Importance of a Novel Oxidative Mechanism for Elimination of Brain Cholesterol

TURNOVER OF CHOLESTEROL AND 24(S)-HYDROXYCHOLESTEROL IN RAT BRAIN AS MEASURED WITH 18O2 TECHNIQUES IN VIVO AND IN VITRO*

(Received for publication, July 14, 1997, and in revised form, September 17, 1997)

Inge Dar Jónsson*§, Dieter Lütjohann†, Olof Breuer‡, Augustinas Sakins‡, and Åke Wennmalm¶

From the *Division of Clinical Chemistry, Karolinska Institutet, Huddinge Hospital, SE-141 86 Huddinge, Sweden and the ‡Division of Clinical Physiology, Sahlgrenska University Hospital, Göteborg University, Göteborg, Sweden

The brain is the most cholesterol-rich organ in the body. Brain cholesterol is characterized by a very low turnover with very little exchange with lipoproteins in the circulation. Very recently we showed that there is a continuous age-dependent flux of 24(S)-hydroxycholesterol from the human brain into the circulation (Lütjohann, D., Breuer, O., Ahlborg, G., Nennesmo, I., Sidén, Å., Diczfalussy, U., and Björkhem, I. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 9799–9804). Here we measured the rate of synthesis of cholesterol as well as the conversion of cholesterol into 24(S)-hydroxycholesterol in rat brain in vivo with use of an 18O2 inhalation technique and mass isomeromer distribution analysis. Cholesterol synthesis was found to correspond to 0.03 ± 0.01% of the pool per h. Conversion of cholesterol into 24(S)-hydroxycholesterol was of a similar magnitude, about 0.02% of the pool per h. Brain microsomes converted endogenous cholesterol into 24(S)-hydroxycholesterol at a similar rate when incubated in the presence of NADPH. When incubated with whole homogenate and subcellular fractions of rat brain, there was no significant conversion of tritium-labeled 24-hydroxycholesterol into more polar products. Plasma from 18O2-exposed rats contained 24(S)-hydroxycholesterol with an enrichment of 18O similar to that in 24(S)-hydroxycholesterol in the brain.

The results suggest that the present 24(S)-hydroxylation mediated mechanism is most important for elimination of cholesterol from the brain of rats. There is a slow conversion of brain cholesterol into 24(S)-hydroxycholesterol with a rapid turnover of the small pool of the latter oxysterol due to leakage to the circulation (half-life of brain 24(S)-hydroxycholesterol is about 0.5 days as compared with 2–4 months for brain cholesterol). It is evident that the 24(S)-hydroxylation greatly facilitates transfer of cholesterol over the blood-brain barrier and that this hydroxylation may be critical for cholesterol homeostasis in the brain.

The largest pool and concentration of cholesterol in the body is found in the brain. Being a constituent of myelin and cell membranes cholesterol is important for the function of this organ and an inborn defect in cholesterol synthesis is associ-

* This work was supported by a grant from the Swedish Medical Research Council and the Osterman Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed: Div. of Clinical Chemistry, Huddinge University Hospital, SE-141 86 Huddinge, Sweden.
cholesterol 24-hydroxylase in rat brain microsomes under enzymological conditions with use of the above 18O2 technique.

In theory, the primary product of the cholesterol 24-hydroxylase in the brain might be eliminated from the brain as such or as a metabolite. To evaluate the latter possibility we have prepared tritium labeled 24-hydroxycholesterol and incubated it with preparations of rat brain under different conditions.

**EXPERIMENTAL PROCEDURES**

**Materials**—Reagents and solvents used were of analytical or high performance liquid chromatography grade. Unlabeled and tritium labeled cholesterol were those used in previous work from this laboratory (8, 9). [4-14C]Cholesterol with a specific radioactivity of 55–60 mCi/mmol was obtained from The Radiochemical Center (Amersham, United Kingdom) and was purified by aluminum oxide chromatography before use.

18O2 (95% isotopic and 98% chemical purity) was obtained from Cambridge Isotope Laboratories, Andover, MA, in break-seal ampoules of atmospheric pressure. 3a-[3H]Labeled 24-hydroxycholesterol was prepared from unlabeled cholesterol as described previously for preparation of other tritium labeled 3β-hydroxy 5-un saturated steroids (12). The material obtained was purified by preparative thin-layer chromatography, using toluene/ethyl acetate as moving phase. The material obtained was purified by preparative thin-layer chromatography, using toluene/ethyl acetate as moving phase. The material obtained was purified by preparative thin-layer chromatography, using toluene/ethyl acetate as moving phase.

**Animals**—Male rats of an outbred Sprague-Dawley strain weighing 200–250 g were used. In some in vitro experiments a rat weighing 750 g and several rats weighing 50 g were used. The animals were given free access to a standard chow and water. The experiments were approved by the animal ethics committee at the Karolinska Institute, Huddinge Hospital.

**Preparation of Subcellular Fractions and Acetone Powder**—The rats were decapitated and their brains (including cerebrum, cerebellum, midbrain, and medulla) were removed. After removal of the meninges and rinsing several times with 0.9% saline, the brains were homogenized in 2.5 volumes of 0.15 M potassium phosphate buffer, pH 7.4, containing 10 mM EDTA with a loosely fitting Teflon pestle in a glass homogenizing tube. The microsomal fraction was prepared by centrifugation at 800 × g, 20,000 × g, and 100,000 × g. The microsomal pellet obtained after the last centrifugation was resuspended in the homogenizing buffer. In some experiments a crude mitochondrial fraction was obtained by recentrifugation of the above 20,000 × g infranatant at 6,500 × g for 20 min and resuspending the pellet in the homogenizing buffer. Acetone powder of some brain microsomal preparations was prepared as described by Shefer et al. (13).

The microsomal fraction obtained as above was found to contain 0.21 ± 0.04 mg of cholesterol/ml and 0.63 ± 0.04 mg of 24(S)-hydroxycholesterol/ml. The mitochondrial fraction contained about 0.50 mg of cholesterol/ml and 1.2 μg of 24(S)-hydroxycholesterol/ml. The acetone powder contained about 25 μg of cholesterol/ml, which was equivalent to 1 ml of the above microsomal fraction.

**Incubation Conditions**—Under standard conditions, 1.5–3 ml of microsomal or mitochondrial fractions were incubated in a total volume of 3 ml of the homogenizing buffer for 2 h at 37 °C together with 3 μl of NADPH. In some incubations with mitochondrial fractions, isocitrate, 7 mM, was also added to the incubation mixture. In some specific experiments, 4-[14C]cholesterol was added to the incubation mixture, 1.5 × 106 cpm, dissolved in 20 μl of aceton, 1 mg of Tween 80, or 4.5 μmol of cycloexodrin.

In some specific experiments, 50 μg of [3H]labeled 24-hydroxycholesterol dissolved in 20 μl of aceton was incubated with brain microsomes, mitochondria, or whole homogenate under the same conditions as above. All incubations were terminated and extracted with chloroform/methanol, 2:1 (v/v).

In several experiments the incubations were performed in a closed system containing about 20% oxygen in nitrogen. The oxygen contained about 70% 18O2 (varying between 60 and 80% in different experiments). The closed tube containing the incubation mixture was first equilibrated with nitrogen under atmospheric pressure. Two syringes were connected to the closed system, one of which contained the appropriate 18O2-containing gas mixture. The 18O2 was then injected into the closed system containing about 20% oxygen in nitrogen. The oxygen contained about 70% 18O2 (varying between 60 and 80% in different experiments).

After incubation by another syringe and analyzed for content of 14O by mass spectrometry (9). The enrichment of 18O was found to be 69 ± 11% under the conditions employed.

**In Vivo Experiments with Rats**—The incubation experiments were performed essentially as described previously (9) but with a modification allowing the rats to be exposed to a relatively constant concentration of 18O2. A pump and filter device was used that continuously freed the 18O-containing gas from carbon dioxide and water vapor. The volume of the system was allowed to decrease in parallel with the consumption of oxygen and elimination of CO2 and water by the filters. Under these conditions it was possible to keep a relatively constant ratio between 16O and 18O during the experiment despite a continuous drop in the O2/N2 ratio. The absolute concentration of oxygen (18O2 + 16O2) thus varied in these experiments between 45 and 16%. In most of these experiments the relative content of 18O2 varied between 60 and 65%.

**Isolation of 24(S)-Hydroxysterol and Cholesterol and Analysis of 18O Enrichment by Combined Gas Chromatography-Mass Spectrometry**—After incubation of the organic solvent, the material in the chlo roform phase obtained in the extraction of the above incubations was subjected to alkaline hydrolysis and solid phase extraction as described previously (14). The oxysterol fractions were converted into trimethyl silyl ether derivatives and analyzed by combined gas chromatography-mass spectrometry using an HP 5970 quadrupole type mass spectrometer (Hewlett-Packard, Palo Alto, CA). Helium was used as carrier gas with a column pressure of 75 kloPascal. Samples (2 μl) dissolved in hexane were injected in a splitless mode. Electron impact ionization at 70 eV was applied. The column temperature was kept at 180 °C for 1 min, subsequently raised at a rate of 35 °C/min to 270 °C, and finally increased 20 °C/min to 310 °C. The mass spectrometer was operated in a selective ion monitoring mode using the molecular ions at m/z 546 and 548 for registration of trimethylsilyl ether of unlabeled and 18O-labeled 24(S)-hydroxysterol, respectively. In the in vivo experiments, also the ion at m/z 550 was followed to exclude the possibility of incorporation of two atoms of 18O in the product. No such incorporation was seen in 24(S)-hydroxysterol. In the analysis of 24(S)-hydroxysterol in serum, the ions at m/z 546 and 548 gave signals too low to allow for accurate analysis, and in these cases the ions at m/z 413 and 415 were used (M-90–43). In other experiments with 24(S)-hydroxysterol with an 18O label at C-24, it was ascertained that the same result was obtained regardless of whether the fragment at m/z 415 or the molecular ion was used for determination of the amount of 18O.

Also in the in vivo experiments in which incorporation of 18O in plasma and hydrosysterols and resuspended in the homogenizing buffer. Acetone powder of some brain microsomal preparations was prepared as described by Shefer et al. (13).

The microsomal fraction obtained as above was found to contain 0.20 ± 0.04 mg of cholesterol/ml and 0.63 ± 0.04 mg of 24(S)-hydroxycholesterol/ml. The mitochondrial fraction contained about 0.50 mg of cholesterol/ml and 1.2 μg of 24(S)-hydroxycholesterol/ml. The acetone powder contained about 25 μg of cholesterol/ml, which was equivalent to 1 ml of the above microsomal fraction.

The mixture used in the analysis of 24(S)-hydroxycholesterol isolated from whole brain or subcellular fractions from brain and about 2% in the case of 24(S)-hydroxysterol isolated from plasma. The unlabeled 24(S)-hydroxysterol used in the above subtraction was isolated from the appropriate control microsomes or control rat exposed to air.

In the determination of the very low enrichment of 18O in cholesterol in the brain of rats exposed to 18O2 in vivo, relatively high amounts of material (micrograms) were isolated and purified by C18 chromatography after alkaline hydrolysis. The material was analyzed as trimethylsilyl ether with the mass spectrometer focused on the M+ 1 ion at m/z 459 and on the M+ 2 ion at m/z 460. Each cholesterol extract was analyzed in quadruplicate. In separate recordings the ratio between the ions at m/z 548 and 415 (see Ref. 9).

In the analysis of 24(S)-hydroxysterol obtained after exposure of a subcellular fraction or a living rat to 18O2, the contribution from the natural isotopeomer cluster of unlabeled 24(S)-hydroxysterol was subtracted from the monitored intensity at m/z 548 or 415 (see Ref. 9). The sensitivity and precision in the analysis of enrichment of 18O in the above measurements are dependent upon the amount and purity of material analyzed and the amount of data collected from each chromatographic peak. The calculations were based on the computer-derived areas of the recordings of the intensities of the different ions. Under the conditions employed the “background noise” in the analysis was found to have an amplitude of approximately ± 0.3% in the case of 24(S)-hydroxysterol isolated from whole brain or subcellular fractions from brain and about ± 2% in the case of 24(S)-hydroxysterol isolated from plasma. The unlabeled 24(S)-hydroxysterol used in the above subtraction was isolated from the appropriate control microsomes or control rat exposed to air.
beled 24(S)-hydroxycholesterol, as well as cholesterol, was assayed by isotope-dilution mass spectrometry with use of deuterium-labeled standards as described previously (14, 15).

Assay of Conversion of 3H-Labeled 24-Hydroxycholesterol and [4-14C]Cholesterol in Vitro—The extract obtained after incubation of 3H-labeled 24-hydroxycholesterol was subjected to thin-layer chromatography using toluene/ethyl acetate, 1:4 (v/v), as solvent. Radioactivity in the different zones was assayed with a Berthold Tracemaster 20 TLC scanner. The extract obtained after incubation of [4-14C]cholesterol was assayed with the same method.

RESULTS

Synthesis of Brain Cholesterol in Vivo—Exposing three rats to oxygen enriched with 60–70% 18O2 for 12–40 h resulted in a significant incorporation of 18O into brain cholesterol (0.1–1.3%). Since the brain homogenates must have been contaminated by blood to some extent (possibly a few percent) the possibility must be considered that part of the above small incorporation of 18O could have been due to contamination by circulating cholesterol. The latter had an enrichment of 18O of 1–3%. In the experiment with the rat exposed to 18O2 for 40 h, the enrichment was found to be 1.3% in brain cholesterol and 3.1% in circulating cholesterol.

Since the total concentration of cholesterol in the brain is 1–1.5% (w/w) and the corresponding concentration in the blood is less than 10% of this in a rat, it is evident that a small contamination with blood cholesterol cannot have a significant effect on the results. A contamination by 2% blood would give a contribution of 18O in brain cholesterol by less than 0.01% under these specific conditions.

Due to the very low turnover of brain cholesterol (4, 5), the accumulation of newly synthesized cholesterol could be assumed to reflect cholesterol synthesis. When taken into account the enrichment of 18O in the inhalation atmosphere and the time of exposure in each experiment, the synthesis rate was calculated to be 0.02, 0.03, and 0.05, respectively, of the cholesterol pool per h in the three rats. This is consistent with a half-life of 2–4 months.

Synthesis and Turnover of 24(S)-Hydroxycholesterol in Vivo—In one previously published single experiment (8), we found that a Sprague-Dawley rat exposed to 18O2 in atmosphere (isotopic purity about 80%) for 3.5 h had incorporated about 11% 18O in 24(S)-hydroxycholesterol in its brain. This would correspond to a turnover of the brain 24(S)-hydroxycholesterol at a rate of at least 4% per h. This is a minimum figure since part of the labeled 24-hydroxycholesterol formed during the time of exposure might have been eliminated.

The above experiment was repeated with another Sprague-Dawley rat that was exposed for 3 h to an atmosphere containing oxygen enriched to about 80% with 18O2. In this case the incorporation of 18O in brain 24(S)-hydroxycholesterol was found to be about 9%, which also corresponds to a turnover of the brain 24-hydroxycholesterol at a rate of at least 4% per h.

In another set of experiments rats were exposed to 18O2 for varying periods of time up to 12 h. The enrichment of 18O in 24(S)-hydroxycholesterol in the brain of these rats was found to increase about linear with time (results not shown).

To calculate true synthesis and turnover of 24(S)-hydroxycholesterol in the brain, the disappearance rate of this steroid must be known. Fig. 1 shows an experiment allowing calculation of this. Two rats were exposed to an atmosphere enriched to about 60% with 18O2 for 12 h and one of the rats was then sacrificed. The other rat was exposed to normal air for additional 15 h prior to sacrifice. 24(S)-Hydroxycholesterol isolated from the brain of the first rat had an 18O content of 21% while that isolated from the brain of the second rat had an 18O content of 9%.

From the results of the experiment with the first rat the accumulation of newly synthesized 24(S)-hydroxycholesterol was calculated to be about 35% of the pool during the 12-h exposure to 18O2 (taking into account that the enrichment of 18O2 in the atmosphere was 60% rather than 100%). Considering that part of the newly synthesized 18O-labeled 24(S)-hydroxycholesterol is eliminated during this period of time, the rate of synthesis must exceed 3% of the pool per h. From the finding that the 18O content in brain 24(S)-hydroxycholesterol had decreased to less than half as a consequence of 15-h exposure to 18O2 in the second rat, it is evident that 24(S)-hydroxycholesterol disappears from the brain at a rate of about 4% of the pool per h (T1/2 = 11 h). Assuming steady-state conditions, the rate of synthesis of 24(S)-hydroxycholesterol would then correspond to about 4% of the pool per h. This would correspond to elimination of cholesterol by this mechanism in the second rat in Fig. 1 at a rate of about 0.015% per h.
plasma sample collected 15 h later from the same rat contained with an 18O content of 56 and 64%, respectively (Fig. 1). A by Dhar et al. oxysterols (see Ref. 9). As shown in Fig. 2, enrichment of 18O content less than 1%. It is evident that there is a much higher contained in 2.4 (11) gave negligible conversions into labeled product with chromatographic properties as 24(S)-hydroxycholesterol, in general, considerably less than 0.2%. In our hands the small extent of conversion of the labeled cholesterol varied considerably in different sets of experiments, regardless of the time of incubation and the mode of addition of the cholesterol (acetone, cyclodextrin, or Tween 80). If it would be possible to remove most of the endogenous cholesterol from brain microsomes with retention of enzymatic activity, a higher conversion of exogenous labeled cholesterol would be expected. Acetone extraction (13) of the brain microsomes gave preparations containing less than 10% of the original content of cholesterol. All these preparations were, however, completely inactive.

Since the microsomal 24(S)-hydroxylase system is likely to be saturated with substrate cholesterol, the possibility was tested to assay the production of 24(S)-hydroxycholesterol by measuring this steroid in brain microsomes before and after incubation without adding substrate. Using the sensitive and accurate method based on isotope dilution mass spectrometry and a deuterium-labeled internal standard (14), the initial amount of 24(S)-hydroxycholesterol was found to be 0.72 μg/ml of the brain microsomal fraction in a typical experiment. After 2 h of incubation this amount had expanded to 0.82 μg and was further increased to 0.86 μg after 20 h of incubation. The increase in amount of 24(S)-hydroxycholesterol (about 50 ng/h/ml) appeared to be approximately linear with time during the first 2 h of incubation. This was confirmed in another experiment in which the amount of 24(S)-hydroxycholesterol increased from 0.64 to 0.72 μg during 2 h of incubation. This increase corresponds to a conversion of about 0.02% of the endogenous cholesterol per h under the conditions employed.

Addition of exogenous cholesterol to the brain microsomes, dissolved in acetone or cyclodextrin, did not further increase the yield of the product in this assay. It is evident that the enzyme system was saturated with substrate under the conditions employed.

Due to the very low degree of conversion and the small expansion of the relatively large endogenous pool of 24(S)-hydroxycholesterol, the above assay was, however, less reproducible. In some experiments there was no linear increase in the amount of 24(S)-hydroxycholesterol.

Another more successful strategy for assay of cholesterol 24(S)-hydroxylase activity was to utilize the fact that a reaction catalyzed by a mixed function oxidase involves introduction of one atom of oxygen (cf. above). 24(S)-Hydroxylation of cholesterol can then be expected to yield a product containing one atom of 18O if the reaction is performed in 18O2 atmosphere. Fig. 3 shows the result of such an experiment. There was a clear enrichment of 18O in 24(S)-hydroxycholesterol and this enrichment increased approximately linear with time during the first 2 h at a rate of about 3% per h. Since the amount of 24(S)-hydroxycholesterol in the microsomal fraction was about 0.7 μg/ml in this experiment, the rate of formation of 18O-labeled 24(S)-hydroxycholesterol was estimated to be about 20 ng/h/ml under the conditions employed. Since the 18O enrichment in the oxygen was about 70% under the conditions employed, the true rate of formation should be about 30 ng/h/ml. When taking into account the concentration of endogenous cholesterol in the brain microsomes, this would correspond to a conversion of about 0.015% cholesterol per h. This degree of conversion is similar to that obtained with the above technique. When NADPH was omitted from the incubations, there was no significant incorporation of 18O in 24(S)-hydroxycholesterol.

The 18O technique was found to be considerably more reproducible than the assay based on expansion of the pool of 24(S)-
hydroxycholesterol. Despite the high costs and the relatively complicated design, the $^{18}$O assay was therefore used in all subsequent experiments.

The mitochondrial fraction of rat brain was also tested for 24(S)-hydroxylase activity by the same method. The rate of incorporation of $^{18}$O in 24(S)-hydroxycholesterol was found to be lower than in the incubations with microsomes, and corresponded to a conversion of the endogenous cholesterol of about 0.003% per h.

In view of a previous report that 24(S)-hydroxycholesterol accumulates in rat brain during maturation (10) and our finding that the flux of 24(S)-hydroxycholesterol from the human brain seems to be age-dependent (8), microsomes were prepared from several brains of young rats weighing only about 50 g. The enrichment of $^{18}$O in 24(S)-hydroxycholesterol in this preparation was found to be about 0.7% per h, corresponding to a conversion of the endogenous cholesterol of about 0.004% per h. Microsomes were also prepared from the brain of a rat weighing about 750 g. In this preparation, endogenous cholesterol was converted into 24(S)-hydroxycholesterol at a rate of about 0.01% per h. The conversion of brain microsomal cholesterol into 24(S)-hydroxycholesterol thus appears to be lower in young rats as compared with older rats.

Experiments with Tritium-labeled 24-Hydroxycholesterol—Whole homogenates as well as microsomal and mitochondrial preparations of brain were incubated with tritium-labeled 24-hydroxycholesterol together with NADPH and/or isocitrate under different conditions (incubation times up to 4 h). The extent of conversion to more polar products was less than 1% in all these experiments.

DISCUSSION

Methodological Aspects—Brain cholesterol metabolism has previously been studied in vivo with use of labeled cholesterol or precursors to cholesterol or with use of tritium-labeled water (for a general review, see Ref. 3). The present in vivo $^{18}$O$_2$ technique has clear merits in relation to these previous methods. Oxygen is consumed by all cells in the body and there is little or no compartmentalization of oxygen. According to our experience (9, 16, 17), exposure of a rat to $^{18}$O$_2$ leads to a rapid incorporation of $^{18}$O in proteins formed by monooxygenation. The labeling appears to reach its maximum already after about 15 min of exposure (16) and it is thus possible to obtain a pulse labeling of a specific compound that can be used for turnover studies. The disadvantage of the technique is that it is expensive and requires combined gas chromatography-mass spectrometry for determination of the enrichment of $^{18}$O in the product.

The $^{18}$O$_2$ technique was used here also for in vitro determin-nation of the very slow conversion of endogenous brain microsomal cholesterol into 24(S)-hydroxycholesterol. The advantage with this technique is obvious when considered that the rate of 24(S)-hydroxylation of cholesterol occurs at a rate of only about 0.02% per h, whereas the pool of 24(S)-hydroxycholesterol has a turnover of about 4% per h. According to our experience a conversion of less than 0.1% of labeled cholesterol is very difficult to assay accurately, whereas it is relatively easy to determine the content of $^{18}$O in 24(S)-hydroxycholesterol after 2 h incubation. Another disadvantage with use of labeled cholesterol is that there is always an uncertainty with respect to the degree of equilibration of this cholesterol with the endogenous pool. It should be noted that the content of cholesterol in brain preparations is considerably higher than in any other organ.

Synthesis of Cholesterol in Vivo—The rate of synthesis of cholesterol in rat brain found here, 0.02–0.05% of the pool per h, corresponding to a half-life of 2–4 months, is of the same magnitude as has been reported previously. With use of an in vivo technique based on disappearance of radioactive cholesterol from rat brain, the half-life was found to be about 4 months (4). With use of an in vitro technique with slices of rat brain, the estimated half-life of cholesterol was found to be about 6 months (5). The turnover of brain cholesterol may vary as a consequence of the age and strain of the rat. There was a relatively great difference in cholesterol synthesis in the three rats studied here, despite the fact that they had about the same age and belonged to the same strain. The most rapid synthesis was obtained in the rat exposed for 40 h to $^{18}$O$_2$. Due to the long exposure, this rat may have been more stressed than the other rats and at present we cannot exclude the possibility that this may affect cholesterol synthesis.

Synthesis and Flux of 24(S)-Hydroxycholesterol—The pool size of 24(S)-hydroxycholesterol in rat brain is about 0.3% of that of cholesterol (8). It is evident from the present results that this pool has a rapid turnover as compared with brain cholesterol. The estimated half-life of 24(S)-hydroxycholesterol was thus found to be about one-half day as compared with about 2–4 months for cholesterol.

In theory there may be several different mechanisms by which the brain can eliminate its 24(S)-hydroxycholesterol: (a) the compound may pass into the cerebrospinal fluid prior to the general circulation; (b) it may be locally metabolized to another steroid that may pass into the cerebrospinal fluid; (c) it may directly pass the blood-brain barrier.

In the previous work (8) we measured the concentration of 24(S)-hydroxycholesterol in human cerebrospinal fluid and found that less than 1% of the total flux of this compound from the brain can occur via the cerebrospinal fluid. The possibility that there is a local metabolism of 24-hydroxycholesterol in the brain was investigated here. No significant conversion of tritium-labeled 24-hydroxycholesterol into the more polar product was obtained. This finding is in agreement with recent results published by Zhang (18). In contrast to 25-hydroxycholesterol and 27-hydroxycholesterol, 24-hydroxycholesterol was not 7a-hydroxylated by cultured rat astrocytes, Schwann cells, and neurons. A very small 25-hydroxylation of 24-hydroxycholesterol occurred in astrocytes. No such conversion could be observed in the present work, however.

We are thus left with the possibility that unmetabolized 24(S)-hydroxycholesterol passes the blood-brain barrier. This is consistent with previous demonstrations that side chain-oxidized steroids are transferred through lipophilic membranes at rates orders of magnitude faster than cholesterol (19).

If there is a flux of unmetabolized 24(S)-hydroxycholesterol...
from the brain into the circulation and if most of the 24(S)-hydroxycholesterol in the circulation originates from the brain, the \(^{18}\)O content in this steroid can be expected to be about the same in the circulation as in the brain in the present \(^{18}\)O\(_2\) experiments. This was also found to be the case (Fig. 2). If most of the circulating 24(S)-hydroxycholesterol originates from the brain, it is also evident that there must be a rapid equilibration between the two pools of 24-hydroxycholesterol over the blood-brain barrier.

The incorporation of \(^{18}\)O into 24(S)-hydroxycholesterol was markedly lower than in the other two circulating oxysterols, 7α-hydroxycholesterol and 27-hydroxycholesterol. In principle, this may be due to differences in rate of formation or elimination of the oxysterol or in the rate of exchange of a pool of newly synthesized material with that in plasma. Evidence has been presented that most of the 7α-hydroxycholesterol in the circulation is formed by the hepatic cholesterol 7α-hydroxylase and that there is a rapid turnover of this compound (20). Evidence has also been presented that most of the 27-hydroxycholesterol present in the circulation is formed extrahepatically and that there is a rapid flux of this steroid to the liver (9, 21).

The most likely explanation for the low incorporation of \(^{18}\)O in the circulating 24(S)-hydroxycholesterol is that the rate of synthesis of this oxysterol in relation to its pool size is lower than for the other oxysterols. Theoretically part of the explanation for the low incorporation of \(^{18}\)O in circulating 24(S)-hydroxycholesterol could be a slow rate of turnover in this compartment. According to preliminary experiments with labeled 24-hydroxycholesterol in humans, however, the half-life of this steroid is very short, less than 1 h.\(^1\) Thus it seems unlikely that a long half-life in the circulation can explain the low incorporation of \(^{18}\)O in circulating 24(S)-hydroxycholesterol. That 7α-hydroxycholesterol and 27-hydroxycholesterol have a short half-life in the circulation of rats is evident from the experiments shown in Fig. 1.

Adrenals are known to contain relatively high concentration of 24(S)-hydroxycholesterol (8), and theoretically part of the 24(S)-hydroxycholesterol in the circulation might be derived from the adrenals. The adrenals are small in relation to the brain, however, and it was shown that the adrenals of Sprague-Dawley rats contain less than 0.3% of the amount of 24(S)-hydroxycholesterol present in the brain. In one of the \(^{18}\)O\(_2\) experiments it was shown that the incorporation of \(^{18}\)O in 24(S)-hydroxycholesterol present in the adrenals was similar to that in 24(S)-hydroxycholesterol isolated from the brain of the same animal. The very small amounts of material prevented, however, a more detailed investigation on the turnover of 24(S)-hydroxycholesterol in the adrenals.

Conversion of Cholesterol into 24(S)-Hydroxycholesterol in Brain Microsomes—The present results confirm two previous reports (10, 11) that brain microsomes contain 24(S)-hydroxylase activity toward cholesterol. Since NADPH and oxygen was required for the enzymatic activity, it seems likely that a species of cytochrome P-450 is involved. Evidence has been presented that brain contains species of cytochrome P-450 capable of 27-, 7α-, and 11β-hydroxylation of steroids (22–24), as well as steroid side chain cleavage (24, 25). Recently Stapleton et al. (26) described a novel cytochrome P-450 (hct-1) that is expressed primarily in the brain. When expressed in cultured cells this enzyme was found to 7α-hydroxylate pregnenolone and dehydroepiandrosterone (27) and had no significant activity toward cholesterol. The species of cytochrome P-450 responsible for 24(S)-hydroxylation of cholesterol has thus not yet been defined.

Under the specific in vitro conditions used here, the rate of 24(S)-hydroxylation of endogenous cholesterol in brain microsomes was found to be similar to the overall rate of 24(S)-hydroxylation of cholesterol obtained in the in vivo experiments (about 0.02% conversion per h). The relatively complicated assay prevented a more detailed characterization of the properties of the enzyme. The marked age-dependent variations in levels of circulating 24(S)-hydroxycholesterol (8) stimulated us to assay cholesterol 24-hydroxylase activity in brain microsomes from very young and old rats. While the activity appeared to be lower in preparations from young rats, the difference was too low to allow definite conclusions. Anyway, this difference is considerably less than the marked difference in levels of 24(S)-hydroxycholesterol in the circulation of infants and adults previously reported (8).

Importance of 24(S)-Hydroxylation for Cholesterol Homeostasis in the Brain—In view of the low exchange with cholesterol-containing lipoproteins in the circulation (3), it is evident that the local synthesis of cholesterol in the brain must be balanced by an elimination that is similar to or slightly lower than this synthesis. In a young subject in which the brain is still growing, the rate of synthesis must be higher than the rate of elimination. The present finding that the rate of removal of cholesterol as 24(S)-hydroxycholesterol from the brain of rats is at least half of the rate of cholesterol synthesis is consistent with the 24(S)-hydroxylase mechanism as the most important pathway for elimination of cholesterol from this organ. Lipoprotein-mediated elimination of unmetabolized cholesterol is thus likely to be less important. Apolipoprotein E has been ascribed a role in redistribution and homeostasis of cholesterol in the brain (3, 28). Astrocytes are thus known to synthesize and secrete apolipoprotein E and there is a flux of apolipoprotein E-containing lipoproteins into the cerebrospinal fluid. It has been calculated (28) that such a clearance of cholesterol could account for the removal of 1–2 mg of cholesterol per day from the human brain. Based on the plasma concentrations of 24(S)-hydroxycholesterol in the internal jugular vein and in a peripheral artery, we have found (8) that the rate of elimination of cholesterol from the human brain by the present mechanism is about 4–6 mg per day. The rate of synthesis of cholesterol in the human brain is not known, however, and we do not know the relation between this synthesis and the two different mechanisms for removal of cholesterol in man.

Extrahepatic tissues and organs contain a sterol 27-hydroxylase and it is known that intracellular cholesterol can be eliminated from such cells to some extent by a mechanism involving conversion of cholesterol into the more polar steroids 27-hydroxycholesterol and 3β-hydroxy-5-cholestenolic acid (21, 29). Also the brain contains a sterol 27-hydroxylase (22) and the possibility must be considered that cholesterol may be eliminated from the brain by this mechanism. The capacity of the sterol 27-hydroxylase in the brain is, however, considerably lower than that of the 24(S)-hydroxylase and this enzymatic activity was found to be too low to be measured by the present technique. The concentration of 27-hydroxycholesterol in the brain is considerably lower than that of 24(S)-hydroxycholesterol, and we have failed to demonstrate a net flux of 27-hydroxycholesterol from the brain into the circulation (8). Presence of a sterol side chain cleavage activity in the brain has been reported (24, 25). As judged from the published in vitro data (25), this activity is, however, several orders of magnitude lower than cholesterol 24(S)-hydroxylase activity and is not likely to be of importance for the overall turnover of cholesterol in the brain. Attempts in our laboratory to measure the low sterol side chain cleavage activity in the brain by the present

---

1 D. Lütjohann and I. Björkhem, unpublished studies.
24(S)-Hydroxylase and Cholesterol Homeostasis in Rat Brain

 technique has failed thus far.

In addition to being a transport form for cholesterol over the blood-brain barrier, 24(S)-hydroxycholesterol is a potent inhibitor of cholesterol synthesis (30). In theory, both removal of cholesterol and inhibition of cholesterol synthesis in the brain may thus be under regulation of the 24(S)-hydroxylase. Overall synthesis of cholesterol in the body is markedly higher in young subjects than in old, and throughout the life of a rat, from a rapidly developing fetus to adult, there is a 10-fold decrease in the rate of synthesis of cholesterol (2). We found a lower cholesterol 24(S)-hydroxylase activity in brain microsomes from young rats as compared with old rats. This finding is consistent with a previous report that the levels of 24(S)-hydroxycholesterol in the brain are lower in young rats than in older rats (10). Whether the lower levels of 24(S)-hydroxycholesterol in the brain of younger rats are of importance for cholesterol synthesis is not known. At the present state of knowledge we cannot exclude that the 24(S)-hydroxylase-mediated elimination of brain cholesterol is intimately coupled to cholesterol synthesis and that the age-dependent variations seen in circulating levels of this oxysterol are secondary to changes in cholesterol synthesis. The relative importance of a regulation of the cholesterol 24(S)-hydroxylase for cholesterol homeostasis in the brain can be evaluated first when the species of cytochrome P-450 responsible for the hydroxylation has been identified and characterized at a molecular level.

Acknowledgments—The skilful technical assistance of Manfred Held and Anita Lövgren is gratefully acknowledged.

REFERENCES
1. Tint, G. S., Irons, M., Elias, E. R., Batt, A. K., Frieden, R., and Chen, T. S. (1994) New Engl. J. Med. 330, 157–113
2. Dietzchy, J. M., Turley, S. D., and Spady, D. K. (1993) J. Lipid Res. 34, 1637–1659
3. Snipes, G. J., and Suter, U. (1997) in Subcellular Biochemistry (Bittman, R., ed) Vol. 28, pp. 173–204, Plenum Press, New York
4. Serouguine-Gautheron, C., and Chevallier, F. (1973) Biochim. Biophys. Acta 316, 244–250
5. Anderson, M., Elmlinger, P. G., Edlund, C., Kristensson, K., and Dallner, G. (1990) FEBS Lett. 269, 15–18
6. Gerdien, E. L., Daniells, P. E., Nguyen, T. S., and Winn, H. R. (1991) In Vitro Cell Dev. Biol. 27A, 312–326
7. De Vries, H. (1995) Characteristics of Blood-Brain Barrier Endothelial Cells in Response to Inflammatory Stimuli. Thesis, Costar Europe Ltd., Badhoevedorp, The Netherlands
8. Litjohann, D., Breuer, O., Ahlborg, G., Nennesmo, I., Siden, A., Diczfalusy, U., and Björkhem, I. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 9799–9804
9. Breuer, O., and Björkhem, I. (1995) J. Biol. Chem. 270, 20278–20284
10. Lin, Y., and Smith, L. L. (1974) J. Biol. Chem. 249, 189–196
11. Dhar, A. K., Teng, J. I., and Smith, L. L. (1973) J. Neurochem. 21, 51–60
12. Aringer, L., and Eneroth, P. (1971) Steroids 18, 381–388
13. Shefer, S., Cheng, F. W., Hauser, S., Batt, A. K., and Salen, G. (1981) J. Lipid Res. 22, 532–536
14. Dzelnetovic, S., Breuer, O., Lund, E., and Diczfalusy, U. (1995) Anal. Biochem. 225, 73–80
15. Björkhem, I., Blomstrand, R., and Svensson, L. (1974) Clin. Chim. Acta 54, 185–193
16. Björkhem, I., and Lewenhaupt, A. (1979) J. Biol. Chem. 254, 5252–5256
17. Benzhin, G., Björkhem, I., Breuer, O., Sakinis, A., and Wennmalm, Å. (1997) Biochem. J. 323, 853–858
18. Zhang, J. (1996) Studies on 7α-Hydroxylation of 25- and 27-Hydroxycholesterol in Extrathoracic Tissues and Cells. Thesis, Karolinska Institutet. Repro Print AB, Stockholm
19. Lange, Y., and Strebel, P. (1995) J. Lipid Res. 36, 1092–1097
20. Björkhem, I., Reinhner, E., Angelin, B., Ewerth, S., Åkerlund, J.-E., and Einarsson, K. (1997) J. Lipid Res. 38, 889–894
21. Lund, E., Andersson, O., Zhang, J., Bahiker, A., Ahlborg, G., Diczfalusy, U., Einarsson, K., Sjöwall, J., and Björkhem, I. (1996) Arterioscler. Thromb. Vasc. Biol. 16, 209–211
22. Pedersen, J. J., Olenbro, H., and Björkhem, I. (1989) Biochem. Int. 18, 615–622
23. Zhang, J., Akwa, Y., Baulieu, E. E., and Sjöwall, J. (1995) Compt. Rend. 318, 345–349
24. Mellon, S. H., and Deschepper, C. F. (1993) Brain Res. 589, 283–292
25. Jung-Testas, I., Hu, Z. Y., Baulieu, E. E., and Robel, P. (1989) Endocrinology 125, 2083–2091
26. Stapleton, G., Steel, M., Richardson, M., Mason, J. O., Rose, K. A., Morris, R. G. M., and Lathe, R. (1996) J. Biol. Chem. 270, 29739–29745
27. Rose, K. A., Stapleton, G., Dott, K., Kienny, M. P., Best, R., Schwarz, M., Russel, D. W., Björkhem, I., Seck, J., and Lathe, R. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 4925–4930
28. Pitas, R. E., Boyles, J. K., Lee, S. H., Foss, D., and Mahley, R. W. (1987) Biochem. Biophys. Acta 917, 148–161
29. Björkhem, I., Andersson, O., Diczfalusy, U., Sevastik, B., Xiu, R.-J., Duan, C., and Lund, E. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 8592–8596
30. Saucier, S. E., Kandutsch, A. A., Gayen, A. K., Swahn, D. K., and Spencer, T. A. (1989) J. Biol. Chem. 264, 6863–6869

2 Björkhem, D. Litjohann, O. Breuer, A. Sakinis, and Å. Wennmalm, unpublished observation.