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THE BIOSYNTHESIS OF 7,22-BISDEHYDROCHOLESTEROL FROM CHOLESTEROL

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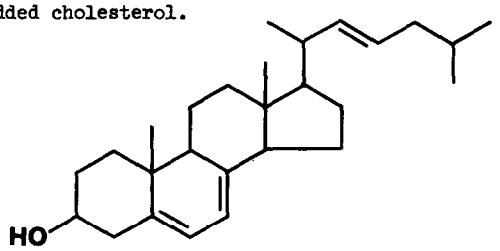
We have isolated and characterized the previously unknown 7,22-bisdehydrocholesterol ($\Delta^{5,7,22}$-cholestatrien-3β-ol)(I-OH). This elusive sterol, whose natural occurrence has been suggested but not confirmed in the past,[2] was the major component of the unsaponifiable lipid fraction obtained from the protozoan Tetrahymena pyriformis when we incubated this organism in a growth medium containing added cholesterol.

I-OH



Thus, in each of 204 flasks a solution of 5 mg of cholesterol in 1 ml of ethanol was dispersed into 500 ml of aqueous proteose peptone-based culture fluid at 80°; the mixtures were cooled to 28° and a seed culture of Tetrahymena pyriformis was added. After these axenic cultures were maintained at 28° for 36-40 hr, the cells (whose population had reached a stationary phase of growth) were collected by centrifugation and lyophilized. The residue (141.5 g) was extracted with chloroform-methanol (2:1, v/v), the extract was filtered and evaporated, and the residual total lipid fraction (14.4 g) was chromatographed on silicic acid with successive elution by 40-60° petroleum ether, 40-60° petroleum ether-diethyl ether (3:1, v/v), and methanol. The second of these eluates was evaporated and the residue was saponified with potassium hydroxide in aqueous methanol. The unsaponifiable lipid fraction was shown by quantitative glc to contain 660 mg of a mixture of sterols (65% yield based on 1.02 g of cholesterol). This mixture was crystallized from methanol, the 350-mg first crop of crystals was acetylated, and the resulting mixture was separated by column chromatography on silicic acid-silver nitrate[3] into the following acetates: cholesteryl (9.5%), 22-dehydrocholesteryl (5%), 7-dehydrocholesteryl (0.5%), and 7,22-bisdehydrocholesteryl(85%). The first two components were rigorously identified by

the agreement of various properties (melting points, $[\alpha]_D$ values, infrared spectra, glc retention times on three columns, and tlc R_f values) of the acetates, and also the free alcohols derived from them by saponification, with the properties of authentic samples. The identification of the 7-dehydrocholesteryl acetate is tentative, since it is based only on glc and tlc comparisons with authentic material. The proof of structure of the major component is outlined below.

The sample of 7,22-bisdehydrocholesteryl acetate (I-OAc) obtained from the column was recrystallized three times from methanol to give 113 mg of material with mp 144.0-144.2°, $[\alpha]_D^{24}$ -78° (c 1, chloroform), and ultraviolet λ_{max} 271.0, 281.5, and 293.0 nm with ϵ_{max} 12,100, 12,700, and 7,100 liter $mole^{-1}$ cm^{-1}, respectively. Saponification of the methanol mother liquors gave 60 mg of I-OH, which was recrystallized three times from methanol to give a 23-mg sample with mp 118-123° (the wide range appears to result from liquid crystal formation), $[\alpha]_D^{24}$ -111° (c 1, chloroform), and ultraviolet λ_{max} 271.0, 281.5, and 293.0 nm with ϵ_{max} 11,400, 12,100, and 6,900 liter $mole^{-1}$ cm^{-1}, respectively. Hydrogenation of I-OAc over Raney nickel in ethyl acetate gave lathosteryl acetate (3β-acetoxy-Δ^7-cholestene), identified by comparisons with authentic samples of infrared spectra and glc retention times on three columns for itself and its saponification product, lathosterol. The formula of I-OAc was established as $C_{29}H_{44}O_2$ by its mass spectrum, which exhibited peaks at m/e 424 (M^+), 364 ($M^+ - CH_3CO_2H$), 349 ($M^+ - CH_3CO_2H - CH_3$), and 253 ($M^+ - CH_3CO_2H - C_8H_{15}$); high-resolution mass measurements of the m/e 364 and 253 peaks showed these fragments to have compositions $C_{27}H_{40}$ (calcd 364.3130, found 364.3127) and $C_{19}H_{25}$ (calcd 253.1956, found 253.1956).[4] Thus, I-OAc has a typical C_{27} sterol carbon skeleton with a 3β-acetoxy group and three units of unsaturation. The ultraviolet data provide unequivocal evidence for a $\Delta^{5,7}$ diene system.[5] Periodate-permanganate oxidation[6] of I-OAc gave isovaleric acid, indicating Δ^{22} unsaturation (see below); this Δ^{22} double bond was shown to be trans by the intense peak at 968 cm^{-1} in the infrared spectrum of I-OAc and also in that of I-OH. The 60-MHz nmr spectrum of I-OAc corroborates its structural assignment.

Incubations of Tetrahymena pyriformis in 10 flasks with a total of 50 mg of 26-^{14}C-cholesterol (8.9 μC/mmole) and 5 liters of culture fluid led to the isolation of 69% of the radioactivity in the total lipid fraction extracted from the cells. Using methods analogous to those described above, pure samples of cholesteryl acetate (2.8 mg), 22-dehydrocholesteryl acetate (1.5 mg), and I-OAc (7.1 mg) were obtained from this material. These three samples had specific activities of 8.8, 9.2, and 8.7 μC/mmole, respectively; the close agreement of these values with that of the starting 26-^{14}C-cholesterol shows that both 22-dehydrocholesterol and I-OH are

derived metabolically from cholesterol in the cells. Insufficient 7-dehydrocholesteryl acetate was obtained in these experiments to determine its specific activity, but qualitatively the extent of labeling suggested a metabolic derivation from cholesterol for this $\Delta^{5,7}$ system also.

We have developed a micro method for determining the presence of Δ^{22} unsaturation in a sterol by glc analysis of the low-molecular weight carboxylic acids produced by periodate-permanganate oxidation.[6] Typically, a mixture of 0.3 ml of a tert-butanol solution containing 0.5-1.0 μmole of the sterol or sterol acetate, 0.3 ml of an aqueous solution 97 mM in sodium periodate and 3 mM in potassium permanganate, and 0.05 ml of aqueous 50 mM potassium carbonate was maintained at 37° overnight, after which the reaction mixture was acidified with sulfuric acid, decolorized with aqueous sodium bisulfite, and extracted with diethyl ether. The ether extract was dried, concentrated to 1 ml, and analyzed quantitatively by glc. Cholesteryl, 7-dehydrocholesteryl, and lathosteryl acetates, which lack Δ^{22} unsaturation, gave no detectable peaks when analyzed by this method; 22-dehydrocholesteryl acetate and I-OAc gave isovaleric acid in 70-80% yield. The validity of this method was confirmed by carrying out appropriately scaled-up versions using the ^{14}C-labeled samples of cholesteryl acetate, 22-dehydrocholesteryl acetate, and I-OAc whose isolation was described above. In each case, a known amount of carrier isovaleric acid (ranging from 110 to 500 mg) was added to the reaction mixture after the oxidation was complete, and the p-bromophenacyl ester derivative was prepared and recrystallized five times. With each successive recrystallization, the ^{14}C content of the p-bromophenacyl isovalerate from the control experiment with cholesteryl acetate steadily approached zero, whereas the specific activities of the p-bromophenacyl isovalerate samples from the 22-dehydrocholesteryl acetate and I-OAc experiments remained high and constant.

The major component of the unsaponifiable material from Tetrahymena pyriformis grown without added sterols is tetrahymanol, a pentacyclic triterpenoid alcohol whose isolation, structure elucidation, and mechanism of biosynthesis we have described earlier;[7] sterols are undetectable in this material. However, in the cholesterol incubation studies reported herein only traces of tetrahymanol were obtained from the cells, equal in amount to that initially present in the seed cultures of the protozoan; thus, added cholesterol leads to the inhibition of the biosynthesis of this squalene cyclization product. Further studies of this inhibition are in progress.

The presence of 22-dehydrocholesterol in the cholesterol-supplemented cells suggests that this $\Delta^{5,22}$ dienol might be an intermediate in the overall conversion of cholesterol to the $\Delta^{5,7,22}$ trienol, I-OH. In view of the tentative evidence for the presence of 7-dehydrocholes-

terol in these cells, we cannot rule out the simultaneous operation of a second pathway for this conversion involving the intermediacy of that $\Delta^{5,7}$ dienol. In accord with this two-pathway hypothesis, we have found that incubation of Tetrahymena pyriformis in the presence of added samples of either of these two dienols results in their efficient transformation to I-OH.

The $\Delta^{5,7,22}$ pattern of sterol unsaturation was known previously only in C-24 alkyl sterols such as ergosterol and 7-dehydrostigmasterol (the 24-methyl and 24-ethyl homologs of I-OH). These and other C_{28} and C_{29} sterols with Δ^{22} double bonds are widely distributed in nature, but prior to our isolation of I-OH and 22-dehydrocholesterol from Tetrahymena pyriformis, the observations of the latter sterol in three different organisms[8] constituted the only examples of the natural occurrence of any sterol with Δ^{22} unsaturation but lacking a C-24 alkyl substituent. We know of only one other explicit report[9] of the conversion of a saturated side chain of a sterol into a Δ^{22} system; our investigations of this unusual type of transformation are continuing.

Full experimental details of all the studies described above will be published elsewhere.

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