Cholesterol 26-hydroxylase activity of hamster liver mitochondria: isotope ratio analysis using deuterated 26-hydroxycholesterol

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Summary Deuterated 26-hydroxycholesterol prepared from diosgenin by modifications of existing methods permitted the determination of mitochondrial cholesterol 26-hydroxylase using endogenous cholesterol as the substrate. Enzyme activity in a group of Syrian hamsters was found to be 10.3 ± 3.7 pmol · min^{-1} · mg $protein^{-1}$. —**Kok, E., and N. B. Javitt.** Cholesterol 26-hydroxylase activity of hamster liver mitochondria: isotope ratio analysis using deuterated 26-hydroxycholesterol. *J. Lipid Res.* 1990. **31:** 735–738.

Supplementary key words diosgenin • GLC-MS

Microsomal cholesterol 7α-hydroxylase and mitochondrial cholesterol-26 hydroxylase represent, respectively, the initial enzymatic steps in the transformation of the sterol ring and side chain of cholesterol in the metabolic pathways leading to the formation of chenodeoxycholic and cholic acids. Pitfalls in the assay of cholesterol 7α-hydroxylase previously described (1) have been reduced by using endogenous microsomal cholesterol as the substrate and deuterated 7α-hydroxycholesterol as an internal standard (2).

We describe the synthesis of a deuterated 26-hydroxycholesterol and its use for the assay of mitochondrial cholesterol 26-hydroxylase activity in hamster liver using endogenous cholesterol as the substrate.

MATERIALS AND METHODS

The preparation of deuterated 26-hydroxycholesterol begins with the Clemmensen reduction of diosgenin (Sigma Chemical, St. Louis, MO) to tetrahydrodiosgenin (16β-26-hydroxycholesterol), followed by chromium trioxide oxidation (3) to yield mostly the 16-oxo derivative and a minor product, 25-formyl-3β-hydroxycholest-5-en-16-one. Although these products are easily separable as described (3), they can be reduced together by the modified Wolff-Kishner reaction to yield 26-hydroxycholesterol and cholesterol, respectively. By substituting ND_2ND_2 and KOD (Merck Stable Isotopes, St. Louis, MO) for hydrazine and KOH, respectively, in the reduction step, deuterium is incorporated into both products. The deuterated cholesterol can be used for the synthesis of 7α-hydroxycholesterol, which can then serve as an internal standard for the previously reported method using isotope ratio mass spectrometry (2). This report concerns the deuterated 26-hydroxycholesterol.

A typical procedure begins with the addition of 2 g of diosgenin to 300 ml of 95% ethanol in a round-bottom flask equipped with a reflux condenser and a side-arm for a dropping funnel. After addition of 200 g of freshly prepared zinc amalgam (4), the flask is heated to the boiling point of the mixture. Concentrated HCl (100 ml) is added dropwise over a 90-min period and refluxing is continued for an additional 15 min. The ethanolic mixture is decanted through fluted filter paper into 2 liters of ice water and allowed to stand overnight in a cold room. The precipitate is collected by Buchner filtration and washed with water until the effluent is neutral. The dried product, mostly tetrahydrodiosgenin, is dissolved in hot ethyl acetate and precipitates as a semicrystalline mass (1.2 g) on cooling.

For oxidation with chromium trioxide, 500–1000 mg of the Clemmenson reduction product is first added to a mixture of 2.48 g of sodium acetate in 90 ml of glacial acetic acid in an Erlenmeyer flask equipped with a stirring bar. In a separate test tube, 80–160 mg of chromium trioxide is dissolved in 0.4 ml of water and diluted with 0.8 ml of glacial acetic acid; this solution is added to the flask with stirring. Although it is recommended that the reaction mixture be stirred at room temperature for 18 h (2), we have followed the oxidation of the tetrahydrodiosgenin at 2-h intervals using TLC with silica gel G and a solvent system of chloroform–methanol 4:1 (v/v) and noted the disappearance of its peak (R_f = 0.44) by 8 h and the appearance of two major derivatives (R_f = 0.50 and 0.73). We think that longer reaction times yield proportionally greater amounts of 25-formyl-3β-hydroxycholest-5-en-16-one.

The reaction mixture is poured into a separatory funnel and two phases are developed by the addition of water saturated with NaCl and a 1:3 mixture of toluene and ethyl acetate. The aqueous phase is discarded and the upper phase is washed with water until neutral and then taken to dryness.

The Wolff-Kishner reduction is carried out in a round-bottom flask equipped with a reflux condenser and a side-arm for a thermometer. The oxidation products (300 mg) are dissolved in 40 ml triethylene glycol, and 1.0 ml of 100% ND_2ND_2 and 800 mg of KOD are added. The temperature is raised slowly to 150°C to avoid excessive foaming. The reaction mixture initially appears amber but slowly loses color. The temperature is maintained at 150°C for 30 min and then raised to 195°C for 2 h, during which time the mixture becomes almost colorless. After

Abbreviations: GLC-MS, gas-liquid chromatography–mass spectrometry; TLC, thin-layer chromatography.

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cooling, it is poured into 250 ml of cold 0.5 N HCl and placed in the cold room overnight. The precipitate is harvested by Buchner filtration, washed until neutral, and dried in a drying oven at 80°C.

The dried reaction products (220 mg) are acetylated with pyridine–acetic anhydride and the reagents are removed by flash evaporation at 80°C. The syrupy residue is dissolved in methanol, and a semicrystalline material forms after the addition of water. The precipitate is collected by Buchner filtration and dried in a drying oven at 60°C.

The sterol acetates are dissolved in a mixture of toluene containing 0.1% ethyl acetate and adsorbed on a 30-g column of neutral alumina (activity grade 0). The column is developed with the same solvent mixture until the cholesterol acetate appears in the eluate (approximately 50–100 ml of solvent). The proportion of ethyl acetate is then increased to 0.5% and elution is continued until 26-hydroxycholesterol diacetate is obtained (usually 200 ml of solvent). After flash evaporation of the solvent, the 26-hydroxycholesterol is crystallized as needles from hot methanol (180 mg, m.p. 129°C).

GLC–MS analysis was done using a Hewlett Packard Model 5970 electron impact quadropole mass detector connected to a Model 5890 gas chromatograph. A standard 25-m CPsil-19 fused silica column (i.d. ± 0.25 mm) (Chrompack, Raritan, NJ) was used with a head pressure of 34.5 kPa.

For analyzing the diacetates of 25- and 26-hydroxycholesterol, temperature programming was at 0.2°C/min, from 270°C to 278°C. The instrument was run in the simultaneous ion-monitoring mode for quantification of 26-hydroxycholesterol and in the scan mode (*m/z* 128 to 650) for complete spectra.

Cholesterol was determined by GLC using 5β-cholestane-3β-ol as an internal standard, and protein was quantified by the method of Lowry et al. (5).

Syrian hamsters (Camm Research Lab Animals, Wayne, NJ) housed under fixed lighting conditions were maintained on a regular chow diet. For the preparation of liver mitochondrial fractions, the animals were acutely anesthetized between 9 and 10 AM with intraperitoneal pentobarbital, the abdomen was opened, and the liver was perfused with ice-cold 0.25 M sucrose containing 1 mM EDTA and buffered to pH 7.4.

After the livers were homogenized in 0.25 M sucrose containing 0.1 M Tris buffer, pH 7.4, mitochondria were prepared following previously described methods (2, 6, 7)

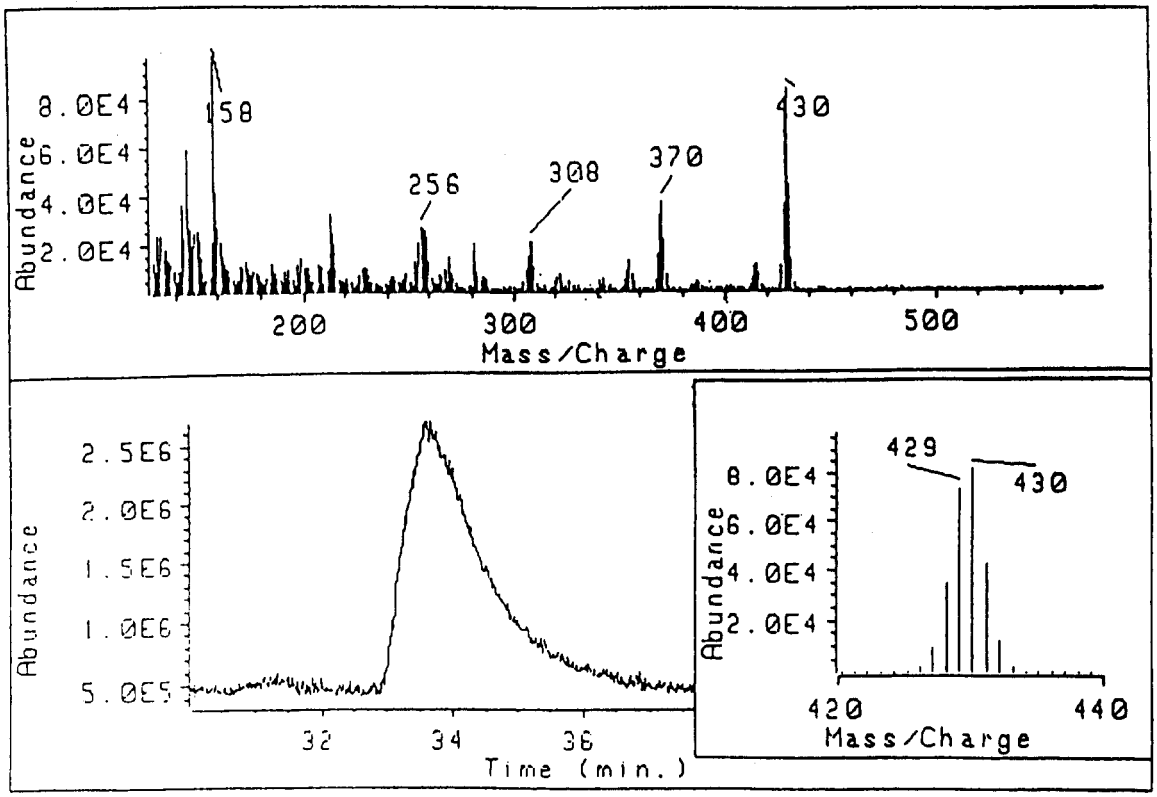

Fig. 1. GLC–MS analysis of deuterium-enriched 26-hydroxycholesterol diacetate. The total ion abundance is shown in the left lower panel with the abundance on the ordinate and time in minutes (min) on the abscissa. The peak at 33.8 min has a total ion current slightly greater than 2.5E6 (2.5×10^6). The abundance of each major ion fragment is shown in the upper panel and the region between *m/z* 420 and 440 is illustrated in greater detail in the lower panel, right insert. The predominant peaks are *m/z* 429 and 430, indicating the introduction of three to four deuterium atoms. The *m/z* 426 peak of protium 26-hydroxycholesterol diacetate [*m/z* = 486–60 (acetate)] is very slightly above background.

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with no significant modifications. After the mitochondrial suspension was warmed to 37°C, the reaction was started by the addition of isocitrate and aliquots were taken at 5-min intervals for 20 min. Each 1.5-ml aliquot contained 22–30 μg/ml of nonesterified cholesterol and 2.5–4.4 mg/ml of protein.

The incubations were terminated by the addition of 0.5 ml of 1 N NaOH. After addition of 1 μg of deuterated 26-hydroxycholesterol dissolved in 20 μl of methanol, the mixture was vortexed and the sterols were extracted into ethyl acetate. The organic fraction was taken to dryness, applied to a silica gel G thin-layer plate, and run in a solvent system of hexane–ethyl acetate 10:1 that leaves 26-hydroxycholesterol at the origin and removes cholesterol and lecithin (8). The sterols were extracted from the silica with methanol and acetylated using a 2:1 mixture of acetic anhydride–pyridine at 80°C for 30 min.

RESULTS AND DISCUSSION

Fig. 1 indicates the retention time of the deuterium-enriched 26-hydroxycholesterol. Reduction in strong base is known to labilize hydrogen atoms on carbons adjacent to the oxo group and therefore substitutions of more than two deuterium atoms usually occur during the reduction (9). The most abundant species are *m/z* 429 and 430, representing the substitution of 3 and 4 deuterium atoms, respectively. The protium 26-hydroxycholesterol, *m/z* = 426 [molecular ion = 486 – 60 (acetate)], constitutes less than 0.25% of the total mass and therefore no correction factor is needed for calculation of the amount of endogenous 26-hydroxycholesterol present. Also, the naturally occurring $M_0 + 4$ peak derived from 26-hydroxycholesterol is usually not detectable and therefore no background subtraction is necessary. Thus, the amount of endogenous 26-hydroxycholesterol that is generated is calculated directly from the ratio of the integrated areas of 426/430 multiplied by the proportion of *m/z* 430 present in the deuterated 26-hydroxycholesterol added to the sample.

Fig. 2 compares the peak of endogenous 26-hydroxycholesterol (*m/z* = 426) found in the mitochondria after incubation to the peaks of the internal standard (1 μg). Addition of cholesterol dissolved in acetone (7) to the mitochondrial suspension did not increase the yield of 26-hydroxycholesterol.

Fig. 3 indicates that the rate of synthesis was linear for the initial 10 min. Using the value obtained at 10

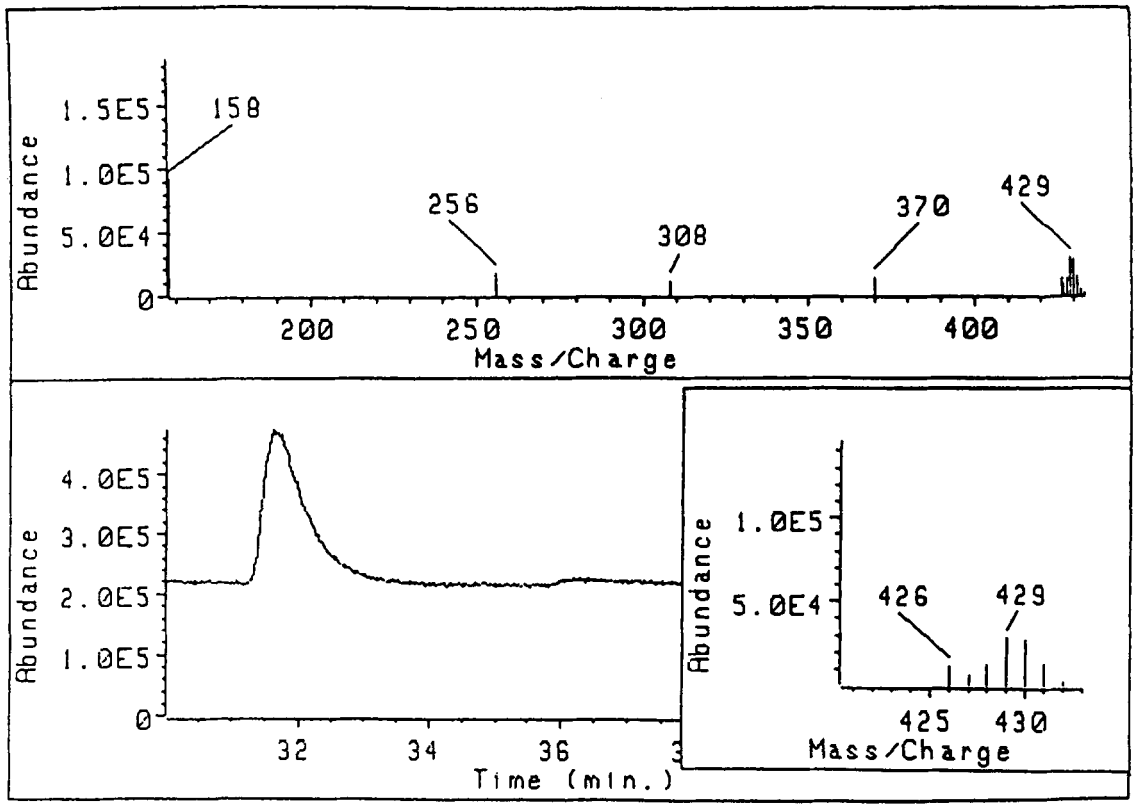

Fig. 2. GLC–MS analysis of 26-hydroxycholesterol diacetate from hamster mitochondria. As compared with Fig. 1, it is apparent that an increase in the abundance of *m/z* 426 relative to *m/z* 429 and 430 has occurred, which indicates the production of 26-hydroxycholesterol from endogenous cholesterol.

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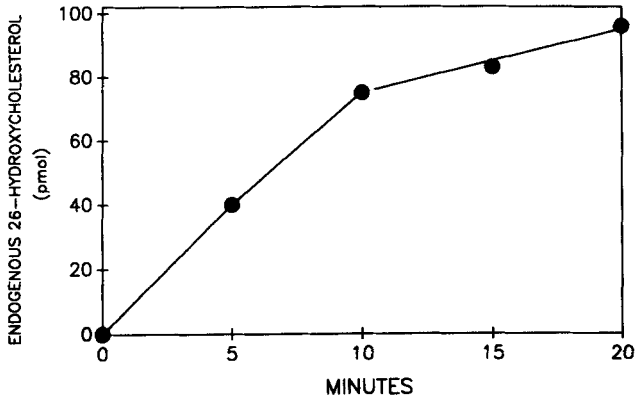

Fig. 3. Mitochondrial cholesterol-26 hydroxylase activity. Using isotope ratio analysis, the rate of production of 26-hydroxycholesterol was found to be linear for the initial 10 min.

min, mean synthesis rate of 26-hydroxycholesterol in five male hamsters was found to be 10.3 ± 3.7 (SD) pmol · min^{-1} · mg $protein^{-1}$.

A previous study of mitochondrial cholesterol 26-hydroxylase activity in rat liver required the addition of 20 μg of [4-^{14}C]cholesterol in acetone to the assay mixture (6). The rate of conversion was 14.7 pmol · min^{-1} · mg $protein^{-1}$ with isocitrate in the incubate as compared with 18.7 pmol · min^{-1} · mg $protein^{-1}$ when NADPH and Ca^{2+} were added. During the course of this study evidence was obtained to indicate that complete mixing of exogenous and endogenous cholesterol does not occur.

The synthesis of a deuterated internal standard eliminates the need for the addition of cholesterol and its vehicle and allows unambiguous identification of the endogenous 26-hydroxycholesterol based on mass spectrum and retention time.

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