

Key regulatory oxysterols in liver: analysis as Δ^4 -3-ketone derivatives by HPLC and response to physiological perturbations

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Abstract A number of oxysterols have been implicated in metabolic regulation. Key among these are (24S),25-epoxycholesterol and (24S)-hydroxycholesterol, high affinity ligands for the nuclear transcription factor liver X receptor α ; 27-hydroxycholesterol, a bile acid synthetic intermediate; and 25-hydroxycholesterol, which has been used to study regulation of lipid metabolism by the sterol regulatory element-binding protein family of transcription factors. Investigation of the physiological importance of these compounds in vivo has been hampered by lack of analytical methods to reproducibly and accurately determine their concentrations in tissues. This article describes a method designed to determine quantitatively the amounts of these important side-chain oxysterols by derivatization to the Δ^4 -3-ketones followed by high performance liquid chromatography. The method was validated with known standards and then was used to determine the concentrations of these oxysterols in rodent liver under various physiological conditions. All four oxysterols were present in the picogram per milligram protein range and have distinct subcellular distributions and responses to physiological perturbations in vivo.—Zhang, Z., D. Li, D. E. Blanchard, S. R. Lear, S. K. Erickson, and T. A. Spencer. **Key regulatory oxysterols in liver: analysis as Δ^4 -3-ketone derivatives by HPLC and response to physiological perturbations.** *J. Lipid Res.* 2001. 42: 649–658.

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The hypothesis that oxysterols regulate cholesterologenesis was advanced by Kandutsch, Chen, and Heiniger (1) after they observed that exogenous, purified cholesterol was ineffective at suppressing the activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase in tissue culture cells (2, 3), whereas numerous oxysterols were potent reductase repressors (4–7). Discovery of a cytosolic oxysterol-binding protein (8) and demonstration of a strong correlation between the binding affinity of oxysterols to this protein and their potency as repressors of

HMG-CoA reductase (9) led Taylor and Kandutsch (10) to propose a model for cholesterol regulation by oxysterols analogous to the mechanism of steroid hormone action.

Specific oxysterols have been identified as ligands for the nuclear transcription factors liver X receptor α (LXR α) and LXR β (11–13). Mangelsdorf's group (11) suggested that oxysterol-LXR-mediated transcriptional regulation might be part of a feedback regulation of crucial metabolic pathways, such as steroid hormone or bile acid biosynthesis. Lehmann et al. (12) proposed that LXR α is activated in the liver by (24S),25-epoxycholesterol (1), that LXR β is activated in the brain by (24S)-hydroxycholesterol (2), and that cholesterol 7 α -hydroxylase (cyp7A1), a rate-limiting enzyme for bile acid synthesis, is an LXR α target. Janowski et al. (14) have described an extended investigation of structural requirements for oxysterol ligands for LXR α and LXR β . 25-Hydroxycholesterol (3) is not a high affinity ligand for the LXR family, but it has been reported (15, 16) to be the preferred ligand for the nuclear transcription factor steroidogenic factor 1, although others (17) dispute this claim.

Oxysterols also may act as suppressors in the transcriptional regulation of expression of a variety of genes involved in cholesterol and fatty acid metabolism via the sterol regulatory element-binding protein (SREBP) family of transcription factors, as described by Brown and Goldstein (18). These and other possible roles of oxysterols as modulators of cholesterol and cellular metabolism are included in a comprehensive review by Schroepfer (19).

Investigation of possible regulatory role(s) for oxysterols

Abbreviations: GC-MS, gas chromatography-mass spectrometry; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme-A; HPLC, high performance liquid chromatography; IR, infrared; LXR, liver X receptor; NMR, nuclear magnetic resonance; SREBP, sterol regulatory element-binding protein.

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in vivo must include development of methods for detection and quantification of these substances in mammalian cells and tissues. This type of analysis is highly challenging because the amounts of oxysterols present are small, typically in the range of a few nanograms per gram of tissue, and because the oxysterols do not possess a strong chromophore to facilitate detection. In addition, many oxysterols arise as adventitious autoxidation products, a fact that for a long time caused their intracellular formation and physiological relevance to be regarded with doubt (20).

In previous work, direct UV detection of oxysterols at 205–210 nm was used to analyze oxysterol concentrations in liver tissue from mice (21) and rats (22), but it became clear that this approach did not provide sufficiently accurate or reproducible quantitative results. The method described herein is based directly on that of Teng and Smith (23), who analyzed oxysterols in human erythrocytes by application of the well-known cholesterol oxidase-catalyzed conversion of 3-hydroxy sterols to their corresponding Δ^4 -3-ketones (24–29).

In the present work, analyses were limited to the four oxysterols shown in **Fig. 1A** on the basis of previous evidence that these four play specific roles in the regulation of cholesterol metabolism. (24*S*),25-Epoxycholesterol (**1**) and (24*S*)-hydroxycholesterol (**2**) had been implicated as potential regulators in our earlier investigations (22, 30) and, as described above, were found to be high affinity ligands for LXR receptors (11–13). 25-Hydroxycholesterol

(**3**) is one of the most potent oxysterols in HMG-CoA reductase repression assays (4–6, 7), and because it is commercially available, it has been widely used as a “representative” oxysterol in studies of mechanisms of regulation of lipid metabolism. It is produced enzymatically in the liver (31), where it can be converted to bile acids (32). Observations consistent with a regulatory role for this oxysterol *in vivo* have been reported (33–35). 27-Hydroxycholesterol [25(*R*),26-hydroxycholesterol] (**4**) is synthesized in many tissues, including the liver, where it also can be converted to bile acids (32).

In this article we describe the development and utilization of methodology for the quantitation of oxysterols **1–4** in liver through application of the cholesterol oxidase reaction coupled with suitable high performance liquid chromatography (HPLC) analytical procedures, including those for identification of (*R*)- and (*S*)-epimers. This work has required the synthesis and characterization of enones **5–8**, the products obtained from **1–4** by the action of cholesterol oxidase (Fig. 1A). All four oxysterols **1–4** were found in both free and esterified form in rodent liver. Their identities were confirmed by gas chromatography-mass spectrometry (GC-MS) analysis. Oxysterols **1** and **2** were shown to be present essentially exclusively as the natural, physiologically relevant *S* epimers.

EXPERIMENTAL

Materials and methods

Chemicals. Chemicals were obtained from Fisher (Pittsburgh, PA) unless indicated otherwise. Solvents for HPLC and extraction were HPLC grade. Water was purified through a nanopure system from Barnsted (Dubuque, IA) and the specific conductance was 18 M Ω -cm. Cholesterol oxidase (from *Streptomyces*), catalase, diosgenin, and 25-hydroxycholesterol (**3**) were obtained from Sigma (St. Louis, MO). Other oxysterols were prepared as described below.

Animals. Male Fischer 344 rats, 4 months (young) of age, 16 months (middle-aged) of age, and 24 months (old) of age, were from the colony maintained by the National Institute of Aging (Bethesda, MD); their livers were provided to us by M. Engler and M. Engler (School of Nursing, University of California, San Francisco, CA). Male Sprague-Dawley rats, 180–200 g (Simonsen, Gilroy, CA) were maintained on standard rodent chow and water ad libitum at the Veterans Affairs Medical Center (San Francisco, CA). In some cases, one group was fed an atherogenic diet (standard rodent chow containing 1% cholesterol, 4% lard, 0.2% cholic acid) for 2–3 weeks while their controls received the standard chow. In other cases, the animals were given mevalonic acid intragastrically as described previously (35) 1 h before death. Their controls received the same volume of vehicle. All protocols were approved by their respective institutional animal use review boards. Liver homogenates and subcellular fractions were prepared as described previously (36). Protein was determined by the biuret reaction (37), using bovine serum albumin as reference standard.

Preparation of nonsaponifiable extracts for analysis of total oxysterols.

Briefly, to a 9-ml sample of rat liver homogenate were added 21 ml of ethanol containing 21 μ g of butylated hydroxytoluene (BHT) and 3 ml of 33% KOH in H₂O. The same relative proportions were used for subcellular fractions. The vessel was flushed with N₂, capped, and heated in a shaking water bath at 79°C for

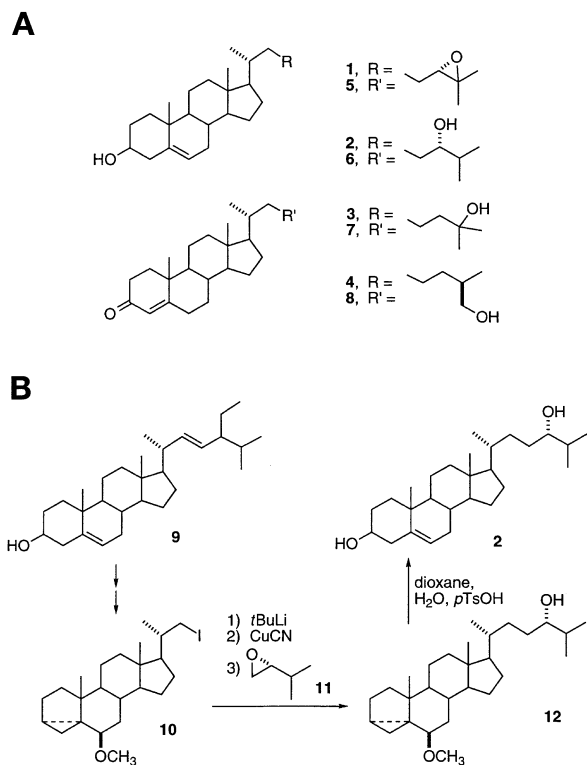


Fig. 1. A: Structures of the four oxysterols for which analytical procedures are described in this article [**1**, (24*S*),25-epoxycholesterol; **2**, (24*S*)-hydroxycholesterol; **3**, 25-hydroxycholesterol; **4**, 25(*R*),26-hydroxycholesterol] and of the enones **5–8** produced by cholesterol oxidase oxidation of **1–4**, respectively. B: Synthesis of (24*S*)-hydroxycholesterol (**2**) from stigmasterol (**9**).

4 h. The cooled mixture was extracted with 3×60 ml of petroleum ether containing BHT at $0.5 \mu\text{g/ml}$. The combined petroleum ether extracts were washed with 10 ml of H_2O and evaporated, using a rotary evaporator. The residue was transferred to a vial with 3×2 ml of CH_2Cl_2 , which was then evaporated under N_2 at room temperature.

Total lipid extraction for analysis of free oxysterols. On the basis of the Bligh-Dyer method (38), to a 9-ml sample of rat liver homogenate was added 27 ml of 2:1 $\text{CH}_3\text{OH}-\text{CHCl}_3$, BHT at $0.5 \mu\text{g/ml}$, and 100 μg of K_2CO_3 . The same relative proportions were used for subcellular fractions. This mixture was allowed to stand for 30 min with intermittent shaking, mixed with 9 ml of CHCl_3 and 7 ml of 0.14 M NaCl, shaken, and allowed to stand for about 30 min for phase separation. The CHCl_3 layer was separated and the aqueous layer was re-extracted with 2 ml of CHCl_3 . The combined CHCl_3 extracts were evaporated under N_2 at room temperature. The residue was dissolved in 1 ml of hexane- CHCl_3 19:1 and loaded onto a silica Sep-Pak cartridge (Waters, Milford, MA) that had been washed with 5 ml of hexane- CHCl_3 19:1. The cartridge was eluted with 10 ml of hexane- CHCl_3 19:1, which was discarded, and then with 10 ml of acetone, which was evaporated under N_2 .

Isolation of oxysterol fraction. To remove cholesterol, the appropriate extract (nonsaponifiable or Bligh-Dyer) was put through a C_{18} Sep-Pak cartridge (Waters) that had been washed with 5 ml of CH_3OH and 5 ml of H_2O , using $\text{CH}_3\text{CN}-\text{CH}_3\text{OH}-\text{H}_2\text{O}$ 60:35:5 (3×1 ml, followed by 5 ml) as eluant. The combined effluent (8 ml) was evaporated under a stream of N_2 at room temperature. The residue was dissolved in 100 μl of methanol and injected into a 5- μm Ultrasphere ODS semipreparative HPLC column (10×300 mm; Beckman, Fullerton, CA) with $\text{CH}_3\text{CN}-\text{methanol}-\text{H}_2\text{O}$ 45:45:10 (v/v/v) as eluant at a flow rate of 4 ml/min. The fraction containing oxysterols 1-4 was collected during the time of 17-24 min, which had been shown to include all the pertinent retention times by use of standard reference oxysterols. This HPLC analysis was done on a Waters instrument consisting of a model 510 solvent delivery system, a model U-6K injector, and a UV-visible detector at 210 nm.

Oxidation of oxysterols to cholest-4-ene-3-ones. The oxysterol fraction was evaporated with a rotary evaporator and transferred to a culture tube with 3×2 ml of CH_2Cl_2 , which was then evaporated under N_2 at room temperature. The oxysterol sample thus obtained was oxidized with cholesterol oxidase essentially according to the method of Teng and Smith (23). To the oxysterol sample dissolved in 50 μl of 2-propanol were added 250 μl of saline and 500 μl of 20 mM phosphate buffer, pH 7.5, containing 0.1% sodium cholate and 0.3% (w/v) Tween 20. The resulting mixture was then gently vortexed and treated with 100 μl of 0.1 M Tris buffer, pH 7.8, containing 60 units of catalase and 100 μl of the same buffer containing 0.4 unit of cholesterol oxidase. The reaction mixture was kept at 37°C for 1 h, and then 300 μl of methanol was added to stop the reaction. The products were extracted with 3×2 ml of petroleum ether, and the extracts were evaporated under N_2 at room temperature. The residue was dissolved in hexane and analyzed by HPLC as described below.

HPLC analysis of oxysterol enone derivatives. HPLC analysis of the enones was conducted with a Waters model 515 pump, model U-6K injector, and model 486 tunable absorbance detector at the λ_{max} of the enones, on a 5- μm Ultrasphere-Si column (4.6×250 mm; Beckman) with hexane-2-propanol 100:2 as eluant at a flow rate of 1 ml/min. The eluent presumed to contain each enone was collected from 1 min before to 1 min after the previously determined retention times of standard enones 5-8. The combined collected enones were rechromatographed in the same manner, using hexane-1-butanol 100:2. Quantification of oxysterol enone derivatives 5-8 was accomplished by

comparing the peak areas of the samples with those of authentic standard samples of 5-8. Separation of the epimers of 24,25-epoxycholest-4-en-3-one and 24-hydroxycholest-4-en-3-one was accomplished on a 5- μm chiral phase Bakerbond DNBPG column (4.6×25 mm; Beckman) with a solvent system of hexane-2-propanol 100:2 at a flow rate of 1 ml/min.

GC-MS analysis. Mass spectra were determined on an HP-5890 GC coupled to a HP-5971 MSD mass spectrometer (Hewlett-Packard, Palo Alto, CA). Helium was used as carrier gas. The column was a 0.25 mm \times 15 m RTX-1 (VWR Scientific Products, West Chester, PA) capillary column with a film thickness of 0.25 μm . A splitless injection was applied with a purge valve on at 1 min. The electron impact ionization energy of the MSD was 70 eV. When enone 5 was analyzed, oven temperature was programmed at 200°C for 2 min, and then increased to 280°C at a rate of $15^\circ\text{C}/\text{min}$, with the temperature of the injector and MS detector set at 280°C . Enone collected from rat liver samples (1-2 μg) was dissolved in 10 μl of CH_2Cl_2 and 1 μl was injected. Collected enones 6-8 (1-2 μg) were converted to trimethylsilyl (TMS) derivatives before GC-MS analysis by heating at 60°C for 15 min with 10 μl of pyridine-hexamethyldisilane-trimethylchlorosilane 3:2:1. The derivatized sample (1 μl) was injected at an oven temperature programmed at 220°C for 2 min, and then increased to 310°C at a rate of $15^\circ\text{C}/\text{min}$, with injector and MS detector temperature at 310°C .

Chemical syntheses

General. Nuclear magnetic resonance (NMR) spectra were taken on a 300-MHz Varian (Palo Alto, CA) spectrophotometer. The chemical shifts are reported in units of δ . Infrared (IR) spectra were recorded with a Perkin-Elmer (Norwalk, CT) 297 IR spectrophotometer. Melting points (mp) were determined with a Thomas-Hoover apparatus and are uncorrected. Thin-layer chromatography was carried out on EM plastic sheets pre-coated with silica gel 60 F-245 (Whatman), or polyester sheets pre-coated with Alumina B F-254 200m (Selecto Scientific, Suwanee, GA). Visualization was obtained with a UV₂₅₄ light source. Flash chromatography was carried out on EM reagent silica gel 60 (230-400 mesh), or aluminum oxide (active neutral, gamma, -60 mesh) (Alfa Aesar, Karlsruhe, Germany). MgSO_4 was used to dry all organic layers. Cholest-5-ene-3,25-diol (3) was purchased from Steraloids (Wilton, NH). TES is *N*-tris(hydroxymethyl) methyl-2-aminoethane-sulfonic acid.

(25R)-26-Hydroxycholesterol (4). By the procedures of Kim et al. (39), diosgenin was converted to 4, mp $173-175^\circ\text{C}$.

(24S)-Hydroxycholesterol (2). Stigmasterol (9) was converted to C-22 iodide (10) by the procedures of Partridge, Faber, and Uskokovic (40), as previously modified (41). *L*-Valine was converted to (*R*)-1,2-epoxy-3-methylbutane (11) as previously described (42, 43). To a solution of 413 mg (0.905 mmol) of 10 in 1 ml of dry ether at -78°C was added dropwise 2.00 ml (2.14 mmol) of 1.07 M *tert*-butyllithium in pentane with stirring under N_2 . After being stirred for 15 min, the mixture was allowed to warm and stand at room temperature for 1 h. It was then cooled to -78°C and added dropwise to a solution of 99.4 mg (1.11 mmol) of CuCN in 1 ml of dry ether at -78°C . The mixture was stirred for 5 min, and allowed to warm slowly for 20-30 min in the air. It was recooled to -78°C , and a solution of 245 mg (2.85 mmol) of 11 in 1 ml of dry ether over 3- \AA molecular sieves was added slowly. The resulting mixture was stirred at -78°C for 5 h, and allowed to stand at room temperature under N_2 overnight. A mixture of 10 ml of saturated NH_4Cl and 1 ml of NH_4OH was added to the mixture and the aqueous layer was extracted with 3×20 ml of ether. The combined organic layers were washed with 2×10 ml of saturated NH_4Cl and 10 ml of water, dried over MgSO_4 , filtered, and evaporated to give 473 mg of yellow resi-

due, which was chromatographed (ethyl acetate–hexane 1:9) to give 213 mg (56%) of **12**: $^1\text{H NMR}$ (CDCl_3) δ 3.31 (m, 4H), 2.75 (t, $J = 2.4$ Hz, 1H), 1.01 (s, 3H), 0.92 (d, $J = 6.6$ Hz, 3H), 0.91 (d, $J = 6.9$ Hz, 3H), 0.88 (d, $J = 6.9$ Hz, 3H), 0.71 (s, 3H) [literature (44) $^1\text{H NMR}$ (CDCl_3) δ 3.29 (s, 3H), 2.75 (m, 1H), 1.01 (s, 3H), 0.94 (d, $J = 6.7$ Hz, 3H), 0.92 (d, $J = 6.7$ Hz, 6H), 0.71 (s, 3H)]. $^{13}\text{C NMR}$ (CDCl_3) 82.6, 76.9, 56.8, 56.7, 56.4, 48.3, 43.6, 43.0, 40.5, 36.2, 35.5, 35.3, 33.6, 33.4, 32.4, 31.0, 30.7, 28.6, 25.2, 24.4, 23.0, 21.7, 19.5, 19.3, 19.1, 17.0, 13.3, 12.5. To 186 mg (0.448 mmol) of **12** in 3 ml of dioxane and 1 ml of water was added 47.7 mg (0.251 mmol) of *p*-toluenesulfonic acid monohydrate. The resulting mixture was stirred for 5 h at 80–85°C and 10 ml of CH_2Cl_2 and 5 ml of water were added. The aqueous layer was extracted with 3×10 ml of CH_2Cl_2 . The combined organic layers were washed with 10 ml of saturated NaHCO_3 , 3×10 ml of water, dried over MgSO_4 , filtered, and evaporated to give 148.4 mg of white solid that was crystallized from CH_3OH to give 120.5 mg (67%) of **2**: mp 175–177.5°C [literature (44) mp 175–177°C], $^1\text{H NMR}$ (CDCl_3) δ 5.36 (m, 1H), 3.50 (m, 1H), 3.32 (m, 1H), 1.02 (s, 3H), 0.94 (d, $J = 6.3$ Hz, 3H), 0.93 (d, $J = 6.6$ Hz, 3H), 0.91 (d, $J = 6.9$ Hz, 3H), 0.69 (s, 3H) [literature (45) $^1\text{H NMR}$ (200 MHz, CDCl_3) δ 5.36 (m, 1H), 3.32–3.58 (m, 2H), 1.00 (s, 3H), 0.94 (d, $J = 6.8$ Hz, 3H), 0.92 (d, $J = 7.6$ Hz, 3H), 0.90 (d, $J = 6.7$ Hz, 3H), 0.68 (s, 3H)]. $^{13}\text{C NMR}$ (CD_3OD) 142.3, 122.6, 78.3, 72.6, 58.3, 57.7, 51.9, 43.6, 43.2, 41.3, 38.7, 37.8, 37.6, 34.7, 33.7, 33.4, 33.2, 32.4, 31.9, 29.4, 25.5, 22.4, 20.0, 19.7, 19.5, 17.7, 12.5.

(24R)-Hydroxycholesterol. Via an analogous coupling with (S)-1,2-epoxy-3-methylbutane (42, 43), iodide **10** was converted to (24R)-hydroxycholesterol, mp 180.5–182°C [literature (44) mp 180–182°C; literature (46) mp 181–183°C]; $^1\text{H NMR}$ (CD_3OD) 5.33 (m, 1H), 3.37 (m, 1H), 3.21 (m, 1H), 1.02 (s, 3H), 0.95 (d, $J = 6.6$ Hz, 3H), 0.90 (d, $J = 6.9$ Hz, 3H), 0.73 (s, 3H); $^{13}\text{C NMR}$ (CD_3OD) 142.4, 122.6, 77.9, 72.6, 58.4, 57.7, 51.9, 43.7, 43.2, 41.3, 38.7, 37.8, 37.2, 35.0, 33.5, 33.4, 33.2, 32.4, 31.7, 29.4, 25.5, 22.4, 20.0, 19.6, 19.4, 18.1, 12.5.

(24S),25-Epoxycholesterol (**1**). Synthesis of **1** was performed as previously described (41).

(24R),25-Epoxycholesterol. Synthesis of this epimeric epoxide was performed as previously described (41).

(25R),26-Hydroxycholesterol-4-en-3-one (**8**). To a solution of 50.2 mg (0.125 mmol) of **4** in 3 ml of ethyl acetate and 1 ml of 1-butanol was added 1.5 ml of 50 mM TES solution (pH 7.5) containing 5 units of cholesterol oxidase and 3,500 units of catalase. The reaction mixture was stirred at room temperature for 60 h, the separated organic layer was evaporated and redissolved in 5 ml of ethanol, and 2 mg of oxalic acid was added. The resulting mixture was stirred at 60°C for 30 min. After evaporation of most of the ethanol, the residue was extracted with 3×2 ml of CH_2Cl_2 . The combined organic layers were dried, filtered, and evaporated to give 51 mg of white solid, which was chromatographed (silica gel, ethyl acetate–hexane 3:7) to give 26.1 mg (52%) of **8**: mp 135.5–136°C [literature (47) 130–133°C; literature (45) 138–139.5°C]; $^1\text{H NMR}$ 5.73 (s, 1H), 3.40–3.53 (m, 2H), 1.18 (s, 3H), 0.91 (d, $J = 6.6$ Hz, 3H), 0.91 (d, $J = 6.6$ Hz, 3H), 0.71 (s, 3H) [literature (48) $^1\text{H NMR}$ (CDCl_3) δ 5.62 (s, 1H), 3.41 (d, $J = 5.5$ Hz, 2H), 1.17 (s, 3H), 0.70 (s, 3H)].

25-Hydroxycholesterol-4-en-3-one (**7**). To a solution of 23.8 mg (0.0592 mmol) of **3** in 1 ml of ethyl acetate was added 1 ml of 50 mM TES solution (pH 7.5) containing 5 units of cholesterol oxidase and 4,900 units of catalase. The mixture was stirred for 2 days at room temperature, diluted with 10 ml of water, and extracted with 3×10 ml of CH_2Cl_2 . The combined organic layers were dried, filtered, and evaporated to give 31.3 mg of a 2:1 mixture (based on $^1\text{H NMR}$ integration) of starting material and **3**. The mixture was redissolved in 0.5 ml of ethyl acetate to which

0.5 ml of *n*-butanol and 1 ml of 50 mM TES solution (pH 7.5) containing 5 units of cholesterol oxidase and 4,550 units of catalase were added. The mixture was stirred for 36 h, and worked up in the manner described above to give 25.0 mg of residue, which was chromatographed (silica gel, ethyl acetate–hexane 1:4) to give 19.0 mg (80%) of **7**: mp 149–150.5°C [literature (49) mp 146–150°C]; IR ν_{max} 3,466, 1,661, 1,613 cm^{-1} ; $^1\text{H NMR}$ 5.73 (s, 1H), 1.22 (s, 6H), 1.19 (s, 3H), 0.93 (d, $J = 6.3$ Hz, 3H), 0.72 (s, 3H). [literature (49) $^1\text{H NMR}$ (CDCl_3) δ 5.73 (s, 1H), 1.21 (s, 6H), 1.18 (s, 3H), 0.93 (d, $J = 6$ Hz, 3H), 0.71 (s, 3H)]; $^{13}\text{C NMR}$ 199.9, 171.9, 124.0, 71.3, 56.2, 56.1, 54.0, 44.6, 42.6, 39.8, 38.8, 36.6, 35.9, 35.8, 34.2, 33.2, 32.2, 29.6, 29.4, 28.4, 24.4, 21.2, 21.0, 18.8, 17.6, 12.2.

(24S)-Hydroxycholesterol-4-en-3-one (**6**). To a solution of 33.9 mg (0.0843 mmol) of **2** in 1 ml of ethyl acetate was added 1 ml of 50 mM TES solution (pH 7.5) containing 5 units of cholesterol oxidase and 4,550 units of catalase. The resulting mixture was stirred at room temperature for 2 days. An additional 1 ml of 50 mM TES solution (pH 7.5) containing 5 units of cholesterol oxidase and 4,900 units of catalase was added. The mixture was stirred at room temperature for 20 h, diluted with 10 ml of water, and extracted with 3×10 ml of CH_2Cl_2 . The combined organic layers were dried, filtered, and evaporated to give 33.0 mg of residue which was chromatographed (silica gel, ethyl acetate–hexane 1:4) to give 24.0 mg (71%) of **6**: mp 140–141.5°C; IR ν_{max} 3,451, 1,672, 1,616 cm^{-1} ; $^1\text{H NMR}$ 5.72 (s, 1H), 3.31 (m, 1H), 1.18 (s, 3H), 0.93 (d, $J = 6.6$ Hz, 3H), 0.93 (d, $J = 6.3$ Hz, 3H), 0.90 (d, $J = 6.9$ Hz, 3H), 0.71 (s, 3H); $^{13}\text{C NMR}$ 200.0, 172.0, 124.0, 76.9, 56.2, 56.1, 54.1, 42.7, 39.9, 38.9, 36.2, 36.0, 35.9, 34.3, 33.4, 33.2, 32.4, 32.3, 31.0, 28.4, 24.4, 21.3, 19.3, 19.0, 17.7, 17.0, 12.2; Anal. Calcd for $\text{C}_{27}\text{H}_{44}\text{O}_2$: C, 80.94; H, 11.07. Found: C, 80.70; H, 11.20.

(24R)-Hydroxycholesterol-4-en-3-one. (24R)-Hydroxycholesterol was converted by the above-described procedure to the corresponding enone, which was used for HPLC analysis without complete characterization.

(24S),25-Epoxycholesterol-4-ene-3-one (**5**). To a solution of 42.4 mg (0.106 mmol) of **1** in 1 ml of ethyl acetate was added 1 ml of 50 mM TES solution (pH 7.5) containing 5 units of cholesterol oxidase and 10,080 units of catalase. The resulting mixture was stirred at room temperature for 24 h, and another 1 ml of 50 mM TES solution (pH 7.5) containing 5 units of cholesterol oxidase was added. The mixture was stirred for an additional 20 h, diluted with 5 ml of H_2O , and extracted with 4×5 ml of CH_2Cl_2 . The combined organic layers were dried, filtered and evaporated to give 42.4 mg of colorless solid, which was chromatographed (neutral alumina, ethyl acetate–hexane 1:9) to give 34.1 mg (81%) of **5**: mp 115.4–116.2°C; IR ν_{max} 1,675, 1,616 cm^{-1} ; $^1\text{H NMR}$ 5.73 (s, 1H), 2.69 (m, 1H), 1.31 (s, 3H), 1.27 (s, 3H), 1.19 (s, 3H), 0.94 (d, $J = 6.6$ Hz, 3H), 0.72 (s, 3H); $^{13}\text{C NMR}$ 199.9, 171.8, 124.0, 65.1, 58.3, 56.2, 56.1, 54.0, 42.6, 39.8, 38.8, 35.9, 35.8, 34.2, 33.1, 32.8, 32.2, 28.3, 25.9, 25.2, 24.4, 21.2, 18.9, 18.7, 17.6, 12.2; Anal. Calcd for $\text{C}_{27}\text{H}_{42}\text{O}_2$: C, 81.35; H, 10.62. Found: C, 81.14; H, 10.64.

(24R),25-Epoxycholesterol-4-en-3-one. (24R),25-Epoxycholesterol was converted by the procedure described above to the corresponding enone, which was used for HPLC analysis without complete characterization.

RESULTS

Synthesis of oxysterols and derived enones

(25R),26-Hydroxycholesterol (**4**) was prepared from diosgenin by the procedures of Schroeffer and co-workers

(39). (24*S*)-Hydroxycholesterol (**2**) was synthesized by the sequence shown in Fig. 1B, involving preparation from stigmasterol (**9**) of iodide **10** by the procedures of Partridge, Faber, and Uskokovic (40), and coupling of metallated **10** with chiral epoxide **11** (42, 43) to afford **12**, which was deprotected to provide **2**. This represents a simplification of the synthesis of **2** reported by Ourisson and coworkers (44), who combined **11** with the C-22 phenylsulfonyl derivative obtained from **10**. (24*R*)-Hydroxycholesterol was prepared in an analogous manner by coupling of metallated **10** with the enantiomer of **11**. (24*S*), 25-Epoxycholesterol (**1**) and its (24*R*)-epimer were prepared as described (41, 50).

Conversion of the oxysterols **1–4** to the corresponding Δ^4 -3-enones **5–8** (Fig. 1A) was accomplished by treatment on a preparative scale with cholesterol oxidase, based on the procedure of Teng and Smith (23). Enones **7** (49) and **8** (45, 47, 48), derived from **3** and **4**, respectively, have been previously prepared and characterized, but enones **5** and **6** had not been previously reported.

Development of analytical protocol

Enones **5–8** have UV λ_{max} at 231 nm in hexane and at 233 nm in hexane containing 2% 2-propanol or 2% 1-butanol, the solvent mixtures ultimately found to be most useful for HPLC analysis. The intensity of absorption is about four times greater than that of the oxysterols **1–4** themselves at 205–210 nm, and there is much less interference from other chromophoric materials at the longer wavelength absorbance of the enones. Response curves were determined for the four enones and an excellent linear relationship between detector response and amount of enone injected into the HPLC apparatus over the range 5 ng to 1 μg was obtained (data not shown). The estimated lower limit of detection for the pure enones is about 2 ng.

Investigation of how best to identify and quantitate the presence of oxysterols **1–4** in rat liver was undertaken next. Rat liver homogenates were prepared and a portion was saponified to determine total (free plus esterified) oxysterol content, and a second portion was extracted for total lipids to determine free oxysterol content. It was found essential to remove the large amount of cholesterol present from either type of extract. Using known amounts of added standard oxysterols it was found that elution from a reversed-phase Sep-Pak cartridge with acetonitrile–methanol–water 60:35:5 allowed essentially total recovery of oxysterols with minimum residual cholesterol. The oxysterol fraction thus obtained was then further purified on a semipreparative reversed-phase HPLC column before conversion of oxysterols **1–4** to the corresponding enones. This oxysterol fraction was then treated with cholesterol oxidase to form a product that was analyzed for enones by normal-phase HPLC using hexane–2-propanol 100:2 as solvent. Under these experimental conditions, the efficiency of conversion of accurately measured, ~100-ng samples of standard oxysterols **1–4** to the corresponding enones **5–8** was determined to be (% \pm SD, $n = 8$): **1**, 83 \pm 7; **2**, 84 \pm 3; **3**, 90 \pm 5; **4**, 86 \pm 6. A parallel oxidation and analysis of a mixture of accurately measured, ~100-ng sam-

ples of oxysterols **1–4** was run, and the results were used to correct for oxidation efficiency.

Figure 2A shows a chromatogram of a typical product after cholesterol oxidase treatment of an oxysterol fraction from a nonsaponifiable liver extract and a chromatogram of a mixture of standard enones **5–8**. It was clear from these results that further purification of the crude oxysterol fraction was necessary to obtain accurate determination of absorption due to enones **5–8**. Accordingly, the eluent containing each enone was collected, and the combined collected enones were reanalyzed by normal-phase HPLC using hexane–1-butanol 100:2 as solvent. By use of standard samples, the recovery of enones from this second HPLC analysis was shown to be as follows: **5**, 97 \pm 2%; **6**,

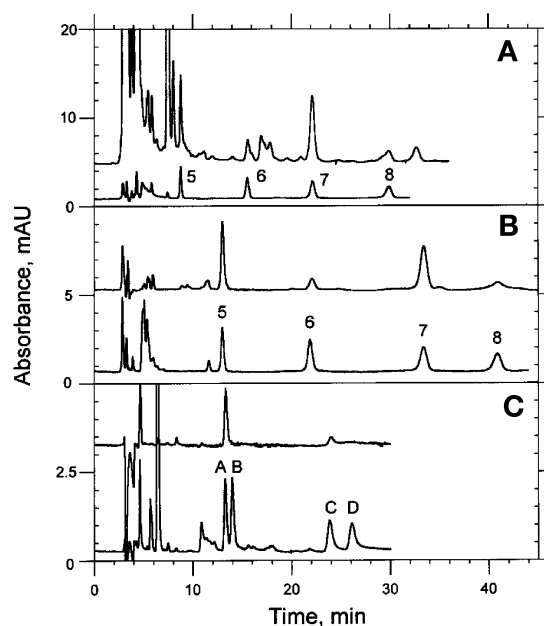


Fig. 2. A: Comparison of high performance liquid chromatography (HPLC) chromatogram of a mixture of enone standards (**5–8**) with that of a sample of rat liver homogenate prepared and pretreated as described in Experimental. Chromatographic conditions were 5 μm Ultrasphere-Si column (4.6 \times 250 mm), hexane–2-propanol 100:2 (v/v) solvent system, 1-ml/min flow rate, and absorbance detector at 233 nm. Amounts of injected standard enones were as follows: (24*S*),25-epoxycholest-4-en-3-one (**5**) 24 ng, (24*S*)-hydroxycholest-4-en-3-one (**6**) 28 ng, 25-hydroxycholest-4-en-3-one (**7**) 28 ng, and 27-hydroxycholest-4-en-3-one (**8**) 24 ng. B: Comparison of HPLC chromatogram of the same mixture of standard enones **5–8** with that of samples of enones collected individually from liver samples and recombined for rechromatography. Chromatographic conditions were the same as in (A) except that the solvent system was hexane–*n*-butanol 100:2. C: Comparison of chiral HPLC chromatogram of a mixture of enone standards with that of enones collected from liver samples. Chromatographic conditions were 5 μm Bakerbond Chiral Phase™ DNBPG (covalent) column (4.6 \times 250 mm), hexane–2-propanol 100:2 (v/v) solvent system, 1-ml/min flow rate, and absorbance detector at 233 nm. Top: Pattern of collected eluents from rat liver samples containing the 24,25-epoxycholest-4-en-3-one and 24-hydroxycholest-4-en-3-one fractions shown in (B). Bottom: The amounts of injected standard epimers were A, (24*S*),25-epoxycholest-4-en-3-one (**5**) 24 ng; B, (24*R*),25-epoxycholest-4-en-3-one, 25 ng; C, (24*S*)-hydroxycholest-4-en-3-one (**6**), 28 ng; and D, (24*R*)-hydroxycholest-4-en-3-one, 25 ng.

97 ± 5%; 7, 93 ± 3%; 8, 93 ± 4% (average of three determinations for each). The cleaner results obtained in this second HPLC analysis are shown in Fig. 2B. Again, the eluent containing each oxysterol enone was collected and saved.

To demonstrate that the amounts of oxysterols 1–4 that were recovered and measured as enones 5–8 reflected the amounts originally present, the entire analytical procedure was conducted eight times on accurately measured, ~100-ng samples of authentic standard oxysterols 1–4. The percent recoveries determined were as follows: 1, 84 ± 10%; 2, 98 ± 10%; 3, 93 ± 7%; 4, 83 ± 9%; after adjustment as described above for the efficiency of conversion in a particular cholesterol oxidase reaction.

To ensure that the enones observed by HPLC analysis had not arisen from oxysterols produced by autoxidation during sample preparation, portions of collected enones 5 and 6 were rechromatographed on a chiral column under conditions that separated them from their (*R*)-epimers. Autoxidation would produce an essentially 1:1 mixture of (*S*)- and (*R*)-epimers, whereas only the (*S*)-forms are produced enzymatically. Figure 2C shows such a chromatogram and contrasts it with one of a mixture of standard (24*S*),25- and (24*R*),25-epoxycholestenone and (24*S*)- and (24*R*)-hydroxycholestenone. Essentially only the (*S*)-isomers were recovered from the liver samples, demonstrating that little, if any, autoxidation had occurred during the analytical procedures.

Finally, the structural identity of the enones formed from rat liver endogenous oxysterols was confirmed by GC-MS. Synthetic standard (24*S*),25-epoxycholestenone (5) and collected, liver-derived 5 showed essentially identical mass spectra, with a molecular ion peak at *m/z* 398, and peaks at *m/z* 229 and *m/z* 124, consistent with fragmentation patterns of other cholestenones (51). (24*S*)-Hydroxycholestenone (6), 25-hydroxycholestenone (7), and 27-hydroxycholestenone (8) were converted to their TMS derivatives for GC-MS analysis, and showed fragmentation patterns similar to those reported (52). The principal peaks were as follows: TMS-6: *m/z* 73 (base peak), 145, and 429; TMS-7: *m/z* 131 (base peak), 196, 343, and 457 (M-15); TMS-8: *m/z* 124, 196 (base peak), 229, 457, and 472 (M⁺).

Oxysterol concentrations in liver

The method described above was used to determine the concentrations of the four oxysterols in rat liver homogenates and subcellular fractions and in mouse liver, with the results presented below. Chiral column HPLC showed that in all cases liver 24,25-epoxycholesterol was exclusively the natural (24*S*)-epimer and that liver 24-hydroxycholesterol was in all cases at least 90%, if not exclusively, the natural (*S*)-epimer (Fig. 2C). The identity of the liver oxysterols was confirmed in all cases by GC-MS.

Subcellular distribution of oxysterols in rat liver. All four oxysterols were present, ranging from 50 to 300 pg/mg protein, in liver homogenates prepared from male Sprague-Dawley rats maintained on a chow diet (control values in Fig. 3). Each oxysterol showed a distinct subcellular distribution (Fig. 4), which in part reflected its site(s) of synthesis.

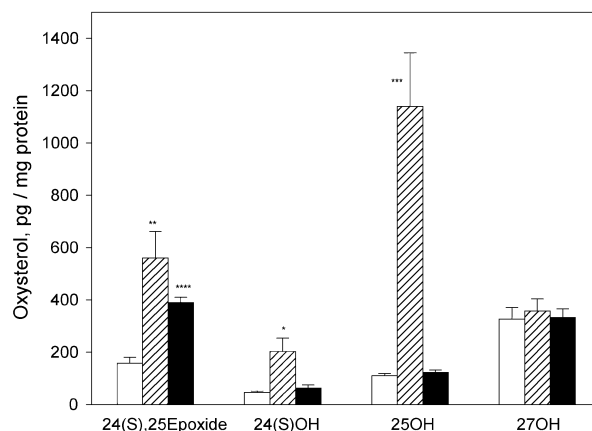


Fig. 3. Effect of feeding an atherogenic diet or mevalonate administration on oxysterol profiles in rat liver homogenates. Rats were fed an atherogenic diet for 2–3 weeks or administered mevalonate intragastrically 1 h before death. Liver homogenates were prepared and analyzed as described in Materials and Methods. Control (open columns); atherogenic diet (hatched columns); mevalonate (solid columns). * Different from control at $P < 0.001$; ** different from control at $P < 0.002$; *** different from control at $P < 0.0001$; **** different from control at $P < 0.00001$.

27-Hydroxycholesterol (4) was higher in mitochondria, an important site of its synthesis via cholesterol 27-hydroxylase (53). 27-Hydroxycholesterol (4), 25-Hydroxycholesterol (3), and (24*S*),25-epoxycholesterol (1) were present at relatively high concentrations in the microsomal fraction, which contains the endoplasmic reticulum, a major site of synthesis for 1 (30) and for both 3 and 4 (31). Small amounts of all the oxysterols were recovered in the cytosol. They were not detected in the floating fat fraction (data not shown), suggesting that these cytosolic oxysterols are metabolically active and not simply storage forms. Both (24*S*),25-epoxycholesterol (1) and (24*S*)-hydroxycholesterol (2) were recovered in nuclei, supporting their proposed roles as the physiological ligands for the resident nuclear transcription factor LXR α (11–13). 27-Hydroxycholesterol (4) was not detected in nuclei and the amounts of 25-hydroxycholesterol (3) were highly variable; in most preparations, it was not detectable in this fraction.

Effects of an atherogenic diet or mevalonate administration on rat liver oxysterol profile. Feeding an atherogenic diet containing cholesterol, cholate, and lard results in hyperlipidemia and downregulation of cholesterol synthesis and catabolism. If these effects are mediated by specific oxysterols, feeding this diet would be expected to result in changes in one or more of the oxysterols implicated in regulation. When rats were fed this diet for 2–3 weeks, an ~3-fold increase in liver homogenate (24*S*),25-epoxycholesterol (1), an ~4-fold increase in (24*S*)-hydroxycholesterol (2), an ~10-fold increase in 25-hydroxycholesterol (3), and no change in 27-hydroxycholesterol (4) were observed (Fig. 3). In preliminary studies, most of the increase in 3 was in microsomes; only 1 and 2 were recovered in nuclei (data not shown).

Administration of a bolus of mevalonic acid intragastrically

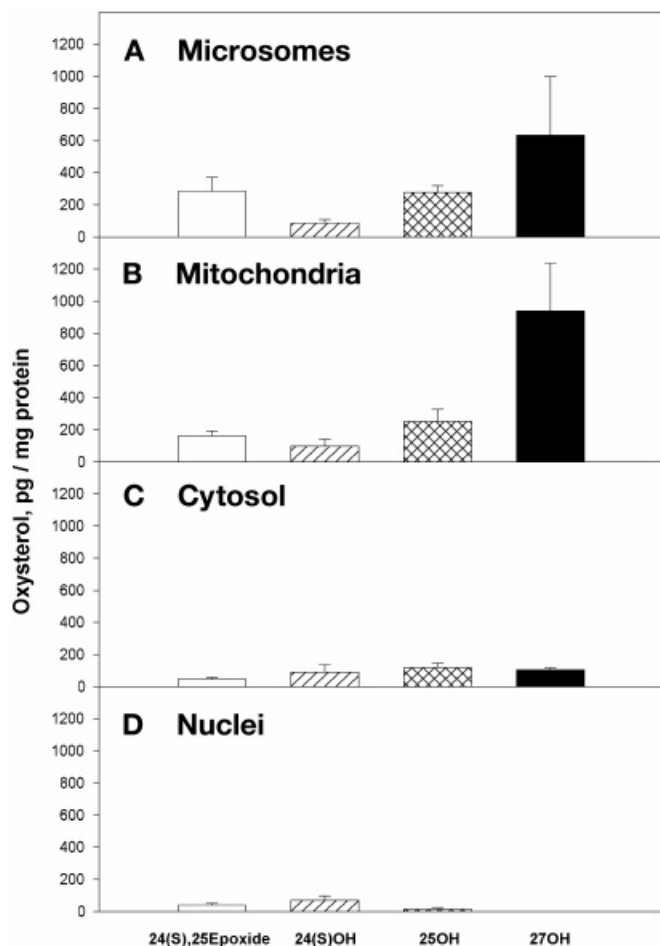


Fig. 4. Subcellular distribution of oxysterols in rat liver. Liver from rats fed a chow diet were fractionated and analyzed as described in Materials and Methods. Microsomes, $n = 6$ experiments; mitochondria, $n = 7$ experiments; cytosol, $n = 7$ experiments; nuclei, $n = 9$ experiments. In each experiment livers from three rats were divided and 5 g of each used for preparation of nuclei, and equal amounts of the remainder for preparation of the other fractions.

cally to rats results in downregulation of HMG-CoA reductase activity (54) and upregulation of cholesterol 7 α -hydroxylase (55) within 2 h. To test the hypothesis that this regulation is mediated specifically by one or more oxysterols, changes in oxysterol concentration were monitored 1 h after mevalonate administration. The only oxysterol that was increased in rat liver homogenates under these conditions was (24*S*),25-epoxycholesterol (1), approximately 2-fold (Fig. 3).

Effects of aging on rat liver homogenate oxysterol content. Aging is associated with changes in cholesterol metabolism. As humans age, their plasma cholesterol is increased (56). This suggests that aging may affect regulation of hepatic cholesterol metabolism and that such regulatory effects might be accomplished by changes in the amounts of signal molecules, such as specific oxysterols in the liver. To study this possibility, we measured amounts of the four total and free oxysterols in livers from young (4 months), middle-aged (16 months), and old (24 months) male Fischer 344 rats. No effects of age were found on the content of the

TABLE 1. Effect of age on concentration of oxysterols in rat liver homogenates^a

	Compound			
	1	2	3	4
A. Total oxysterol content				
	<i>pg total oxysterol/mg protein</i>			
Young ($n = 5$)	80 \pm 11	35 \pm 5	360 \pm 60	400 \pm 40
Middle ($n = 5$)	62 \pm 9	46 \pm 20	280 \pm 10	390 \pm 110
Old ($n = 5$)	85 \pm 17	50 \pm 10	340 \pm 140	540 \pm 80
B. Percent oxysterol ester				
	<i>% ester</i>			
Young ($n = 5$)	46 \pm 8	76 \pm 6	66 \pm 4	72 \pm 2
Middle ($n = 5$)	41 \pm 5	81 \pm 5	90 \pm 2	77 \pm 3
Old ($n = 5$)	33 \pm 10	76 \pm 4	84 \pm 10	82 \pm 3

^a Samples of rat liver were homogenized and saponified or extracted, and the concentrations of the oxysterols were determined as described in Experimental. Results are expressed as means \pm SE. No significant differences among the age groups were found. The percentage of 1 as ester was less than that of 2–4 at $P < 0.01$ except in the young rats, where the difference between 1 and 3 was at $P < 0.05$.

four oxysterols in rat liver homogenates (Table 1), and the amounts of free or esterified oxysterols also did not vary significantly with age.

Mouse liver. An earlier report (21) concluded that (24*S*), 25-epoxycholesterol (1) was not present in mouse liver. Because this contrasts with the finding of 1 in rat liver, we applied the current analytical protocol to samples of liver from mice of three strains, 129/SVJ, C57BL/6J, and C3HeB/FeJ,² the strain used in the previous study (21). *S*-Epoxy 1 was indeed present in all three strains in amounts similar to those found in rat liver (data not shown).

DISCUSSION

Several approaches to development of methods for analysis of low concentrations of oxysterols have been described in addition to the direct UV detection at short wavelength mentioned earlier (21, 22). Breuer and Björkhem (57) have conducted extensive analyses of oxysterols in biological samples by use of GC-MS. Two factors dictated against our choosing to use this approach for analysis of liver oxysterols. First, our early investigations had a major focus on (24*S*),25-epoxycholesterol (1) (58, 59), and preliminary work suggested that this compound did not survive the temperature required for GC analysis (J. A. Nelson and T. A. Spencer, unpublished observation). Second, MS cannot distinguish between stereoisomers, such as (24*S*)-hydroxycholesterol and its (24*R*)-epimer, and the ability to do so was deemed essential to ensure that the detected oxysterols were the natural, physiologically relevant stereoisomers and not autoxidation products.

Schroepfer and co-workers (60) developed a method

² The samples of mouse liver were graciously provided by Drs. David Mangelsdorf and Daniel Peet, University of Texas Southwestern Medical Center (Dallas, TX).

for oxysterol analysis involving acetylation of mixtures with [³H]acetic anhydride in order to convert the oxysterols to radiolabeled, and therefore readily quantitated, 3β-acetate derivatives. An elaborate apparatus is used in this method to avoid autoxidation of biological samples during saponification and extraction for derivatization and HPLC analysis. This approach gives good results, even at the nanogram level, but was judged to be too cumbersome, expensive, and time-consuming to be used on a large number of samples.

Benzoylation of oxysterols has been used to enhance UV detectability by several groups (61–64), although none has reported analyses below the microgram level. Despite considerable effort, we were unable to develop such an analytical protocol that would allow accurate, reliable determination of oxysterols in liver samples because there were too many nonoxysterol materials present that also become benzoylated, resulting in complex HPLC traces. The needed enhancement of UV detectability was achieved by application of the well-known cholesterol oxidase reaction (23–29) to oxysterols 1–4 to form the corresponding Δ⁴-3-ketones 5–8. This reaction occurs completely selectively with 3-hydroxy sterols and produces products with a strong chromophore at λ_{max} 240_{ethanol}–230_{hexane}.

This method has established unequivocally that all four of the key selected oxysterols, (24*S*),25-epoxycholesterol (1), (24*S*)-hydroxycholesterol (2), 25-hydroxycholesterol (3), and 27-hydroxycholesterol (4), are present in both free and esterified form in rat liver. Oxysterols 1 and 2 are present essentially to the exclusion of their (24*R*)-epimers. In previous analyses for regulatory oxysterols, we identified (24*S*),25-epoxycholesterol (1) and 25-hydroxycholesterol (3) in cultured fibroblast cells (58) and 1 in human liver (59). In another, earlier study with the goal of detecting all oxysterols in mouse liver that repressed HMG-CoA reductase activity, hydroxycholesterols 2, 3, and 4 (as well as C-7 oxygenated cholesterols) were identified as potential endogenous regulatory compounds (21). However, no (24*S*),25-epoxycholesterol was detected in the livers of the C3HeB/FeJ mice used in that study by the analytical protocol then used (21). Now we have shown conclusively that (*S*)-epoxide 1 is indeed present in livers from C3HeB/FeJ and two other strains of mice.

Age had little effect on either rat liver total oxysterol content or the amount in esterified form (Table 1). However, a relatively larger fraction (60–70%) of (24*S*),25-epoxycholesterol (1) was in the free form as compared with ~25% or less of the three hydroxycholesterols. All four oxysterols are substrates for the intracellular cholesterol-esterifying enzymes in the liver, the acyl-coenzyme A:cholesterol acyltransferases 1 and 2 (65). By analogy with the steroid hormones, it is likely that only the free form of an oxysterol is a ligand for its nuclear receptor(s). Thus, epoxide 1 may have a unique regulatory role. The biosynthetic origin of 1 is different from that of the three hydroxycholesterols 2–4, being synthesized from mevalonate via squalene dioxide (30), not from cholesterol.

The higher proportion of the three hydroxycholesterols 2–4 in the esterified form may reflect their presence in

organelles of the endocytic or selective uptake pathways, because none of the four oxysterols was detectable in the floating fat layer or lipid droplet storage compartment (data not shown). This suggests that some of the hepatic esterified hydroxycholesterols are of extrahepatic origin. Most of the (24*S*)-hydroxycholesterol (2) in the circulation is thought to be derived from the brain (66), while most of the 27-hydroxycholesterol is thought to originate in the lungs (67). It has been proposed that as a part of reverse cholesterol transport these two oxysterols are removed by the liver, where they enter the bile acid synthetic pathway.

The subcellular distribution of the four oxysterols reflects not only their sites of synthesis, but also suggests potential mechanisms by which they act as regulatory/signaling molecules. The finding that both (24*S*),25-epoxycholesterol (1) and (24*S*)-hydroxycholesterol (2) were recovered in nuclei supports their proposed roles as the physiological high affinity ligands for the resident nuclear transcription factor LXRα (11–13). The absence of detectable 27-hydroxycholesterol (4) in the nuclear fraction suggests that it does not play a role in transcriptional regulation at the nuclear level, as does the variable and inconsistent recovery of 25-hydroxycholesterol (3) in this compartment. Recovery of all four oxysterols in the microsomal fraction, which is enriched in endoplasmic reticulum, suggests that, at some critical concentration, one or all of them may downregulate the sterol-sensitive proteolytic cleavage of the SREBPs described by Brown and Goldstein (18).

Of these four oxysterols, 27-hydroxycholesterol (4) appears unlikely as a mediator of the effects of feeding an atherogenic diet containing cholesterol, cholate, and lard because no change in the amount of 4 was observed. In preliminary studies of the effects of this diet on subcellular distribution, most of the 25-hydroxycholesterol (3) was recovered in microsomes; only epoxide 1 and (24*S*)-hydroxycholesterol (2) increased in the nuclear fraction, supporting the notion that these two oxysterols specifically are important in transcriptional regulation at the nuclear level in response to a cholesterol-rich diet. The results also suggest that 25-hydroxycholesterol (3), at least under this dietary condition, participates in maintaining the SREBPs in their holo (endoplasmic reticulum) form.

Administering a bolus of mevalonic acid intragastrically to rats has been shown to result in downregulation of HMG-CoA reductase activity (54) and upregulation of cholesterol 7α-hydroxylase (55) within 2 h. The approximately 2-fold increase in (24*S*),25-epoxycholesterol (1) in liver homogenate with no change in the other oxysterols measured at 1 h after mevalonate administration suggests that 1 is a key mediator of these effects of mevalonate. The data suggest that this oxysterol specifically downregulates HMG-CoA reductase posttranslationally at the endoplasmic reticulum level by triggering degradation (68) and transcriptionally by inhibiting activation of the SREBPs by proteolytic clip (18). The increased amount of epoxide in whole homogenate may also reflect increased amounts in nuclei where, as a high affinity LXRα ligand, (24*S*),25-

epoxycholesterol could upregulate *cyp7A1* gene expression. This interpretation is consistent with the increased transcription of this gene observed 2 h after mevalonate administration (55). While it is, of course, possible that as yet unidentified oxysterols may play regulatory roles, the results of this study lend support to the hypothesis that the specific oxysterols investigated are physiologically important in the regulation of cholesterol metabolism. ■

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