Dehydrogenation of Sterols by the Protozoan Tetrahymena pyriformis

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SUMMARY

Evidence is provided that Tetrahymena pyriformis is capable of converting 5α-cholest-7-en-3β-ol, 5α-cholestan-3β-ol, and cholest-5,24-dien-3β-ol (desmosterol) to products having a 5,7,22-triene system. In the case of desmosterol the C-24 double bond is largely retained. Studies with [6α-3H]- and [6β-3H]5α-cholest-7-en-3β-ol indicate that the introduction of the 5 double bond in these compounds proceeds with the abstraction of the 5α- and 6α-hydrogens. The 6β-tritium atom is completely retained. The introduction of the 5,6 double bond is accompanied by a considerable isotope effect. This is in contrast to the previously reported observations that the introduction of the 7 and 22 double bonds does not involve an isotope effect.

The protozoan Tetrahymena pyriformis has been shown to convert mevalonic acid (MVA) to tetrahymanol (I) via a non-oxidative proton-initiated cyclization of squalene (1-4). No evidence has been found for the production of cholesterol or related sterols by this organism or for an absolute sterol nutritional requirement (Reference 5 and references therein). However, it has been demonstrated that exogenous cholesterol inhibits tetrahymanol production and is itself efficiently metabolized to the cholesta-5,7,22-triene-3β-ol (IIa) by the organism (6-10).

We have proven that the dehydrogenations proceed by the abstraction of the C-7β and C-8β hydrogens and of the 22-pro-R hydrogen (7, 8). Our results were subsequently confirmed by other investigators (9, 10) who also showed that a 23-pro-S proton is removed in the trans Δ2 formation (9). It is worthy of note that the C-7 and C-22 double bond formations proceed with the removal of cis-orientated hydrogens. Several mechanisms were suggested for cis dehydrogenations of sterols (7, 8, 10, 11).

The present work was undertaken with the aim of defining the structural features required for sterols to act as substrates for the dehydrogenases. With this in mind we explored the ability of the organism to dehydrogenate 5α-cholest-7-en-3β-ol (IIa) and 5α-cholestan-3β-ol (IV). Since both sterols were converted to the triene (IIa), the stereochemistry of introduction of the 5(6) double bond in (IIa) was then established.

EXPERIMENTAL PROCEDURE

Physical Methods and Materials—Melting points were taken on a hot stage apparatus and are corrected. Infrared spectra were recorded on a Perkin-Elmer instrument. Ultraviolet spectra were recorded on a Perkin-Elmer model 202 spectrophotometer. Radioactive samples were counted on a Nuclear-Chicago Mark I automatic liquid scintillation counter; the samples were dissolved in 15 ml of a scintillation solution of toluene containing 4 g of 2,5-diphenyloxazole and 100 mg of 1,4-bis[2(5-phenyloxazolyl)]benzene per liter.

Plates for thin layer chromatography were made from silica gel HFX4420 and activated at 100° for 3 hours before use. Silver nitrate-impregnated plates were made from 30 g of silver nitrate.
and 210 ml of water per 100 g of silica. The plates were dried, activated, and stored in the dark. Solvent systems used were: I, ethyl acetate-hexane (1:9); II, acetone-chloroform (2:98). Plates were scanned for radioactivity with a Vanguard model 885 instrument.

Bacto-Tryptone and Proteose-Peptone were obtained from Difco.

[26-14C]Cholesterol and [26-14C]desmosterol were obtained from New England Nuclear and were used without further purification. The preparations of [6a-3H]cholest-7-en-3β-ol, [6β-3H]cholest-7-en-3β-ol, and [1,7,15,22,26-14C]cholest-7-en-3β-ol have been described (12).

Growth of T. pyrijiformis and Incorporation of Steroles—The double culture technique was used for incorporation of radioactive substrates (1-3). A fresh inoculum of T. pyrijiformis (100 ml) was prepared by incubation in the normal medium without added glucose at 28-30°C for 48 hours. The sterol under investigation (2.5 to 3 mg) was dissolved in ethanol (1 ml) and added to a sterilized 1-liter solution of the growth medium in a 2.8-liter Fernbach flask. The medium was then supplemented with 20% glucose solution (50 ml) and 100 ml of the fresh inoculum of T. pyrijiformis. The flask was shaken vigorously at 28-30°C in the dark for 72 hours. The cultures were cooled to 4°C for 2 hours and the cells were harvested by continuous centrifugation. The wet cells from each flask were saponified with KOH (5 g) in aqueous ethanol (1:1, 30 ml) at reflux for 2 hours in an atmosphere of nitrogen. The volume of the cooled solution was reduced under vacuum and the nonsaponifiable material was isolated by extracting several times with hexane. The organic phase was cautiously diluted with methanol and the solvents were evaporated under vacuum and the nonsaponifiable material was isolated by extracting several times with hexane. The organic phase was cautiously diluted with methanol and the solvents were evaporated. The sterols were separated and purified as described below, all operations being performed in subdued light.

Incubation of [6-3H]6α-Cholest-7-en-3β-ol with T. pyrijiformis—In a preliminary experiment [6α-3H]cholest-7-en-3β-ol (12) (IIa) (3.8 x 10^5 dpm) and [6β-3H]cholest-7-en-3β-ol (12) (IIb) (3.8 x 10^5 dpm) were mixed and diluted with nonradioactive cholest-7-en-3β-ol (3 mg). Incubation with the protozoan was performed as described above (1 liter of medium) and the non-saponifiable material was acetylated with acetic anhydride-pyridine (1:1, 2 ml) at room temperature overnight. The solution was cautiously diluted with methanol and the solvents were removed under a stream of nitrogen. The crude product was purified by preparative thin layer chromatography on silica gel in System I and the single unresolved band (2.2 x 10^4 dpm) was eluted with ethyl acetate.

The radioactive material was fractionated by thin layer chromatography on silica gel-silver nitrate (System I). The two developed plate showed two major radioactive zones, the upper one corresponding to cholest-7-en-3β-ol acetate and the lower to cholest-5,7,22-trien-3β-ylacetate. After elution with ethyl acetate the upper band yielded 1.2 x 10^4 dpm while the lower band contained 6 x 10^4 dpm and possessed an ultraviolet spectrum characteristic of the 5,7-diene system (λmax 261.5, 271.5, 282, 294 nm).

Incubation of [6β-3H, 14C]Cholest-7-en-3β-ol (IIb) with T. pyrijiformis—[6β-3H]cholest-7-en-3β-ol (12) (IIb) was mixed with [1,7,15,22,26-14C]cholest-7-en-3β-ol (12) (5.5 x 10^5 dpm of 14C) and diluted with nonradioactive material (6 mg). The mixed radioactive [6β-3H, 14C]cholest 7 en 3β ol (6 mg) (3H:14C ratio 14.2) was incubated with T. pyrijiformis in two 1-liter cultures as described above, at 28-30°C for 72 hours. The cells were harvested and saponified. The sterols were extracted with hexane and acetylated. Purification of the sterol acetates by preparative thin layer chromatography (System I) gave a single radioactive zone (8 x 10^5 dpm of 14C) which was eluted and re-chromatographed on silica gel-silver nitrate in the same solvent system. Scanning of the plate revealed two major bands corresponding in Rf value to cholest-7-en-3β-ol acetate (1.0 x 10^6 dpm of 14C) and cholest-5,7,22-trien-3β-ylacetate (3.1 x 10^6 dpm of 14C).

The upper band was further purified by thin layer chromatography and after dilution with nonradioactive cholest-7-en-3β-ol acetate was crystallized four times to give a constant specific activity of 8.8 x 10^4 dpm of 14C per mmole; 3H:14C ratio 13.6.

The lower band was also purified by rechromatography. Its structure was defined as cholest-5,7,22-trien-3β-ylacetate by its spectral properties (6), λmax 262, 271, 282, 294 nm; mass spectrum, m/e 364 (M-60, base peak); 349, 253, 249, 238, 237, 211, 199, 157, 143, 109. An aliquot of the material was diluted with nonradioactive cholest-5,7,22-trien-3β-ylacetate and crystallized giving [6-3H, 14C]cholest-5,7,22-trien-3β-ylacetate acetate and crystallized giving [6-3H, 14C]cholest-5,7,22-trien-3β-ylacetate acetate (m.p. 142-144°C (literature 6°C)); specific activity 1.2 x 10^5 dpm of 14C per mmole; 3H:14C ratio 14.5.

Incubation of [6-3H, 14C]Cholest-7-en-3β-ol (IIIb) with T. pyrijiformis—A mixture of [6-3H]cholest-7-en-3β-ol (IIIa) and [1,7,15,22,26-14C]cholest-7-en-3β-ol (3.5 x 10^5 dpm of 14C; 3H:14C ratio 25.7) was diluted with nonradioactive material (6 mg) and incubated with T. pyrijiformis as described above. After acetylation and purification by thin layer chromatography the radioactive product (2.7 x 10^6 dpm of 14C) was further fractionated on silica gel-silver nitrate (System I) to give two major bands. The upper band was eluted (0.6 x 10^4 dpm of 14C) and crystallized with authentic cholest-7-en-3β-yl acetate to a specific activity of 3.8 x 10^5 dpm of 14C per mmole; 3H:14C ratio 60.3.

The lower band was purified by thin layer chromatography as described above. The structure of the compound was confirmed as 11β by ultraviolet and mass spectrometry: λmax 262, 271, 282, 293.5 nm; mass spectrum, m/e 364 (M-60, base peak), 349, 253, 249, 238, 237, 211, 199, 157, 143, 109. An aliquot of the material was diluted with nonradioactive cholest-7-en-3β-ylacetate (1.0 x 10^7 dpm per mmole) was added to a growing culture of T. pyrijiformis and diluted with nonradioactive cholest-7-en-3β-ylacetate to a specific activity of 5.6 x 10^5 dpm of 14C per mmole; 3H:14C ratio 40.3.

Synthesis of [26-14C]Cholestanol (IV)—Nonradioactive cholestanol (90 mg) was mixed with [26-14C]cholesterol in ethyl acetate (10 ml). Perchloric acid (2 μl) and platinum oxide (50 mg) were added and the mixture was shaken in an atmosphere of hydrogen. The solution was filtered and shaken with NaHCO3 solution after which it was washed and dried (Na2SO4). Cholestanol was isolated by preparative thin layer chromatography on silica gel-silver nitrate (System II, developed twice at 4°C) and elution with ethyl acetate. The product was crystallized twice from ether-methanol to give [26-14C]cholestanol (18 mg, m.p. 141-142°C, specific activity 5.6 x 10^4 dpm per mmole); mass spectrum, m/e 388 (M+, base peak), 373, 355, 331, 262, 257, 234, 233, 217, 215.

Incubation of [6-3H, 14C]Cholestanol with T. pyrijiformis—The purified [26-14C]cholestanol (2.5 mg; 4.1 x 10^4 dpm; specific activity 5.6 x 10^4 dpm per mmole) was added to a growing culture of T. pyrijiformis in 1 liter of medium, as described above. The cells were then harvested and saponified, and the sterols were extracted with hexane. The recovered sterols (2.3 x 10^6 dpm) were purified by preparative thin layer chromatography (System I) to give a single unresolved radioactive zone, which was fractionated by thin layer chromatography on silica gel-silver
The dehydrogenases of *T. pyriformis* was carried out. We noted that the protozoan efficiently converted the Δ^1-3β-ol to cholesta-5,7,22-trien-3β-ol. This posed the question of the stereochemical course of the introduction of the Δ^\(5\) bond by *T. pyriformis* and the problem was solved with the use of precursors labeled stereospecifically with tritium at the 6α and 6β positions.

In two separate experiments [6\(^-\)H\(^{-}\)14C]cholest-7-en-3β-ol (IIa) and [6\(^+\)H\(^{-}\)14C]cholest-7-en-3β-ol (IIb) were added to actively growing cultures of the organism. After 48 hours the sterols were isolated and acetylated. Purification first on silica gel plates and then on silver nitrate-impregnated silica gel gave, in each case, a major product identified as cholesta-5,7,22-trien-3β-yl acetate (IIb) (70 to 75% of 14C-labeled material incorporated into the cells). The trienes were characterized by their mass and ultraviolet spectra, chromatographic properties, and cocrystallization with authentic material. Unreacted cholest-7-en-3β-yl acetate (IIb) (20 to 25% of 14C-labeled material incorporated into the cells) was identified by chromatography and cocrystallization with an authentic sample. The results are summarized in Table I. It is evident that while the 6β tritium of (IIb) was retained in the derived triene the 6α tritium of (IIa) was lost in the course of triene formation.

To define the need of a C-7 double bond in the substrate the metabolism of [26\(^+\)H\(^{-}\)]cholesterol (III) was investigated under similar conditions. Although a lower incorporation of the radioactivity into the cells (57% of added 14C radioactivity) was observed, acetylation of the nonsaponifiable material and separation by thin layer chromatography showed a similarly efficient conversion of the substrate into cholesta-5,7,22-trien-3β-ol (69% of the incorporated 14C). The product was again identified by thin layer chromatography and ultraviolet and mass spectrometry.

The results show that the dehydrogenase systems of *T. pyriformis* do not require the presence of a double bond in the steroid substrate and can efficiently metabolize cholesterol, cholesterol, and cholest-7-en-3β-ol to cholesta-5,7,22-trien-3β-ol. The introduction of the Δ^\(5\) double bond involves loss of the cis-oriented 5α- and 6α-protons, the 6β-proton being retained. Clearly, this is another example of an over-all cis dehydrogenation at nonactivated carbon atoms and follows the same over-all steric course as the 5,6 dehydrogenations involved in the biosynthesis of cholesterol in rat livers and ergosterol in yeast.

The results (Table I) also show that, in the case of the [6α\(^-\)H\(^{-}\)]cholest-7-en-3β-ol (IIa) (14:14C ratio 25.7), removal of the 6α-proton involves a significant isotope effect. While complete loss of tritium is apparent in the trienyl acetate (IIc) (14:14C ratio 0.43), the recovered cholest-7-en-3β-yl acetate (IIe) has a considerably higher 14C:14C ratio (60.5) than the starting material. Worthy of note is the fact that no isotope effect was observed in the removal of the 7β- or 22-pro-R-protons of cholesterol by *T. pyriformis* (7-9). A similar isotope effect was first observed in the conversion of cholest-7-en-3β-ol to cholesta-5,7,22-dienol in a rat liver enzyme system (13). This effect may reflect a similar enzymic process in these two cases.

We have also incubated [24\(^+\)H\(^{-}\)]desmosterol (V) (approximately 5 \times 10^6 dpm) with *T. pyriformis* and obtained a mixture of dehydrogenated products (3.0 \times 10^6 dpm). Mass spectrometry indicated the products to be cholesta-5,7,22,24-tetraen-3β-ol (m/e 362 (M-CH\(=\)COOH)) and a smaller amount of cholesta-5,7,24-trien-3β-ol.

Recently Nes et al. (15) investigated the ability of *T. pyriformis* to dehydrogenate C-5,24(28) unsaturated steroidal dienes.
They noted that while the presence of C-24 ethylene or trans-C-24(28)-ethylidene moieties do not interfere with the dehydrogenation at C-22, a cis-C-24(28)-ethylidene group prevents the introduction of the C-22 double bond. They concluded that dehydrogenation at C-22 can occur when carbons C-20, 22, 23, 24, 28, and 29, if present, can lie in one plane, the other side of the plane being exposed for the abstraction of cis-oriented C-22 and C-23 hydrogens.

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