circulation, indicating that there is a considerable extracerebral cholesterol 24-hydroxylase activity in mice (18).

We have suggested that plasma levels of (24*S*)-hydroxycholesterol may reflect cholesterol homeostasis in the brain, and that these levels can be used as a marker for pathological and/or developmental changes in the brain (19). A prerequisite for this is, however, that the proportion of (24*S*)-hydroxycholesterol in the human circulation originating from extracerebral sources is negligible. This has not yet been established with certainty.

Information about the origin of the oxysterols in the circulation may be obtained by isotope experiments, whereby the incorporation of labeled cholesterol into in vivo biosynthethic pools is monitored. Because the hepatic cholesterol pool is in rapid equilibrium with the circulation (20) oxysterols formed in the liver are likely to contain about the same amount of label as plasma cholesterol at any time. In general, extrahepatic pools of cholesterol would be expected to incorporate label at a slower rate than plasma cholesterol. Thus oxysterols formed from such pools should contain less label than plasma cholesterol during an early phase of an experiment but similar or more label than plasma cholesterol during later phases.

Because of the presence of the efficient blood–brain barrier, there is little direct cholesterol exchange between the brain and the circulation. In accordance with this, little or no incorporation of label in brain cholesterol was found in experimental animals (21–23) as well as humans (24) after administration of labeled cholesterol. Oxysterols formed in the brain of experimental animals or humans after infusion of labeled cholesterol would therefore be expected to contain little or no label.

In the present work we test the hypothesis that the three major oxysterols in the circulation originate from functionally distinct pools of cholesterol. In particular, we wanted to clarify to what extent circulating 27-hydroxycholesterol originates from extrahepatic sources, and to what extent circulating (24*S*)-hydroxycholesterol originates from the brain. This investigation was performed by administering deuterium-labeled cholesterol to a human, and monitoring the rate of incorporation of label in circulating cholesterol and oxysterols. To draw conclusions from the incorporation of label in such an experiment, the magnitude of the half-life of the oxysterols must be known. This was estimated by exposing a human volunteer to $^{18}O_2$-enriched air, and measuring the incorporation of ^{18}O in oxysterols formed during the time of exposure.

MATERIALS AND METHODS

Materials

All organic solvents used were of gas chromatography or high performance liquid chromatography grade. [26,26,26,27,27,-27-$^2H_6$]cholesterol with an isotopic purity of about 95% and a chemical purity of more than 99% was obtained from Medical Isotopes (Concord, NH). [^{14}C]cholesterol was obtained from Radiochemical Centre (Amersham, England). Lipids (for liposomes) were from Avanti Polar Lipids (Birmingham, AL). $^{18}O_2$ was from Larodan Fine Chemical (Malmö, Sweden) and was mixed with $^{16}O_2$ prior to use (25).

Exposure to $^{18}O_2$-enriched air

Incorporation of $^{18}O_2$ into oxysterols was performed essentially as described (25). Briefly, a healthy male volunteer was exposed for 30 min to room air enriched with a mixture of $^{16}O_2$ and $^{18}O_2$ (in a ratio of about 3:1), in a closed breathing circuit. Blood was sampled from the antecubital vein before the experiment and up to 60 min after the completion of the exposure (**Fig. 2**). Plasma was separated and stored at −20°C until required for analysis. Inhalation of the mixture did not produce any adverse affects in the subject.

Preparation of [26,26,26,27,27,27-$^2H_6$]-cholesterol-enriched liposomes

Cholesterol-rich liposomes were prepared essentially as described previously (26).

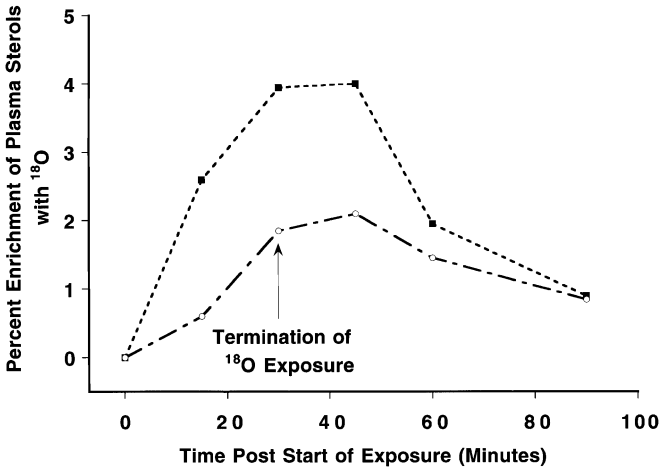

Fig. 2. Percent enrichment of 7α-hydroxycholesterol (solid squares) and 27-hydroxycholesterol (open circles) with ^{18}O in a subject during and after exposure to ambient air selectively enriched with 24% $^{18}O_2$ (for experimental details see Materials and Methods).

L-α-Phosphatidylcholine, 1,2-dioleyl-*sn*-glycero-3-phosphate, and [$^2H_6$]cholesterol at a molar ratio of 9.45:1:9.40 were dissolved in chloroform. The final concentration of cholesterol was 7 g/l. The solvent was evaporated, first under vacuum and then under a gentle stream of nitrogen, to remove any residual solvent.

Aliquots of the mixture corresponding to 1.75 g of [$^2H_6$]cholesterol were then hydrated with 250 ml of physiological saline. Multilamellar vesicles were formed by vortexing the lipid-aqueous mixture. The crude suspension was transferred to an extruder (LipsoFast 50; Avestin, Ottawa, Canada) and extruded twice under nitrogen through two stacked polycarbonate filters of 1-μm pore size. Liposome fractions of 250 ml were collected in 500-ml infusion bottles, which were sealed under sterile conditions.

[$^2H_6$]cholesterol concentrations were determined before and after filtration to determine the incorporation efficiency. The total phospholipid content was 16 mg/ml.

Sealed flasks containing 250 ml of [$^2H_6$]cholesterol-enriched liposomes were sterilized by autoclaving at 121°C for 20 min. Approximately 5 million cpm of [^{14}C]cholesterol was distributed between the prepared liposomes.

All liposome preparations were carried out using aseptic technique.

Infusion of [26,26,26,27,27,27-$^2H_6$]-cholesterol-enriched liposomes

Ten grams of [$^2H_6$]cholesterol-enriched liposomes was infused into a single healthy normolipidemic male volunteer, aged 58 years, weighing 84 kg with a body mass index of 25. Aliquots of the liposomes (250 ml) were infused at a rate of 1 ml/min during six separate periods, during a total period of 55 h. Infusion of the mixture did not produce any adverse affects, and there was no effect on plasma levels of transaminases or alkaline phosphatase during the infusions.

Fasting blood samples were taken preinfusion, daily for 9 days after the first infusion, and then every second day from day 11 to day 31. A single cerebrospinal fluid sample was taken on day 3.

Cholestyramine treatment (8 g three times daily) was initiated on day 21 of the experiment and was continued to day 31.

Precipitation of low density lipoprotein; incorporation of [$^2H_6$]cholesterol into high density lipoprotein

Five hundred microliters of low density lipoprotein (LDL) and very low density lipoprotein (VLDL) precipitant (Boehringer Mannheim, Indianapolis, IN) was added to 200 μl of plasma, and mixed gently. The mixtures were allowed to stand for 10 min at room temperature before centrifugation at 4,000 rpm for 10 min. The clear supernatant was removed and analyzed by gas chromatography-mass spectrometry (GC-MS) as described below.

Measurement of the isotope effect in the 27-hydroxylation of [26,26,26,27,27,27-$^2H_6$]cholesterol by CYP27

Purified human sterol 27-hydroxylase was incubated at 37°C in the presence of adrenoxin, adrenoxin reductase, NADPH, and an eqimolar mixture of [26,26,26,27,27,27$^2H_6$]cholesterol and unlabeled cholesterol. The conditions of the reaction were as previously described (27, 28).

The reaction mixture was extracted with 10 ml of chloroform–methanol 1:1 (v:v) and analyzed by GC-MS as described below.

Analysis by GC-MS

The content of deuterium in plasma and cerebrospinal cholesterol and oxysterols was measured by the same general method as that previously described for determination of absolute levels of these compounds with isotope dilution-mass spectrometry (28), with the following modification. No internal standard was added to the samples because of the fact that only the relative proportions of each isotopomer were of interest. The *m/z* values of the ions monitored were as follows: cholesterol, 458; [$^2H_6$]cholesterol, 464; 7α-hydroxycholesterol, 456; 7α-[$^2H_6$]-hydroxycholesterol, 462; (24*S*)-hydroxycholesterol, 456; (24*S*)-[$^2H_6$]hydroxycholesterol, 462; 27-hydroxycholesterol, 456; and 27-[$^2H_5$]hydroxycholesterol, 461. The sensitivity of the method for detection of $^2H_6$ enrichment was estimated to be about 0.2% for cholesterol and about 0.5% for the different oxysterols.

After precipitation of LDL cholesterol the content of ^2H in high density lipoprotein (HDL) cholesterol was measured as described above. The approximate content of ^2H in LDL cholesterol was estimated from this and the content of ^2H in total cholesterol together with the information about relative concentration of HDL and LDL cholesterol (see below). The small amounts of cholesterol present in VLDL cholesterol were neglected in these calculations.

The content of ^{18}O in the different oxysterols formed during the $^{18}O_2$ exposure was determined by combined GC-MS as described previously (29). The sensitivity of the method for detection of a content of ^{18}O in the different oxysterols was estimated to be about 0.5% under the conditions used.

Measurement of total and HDL cholesterol

Total cholesterol was measured by an enzymatic spectrophotometric method based on cholesterol oxidase from Boehringer Mannheim. HDL cholesterol was measured by the method described by Sugiuchi et al. (30) with use of polyethylene glycol-modified enzymes and sulfated α-cyclodextrin.

Ethical aspects

The two human experiments were approved by the local research ethics committees of Sahlgrenska University Hospital (Gothenburg, Sweden) and Huddinge University Hospital (Huddinge, Sweden) ($^{18}O_2$ experiment and [$^2H_6$]cholesterol experiment, respectively). Informed consent of each participant was obtained before each experiment.

RESULTS

Enrichment of circulating oxysterols with ^{18}O after exposure of a human subject to $^{18}O_2$

Figure 2 shows the result of an experiment in which a human subject inhaled a mixture containing 47% oxygen

and 53% nitrogen enriched with 24% ^{18}O for 30 min. After 15 min of exposure a highly significant enrichment of plasma 7α-hydroxycholesterol and plasma 27-hydroxycholesterol ^{18}O was observed. The maximal enrichment of ^{18}O in each steroid occurred 15 min after termination of the exposure to the $^{18}O_2$-enriched air. During the next 45 min there was a rapid reduction in the content of ^{18}O in the two steroids.

From the rate of this reduction it can be concluded that the half-life of both steroids in the circulation must be less than 1 h (less than 0.5 h for 7α-hydroxycholesterol and less than 0.75 h for 27-hydroxycholesterol). In contrast, no significant enrichment of either plasma cholesterol or (24*S*)-hydroxycholesterol was observed. Under the experimental conditions used the enrichment of each of these sterols was estimated to be not more than than 0.2%, which is consistent with circulating half-lives considerably longer than that of plasma 7α- or 27-hydroxycholesterol.

An additional experiment was performed, with the same design as described above, in another subject but with lower exposure to $^{18}O_2$. The percent enrichments of 7α- and 27-hydroxycholesterol with ^{18}O were considerably lower in this experiment, but the rates of disappearence of the ^{18}O label after the exposure were similar to the above-described experiment.

Enrichment of circulating cholesterol and oxysterols with ^2H after intravenous infusion of [$^2H_6$]cholesterol in a human subject

Figure 3 shows the results of an experiment in which 10 g of [$^2H_6$]cholesterol was infused in a human subject over a period of 55 h. The infusion caused a transient increase in total cholesterol concentration of 15–20% and a transient decrease in serum lathosterol of about 50% during the first week of the experiment (results not shown). To accelerate metabolism and elimination of the labeled cholesterol cholestyramine treatment was initiated on day 24 of the experiment. This treatment elicited a reduction in

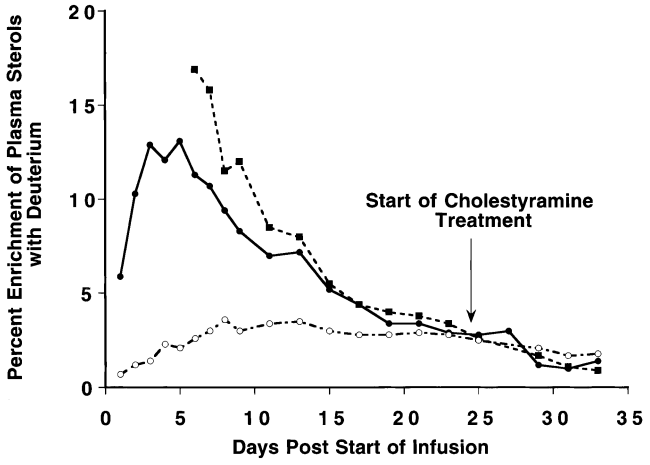

Fig. 3. Percent enrichment of circulating cholesterol (solid circles), 7α-hydroxycholesterol (solid squares), and 27-hydroxycholesterol (open circles) with ^2H during and after infusion of 10 g of [$^2H_6$]cholesterol in a healthy volunteer (for experimental details see Materials and Methods).

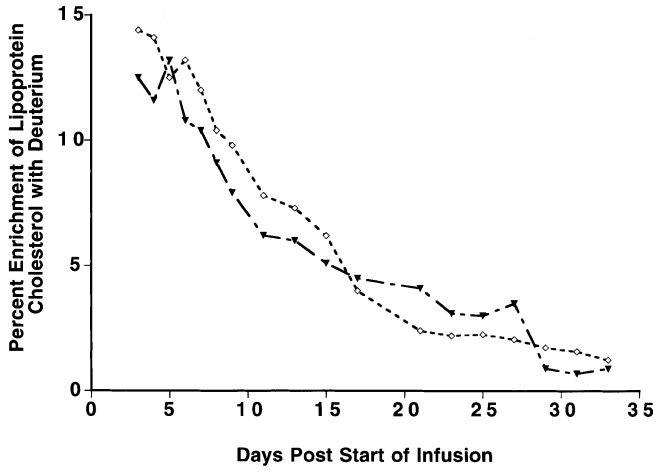

Fig. 4. Percent enrichment of circulating HDL cholesterol (open diamonds) and LDL cholesterol (solid inverted triangles) with ^2H after infusion of [$^2H_6$]labeled cholesterol in a healthy volunteer (for experimental details see Materials and Methods).

LDL cholesterol of about 20%, an about 2-fold increase in serum lathosterol, and an about 4-fold increase in serum 7α-hydroxycholesterol (results not shown).

The maximal enrichment of plasma cholesterol with ^2H, 13%, occurred 4 days after the start of the infusion. After this period of time, there was a slow decrease in content of ^2H with a half-life of about 13 days. The enrichment of LDL and HDL cholesterol was similar most of the time, reflecting a rapid exchange of cholesterol between these lipoproteins (**Fig. 4**). However, during the first 2 weeks after the start of the experiment the enrichment was somewhat higher in HDL than in LDL, whereas during the next 10 days the reverse was observed. After the initiation of cholestyramine treatment the ^2H enrichment became higher in HDL than in LDL.

A small amount of [^{14}C]cholesterol was infused in tandem with the [^2H]cholesterol. The ^{14}C label in plasma decreased in parallel with the decrease in ^2H content in plasma cholesterol (results not shown).

At the time of the maximum enrichment of plasma cholesterol, a sample of cerebrospinal fluid was collected and analyzed with respect to incorporation of ^2H in cholesterol. The content of ^2H was found to be less than 0.5%.

During the sterilization of the infusion mixture some contamination with 7α-[$^2H_6$]hydroxycholesterol occurred, corresponding to less than 0.1% of the infused cholesterol. This may explain why the ^2H enrichment of circulating 7α-hydroxycholesterol appeared to be higher than that of cholesterol during the first week of the experiment. However, after this period of time the ^2H enrichment of 7α-hydroxycholesterol closely followed that of plasma cholesterol. This was also the case after initiation of cholestyramine treatment (Fig. 3).

The enrichment of plasma 27-hydroxycholesterol with ^2H was considerably lower than that of cholesterol and 7α-hydroxycholesterol during the first 3 weeks of the experiment (Fig. 3 and **Fig. 5**). Plasma 27-hydroxycholesterol ^2H content increased slowly during the first 10 days and re-

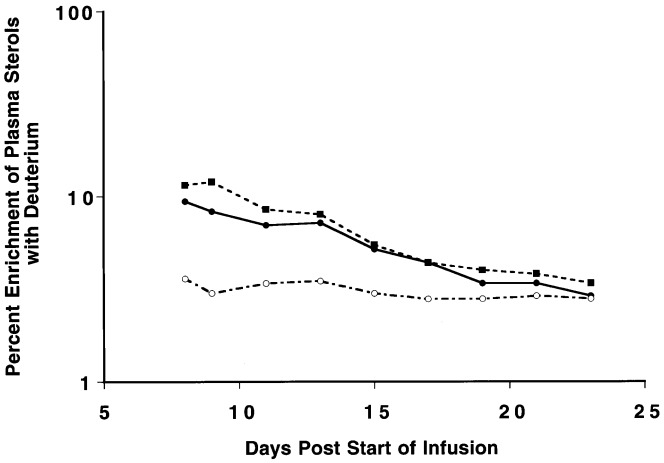

Fig. 5. Percent enrichment of circulating cholesterol (solid circles), 7α-hydroxycholesterol (solid squares), and 27-hydroxycholesterol (open circles) (log scale) with ^2H during days 8–23 after infusion of [$^2H_6$]cholesterol in a healthy volunteer (for experimental details see Materials and Methods).

mained about constant during the following 2 weeks. Subsequently there was a slow decrease in ^2H enrichment.

After day 25 of the experiment, the ^2H enrichment of 27-hydroxycholesterol was higher than that of cholesterol and 7α-hydroxycholesterol, and on day 31 it was almost double that of plasma cholesterol and plasma 7α-hydroxycholesterol (Fig. 3).

No significant enrichment of (24*S*)-hydroxycholesterol with ^2H could be detected at any stage of the experiment. Under the specific conditions used an enrichment of 0.5% or more would have been detected.

Demonstration of the lack of a significant isotope effect in the 27-hydroxylation of [$^2H_6$]cholesterol

Significant isotope effects associated with cytochrome P450-mediated hydroxylations of deuterium-labeled steroids have previously been described (31). The existence of such an isotope effect would thus influence the CPY27-mediated hydroxyation of cholesterol, and so reduce the ^2H enrichment of circulating 27-hydroxycholesterol. Such an isotope effect has in fact been demonstrated in the 27-hydroxylation of [$^2H_3$]cholesterol by mouse liver mitochondrial sterol 27-hydroxylase (32). In the current study, incubation of a mixture of authentic and deuterated cholesterol with CYP27 yielded an almost identical mixture of unlabeled and $^2H_5$-labeled 27-hydroxycholesterol products. It is unlikely that there is a significant kinetic isotope effect present in this system.

DISCUSSION

Use of $^{18}O_2$- and ^2H-labeled cholesterol as tools for studies of cholesterol metabolism in humans

Previously we have used $^{18}O_2$ to study formation of bile acids and oxysterols from cholesterol (29, 33, 34) as well as synthesis of NO (35) in experimental animals. This technique relies on isotopic labeling facilitated by the introduction of one atom of oxygen derived from molecular oxygen into the product in connection with cytochrome P450-catalyzed reactions.

The $^{18}O_2$ technique was used to study the in vivo synthesis and oxidation of NO (25). An identical approach was used to study the formation of oxysterols in a healthy volunteer. While longer exposure time and a higher excess of $^{18}O_2$ would have been preferred, the conditions used were sufficient to obtain a significant incorporation of isotope in two of the three major oxysterols in the circulation and to allow an estimation of the half-life of these steroids.

To our knowledge [^2H]cholesterol has not been used previously in studies of cholesterol homeostasis or metabolism in humans. This is probably a consequence of both the high cost and the difficulty of introducing the isotope-labeled cholesterol in amounts sufficiently high to allow accurate measurements of isotope ratios. Because an oral load would lead to high losses of the expensive isotope, we administered the labeled cholesterol intravenously, using a preparation of unilamellar phospholipid vesicles (26). Similar preparations have been used to intravenously administer high doses of lipophilic drugs (26). A problem with this approach was the necessary sterilization of the material, leading to the presence of auto-oxidatively formed 7-oxygenated oxysterols in the infusion mixture.

To be able to measure the decay of ^2H enrichment of the different oxysterols over long periods and with sufficient accuracy, a relatively large dose of labeled cholesterol was administered, corresponding to about 10% of the total body pool. As expected the infusion led to a significant but transient increase in total circulating cholesterol. With the possible exception of the first week of the experiment, the transient expansion of the plasma pool of cholesterol should not significantly affect formation of the oxysterols.

It should be noted that the small difference in physical properties between unlabeled and $^2H_6$-labeled cholesterol is unlikely to cause any physiological or adverse effects under the conditions used. The possibility of a primary kinetic isotope effect in the 27-hydroxylation of [$^2H_6$]cholesterol by a purified preparation of human sterol 27-hydroxylase was investigated. Under the in vitro system used, the presence of a significant isotope effect was not demonstrated.

On the basis of analysis of die-away curves, the body pool of cholesterol has been divided into three functionally distinct pools (20). These include a rapidly miscible pool, a more slowly exchangeable pool, and a pool of cholesterol that is either slowly miscible or nonexchangeable.

In the baboon, the size of the three pools is about 20%, 40%, and 35%, respectively (20). The first pool includes cholesterol in the liver, lung, small intestine, spleen, and blood. The second pool includes cholesterol in adipose tissues, skeletal muscle, skin, and a number of other tissues. The third pool includes cholesterol in the brain and in bone (20). It may take a period up to about 2 months before the second pool of cholesterol is in complete equilibrium with plasma cholesterol (36).

In the present experiment one would expect a phase

dominated by a rapid flux of liposomal or HDL-associated [$^2H_6$]cholesterol to the liver, followed by a transport of labeled cholesterol from the liver to different extrahepatic tissues. The labeling of the different extrahepatic pools would be expected to take different times and the essentially nonexchangeable pool would not be labeled at all or labeled to a small extent. In the latter phase of the experiment, in particular after initiation of cholestyramine treatment, one would expect a reverse transport of labeled cholesterol from the extrahepatic tissues to the liver.

Theoretically, HDL is likely to be the most important transporting lipoprotein in the first and third phases and LDL in the second phase. A rapid exchange of cholesterol between different lipoproteins is, however, known to occur, and such exchange would be expected to counteract differences in label between the two different lipoproteins. In accordance with these expectations, the content of ^2H was found to be somewhat higher in HDL than in LDL during the first 2 weeks and during the last 10 days of the experiment. During the period in between, the content of ^2H was higher in LDL than in HDL (Fig. 4).

The decay curve of the [^2H]cholesterol obtained in the present experiment followed a pattern similar to that previously described after administration of [^{14}C]cholesterol to humans (36). It has been shown that over periods as long as 50 days, cholesterol disappears from the circulation at a series of exponential rates rather than at a single rate. Thus no true body turnover rate can be expressed from the plasma disappearance curves during this period. After this period of time, labeled cholesterol declines at an exponential rate with a half-life ranging from 58 to 75 days (36). In the present work this latter stage of the disappearance of the labeled cholesterol was not followed.

The brain is the most significant pool of cholesterol in the body that is not exchangeable with plasma cholesterol (20). As one would expect, little ^2H enrichment of cerebrospinal fluid cholesterol was observed after infusion with [$^2H_6$]cholesterol (about 3% of that in plasma cholesterol). In this connection it is of interest that administration of [^{14}C]cholesterol to terminally ill patients resulted in incorporation of ^{14}C label in the brain that was less than 3% in almost all patients (24). However, it cannot be excluded that a small part of the cholesterol in the cerebrospinal fluid is derived from a pool of cholesterol that is distinct from that in the brain. The (24*S*)-hydroxycholesterol in the cerebrospinal fluid, believed to be derived from brain cholesterol only (see below), contained no detectable amounts of ^2H.

Formation of 7α-hydroxycholesterol

Cholesterol 7α-hydroxylase is located exclusively in the liver (10). In this connection it may be mentioned that we have been unable to demonstrate a difference in concentration of 7α-hydroxycholesterol in the peripheral arteries and in the hepatic veins of healthy volunteers (I. Björkhem, unpublished observation). This finding is consistent with a flux of 7α-hydroxycholesterol from the liver that is similar to the uptake of the same steroid in the same organ, indicating that there is little or no extrahepatic formation of 7α-hydroxycholesterol. A cytochrome P450-mediated conversion of [$^2H_6$]cholesterol into 7α-hydroxycholesterol would result in a product identical to that of a nonenzymatic reaction, and it is not possible to discriminate between these different types of reactions and the results obtained.

As estimated by the ^{18}O enrichment, the half-life of plasma 7α-hydroxycholesterol is less than 1 h. Thus it can be concluded that the turnover of this steroid is not a critical determinant of the enrichment of 7α-hydroxycholesterol with deuterium.

During the 55-h infusion period, the content of ^2H in circulating 7α-hydroxycholesterol appeared to be markedly higher than that in circulating cholesterol (results not shown). This is most likely due to contamination with artifactually formed 7α-[$^2H_6$]hydroxycholesterol and the relative dilution of infused [$^2H_6$]cholesterol and 7α-hydroxycholesterol by their endogenous counterparts.

One would expect the immediate postinfusion period to be characterized by a higher enrichment in liver cholesterol than plasma cholesterol because of the dilution of the latter with extrahepatic cholesterol. Thus compounds formed from liver cholesterol would be expected to be enriched to a slightly higher degree than plasma cholesterol. This is indeed the case in the postinfusion period, during which a slightly higher content of ^2H in circulating 7α-hydroxycholesterol than in plasma cholesterol is observed in most of the samples analyzed. As 7α-hydroxylation is the first step in bile acid biosynthesis, it is apparent that part of the cholesterol utilized for bile acid biosynthesis in the liver is not in complete equilibrium with plasma cholesterol. In experimental animals with upregulated bile acid biosynthesis, newly synthesized cholesterol seems to be preferred for bile acid biosynthesis (34). From this point of view, it is interesting that the difference in label between 7α-hydroxycholesterol and cholesterol in plasma decreased to about zero in the samples collected after initiation of the cholestyramine treatment, a treatment known to upregulate cholesterol 7α-hydroxylase.

Formation of 27-hydroxycholesterol

The short half-life of plasma 27-hydroxycholesterol observed in the $^{18}O_2$ experiment shows that the rate of turnover of this oxysterol cannot be a critical factor in the ^2H enrichment of 27-hydroxycholesterol.

In a previous work we showed that there is a net flux of 27-hydroxycholesterol and its metabolites from extrahepatic organs to the human liver (14). The total flux of 27-oxygenated steroids to the liver was estimated to be about 25 mg/day in healthy volunteers (14). Because there is a significant pathway from cholesterol to bile acids in the liver, which includes 27-hydroxycholesterol as an intermediate (37), it is reasonable to assume that part of the 27-hydroxycholesterol in the circulation may originate from the liver. The slow increase in deuterium enrichment of 27-hydroxycholesterol during the first week after the infusion, as well as the finding that enrichment of 27-hydroxycholesterol was higher than that of cholesterol during the late

phase of the experiment, is consistent with an extrahepatic origin of most of this oxysterol in the circulation.

The relatively high enrichment of plasma 27-hydroxycholesterol with ^{2}H during the latter phase of the experiment may thus reflect the higher content of ^{2}H in peripheral cholesterol than in plasma cholesterol. As estimated from the content of ^{2}H in plasma 27-hydroxycholesterol and cholesterol during the first phase of the experiment, less than 20% of the circulating 27-hydroxycholesterol is likely to originate from the liver or from other organs containing cholesterol pools equilibrated with plasma cholesterol.

Formation of (24*S*)-hydroxycholesterol

Theoretically, the absence of significant ^{2}H enrichment in plasma (24*S*)-hydroxycholesterol may have three different explanations. First, all circulating (24*S*)-hydroxycholesterol is formed from newly synthesized cholesterol and is unexchangeable with preformed cholesterol. Second, the turnover of (24*S*)-hydroxycholesterol may be too slow to allow an exchange with the labeled cholesterol. Third, (24*S*)-hydroxycholesterol originates from a pool of cholesterol that is not exchangeable with plasma cholesterol. The first explanation seems highly unlikely, and has been completely excluded in experimental animals exposed to $^{18}O_2$ (29). The second explanation is also unlikely, as we have shown that racemic 24-hydroxycholesterol has a half-life of between 10 and 14 h in human volunteers (15), which is short in relation to the duration of the present study. The results of the current $^{18}O_2$ experiment are consistent with a half-life of the (24*S*)-isomer of 24-hydroxycholesterol that is considerably longer than that of 7α- or 27-hydroxycholesterol. The third explanation is the most likely, that (24*S*)-hydroxycholesterol is formed from a pool of cholesterol that is essentially unexchangeable with plasma cholesterol. Such pools are present in brain and bone, but may also exist in skin, skeletal muscle, and adipose tissues (20). Of these organs and tissues only the brain contains cholesterol (24*S*)-hydroxylase (16, 18). The results are thus consistent with a cerebral origin of at least 95% of circulating (24*S*)-hydroxycholesterol.

This situation is clearly different from that occurring in experimental animals. In studies in which mice were fed a diet supplemented with [$^{2}H_6$]cholesterol we found an incorporation of ^{2}H in plasma (24*S*)-hydroxycholesterol that was about half that of cholesterol (38). This is consistent with an extracerebral origin of about half the circulating (24*S*)-hydroxycholesterol in mice. Part of the extracerebral formation of (24*S*)-hydroxycholesterol in the mouse may be due to a different enzyme than brain cholesterol 24-hydroxylase (CYP46), as disruption of the gene coding for the this enzyme in mice did not result in complete disappearance of (24*S*)-hydroxycholesterol from the circulation (18).

The major oxysterols in human circulation are derived from different pools of cholesterol

It is evident from the present results that 7α-hydroxycholesterol is derived from the rapidly miscible pool of cholesterol (most probably the liver), 27-hydroxycholesterol from the more slowly exchangeable pool, and (24*S*)-hydroxycholesterol from the pool that is essentially unexchangable (**Fig. 6**). Both of these side chain-oxidized oxysterols are continuously fluxing from extrahepatic sources to the liver, and this flux is likely to be of importance for the total body cholesterol homeostasis. We have shown that the hepatic uptake of 27-oxygenated steroids is about 25 mg per 24 h in healthy volunteers, whereas the corresponding uptake of (24*S*)-hydroxycholesterol is about 7 mg per 24 h. If it is assumed that the side chain-oxidized oxysterols are converted into bile acids in the liver, about 6% of the total formation of bile acids occurs from extrahepatically formed oxysterols.

The extrahepatic origin of most circulating 27-hydroxycholesterol is of importance in relation to the hypothesis that its flux to the liver reflects an antiatherogenic mechanism that is of importance for development of atherosclerosis. It is well documented that a lack of sterol 27-hydroxylase may lead to premature atherosclerosis in spite of normal circulating levels of cholesterol (39). Provided that the hepatic clearance of 27-hydroxycholesterol is similar in different subjects, it is evident from the results of the present study that plasma levels of 27-hydroxycholesterol are likely to reflect the capacity of extrahepatic sterol 27-hydroxylase to eliminate cholesterol.

The most important result of the present investigation is the unique cerebral origin of (24*S*)-hydroxycholesterol in the circulation in humans. The findings strongly support the contention that circulating levels of (24*S*)-hydroxycholesterol can be used to evaluate cholesterol homeostasis in the human brain. However, the capacity of the liver to metabolize (24*S*)-hydroxycholesterol is also an important determinant of the plasma levels of this oxysterol, and we have shown that age-dependent variations in the concentration of (24*S*)-hydroxycholesterol are related to the size of the liver (40).

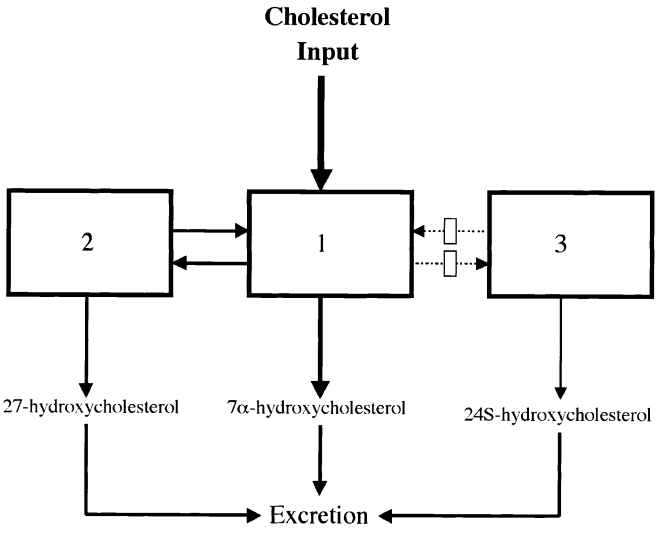

Fig. 6. Formation of oxysterols from the three different pools of cholesterol in the body: a rapid miscible pool (pool 1), a slowly exchangeable pool (pool 2), and a pool that is hardly exchangeable at all (pool 3).

As the expression of cholesterol (24*S*)-hydroxylase is mainly localized to neuronal cells, and (24*S*)-hydroxycholesterol is mainly located in myelin, an expected consequence of active neurodegeneration would be a transient increase in the flux of (24*S*)-hydroxycholesterol across the blood–brain barrier. In support of this hypothesis we have observed slightly but significantly increased levels of circulating (24*S*)-hydroxycholesterol in a specific population of Alzheimer patients (19). In advanced neurodegeneration the decrease in neuronal mass could result in a reduced flux of (24*S*)-hydroxycholesterol, and we have observed a negative correlation between mental capacity and circulating levels of (24*S*)-hydroxycholesterol in these patients. Further studies on the possibility of using (24*S*)-hydroxycholesterol as a biochemical marker in neurodegenerative diseases are now in progress.

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