review

26-Hydroxycholesterol: synthesis, metabolism, and biologic activities

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Abstract Cholest-5-ene-3β,26-diol (26-hydroxycholesterol) is synthesized by a mitochondrial P-450 enzyme that appears to be widely distributed in tissues. Together with other C-27 steroid intermediates it is transported to the liver and metabolized to bile acids. Although 26-hydroxycholesterol is transported in plasma lipoproteins mostly as the fatty acid ester, neither its assembly and orientation within lipoproteins nor its mechanism of transport across the sinusoidal liver membrane is known. Cell culture studies indicate that 26-hydroxycholesterol can inhibit both cholesterol synthesis and low density lipoprotein (LDL) receptor activity. Inhibition of DNA synthesis also occurs and may not be related to the reduction in HMG-CoA reductase activity. The relationship of these in vitro activities to the physiologic role(s) of 26-hydroxycholesterol remains to be clarified. A clue to its biologic role is the knowledge that markedly decreased 26-hydroxylase activity appears to be the molecular basis of cerebrotendinous xanthomatosis, an inborn error of metabolism characterized by a significant decrease in 26-hydroxycholesterol and bile acid synthesis and an increase in cholesterol synthesis. **—Javitt, N. B.** 26-Hydroxcholesterol: synthesis, metabolism, and biologic activities. *J. Lipid Res.* 1990. **31:** 1527–1533.

Supplementary key words 7α-hydroxycholesterol • oxysterol • monohydroxy bile acids • cholesterol 7α-hydroxylase • cerebrotendinous xanthomatosis

Cholest-5-ene-3β,26-diol is most commonly referred to as 26-hydroxycholesterol. It is known that the compound normally circulates in plasma (1–3), and an enzyme catalyzing its synthesis from cholesterol is present in human liver (4). Most recently, the mRNA that codes for synthesis of the enzyme was identified in a variety of tissues (5), a finding that suggests that this endogenous hydroxysterol is widely distributed.

In vitro studies indicate that 26-hydroxycholesterol has a number of potent biologic activities (6–9). Although it has been difficult to establish on the basis of in vivo studies that these activities have a physiologic counterpart, it is currently believed that the virtual absence of 26-hydroxylase activity for C-27 sterols is the molecular basis of an inborn error of metabolism described phenotypically as cerebrotendinous xanthomatosis (10). As a consequence, 26-hydroxycholesterol and bile acid synthesis decrease and an increase in cholesterol synthesis, together with tissue deposition and accelerated atherosclerosis, occurs. The interrelationships of these metabolic events require elucidation.

The purpose of this review is to summarize the knowledge that has been accumulated in regard to 26-hydroxycholesterol, with particular emphasis on the critical issues that will need to be resolved before its role in human metabolism can be defined.

Nomenclature

Two stereoisomers, (25R)- and (25S)-26-hydroxycholesterol, exist (**Fig. 1**). The (25R) isomer, the naturally occurring compound, is referred to as cholest-5-ene-3β,27-diol in an alternate scheme of nomenclature (11). The basis of the isomerism is that the isopropyl methyl groups of cholesterol are not equivalent and the stereospecific oxidation of only one of these groups causes the C-25 carbon to become asymmetric. Because the three-dimensional model of the metabolite is not superimposable on its mirror image, the C-25 carbon of cholesterol is termed a prochiral center.

Chemical Synthesis of 26-Hydroxycholesterol

A practical method for synthesizing 26-hydroxycholesterol from kryptogenin (cholest-5-ene-3β,26-diol,16-22-dione) was developed using a two-step reduction procedure. A Clemmensen reduction (Zn/Hg amalgam and HCl), yielded a mixture of 26-hydroxycholesterol, 26-hydroxycholesterol-16-one (12), and 26-hydroxycholesterol-22-one (13). Because the 16-oxo intermediate cocrystallizes with 26-hydroxycholesterol, a second reduction step using hydrazine and KOH (a Wolff-Kishner reduction) was done to reduce the keto group to yield 26-hydroxycholesterol.

Chromatographic methods that separate the 16-one from 26-hydroxycholesterol now exist (14), so that the se-

Abbreviations: LDL, low density lipoprotein

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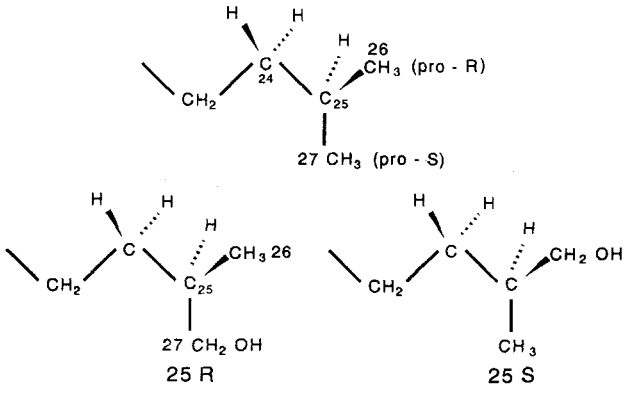

Fig. 1. Stereochemistry for 26-hydroxycholesterol. The C-25 of 26-hydroxycholesterol is a prochiral center since stereospecific substitution of the terminal methyl groups, designated pro-R and pro-S, yields two diastereoisomers. The mitochondrial fraction of liver hydroxylates the methyl group of cholesterol in position C-27 (th 25-*pro*-S methyl group); however, by convention the product is usually referred to as 26- rather than 27-hydroxycholesterol. Because of the change in priority after the hydroxylation, the complete designation is (25R)-26-hydroxycholesterol. The other isomer, (25S)-26-hydroxycholesterol, also exists in nature, but less is known concerning its formation. A question that has not been resolved entirely is the stereospecificity of the 26-hydroxylation of the 5β-cholestane series (diol and triol). It is possible that these intermediates in bile acid synthesis undergo side chain hydroxylation in both the microsomes and the mitochondria to yield both (25R) and (25S) isomers (see references #11 and #26).

cond reduction step is not essential. Kryptogenin, however, is no longer commercially available.

Diosgenin ([25R],5-spirosten-3β-ol), which is readily available (Sigma, St. Louis, MO), can be substituted for kryptogenin (15). However, the Clemmensen reduction yields the intermediate, cholest-5-ene-3β,16β,26-triol, and the hydroxyl group at C-16 must be oxidized to a keto group prior to the Wolff-Kishner reduction. The oxidation step utilizes chromium trioxide in glacial acetic acid buffered with sodium acetate. Although none of the hydroxyl groups is protected, an acceptable yield of cholest-5-ene-3β,26-diol,16-one is obtained. However, other investigators (16, 17) prefer to protect the 3β-ol and 26-ol prior to oxidation and thus obtain only the 16-one intermediate.

Diosgenin contains varying amounts of yamogenin (17), which is the source of small amounts of (25S)-26-hydroxycholesterol. Yamogenin can be separated from diosgenin as the acetate prior to reduction (17), or alternatively, the diastereoisomers of 26-hydroxycholesterol can be separated chromatographically (18).

Formation of epimers at C-20 and C-25 have been reported to occur during the reduction of kryptogenin (13), largely because of the presence of the C-22 oxo intermediate. A similar loss of stereospecificity of the final products has not been reported when diosgenin is used, but because of the strong acid and alkaline conditions that are used, one needs to be aware of the possiblity that these rearrangements can occur.

Most biologic studies have been performed using crystalline 26-hydroxycholesterol obtained by variations of the above procedures and therefore uncertainty exists in regard to the proportion of various stereoisomers that may have been present.

A stereospecific synthesis of (25R)-26-hydroxycholesterol beginning with pregnenolone has been described (19). A relatively large number of steps are required and the yield is apparently very low.

Physical Properties

26-Hydroxycholesterol is readily crystallized from ethyl acetate as fine needles (mp 178–179°C), and the diacetate derivative also yields fine needles (mp 129°C) when crystallized from methanol. However, these procedures do not eliminate the presence of stereoisomers. High-pressure liquid chromatographic analysis of the product obtained from diosgenin indicates that approximately 80% is the naturally occurring (25R) isomer (18).

Little is known about the orientation of 26-hydroxycholesterol at oil–water interfaces or in phospholipids or mixtures of phospholipids and cholesterol. Infrared spectrometric analysis gave evidence for a 1:1 hydrogen bonding between 26-hydroxycholesterol and trilaurin, findings similar to those for cholesterol (20).

It is expected that because polar groups are present on both ends of the sterol, its orientation in membranes would be very different from that of cholesterol. However, no direct evidence to support this prediction currently exists. The chromatographic behavior of 26-hydroxycholesterol in virtually all systems that have been studied is very different from that of other C-27 diols and triols in which substitutions are all on the steroid ring rather than the side-chain. In an extensive study of dioxygenated C-27 sterols by Aringer (21), side-chain substitutions greatly increased the retention time as compared with ring substitutions; among the side-chain substituted C-27 sterols, the increase in retention time was a function of the distance of the hydroxyl group from the ring. Thus, in most chromatographic systems, the retention time of 26-hydroxycholesterol is longer than that of 25-hydroxycholesterol, which is in turn longer than that of 24- and 22-hydroxycholesterol.

Perhaps because of the physical properties of 26-hydroxycholesterol, a vehicle suitable for biologic studies that solubilizes 26-hydroxycholesterol in amounts sufficient to permit studies over a full range of substrate concentrations has not been developed. As compared with cholesterol, its solubilization in vehicles such as Tween 20 is much more limited. Although much greater amounts can be dissolved in acetone, ethanol, or dimethyl formamide, precipitation occurs almost immediately after dilution in the aqueous medium. Thus, more definitive studies will not only require the use of material of known stereospecificity but also the development of vehicles that can be considered more physiologic.

Biosynthesis of 26-Hydroxycholesterol

Using chemically synthesized 26-hydroxycholesterol as a standard, it was possible to identify radioactive 26-hydroxycholesterol as a metabolite of [4-^{14}C]cholesterol after its incubation with mouse liver mitochondria (22). Studies of the stereospecificity of the hydroxylation using cholesterol (23, 24) and 5β-cholestane-3α,7α,12α-triol (11) in the rat indicate that only one of the two terminal methyl groups of cholesterol is hydroxylated. Because this group is derived from the C-3′ of mevalonate, it has been described as the C-27 methyl group and the enzyme that catalyzes its oxidation is referred to as a C-27 sterol monooxygenase (11). However, in most studies, the stereospecificity was not determined but the 26-hydroxylase convention was followed. It is, however, currently believed that only one stereoisomer is generated. The same mitochondrial enzyme is also thought to hydroxylate the terminal methyl group of all the C-27 sterol intermediates in bile acid synthesis. Rates of hydroxylation of 5β-cholestane-3α,7α-diol and 5β-cholestane-3α,7α,12α-triol are much greater than that of cholesterol, a finding that is consonant with the concept that the major role of the enzyme is to initiate side-chain degradation of 7α-hydroxylated intermediates in bile acid synthesis that are formed in the microsomes from cholesterol.

Purification of the mitochondrial 26-hydroxylase indicates that it is a P-450 enzyme (25) that requires the presence of both ferrodoxin and ferrodoxin reductase in addition to NADPH for hydroxylation to proceed (26).

Microsomal 25- and 26-hydroxylase have been reported in rat and human liver (27, 28), but no activity has been found using cholesterol as a substrate. Therefore, the mitochondrial enzyme appears to be the sole source for 26-hydroxycholesterol.

A homogenous preparation of the mitochondrial enzyme from rabbit liver has led to isolation of a cDNA clone, a portion of which was modified and transfected into COS-M6 cells (5). Expression of enzyme activity was obtained based on the metabolism of 5β-cholestane-3α,7α, 12α-triol to the 26-tetrol. Although cholesterol was not tested as a substrate, the investigators interpreted their findings to support the concept that 26-hydroxycholesterol is widely distributed in cells and participates in the regulation of cholesterol synthesis.

It was also noted that the transfected COS-M6 cells further metabolized the tetrol to the C-27 cholestanoic acid, an activity not previously known to occur in extrahepatic tissues. Thus, both 26-hydroxycholesterol and C-27 bile acids that normally circulate in plasma (29) can be derived from extrahepatic sources.

Isolation of the appropriate cDNA clone depended on the preparation of oligonucleotide probes based on the sequence of 15 amino acids beginning with the N-terminal end of the enzyme. The cDNA was also found to hybridize with a sterol 26-hydroxylase mRNA of approximately 1.9 kilobases that was detected in liver, duodenum, adrenal gland, lung, kidney, and spleen.

Biologic Activities of 26-Hydroxycholesterol

Inhibition of cholesterol synthesis. With the finding that 26-hydroxycholesterol is a normal constituent of low density lipoproteins (LDL) and other lipoproteins (1) and that its concentration in plasma in markedly diminished in cerebrotendinous xanthomatosis (30), the stage was set to determine whether this endogenously derived hydroxysterol down-regulated HMG-CoA reductase activity.

Studies using Chinese hamster ovary cells (6), and HepG2 cells (9) all indicated that the compound inhibits cholesterol synthesis. However, the concentration needed to inhibit cholesterol synthesis in vitro appeared to be greater than the amounts that occur endogenously, a finding that led to the speculation that 26-hydroxycholesterol plays no role in the down-regulation of cholesterol synthesis that occurs after the internalization of LDL (31). The question remains moot, since in all studies thus far the method of delivery of 26-hydroxycholesterol to the cell was not physiologic. In analogy with LDL receptor-mediated transport as compared with non-receptor-mediated transport, down-regulation of HMG-CoA reductase activity by 26-hydroxycholesterol may also require a specific pathway for maximum effect on the expression and activity of the enzyme.

With the exception of 7α-hydroxycholesterol, which was found not to inhibit cholesterol synthesis (9), the findings appear to mimic the known inhibitory effects of a variety of other "oxysterols" (32).

Among these "oxysterols" is 25-hydroxycholesterol, a known autooxidation product of cholesterol (33) that is available commercially and has been used extensively for studies of the regulation of cholesterol synthesis. There is some evidence to indicate that it may be biosynthesized in cultured fibroblasts (34), but when efforts were made to exclude autooxidation, the compound could not be identified in normal plasma (1, 3).

The biological activity of these "oxysterols" has been linked to their binding affinity for a specific cytosolic receptor protein that in combination with the sterol will decrease synthesis of HMG-CoA reductase (32). However, a modified view can be proposed based on the careful study of 3β-hydroxy-5α-cholest-8(14)-ene-15-one. The decrease in HMG-CoA reductase activity by this sterol is dependent at least in part on the 26-hydroxylation (35) that occurs after its administration. In view of the broad substrate specificity of the mitochondrial P-450 C-26-hydroxylase and its presence in many mammalian cells including fibroblasts (36), it is possible that inhibitory "oxysterols" have in common the presence of a hydroxyl group on the side chain.

Recently it was proposed that 25-hydroxycholesterol induces the synthesis of a nuclear protein that binds to the

sterol-regulatory element region of the gene promoter that regulates synthesis of HMG-CoA reductase (37). Identification of the nuclear protein was dependent on the synthesis of an oligonucleotide probe that was modeled after the known sequence of the sterol-regulatory element and was used to screen a λgt11 cDNA library from HepG2 cells. A positive clone was isolated and the complete nucleotide and derived amino acid sequence were determined. The derived protein contains zinc finger repeats characteristic of DNA-binding proteins. However, binding to only one strand of the DNA was found and therefore the mechanism by which the regulatory effect occurs is not well understood. The decrease in cholesterol synthesis that occurred in the presence of 25-hydroxycholesterol was associated with an increase in the concentration of both the nuclear binding protein and the mRNA coding for its synthesis, thus providing circumstantial evidence for a role in the regulation of sterol metabolism (37).

Detailed studies of the mechanisms by which sterols modulate cholesterol synthesis are in a relatively early stage of development. It may be premature to lump together their mechanism of action, just as it would have been short-sighted to lump together the activity of lipoproteins because they all contain cholesterol, phospholipid, and protein.

Inhibition of DNA synthesis. Thymidine incorporation into DNA and HMG-CoA reductase activity were studied in phytohemagglutinin-stimulated lymphocytes after the addition of different side-chain-hydroxylated C-27 sterols (7). None of the sterols used was cytotoxic as judged by Trypan Blue exclusion and total cell counts. At a medium concentration of 12.5 μM, inhibition of HMG-CoA reductase activity ranged from 51 to 96% in contrast to inhibition of DNA synthesis, which ranged from 0 to 28%. Both 25- and 26-hydroxycholesterol caused 96% inhibition of cholesterol synthesis but only 9–18% inhibition of DNA synthesis. At a higher concentration (62.5 μM) inhibition of DNA synthesis ranged from 74 to 97%. Also of interest is the finding that at the higher concentration some sterols such as 22(S)-hydroxycholesterol inhibited HMG-CoA reductase activity (95%) to a much greater extent than DNA synthesis (3–12%). The investigators concluded that the inhibition of DNA synthesis is not related to the suppression of HMG-CoA reductase activity.

Regulation of LDL-receptor activity. A dose-dependent effect of 26-hydroxycholesterol on LDL receptor-mediated transport by human fibroblasts was reported (8). At a concentration of 1 μM, either (25S)- or (25R)-26-hydroxycholesterol reduced the number of LDL binding sites with no change in ligand affinity.

Metabolism of 26-Hydroxycholesterol

Initially, only chenodeoxycholic acid was identified as a metabolite of parenterally administered radioactive 26-hydroxycholesterol in the rat with a bile fistula (38). In another study, which used chemically synthesized 26-hydroxycholesterol of much higher specific activity, it was found that both cholic and muricholic acids were also metabolites (39). In hamsters (40), rabbits (16), and humans (41), species that do not synthesize muricholic acids, the proportions of chenodeoxycholic and cholic acids derived from 26-hydroxycholesterol can account for the proportions of these bile acids normally found in bile. However, 7α-hydroxycholesterol is also metabolized to chenodeoxycholic and cholic acids, and therefore the presence of these end-products does not provide evidence as to which of the precursors makes the major contribution to bile acid synthesis.

Monohydroxy bile acids such as 3β-hydroxy-5-cholenoic acid and 3β-hydroxy-5-cholestenoic acid, which are normally present in plasma (29, 42), are derived exclusively via 26-hydroxycholesterol. 3β-Hydroxy-5-cholenoic acid has been identified as a metabolite of 26-hydroxycholesterol in liver cell culture (43) together with 3α-hydroxy-5β-cholanoic acid (lithocholic acid). Although lithocholic acid has been identified in newborn plasma and meconium (44, 45), it is also derived from the 7α-dehydroxylation of chenodeoxycholic acid as a result of normal intestinal bacterial activity, which is probably the major source of this bile acid in adults.

Quantitative Aspects of Bile Acid Synthesis

The major source of chenodeoxycholic and cholic acids is currently thought to be via a pathway beginning with 7α-hydroxylation of cholesterol. These two bile acids account for more than 95% of the total amount of bile acid produced by the liver and thus represent the major metabolite pathway for the disposal of cholesterol as acidic metabolites.

There is evidence in humans (46) and other species (47) to indicate that cholesterol 7α-hydroxylase activity is induced by either external biliary drainage or the administration of bile acid sequestrants such as cholestyramine. Based on this knowledge and also on data indicating that 7α-hydroxylated steroid intermediates are rapidly metabolized to bile acids, the concept that cholesterol 7α-hydroxylase is the rate-limiting enzyme for the daily production of chenodeoxycholic and cholic acids is widely accepted.

However, the paradigm was developed (38) before it was known that 26-hydroxycholesterol can also be a source for both bile acids and that its synthesis may not be confined to the liver. Knowledge of the daily production rate of 26-hydroxycholesterol will need to be derived from kinetic studies based on the administration of a tracer stereospecific 26-hydroxycholesterol administered in a physiological vehicle such as a lipoprotein. Based on information of this type, one can evaluate the quantitative contribution of 26-hydroxycholesterol to bile acid synthesis. Establishing the proportion of 26-hydroxycholesterol

that is derived from extrahepatic sources would also indicate its quantitative role in the return of cholesterol from the tissues to the liver.

A related question is the relationship of the enzyme catalyzing the 7α-hydroxylation of 26-hydroxycholesterol to cholesterol 7α-hydroxylase, which is considered to be the rate-limiting enzyme in chenodeoxycholic and cholic acid synthesis. If 7α-hydroxylation of all C-27 sterols is governed by the same enzyme, then they compete with cholesterol for bile acid synthesis. Alternatively, if a family of discrete P-450 steroid 7α-hydroxylases exists, then bile acid production from cholesterol and from 26-hydroxycholesterol is independently regulated. The most purified cholesterol 7α-hydroxylase from rat liver has no activity toward 3β-hydroxy-5-cholenoic acid (48), the monohydroxy bile acid metabolite of 26-hydroxycholesterol that in humans is metabolized to chenodeoxycholic acid (49). Based on this finding, it is possible that 7α-hydroxylation of 26-hydroxycholesterol is separately regulated and determines the metabolic fate of intracellular 26-hydroxycholesterol.

Working Hypothesis

To develop a working hypothesis for evaluating the biological role of 26-hydroxycholesterol, it is useful to try to relate its biologic effects in vitro to the inborn error of metabolism in which the enzyme catalyzing its synthesis is deficient. The in vitro data indicating that 26-hydroxycholesterol inhibits cholesterol synthesis correlates with the knowledge that cholesterol synthesis is approximately 30% greater than normal in individuals with cerebrotendinous xanthomatosis (50). However, the reduction in bile acid synthesis that occurs may also function as a stimulus to increase cholesterol synthesis via a mechanism similar to that occurring following bile acid diversion or the administration of resins that bind bile acids.

The reduced LDL receptor activity by 26-hydroxycholesterol is consistent with the hypothesis that it is coordinately regulated with HMG-CoA reductase activity via a similar sterol regulatory element on both genes (51) (**Fig. 2**) and with the report that the rate of LDL turnover is increased in cerebrotendinous xanthomatosis (52). In this in vivo kinetic study it was not possible to identify the tissues responsible for the increased LDL receptor activity.

It is difficult to rationalize coordinate inhibition of both activities in liver cells that have the special function of bile acid synthesis. In hepatocytes, inhibition of cholesterol synthesis should favor utilization of the cholesterol returning in lipoproteins for bile acid synthesis, and induction rather than suppression of the LDL receptor activity might be expected. Perhaps this latter activity is dependent on the intracellular concentration or distribution of

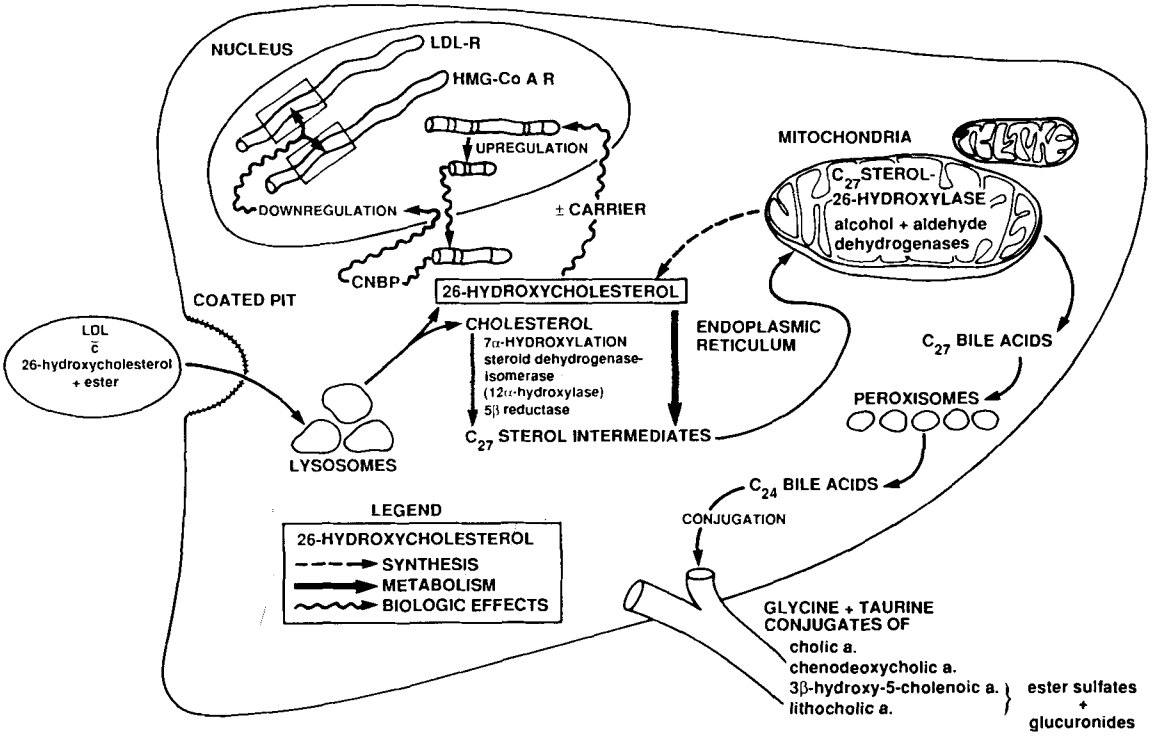

Fig. 2. Role of 26-hydroxycholesterol in cholesterol and bile acid metabolism. Cellular levels of 26-hydroxycholesterol in the liver are dependent on both mitochondrial synthesis and transport from extrahepatic tissues via plasma proteins. Metabolism to bile acids provides an undetermined fraction of total daily synthesis from cholesterol and reduces its regulatory effects on LDL receptor and cholesterol synthesis. Repression of transcription by 26-hydroxycholesterol is postulated to occur by induction of a repressor cellular nucleic acid binding protein (CNBP) that bonds to the sterol-regulatory element (SRE) at the promoter region of the LDL-R and HMG-CoA-R genes.

26-hydroxycholesterol. Hepatocytes metabolize 26-hydroxycholesterol to bile acid and therefore the intracellular concentration remains low and suppression of LDL receptor activity may not occur.

It is hoped that this working hypothesis will help in designing studies that can further elucidate the biological role of 26-hydroxycholesterol in cholesterol metabolism. Knowledge that this hydroxycholesterol is normally found in relatively large amounts in meconium (53) and that its metabolite, 3β-hydroxy-5-cholenoic acid, is found in amniotic fluid and represents 38% of the total bile acids that are detected during the last trimester of pregnancy (54) suggests possible additional roles perhaps related to the modulation of cell growth and turnover.

I wish to thank Suzanne Javitt for editing and preparing the manuscript. The author's research is supported by a grant from the National Institutes of Health (DK-32995).

Manuscript received 9 February 1990 and in revised form 3 April 1990.

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