Steric effects at C-20 and C-24 on the metabolism of sterols by *Tetrahymena pyriformis*

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Abstract Cultures of *Tetrahymena pyriformis* were incubated with various sterols and the extent of dehydrogenation at C-7 and C-22 was determined. The sterols incubated were desmosterol, 22-dehydrodesmosterol, 24-methyldesmosterol, 24α-methylcholesterol (campesterol), 24-methylenecholesterol, halosterol (26,27-bisnorcampesterol, also known as 24,24-dimethylchol-5-en-3β-ol, a naturally occurring C_{26}-sterol), and 20-isohalosterol. 20-Isohalosterol was not metabolized, while products with Δ^7- and Δ^{22}-bonds were formed from halosterol and all of the other sterols studied. This confirms an earlier conclusion, based on results with 20-isocholesterol and cholesterol, that inversion of the configuration from 20(*R*) to 20(*S*) completely prevents metabolism both in the nucleus and the side chain. On the other hand, changes in the electronics or stereochemistry at C-24 had a direct affect only on metabolism in the side chain. The presence of a methyl group at C-24 reduced the yield of metabolites with a Δ^{22}-bond relative to those with a Δ^7-bond producing an accumulation of 7-dehydro metabolite. A double bond at position-24 counteracted this steric effect, presumably by enhancing the rate of dehydrogenation, and a $\Delta^{24(28)}$-bond was more effectve than was a $\Delta^{24(25)}$-bond.—**Nes, W. R., J. M. Joseph, J. R. Landrey, S. Behzadan, and R. L. Conner.** Steric effects at C-20 and C-24 on the metabolism of sterols by *Tetrahymena pyriformis*. *J. Lipid Res.* 1981. **22:** 770–777.

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The protozoan *Tetrahymena pyriformis*, although incapable of sterol biosynthesis, can, among other things, dehydrogenate exogenously supplied sterols both at C-7 and at C-22 (1–3) as shown in **Fig. 1** for cholesterol. These dehydrogenations, especially the latter, also occur in various other living systems. For instance, introduction of the Δ^{22}-bond occurs widely in algae and higher plants (4); a Δ^{22}-sterol has recently been isolated from the urine of a young girl with congenital adrenal hyperplasia (5); and mammals can desaturate cholesterol to 7-dehydrocholesterol (6) which acts as a precursor to vitamin D. However, the presence of endogenously biosynthesized sterol complicates the study of these reactions in mammals and most of the other eukaryotes. This problem is obviated by work with *T. pyriformis*, and permits this protozoan to be a useful experimental model for the study of the effects of structure on sterol metabolism.

Earlier investigations (2, 3, 7) lead us to believe that the following rules are among those that govern the introduction of the Δ^7- and Δ^{22}-bonds. *A*) At the moment the sterol becomes bound to enzyme, C-22 must lie to the right in the usual view of the molecule (3). *B*) Binding of protein occurs on the front (β-face) of the sterol (without excluding concomitant binding on the rear) in such a way that bulk on C-20 cannot exceed the size of an H-atom in front (3). *C*) The length of the longest array of carbon atoms on C-20 in the sterol side chain must conform to the natural length which is 4–6 carbon atoms (7). *D*) The introduction of the Δ^{22}-bond, but not of the Δ^7-bond, is hindered by a substituent at C-24 (2). *E*) The presence or absence of branching at C-20 and C-25, i.e., whether or not C-21 and C-27 (when C-26 is taken to be the end of the chain) are present, is of minor sig-

Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography; MS, mass spectroscopy; UV, ultraviolet; SC, side chain; ^{1}H-NMR, proton nuclear magnetic resonance spectroscopy; RRT, GLC retention time relative to cholesterol for sterols and relative to cholesteryl acetate for steryl acetates; HPLC, high performance liquid chromatography; α,K′/K′ for cholesterol in HPLC; K′, (elution vol. − dead vol.)/dead vol.

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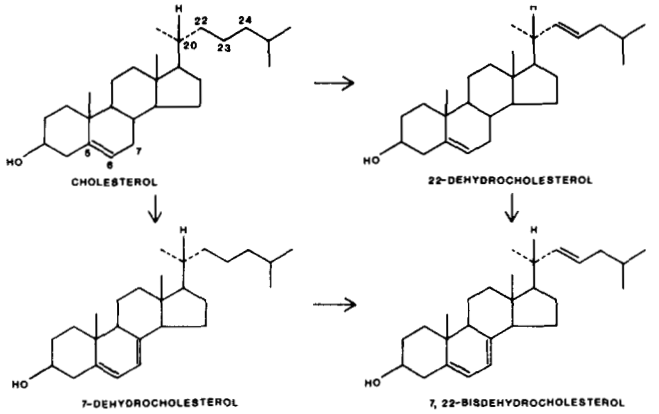

Fig. 1. Dehydrogenation of cholesterol by *T. pyriformis*.

nificance (7), and *F*) oxygen on C-20 is very deleterious (7).

Rule *D* rests on an observed (2) decrease in the amount of introduction of the Δ^{22}-bond when a 24-ethylidene group is present, but the rule is rendered somewhat uncertain by our finding (2) that a 24-methylene group does not decrease dehydrogenation at C-22. This discrepancy could be due either to a lack of steric hindrance by a C_1-group at C-24 or to an electronic enhancement in dehydrogenation at C-22 caused by the double bond at C-24. In order to examine this problem as well as to increase our knowledge of rules *A* and *B*, which together require that inversion of the configuration at C-20 should inhibit or prevent dehydrogenation at both C-7 and C-22, we have reexamined 24-methylenecholesterol and also studied the metabolic fates of several additional sterols. They are: *a*) 24α-methylcholesterol (campesterol, biosynthesized (4) by many plants) which allows the influence of a methyl group at C-24 to be examined in the absence of a double bond in the side chain; *b*) the naturally occurring (8) 24-dehydro derivative of campesterol (24-methyldesmosterol, tentatively thought (9) to be a biosynthetic intermediate to campesterol) from which the combined influence of the methyl group and a double bond at C-24 can be assessed; *c*) halosterol (a C_{26}-analog of campesterol lacking C-26 and C-27 which occurs in the marine environment (4)) which offers a second opportunity to examine hindrance from a methyl group; and *d*) the 20(*S*)-isomer of the latter, 20-isohalosterol, which permits examination of the effect of stereochemistry at C-20. In addition, through the use of desmosterol and 22-dehydrodesmosterol, intermediates to cholesterol in insects (4), it was possible to examine the effect of the $\Delta^{24(25)}$-bond on metabolism in the absence of a substituent at C-24. The structures of the sterols examined are given in **Fig. 2.**

MATERIALS AND METHODS

Substrates

24α-Methylcholesterol (campesterol), shown earlier to be stereochemically pure (10), was obtained from Applied Science Laboratories, State College, PA. 24-Methyldesmosterol was prepared from 24-methylenecholesterol (isolated from the pollen of the Saguaro cactus) by isomerization of the acetate with iodine in benzene (22 mg/15 ml) at reflux for 8 hr. This procedure gave a mixture of starting material (very minor) and its Δ^{23}- and $\Delta^{24(25)}$-isomers (roughly 1:1). The mixture was separated into its components by argentation thin-layer chromatography (10% $AgNO_3$ on silica gel G, chloroform–petroleum ether–acetone, 75:25:0.75). The structure was confirmed by the presence of a base peak in the mass spectrum at m/e 296 for $M^+ - HOAc - (CH_3)_2C = C(CH_3)_2$ corresponding to the expected allylic cleavage at the 22(23)-bond. The Δ^{23}-acetate gave a peak at m/e 283 for $M^+ - HOAc - (CH_2 - CH = C(CH_3) - CH(CH_3)_2)$ corresponding to allylic cleavage at the 20(22)-bond. Hydrolysis of the $\Delta^{24(25)}$-acetate gave the alcohol (RRT 1.47). The Δ^{23}-alcohol had RRT 1.24, and its acetate moved slower and was distinctly separated from the $\Delta^{24(25)}$-acetate in the argentation TLC. The $\Delta^{24(28)}$-acetate migrated still slower. Halosterol (RRT 0.78) and 20-isohalosterol (RRT 0.69) were prepared from pregnenolone by a Grignard reaction with isoamyl

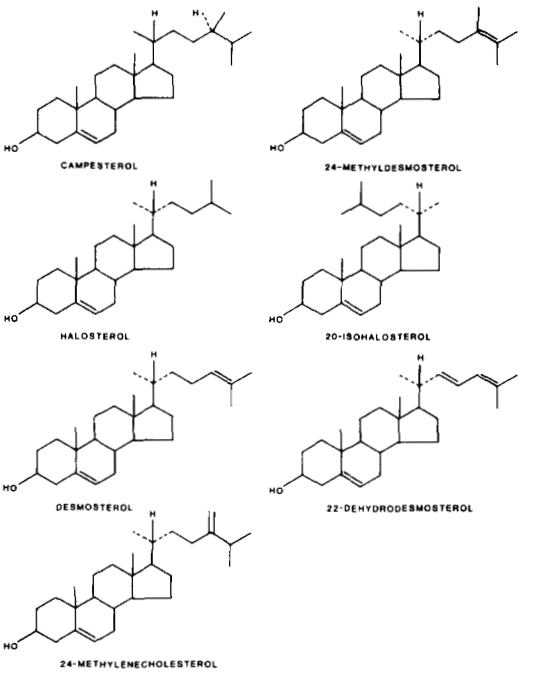

Fig. 2. Structures of sterols with which *T. pyriformis* was incubated.

bromide, dehydration, and reduction by the procedure described earlier for the preparation of cholesterol and 20-isocholesterol (11). The 20(*R*)- and 20(*S*)-isomers were separated by chromatography on alumina, the latter moving the faster. Their structures were proven by ^{1}H- and ^{13}C-NMR spectroscopy. Details of the synthesis and physical properties will be presented elsewhere, but spectroscopic and other analysis of the material obtained from *T. pyriformis* cells after incubation, which is presented in the present study, also constitutes proof of structure. Desmosterol was purchased from Calbiochem, La Jolla, CA, and purified by argentation chromatography. 22-Dehydrodesmosterol was isolated from insects (12). Steryl acetates were prepared in pyridine-acetic anhydride. Hydrolysis to free sterols was achieved with 5% KOH in methanol for 30 min.

Growth of *Tetrahymena pyriformis* W

Cultures of *Tetrahymena pyriformis* W were grown in 2800-ml Fernbach flasks which contained 500 ml of an enriched proteose-peptone medium (1). Five mg of sterol dissolved in 1 ml of absolute ethanol was added to the medium, and incubations were performed in 500-ml batches. Cells were harvested after a growth period of 22–24 hr at 25 ± 1°C. Procedures for estimation of cell numbers, harvesting, lyophilization of cells, and extraction of lipids have been described (1). The amount of each sterol used is given in the section on Results.

Isolation of sterols

Lyophilized cells were extracted with acetone for 18 hr in a Soxhlet apparatus and the extracted material was saponified in 5% methanolic KOH at reflux for 30 min to give ether-soluble neutral lipids. The neutral lipids were examined by GLC and UV spectroscopy, and the results comprised the principal but not the only means of quantitating the amounts of different metabolites. After chromatography of the neutral lipids on alumina, the fractions were monitored by GLC and UV spectroscopy. Appropriate fractions were combined for further study, e.g., by reversed phase or argentation chromatography or by spectroscopy. This investigation spanned several years, which influenced the choice of methodology. Only recently, for instance, did we have the reversed phase system operative.

Chromatography

Adsorption chromatography was performed on neutral alumina which was dried overnight at 120°C and then deactivated with water (3% w/v, except for the work with the C_{26}-sterols in which case 10% w/v was used). The eluent was ether graded into hexane. Reversed phase chromatography was performed on a column of lipophilic dextran (Lipidex 5000, Packard Instrument Co.) with 5% hexane in methanol. Gas–liquid chromatography was performed on a 6-foot U-column of 1% XE-60 on Chromosorb W at 235°C with detection by flame ionization. Retention times (RRT) for alcohols are given relative to that of cholesterol and for acetates relative to cholesteryl acetate. The stationary phase for argentation thin-layer chromatography, unless otherwise noted, was 10% of $AgNO_3$ on silica gel (w/w), and the mobile phase was chloroform–hexane–acetone 100:40:0.75 (v/v). High performance liquid chromatography was performed on a μBondapak C-18 column, 0.3 × 30 cm (Waters Associates, Milford, MA), and the liquid phase was 100% acetonitrile moving at 1 ml/min. A variable wavelength detector (Spectromonitor III, Laboratory Data Control, Riviera Beach, FL) was used; it was set at the wavelength(s) indicated in the section on results.

Spectrometry

Mass spectra were obtained at or near 90°C, V_1 = 50 volts, V_A = 175 volts, and V_M = 2000 volts by the electron impact procedure with a direct probe on a Perkin-Elmer instrument (Model RMU-6E) at Morgan Schaffer of Montreal, Canada. Nuclear magnetic resonance spectroscopy for ^{1}H was performed at 220 MHz on a Varian Associates instrument (Model M-66) at ambient temperature in $CDCl_3$ with $Si(CH_3)_4$ as internal standard. A Cary (Model 15) spectrometer was used for ultraviolet spectroscopy. Samples were dissolved in ethanol.

Crystallization and melting points

Methanol was used for all crystallizations. Melting points were determined on a Kofler hot stage under a microscope fitted with a polarizer.

RESULTS

Incubation with 20-isohalosterol

The total protozoan population from incubation with 90 mg of 20-isohalosterol (20(*S*)-24,24-dimethylchol-5-en-3β-ol) was 1.23×10^9 cells yielding a dry weight of 3.45 g. The neutral lipids (69.1 mg) after saponification showed a single GLC peak at RRT 0.69 (authentic substrate: 0.69) and no UV absorption typical of $\Delta^{5,7}$-sterols. The sterol (2.5 mg) obtained after chromatography on alumina also showed RRT 0.69, no ultraviolet absorption, and the following

additional constants: mp 139–140°C (authentic: 139–140°C), and MS: m/e 372 (M^+, 100), 357 ($M^+ - CH_3$, 41), 354 ($M^+ - H_2O$, 60), 339 ($M^+ - CH_3 - H_2O$, 57), 313 ($M^+ - C_3H_7O$, 12), 287 ($M^+ - C_5H_9O$, 72), 273 (M^+ – SC, 37), 261 ($M^+ - C_7H_{11}O$, 100), 255 (M^+ – SC – H_2O, 49), 231 (M^+ – SC – C_3H_6, 49), 229 (M^+ – SC – C_3H_8, 22), and 213 (M^+ – SC – C_3H_6 – H_2O, 82). These various physical properties agree with those of unchanged substrate. No peaks for the two possible dienols (mol wt 370) or the trienol (mol wt 368) were observed either in the mass spectrum or in the gas–liquid chromatogram.

Incubation with halosterol

From 2.94×10^9 cells (dry wt, 8.6 g) after incubation with 110 mg of halosterol (20(*R*)-24,24-dimethylchol-5-en-3β-ol) we obtained 139 mg of neutral lipid after saponification which showed two peaks in GLC at RRT 0.77 and 0.94 of nearly the same intensity. The first peak corresponded to the RRT of substrate (RRT 0.78) but, as demonstrated subsequently, it also contained $\Delta^{5,7,22}$-trienol (calculated RRT 0.82) which was not resolved due to the small amount. The second peak had the expected RRT of the $\Delta^{5,7}$-dienol (calculated 0.93). The $\Delta^{5,22}$-dienol (calculated 0.69) was not evident, but a trace was identified by other means (see below). The UV spectrum showed λ_{max} 272, 282, and 293 nm with extinction coefficients indicating 60% of the recovered sterol contained the $\Delta^{5,7}$-grouping.

From the leading edge of the sterol band in chromatography of the neutral lipids on alumina we obtained 8.1 mg of pure substrate, mp 152–153°C (authentic: 152–153°C); MS: m/e 372 (M^+, 100), 357 ($M^+ - CH_3$, 32), 354 ($M^+ - H_2O$, 46), 339 ($M^+ - CH_3 - H_2O$, 34) 313 ($M^+ - C_3H_7O$, 5), 287 ($M^+ - C_5H_9O$, 8), 273 (M^+ – SC, 22), 261 ($M^+ - C_7H_{11}O$, 59), 255 (M^+ – SC – H_2O, 27), 231 (M^+ – SC – C_3H_6, 24), and 213 (M^+ – SC – C_3H_6 – H_2O, 40); ^{1}H-NMR: 0.68 (s, C-18), 1.01 (s, C-19), 0.91 (d, J = 6.5 Hz, C-21), and 0.86 and 0.87 (d, J = 6.5 Hz, gem-dimethyl group) ppm. The spectra agreed with those of authentic substrate.

The sterol remaining in the chromatographic band after removal of the substrate in the leading edge was composed of residual substrate, the trienol, and the two dienols as shown by separation on a column of lipophilic dextran. The fastest moving component was the trienol (7,22-*trans*-bisdehydrohalosterol, 24,24-dimethylchola-5,7,22-*trans*-trien-3β-ol): RRT 0.79; 5 mg after crystallization; mp 122–126°C; UV: 272 (ϵ 10,000), 282 (ϵ 11,000), and 293 (ϵ 6,200) nm; MS: m/e 368 (M^+, 91), 353 ($M^+ - CH_3$, 6), 350 ($M^+ - H_2O$, 9), 335 ($M^+ - CH_3 - H_2O$, 100), 309 ($M^+ - C_3H_7O$, 54), 271 (M^+ – SC, 23), 251 (M^+ – SC – H_2O – 2H, 9), and 211 (M^+ – SC – C_3H_6 – H_2O, 38); ^{1}H-NMR: 0.62 (s, C-18), 0.94 (s, C-19), 1.01 (d, J = 6.5 Hz, C-21) and 0.95 (d, J = 6.5 Hz, gem-dimethyl group) ppm. From the tail end of the trienol band, we obtained fractions enriched in 22-*trans*-dehydrohalosterol (24,24-dimethylchola-5,22-*trans*-dien-3β-ol) with RRT 0.67 (calculated 0.69), but the small amount (1% of recovered sterol) and the fact that the trienol was also present (40% of the mixture) precluded further characterization.

Following the $\Delta^{5,7,22}$-trienol-/-$\Delta^{5,22}$-dienol band were fractions containing only the $\Delta^{5,7}$-dienol (7-dehydrohalosterol, 24,24-dimethylchola-5,7-dien-3β-ol): RRT 0.94; 18 mg after crystallization; mp 132–134°C; UV: λ_{max} 272 (ϵ 9,400), 282 (ϵ 10,000), and 293 (ϵ 6,000) nm; MS: m/e 370 (M^+, 84) 355 ($M^+ - CH_3$, 7), 352 ($M^+ - H_2O$, 12), 337 ($M^+ - CH_3 - H_2O$, 100), 311 ($M^+ - C_3H_7O$, 52), 271 (M^+ – SC, 14), 253 (M^+ – SC – H_2O, 16), 229 (M^+ – SC – C_3H_6, 6), and 211 (M^+ – SC – C_3H_6 – H_2O, 26); ^{1}H-NMR: 0.61 (s, C-18), 0.94 (s, C-19), 0.93 (d, J = 6.5 Hz, C-21), and 0.86 and 0.87 (d, J = 6.5 Hz, gem-dimethyl group) ppm. Finally, a band containing substrate (mp 152–153°C, RRT 0.77) was eluted.

Based on an analysis of the various separations and physical properties (especially GLC and UV), the composition of the metabolic mixture was 23% $\Delta^{5,7,22}$-trienol, 2% $\Delta^{5,22}$-dienol, and 75% $\Delta^{5,7}$-dienol.

Incubation with campesterol

From 1.2×10^{10} cells (14 g dry wt) 2.06 g of acetone-extractable material was obtained after incubation with 96 mg of campesterol (24α-methylcholesterol). This yielded 262 mg of neutral lipids following saponification. Chromatography on alumina produced two distinct but incompletely resolved distributions of sterol based on the weight of each fraction. The first peak (combination of fractions: 10 mg) was composed of substrate, RRT 1.28, mp 157–158°C, and a smaller amount (10%) of 7-dehydrocampesterol (24α-methylcholesta-5,7-dien-3β-ol) with RRT 1.55 and UV peaks at λ_{max} 272, 282, and 294 nm. The second peak (combination of fractions: 57 mg) was 7-dehydrocampesterol (RRT 1.52) with a smaller amount of material with RRT 1.27 in a ratio of 2:1, respectively. The material with RRT 1.27 was shown to be a mixture of 7,22-bisdehydrocampesterol (24-epiergosterol, 24α-methylcholesta-5,7,22-*trans*-trien-3β-ol, calculated RRT 1.31) and a small amount of substrate (RRT 1.28). When ergosterol (RRT 1.32) was added to a small aliquot, the earlier GLC peak was enhanced without resolution into two peaks. The UV spectrum of the material in the second peak from

the alumina chromatogram showed strong absorption at 272, 282, and 293 nm. The intensity corresponded to 89% of that expected for pure $\Delta^{5,7}$-sterol. The mixture must have contained 11% of substrate and therefore must have represented a third of the GLC peak with RRT 1.27. No cholestatrienol (RRT 1.02) was present.

The material from the second peak in the alumina chromatogram was acetylated and submitted to argentation thin-layer chromatography. Three bands were found. Based on UV and GLC, the slowest moving one (band *1*) was mostly the $\Delta^{5,7,22}$-trienol with a much smaller amount of the $\Delta^{5,7}$-dienol as judged by GLC; band *2* was pure $\Delta^{5,7}$-dienol; and the fastest moving band (*3*) was pure substrate. The region of the thin-layer plate between bands *2* and *3* contained traces of a mixture which, however, contained 25% of a material with RRT 1.10 which may have been the $\Delta^{5,22}$-dienyl acetate (calculated RRT 1.08). Insufficient material was present to study it further. From repeated and sequential chromatography, band *3* gave 9 mg of substrate acetate (RRT 1.29); band *2* gave 24 mg of $\Delta^{5,7}$-dienyl acetate; band *1* yielded 6 mg of $\Delta^{5,7,22}$-trienyl acetate. The $\Delta^{5,7}$-substance (7-dehydrocampesteryl acetate) was crystallized giving 6 mg with mp 121–123°C and RRT 1.52. This entire sample was used for ^{1}H-NMR: 0.62 (s, C-18), 0.95 (s, C-19), 0.93 (d, C-21), 0.78 and 0.80 (d, d, J = 7 Hz, C-26 and C-27), and 0.85 (d, J = 7 Hz, C-28) ppm. Although a mass spectrum was not obtained, the structure of the material is unequivocal. Among other things, the NMR spectrum showed the absence of a Δ^{22}-bond by the position of the signal for C-21 and the presence of a $\Delta^{5,7}$-grouping by the positions of the signals for C-18 and C-19. In addition, the UV and GLC agreed with expectation as already mentioned. The trienyl acetate (24-epiergosteryl acetate) was used without crystallization for spectroscopy, ^{1}H-NMR: 0.63 (s, C-18), 0.95 (s, C-19), 103 (d, J = 7 Hz, C-21), 0.84 and 0.83 (d, d, J = 7 Hz, C-26 and C-27), and 0.92 (d, J = 7 Hz, C-28) ppm; MS: m/e (assignment) 438 (M^+), 378 (M^+ – HOAc), 363 (M^+ – HOAc – CH_3), 253 (M^+ – HOAc – SC) and 211 (M^+ – HOAc – SC – C_3H_6).

The various results (prior to argentation chromatography in which losses were involved) show that of the metabolites (without regard for an unquantitated trace of 22-dehydrocampesterol) 75% was $\Delta^{5,7}$-dienol and 25% was $\Delta^{5,7,22}$-trienol.

Incubation with 24-methyldesmosterol

T. pyriformis was incubated with 38 mg of 24-methyldesmosterol (24-methylcholesta-5,24(25)-dien-3β-ol) yielding 1.64×10^9 cells (5.0 g dry wt). The cells gave 0.8 g of acetone-extractable material from which 445 mg of material was obtained. This material, after saponification, yielded 152 mg of neutral lipid that was chromatographed on alumina. The sterol fractions showed two poorly resolved peaks. The first (2 mg) was mostly substrate (RRT 1.47; authentic: 1.47) together with a small percent of material in about equal amounts with RRT 1.64 (unidentified) and 1.77 (see below). The UV spectrum showed two bands, one of λ_{max} 241 nm and one with λ_{max} 272, 282, and 293 nm in a ratio (ϵ_{241} to ϵ_{282}) of 13 to 12.

The fractions in the second peak (10 mg) were principally composed of material with RRT 1.77; the remainder was substrate (ca. 20%) and an incompletely identified substance with RRT 1.94 (ca. 10%). The material with RRT 1.77 was subsequently shown to be a mixture of $\Delta^{5,7,24}$-trienol and $\Delta^{5,7,22,24}$-tetraenol. The UV spectrum showed two chromophores were present (λ_{max} 241 with shoulders at 235 and 248 and λ_{max} 272, 282, and 293 nm) in a ratio of intensities (ϵ_{241} to ϵ_{282}) of 16 to 12, respectively. If we take 12,000 as ϵ_{282} for the $\Delta^{5,7}$-chromophore and 29,000 as ϵ_{241} for the $\Delta^{22,24}$-chromophore (see incubation with 22-dehydrodesmosterol from which the $\Delta^{5,7,22,24}$-tetraenol was isolated in a pure state), the ratio of $\Delta^{5,7,22,24}$-tetraenol (24-methylcholesta-5,7,22-*trans*,24(25)-tetraen-3β-ol) to $\Delta^{5,7,24}$-trienol (24-methylcholesta-5,7,-24(25)-trien-3β-ol) in the total of metabolites was close to 53 to 47, respectively. The tetraenol and trienol were partially separated by crystallization.

The crystalline material weighed 3 mg and was characterized by GLC, UV, and MS. It had RRT 1.77 with only a few percent of substrate (RRT 1.47) and material with RRT 1.94. The UV spectrum (ϵ_{241} to ϵ_{282}:1.00) showed the crystals were primarily composed of the $\Delta^{5,7,24}$-trienol. This was corroborated by the mass spectrum. Except for 4% of substrate and 4–5% each of higher molecular weight species (mol wt 410 and 412), the spectrum was composed of m/e (assignment, relative intensity) 396 (M_1^+, 100), 394 (M_2^+, 29), 363 (M_1^+ – CH_3 – H_2O, 88), 361 (M_2^+ – CH_3 – H_2O, 21), 337 (M_1^+ – C_3H_7O, 38), 335 (M_2^+ – C_3H_7O, 4), 314 (M_1^+ – C_6H_{10} from the side chain, 28), 271 (M_1^+ and M_2^+ – SC, 29), 269 (M_1^+ and M_2^+ – SC – 2H, 15), 253 (M_1^+ and M_2^+ – H_2O – SC, 33), 251 (M_1^+ and M_2^+ – H_2O – SC – 2H, 36), 211 (M_1^+ and M_2^+ – H_2O – SC – C_3H_6 from ring D, 43), and 209 (M_1^+ and M_2^+ – H_2O – SC – C_3H_6 – 2H, 21). The relative amount of tetraenol may have been somewhat higher than the ratio of M_2^+ to M_1^+ indicates, since Δ^{22}-sterols undergo allylic cleavage at the 17(20)-bond more readily than do those without this feature.

The mother liquor from the crystallization was subjected to argentation TLC. The slowest moving substance (minor) had RRT 1.94 and a UV spectrum consisting only of the $\Delta^{5,7}$ chromophore. This may have been the substance with mol wt 410 for which a structure based on the addition of oxygen to the side chain of the tetraenol is reasonable, but too little sample was available for further study. The principal (faster moving) band in the TLC had RRT 1.77 and was a mixture of $\Delta^{5,7,22,24}$-tetraenol and $\Delta^{5,7,24}$-trienol in a 3:1 ratio based on the UV spectrum (ϵ_{241} to ϵ_{282}:1.83). Substrate was identified by GLC as the fastest moving component. Since the material in the mother liquor was primarily the tetraenol, while the crystals were principally the trienol, and since the major component of both mother liquor and crystals had RRT 1.77, this RRT must have applied to both tetraenol and trienol.

Incubation with desmosterol

T. pyriformis was incubated with 25 mg of recrystallized desmosterol yielding 1.45×10^9 cells (4.32 g dry weight). From this, 684 mg of lipid was obtained which was saponified and yielded 90 mg of non-saponifiables. The sterols (12.3 mg) recovered were equivalent to 49% of the added desmosterol. On an SE-52 column two peaks were observed, RRT 1.10 and 1.33. The steryl acetates were partially resolved by argentation chromatography into three fractions: *1*) unmetabolized desmosteryl acetate, RRT 1.06; *2*) a component that eluted from the silver column shortly after desmosterol acetate (RRT 1.12) which had an RRT identical to that of 22-dehydrodesmosterol; and *3*) an acetate that eluted from $AgNO_3$ with 50% ether in hexane, RRT 1.30, (RRT of 7,22-bisdehydrodesmosterol, 1.30). The 22-dehydrodesmosterol showed UV absorption (λ_{max} 241 nm) which was the same as that for the $\Delta^{22,24}$-conjugated diene. The product identified as 7,22-bisdehydrodesmosterol showed the presence of two chromophores: λ_{max} 241 nm with shoulders at 235 and 248 nm indicative of the $\Delta^{22,24}$-diene system and λ_{max} 272, 282, and 293 nm indicative of the $\Delta^{5,7}$-conjugated system. No metabolite was observed that would have corresponded to 7-dehydrodesmosterol. Of the metabolites observed, 22-dehydrodesmosterol accounted for 37% of the total and 7,22-bisdehydrodesmosterol accounted for 63%.

Incubation with 22-dehydrodesmosterol

Primarily in order to assess the UV spectrum of the $\Delta^{22,24}$-diene system in the presence of the $\Delta^{5,7}$-grouping, *T. pyriformis* was incubated with 8 mg (2 mg/500-ml flask) of 22-dehydrodesmosterol yielding 8×10^8 cells (1.32 g dry weight). From this, 160 mg of material was obtained that was saponified to yield 52 mg of neutral lipids. After adsorption chromatography, the sterol-containing fraction showed only one component (RRT 1.33) by GLC on SE-52. The recovered sterol amounted to 1.84 mg which is equivalent to 23.7% of the substrate used in the incubation. Argentation chromatography of the acetylated sterols showed only traces of 22-dehydrodesmosterol acetate (RRT 1.12).

The UV spectrum of the pure $\Delta^{5,7,22,24}$-acetate (RRT 1.30) showed that two chromophores were present at λ_{max} 241 nm (ϵ = 29,000) with shoulders at 235 and 248 nm and λ_{max} 272, 282 (ϵ = 11,910) and 293 nm. The retention time and the UV spectrum indicated that this sterol was 7,22-bisdehydrodesmosteryl acetate. The mass spectrum showed m/e 422 and fragmentation consistent with the assigned structure. The tetraene constituted >95% of the recovered sterol.

Incubation with 24-methylenecholesterol

In a previous paper (2) it was reported that incubation of *T. pyriformis* with 24-methylenecholesterol gave rise exclusively to the 7,22-bisdehydro derivative. In the incubation reported, a small amount of the substrate (2 mg/500 ml medium) was incubated for a long period of time (40 hr). In the present work, the ciliates were incubated with 5 mg sterol/500 ml medium for 24 hr in order to compare the results with those reported in the present investigation. Once again the principal metabolite was 7,22-bisdehydro-24-methylenecholesterol (75%), but smaller amounts of 22-dehydro-24-methylenecholesterol (18%) and 7-dehydro-24-methylenecholesterol (7%) were observed. Quantitation and identifications were made primarily by HPLC using a variable wavelength UV detector at 205, 240, and 280 nm. The tetraene was observed at all 3 wavelengths (K′ = 3.0, α = 0.45), the 22-dehydro derivative was detected at 205 and 240 nm (K′ = 3.93, α = 0.58) but it was not detectable at 280, and the 7-dehydro derivative was detected at 205 and 280 nm (K′ = 4.0, α = 0.59) but not at 240. The λ_{max} for the $\Delta^{22,24}$- and $\Delta^{5,7}$-diene systems is near 240 and 280 nm, respectively. Recovered substrate was observed only at 205 nm (α = 0.75) and accounted for 40% of the total sterol recovered. The GLC data showed the presence of the tetraenol (RRT 1.51, 90% of the metabolites) and the $\Delta^{5,7,24(28)}$-trienol (RRT 1.65, 10%). Identification of the $\Delta^{5,22,24(28)}$-trienol by GLC was unclear.

DISCUSSION

Stereochemistry at C-20

The requirement for C-22 to lie on the right (Rule *A*, see Introduction) is derived from the observation (3) that, when C-21 and C-22 are fixed in space by a $\Delta^{17(20)}$-double bond, only the isomer, E-17(20)-dehydrocholesterol, with C-22 to the right is metabolized giving principally the $\Delta^{5,7,17(20),22}$-tetraenol. Thus, sterols such as cholesterol in which C-20 is tetrahedral must assume the conformation about the 17(20)-bond with C-22 to the right when bound to protein. This places the 20α-H-atom of cholesterol on the front of the molecule. If, then, the configuration is inverted, while C-22 is maintained on the right, C-21 is on the front. Since 20-isocholesterol is not metabolized (3), the bulk of the methyl group (C-21) on the front must have prevented binding to protein (Rule *B*). This analysis was corroborated (3) by strong inhibition or complete prevention of metabolism when a 20-methyl group or 20α-hydroxyl group was added to cholesterol. The present finding that 20-isohalosterol is not metabolized is a further indication of the validity of Rules *A* and *B*. If 20-isohalosterol (or 20-isocholesterol) were to assume a conformation with C-22 to the left (Rule *A*), no metabolism should occur, but metabolism should also not occur even in the correct conformation with C-22 to the right owing to the bulk of C-21 on the front (Rule *B*). The conformation with C-22 to the right, incidentally, is theoretically the most stable one (3), and x-ray diffraction studies of various sterols with a 20α-H-atom have shown this conformation to exist in the crystalline state (13). Our interpretation of the data to mean binding occurs on the β-face is consistent with the fact that dehydrogenation at C-7 requires elimination of the 8β-proton; this proton almost certainly is not removed without being abstracted by a proton-acceptor on the enzyme. However, none of our data preclude concomitant binding on the rear (α-face of the nucleus). Indeed, evidence exists for the latter. Mulheirn, Aberhart, and Caspi (14) found that, when lathosterol (cholest-7-en-3β-ol) proceeds to 7,22-bisdehydrocholesterol in *T. pyriformis*, dehydrogenation at C-5 occurs with loss of the 5α- and 6α-H-atoms. It therefore appears highly probable that the dehydrogenases have a cleft into which the sterol fits and that binding occurs on both sides of the nucleus.

TABLE 1. Influence of sterol structure on dehydrogenation

Substrate	Percent of Metabolites $\Delta^{5,7}$	$\Delta^{5,22}$	$\Delta^{5,7,22}$	Reference
Cholesterol	3	15	82	1[a]
26(or 27)-Norcholesterol			92	7
26,27-Bisnorcholesterol	25	31	44	7
Z-24-Ethylidenecholesterol	33		67	2
E-24-Ethylidenecholesterol	91		9	2
24-Methylenecholesterol	7	18	75	This paper
24-Methyldesmosterol	24		76	This paper
24α-Methylcholesterol	59		41	This paper
Halosterol	75	2	23	This paper
20-Isohalosterol				This paper
Desmosterol		37	63	This paper
22-Dehydrodesmosterol			>95	This paper

[a] Also unpublished observations by Landrey, J. R., W. R. Nes, and R. L. Conner.

Stereochemistry at C-24

Inhibition of dehydrogenation at C-22 by a substituent at C-24 (Rule *D*) is derived primarily from the very strong effect that inversion of the configuration at C-28 was found to have when the isomeric 24-ethylidenecholesterols were examined (2). In the case of the E-isomer (fucosterol), C-29 is *cis* with respect to C-22, and most of the mixture of metabolites consisted of the 7-dehydro derivative. Inversion of the configuration to give the Z-isomer (isofucosterol) reversed the relative amounts of dehydrogenation at C-7 and C-22, and the 7,22-bisdehydro derivative became the major product (**Table 1**). However, even in the latter case more $\Delta^{5,7}$-metabolite accumulated than is found (1, 7) with cholesterol and other sterols, e.g., 26(or 27)-norcholesterol, that lack substitution at C-24 (Table 1). Consequently, substitution of an ethylidene group itself at C-24 probably inhibits dehydrogenation at C-22, and the presence of a methyl group toward C-22 in the E-isomer presumably only enhanced the steric effect. Our present results are in agreement with this interpretation, because a substituent as small as CH_3 at C-24 was also found to be sterically hindering. Halosterol and campesterol (24α-methylcholesterol) both yielded primarily their 7-dehydro derivatives, while cholesterol and other sterols with a saturated side chain and no substituent at C-24 primarily yielded 22-dehydro- and 7,22-bisdehydro derivatives even when the side chain was shortened, as in the case of 26,27-bisnorcholesterol (Table 1).

The isolation of the $\Delta^{5,7,22}$-sterol (24-epiergosterol) from the incubation with campesterol, which was reported in a preliminary communication (15) in another connotation, represents the first description of this interesting compound. The ^{1}H-NMR spectrum can be distinguished from that of ergosterol, and this difference permitted us to show (15) the presence of 24-epiergosterol as a naturally occurring companion of ergosterol in a lower tracheophyte as well as to show (15) that fungal ergosterol is not accompanied by the epimer.

Influence of a double bond at C-24

Less than half of the metabolites of 24α-methylcholesterol contained the Δ^{22}-bond, but, when a double bond either at position-24(28) (24-methylenecholesterol) or position-24(25) (24-methyldesmosterol) was introduced, more than three-quarters of the metabolic mixture consisted of sterols with a Δ^{22}-bond. This leads us to believe that a double bond ($\Delta^{24(28)}$ or $\Delta^{24(25)}$) that is allylic to the position of dehydrogenation (C-22, C-23) enhances the latter reaction electronically, thereby acting counter to the effect of steric hindrance. As expected from this conclusion, desmosterol, which lacks a 24-methyl group but has a $\Delta^{24(25)}$-bond, yielded metabolites, all of which contained the Δ^{22}-bond. The accumulation of more $\Delta^{5,22}$-sterol than is found with cholesterol may reflect a preference in the specificities of the dehydrogenases for a sequence in which the Δ^{7}-bond is introduced before introduction of the Δ^{22}-bond which would mean the $\Delta^{5,22}$-sterol might proceed to the trienol slower than would the $\Delta^{5,7}$-sterol. However, as shown by the conversion of 22-dehydrodesmosterol to the tetraenol, the Δ^{22}-bond does not prevent dehydrogenation at C-7.

The differences in the electronic enhancement of dehydrogenation at C-22 by a $\Delta^{24(28)}$- or $\Delta^{24(25)}$-bond can be rationalized by our assuming a repressive inductive effect from the substituents on the double bond. The data are in accord with an increase in the rate of dehydrogenation when the double bond terminates with two H-atoms (24-methylenecholesterol) compared to two methyl groups (24-methyldesmosterol). This suggests that the mechanism of dehydrogenation may not involve creation of a positive charge on C-23.

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