In vivo formation of 25-hydroxycholesterol from endogenous cholesterol after a single meal, dietary cholesterol challenge[1]

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Abstract The role of oxysterols as regulatory molecules in the suppression of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity was investigated in the intact rat in response to an acute dietary cholesterol challenge. When rats were fed highly purified cholesterol as a single meal at a level of 5% of the diet, maximal inhibition of enzyme activity (66%) occurred 120 min after the completion of the meal. Furthermore, when nonsaponifiable liver extracts were chromatographically resolved and analyzed by high performance liquid chromatography (HPLC) and capillary gas chromatography–mass spectrometry (GC–MS), 25-hydroxycholesterol was identified in the livers of rats 120 min after the completion of the single cholesterol meal. Significantly, only barely detectable amounts of 25-hydroxycholesterol were observed in the livers from control rats fed a sterol-free diet. The biosynthetic origin of 25-hydroxycholesterol was investigated with the use of deuterated water. Rats were fed deuterium oxide (33%) ad libitum for 3 days and then killed 120 min after the completion of a single cholesterol meal. As before, 25-hydroxycholesterol was detected in the livers from cholesterol-fed rats, but not to a significant extent in livers from control-fed rats receiving a sterol-free diet. Isotope ratio mass spectrometry revealed that the fractional incorporation of deuterium into 25-hydroxycholesterol (21%) was less than that observed for cholesterol (24%) isolated from the same livers, indicating that 25-hydroxycholesterol was produced endogenously from exogenous cholesterol and not from autoxidation of cholesterol. In a separate experiment it was also shown that [^{3}H]mevalonate was incorporated into 25-hydroxycholesterol after a single meal cholesterol challenge, but was barely detected in the livers of control rats. The evidence obtained in the present article supports the hypothesis that 25-hydroxycholesterol is endogenously produced from cholesterol at early time intervals after an acute dietary cholesterol challenge. In addition, rat liver HMG-CoA reductase was inhibited by the administration of a single intragastric dose (1 μg/kg) of an aqueous solution of 25-hydroxycholesterol. Thus, the results provide strong support for the conclusion that 25-hydroxycholesterol plays a significant role in the in vivo regulation of rat liver cholesterol biosynthesis after an acute dietary cholesterol challenge.—**Johnson, K. A., C. J. Morrow, G. D. Knight, and T. J. Scallen.** In vivo formation of 25-hydroxycholesterol from endogenous cholesterol after a single meal, dietary cholesterol challenge. *J. Lipid Res.* 1994. **35:** 2241–2253.

Supplementary key words cholesterol biosynthesis • regulation • mass spectrometry • oxysterol

Much has been learned concerning the uptake of cholesterol from lipoproteins by cells via receptor-mediated endocytosis in health and disease (1). However, once cholesterol enters the cell or is endogenously formed within the cell, the nature of the intracellular sterol signals involved in the in vivo regulation of cholesterol biosynthesis after a dietary cholesterol challenge has remained a mystery.

A pioneer study by Schoenheimer and Breusch (2), based on a sterol balance technique, provided the first evidence that the addition of cholesterol to the diet significantly reduced the ability of an animal to synthesize cholesterol. The demonstration that the addition of cholesterol to the diet inhibits the conversion of [^{14}C]acetate into cholesterol in liver slices was made by Gould et al. (3–5), and was confirmed by studies from several laboratories (6–11). Siperstein and co-workers (12, 13) inferred that the principal regulated step in cholesterol biosynthesis was the conversion of HMG-CoA to mevalonic acid. The first direct demonstration that cholesterol feeding blocked HMG-CoA reductase was made by Linn (14) and was confirmed by Shapiro and Rodwell (15). Thus, HMG-CoA reductase is recognized as a major regulated enzyme in the biosynthesis of cholesterol, and its activity precisely reflects overall cholesterol biosynthesis in the liver (16).

Based upon in vitro cell culture experiments, Kandutsch, Chen, and Heiniger (17) proposed that the regulation of cholesterol biosynthesis was achieved not by cholesterol itself, but by oxygenated derivatives of cholesterol. The activity of these oxysterols was measured

Abbreviations: HMG, 3-hydroxy-3-methylglutaryl; HPLC, high performance liquid chromatography; GC–MS, gas chromatography–mass spectrometry; TLC, thin-layer chromatography; SIM, single ion monitoring.

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by their ability to suppress HMG-CoA reductase activity in various cultured cell lines when added to the cell culture media. Werthessen and colleagues (18) also proposed that oxygenated sterols might be involved in the regulation of cholesterol metabolism. In addition, various sterol 24,25-epoxides have been shown to suppress HMG-CoA reductase activity when added to the cell culture media (19–21). In general, these oxygenated derivatives were significantly more potent as down-regulators of HMG-CoA reductase than was cholesterol (17). Evidence has also been obtained showing that the formation of sterols, other than cholesterol, can be associated with the suppression of HMG-CoA reductase activity (22, 23). For example, Saucier et al. (24) showed that when a non-saponifiable extract from cultured Chinese hamster lung (Dede) cells was fractionated by HPLC and the fractions were assayed for their ability to suppress HMG-CoA reductase activity in cultured L cells, most of the inhibitory activity was associated with the 24(S),25-epoxycholesterol and 25-hydroxycholesterol chromatographic regions from these cultured cells.

Although evidence exists to support the participation of oxysterols in the regulation of cholesterol biosynthesis in various in vitro cultured cell systems, there is also evidence that does not support a role for oxysterols in the regulation of cholesterol biosynthesis in cultured cells (25). In these studies, which used a cholesterol auxotroph (CHO-215 cells) and egg lecithin-highly purified cholesterol ($\geq$ 99.5%) liposomes, it was shown that liposomal cholesterol ($>$ 30 μg/ml) was capable of down-regulating HMG-CoA reductase. Under the conditions of the experiment there was no evidence to support the synthesis of oxysterol derivatives such as 25-hydroxycholesterol. It was concluded (25) that either oxysterols more potent than 25-hydroxycholesterol were synthesized and/or cholesterol served to initiate the regulatory response.

Thus, studies with cultured cell systems both support and do not support the hypothesis that oxysterols are the initiating signals in the regulation of cholesterol biosynthesis.

There is a paucity of evidence as to the participation of oxysterols in the in vivo regulation of cholesterol biosynthesis, especially in response to an acute cholesterol challenge. Short-term in vivo studies are critical to the understanding of cholesterol regulation by oxysterols as Saucier et al. (24) have shown that, in the presence of added 25-hydroxycholesterol, HMG-CoA reductase activity declines in L-cells with an estimated half-life of approximately 1 h. The half-life of hepatic reductase has been estimated to range from 1 to 4 h, in vivo (26). Therefore, in the present study we have conducted in vivo experiments at early time intervals using a single-meal, cholesterol-fed rat model (27).

A potential difficulty in dietary sterol studies is the fact that a number of oxysterols can be formed by the autoxidation of cholesterol itself (28, 29), thus raising the concern that oxysterols might be artifacts and not true endogenous regulators of sterol biosynthesis (28). Therefore, any in vivo assessment of the regulatory significance of oxysterols must address the issue of autoxidation.

The present article demonstrates the endogenous formation of 25-hydroxycholesterol 120 min after the completion of a single cholesterol meal dietary challenge. In addition, a single dose of 25-hydroxycholesterol (1 μg/kg), administered by intragastric tube, produced inhibition of liver HMG-CoA reductase at both 3 and 16 h after the single dose. The results are consistent with the conclusion that 25-hydroxycholesterol plays a significant role in the in vivo regulation of cholesterol biosynthesis in rat liver after an acute cholesterol dietary challenge. A preliminary account of these findings has appeared (30).

MATERIALS AND METHODS

Source of materials

Cholesterol (purified through the dibromide) was purchased from Eastman Kodak. 25-Hydroxycholesterol, 7α-hydroxycholesterol, 7β-hydroxycholesterol, 7-ketocholesterol, 7-dehydrocholesterol, and desmosterol were all purchased from Steraloids Inc. 26-Hydroxycholesterol was a gift from Dr. Norman Javitt (New York University Medical Center). Lanosterol-24,25-epoxide, squalene-2,3-epoxide, and squalene diepoxide were prepared by chemical synthesis (31, 32). Squalene was purchased from Eastman Organic Chemicals. Lanosterol and dihydrolanosterol were purchased from Sigma.

Tri-Sil Z, the reagent used to form the trimethylsilyl ether sterol derivatives, was purchased from Pierce Chemical Company. Deuterium oxide (99.8%) was obtained from Aldrich. Reverse-phase silica gel (C_{18} bonded phase, 40 μm layer) was purchased from J. T. Baker. Merit Radioassay Medium was purchased from Isolabs Inc. NADPH was obtained from Sigma. [3-^{14}C]HMG-CoA (6.83×10^{-4} mCi/ml, 2.43 mM) was obtained from New England Nuclear. R,S[5-^{3}H]mevalonate (2.1 mM, 9.23 mCi/ml) was prepared by Dr. Andrzej Pastuszyn (University of New Mexico) by the method of Keller (33).

All solvents used were HPLC grade. All other materials were reagent grade obtained from commercial sources.

Synthesis of oxysterol standards

Cholesterol-24,25-epoxide was synthesized by the method of Boar, Lewis, and McGhie (32), except that desmosterol was used as the starting material instead of lanosterol. 25-Hydroxylanosterol was prepared by the method of Rodewald and Jagodzinski (34).

Preparation of highly purified cholesterol

Commercially available dibromide-purified cholesterol was further purified by bromination of the 5,6-double bond to yield the saturated 5,6-dibromo-cholestan-3β-ol

by the method of Fieser (35). Regeneration of the double bond by neutral debromination and recrystallization from methanol was performed by the method of Schoenheimer (36) to give highly purified cholesterol (m.p. 148–149°C). This method has been previously shown to give cholesterol that is both pure and stable (37). Capillary GC–MS analysis of the cholesterol purified in this manner was unable to detect the presence of any contaminating sterol (see Results).

Animals

Male Sprague-Dawley rats (80–100 g) were obtained from Harlan Inc. and were housed two animals per cage in a windowless room artificially lit between 8 PM–8 AM (with 8 AM–8 PM dark). Rats were maintained on a commercially available diet (Teklad). Food and water were available ad libitum. Animals weighed 150–200 g at the time of killing.

Sterol-free, synthetic chow diet

The synthetic rat chow was prepared by adding oleic acid (Fisher) to powdered, sterol-free rat chow (ICN Nutritional Biochemicals) and mixing thoroughly to give a moist powder with the following composition: 43% vitamin-free casein, 43% cornstarch, 8% oleic acid, 3% alphacel, 2% salt, and 1% vitamins. The cholesterol-supplemented diet was prepared immediately before use from the synthetic rat chow by adding purified cholesterol (prepared as described above) to give a 5% cholesterol powdered rat chow. GC–MS analysis showed the cholesterol-supplemented rat chow to be free of contaminating sterols (see Results).

Cholesterol feeding

Twelve male Sprague-Dawley rats were housed in pairs as described above. Five days prior to killing the rats were weighed and paired to give pairs of individual rats of similarly matched weight, and the pelleted Teklad rat chow was replaced with synthetic, sterol-free rat chow. The powered sterol-free rat chow in heavy metal bowls was placed on the floor of each rat cage. On the day of the experiment, the rats were fasted for 6 h (5 AM–11 AM). At 11 AM, three pairs of rats were fed synthetic rat chow and three pairs of rats were fed synthetic rat chow containing highly purified cholesterol (5%), ad libitum, for 60 min. (The highly purified cholesterol was first ground to a fine powder with a mortar and pestle and then mixed with the synthetic, sterol-free diet.) All food was removed from the animals at 12 noon and the amount of food consumed was measured and recorded. All rats were killed by decapitation at 2 PM (mid-dark).

The fasting/feeding schedule described here was for a 3-h experiment. For experiments at other time intervals, the fasting/feeding schedule was adjusted accordingly so that rats were always killed at mid-dark.

Deuterium oxide administration

Deuterium oxide (99.8%) was diluted with distilled water to give deuterium oxide (33% by weight) and was given, ad libitum, to the rats that were to be cholesterol-fed 3 days prior to sacrifice. The control rats received distilled water ad libitum. At the time of killing, all water was removed and the amount consumed was measured and recorded. No significant difference existed in the amount of water consumed between the control animals and those given 33% deuterium oxide.

[5-^{3}H]Mevalonate labeling

Pairs of rats were housed and fed as described above for the cholesterol feeding experiments. After the single cholesterol meal and 60 min prior to killing, one rat from each pair of control-fed and cholesterol-fed rats was given an intraperitoneal injection (0.5 ml) of R,S [5-^{3}H]mevalonolactone (2.0 mCi/ml in a 0.9% saline solution, 0.45 mM), prepared by evaporating the 675 μl of toluene from R,S [5-^{3}H]mevalonolactone (9.23 μCi/μl in toluene, 2.1 mM) under a stream of nitrogen and dissolving the resulting residue in aqueous 0.9% saline solution (3.12 ml).

Administration of 25-hydroxycholesterol

Pairs of rats were housed and fed as described above except that Teklad rat chow was fed ad libitum during the entire experiment. Aqueous solutions of 25-hydroxycholesterol were prepared by making a stock solution of 25-hydroxycholesterol (20 μM) in distilled water. This was done by sonicating recrystallized 25-hydroxycholesterol in distilled water for 30 min. Appropriate dilutions of the stock solution were performed to give solutions of appropriate lower concentrations. Syringes (2 ml), equipped with ball-tipped curved needles, were used to give each rat a single intragastric dose of aqueous 25-hydroxycholesterol (in 0.5 ml distilled water), either 3 or 16 h prior to killing. Control rats were tube-fed 0.5 ml of distilled water in an identical manner. All rats were killed at mid-dark and excised livers were used to prepare microsomes for the assay of HMG-CoA reductase activity.

Buffers

All rat liver microsomal isolations and incubations were performed using buffer A (0.25 M sucrose, 0.05 M potassium chloride, 0.03 M ethylenediaminetetraacetate, 0.03 M potassium phosphate dibasic, 0.01 M potassium phosphate monobasic, 0.01 M dithiothreitol, pH 7.2).

Preparation of anion exchange columns

Resin used for the separation of HMG-CoA from mevalonate in rat liver microsomal preparations was prepared by converting the chloride salt form of Dowex-1 anion exchange resin (dry mesh 200–400, Sigma) to the hydroxide form by the addition of 1 N sodium hydroxide (20 volumes), followed by draining and rinsing with dis-

tilled water (5 volumes). Further treatment with 1 N formic acid (3 to 4 volumes) produced the formate salt. After rinsing with distilled water (5 volumes) and draining off the liquid, the damp resin was combined with distilled water (3 parts by weight) and the resulting resin slurry (5 ml) was poured into a polystyrene column (QS-J Isolab, Inc.) and allowed to drain. Dimensions of the settled resin were 0.7 × 4.0 cm. Columns were stored in the dark at 4°C with the resin in the column submerged in distilled water.

Preparation of liver microsomes

Rats were killed by decapitation and excised livers from pairs of animals were quickly chilled in buffer A, cut into small pieces, and homogenized in buffer A (2 ml/g of liver) with a tight-fitting, motor-driven Teflon pestle. The homogenate was centrifuged twice at 17,000 *g* at 4°C for 30 min in a Sorval RC-2B centrifuge. After each centrifugation, the top two-thirds of the supernatant was collected by pipette and the remaining homogenate was discarded. The supernatant from the second centrifugation was centrifuged at 240,000 *g* at 4°C (70.1 Ti rotor) for 30 min in a Beckman L8-M ultracentrifuge. The soluble supernatant was discarded and the microsomal pellet was homogenized in buffer A (1 ml/g original liver). Three additional centrifugations and resuspensions were conducted at 240,000 *g* at 4°C for 30 min. Each time the supernatant was discarded and the microsomal pellet was homogenized in buffer A (0.5 ml/g original liver). After the final centrifugation, the microsomal pellet was homogenized and diluted with buffer A to approximately 1.0 mg protein/ml homogenate. Protein determinations of microsomal suspensions were performed by the method of Bradford (38). Microsomes were either used immediately or frozen in liquid nitrogen and stored at −80°C.

Assay of HMG-CoA reductase activity

HMG-CoA reductase activity was assayed by a modification of the method described by Avigan, Bhathena, and Schreiner (39). The technique as modified can handle large numbers of assays and is more economical. Briefly, rat liver microsomal suspensions (200 μl of a microsomal suspension containing approximately 1.0 mg protein/ml) were used to assay each sample for HMG-CoA reductase activity. Each liver pair was assayed in triplicate and assays always included three blanks (200 μl buffer A in each sample). Samples were preincubated at 37°C in a shaking water bath for 20 min. NADPH (20 μl of a 26.2 mM solution in buffer A) and [3-^{14}C]HMG-CoA (20 μl of a 25.5 mM solution in buffer A, 6.83 × 10^{-4} μCi/μl) were added to each sample and incubated for 30 min in a shaking water bath at 37°C. The enzymatic reaction was stopped by the addition of 12 M HCl (30 μl) to each sample. [5-^{3}H]mevalonate (100 μl, 5.36 μM, 2.05 × 10^{-3} μCi/μl) in distilled water was added to each sample as an internal standard. After room temperature incubation for a minimum of 1 h, each sample was eluted through a polystyrene column containing a Dowex-1 resin slurry (5 ml, formate form) and allowed to drain into a collection vial. This procedure separates mevalonolactone (eluted from the column by distilled water) from HMG-CoA and HMG (which are retained on the resin). Each column was further eluted with distilled water (2 ml). The radioactivity of each eluate was measured in a scintillation spectrometer after the addition of Merit Radioassay Medium (10 ml). Enzyme activities were calculated using the internal standard method of Goldfarb and Pitot (40).

Saponification and extraction of livers

Livers from pairs of rats (typically six to eight pairs) were frozen at liquid nitrogen temperature immediately after excision. Livers from rats fed either synthetic (sterol-free) rat chow, or synthetic rat chow containing highly purified cholesterol (5%) as a single meal were combined, respectively (approximately 70 g, wet weight) and saponified by treatment with a solution containing 50% aqueous KOH (200 ml) and 95% ethanol (300 ml) at reflux temperature for 3 h in the dark in an atmosphere of nitrogen. A small amount (approximately 0.001%) of butylated hydroxytoluene was added as an antioxidant. The mixture was then cooled and extracted three times with hexane (200-ml portions). The hexane layers were combined, washed twice with distilled water (100-ml portions) and once with an aqueous saturated NaCl solution (100 ml), dried over magnesium sulfate, and evaporated under vacuum to give a nonsaponifiable product. This nonsaponifiable product was dissolved in toluene (2 ml) and stored in the dark at 4°C in a Teflon-capped glass vial. A small aliquot was removed for GC–MS analysis.

Reverse-phase flash column chromatography

Flash column chromatography by the method of Still, Kahn, and Mitra (41) was used to remove the majority of the relatively large amount of cholesterol present in the nonsaponifiable liver extracts. A glass column (3.5 × 60 cm) was fitted with a fritted glass bottom and a Teflon stopcock placed at the bottom of the column. The top of the column had a glass reservoir (500 ml) and a screw-clamped, rubber gasket-lined glass hose attachment for a nitrogen line. The column was filled with dry silica gel (C_{18} bonded-phase) to a height of 15 cm, and equilibrated with methanol (100%) under nitrogen pressure (4 psi). The nonsaponifiable liver extract, dissolved in toluene, was loaded onto the column and eluted with methanol (100%) under nitrogen pressure. The flow rate was adjusted so that the solvent head above the absorbent bed dropped approximately 5 cm/min. Fractions (18 ml) were collected manually (typically 36 fractions).

Reverse-phase thin-layer chromatography was used to analyze a small aliquot (5 μl) from each fraction. All fractions containing cholesterol (later eluting) were com-

bined. All fractions eluting earlier than cholesterol (more polar than cholesterol) were combined and concentrated under vacuum. The column was washed with chloroform–methanol 2:1 (v:v) (250 ml) and then with methanol (100%, 250 ml). Both column washes were added to the cholesterol-containing fractions and concentrated under vacuum. The cholesterol-containing fraction and the polar sterol-containing fraction were each dissolved in toluene (100 μl) and stored in Teflon-lined screw-capped glass vials. A small aliquot from each fraction was removed for GC–MS studies.

Studies using authentic standard 25-hydroxycholesterol revealed a 97% recovery during this saponification and chromatographic fractionation process.

Thin-layer chromatography

The behavior of various sterols on a reverse-phase TLC slide is comparable to that for reverse-phase flash column chromatography when the solvent is the same for each system. Thus, reverse-phase TLC slides were used to assay each flash column fraction and determine its content. Glass capillary tubes (5 μl) were used to remove an aliquot from each reverse-phase flash column fraction which was then spotted onto a reverse-phase TLC slide. Synthetic standards were spotted alongside the fraction aliquots. The slides were developed in an air-tight glass chamber containing methanol (100%) and visualized by briefly immersing the slide in phosphomolybdic acid (5% in methanol) and then heating the slide on a hot plate until visualization was complete. The differences in R_f values between cholesterol and oxysterols were greater than 0.1; for example, the R_f values for cholesterol and 25-hydroxycholesterol were 0.22 and 0.46, respectively. According to Still et al. (41), differences in R_f values from TLC greater than 0.1 warrant the use of flash column chromatography for the separation of components in samples up to 360 mg.

Normal-phase HPLC

HPLC was performed at room temperature with a Waters liquid chromatograph (Model ALC/GPC), equipped with a refractive index detector, using a μPorasil normal-phase column (3.9 × 30 cm, Millipore, Waters Chromatography Division) and eluting with hexane–toluene–2-propanol 68.5:30:1.5 at a flow rate of 2 ml/min. This chromatographic system is a modification of that described by Saucier et al. (42).

For the [5-^{3}H]mevalonate labeling experiment, an aliquot (25 μl) of the concentrated polar sterol fraction from reverse-phase flash chromatography was fractionated by HPLC. Fractions for scintillation counting were collected on a LKB 2112 Redirac fraction collector and were taken to dryness under nitrogen. After the addition of Merit Radioassay Medium (2 ml) to each fraction, the radioactivity of each fraction was measured in a scintillation spectrometer.

For GC–MS studies the polar sterol fraction from the flash column was fractionated by normal-phase HPLC into two fractions, which were collected manually (0–6 min, and 6–30 min) from several injections, and were subsequently combined and concentrated. A small aliquot from the later eluting fraction (more polar than cholesterol) was removed for GC–MS analysis; the remaining sample was reinjected, and 60 fractions were collected (30 sec/fraction) using an LKB 2112 Redirac fraction collector. Various fractions corresponding in retention time to polar sterol standards were combined, concentrated, and analyzed by GC–MS. Recovery during this chromatographic procedure was determined using synthetic 25-hydroxycholesterol and analyzing appropriate fractions by GC–MS. Recovery of 25-hydroxycholesterol was determined to be approximately 88%.

Formation of sterol trimethylsilyl ether derivatives

Sterols, dissolved in toluene, were derivatized by the addition of Tri-Sil Z (trimethylsilylimidazole in pyridine, 1.5 mEq/ml) to the sterol in a Teflon-lined screw-capped glass vial at room temperature for a minimum of 10 min. This reagent has been shown by Sakauchi and Horning (43) to be an all-purpose reagent for unhindered to highly hindered steroids. It reacts quickly and smoothly with hydroxyls and carboxylic acids, but not with amines (44).

Gas chromatography–mass spectrometry

Capillary gas chromatography mass spectrometry (Gc–MS) was performed on a Hewlett-Packard 5890 gas chromatograph/5970 mass spectrometer utilizing on-column injection onto a bonded-phase cross-linked capillary column (J&W Scientific) of either 5% phenylmethylsilicone (15 m × 0.322 mm, 1.0 μm film thickness) or a 50% phenylmethylsilicone (15 m × 0.244 mm, 0.25 μm film thickness) with a helium carrier gas flow rate of 30 cm/sec and an ionizing voltage of 70 eV. Column temperatures were programmed from 100 to 270°C at 20°C/min. Exceptions to this temperature program are described with the experiments in which they occurred. Sample volumes analyzed by GC–MS typically ranged from 0.2 to 2.0 μl.

Both the 5% and 50% phenylmethylsilicone capillary columns gave baseline separation of a variety of sterols when analyzed as TMS ethers (data not shown).

RESULTS AND DISCUSSION

Experimental design

The present investigation used the single cholesterol meal model in an in vivo evaluation of the oxysterol ques-

tion in the rat. A number of features of this design were critical in order to eliminate or at least minimize autoxidation. First, the cholesterol used in these studies was highly purified (see Materials and Methods). Second, a sterol-free synthetic diet was used (casein, corn starch, oleic acid, and vitamins) for 5 days prior to the single meal feeding. Third, in vitro saponification of the rat livers was conducted in the dark, under nitrogen, and in the presence of the antioxidant butylated hydroxy toluene (BHT).

Figure 1 shows the results of an assessment of the purity of the highly purified cholesterol used in the single meal feeding studies. When analyzed by capillary GC-MS procedures designed to maximize the detection of oxysterols such as 7α-hydroxycholesterol, 7β-hydroxycholesterol, 7-ketocholesterol, and 25-hydroxycholesterol, no oxysterols were detected either in the cholesterol itself (Fig. 1A and B) or in a lipid extract of the synthetic diet containing 5% highly purified cholesterol (Fig. 1C and D). The synthetic diet, containing 5% highly purified cholesterol, was also analyzed by the same analytical procedures used to analyze the rat livers, i.e., saponification, extraction, and chromatography. Again, sensitive GC-MS procedures did not detect the presence of oxysterols in this diet (data not shown).

When three pairs of rats were fed a single meal consisting of either the sterol-free synthetic diet (controls) or the synthetic diet containing highly purified cholesterol (5%), substantial inhibition (66%) of liver HMG-CoA reductase activity occurred (P = 0.0001) 120 min after the completion of the single cholesterol meal.

Oxysterol content of rat livers 120 min after the completion of a single cholesterol meal

Three pairs of rats were each fed either synthetic rat chow or synthetic rat chow containing highly purified cholesterol (5%) as a single meal. **Figure 2A** shows the GC-MS-SIM chromatogram obtained on the HPLC fractionated oxysterol extract from control-fed rats. A very small peak, which may represent a trace quantity of 25-hydroxycholesterol, was detected (Fig. 2A) in the controls. Figure 2B shows the same analysis performed on the fractionated liver extract from the cholesterol-fed rats. 25-Hydroxycholesterol (34.57 min retention time) was ob-

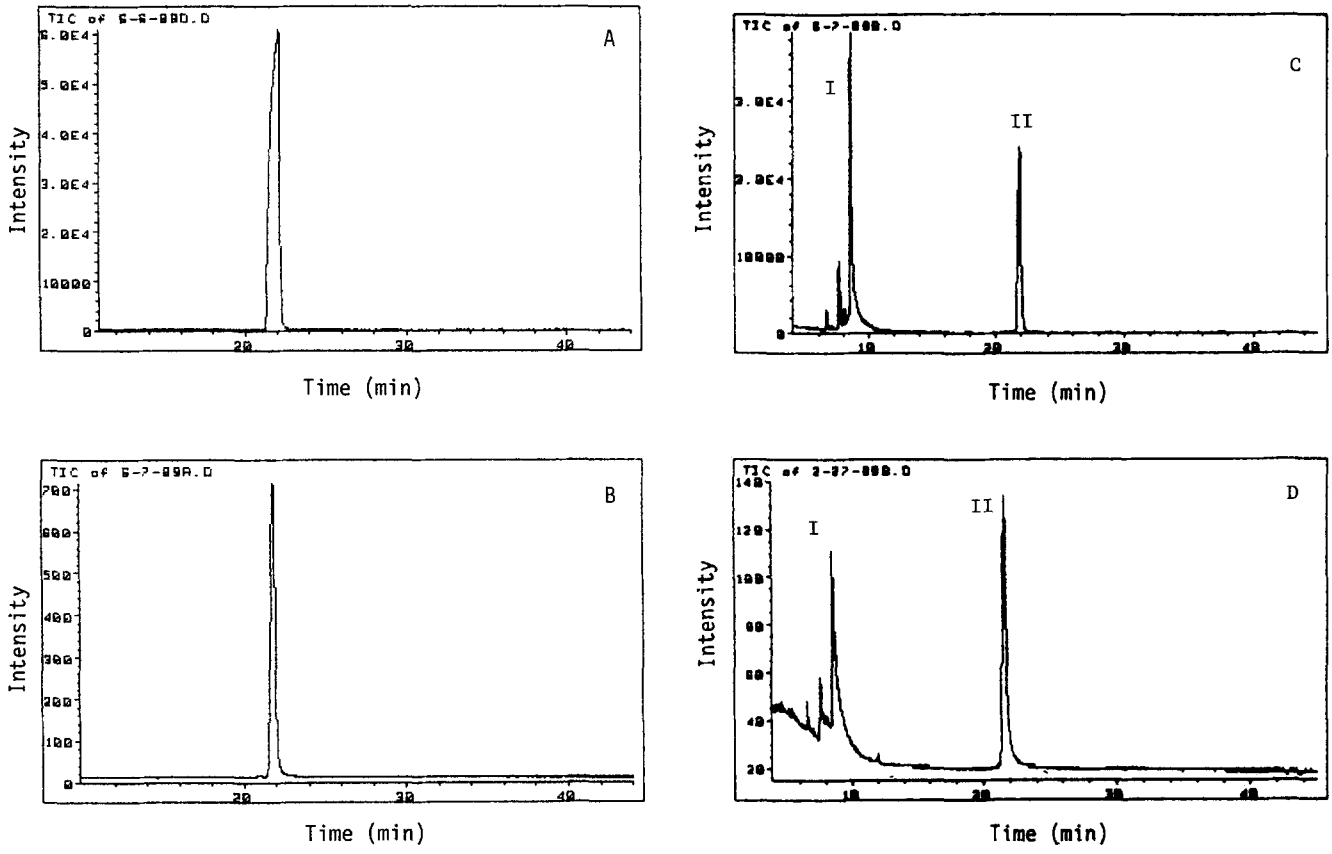

Fig. 1. GC-MS analysis of purified cholesterol and of cholesterol-supplemented synthetic rat chow. Purified cholesterol (2 μg) and cholesterol extracted from rat chow was converted to the di-TMS derivative and was analyzed by capillary GC-MS using a phenylmethylsilicone (5%) column. A: GC-MS chromatogram of purified cholesterol obtained by scanning the mass from 40 to 500 a. m. u. B: GC-MS chromatogram of purified cholesterol obtained by monitoring the ions of nominal mass 131, 271, 456, and 472. In the experiments shown in panels C and D, the 5% cholesterol synthetic rat chow (5 g) was extracted with toluene and examined by GC-MS as above. Analyses were performed on a 1-μg aliquot of TMS derivatized material. Peaks labeled I and II were identified as fatty acids (tetradecanoic acid, octadecanoic acid) and cholesterol, respectively.

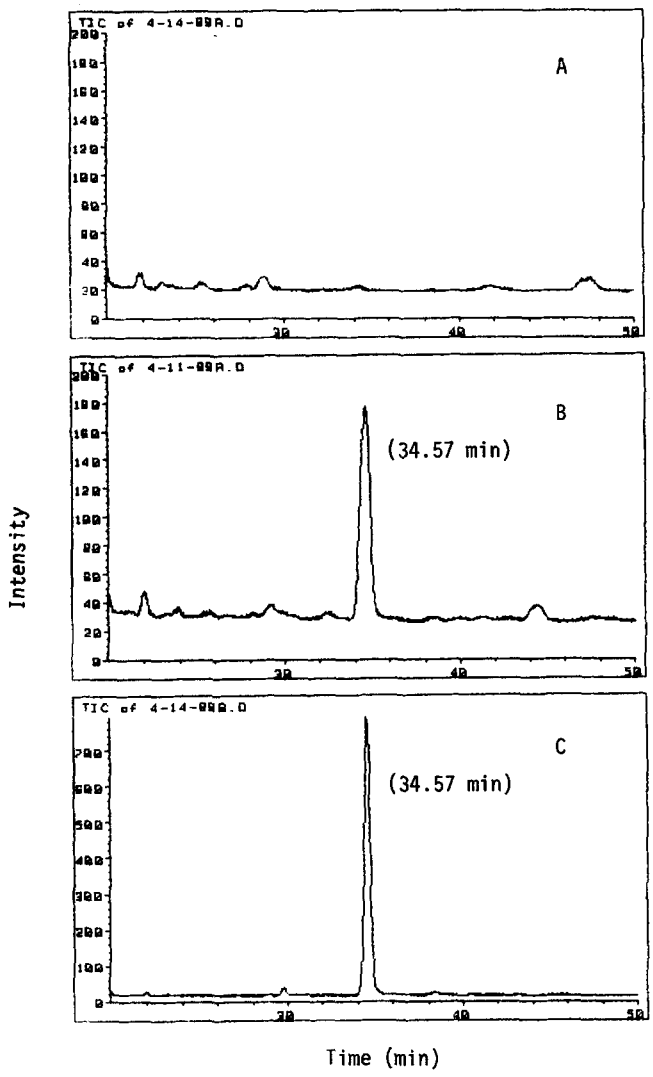

Fig. 2. GC-MS analysis of HPLC fractions corresponding in retention time to 25-hydroxycholesterol. The oxysterol fraction, recovered from a μPorasil normal-phase HPLC fractionation of the non-saponifiable extract obtained from the livers of control-fed or cholesterol-fed rats, was rechromatographed on a μPorasil normal-phase column using hexane–toluene–2-propanol 68.5: 30: 1.5 at a flow rate of 2 ml/min. Fractions 24–26 were combined, concentrated, and derivatized with Tri-Sil Z and analyzed by GC-MS using on-column injection onto a DB-5 capillary column (15 m × 0.322 mm, 1.0 μm film thickness), temperature programmed from 100 to 270°C at 20°C/min. Ions 129, 131, 271, and 456 were monitored. A: GC-MS trace of HPLC fractions 24–26 from control-fed rats; B: GC-MS trace of HPLC fractions 24–26 from cholesterol-fed rats; C: GC-MS trace of HPLC fractions 24–26 from authentic 25-hydroxycholesterol.

served and was strikingly increased when compared to the control-fed rats (Fig. 2A). Figure 2C shows the results of the same analysis performed on authentic reference 25-hydroxycholesterol-di-TMS. The retention time for authentic 25-hydroxycholesterol-di-TMS (34.57 min) was identical to the retention time observed for the 25-hydroxycholesterol isolated from the livers of the cholesterol-challenged rats (Fig. 2B, 34.57 min). These results demonstrate that the livers of rats killed 120 min after the completion of a single cholesterol meal contained a substance tentatively identified as 25-hydroxycholesterol.

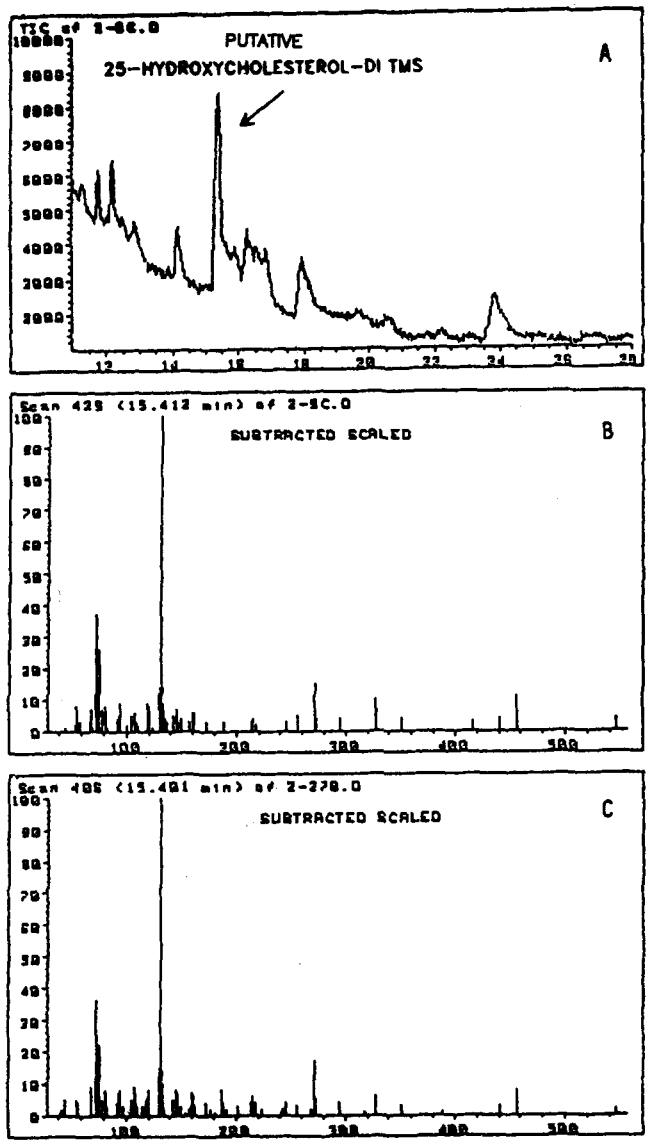

Fig. 3. Capillary GC-MS full-spectrum evidence demonstrating the presence of 25-hydroxycholesterol in the livers of single meal, cholesterol-fed rats. The polar sterol fraction, recovered from reverse-phase preparative column chromatography (100% methanol) of the non-saponifiable extract [obtained from the livers of six rats, 200 g each, killed 120 min after the completion of a single cholesterol meal], was rechromatographed on a μPorasil normal-phase column with hexane–toluene–2-propanol 68.5: 30: 1.5 at a flow rate of 2 ml/min. The oxysterol fraction from the normal-phase column was concentrated to 30 μl. A 5-μl aliquot was derivatized with Tri-Sil Z and analyzed by capillary GC-MS by scanning the mass range from 40 to 550 a. m. u. using on-column injection onto a DB-17 capillary column (15 m × 0.244 mm, 0.25 μm film thickness) temperature programmed from 200 to 270°C at 20°C/min. A: GC-MS trace of the oxysterol fraction from the livers of single-meal, cholesterol-fed rats; B: mass spectrum of putative 25-hydroxycholesterol-di-TMS from the livers of the cholesterol-fed rats; C: mass spectrum of authentic 25-hydroxycholesterol-di-TMS ether.

GC-MS studies using SIM techniques (as in Fig. 2) offer the advantage of greater sensitivity in terms of detection limits, when compared with GC-MS studies performed in the scanning mode (scanning all ions from 40 to 550 amu). However, SIM studies are not as definitive, as a full mass spectrum is not obtained. Therefore, an effort was made to obtain full spectra of oxysterols produced as a result of cholesterol feeding. The single meal cholesterol-feeding experiment was repeated, again killing the rats 120 min after the completion of the single meal. GC-MS analysis was performed in the scanning mode.

GC-MS analysis of the oxysterol fraction, obtained from the livers of rats fed a single cholesterol meal, revealed a single GC peak (**Fig. 3A**) that had a full mass spectrum (Fig. 3B) virtually identical to the mass spectrum of authentic reference 25-hydroxycholesterol-di-TMS (Fig. 3C). The mass spectrum shown in Fig. 3B for the endogenously occurring oxysterol was obtained at 15.41 min, very close in retention time to the mass spectrum of authentic reference 25-hydroxycholesterol (Fig. 3C, 15.40 min).

Quantitation of sterols

Studies conducted to detect the presence of other oxysterols, using full scanning and SIM GC-MS techniques as described above, were performed on the nonsaponifiable extracts and chromatographically resolved fractions from the livers of control and single meal cholesterol-fed rats. At the time interval studied (120 min after the completion of the single meal), no evidence was obtained to support the presence of lanosterol-24,25-epoxide, cholesterol-24,25-epoxide, 24-hydroxycholesterol, 5α-lanost-8-en-3β,32-diol (or the corresponding 32 aldehyde) in either the control liver extract or the liver extract from the cholesterol-fed rats. The concentrations of sterols (and squalene), identified in control and cholesterol-challenged rats (per gram of rat liver), are shown in **Table 1.** Cholesterol increased in the livers of the cholesterol-fed rats by 15% with the single meal cholesterol challenge (1,550,000 ng/g liver vs. 1,350,000 ng/g liver). Of the oxysterols measured, only 25-hydroxycholesterol appeared to change significantly; with the cholesterol challenge, it was 40 ng/g liver; in the control animals, it was less than 5 ng/g liver (the quantifiable detection limit of the GC-MS SIM analytical procedure). Thus, of the oxysterols measured, only 25-hydroxycholesterol changed significantly and the change was substantial, at least 8-fold. (Using the relative peak areas of the GC-MS peaks identified as 25-hydroxycholesterol in Fig. 2, there was a 14-fold increase in 25-hydroxycholesterol after the single cholesterol meal challenge.) Furthermore, this concentration (40 ng/g of liver) is in the concentration range already known from cell culture experiments to be effective in the suppression of HMG-CoA reductase [equivalent to 0.1 μM which is close to the IC_{50} of 25-hydroxycholesterol in cultured fibroblasts (0.05–0.17 μM)] (29).

Incorporation of [^{3}H]mevalonate into sterols by rats fed a single cholesterol meal

This experiment was conducted to test whether or not 25-hydroxycholesterol was endogenously synthesized from tritium-labeled mevalonate, when rats were challenged with a single cholesterol meal. **Figure 4** shows the HPLC chromatogram from this experiment. Two major peaks (II and III) were seen in the oxysterol fraction in the control (Fig. 4A) and corresponded in mobility to 7-ketocholesterol and 7α-hydroxycholesterol, respectively. 25-Hydroxycholesterol (fractions 10–13) was labeled to only a minor extent in the livers of control-fed rats (peak I). However, the HPLC chromatogram for the oxysterol fraction obtained from the livers of the cholesterol-challenged rats (Fig. 4B) showed the presence of a new peak identified as radioactive 25-hydroxycholesterol (peak I, Fig. 4B), based upon its chromatographic mobility relative to reference 25-hydroxycholesterol. In addition peaks II and III (Fig. 4B), which are also seen in the control extract, were labeled with tritium to a greater

TABLE 1. Concentration of oxysterols and squalene in the livers of single meal control and cholesterol-fed rats

Compound	Control-fed (ng/g liver)[a]	Cholesterol-fed (ng/g liver)[a]	Cholesterol-fed/ Control-fed
Squalene	48,000	35,500	0.74
Cholesterol	1,350,000	1,550,000	1.15
7-Dehydrocholesterol	112	107	0.96
7α-Hydroxycholesterol	265	222	0.84
7β-Hydroxycholesterol	457	272	0.60
6-Hydroxycholest-4-en-β-ol[b]	169	141	0.83
25-Hydroxycholesterol	$\leq$5[d]	40	$\geq$8.00
26-Hydroxycholesterol	89	91	1.02
26-Hydroxycholest-4-en-3β-ol[c]	14	17	1.21
26-Hydroxycholestanol[c]	88	85	0.97

[a] Liver mass from control-fed rats, 77 g; liver mass from cholesterol-fed rats, 76 g.
[b] Quantitated relative to 7β-hydroxycholesterol.
[c] Quantitated relative to 26-hydroxycholesterol.
[d] Limit of quantitation was determined to be approximately 5 ng of 25-hydroxycholesterol/g liver.

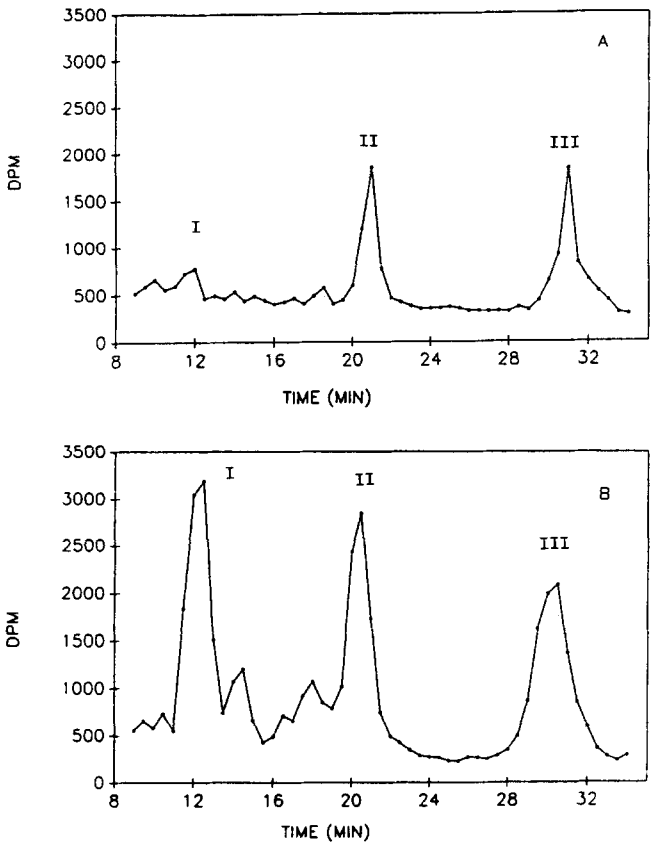

Fig. 4. Chromatographic separation of biosynthetically labeled oxysterols. One rat from each of three pairs of control-fed and three pairs of cholesterol-fed rats was injected intraperitoneally with [5-^{3}H]mevalonate (2 mCi/ml, 0.45 mCi/rat) 60 min prior to killing (60 min after the completion of the single meal). The polar sterol fraction, recovered from reverse-phase preparative column chromatography fractionation (100% methanol) of the nonsaponifiable extract from the livers of control and cholesterol-fed rats, was rechromatographed on a μPorasil normal-phase column with hexane–toluene–2-propanol 68.5:30:1.5 at a flow rate of 2 ml/min. Each fraction was assayed for radioactivity. A: oxysterol fraction from the livers of control-fed rats; I, 25-hydroxycholesterol; II, 7-ketocholesterol, III, 7α-hydroxycholesterol; B: oxysterol fraction from the livers of cholesterol-fed rats; I, 25-hydroxycholesterol; II, 7-ketocholesterol, III 7α-hydroxycholesterol.

extent than in the control (Fig. 4A). This is consistent with a stimulation of bile acid formation in response to the cholesterol challenge. Previous studies by Erickson et al. (45) showed that labeled mevalonate is quickly taken up by the liver and rapidly converted to cholesterol. Therefore, the results shown in Fig. 4A and B demonstrate the in vivo formation of 25-hydroxycholesterol from labeled mevalonate in response to a cholesterol challenge.

Incorporation of deuterium-labeled water into sterols by rats fed a single cholesterol meal

The biosynthetic origin of 25-hydroxycholesterol was investigated further with the use of deuterated water. As can be seen in **Table 2,** cholesterol showed a 24% enrichment in deuterium due to the incorporation of deuterium from deuterium-labeled water that occurred during cholesterol biosynthesis, in vivo. Biosynthetic precursors of cholesterol were labeled to a greater extent: squalene, 52% enrichment; 4-methyl-cholest-8-en-3β-ol, 33% enrichment; Δ^7-cholesten-3β-ol, 28% enrichment; and 7-dehydrocholesterol, 26% enrichment. Metabolites formed from cholesterol were labeled to a similar or lesser extent than cholesterol; cholestan-3β-ol, 24%; 7α-hydroxycholesterol, 19%; and 26-hydroxycholesterol, 7%. It is of interest to note that 25-hydroxycholesterol (present in the livers of rats 120 min after completion of the single cholesterol meal challenge) showed 21% enrichment in deuterium (Table 2), a finding that is consistent with its being biosynthesized, in vivo, from cholesterol. It is reasonable that 25-hydroxycholesterol contained approximately 10% less deuterium (21%) relative to cholesterol (24%), in view of the fact that studies by Lakshmanan and Veech (46) showed that rats given tritium-labeled water incorporated tritium into cholesterol at ten locations (one of which was at C-25). Thus, hydroxylation of cholesterol at this position would result in the loss of one deuterium atom (10% of the total).

7β-Hydroxycholesterol is a well-known autoxidation product of cholesterol (28). It was found in the liver extracts of both the control-fed animals and the cholesterol-

TABLE 2. Isotope ion mass spectrometric measurement of deuterium incorporation into sterols and squalene isolated from the livers of rats administered deuterium oxide (33%), ad libitum, for 3 days and challenged with a single cholesterol meal

Compound	Fractional Enrichment[a]
	%
Precursors	
Squalene	52
4-Methyl-cholest-8-en-3β-ol	33
Cholest-7-en-3β-ol	28
Cholest-5,7-dien-3β-ol	26
Cholesterol	24
Metabolites of cholesterol	
Cholestan-3β-ol	24
7α-Hydroxycholesterol	19[b]
26-Hydroxycholesterol	7[b]
25-Hydroxycholesterol	21[c]
Autoxidation product	
7β-Hydroxycholesterol	0

Rats were fed either a single cholesterol meal or a single sterol-free meal as described in the Materials and Methods.

[a] Values reported are a relative percent of the molecular ion [M] unless stated otherwise; fractional enrichment = [M + 1 ion abundance relative to the molecular ion (M) from the liver extracts of D_2O watered rats − M + 1 ion abundance relative to [M] from liver extracts of control watered rats]/M + 1 ion abundance relative to M from the liver extracts of D_2O watered rats.

[b] Fragment ion [M-90] and its isotope ion [M-90 + 1] were used in fractional enrichment calculations.

[c] Ion abundances from authentic 25-hydroxycholesterol used in place of ion abundances from the control liver extract (animals received a single sterol-free meal) in fractional enrichment calculations.

challenged rats. It is clearly not biosynthesized, in vivo, by the animals as no deuterium from the deuterium-labeled water was incorporated (Table 2, no enrichment). Furthermore, it clearly was not formed by autoxidation of cholesterol, in vitro, during the saponification, extraction, and chromatographic procedures, as this would have yielded a deuterium-labeled substance. This finding shows conclusively that significant cholesterol autoxidation did not occur during the saponification, chromatography, or concentration procedures. The most likely source for this substance was the commercial rat chow diet eaten by the animals prior to placing them on the synthetic, sterol-free diet. 7-Ketocholesterol was detected in the livers of control-fed rats, but not in the livers of cholesterol-fed rats; the levels of this sterol can vary widely as previously reported (47).

Exogenously administered 25-hydroxycholesterol is known to be a potent inhibitor of sterol biosynthesis in a variety of cells in culture, in perfused liver and in intact rats (45). It also has been detected in human liver by Smith et al. (48). In addition, 25-hydroxycholesterol has a marked affinity for the oxysterol binding protein (OSBP) (24) and has been shown to suppress the synthesis of HMG-CoA reductase by altering the level of its mRNA (49). Yet the physiological relevance of this oxysterol, both in vitro and in vivo, has been unclear as 25-hydroxycholesterol can also be a product of cholesterol autoxidation (28). The experiments described in the present study preclude the autoxidative origin of 25-hydroxycholesterol, as it was detected in only trace quantities in the livers from control-fed rats (Fig. 2) and it was not present in the cholesterol diet (Fig. 1) when both of these were subjected to the same analytical procedures used to analyze the livers from the cholesterol-fed rats. In addition, tritium from [5-^{3}H]mevalonate and deuterium from deuterated water were shown to be incorporated into 25-hydroxycholesterol, in vivo, in livers of single-meal cholesterol-fed rats (Figs. 4 and Table 2). This is important since, after our initial report of the presence of 25-hydroxycholesterol in the livers of single-meal cholesterol-fed rats (30), Saucier et al. (47) in a 24- and 48-h longer-term study reported the occurrence of 24-hydroxycholesterol and 25-hydroxycholesterol in the livers of cholesterol-fed mice. This study differs from the experiments described here in two important respects. First, the cholesterol-supplemented diet was not shown to be free of contaminating oxysterols. Second, the cholesterol feeding was conducted over a 1- or 2-day period, rather than a single 60-min meal as in the present study. A 60-min feeding period has the advantage that, when one looks 120 min after completion of the meal, early regulatory events can be observed. It should be noted that 24-hydroxycholesterol was not detected in the present study (Table 2). 24(S)-Hydroxycholesterol is known to be present in brain (50) and brain is a frequent source for commercial cholesterol. It is possible that this compound may have been present in the cholesterol used in the mouse study (47), as this cholesterol was recrystallized only once and it is known that this technique is not sufficient to remove oxysterol impurities (28). Alternatively, 24-hydroxycholesterol may be formed, in vivo, at the longer time intervals (24 and 48 h) used in this study (47). In addition, recent studies conducted by Lund, Breuer, and Björkhem (51), using cholesterol labeled with deuterium in the sidechain, have presented evidence that supports the conclusion that neither 24- nor 27-hydroxylation is involved in the down-regulation of HMG-CoA reductase in cholesterol-fed mice. When using [24-$^{2}H_2$]- or [23,23,24,24,25-$^{2}H_5$]-labeled cholesterol as a substrate for mouse liver mitochondria, which catalyze, in vitro, the 24-hydroxylation of cholesterol, an isotope effect of about 4.5 was observed. When using [26,26,26,27,27,27-$^{2}H_6$]-labeled cholesterol substrate, a kinetic isotope effect of about 2.5 was observed for the 27-hydroxylation. Use of these deuterium-labeled cholesterol species thus allowed a specific suppression of the rate of 24- and 27-hydroxylation. Feeding mice unlabeled pure cholesterol in the diet for 24 h inhibited the hepatic HMG-CoA reductase activity by about 50%. The same degree of suppression was obtained after feeding with [23,23,24,24,25-$^{2}H_5$]- and [26,26,26,27,27,27-$^{2}H_6$]-labeled cholesterol. Were mitochondrial 24- and 27-hydroxylation of importance, one would expect reduced suppression of HMG-CoA reductase when feeding these deuterium-labeled substrates, due to the documented isotope effects. As this was not observed (51), it is concluded that neither 24-hydroxylation nor 27-hydroxylation are critical for the cholesterol-induced down-regulation of HMG-CoA reductase in mouse liver (51). In addition, Saucier et al. (52) have shown that the intragastric administration of 24(S)-hydroxycholesterol did not lower the level of hepatic HMG-CoA reductase activity, which contrasts the findings of the present investigation for 25-hydroxycholesterol (see below).

Activity of liver microsomal HMG-CoA reductase after the intragastric administration of a single dose of 25-hydroxycholesterol

The next question addressed was whether the administration of a small single dose of 25-hydroxycholesterol to rats would produce inhibition of liver microsomal HMG-CoA reductase. As shown in **Table 3,** either 3 or 16 h after the administration of a single intragastric dose of 25-hydroxycholesterol a substantial down-regulation was observed for liver HMG-CoA reductase activity, the rate-limiting enzyme in cholesterol biosynthesis. It should be noted that 25-hydroxycholesterol is a relatively potent inhibitor of HMG-CoA reductase with 48% inhibition being observed 16 h after a single dose of 1 μg/kg. This corresponds to the administration of 0.5 ml of a 1.0 μM aqueous solution of the compound. This concentration is

TABLE 3. Activity of liver microsomal HMG-CoA reductase after the intragastric administration to rats of a single dose of aqueous 25-hydroxycholesterol

Single Dose of 25-Hdroxycholesterol[a]	Time	HMG-CoA Reductase Specific Activity[b]	Inhibition
μg/kg	h		%
0	3	1.10 ± 0.09	
1	3	0.87 ± 0.09	21[c]
0	16	1.31 ± 0.10	
0.01	16	1.39	0
0.1	16	0.93	29
1	16	0.68	48[c]
10	16	1.18	10
20	16	1.06	19

[a] Pairs of rats received a single dose of 25-hydroxycholesterol, dissolved in water (0.5 ml), by intragastric tube as described in Materials and Methods. The rats were killed at mid-dark, either 3 or 16 h after the administration of the single dose.

[b] Three pairs of rats were used for the control (0.5 ml of water administered by intragastric tube), and one pair of rats was used for each of the doses shown in the 16-h experiment. In the 3-h experiment, three pairs of rats were used for the control as well as for the 25-hydroxycholesterol (1 μg/kg). The specific activity of the control is shown ± the standard error of the mean.

[c] For the 1 μg/kg dose at 16 h, $P = 0.004$; for the 1 μg/kg dose at 3 h, $P = 0.006$.

near the limit of the water solubility of 25-hydroxycholesterol. At higher doses actually less inhibition of the reductase was observed (Table 3). Even at early time intervals (3 h), 21% inhibition of HMG-CoA reductase was observed after a single dose of 1 μg/kg ($P = 0.004$). These studies demonstrate that 25-hydroxycholesterol, dissolved in water, is an extremely potent inhibitor of liver HMG-CoA reductase in the intact rat.

Regulation of liver microsomal HMG-CoA reductase

Figure 5 shows a diagram of a regulatory scheme that is consistent not only with the results obtained here, but also with the results obtained by several other investigators. The results of the present study suggest the presence of a cholesterol-25-hydroxylase enzyme system that may be activated in response to a dietary cholesterol challenge. Evidence for the presence of this enzyme in liver has been reported (53–55). Also, a cytochrome P450 cholesterol 25-hydroxylase has been identified in rat adrenal, lung, testis, and heart (56) and a purified enzyme from pig liver mitochondria capable of 27, 25, and 24-hydroxylation (1:0.2:0.04) has been described by Lund et al. (57). These findings, coupled with the results presented here, support the formation of 25-hydroxycholesterol from cholesterol by a cytochrome P450 cholesterol 25-hydroxylase (Fig. 5). 25-Hydroxycholesterol reportedly affects HMG-CoA reductase enzyme concentration by inhibiting HMG-CoA reductase enzyme synthesis (58, 59) and/or by increasing the rate of degradation/inactivation of preformed enzyme (60–62).

Reports by Gupta, Sexton, and Rudney (63, 64) described the treatment of rat intestinal epithelial cells (IEC-6) with ketoconazole, a known cytochrome P450 inhibitor. Ketoconazole abolished the inhibition of reductase activity by cholesterol derived from low density lipoprotein (LDL). The drug did not affect the ability of the cells to take up and degrade LDL, nor did it affect the intracellular movement of unesterified cholesterol derived from LDL. As ketoconazole blocks the formation of endogenous polar sterols (63, 64), it is possible that this compound prevents the LDL-mediated suppression of reductase activity by inhibiting the cytochrome P450-dependent formation of endogenous 25-hydroxycholesterol induced by LDL cholesterol. Consistent with this premise Takagi et al. (65) have shown that ketoconazole blocks serum lipoprotein-mediated regulation of the LDL receptor gene, studied using a LDL promoter–chloramphenicol acyltransferase (CAT) fusion gene construct (pLDR-CAT 6500) transfected into JEG-3 choriocarcinoma cells. As down-regulation of this LDL promoter activity by 25-hydroxycholesterol was not blocked by ketoconazole (65), this oxysterol may circumvent the ketoconazole block of a cytochrome P450 system catalyzing the oxidation of cholesterol to 25-hydroxycholesterol.

The present article demonstrates that 25-hydroxycholesterol is endogenously produced from cholesterol at early time intervals after an acute dietary cholesterol challenge (Figs. 2–4). The endogenously produced 25-hydroxycholesterol is present in the liver of the cholesterol-challenged animals in sufficient amounts [Table 1, 40 ng/g liver,

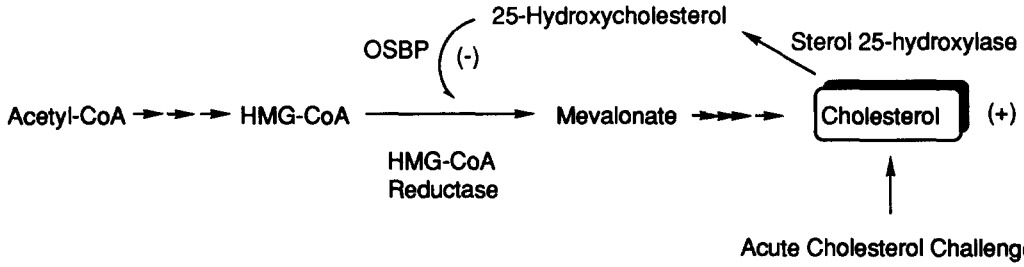

Fig. 5. Diagram postulating the in vivo regulation of cholesterol biosynthesis by endogenously produced 25-hydroxycholesterol after a dietary cholesterol challenge.

amounts nearly identical to the concentration required in cultured cells to produce 50% inhibition of HMG-CoA reductase (29)] to initiate ensuing regulatory events in the down-regulation of HMG-CoA reductase and, therefore, of liver cholesterol biosynthesis.

By analogy, the experimental findings presented here are consistent with all three of the Henle-Koch postulates (66), i.e., 25-hydroxycholesterol is increased approximately 8-fold after a single cholesterol meal (first postulate), compared to just detectable trace levels of 25-hydroxycholesterol in the livers of rats fed a single cholesterol-free meal (second postulate) (Table 2). In addition, inhibition of rat liver microsomal HMG-CoA reductase was produced by the administration of a single intragastric dose (1 μg/kg) of 25-hydroxycholesterol, both 3 and 16 h after the single dose (third postulate) (Table 3). The Henle-Koch postulates have been used widely for testing possible cause and effect relationships (66).

The experimental findings presented here provide strong support for the conclusion that endogenously formed 25-hydroxycholesterol plays a significant role in the in vivo regulation of cholesterol biosynthesis, after an acute dietary cholesterol challenge.

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