5α-Cholest-8(14)-en-3β-ol-15-one, a potent regulator of cholesterol metabolism: occurrence in rat skin

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Abstract 5α-Cholest-8(14)-en-3β-ol-15-one is a potent inhibitor of cholesterol biosynthesis which has significant hypocholesterolemic activity upon oral administration to animals. Described herein are the results of experiments that indicate the presence of the 15-ketosterol in rat skin. The 15-ketosterol was, after purification by medium pressure liquid chromatography on Lichroprep RP-8 columns, thin-layer chromatography on silica gel G, and reverse phase high performance liquid chromatography, characterized by gas–liquid chromatography–mass spectrometry in the form of its trimethylsilyl ether derivative. The use of an internal standard containing both tritium and deuterium permitted the determination of the levels of the 15-ketosterol by mass fragmentography. The results of five separate analyses of portions of the skin of a male Sprague Dawley rat showed a mean value of 84.5 ± 4.1 (SEM) ng per g. Analyses of hair samples of ten male Sprague Dawley rats indicated a mean level of 143 ± 19 (SEM) ng per g of hair. Most (~72%) of the 15-ketosterol in hair was esterified. This report constitutes the first isolation of the 15-ketosterol from animal tissues.—**Emmons, G. T., J. St. Pyrek, R. Dam, M. Martin, K. Kudo, and G. J. Schroepfer, Jr.** 5α-Cholest-8(14)-en-3β-ol-15-one, a potent regulator of cholesterol metabolism: occurrence in rat skin. *J. Lipid Res.* 1988. **29:** 1039–1054.

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5α-Cholest-8(14)-en-3β-ol-15-one (**I**) is a potent inhibitor of sterol synthesis in mammalian cells in culture and lowers the levels of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity in these cells (1–3). The levels of activity of two other key enzymes involved in the enzymatic formation of mevalonic acid, i.e., cytosolic acetoacetyl-CoA thiolase and HMG-CoA synthase, have also been shown to be suppressed by the 15-ketosterol **I** in CHO-K1 cells (2). In addition to its effects on sterol synthesis, dietary administration of **I** has been shown to cause a marked inhibition of the intestinal absorption of exogenous cholesterol in rats (4). **I** has been shown to serve as a substrate for the enzyme acyl coenzyme A:cholesterol acyl transferase (ACAT) of microsomes of rat liver and jejunum and to inhibit the oleoyl coenzyme A-dependent esterification of cholesterol in microsomes of liver and jejunum (5). The 15-ketosterol **I** has been shown to have marked hypocholesterolemic activity upon oral administration to rats (6, 7), mice (6), baboons (8), and rhesus monkeys (9). The reduction of the levels of total serum cholesterol in the rhesus monkeys has been shown to be associated with a lowering of the levels of low density lipoprotein (LDL) cholesterol and LDL protein and with an elevation of the levels of high density lipoprotein (HDL) cholesterol and HDL protein (9). The increase in HDL cholesterol levels, induced by the administration of **I**, has been found to be associated with a shift in the HDL profile to one in which the HDL_2 species predominated (9). All of the observed effects of **I** noted above are believed to be beneficial for the treatment and/or prevention of atherosclerosis. Another novel feature of **I** is its efficient conversion to cholesterol, a transformation that has been shown to occur in vitro in rat liver homogenate preparations (10) and in vivo upon oral or intravenous administration to rats (11–13) and baboons (14, 15). Metabolism of **I** to 5α-cholesta-8,14-dien-3β-ol (16) and 5α-cholest-7-en-3β-ol (10, 16) has been shown in rat liver homogenate preparations and a scheme for the overall conversion of **I** to cholesterol has been presented (16). While cholesterol and cholesteryl esters represent the major metabolites found in blood and in tissues of rats after the oral or intravenous administration of **I** (11–13), a quantitatively more important fate of **I** (or of cholesterol formed from **I**) is conversion to polar metabolites that are very rapidly excreted in bile (12). In the course of these studies

Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; ACAT, acyl coenzyme A:cholesterol acyltransferase; LDL, low density lipoprotein; HDL, high density lipoprotein; UV, ultraviolet; PPO, 2,5-diphenyloxazole; TMS, trimethylsilyl; BSTFA, bis(trimethylsilyl)trifluoroacetamide; TLC, thin-layer chromatography; MPLC, medium pressure liquid chromatography; HPLC, high performance liquid chromatography; GLC, gas–liquid chromatography; MS, mass spectra or mass spectrometry; NMR, nuclear magnetic resonance; MF, mass fragmentography; NSL, nonsaponifiable lipids.

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we noted, at 48 hr after the intravenous administration of [4-^{14}C]-labeled **I** to a bile duct-cannulated rat, the presence of small but significant amounts of labeled **I** in skin (12). This result, coupled with the well-documented presence of high steady-state levels of sterol precursors of cholesterol in skin (17, 18 and references cited therein) and the results of other preliminary experiments, provided the stimulus for the present study.

Described herein are results of experiments which indicate the presence of **I** in rat skin and in rat hair.

EXPERIMENTAL PROCEDURES AND RESULTS

General

Melting points were recorded on a Thomas Hoover apparatus using sealed, evacuated capillary tubes. Ultraviolet (UV) determinations were made using an IBM 9430 spectrophotometer (Danbury, CT) with hexane or isopropanol as the solvent. Radioactivity was measured using a Packard TriCarb Model 4640 liquid scintillation spectrometer (United Technologies, Downers Grove, IL). 2,5-Diphenyloxazole (PPO) was purchased from Beckman Instruments, Inc. (Fullerton, CA). Radioactivity was assayed using PPO (0.4%) in toluene, PPO (0.4%) in a toluene–ethanol 2:1 mixture, or Scintisol (Isolab; Akron, OH). Trimethylsilyl (TMS) ether derivatives of the sterols were prepared by minor modifications of procedures described previously (19) using bis(trimethylsilyl)trifluoroacetamide (BSTFA)–pyridine–ethyl acetate 1:1:1 for 30 min at 65°C unless specified otherwise.

Thin-layer chromatography (TLC) was performed using prewashed (with ethyl acetate) plates of silica gel (0.25 mm and 1.0 mm in thickness) which were obtained from Analtech, Inc. (Newark, DE). Components on the plates were visualized after spraying with molybdic (5%) in 10% sulfuric acid with subsequent heating of the plates (20) or by spraying the plates with water. Radio-TLC analyses were carried out as described previously (21).

Silicic acid column chromatography was carried out using either Unisil (100–200 mesh; Clarkson Chemical Company, Inc.; Williamsport, PA) or silica gel (60–200 mesh; J. T. Baker Chemical Company; Phillipsburg, NJ). Medium pressure liquid chromatography (MPLC) was performed using two Lichroprep RP-8 columns (10 mm × 240 mm; EM Science; Darmstadt, West Germany) connected in tandem. Methanol–water 9:1 was used for sample injection (2 ml) and for elution of the column. In some cases the sample was filtered through glass wool prior to injection to remove particulate matter. In these cases, only slight (generally less than 10%) loss of the labeled internal standard (vide infra) was observed. The flow rate was 3.0 ml per min and fractions were collected every 2 min. High performance liquid chromatography (HPLC) was carried out using a system consisting of an LC pump (Kontron Instruments; Everett, MA) a column and a V_4 variable wavelength detector (ISCO Inc.; Lincoln, NE) or using a Waters M-600 gradient system with a Model 490 programmable multiple wavelength detector (Waters Associates, Inc.; Milford, MA). The following HPLC columns (obtained from Custom LC (Houston, TX)) were used: Spherisorb 5 μm (4.6 mm × 250 mm), semi-preparative Spherisorb 5 μm (9.2 mm × 250 mm), and Spherisorb ODS-II 5μm (4.6 mm × 250 mm).

Gas–liquid chromatographic (GLC) analyses were made using either a Shimadzu GC9A (Shimadzu Corporation; Kyoto, Japan) or a Perkin Elmer Sigma 2000 (Perkin Elmer; Norwalk, CT) chromatograph, both equipped with flame ionization detectors. With the former unit, injections were made in the split mode (injector temperature, 290°C) under isothermal (260°C) conditions using a 0.1-μm DB-1 fused silica capillary column (0.25 mm × 30 m; J & W Scientific; Rancho Cordova, CA) (condition A). The Sigma 2000 unit was equipped with a 0.1-μm DB-5 fused silica capillary column (0.3 mm × 30 m; J & W Scientific; Rancho Cordova, CA) and injections were made in a splitless mode (injector temperature, 285°C) with temperature programming from 150°C (1 min hold) to 230°C at 30°C per min followed by a 2°C per min increase to 290°C (condition B). Mass spectra (MS) were measured under electron impact conditions at 20 eV using solid probe introduction into a Shimadzu QP-1000 spectrometer (Shimadzu Corporation; Kyoto, Japan). Combined GLC–MS analyses were made on an Extreal ELQ-400 quadrupole mass spectrometer (Extrel Corporation; Pittsburg, PA) which was interfaced with a Varian 3500 gas chromatograph (Varian; Walnut Creek, CA) equipped with a 0.1-μm DB-5 fused silica capillary column (either 0.25 mm × 11 m or 0.25 mm × 15 m). Sample injections were made in a splitless mode (injector temperature, 285°C) in decane with temperature programming from 150°C (with 1 min hold) to 260°C at 40°C per min (condition C). ^{13}C Nuclear magnetic resonance (NMR) spectra were recorded on an IBM AF300 spectrometer under conditions described in detail previously (19). $CDCl_3$ was used as the solvent.

Sodium borodeuteride (99.8 atom % D), deuterium oxide (99.8 atom % D), and neutral aluminum oxide (activated; Brockman grade I) were purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI). Sodium [^{3}H]borohydride (25 mCi, 283 per mmol) was obtained from Amersham Company (Arlington Heights, IL). Ovalbumin (grade V) was obtained from the Sigma Chemical Company (St. Louis, MO). 15-Ketosterol, **I**, melting at 148–149°C (lit., 147.5–149.0°C (1, 22–24)), was prepared by minor modifications of procedures described previously (1, 24) and showed a single component (> 99%) on TLC (solvent, 30% ethyl acetate in hexane), GLC (3% OV-17 on Gas Chrom Q), and HPLC (5 μm Spherisorb ODS-II; solvent, methanol). The preparation and characterization of the palmitoleic acid ester of **I** have been described elsewhere (25). 5α-

Cholest-8(14)-ene-7α,15α-diol-3-one, melting at 122–123°C (lit., 121.5–122.5°C (9, 23), 121.0–122.5°C (22)), was prepared as described previously (22). Capillary GLC (30 m DB-1 column) of the di-TMS derivative showed a purity in excess of 99%.

[2,2,3α,4,4-$^2H_5$]5α-Cholest-8(14)-en-3β-ol-15-one

To 5α-cholest-8(14)-ene-7α,15α-diol-3-one (5.0 g) in benzene (500 ml) was added neutral aluminum oxide (30 g) and potassium *t*-butoxide (5 g). D_2O (5 ml) was added to the stirred mixture through a septum under nitrogen. The resulting mixture was stirred for 30 min at room temperature and then heated (heating mantle) and the D_2O was then removed by distillation as an azeotrope with benzene and collected in the side arm of a Dean Stark apparatus. After cooling of the reaction mixture to room temperature, fresh D_2O (5 ml) was added and the procedure was repeated 6 times. After the final exchange, the mixture was cooled to room temperature and filtered through a cotton plug under nitrogen pressue. A portion of the crude deuterated product was removed for reduction with NaB^3H_4 (vide infra) and a larger portion (2.0 g) in isopropanol (40 ml) was treated with NaB^2H_4 (200 mg in 0.5 ml of D_2O). After 72 hr at room temperature, 0.5% acetic acid (20 ml) was carefully added and, after the bulk of the isopropanol was removed under reduced pressure, the resulting mixture was extracted 4 times with ethyl acetate (40-ml portions). Evaporation of the solvent under reduced pressure gave 1.8 g of solid material which, upon GLC (condition A) of the TMS derivative (obtained by silylation of the sample for 2 hr at 120°C), showed only two components that had the same retention times as the tri-TMS derivative of 5α-cholest-8(14)-ene-3β,7α,15α-triol (87%) and 5α-cholest-8(14)-ene-3α,7α,15α-triol (13%). No unreacted starting material was observed. GLC–MS of the TMS derivative of the product showed that the two components had similar mass spectra. Both components showed molecular ions at *m/z* 639, corresponding to the incorporation of five atoms of deuterium, and ions at *m/z* 624 ($M-CH_3$), 549 (M-TMSOH; base peak), 459 (M-TMSOH-TMSOH), 329, 256 (M-TMSOH-TMSOH-TMSOH-side chain), and 219. A portion (110 mg) of the mixture of the two epimeric triols was treated with concentrated HCl in 95% ethanol for 1 hr (22). The major product was purified by preparative TLC (two, 20 × 20 cm plates) each developed 3 times in hexane–ethyl acetate 7:3 at 4°C. After visualization of the components on the plates following spraying with water, the major component (R_f 0.42) was recovered from the plates and recrystallized from methanol–water to give [2,2,3α,4,4-$^2H_5$]-**I** which melted at 145.5–146.5°C (lit., 147.5–149.0°C (1, 22–24)). GLC of the TMS derivative on a 30-m DB-1 column and on a 15-m DB-5 column and HPLC on a Spherisorb column (solvent, 20% ethyl acetate in hexane at 2 ml per min) indicated one major (> 99%) component with the same retention time as the TMS derivative of authentic **I**. The crystalline product was found to have less than 0.1% of 5α-cholest-8(14)-en-3α-ol-15-one. The mass spectrum of the labeled **I** showed a molecular ion at *m/z* 405 (100%), corresponding to the incorporation of five atoms of deuterium, and prominent ions at *m/z* 390 ($M-CH_3$; 25%), 387 ($M-H_2O$, 18%), 372 ($M-H_2O-CH_3$, 43%), 292 (M–side chain, 23%), 274 ($M-H_2O$–side chain, 97%) and 256 ($M-H_2O-H_2O$–side chain, 19%). The calculated isotopic composition was d_5 87%, d_4 12%, and d_3 < 1%. For a compound with five deuterium atoms with equal labeling at each of the five positions, the observed value of 87% d_5 species corresponds to a 97.5% deuteration at each of the five sites. The results of ^{13}C NMR analysis were compatible with the assigned structure (26) and the specified locations of the deuterium atoms.

[3α-^{3}H][2,2,4,4-$^2H_4$]5α-Cholest-8(14)-en-3β-ol-15-one

[2,2,4,4-$^2H_4$]5α-Cholest-8(14)-ene-7α,15α-diol-3-one (100 mg), obtained as described above, was reacted with NaB^3H_4 (25 mCi; 3.35 mg, according to supplier) in isopropanol (3 ml) for 1 hr at room temperature. Excess NaB^2H_4 (20 mg) was then added and the reaction mixture was maintained overnight at room temperature. Acetic acid (0.5%) was carefully added and the resulting mixture was thoroughly extracted with ethyl acetate until no further radioactivity was recovered in the organic extract. The combined extracts were evaporated to dryness under nitrogen; a portion (83 mg) of the recovered residue was dissolved in 95% ethanol and reacted with concentrated HCl for 1 hr (22). [3α-^{3}H][2,2,4,4-$^2H_4$]-**I** was purified by two passes through a Unisil column (1.5 × 60 cm) using mixtures of hexane and ethyl acetate as the eluting solvent. The doubly labeled 15-ketosterol (33.4 mg) had a specific activity of 74.1 mCi per mmol. HPLC on a Spherisorb column (solvent, 20% ethyl acetate in hexane at a flow rate of 1 ml per min; UV detection at 260 nm) showed a single radioactive component with the same retention time as that of the UV-absorbing species and that of authentic **I** (**Fig. 1**). Less than 0.01% of the recovered ^{3}H eluted in the region corresponding to the mobility of 5α-cholest-8(14)-en-3α-ol-15-one in this system. GLC of the TMS derivative on a 15-m DB-5 column showed a single (> 99%) component. GLC–MS of the TMS derivative showed a molecular ion at *m/z* 476 (100%), corresponding to an incorporation of four atoms of deuterium. Other prominent ions were found at *m/z* 461 ($M-CH_3$, 26%), 386 (M-TMSOH, 17%), 371 (M-TMSOH-CH_3, 87%), 345 ($M-H_2O$–side chain, 26%), 273 (M-TMSOH–side chain, 35%), 255 (M-TMSOH-H_2O–side chain, 70%). The calculated isotopic composition was: d_5 0.7%, d_4 90.8%, d_3 8.2%, and d_2 0.2%. For a compound with four deuterium atoms with equal labeling at each of the four positions, the observed value of 90.8% d_4 species corresponds to a 97.5% deuteration at each of the four sites.

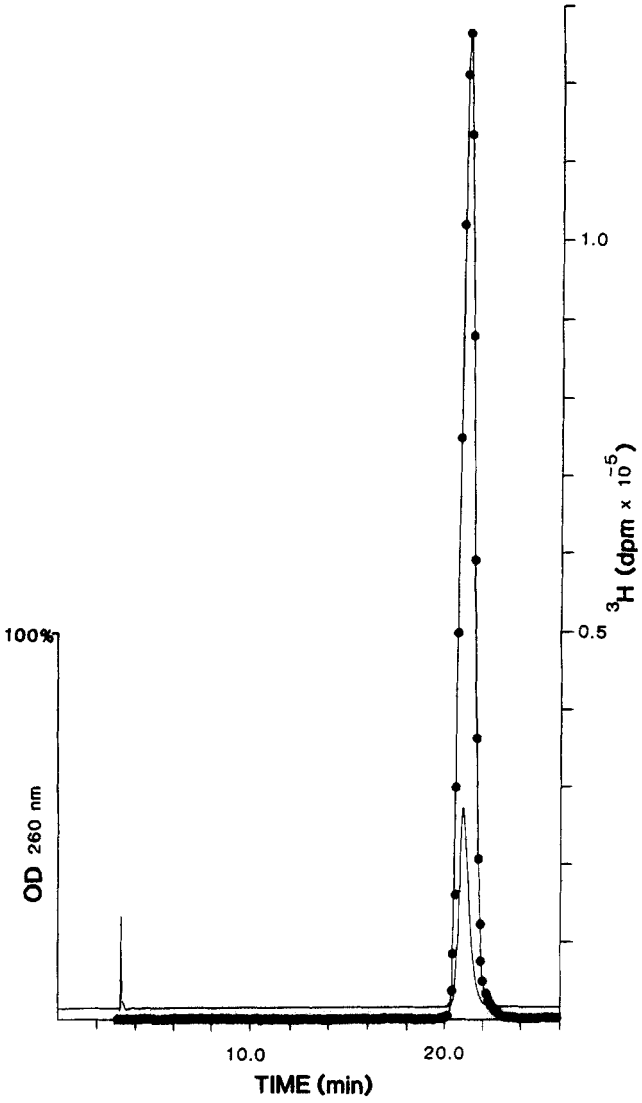

Fig. 1. HPLC of [3α-^{3}H][2,2,4,4-$^2H_4$]5α-cholest-8(14)-en-3β-ol-15-one on an analytical Spherisorb column monitored by assay of ^{3}H and absorbance at 260 nm (0.1 AUFS), using 20% ethyl acetate in hexane as the eluting solvent; (●), radioactivity.

The results of ^{13}C NMR analysis were compatible with the assigned structure (26) and the specified locations of the deuterium atoms.

Stearic acid ester of [3α-^{3}H][2,2,4,4-$^2H_4$]5α-cholest-8(14)-en-3β-ol-15-one

[3α-^{3}H][2,2,4,4-$^2H_4$]-**I** (4.35 mg; 0.8 mCi) in CH_2Cl_2 (2 ml) was condensed with stearic acid (4.3 mg) under anhydrous conditions in the presence of dicyclohexyl carbodiimide (5 mg) and dimethylaminopyridine (1 mg) at room temperature for 2 hr. Water was added and the resulting mixture was thoroughly extracted with hexane. Quantitative recovery of ^{3}H was observed. A portion (344 μg) of this material was subjected to TLC (solvent, 10% ethyl acetate in hexane). Almost all (> 99%) of the ^{3}H had the mobility of the palmitoleic acid ester of **I** and less than 0.1% of the ^{3}H had the mobility of **I**. The radiopurity of the ester was in excess of 99% as judged by radio-TLC (solvent, 10% ethyl acetate in hexane).

Internal standard A

Samples of [2,2,3α,4,4-$^2H_5$]-**I** and [3α-^{3}H][2,2,4,4-$^2H_4$]-**I** were mixed to give a sample designated as internal standard A. The specific activity of this sample was 41.4 mCi per mmol. The radiopurity was judged to be in excess of 99% on the basis of HPLC on a Spherisorb column (4.6 mm × 250 mm; solvent, 20% ethyl acetate in hexane at 1 ml per min). The same analysis showed a single species absorbing at 260 nm which had the same mobility as that of authentic **I**. GLC–MS of the TMS derivative showed a single component (> 99%) with the same retention time of the TMS derivative of authentic **I**. The mass spectrum of the TMS derivative of internal standard A showed the following isotopic composition: d_5 44%, d_4 52%, d_3 < 4%. The abundance of ions corresponding to d_0 species was less than 0.1% that for the d_5 species.

Internal standard B

Samples of [2,2,3α,4,4-$^2H_5$]-**I** and [3α-^{3}H][2,2,4,4-$^2H_4$]-**I** were mixed and treated with a 1:1 mixture of acetic anhydride and pyridine for 1 hr at 70°C. After evaporation of the solvent, a portion of the resulting product was subjected to radio-TLC (solvent, 20% ethyl acetate in hexane) along with authentic samples of **I** and 3β-acetoxy-5α-cholest-8(14)-en-15-one. GLC–MS showed a single component (> 99%) which had the same retention time as an authentic sample of 3β-acetoxy-5α-cholest-8(14)-en-15-one. The mass spectrum of the acetate derivative of the doubly labeled 15-ketosterol showed the following isotopic composition: d_5 44%, d_4 53%, and d_3 < 3%. The abundance of ions corresponding to d_0 species was less than 0.1% that for the d_5 species. This sample was designated as internal standard B.

Internal standard C

The preparation and characterization of [2,2,3α,4,4,7,7,9α,16,16-$^2H_{10}$]-**I** have been described previously (19). This sample was designated as internal standard C. Its isotopic composition was as follows: d_{10} 54%, d_9 35%, and d_8 11%. For a compound with ten deuterium atoms with equal labeling at each of the ten positions, the observed value of 54% d_{10} species corresponds to a 94% deuteration at each of the ten sites.

Experimental design

The experimental design used for the determination of the levels of **I** in rat skin and in rat hair is outlined in **Fig. 2**. The results of preliminary experiments indicated that the probable concentrations of **I** in skin were very low, requiring a sensitive detection system, such as GLC–MS, and

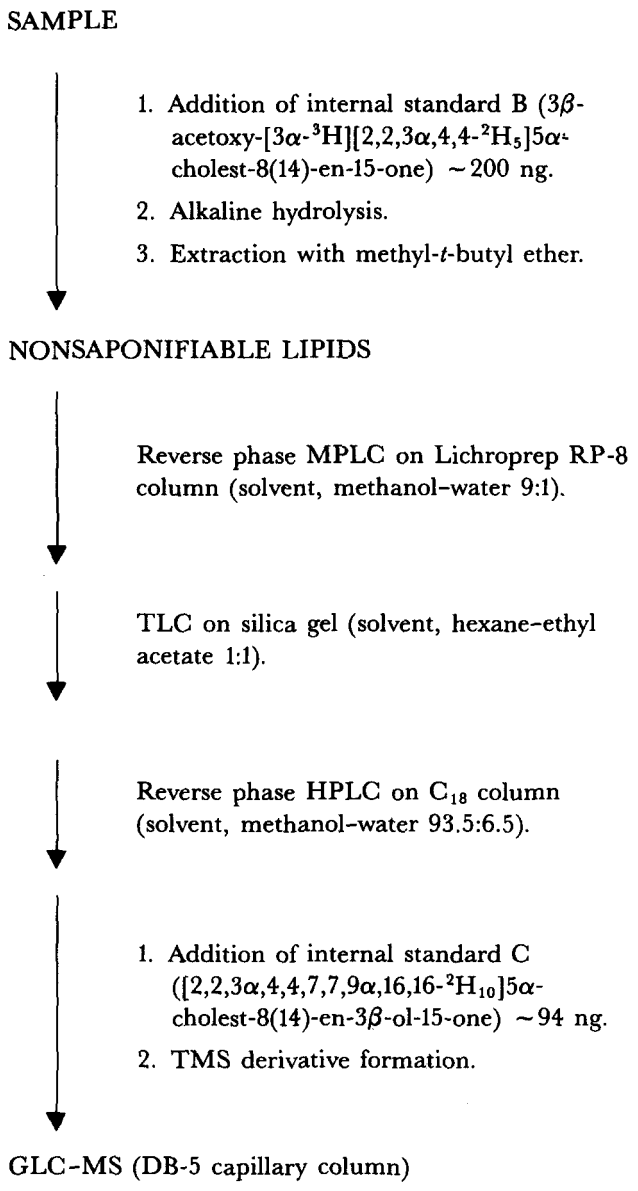

Fig. 2. Experimental design for the determination of total 5α-cholest-8(14)-en-3β-ol-15-one.

extreme attention in preparation of samples. Hydrolysis under alkaline conditions with subsequent isolation of the nonsaponifiable lipids (NSL) represents a classical initial step in the detection and quantitation of individual sterols. The case of **I** presented a special problem in this respect since this sterol, especially in low concentrations, has been found to be notably unstable to commonly employed saponification conditions (10). The base-catalyzed decomposition of **I** can be partially suppressed by conducting the alkaline hydrolysis under mild conditions (14) and/or in the absence of oxygen (16).

The GLC-MS detection and characterization of minor components of the NSL of skin required very extensive fractionation of a complex mixture of sterols and other constituents. Removal of the bulk of the nonpolar sterols in skin was effected by reverse phase MPLC followed by TLC on silica gel plates. Reverse phase HPLC was required to remove residual nonpolar constituents and to provide partial fractionation of **I** from other constituents prior to GLC-MS.

The GLC-MS quantitation of **I** employed internal standards that were almost completely enriched with deuterium at carbon atoms 2 and 4 and labeled with either ^3H or ^2H at the 3α-position. The resulting internal standards (obtained upon admixture of [2,2,3α,4,4-$^2H_5$]-**I** and [3α-^3H][2,2,4,4-$^2H_4$]-**I**) provided samples containing 44% and 52% (internal standard A) and 44% and 53% (internal standard B) of the d_5 and d_4 species, respectively, levels of deuteration which were very favorable for their use in mass fragmentography (MF). The use of an internal standard labeled not only with ^2H but also with moderate levels of ^3H in the same molecule provided a very sensitive and accurate means for the localization of **I** in the various chromatographic procedures used in the preparation of the sample for final quantitation by GLC-MS. Moreover, the ^3H-labeled internal standard permitted estimation of the recovery of **I** at the individual steps in its purification prior to GLC-MS. It is important to note that the doubly labeled internal standards (internal standards A and B) were prepared in such a way that negligible amounts of d_0 species were present, thereby simplifying the determination of small quantities of **I** by mass fragmentography.

A carrier, internal standard C (94 ng), was also added to the samples prior to TMS formation and GLC-MS to improve GLC performance. This addition provided a constant amount of a carrier independent of the recovered amount of **I** (both labeled and unlabeled). The very high extent of deuteration in internal standard C permitted its use for this purpose without compromising the quantitation of the **I** by MF. For the calculation of the amount of **I** in the samples the following equations were used.

$$a_u = a_d \times \frac{A_{472} + A_{473}}{A_{475} + A_{476} + A_{477} + A_{478}}$$

a_u = the amount of unlabeled species
a_d = the amount of labeled internal standard B (nmol)
a_N = the area for the mass fragmentogram of *m/z* N with the retention time of the TMS derivative of **I** *Eq. 1*

$$CA_{478} = A_{478} - \left(A_{481} \times \frac{A_{478}^*}{A_{481}^*}\right)$$

CA_{478} = the corrected area for the mass fragmentogram of *m/z* 478 due to the contribution from the d_6 species in internal standard C (see explanation below) *Eq. 2*

A_N^* = the area for the mass fragmentogram of *m/z* N from a separate analysis of the TMS derivative of internal standard C.

$$a_u = a_d \times \frac{A_{472} + A_{473}}{A_{475} + A_{476} + A_{477} + CA_{478}} \qquad \textit{Eq. 3}$$

Since the molecular ion is the ion of highest abundance (base peak) in the mass spectrum of the TMS derivative of **I** and the level of d_0 species in the internal standards is negligible, the standard equation used for isotope dilution methods can be simplified to the formula presented above (equation 1). A correction was made for the contribution of d_6 species derived from internal standard C (equation 2). The ratio of the area of the peaks from the *m/z* 478 and 481 ion traces from a number of mass fragmentography analyses of internal standard C was fairly constant (0.05 ± 0.002 (SEM; N = 30)). The contribution of the d_6 species was determined by measurements of the peak area for the *m/z* 481 ion trace from the sample multiplied by the ratio of the peak areas in the *m/z* 478 and 481 ion traces from a separate analysis of internal standard C made on the same day as the analysis of the sample. The average contribution of the signal at *m/z* 478 due to the d_6 species to the sum of the peak areas from the ion traces (*m/z* 475–478) was 1.6% and was never greater than 2.8% of the total value used to represent the internal standard B in the sample ($A_{475} + A_{476} + A_{477} + CA_{478}$). Equation 3 was used to determine the amount of **I** in the samples.

Studies of the levels of 5α-cholest-8(14)-en-3β-ol-15-one (free plus esterified) in rat skin

The pelt (~68 g) of a male Sprague-Dawley rat (~300 g in weight) was cut into 1-cm^2 square sections which were then stored at −70°C. Five separate samples of varying weight (2.0 to 6.9 g) were, after the addition of the doubly labeled internal standard B (~200 ng), heated at 70°C for 1 hr with 10% KOH in 95% ethanol (20 ml) in a sealed tube under argon. After cooling to room temperature, water (20 ml) was added and the resulting mixture was extracted twice with methyl-*t*-butyl ether (40-ml portions). The combined ether extracts were washed twice with water (40-ml portions) and the solvent was evaporated under reduced pressure and the residue was dried by evaporation with benzene (~200 ml). The average recovery of the added labeled internal standard in the NSL was 79% (range, 61 to 96%). The major portion of the NSL was subjected to MPLC as described above. Almost all (92–99%) of the ^3H eluted as a discrete peak (center at fractions 23–24) which contained over 90% of the applied ^3H in each case. The fractions corresponding to the major peak of ^3H were pooled and subjected to TLC on silica gel G (solvent, hexane–ethyl acetate 1:1). The material in a 1-cm zone (R_f 0.39–0.45) corresponding to the mobility of **I** (as monitored by TLC of internal standard C applied to a separate lane) was scraped from the plate and eluted from the silica gel with a recovery of the ^3H ranging from 85 to 95%. The material with the mobility of **I** on TLC was further subjected to reverse phase HPLC using methanol–water 93.5:6.5 as the eluting solvent at a flow rate of 1 ml per min. Fractions were collected each minute. Most (85–90%) of the ^3H was recovered as a single peak with the same retention time (~12.5 min) as that of authentic **I** (as determined by separate injections of internal standard C). The bulk of this material was usually recovered in a single fraction. The HPLC elution profile for one of the samples is presented in **Fig. 3**. Mass was monitored by absorbance at 260 nm and ^3H was monitored by assay of a small portion (~1%) of the fractions. While the bulk of the ^3H (89%) was recovered at 12.0 to 13.0 min (corresponding to the mobility of **I**), the profile of absorbance at 260 nm showed the presence of large amounts of material other than **I**. Of the initial amounts of internal standard B which were added to the skin samples, approximately 40% was available for GLC–MS analysis after saponification, purification by MPLC, TLC, and reverse phase HPLC. After the addition of the internal standard C (94 ng) the samples were silylated and ~20% was subjected to GLC–MS (condition C). **Fig. 4** (lower panel) shows the total ion current (*m/z* 200 to *m/z* 600) obtained for the sample illustrated in Fig. 3. In addition, this separation was monitored at *m/z* 477 (molecular ion of TMS derivative of internal standard B) and 472 (molecular ion of TMS derivative of **I**). This permitted the localization of the derivative of **I** within the complex mixture of minor sterols still present in the HPLC fraction corresponding to **I**.

Fig. 5 (middle panel) shows the averaged mass spectrum (*m/z* 200 to *m/z* 500) of the material detected in scans 191 and 192 (the center of the GLC peak, Fig. 4, bottom panel). The mass spectrum was, apart from the small cluster of ions around *m/z* 476 and 477 (residue of d_4 and d_5 species), essentially identical to that of TMS derivative of authentic **I** (bottom panel). This finding provided very strong additional evidence indicating that the material obtained from rat skin that had the same chromatographic behavior as **I** on MPLC, TLC, reverse phase HPLC and, as the TMS derivative, on GLC is, in fact, the $\Delta^{8(14)}$-15-ketosterol. Moreover, further analysis of the mass spectrum of the TMS derivative of the sample derived from the rat skin provides additional evidence on this matter. A number of individual ions in the mass spectrum of the TMS derivative of the sample obtained from the rat skin (Fig. 5, middle panel) are of considerable diagnostic significance. Moreover, considerable supporting evidence exists with respect to the composition and the origin of these ions (19). Ions in the high mass region of the spectrum were those at *m/z* 472 (M), 457 (M–CH_3), 454 (M–H_2O), 382 (M–TMSOH), 367 (M-TMSOH–CH_3), and 341 (M–H_2O–side chain). Ions in the lower mass range included those at *m/z* 287 (no assignment), 269 (M-TMSOH-side chain), and 251 (M–H_2O-TMSOH-side chain). Two ions, those

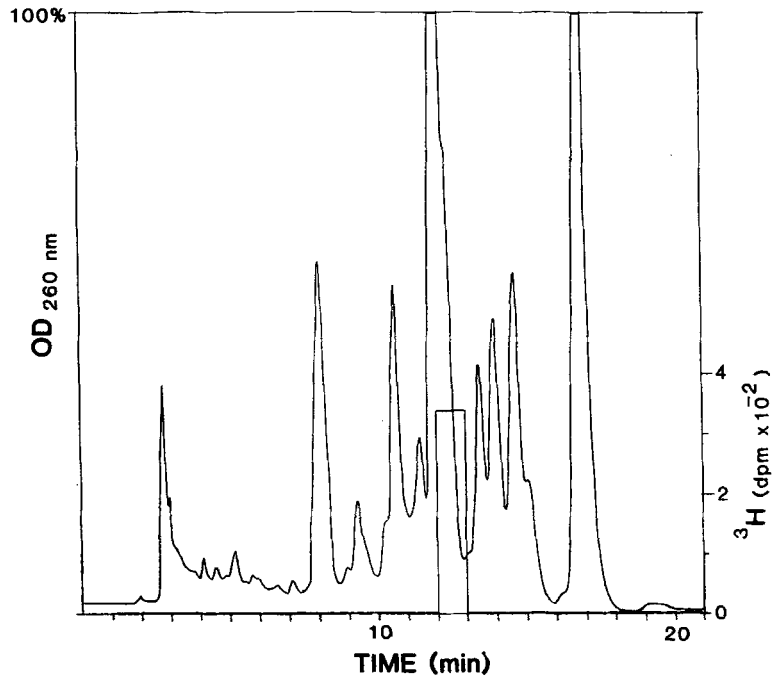

Fig. 3. C_{18} HPLC elution profile monitored by assay of ^3H and absorbance at 260 nm (0.05 AUFS) of nonsaponifiable lipids of rat skin containing [3α-^3H][2,2,4,4-$^2H_4$]5α-cholest-8(14)-en-3β-ol-15-one (derived from internal standard B) after preliminary purification by MPLC and TLC. The eluting solvent was methanol–water 93.5:6.5.

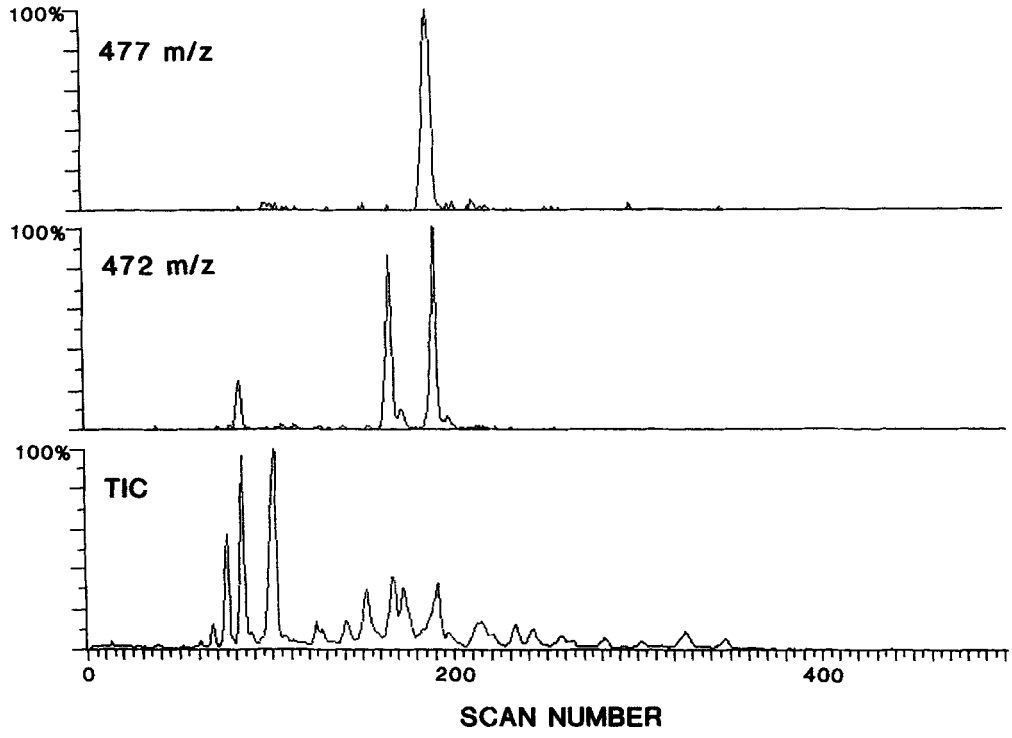

Fig. 4. Capillary GLC–MS of the TMS derivative of sample derived from rat skin (containing internal standards B and C) after purification by MPLC, TLC, and HPLC (Fig. 3). The lower panel shows the profile obtained by monitoring of the total ion current (*m/z* 200 to 600). The middle and upper panels show the profiles obtained upon monitoring of the ions at *m/z* 472 and *m/z* 477, respectively. Scans were recorded from 4.0 to 9.6 min.

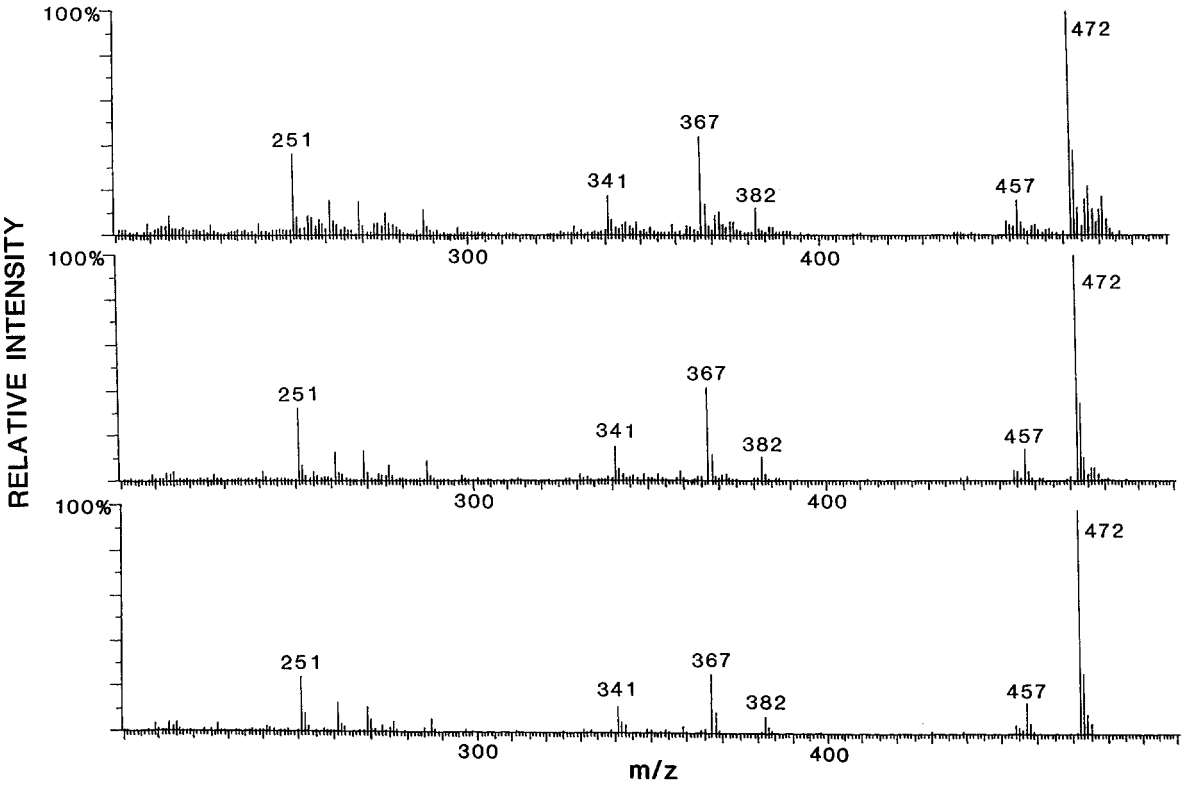

Fig. 5. Mass spectra (*m/z* 200 to *m/z* 500) of TMS derivative of sample obtained from rat skin (containing internal standards B and C) after purification by MPLC, TLC, HPLC (Fig. 3), and capillary GLC (Fig. 4). The middle panel shows the averaged partial mass spectrum of the material detected in scans 191 and 192 (Fig. 4) which correponds to the peak of the material with the chromatographic behavior of the TMS derivative of the 15-ketosterol on capillary GLC as detected by monitoring at *m/z* 472. The top panel shows the averaged partial mass spectrum for the scans between 183 and 195 (Fig. 4) which corresponds to the bulk of the peak observed in the recording of the total ion current (Fig. 4). The bottom panel shows the partial mass spectrum of the TMS derivative of an authentic sample of 5α-cholest-8(14)-en-3β-ol-15-one in an independent experiment.

at *m/z* 276 and 261, are of special diagnostic significance (19) and correspond to ions arising from rings C and D and the alkyl side chain (19).

Also shown in Fig. 5 (top panel) is the averaged mass spectrum (*m/z* 200 to *m/z* 500) for the scans between 183 and 195, corresponding to the bulk of the GLC peak which was detected at *m/z* 477 and at *m/z* 472 and in the total ion current (Fig. 4). This mass spectrum shows all ions of the TMS derivative of unlabeled **I** in addition to ions corresponding to internal standard B and C.

The levels of **I** observed in the five samples of rat skin are presented in **Table 1**. The mean level of **I** in the skin samples was 84.5 ± 4.1 (SEM) ng per g of skin.

Studies of the levels of 5α-cholest-8(14)-en-3β-ol-15-one (free plus esterified) in rat hair

The hair of ten Sprague Dawley rats (average body weight ~338 g) was removed and stored in sealed plastic bags at −70°C. Samples (1.0–2.0 g) from the ten animals were individually assayed for the levels of **I** as described above for rat skin. The doubly labeled internal standard B (205 ng) was added prior to saponification. The average recovery of the added labeled internal standard in the NSL was 87%. A portion of the NSL was subjected to GLC analysis (condition B) for determination of the levels of cholesterol in the rat hair samples. A major portion of the NSL was then processed as in the case of the rat skin by MPLC, TLC, and reverse phase HPLC (Fig. 2). The average recoveries of ^{3}H at each step were as follows: after MPLC, 90%; after TLC, 95%; and after reverse phase HPLC, 89%. Of the initial amounts of internal standard B which were added to the hair samples, approximately 42% (range, 31 to 47%) was available for GLC–MS. After the addition of internal standard C (94 ng), the samples were silylated and ~20% of the resulting TMS derivative was subjected to GLC–MS (condition C).

The analyses of the hair samples are illustrated with the following description of the analysis of one of the hair samples. A portion (0.80 g) of the hair from one of the animals was, after the addition of the doubly labeled internal standard B (205 ng), treated in the same manner as described for the case of rat skin. The recovery of ^{3}H of the internal standard in the NSL was 80%. After MPLC and TLC the recoveries were 92% and 89%, respectively. The material

TABLE 1. Levels of total 5α-cholest-8(14)-en-3β-ol-15-one in samples of rat skin from a male Sprague-Dawley rat

Sample	5α-Cholest-8(14)-en-3β-ol-15-one	
	Total	Concentration
g	*ng per sample*	*ng per g*
2.0	199	99.5
4.0	318	79.5
4.1	310	75.6
5.4	446	82.6
6.9	590	85.5
Mean ± SEM		84.5 ± 4.1

with the mobility of **I** on TLC was further subjected to reverse phase HPLC using methanol–water 94:6 as the eluting solvent at a flow rate of 1 ml per min. Fractions were collected each min from 2.6 to 22.6 min and small portions (~1%) were removed for assay of ^3H. The chromatogram is shown in **Fig. 6**. The recovery of applied ^3H in fractions 12 and 13, corresponding to the mobility of authentic **I**, was 92%. Of the initial amount of the internal standard B which was added to the hair sample, 39% was available for GLC–MS. The material recovered in fraction 12 from the HPLC separation was, after the addition of internal standard C (94 ng), silylated. Approximately 20% of the resulting TMS derivative was subjected to GLC–MS using GLC condition C.

Fig. 7 shows the chromatogram obtained by monitoring the total ion current (*m/z* 100 to *m/z* 600). As in the case of the rat skin (Fig. 4) and despite the extensive purification of the sample by MPLC, TLC, and HPLC prior to GLC, the chromatogram derived from monitoring the total ion current shows a large number of components. Fig. 7 also shows the averaged mass spectrum (*m/z* 100 to *m/z* 500) of the material detected in scans 225 and 226 (corresponding to the peak of the material with the chromatographic behavior of the TMS derivative of **I** on GLC as detected by monitoring at *m/z* 472 or the total ion current (*m/z* 100 to *m/z* 600) as shown in the chromatogram). The mass spectrum was very similar to that observed with the TMS derivative of an authentic sample. Ions of low abundance were observed at *m/z* 476 and 477 in the sample from rat hair that were due to the d_4 and d_5 species in internal standard B which was added prior to saponification. The high degree of correspondence of the mass spectrum of the TMS derivative of the sample derived from rat hair with that obtained from the TMS derivative of an authentic sample of **I** provides very strong additional evidence indicating that the material obtained from rat hair which had the same chromatographic behavior as **I** on MPLC, TLC, reverse phase HPLC, and, as the TMS derivative, on GLC is, in fact, the $\Delta^{8(14)}$-15-ketosterol.

The results of the MF analysis indicated the presence of 228 ng of **I** in the 0.80-g sample of rat hair. **Table 2** presents the results of identical studies of the levels of **I** in rat hair obtained from ten rats. The mean concentration of **I** was 143 ± 19 (SEM) ng per g of hair. Also presented in Table 2 are the levels of cholesterol in the hair obtained from eight of the rats. The average concentration of cholesterol was 2.54 ± 0.10 (SEM) mg per g of hair. The concentration of **I** was ~0.006% that of cholesterol.

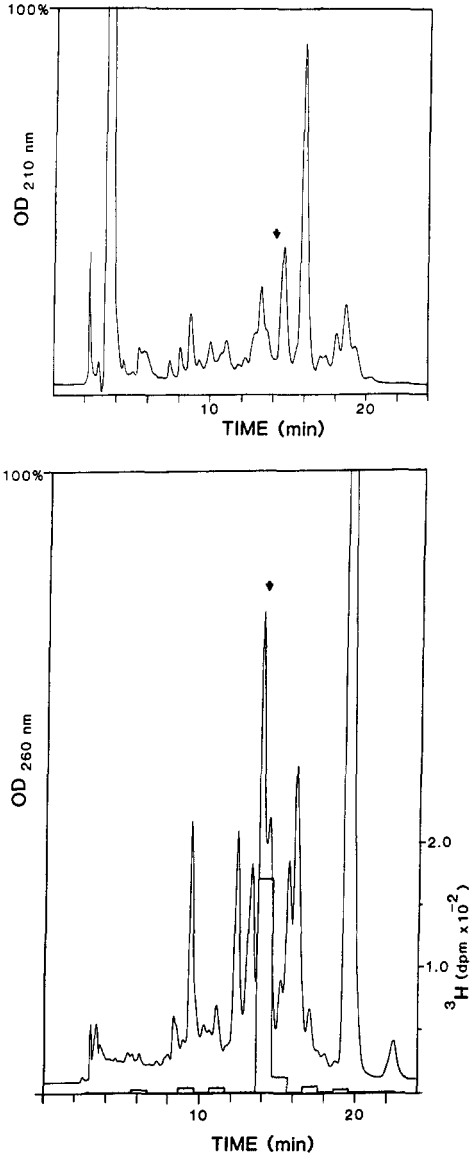

Fig. 6. C_{18} HPLC elution profiles monitored by assay of ^3H and absorbance at 210 nm (above, 0.5 AUFS) and 260 nm (below, 0.1 AUFS) of nonsaponifiable lipids of rat hair containing [3α-^3H][2,2,4,4-$^2H_4$]5α-cholest-8(14)-en-3β-ol-15-one (derived from internal standard B) after preliminary purification by MPLC and TLC. The eluting solvent was methanol–water 94:6 and fractions were collected every minute from 2.6 min to 22.6 min. The arrows indicate the elution time of internal standard C from a separate injection.

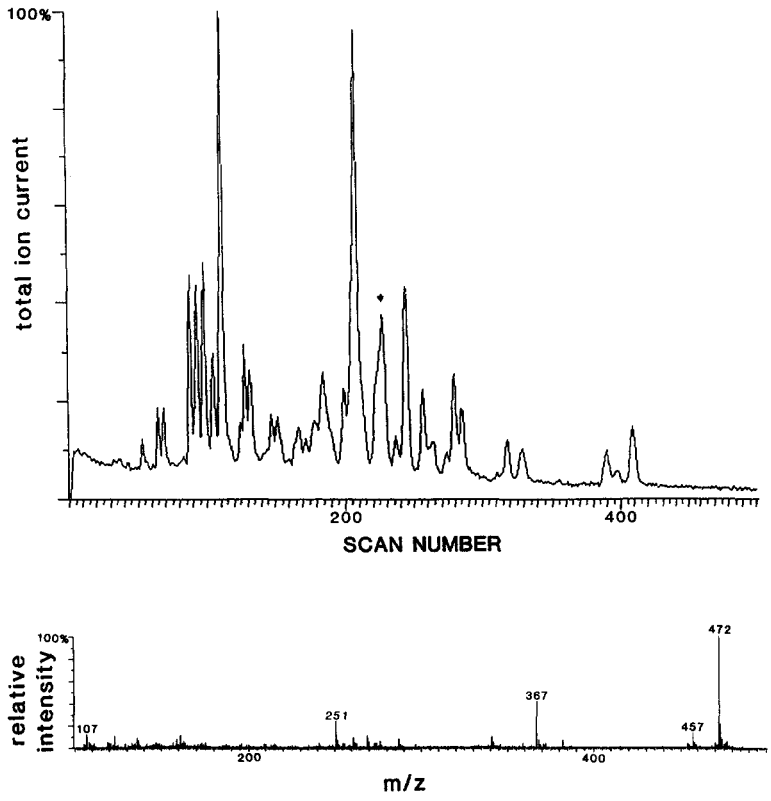

Fig. 7. Capillary GLC-MS of the TMS derivative of sample derived from rat hair (containing internal standards B and C) after purification by MPLC, TLC, and HPLC (Fig. 6). Shown is the profile obtained upon monitoring of the total ion current (above, *m/z* 100 to *m/z* 600). Scans were recorded from 6.0 to 13.0 min and the peak containing the internal standards is marked with the arrow. The bottom panel shows the averaged mass spectrum of the material detected in scans 225 and 226 (above) which correspond to the peak of the material with the chromatographic behavior of the TMS derivative of the 15-ketosterol.

State of esterification of 5α-Cholest-8(14)-en-3β-ol-15-one in rat hair

To a large sample of rat hair (41 g) from male Sprague-Dawley rats was added the stearic acid ester of [3α-^{3}H][2,2,4,4-$^2H_4$]-**I** (1.37 μg; 3.35 × 10^5 dpm) in benzene (20 μl) and internal standard A (0.89 μg; 2.01 × 10^5 dpm) in ethyl acetate (200 μl). This material was then extracted twice with chloroform-methanol 2:1 (600-ml portions) and filtered through prewashed Whatman #1 paper. The hair on the filter was then washed 5 times with chloroform (100-ml portions). After reduction of the volume of the extract to ~500

TABLE 2. Levels of 5α-cholest-8(14)-en-3β-ol-15-one and of cholesterol in samples of rat hair from male Sprague-Dawley rats

Rat	Sample	5α-Cholest-8(14)-en-3β-ol-15-one		Cholesterol Concentration
		Total	Concentration	
	g	*ng*	*ng per g*	*mg per g*
1	0.66	68	103	
2	1.05	147	140	2.51
3	1.17	122	104	2.36
4	2.17	214	99	
5	0.87	63	72	2.93
6	0.98	160	163	2.57
7	0.80	228	285	2.73
8	0.69	122	177	2.81
9	0.84	129	154	2.09
10	0.92	118	128	2.33
Mean ± SEM			143 ± 19	2.54 ± 0.10

ml, the solution was washed once with water (240 ml) and the solvent was evaporated under reduced pressure. Recovery of the added ^3H in the lipid extract was 97%. A small portion of this material was utilized for determination of free cholesterol by GLC. Another small portion of this material was saponified (10% KOH in 95% ethanol at 70°C for 1 hr) and the NSL recovered after extraction with methyl-*t*-butyl ether were subjected to GLC for determination of total cholesterol. A major portion (94%) of the material in the lipid extract of the rat hair was subjected to silica gel (60–200 mesh) column (100 cm × 2 cm) chromatography. The column was successively eluted with toluene (fractions 1 through 197), 5% ether in toluene (fractions 198 through 250), 10% ether in toluene (fractions 251 through 400), 20% ether in toluene (fractions 401 through 475), and finally chloroform–methanol 2:1. Fractions were collected every 32 min at a flow rate of 0.375 ml per min. Aliquots were taken for assay of radioactivity. Recovery of radioactivity applied to the column was 97%. The ^3H of the internal standard of the stearic acid of [3α-^3H][2,2,4,4-$^2H_4$]-I was eluted in fractions 165–200 while that of internal standard A was recovered in fractions 392–428.

Fractions 165–200 were pooled and, after evaporation of the solvent, dried. A portion of this material was saponified with 5% KOH in 95% ethanol for 30 min at 65°C under argon and the NSL were subjected to MPLC (78% recovery) and TLC (95% recovery) as described above for the case of rat skin. The material recovered from the TLC was further subjected to reverse phase HPLC using methanol–water 9:1 as the eluting solvent at 1 ml per min. Most of the ^3H eluted at 27–28 min and this material was, after addition of internal standard C (47 ng), silylated. Approximately 20% of the resulting TMS derivative was subjected to GLC–MS (condition C). The chromatogram obtained by monitoring of total ion current (*m/z* 100 to *m/z* 600) showed a large number of components (**Fig. 8**). The peak seen in this chromatogram between scans 180 and 195 contained the TMS derivative of internal standard C (centered

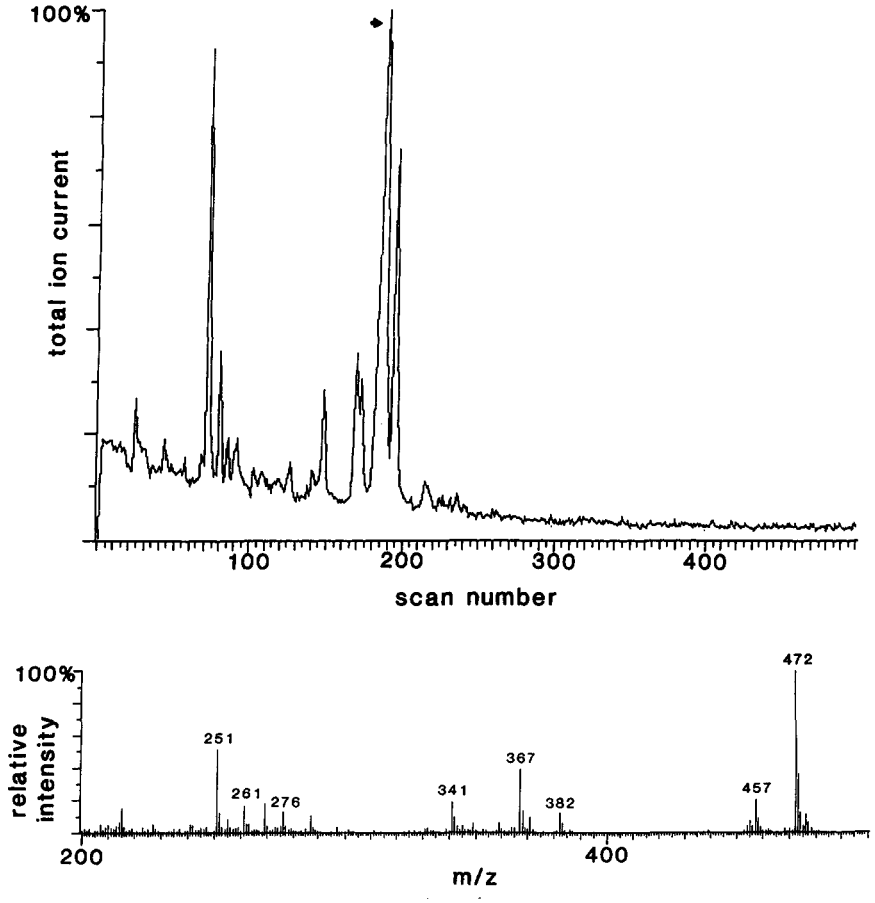

Fig. 8. Capillary GLC–MS of the TMS derivative of sample derived from solvent-extracted rat hair (containing [3α-^3H][2,2,4,4-$^2H_4$]5α-cholest-8(14)-en-3β-ol-15-one from the stearic acid ester and internal standard C) after purification by silica gel chromatography, saponification of pooled fractions 165–200 and then further purification by MPLC, TLC, and HPLC of the nonsaponifiable lipids. Shown is the profile obtained upon monitoring of the total ion current (above, *m/z* 100 to *m/z* 600). Scans were recorded from 6.0 to 14.4 min. The bottom panel shows the averaged mass spectrum of the material detected in scans 187 and 188 (above) which corresponds to the peak of the material with the chromatographic behavior of the TMS derivative of the 15-ketosterol.

at scan 181) and the d_4 species from the stearic acid ester of **I** (centered at scan 185). Also shown in Fig. 8 is the averaged mass spectrum of this material (scans 187 and 188). The spectrum is similar to that of the TMS derivative of authentic **I** (Fig. 5). Also present in this mass spectrum was the molecular ion (*m/z* 476) derived from the internal standard. The internal standard of the stearic acid ester of the doubly labeled **I** used in this experiment contained four atoms of deuterium. Accordingly, the calculation of the amount of **I** in the sample involved the sum of the peak areas of the ion profiles at *m/z* 475, 476, and 477 (rather than those from *m/z* 475–478 for the case of the experiments described above which involved the use of internal standard B). The calculated amount of **I** associated with its esters derived from 41 g of rat hair was 1,544 ng.

The contents of fractions 392 through 428 (corresponding to the mobility of free **I**) from the silica gel column chromatographic separation of the rat hair lipids were subjected to MPLC as described above. Of the ^3H applied to the MPLC column, ~77% had the mobility of **I**. A portion of this material was separated by semi-preparative Spherisorb HPLC (5% isopropanol in hexane at 2 ml per min) and the contents of the fractions (90% of ^3H applied to the column) corresponding to the mobility of **I** were subjected to reverse phase HPLC. The contents of the fractions (83% of ^3H applied to the C-18 column) corresponding to the mobility of **I** were pooled and, after the addition of internal standard C (94 ng), silylated. Approximately 10% of the resulting TMS derivative was subjected to GLC-MS (condition C). The chromatogram (**Fig. 9**) obtained by monitoring of the total ion current (*m/z* 100 to *m/z* 600) showed a number of components. The peak seen in this chromatogram between scans 180 and 195 corresponds to the TMS derivative of the deuterated (internal standards A and C) and undeuterated **I** (as determined by MF). The averaged mass spectrum of scans 191 and 192 is also presented in Fig. 9 and shows the characteristic fragment ions of **I**. The extract of the 41 g of rat hair contained 588 ng of free **I**. Thus, of the total **I** recovered in the extract of the rat hair, ~72% was associated with its esters.

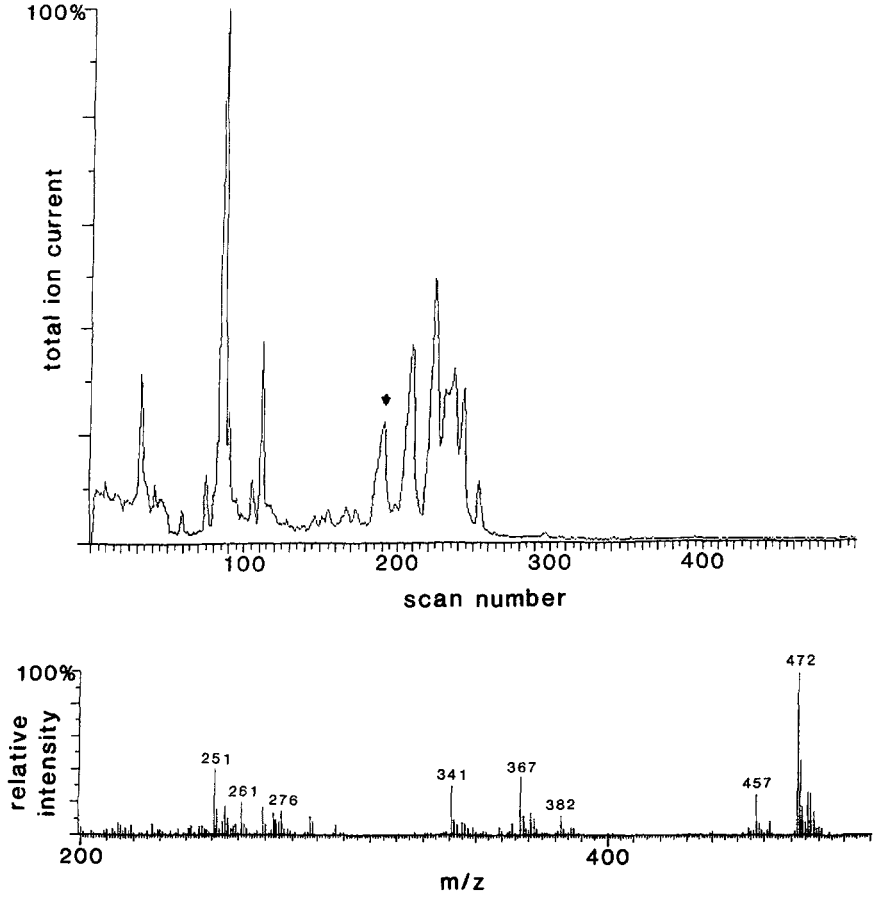

Fig. 9. Capillary GLC-MS of the TMS derivative of sample derived from solvent-extracted rat hair (containing internal standards A and C) after purification by silica gel chromatography (pooled fractions 391–428), MPLC, semi-prep silica gel HPLC, and C_{18}-HPLC. Shown is the profile obtained upon monitoring of the total ion currents (above, *m/z* 100 to *m/z* 600, from 6.0 to 14.4 min). The bottom panel shows the averaged mass spectrum of the material detected in scans 191 and 192 (above) which corresponds to the peak of the material with the chromatographic behavior of the TMS derivative of the 15-ketosterol.

The amounts of free and esterified cholesterol in the extract of the 41 g of rat hair were 13.2 mg and 25.2 mg, respectively. Thus, of the total cholesterol recovered in the extract of the rat hair, ~66% was associated with its esters.

The extraction conditions employed in this study were intentionally very mild so as to avoid autooxidation and other decomposition of the sample which might be anticipated through the use of more drastic extraction procedures. As a consequence of this design, the recoveries of total I and total cholesterol were considerably less than that anticipated on the basis of the previous analyses of the individual samples of rat hair. However, the recoveries of total I and total cholesterol from the rat hair were comparable, i.e., 38% and 37%, respectively.

Additional control experiments and precautions to insure validity of experiments

In the determinations of the amounts of I in rat skin and rat hair the amount of the doubly labeled internal standard B added to the sample was ~200 ng. The amount of d_0-I present in internal standard B was less than 0.1% that of the d_5 species or such that the contribution of the d_0 species in internal standard B in the analysis of I in the skin or hair samples was less than 0.1 ng, a value considerably less than the estimated lower limit of detection of ~0.4 ng of I. While a background level of ~0.4 ng per sample was anticipated, a higher level (~4 ng per sample or ~2 ng in the final GLC–MF determination) was consistently observed. This "background" level was determined by periodic inclusion of control experiments involving the processing of internal sample B through the various steps used in the isolation and determination of I as described above for the skin and hair samples. Due to the instability of I under alkaline conditions (10) a slight modification was introduced into the procedure outlined in Fig. 2 for the control experiments. We found that adding ovalbumin (~200 mg) dissolved in a small amount of water (1–2 ml) to the ethanolic KOH solution for the hydrolysis of internal standard B (~200 ng) diminished the extent of the base-catalyzed decomposition of I. The recovery of ^{3}H, derived from internal standard B, in the extract after saponfication ranged from 60 to 90% when ovalbumin was added while only 10–20% was usually recovered without this addition. After passing the doubly labeled internal standard through the same chromatographic procedures outlined in Fig. 2, the amount remaining for GLC–MF analysis ranged from 30 to 50% of the amount of ^{3}H added prior to saponification. In a separate experiment, the amount of I detected in the ovalbumin was less than 0.8 ng per 200 mg of ovalbumin. As noted above for the case of rat skin and rat hair, the recovery of the internal standard after saponification without the addition of ovalbumin was high (~80–90%). The factor(s) present in rat skin and rat hair which appear to suppress the decomposition of I during alkaline hydrolysis is not known and was not investigated further.

As noted above, a "background" of ~4 ng per sample was observed in the GLC–MF analyses of the control samples. While the source of this consistent "background" at *m/z* 472 in the determination of I was not established, it is important to note that the amounts of I actually detected in the final GLC–MF analyses of the samples from skin and hair were very considerably in excess of this "background." For example, the actual amounts (ng) of I detected in the five analyses of rat skin and the ten analyses of rat hair were 17 to 149 times this "background" level.

Other individuals of this research group have, over the past decade, been involved in the chemical synthesis of I for use in chemical and biological experiments. To rule out the possibility of contamination of biological samples, glassware, solvents, equipment, etc. with I derived from this source, a separate laboratory (in which no experimentation with I had been pursued previously and three floors distant from the synthetic laboratory) was provided for the completion of the experiments described herein. The laboratory was equipped with new glassware, solvents, chromatographic units, etc. In addition to the controls noted above, frequent GLC–MS "blank" analyses were made on samples of solvents and reagents during the course of the analysis of the samples of skin and hair.

Since 5α-cholest-8(14)-en-3β-ol has been detected in rat skin (27, 28) and since I is a possible autooxidation product of 5α-cholest-8(14)-en-3β-ol, an analysis was made of a sample of rat hair (4.9 g) to which 520 μg of 5α-cholest-8(14)-en-3β-ol was added prior to saponification. The measured level of I in the hair sample was unaffected by the presence of the added 5α-cholest-8(14)-en-3β-ol, a finding indicating that I found in rat skin and hair was not generated from 5α-cholest-8(14)-en-3β-ol by autooxidation during the procedures used in the purification and quantitation of I.

DISCUSSION

The results presented herein demonstrate the presence of 5α-cholest-8(14)-en-3β-ol-15-one (I) in rat skin and hair. The mean level of I in ten rat hair samples was 143 ± 19 (SEM) ng per g. Approximately 72% of I in rat hair corresponded to esters of I. The identification of I in rat hair and rat skin was based upon its chromatographic behavior on reverse phase MPLC on Lichroprep RP-8 column, TLC on silica gel plates, reverse phase HPLC on a C_{18} column, and (as its TMS derivative) GLC on a DB-5 column and the correspondence of the mass spectrum of its TMS derivative to that of the TMS derivative of an authentic sample of I. This is the first report of the isolation of I from natural sources. It also represents the first isolation of a C_{27} 15-oxygenated 3β-hydroxy-5α-sterol from animal tissues. The potential importance of this finding derives from the very high potency of I in the regulation of the formation of mevalonic acid. The 15-ketosterol I, by

virtue of its control of mevalonate formation, represents a potential natural regulator of the synthesis of cholesterol and other mevalonate-derived products and thereby represents a potential new regulator of cell replication.

It is important to note that the natural occurrence and/or enzymatic formation of a number of 15-oxygenated steroid hormones has been described previously. The microbiological oxidation of a variety of steroid hormones to give 15-hydroxylated analogs has been very extensively studied (29–36). Similarly, the enzymatic formation of 15-hydroxylated C_{18}, C_{19}, and C_{21} steroids in animal tissues (or enzyme preparations derived therefrom) and/or their occurrence in urine has been reported previously (37–50). While 15-hydroxylation of bile acids by a number of molds has been reported (51), the formation of 15-oxygenated bile acids in animals has not, to our knowledge, been documented. 15-Oxygenated steroidal derivatives in plants are well known and include a number of cardiac glycosides (52, 53). In addition, the presence of a 15-oxygen function in various oogoniols is worthy of note. These oxygenated C_{29} sterols act as sex hormones in the water mold *Achyla* and reportedly induce the formation of oogonial branches at concentrations as low as 50 ng per ml (54–57 and references cited therein). The occurrence of 15-oxygenated sterols has also been reported in starfish (58, 59). Several 15α-hydroxysterols, substituted on the 15α-hydroxyl group with an N-acetylglucosamine function, have been isolated from a fish *Pardachirus pavoninus* (60). These substituted 15-oxygenated sterols, termed pavoninins, have been reported to be lethal to other fish and to have shark-repelling activity (60). Aringer (61) has reported the 15β-hydroxylation of 5β-cholestan-3α-ol and 24α-ethyl-5β-cholestan-3α-ol upon incubation with the 18,000 *g* supernatant fraction of rat liver homogenates. The hydroxylation was reported to be specific for these 3α-hydroxy-5β-sterols.

The precise mode(s) of origin of **I** in rat skin and hair is not known. 5α-Cholest-8(14)-en-3β-ol represents a potential precursor of **I**. We have previously demonstrated the presence of 5α-cholest-8(14)-en-3β-ol in rat skin (27, 28) and shown its convertibility to cholesterol in rat liver homogenate preparations (28, 62). It has been proposed that the overall conversion of the $\Delta^{8(14)}$-sterol to cholesterol proceeds via an intermediary $\Delta^{8(14)}$-3β,15-dihydroxysterol (63). The results of previous studies have shown that both 5α-cholest-8(14)-ene-3β,15α-diol and 5α-cholest-8(14)-ene-3β,15β-diol serve as efficient substrates for cholesterol formation in rat liver homogenate preparations (63–65). The conversion of the $\Delta^{8(14)}$-3β,15-hydroxysterols to cholesterol proceeds via the 5α-cholesta-8,14-dien-3β-ol which is then successively converted to 5α-cholest-8-en-3β-ol, 5α-cholest-7-en-3β-ol, 7-dehydrocholesterol, and cholesterol (63, 65). Dehydrogenation of either of the $\Delta^{8(14)}$-3β,15-dihydroxysterols would yield **I**. It is also important to note that **I** could represent an autooxidation product of 5α-cholest-8(14)-en-3β-ol. The results presented herein indicate that autooxidation of the $\Delta^{8(14)}$-sterol to give **I** did not occur during the procedures used for the isolation and quantitation of **I** in rat skin and hair. However, the possibility remains that some or all of **I** found in rat skin and hair arose by autooxidation of 5α-cholest-8(14)-en-3β-ol prior to the death of the animal. Thus, 5α-cholest-8(14)-en-3β-ol could serve as a precursor for the enzymatic formation of **I** or for its autooxidation to give **I**. The possibility that **I** of rat skin and hair arises by the action of bacteria or other microorganisms can also be considered.

Irrespective of the mechanism of origin of **I**, its occurrence in rat skin and hair raises the possibility of its role in the regulation of sterol and isoprenoid synthesis in skin and its potential role in the control of cell replication in this important organ.

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