Down-Regulation of Hepatic HMG-CoA Reductase in Mice by Dietary Cholesterol: Importance of the Δ^5 Double Bond and Evidence That Oxidation at C-3, C-5, C-6, or C-7 Is Not Involved†

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ABSTRACT: It has been suggested that the down-regulation of hepatic HMG-CoA reductase by dietary cholesterol requires modification of the cholesterol molecule before it can exert its suppressive action. In a recent study [Lund, E., Breuer, O., & Björkhem, I. (1992) *J. Biol. Chem. 267*, 25092–25097], we showed that side-chain hydroxylation is not likely to be of importance for this down-regulation in male C57BL/6J mice. In this study, we studied the possibility that modification of cholesterol in the region around the Δ^5 double bond is required for the suppression. It was shown that cholestanol, which does not have a Δ^5 double bond but is otherwise identical to cholesterol, is a poor suppressor of HMG-CoA reductase activity. Groups of mice were fed with diets containing cholestanol, epicholesterol, 6-methylcholesterol, 6-fluorocholesterol, [3α-^{2}H]cholesterol, and [7,7-$^2H_2$]cholesterol with control groups fed cholesterol or a cholesterol-free diet. These cholesterol analogues were selected to interefere with potential *in vivo* modifications and to clarify structural requirements for the down-regulation. After sacrifice, the hepatic HMG-CoA reductase activity was assayed. Cholesterol, 6-methylcholesterol, and 6-fluorocholesterol were efficient suppressors whereas cholestanol and epicholesterol only had a low suppressive capacity. Differences in the degree of absorption from the intestine or degree of esterification were too small to explain the differences in HMG-CoA reductase suppressing capacity. The two deuterated cholesterol species had a suppressive capacity similar to that of unsubstituted cholesterol. The results seem to exclude that a transformation of cholesterol in the region C-3 to C-7 is required for down-regulation of HMG-CoA reductase by dietary cholesterol and show that the Δ^5 double bond is essential. The results are consistent with the possibility that cholesterol itself is the most important suppressor of HMG-CoA reductase, at least in the specific strain of mice studied.

HMG-CoA reductase, the key enzyme in cholesterol biosynthesis, is subjected to highly elaborate regulation [for a review, see Goldstein and Brown (1990)]. In mammalian liver, the enzyme is efficiently down-regulated by dietary cholesterol. The mechanism behind this regulation has not yet been defined in detail.

The *in vivo* down-regulation of HMG-CoA reductase by dietary cholesterol can be mirrored in various cell systems by LDL. While cholesterol in itself is a poor inhibitor in these systems, several oxidized derivatives of cholesterol are efficient suppressors of HMG-CoA reductase [for a review, see Smith and Johnson (1989)]. 25-Hydroxycholesterol[1] is one of the most potent suppressors and gives the same effect as LDL not only in the suppression of HMG-CoA reductase but also in the inhibition of other enzymes and receptors involved in cholesterol biosynthesis (Chang & Limanek, 1980). In addition, in mutant cell lines resisting regulation of HMG-CoA reductase by 25-hydroxycholesterol, also the suppression by LDL is lost (Metherall et al., 1989). It has also been reported that ketoconazol is able to abolish the suppressive effect of HMG-CoA reductase by LDL (Gupta et al., 1985). Since ketoconazol is a cytochrome P-450 inhibitor, the finding is consistent with the possibility that cytochrome P-450-dependent hydroxylation of cholesterol is necessary prior to the down-regulation of HMG-CoA reductase by LDL. However, firm conclusions can be drawn from this type of experiment only if it can be convincingly shown that ketoconazol is a highly specific cytochrome P-450 inhibitor without effect on a number of other enzymes or receptors of importance. An oxysterol-binding protein has been identified, and a correlation has been demonstrated between the affinity of this protein to different oxysterols and the potential of the oxysterols to down-regulate HMG-CoA reductase in cell cultures (Taylor et al., 1984).

Observations like these have led to the widely-held view that cholesterol must be converted into an oxysterol before it can exert its suppressive action on HMG-CoA reductase. It has been suggested that (cytochrome P-450 dependent) side-chain hydroxylation of cholesterol may be of importance in the regulation of HMG-CoA reductase (Saucier et al., 1989; Rennert et al., 1990; Javitt et al., 1981). However, most studies on the regulation of HMG-CoA reductase and especially on the potential involvement of oxysterols have been performed on isolated cell systems whereas there are few studies on cholesterol biosynthesis in intact animals. The possibility that side-chain oxygenation of cholesterol is critical for the cholesterol-induced down-regulation of HMG-CoA reductase in mouse liver was recently investigated in our laboratory (Lund et al., 1992b). Utilizing the fact that side-chain

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[1] Abbreviations: cholestanol, 5α-cholestan-3β-ol; 6-methylcholesterol, 6-methylcholest-5-en-3β-ol; 6-fluorocholesterol, 6-fluorocholest-5-en-3β-ol; epicholesterol, cholest-5-en-3α-ol; 25-hydroxycholesterol, cholest-5-ene-3β,25-diol; 7α-hydroxycholesterol, cholest-5-ene-3β,7α-diol; 7β-hydroxycholesterol, cholest-5-ene-3β,7β-diol; 7-oxocholesterol, 3β-hydroxycholest-5-en-7-one; cholesterol 5,6α-epoxide, 5,6α-epoxy-5α-cholestan-3β-ol; cholesterol 5,6β-epoxide, 5,6β-epoxy-5β-cholestan-3β-ol.

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hydroxylations of specific deuterated cholesterol species are associated with a kinetic isotope effect in a specific strain of mice, we showed that side-chain hydroxylations are not likely to be involved in cholesterol-induced down-regulation of the enzyme in mouse liver.

Interestingly, we (Björkhem et al., 1985) and others (Kandutsch & Packie, 1970) have found that dietary cholestanol, the 5,6–saturated analogue of cholesterol, is a poor inhibitor of HMG-CoA reductase. This suggests that the Δ^5 double bond is necessary for the down-regulation of the enzyme. If an intermediate conversion of cholesterol is necessary for the down-regulation, this may thus be dependent upon the Δ^5 double bond. Epoxidation of the Δ^5 double bond or allylic oxidation in the 7-position give oxysterols that are known to be efficient inhibitors of cholesterol synthesis in various cell systems (Smith & Johnson, 1989; Smith, 1981).

Mice were fed with cholesterol, cholestanol, [7,7-$^2H_2$]cholesterol, [3α-^{2}H]cholesterol, 6-fluorocholesterol, 6-methylcholesterol, and epicholesterol followed by assay of hepatic HMG-CoA reductase. These cholesterol analogues were selected to interfere with potential *in vivo* oxidations and to define structural requirements for the down-regulation of the enzyme. Since differences in the suppressive capacity may be related to differences in the degree of absorption, we have measured the recovery of the different steroids in the circulation after oral administration. We have also evaluated the absorption of the different steroids in a lymph fistula rat.

The possibility that there are differences with respect to degree of esterification of the different steroids was also investigated.

In order to estimate isotope effects *in vivo* for the transformation of 7-deuterated cholesterol into 7-oxygenated species, two mice were fed a mixture of [4-^{14}C]cholesterol and [6-^{3}H-7,7-$^2H_2$]cholesterol. 7-Oxocholesterol was isolated from the livers, and the ratio of ^{3}H to ^{14}C was determined.

MATERIALS AND METHODS

Compounds for Feeding Experiments. 6-Fluorocholesterol was a gift from Du Pont Merck Pharmaceutical Co., Wilmington, DE. The synthesis and properties of this compound are described previously (Boswell, 1980). [7,7-^{2}H]Cholesterol was synthesized as described (Lund et al., 1992a).

All compounds were analyzed with GC-MS, and the spectra and GC retention times were consistent with those expected.

Synthesis of Cholest-5-en-3α-ol (Epicholesterol). To a solution of potassium *tert*-butoxide (600 mg) in 4 mL of *tert*-butanol was added a solution of 4-cholesten-3-one (200 mg) in 4 mL of *tert*-butanol and 4 mL of dry tetrahydrofuran under an argon atmosphere. The mixture was stirred for 2 h at 50 °C. A solution of acetic acid, 2.5 mL in water (7.5 mL), was added. The mixture was stirred for 1 min, and the product, cholest-5-en-3-one, was isolated by work-up with diethyl ether, dilute sodium bicarbonate, and water and dried over $MgSO_4$. This compound was immediately dissolved in 10 mL of THF. The solution was cooled to −75 °C under an argon atmosphere; 0.8 mL of potassium tri-*sec*-butylborohydride, 1 M in tetrahydrofuran, was added, and the solution was stirred for 1 h. An aqueous solution of saturated ammonium sulfate (1 mL) was added, and the mixture was slowly allowed to reach room temperature. After work-up with diethyl ether, hydrochloric acid, and water, 150 mg of crude cholest-5-en-3α-ol was isolated. This compound was purified on aluminum oxide and by HPLC; total yield of pure product, 80 mg. The mass spectrum (as TMS ether) was virtually identical to that of cholesterol, but it eluted earlier on a methyl silicone GC column. The configuration at C-3 was established with proton NMR.

Synthesis of [3α-^{2}H]Cholesterol. Cholest-5-en-3-one was synthesized as above. This compound was reduced using 3 equiv of sodium borodeuteride in ethanol. The product was worked-up using diethyl ether, water, and dilute hydrochloric acid, and purified as above.

Synthesis of 6-Methylcholest-5-en-3β-ol. Methylmagnesium iodide was prepared by mixing magnesium (25 mg) and methyl iodide (50 μL) in 6 mL of dry diethyl ether with stirring of the solution until the reaction apparently had stopped and there was a greyish semi-opaque solution. 3β-*tert*-Butyldimethylsilyloxy-5α-cholestan-6-one, prepared as described (Corey & Venkateswarlu, 1972) from 200 mg of 3β-hydroxy-5α-cholestan-6-one, was dissolved in dry diethyl ether (4 mL) and added to the solution of methylmagnesium iodide. After being stirred for 4 h, 3 mL of moist acidic diethyl ether was added. Work-up with diethyl ether, water, and dilute hydrochloric acid yielded 180 mg of crude product containing 80% (GC) 3β-*tert*-butyldimethylsilyloxy-6-methyl-5α-cholestan-6-ol (configuration at C-6 not determined). This material was purified on aluminum oxide, yielding 120 mg of pure (GC) product. The mass spectrum was consistent with that expected. The compound was dehydrated using phosphorus oxychloride as described (Corey & Gregoriou, 1958), yielding 90 mg of pure 3β-*tert*-butyldimethylsilyloxy-6-methylcholest-5-ene after chromatography on aluminum oxide. The *tert*-butyldimethylsilyloxy group was removed using tetrabutylammonium fluoride in tetrahydrofuran (Corey & Venkateswarlu, 1972). The product, 6-methylcholest-5-en-3β-ol (70 mg), showed a mass spectrum (as TMS ether) consistent with that expected. Ions detected included those at *m/z* 472 (M, 22%), 457 (M–15, 10%), 382 (M–90, 39%), 367 (M–90–15, 100%), 343 (M–129, 59%), and 269 (ABCD ring ion, 5%). The locations of the methyl group and the double bond were established by proton NMR.

Synthesis of [3β-^{3}H]Epicholesterol. Cholest-5-en-3-one was synthesized as described above and reduced using solid sodium [^{3}H]borohydride in ethanol. The [3β-^{3}H]epicholesterol formed was separated from the much more abundant [3α-^{3}H]cholesterol using TLC (ethyl acetate/toluene, 7:3 v/v).

Synthesis of [3α-^{3}H]Cholestanol. Cholestan-3-one was reduced using solid sodium [^{3}H]borohydride (Amersham PLC, U.K.) in ethanol. The product, [3α-^{3}H]cholestanol, was purified with TLC (ethyl acetate/toluene, 7:3 v/v).

Synthesis of [6-^{3}H-7,7-$^2H_2$]Cholesterol. This compound was synthesized in the same way as [6,7,7-$^2H_3$]cholesterol in Lund et al. (1992a), with sodium [$^2H_4$]borohydride replaced by sodium [$^3H_4$]borohydride; total yield, 4.2 mg/300 Mcpm (600 Mdpm).

Treatment of 6-Fluorocholesterol with an Epoxidizing Agent. *m*-Chloroperbenzoic acid was added in parallel in small and equivalent portions to two chloroform solutions of equivalent amounts of cholesterol and 6-fluorocholesterol, respectively. The reactions were continuously monitored with GC. When all cholesterol had been epoxidized, no conversion of 6-fluorocholesterol could be detected.

Animals. Male C57BL/6J mice, 9 weeks at delivery, were used at ages 11–13 weeks. The diet was pelleted R3 or R36 standard food (Lactamin AB, Sweden). Several days before the mice were used in experiments, the pellets were replaced with the same diet in powderized form. The mice were then fed individually with 6 g of food/day. Only the animals consuming all the food were used in the experiments, as starving is known to result in lower HMG-CoA reductase activities

(Bucher et al., 1959). Six grams of steroid-containing diet blend was composed of 5.45 g of diet powder, 0.55 g of peanut oil (cholesterol-free), and 7.06 μmol of steroid (corresponding to 2.7 mg of cholesterol). The steroids were quantitated and purity-checked with GC. These amounts correspond to 10 g of powder, 1 g of peanut oil, and 5 mg of cholesterol. Groups of mice were also fed diet mixtures containing 50% and 100% higher amounts of 6-methylcholesterol and cholestanol, respectively. With the exception for 6-fluorocholesterol, which was pure on delivery, all steroids were purified with HPLC immediately prior to the dietary experiments (Lund et al., 1992b). The control diet was composed in the same way with the exception of the absence of steroid. The mice were fed individually in the morning with 6 g of the steroid-containing diet and killed 24 h later. The liver was removed, and HMG-CoA reductase activity was assayed immediately as described previously (Angelin et al., 1984).

For evaluation of the degree of absorption and recovery of the steroids in the circulation, mice were fed a mixture of [4-^{14}C]cholesterol (15 Mcpm) and [3α-^{3}H]cholestanol (45 Mcpm), or a mixture of [4-^{14}C]cholesterol (2 Mcpm) and [3β-^{3}H]epicholesterol (6 Mcpm), or a mixture (about 1:1, w/w, total concentration in diet, about 0.1%) of 6-fluorocholesterol and 6-methylcholesterol with about 10 Mcpm of [^{14}C]cholesterol. The steroids were added to the diet in the same way as above, but the total amount of food was 2–3 g. The mice starved the night before the experiment. The food was given at 8.30 a.m., the mice were allowed to eat ad libitum, and a serum sample was taken at 4.30 p.m. The serum was mixed with 5 volumes of saline and extracted with 25 volumes of chloroform/methanol (2:1). The organic phase was evaporated to dryness and purified on Bond-Elut NH_2 columns as described (Kaluzny et al., 1985). Only the neutral lipid phase was collected. For estimation of the recovery of epicholesterol and cholestanol, the samples were counted in a Rackbeta (Wallac Oy, Turku, Finland) scintillation counter. The recovery of epicholesterol and cholestanol relative to cholesterol was determined by dividing the ratio of ^{3}H to ^{14}C obtained from the serum sample by the ratio of ^{3}H to ^{14}C obtained from the steroid mixture given in the diet. For determination of the recovery of 6-methylcholesterol and 6-fluorocholesterol, the samples were purified with analytical reversed-phase HPLC (Lund et al., 1992a) after purification on Bond-Elut columns. One fraction containing cholesterol [retention time (rt) 30.3 min] was collected, as well as one containing 6-methylcholesterol (rt 31.6 min) and another containing 6-fluorocholesterol (rt 26.0 min). The radioactivity in the cholesterol fraction was assayed. The fractions containing the 6-substituted sterols were pooled, and 2 μg of [25,26,26,26,27,27,27-$^{2}H_7$]cholesterol (Larodan AB, Malmö, Sweden) was added to this pooled fraction, as well as to a small fraction of the steroid mixture given in the diet containing approximately the same amount of radioactivity. These samples were assayed with GC-MS (Lund et al., 1992a) in the selected ion monitoring mode after conversion to trimethylsilyl ether. The ions used were m/z 465 ([25,26,26,26,-27,27,27-$^{2}H_7$]cholesterol), m/z 472 (6-methylcholesterol), and m/z 476 (6-fluorocholesterol). Additional full-spectrum analyses were performed in order to ascertain the identities of the recovered compounds. The relative recovery of the 6-substituted steroids was calculated as follows: recovery = $[(s_{se}/ch_{se})/(s_{di}/ch_{di})]^{14}C_{di}/^{14}C_{se}$, where s denotes the area of peak corresponding to the 6-substituted sterol in the ion chromatogram, ch denotes the corresponding area for [25,-26,26,26,27,27,27-$^{2}H_7$]cholesterol, and the suffix se denotes a serum sample whereas the suffix di denotes a sample from the steroid mixture added to the diet. ^{14}C denotes ^{14}C activity.

The degree of esterification of the different steroids was determined as follows: 1.7 mg of epicholesterol, 1.4 mg of 6-fluorocholesterol, 1.8 mg of 6-methylcholesterol, and 6 Mcpm of [4-^{14}C]cholesterol were mixed in 4 mL of toluene, and a 15-μL sample was drawn, whereafter the mixture was dried under a stream of argon. The sample was diluted to 150 μL, 15 μL was drawn again, and 2 μg of [26,26,26,27,27,-27-$^{2}H_6$]cholesterol was added. Diet was prepared from the steroid mixture, 4 g of R3 powder, and 0.4 g of peanut oil. One mouse was subjected to this blend for 8 h. The liver was removed, and a 10% homogenate was prepared (Akerlund & Björkhem, 1990). The homogenate was extracted with chloroform/methanol (2:1, v/v), and a small sample was chromatographed with TLC using toluene/ethyl acetate (6:4, v/v) as mobile phase. The TLC plate was analyzed with a Berthold Tracemaster radioactivity scanner. Cholesterol esters appeared as a single peak at R_f = 0.79 and free cholesterol at R_f = 0.43. Thus, the degree of esterification of newly administered cholesterol could be determined. The rest of the extract was divided in two halves which were evaporated to dryness in vacuo. One of the halves was then boiled under reflux for 1 h in 2 M NaOH in ethanol/water and extracted with diethyl ether. The extract was evaporated to dryness in vacuo, and several small portions of it were purified as described (Lund et al., 1992a) using the analytical reverse-phase method. The eluates were collected in two fractions: one from 21 min to the beginning of the cholesterol peak (by UV detection) and from the end of the cholesterol peak and 3.5 min more. This fraction contained the steroids fed except for cholesterol. In the other fraction, the cholesterol was collected. The same HPLC procedure was applied to the nonsaponified material. The activity of the cholesterol fractions was determined by liquid scintillation counting. To the fractions containing the other steroids was added 1 μg of [26,26,26,27,27,27-$^{2}H_6$]cholesterol. After evaporation and conversion to TMS ethers, these samples and the sample drawn from the initial steroid mixture were analyzed with GC-MS in the selected ion monitoring mode using deuterated cholesterol as internal standard. The following ions were used (m/z): 6–methylcholesterol, 472; 6-fluorocholesterol, 476; epicholesterol, 458; [26,26,26,27,27,27-$^{2}H_6$]cholesterol, 464. Additional full-spectrum analyses were performed in order to ascertain the identities of the recovered compounds. Thus, the amounts of the different steroids in the fractions were determined. Combined with the activity of the cholesterol fraction from the saponified and the unsaponified extract and the degree of esterification of cholesterol, the degree of esterification of each individual steroid could be calculated. The degree of esterification of cholestanol was determined for another mouse the same way as for cholesterol, using 30 Mcpm of [3α-^{3}H]cholestanol as the food additive.

Determination of the Absorption of the Steroids in a Lymph Fistula Rat. A 150-g rat was lymph-fistulated as described (Akerlund & Björkhem, 1990). At 6.30 p.m., the rat was given a diet mixture containing 6.90 mg of [26,26,26,27,-27,27-$^{2}H_6$]cholesterol, 7.87 mg of epicholesterol, 7.04 mg of cholestanol, 6.54 mg of 6-fluorocholesterol, 7.09 mg of 6-methylcholesterol, and 10 Mcpm of [4-^{14}C]cholesterol in 1.35 g of peanut oil and 13.5 g of R36 powder. The lymph was collected continuously in approximately 2-h fractions until 1 p.m. the day after. The lymph fractions were diluted with water and extracted with chloroform/methanol (2:1, v/v). The extracts were dried under an argon stream; the residue

was converted to TMS ethers and analyzed with GC-MS the same way as for the esterification samples (m/z 460 was used for cholestanol). Also a small amount of the intial steroid mixture was analyzed. The ratio between each steroid to [26,26,26,27,27,27-$^2H_6$]cholesterol in the lymph fractions was found to be about constant in the different samples, with the exception of epicholesterol, and was taken as a measure of the absorption of the steroid compared to cholesterol.

Demonstration of an Isotope Effect in Vivo for the Conversion of [7,7-$^2H_2$]Cholesterol to 7-Oxocholesterol. Mouse diet was prepared from 20 g of R36 powder, 2.0 g of peanut oil, 150 Mcpm of [6-^{3}H-7,7-$^2H_2$]cholesterol, and 50 Mcpm of [4-^{14}C]cholesterol. Two mice were fed this blend during 2 days. The livers were then removed, and 10% homogenates were prepared (Akerlund & Björkhem, 1990), which were extracted with chloroform/methanol (2:1); 200 μg of unlabeled 7-oxocholesterol was added to the extracts in order to facilitate the detection and minimize unspecific loss during the purification. The extracts were purified with HPLC using a semipreparative reversed-phase system (Lund et al., 1992a). Fractions were collected around the retention time of 7-oxocholesterol. These fractions were rechromatographed with TLC twice, first using 3:7 and then 15:85 (toluene/ethyl acetate, v/v) as mobile phase. Finally, the 7-oxocholesterol spots (one for each mouse) were scraped and analyzed in a scintillation counter. Backgrounds were scraped just below the 7-oxocholesterol spot.

NMR. Proton NMR was run on a Bruker AM-400 instrument at 400 MHz. The spectra were obtained from chloroform solution with tetramethylsilane as a reference zero.

RESULTS

Absorption, Recovery, and Esterification of the Cholesterol Analogues. Mice were fed a diet containing a mixture of [^{3}H]cholestanol and [^{14}C]cholesterol or a mixture of [^{3}H]-epicholesterol and [^{14}C]cholesterol. The ratio of ^{3}H to ^{14}C in the mixtures was determined by liquid scintillation counting prior to preparation of the diet. Blood samples were drawn from the mice after 8 h. After work-up of the serum, the ratio of ^{3}H to ^{14}C was determined again. These experiments were performed in triplicate, and the results are shown in Table 1. The recovery of cholestanol was found to be 33% of that of cholesterol, and the recovery of epicholesterol was found to be 44% of that of cholesterol.

In order to evaluate the recovery of the other steroids in the circulation, 6-methylcholesterol, 6-fluorocholesterol, and [^{14}C]-cholesterol were administered in the same way to mice. The ratio of the compounds was determined prior to administration and in the serum samples after work-up by GC-MS and liquid scintillation counting. The degree of recovery of 6-substituted steroid relative to cholesterol was calculated. Also, these experiments were performed in triplicate, and the results are shown in Table 1. The recovery of 6-methylcholesterol was found to be 22% of that of cholesterol, and the recovery of 6-fluorocholesterol was found to be 137% of that of cholesterol.

In order to ascertain that the recovery data correspond well to the absorption, experiments with lymph fistula mice would have been suitable. It was not possible to use such an animal model, however. Since species differences could be expected to be small in this respect, a lymph fistula rat was used. Such a rat was fed a diet containing the different steroids investigated together with [26,26,26,27,27,27-$^2H_6$]cholesterol. Lymph samples were drawn approximately every 2 h, and were analyzed with GC-MS. The ratio of steroid to [26,26,26,-27,27,27-$^2H_6$]cholesterol was taken as a measure of the absorption of different steroids related to cholesterol. The results are given in Table 1. It was found that the recovery data corresponded very well to the absorption data, with the exception of epicholesterol. The absorption of epicholesterol was 163% of that of cholesterol and varied greatly with time.

Table 1: Recovery and Absorption of Dietary Steroids

steroid	recovery (in mouse) rel to cholesterol (%)[a]	absorption (in rat) rel to cholesterol (%)[b]
epicholesterol	44 ± 7	163 ± 64
cholestanol	33 ± 2	39 ± 7
6-fluorocholesterol	137 ± 0	126 ± 10
6-methylcholesterol	22 ± 2	34 ± 7

[a] For the determination of recovery, mice were given a mixture of [4-^{14}C]cholesterol and [3α-^{3}H]epicholesterol, or a mixture of [4-^{14}C]cholesterol and [3α-^{3}H]cholestanol in the diet as described under Materials and Methods. The ^{3}H and ^{14}C activity was assayed in the steroid mixture added to the diet and in worked-up serum samples from mice, and the recovery of epicholesterol and cholestanol relative to cholesterol was calculated. Similarly, a mixture of [4-^{14}C]cholesterol, 6-fluorocholesterol, and 6-methylcholesterol was added to the diet as described under Materials and Methods. The ratio of 6-fluorocholesterol and 6-methylcholesterol to [4-^{14}C]cholesterol in the initial mixture and in processed serum samples was determined using liquid scintillation counting and GC-MS with [$^2H_7$]cholesterol as internal standard. All experiments were performed in triplicate. [b] For the determination of absorption, a lymph fistula rat was given a mixture of the steroids listed in the table together with [26,26,26,27,27,27-$^2H_6$]cholesterol. Lymph was collected continuously, and the lymph reservoir was changed every approximately 2 h so that in total nine lymph fractions were obtained. The ratio of steroid to [26,26,26,27,27,27-$^2H_6$]cholesterol was determined with GC-MS for each fraction and taken as a mesure of the absorption of the steroid relative to cholesterol. The results are given as mean ± SEM (recovery) and mean ± SD (absorption).

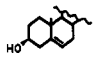
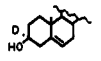
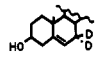
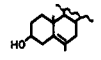
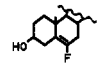
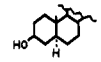
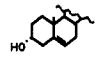
As free and esterified steroids can be supposed to have very different impacts on the regulation of HMG-CoA reductase, the degree of esterification of the newly administered (after 8 h) steroids was determined. It appeared that after 8 h, 20% of the cholesterol had been esterified whereas all of the other steroids were entirely in the free form.

Demonstration of an Isotope Effect in the Conversion of [7,7-$^2H_2$]Cholesterol into 7-Oxocholesterol in Vivo. The oxidation of cholesterol into 7-oxocholesterol in the liver occurs in the microsomes, and a marked isotope effect ($k_H/k_D \approx 15$) has been demonstrated in this conversion when the two allylic hydrogen atoms are replaced by deuterium (Lund et al., 1992a). It was considered desirable to demonstrate that there is such an isotope effect also under *in vivo* conditions.

Two mice were fed a mixture of [4-^{14}C]cholesterol and [6-^{3}H-7,7-$^2H_2$]cholesterol for 2 days. 7-Oxocholesterol was isolated from the liver, and the ratio of ^{14}C to ^{3}H was determined. The activity in the isolated 7-oxocholesterol was low, 3–6 times above the background. The isotope effect associated with the formation of 7-oxocholesterol from [6-^{3}H-7,7-$^2H_2$]cholesterol was calculated as follows: $k_H/k_D = (^{14}\text{C activity}_{liver}/^{14}\text{C activity}_{diet})(^3\text{H activity}_{diet}/^3\text{H activity}_{liver})$, and was found to be 5 and 3, respectively.

Effect of Feeding of Cholesterol Analogues on the HMG-CoA Reductase Activity. Groups of mice were fed cholesterol, epicholesterol, cholestanol, 6-methylcholesterol, 6-fluorocholesterol, [7,7-$^2H_2$]cholesterol, or [3α-^{2}H]cholesterol, and the hepatic HMG-CoA reductase activity was assayed. The results are presented in Table 2. No differences in the degree of suppression of HMG-CoA reductase activity were found between cholesterol, [7,7-$^2H_2$]cholesterol, or [3α-^{2}H]cholesterol. 6-Fluorocholesterol was found to be an effective suppressor of HMG-CoA reductase activity. 6-Methylcholesterol possessed a suppressive capacity of about 70% of that of cholesterol, without regard taken to the lower degree of recovery in the circulation.

Table 2: Suppression of Hepatic HMG-CoA Reductase by Different Steroids[a]

Steroid	n	HMG-CoA reductase activity (pmol/min,mg)	% of controls
None	14	359 ± 33	100 ± 9
 cholesterol	14	120 ± 14	33 ± 4
 [3α-^{2}H]-cholesterol	8	133 ± 22	37 ± 6
 [7,7-$^2H_2$]-cholesterol	10	114 ± 20	32 ± 6
 6-methyl-cholesterol	9	205 ± 31	57 ± 9
 6-fluoro-cholesterol	8	101 ± 13	28 ± 4
 cholestanol	10	293 ± 35	82 ± 10
 epicholesterol	9	318 ± 47	89 ± 13

[a] Groups of mice were fed a steroid-containing diet as described under Materials and Methods. After 24 h, the mice were sacrificed, the liver was removed, and the hepatic HMG-CoA reductase activity was assayed. In the table, the mean and SEM are given for the different groups of mice, as well as a truncated structural formula for each steroid.

Epicholesterol and cholestanol were found to be poor suppressors of HMG-CoA reductase activity.

Feeding four mice with a double-fold higher concentration of cholestanol in the diet did not result in a higher suppression of HMG-CoA reductase (99 ± 21%, normalized in the same way as in Table 2). Feeding four mice with a 50% higher concentration of 6-methylcholesterol in the diet resulted in a more efficient suppression of HMG-CoA reductase (22 ± 2%, normalized in the same way as in Table 2).

DISCUSSION

Requirement of the Δ^5 Double Bond in Cholesterol for Suppression of HMG-CoA Reductase. In agreement with a previous study from our laboratory (Björkhem et al., 1985), it was shown that dietary cholestanol was a poor suppressor of hepatic HMG-CoA reductase. This was not due to the lower degree of absorption compared to cholesterol, since increasing the amount of cholestanol in the diet did not result in a higher degree of suppression. In a previous study (Kandutsch & Packie, 1970), dietary cholestanol was found to suppress cholesterol biosynthesis, although to a considerably lesser extent than did cholesterol. In those experiments, the load of the steroid was much higher than in the present study.

6-Methylcholesterol was found to be a potent suppressor of HMG-CoA reductase, in particular when taking into account the low absorption and recovery of this compound. The steric effect of introducing a bulky methyl group at C-6 is considerable, and it is evident that the necessity of the Δ^5 double bond for the down-regulation of the HMG-CoA reductase is not related to stereochemical factors.

Evidence That Allylic Oxidations or Epoxidations Are Not Involved in the Suppression of HMG-CoA Reductase. Certain chemical transformations are made possible or facilitated through the presence of the double bond in cholesterol. The most important are allylic oxidation, yielding 7α- and 7β-hydroxycholesterol and 7-oxocholesterol, and epoxidation to yield cholesterol 5,6(α or β)-epoxide. A soybean lipoxygenase–linoleic acid system (Lund et al., 1992a) together with unsaturated fatty acid was found to convert 6-methylcholesterol into 7- and 5,6-oxygenated steroids about as efficiently as cholesterol. Thus, the results with dietary 6-methylcholesterol do not exclude that allylic oxidation or epoxidation is involved in the cholesterol-induced down-regulation of HMG-CoA reductase.

In vivo, 7α-hydroxycholesterol can originate from the hepatic cholesterol 7α-hydroxylase. This enzyme is active both on cholesterol and on cholestanol (Shefer et al., 1968). The cholesterol 7α-hydroxylase is strictly located in the liver whereas cholesterol biosynthesis occurs in all tissues. In view of this, it seems unlikely that this enzyme is critical for regulation of HMG-CoA reductase.

In addition to enzymatic oxidation of cholesterol at C-7, there may be unspecific allylic oxidations secondary to lipid peroxidation or the action of lipoxygenases on unsaturated fatty acids (Smith, 1981). We have shown that such unspecific oxidation of cholesterol to 7-oxygenated products is associated with marked isotope effects if the 7-hydrogens are replaced by deuterium (Lund et al., 1992a). Consequently, there must be a slower formation of 7-oxygenated compounds from 7-deuterated cholesterol than from unlabeled cholesterol. As shown here, the formation of 7-oxocholesterol from [6-^{3}H-7,7-$^2H_2$]cholesterol was associated with an isotope effect also in vivo. Since the suppression of HMG-CoA reductase by [7,7-$^2H_2$]cholesterol and unlabeled cholesterol was virtually identical, it is evident that this type of 7-oxygenation cannot be of importance for the down-regulation of HMG-CoA reductase by dietary cholesterol.

The possibility that an epoxidation of cholesterol is needed to suppress the HMG-CoA reductase seems also excluded by the experiments with 6-fluorocholesterol. The steric effect of the introduction of a fluoro atom in the 6-position is comparable to the effect of insertion of a methyl group. 6-Fluorocholesterol suppressed HMG-CoA reductase efficiently despite the strongly electronegative properties of the fluorine atom that prevent epoxidation. This observation gives further support for the contention that 7-oxygenation is not critical for the down-regulation of HMG-CoA reductase by dietary cholesterol, since the susceptibility of 6-fluorocholesterol to allylic oxidation can be expected to be lower than that of cholesterol. A linoleic acid–soybean lipoxygenase system (Lund et al., 1992a) was unable to epoxidize 6-fluorocholesterol as determined with GC-MS. 6-Fluorocholesterol was also not epoxidized by *m*-chloroperbenzoic acid (MCPBA) under conditions which readily epoxidize cholesterol.

Evidence That Oxidation of the 3β-Hydroxyl Group Is Not Involved in the Suppression of the HMG-CoA Reductase. Transformation of cholesterol to 4-cholesten-3-one, either by unspecific lipid peroxidation or by a specific 3β-hydroxysteroid dehydrogenase, must also be considered as a possible intermediate step in the cholesterol-induced down-regulation of the HMG-CoA reductase. Such an oxidation should be markedly reduced if there is an electronegative group in the 6-position, and thus the results obtained with 6-fluorocholesterol do not support that oxidation of the 3β-hydroxyl group is of importance. Oxidation of a 3β-hydroxy-Δ^5-steroid with the 3α-hydrogen replaced with deuterium can be expected to be associated with a significant isotope effect. Such an isotope effect has previously been observed in the oxidation of [3α-

^{3}H]-7α-hydroxycholesterol into 7α-hydroxy-4-cholesten-3-one by rat liver microsomes (Björkhem, 1969) and in the oxidation of [3α-^{3}H]cholesterol in intestinal bacteria (Björkhem & Gustafsson, 1971). The enzymatic oxidation of cholesterol into 4-cholesten-3-one in liver microsomes is very slow (Björkhem & Karlmar, 1974), and it was thus difficult to demonstrate *in vitro* that there is an isotope effect in the enzymatic oxidation of [3α-$^2H_2$]cholesterol into 4-cholesten-3-one. Introduction of deuterium in the 3α-position did, however, not change the suppressive capacity of cholesterol to down-regulate HMG-CoA reductase. The combined results with 6-fluorocholesterol and [3α-^{2}H]cholesterol seem to exclude the possibility that oxidation of the 3β-hydroxyl group is of importance in the regulation.

In order to evaluate the relative importance of the orientation of the 3-hydroxyl group for the cholesterol-induced down-regulation of HMG-CoA reductase, one group of mice was fed an epicholesterol-containing diet. Epicholesterol was found to be a poor suppressor. It thus seems that the orientation of the 3-hydroxyl group is of critical importance for the down-regulation of HMG-CoA reductase and that the HMG-CoA reductase suppressing capability of Δ^5 steroids is dependent upon the presence of a 3β-hydroxyl group. However, due to the inconsistencies between recovery and absorption data, it is difficult to draw firm conclusions about the importance of the 3β-hydroxyl group.

Is Any Conversion of Cholesterol Required in Connection with Suppression of HMG-CoA Reductase? According to the results of the present as well as previous work (Lund et al., 1992b; Erickson & Nes, 1982), the following structural features of cholesterol are critical for the suppression of the hepatic HMG-CoA reductase in mice: (i) The Δ^5 double bond must be present, and steric features at the 5,6-positions are relatively unimportant as long as the double bond is preserved. (ii) It is likely that the molecule must contain a 3β-hydroxyl group. (iii) The structure of the side chain is less important. Even when there is a total absence of the side chain, 3β-hydroxy-Δ^5-steroids seem to suppress the enzyme activity (Erickson & Nes, 1982).

The above requirements coupled to our demonstration that epoxidation at 5,6 or oxidation at C-7 or C-3 is not involved in the regulation are consistent with the possibility that cholesterol itself rather than an oxidized metabolite is the most important suppressor of HMG-CoA reductase. The much higher capacity of some oxysterols as compared to cholesterol to suppress cholesterol synthesis in various cell systems may be related to the higher solubility of oxysterols and the presence of a specific binding protein.

As for (i), this feature seems to be critical for the effect of cholesterol, but it is not an absolute requirement for sterol-induced down-regulation of HMG-CoA reductase. Certain synthetic 5α-stanols are very potent *in vivo* down-regulators of the enzyme (Swaminathan et al., 1992). These compounds typically contain an oxo group at C-15 and a $\Delta^{8(14)}$ double bond. It seems likely that the Δ^5 double bond is crucial for the normal physiological down-regulating activity of cholesterol and closely related compounds but that its absence can be compensated for by other structural features. Whether the above synthetic sterols at all down-regulate HMG-CoA reductase by the same mechanisms as dietary cholesterol remains, however, unclear.

In a recent study using cell cultures (Taylor, 1992), different oxysterols were tested for their ability to promote the degradation of a fusion protein (HMGal) containing the membrane domain of HMG-CoA reductase and β-galactosidase (Chun & Simoni, 1992). A good correlation was obtained between binding affinity to the oxysterol receptor and HMGal degradation. Interestingly, of the compounds tested, the four most potent with respect to the HMGal-degrading activity all possessed a 3β-hydroxy-Δ^5-structure. The fifth and sixth most active compounds contained a 3β-hydroxy-5α-H-structure. However, these compounds had a very high binding affinity to the oxysterol receptor and may therefore compensate a lower inherent HMGal-degrading activity by more efficient transport to an active site. In another cell culture study (Gupta et al., 1989), vitamin D_3 analogues were tested for their effect on cholesterol biosynthesis. All these secosteroids contain the 3β-hydroxy-Δ^5-structure. It was shown that vitamin D_3, 25-hydroxyvitamin D_3, and 24,25-dihydroxyvitamin D_3 all were effective suppressors of HMG-CoA reductase whereas 1,25-dihydroxyvitamin D_3 showed a more ambiguous behavior. Introduction of a hydroxyl group in the 1-position corresponds to a considerable perturbance in the A-ring of the molecule, and it is possible that binding to a suppressive site requires this part of the molecule to be hydrophobic. While this observation is interesting, it must also be pointed out that the overall three-dimensional structure of these secosteroids is quite different from that of cholesterol.

To summarize, it is evident that the Δ^5 double bond is of essential importance for the down-regulation of HMG-CoA reductase by dietary cholesterol and that allylic oxidations or epoxidations are not involved. Since side-chain modifications also seem to be of little importance, it is tempting to suggest that it is cholesterol itself rather than an oxidized metabolite that is the most important suppressor. If the structure of the A- and B-rings of cholesterol is most important for the suppression, it would be of interest to study the possibility that, e.g., 10β-methyldecal-5-en-3β-ol could be used for suppression of HMG-CoA reductase *in vivo*. Experiments with this and analogous compounds have recently been initiated in our laboratory.

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