The Conversion of Cholesterol to $\Delta^{5,7,22}$-Cholestatrien-3$\beta$-ol by Tetrahymena pyriformis*

(Received for publication, October 21, 1968)

R. L. Conner, F. H. Mallory,‡ J. R. Landrey, and C. W. L. Itengar

From the Departments of Biology and Chemistry, Bryn Mawr College, Bryn Mawr, Pennsylvania 19010

SUMMARY

The protozoon Tetrahymena pyriformis was incubated in a growth medium containing added unlabeled cholesterol or 26,27,28,29-4C-cholesterol with the following consequences: (a) The biosynthesis of tetrahymanol, a pentacyclic triterpenoid alcohol produced in the organism by squalene cyclization, was inhibited, and (b) the cholesterol was accumulated by the cells and converted metabolically to a mixture of other sterols containing $\Delta^{5,7,22}$-cholestatrien-3$\beta$-ol, (7,22-bisdehydrocholesterol) as the major component, together with 22-dehydrocholesterol and possibly a small amount of 7-dehydrocholesterol. These three sterols, along with unconverted cholesterol, were obtained in the unsaponifiable lipid fraction from the cells, and were separated from one another by silicic acid-silver nitrate column chromatography of the corresponding acetylated derivatives. The 22-dehydrocholesterol and the recovered cholesterol were rigorously identified by comparisons of various chromatographic, spectral, and other properties of the alcohols and their acetates with those of authentic samples. The tentative identification of the 7-dehydrocholesterol was based on chromatographic comparisons of the acetate derivative with authentic material. The structure of the 7,22-bisdehydrocholesterol, a previously unknown compound, was established on the basis of ultraviolet, infrared, nuclear magnetic resonance, and mass spectral data for both the free sterol and its acetate derivative with authentic samples. The conversion of cholesterol to 7,22-bisdehydrocholesterol in the cells proceeds by way of either 7-dehydrocholesterol or 22-dehydrocholesterol as intermediate.

A requirement for trace amounts of sterols is often encountered in the phylum Protozoa, and several detailed nutritional studies have been carried out with the class Ciliata. A relationship between the molecular structure of the sterol and its biological activity has been shown for Paramecium aurelia (2) and for some members of the genus Tetrahymena (3–5). The species T. pyriformis has no sterol nutritional requirement; however, three types of observation indicate the importance of sterols in the physiology of this ciliated protozoan. First, the addition of sterols, such as cholesterol or stigmasterol, elevates the growth rate of this organism in a chemically defined medium (6). Second, an absolute sterol nutritional requirement can be induced in this protozoan by a number of growth inhibitors; these include triparanol, a compound known to block cholesterol biosynthesis in other organisms (7–9), 2,4-dinitrophenol, an oxidative phosphorylation-uncoupling agent (6), and a number of other substances the mode of action of which is unknown, such as substituted purines (10), colchicine (11), and steroids of the pregnane series (11, 12). Third, cholesterol influences both orthophosphate flux (13) and hypoxanthine excretion (14) in these cells. Such findings are in accord with the suggestion that certain sterols or their derivatives may serve in a metabolic capacity in T. pyriformis. As part of a program to evaluate this possibility, we have sought detailed information about the chemical fate of cholesterol when it is added to the growth medium in which this organism is incubated.

Extensive accumulation and concentration of exogenous cholesterol by this protozoan form has been noted, and some of the sterols accumulated have been identified as 7,22-bisdehydrocholesterol, 7,22-dehydrocholesterol, and triparanol. These sterols are also accumulated by Paramecium aurelia, and there is evidence that some of the sterols present in this protozoan are derived from the added cholesterol.

* This work was supported by Grant GB-4695 from the National Science Foundation and by the Bryn Mawr College Fund for the Coordination of the Sciences. A preliminary report of these investigations was presented at the Second International Conference on Protozoology held in London in 1965, and has appeared in abbreviated form (1). We are pleased to acknowledge the receipt of Research Instrument Grants from the National Science Foundation for the purchase of the Cary model 14 ultraviolet spectrometer (Grant GP-1667), the Varian A-56/60A nuclear magnetic resonance spectrometer (Grant GP-5431), and the Perkin-Elmer model 257 infrared spectrometer (Grant GP-8271).

‡ Alfred P. Sloan Research Fellow, 1964 to 1968.

1 The trivial names used are: cholesterol, $\Delta^{5}$-cholesten-3$\beta$-ol; stigmasterol, $\Delta^{5,22}$-stigmastadien-3$\beta$-ol; triparanol, 1-[p-(3-dimethylaminoethoxy)phenyl]-1-(p-tolyl)-2-(p-chlorophenyl)ethanol; 7,22-bisdehydrocholesterol, $\Delta^{5,22}$-cholestadien-3$\beta$-ol; 22-dehydrocholesterol, $\Delta^{5,22}$-cholestadien-3$\beta$-ol; ergosterol, $\Delta^{5,22}$-ergostatrien-3$\beta$-ol; 7-dehydrocholesterol, $\Delta^{5,7}$-cholestadien-3$\beta$-ol; lanosterol, $\Delta^{7}$-cholesten-3$\beta$-ol; tetrahymanol, $\Delta^{3,7}$-cholesten-3$\beta$-ol.
the characteristics of this process have been described (15); examination of the unsaponifiable lipids from the cells after cholesterol accumulation indicated the formation of products more polar than cholesterol.

The present report includes a description of the isolation and identification of some of the products that arise as a result of cholesterol metabolism in T. pyriformis. The major product has been established as Δ⁷,¹²-cholestatrien-3β-ol (7,22-bisdehydrocholesterol) (see Fig. 1). We believe this to be the first substantiated report of this sterol.

**EXPERIMENTAL PROCEDURE**

**Materials**

Commercial samples of cholesterol (Sigma) were purified by way of the dibromide by the method of Wissel (16), as modified by Franta et al. (17); the regenerated cholesterol was recrystallized from ethanol to give material with a melting point of 150-151° (literature m.p., 149° (18)). This material contained no detectable impurities as judged by two methods: thin layer chromatography on Silica Gel G (Drikanam) plates that were developed with a variety of solvent systems and visualized by iodine staining, and gas-liquid chromatography on three different columns as described in the next section. The purified cholesterol was stored at -20° as a methanol paste in a nitrogen atmosphere (18).

The 26-¹⁴C-cholesterol (Schwarz BioResearch) used was combined with purified, unlabeled cholesterol, and was carried through the purification procedure described above. The purity of the resulting material was established by TLC* with plates coated with Silica Gel G and developed with ethyl acetate-benzene (1:1, v/v); an autoradiogram with Kodak Royal Blue x-ray “no screen” film showed a single spot with an appropriate RF value (0.72) for cholesterol.

A small sample of 22-dehydrocholesterol was kindly provided for comparison purposes by W. E. Robbins and J. N. Kaplanis, Agriculture, Beltsville, Maryland. We were informed (by W. E. Robbins) that this was a sample of material that had been prepared biosynthetically by feeding ergosterol to Blattella germanica as described by Clark and Bloch (19), and that it had a melting point of 137-138° (literature m.p., 133.5-134.0° (20)), an infrared spectrum identical with that reported for lathosterol (23), and that it had a melting point of 124-125° (literature m.p. 123° (18)), and its infrared spectrum agreed with that reported for lathosterol (18). This sample was found by GLC (SE-52 column) to consist of 96% lathosterol with a 4% component that has previously been suggested to be the Δ⁶¹⁴ isomer (17).

Sterols were acetylated by treatment with acetic anhydride and pyridine; the resulting acetates were recrystallized from methanol. The authentic sterol acetates prepared in this way include cholesteryl acetate (m.p. 115-115.5°, literature m.p. 116° (18)), 7-dehydrocholesterol acetate (m.p. 129-129.5°, literature 130° (18)), ergosterol acetate (m.p. 176-178°, literature m.p. 181° (18)), lathosterol acetate (m.p. 119°, literature m.p. 119° (18)), and 22-dehydrocholesterol acetate. These compounds showed single GLC peaks on the three different columns used in this work (except for the lathosterol acetate, which contained a minor component as in its lathosterol precursor), and also showed single spots on Silica Gel H-silver nitrate TLC plates, as described in a later section.

Samples of pure tetrahymanol (m.p. 315–317°) and tetrahymanyl acetate (m.p. 303–305°) were obtained by previously described procedures (24).

All organic solvents were redistilled before use. The petroleum ether had a boiling range of 40–60°.

All melting point values determined in this work were measured in evacuated capillaries in an oil bath and are uncorrected.

**Methods**

**Gas-Liquid Chromatography**—Analyses by GLC utilized an F & M model 400 gas chromatograph equipped with a hydrogen flame ionization detector and a disc integrator, with nitrogen as the carrier gas at a flow rate of about 100 ml per min. Sterols and sterol acetates were analyzed with glass columns (3 mm internal diameter) of the following three types: a 6-foot column packed with 80 to 100 mesh GasChrom S coated with 0.75% SE-52 silicone gum rubber (Hewlett-Packard), a 2-foot column packed with 80 to 100 mesh GasChrom S coated with 2% neopentyl glycol succinate (Hewlett-Packard), and a 6-foot column packed with 80 to 100 mesh Diaport S coated with 3% QF-1 (Applied Science Labs). Carboxylic acids were analyzed with a 6-foot stainless steel column (3 mm internal diameter, Hewlett-Packard) packed with 60 to 80 mesh Diaport S coated with 10% diethylene glycol succinate and 2% phosphoric acid. These four columns will hereafter be designated SE-52, NGS, QF-1, and DGS, respectively. The retention times of the sterols and sterol acetates were calculated relative to cholestane; those of the carboxylic acids were calculated relative to acetic acid. Relative retention time values are given for various authentic samples of these compounds in Tables I and II. Tetrahymanol and its acetate were analyzed with the SE-52 column at 250°; the retention times relative to cholestane were 5.1 and 6.6, respectively.

Quantitative analyses by disc integration of peak areas were based on calibrations with solutions containing known amounts of authentic samples.

**Growth of Tetrahymanol**—Axenic cultures of T. pyriformis W were maintained in a peptone-based culture fluid, and the cells were harvested by methods described previously (25, 26). In a typical experiment involving cholesterol supplementation of the growth medium, a solution of 5 mg of cholesterol in 1 ml

* The abbreviations used are: TLC, thin layer chromatography; GLC, gas-liquid chromatography.
Retention times relative to cholestane for series of sterols and sterol acetates determined by GLC analysis on three different columns

Authentic samples were used; their preparations are described in "Materials." Results are the average of three determinations. Absolute retention times of cholestane were SE-52, 3.0 min; NGS, 1.1 min; QF-1, 6.5 min. Details regarding the columns are given in the text. Oven temperatures were SE-52, 225°; NGS, 215°; QF-1, 205°.

<table>
<thead>
<tr>
<th>Compound</th>
<th>SE-52</th>
<th>NGS</th>
<th>QF-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td>3.00</td>
<td>6.0</td>
<td>3.06</td>
</tr>
<tr>
<td>Cholesterol acetate</td>
<td>2.71</td>
<td>6.1</td>
<td>3.73</td>
</tr>
<tr>
<td>7-Dehydrocholesterol</td>
<td>2.15</td>
<td>8.1</td>
<td>3.54</td>
</tr>
<tr>
<td>7-Dehydrocholesterol acetate</td>
<td>3.01</td>
<td>6.7</td>
<td>5.42</td>
</tr>
<tr>
<td>Ergosterol acetate</td>
<td>1.79</td>
<td>5.7</td>
<td>2.81</td>
</tr>
<tr>
<td>Lathosterol acetate</td>
<td>2.50</td>
<td>4.9</td>
<td>4.28</td>
</tr>
<tr>
<td>Ergosterol acetate</td>
<td>2.34</td>
<td>8.4</td>
<td>3.48</td>
</tr>
<tr>
<td>Lathosterol acetate</td>
<td>3.30</td>
<td>7.6</td>
<td>5.35</td>
</tr>
<tr>
<td>Ergosterol</td>
<td>2.46</td>
<td>9.0</td>
<td>3.84</td>
</tr>
<tr>
<td>Ergosteryl acetate</td>
<td>3.44</td>
<td>8.0</td>
<td>5.37</td>
</tr>
</tbody>
</table>

Relative retention times for series of aliphatic acids determined by GLC analysis on DGS column

Details regarding the column are given in the text. The oven temperature was 115°, and the absolute retention time of acetic acid was 5.2 min.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Relative retention time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>1.0</td>
</tr>
<tr>
<td>Propionic acid</td>
<td>1.4</td>
</tr>
<tr>
<td>Isobutyric acid</td>
<td>1.4</td>
</tr>
<tr>
<td>n-Butyric acid</td>
<td>1.8</td>
</tr>
<tr>
<td>Isovaleric acid</td>
<td>2.1</td>
</tr>
<tr>
<td>n-Valeric acid</td>
<td>2.6</td>
</tr>
</tbody>
</table>

of ethanol was dispersed aseptically into 500 ml of previously autoclaved culture fluid that had been reheated to 80°; after the mixture was cooled to 28°, the seed culture of cells was added. In these experiments, as well as in control experiments without added cholesterol, the cell population was claimed by centrifugation after a growth period of 36 to 40 hours (stationary phase) at 28 ± 1°. The residual culture fluid was removed from the organisms in the cup of the continuous flow centrifuge by rinsing with distilled water. The cells were lyophilized and stored in the dark at -20° under nitrogen until the lipids were extracted.

**Lipid Extraction and Isolation of Unsaponomifiable Fraction**—The cellular residue after lyophilization was extracted three times with a chloroform-methanol mixture (2:1, v/v) at about 50°, with a total of at least 20 ml of solvent per g of residue. After the extract was filtered through sintered glass to remove cellular debris, the solvent was removed under reduced pressure with a rotary evaporator. This total lipid fraction was dissolved in petroleum ether (10 ml/10 g of packing), and the filtrate was subjected to column chromatography on silicic acid to remove phospholipids. The column (25 mm internal diameter) was prepared with a petroleum ether mixture of acetates was separated into its components by column chromatography on a mixture of silicic acid (Unisil, 100 to 200 mesh, Clarkson Chemical Company, Williamsport, Pennsylvania) and silver nitrate (4:1, w/w) by the method of Vroman and Cohen (27). The columns (25 mm internal diameter, containing 6 g of packing per 20 mg of sterol acetate) were eluted with a graded series of benzene-petroleum ether mixtures, and the fractions were monitored by GLC.

**Thin Layer Chromatography**—The sterol acetates encountered in the incubation studies described herein were readily separated from one another by TLC, with plates coated with a slurry consisting of 25 g of Silica Gel H (Brinkmann), 6.25 g of silver nitrate, and 60 ml of water (27). The plates were air dried and then activated at 140-150° for 40 min. A solvent system of petroleum ether-benzene (1:1, v/v) was used for development, and the acetates were detected by spraying with phosphomolybdic acid in ethanol (10 g/100 ml), followed by heating at 160° for 20 min; the acetates from the 26-3H-cholesterol incubations were also detected by autoradiography.

**Oxidation of Unsaturated Side Chains**—A method was developed for determining the presence or absence of Δ5 unsaturation in a sterol side chain by GLC analysis of the low molecular weight aliphatic acids produced by periodate-permanganate oxidation (28, 29). In a typical case, about 0.5 to 1.0 μmole of the sterol or sterol acetate was dissolved in 0.5 ml of tert-butyl alcohol, to which was added 0.05 ml of aqueous 50 mM potassium carbonate and 0.3 ml of an aqueous solution that was 97 mM in sodium periodate and 3 mM in potassium permanganate; the flask was stoppered and the reaction was allowed to proceed overnight at 37°. The reaction mixture was then acidified with dilute sulfuric acid, decolorized by the dropwise addition of aqueous sodium bisulfite, and extracted twice with 2-ml portions of diethyl ether. The ether extracts were combined and dried over anhydrous sodium sulfate. The mixture was filtered, and the volume of the filtrate was reduced to 1 ml by evaporation under a stream of nitrogen. The ether solution was analyzed quantitatively for its carbonylic acid content by GLC (DGS column) on the basis of comparisons with solutions of known concentra-

*Further details of this method will be published elsewhere.*
tions of authentic samples (see Table II). Trace amounts of an unidentified substance having a relative retention time near that of propionic acid or isobutyric acid were observed in control oxidations carried out in the absence of a sterol or sterol acetate; this minor complication did not interfere with the analysis for side chain unsaturation, since a blank correction could be made. Authentic samples of several sterol acetates lacking Δ7 unsaturation were oxidized as a test of the method; cholesterol acetate, 7-dehydrocholesteryl acetate, and lathosterol acetate gave rise to no products detectable by GLC.

Preparation of p-Bromophenacyl Isovalerate—Samples of 14C-labeled cholesteryl acetate (2.8 mg), 22-dehydrocholesteryl acetate (1.5 mg), and 7,22-bisdehydrocholesteryl acetate (7.1 mg) were obtained from T. pyriformis that had been incubated with exogenous 26,14C-cholesterol (see "Results"). These three samples were each oxidized by appropriately scaled up versions of the analytical periodate-permanganate method described in the previous section. After the three reaction mixtures were acidified and decolorized, carrier isosoloric acid (Matheson, Coleman and Bell) was added (200, 110, and 500 mg of carrier for the oxidations of cholesteryl, 4 22-dehydrocholesteryl, and 7,22-bisdehydrocholesteryl acetates, respectively). In each case, the resulting mixture was extracted with three 25-ml portions of diethyl ether, the ether was evaporated from the extract, and the residual isosoloric acid was converted to p-bromophenacyl isovalerate (30).

Radioactivity Measurements—A Packard model 3310 liquid scintillation spectrometer equipped with an external standard was used to determine 14C. The scintillation fluid consisted of 4 g of 2,5-diphenyloxazole and 0.5 g of 1,4-bis(5-phenyl-2-oxazolyl)benzene per liter of toluene. Counts were recorded for background and were converted to disintegrations per min with the appropriate standards and efficiency factor.

Other Analyses—Ultraviolet spectra were determined in ethanol solutions with a Cary model 14 spectrometer. Infrared spectra were determined in potassium bromide discs with a Perkin-Elmer model 207 spectrometer. The mass spectrum of 7,22-bisdehydrocholesteryl acetate was determined with an Associated Electrical Industries Ltd. MS-9 double focusing spectrometer. Nuclear magnetic resonance spectra were determined in deuteriochloroform solution with a Varian A-56/60A spectrometer; peak positions are reported in Hertz downfield of tetramethylsilane as an internal standard. Specific rotations were based on measurements with a Zeiss photoelectric precision polarimeter of optical rotations for the sodium D line with chloroform solutions containing 10 mg of the sterol or sterol acetate per ml. The procedure described by Cook (31) for the Liebermann-Burchard reaction was used for quantitative detection and differentiation of fast acting (Δ7) and slow acting sterols.

RESULTS

Sterol Content of Cells—When T. pyriformis was grown in the absence of added sterols, the major unsaponifiable component was tetrahymanol, a pentacyclic triterpenoid alcohol the isolation, structure, and mechanism of biosynthesis of which have been the subjects of earlier publications from our laboratory (24, 32-36).

However, only trace amounts of tetrahymanol were found (Table III) in the unsaponifiable fraction from cells that were grown in the presence of added cholesterol. Under these conditions, this fraction contained a mixture of sterols, the identities of which were established by the work to be described below. The presence of cholesterol, 22-dehydrocholesterol, and 7,22-bisdehydrocholesterol in this mixture was rigorously demonstrated, and evidence for the presence of 7-dehydrocholesterol was also obtained. The amount of tetrahymanol in the cholesterol-supplemented cells corresponded approximately to that initially present in the seed culture used to inoculate the flask. That the biosynthesis of tetrahymanol in the cells was inhibited by the added cholesterol and not by the ethanol used to disperse it in the culture fluid was shown by appropriate control experiments (see Table III). The possibility that the dehydro and bisdehydro derivatives of cholesterol might have been artifacts arising from some process not involving the cells was ruled out by control experiments. Thus, uninoculated flasks of culture fluid containing added cholesterol were maintained at 28° ± 1° for 36 to 40 hours, after which the water was removed by sublimation under reduced pressure and the residue in each case was extracted with a chloroform-methanol mixture (2:1, v/v); the only sterol detected in these extracts by GLC analysis (SE-52 and NGS columns) was cholesterol. Similar control experiments without added cholesterol showed that the culture fluid itself is not the source of any detectable quantity of sterols.

Large Scale Metabolic Conversions of Cholesterol—The incubation of T. pyriformis with added cholesterol was carried out on a large scale (a total of 102 liters of culture fluid) in order that sufficient amounts of the resulting sterols could be isolated to allow their unequivocal identification. These incubations yielded 141.5 g of lyophilized cellular residue from which 14.4 g (10%, w/w) of lipid were extracted. After separation of this material by silicic acid chromatography and saponification of the resulting 1.1 g of neutral lipid, a total of 600 mg of sterols (GLC analysis, SE-52 column) was isolated. This unsaponifiable fraction was dissolved in warm methanol, the solution was chilled at 4°, and a 350-mg crop of crystals was collected by suction filtration. The sterols remaining in the filtrate were isolated by precipitation with digitonin and subsequent cleavage of the digitonides. The crystalline material was acetylated and separated by column chromatography on silicic acid-silver nitrate. Table IV shows the results of one of these separations. The appropriate fractions from these columns were combined and the solvents were evaporated to give separate samples of each of the components.

Proof of Structure of 7,22-Bisdehydrocholesterol—The sample of 7,22-bisdehydrocholesterol acetate was recrystallized three times from methanol to give 113 mg of crystals with the properties listed in Table V. The purity of this acetate was established by the following observations: essentially the same sharp melting point was found after each recrystallization, a single spot (Rf 0.10) was detected by TLC on Silica Gel II-silver nitrate, only one GLC peak was observed on each of three different columns (SE-52, NGS, and QF-1), and quantitative GLC analysis (SE-52 column) showed the peak area to be 98% of that expected for an equivalent amount of cholesteryl acetate as a reference standard. A portion of the acetate was saponified and the product was recrystallized three times from methanol to give 23 mg of material having the properties given in Table V. The melting behavior of this sterol suggested the formation of a liquid crystal: at temperatures around 117°-119° the crystals changed phase to
give an opaque liquid, which became clear only as the temperature was raised to around 122-124°. The purity of this compound was shown by observing only a single GLC peak on each of the usual three columns, with the peak on the SE-52 column having 97% of the expected area based on cholesterol as a standard.

The mass spectrum of the acetate exhibited a low intensity

**Table III**

Tetrahymanol content of *T. pyriformis* grown in peptone-based culture fluid with and without cholesterol supplementation

<table>
<thead>
<tr>
<th>Culture fluid additive</th>
<th>No. of cultures assessed</th>
<th>Tetrahymanol content μg/10⁶ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>5</td>
<td>1070 ± 90⁸</td>
</tr>
<tr>
<td>Ethanol</td>
<td>6</td>
<td>1146 ± 197</td>
</tr>
<tr>
<td>Cholesterol + ethanol</td>
<td>5</td>
<td>15 ± 6</td>
</tr>
</tbody>
</table>

⁸ Standard deviation.

**Table IV**

Resolution by column chromatography of mixture of acetates obtained from *T. pyriformis* after incubation with added cholesterol

A 208-mg mixture of acetates prepared by acetylation of the recrystallized unsaponifiable material was dissolved in 100 ml of petroleum ether and applied to a column (26 mm internal diameter) packed with 60 g of silicic acid-silver nitrate. The column was developed with 230-ml portions of various benzene-petroleum ether mixtures as eluents.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Benzene in eluent</th>
<th>Amount of acetate mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>1.4 Tetrahymanol</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>0.1 Tetrahymanol</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>10.8 Cholesteryl</td>
</tr>
<tr>
<td>4</td>
<td>20</td>
<td>0.8 Cholesteryl</td>
</tr>
<tr>
<td>5</td>
<td>25</td>
<td>0.8 22-Dehydrocholesterol</td>
</tr>
<tr>
<td>6</td>
<td>30</td>
<td>8.4 22-Dehydrocholesterol</td>
</tr>
<tr>
<td>7</td>
<td>35</td>
<td>0.8 22-Dehydrocholesterol</td>
</tr>
<tr>
<td>8</td>
<td>50</td>
<td>1.0 7-Dehydrocholesterol</td>
</tr>
<tr>
<td>9</td>
<td>50</td>
<td>1.0 7,22-Bisdihydrocholesterol</td>
</tr>
<tr>
<td>10</td>
<td>75</td>
<td>130.7 7,22-Bisdihydrocholesterol</td>
</tr>
<tr>
<td>11</td>
<td>75</td>
<td>23.7 7,22-Bisdihydrocholesterol</td>
</tr>
<tr>
<td>12</td>
<td>100</td>
<td>3.1 7,22-Bisdihydrocholesterol</td>
</tr>
</tbody>
</table>

* The identities of these acetates were established by methods described in later sections of this paper. The various eluent fractions were monitored by GLC with the use of relative retention time values from SE-52, NGS, and QF-1 columns. Amounts of the acetates were determined by quantitative GLC analysis (SE-52 column). The over-all recovery was 101 mg, which is 92% of the amount of the acetate mixture applied to the column.

The ultraviolet spectral data reported in Table V for both the alcohol and its acetate clearly and specifically indicate the presence in these compounds of Δ⁷ unsaturation (37, 38). The third unit of unsaturation was shown to be a Δ² double bond by
the observation that the periodate-permanganate oxidation of the acetate gave isovaleric acid (relative retention time, 2.1) as the only product detectable by GLC (DGS column); the identity of the isovaleric acid produced in this oxidation was confirmed by C-labeling studies described in a later section. The \( \Delta^2 \) double bond was shown to have the trans configuration by the infrared spectrum of the triene acetate; the Liebermann-Burchard color reactions of the acetate indicated only slow acting material that responded with a color intensity within experimental error of that of authentic cholesteryl acetate and 22-dehydrocholesterol acetate, and also to show single peaks on co-chromatography with authentic samples.

The identification is supported by three additional observations: the acetate was shown by GLC (DGS column) to give isovaleric acid (relative retention time, 2.1) as a result of periodate-permanganate oxidation; there was none of the characteristic absorption of a \( \Delta^5 \)-diene in the ultraviolet spectrum of the acetate; the Liebermann-Burchard color reactions of the acetate at two concentrations indicated only slow acting material that responded with a color intensity within experimental error of that of cholesteryl acetate as a standard (see Table VI).

**Tentative Identification of 7-Dehydrocholesterol**—The material presumed to be 7-dehydrocholesterol acetate was eluted from the silicic acid-silver nitrate columns accompanied by some \( \Delta^5 \)-dehydrocholesterol acetate (e.g. Fraction 9 in Table IV). Both column chromatography and TLC were used in an attempt to purify the supposed 7-dehydrocholesterol acetate. Although these attempts failed to give a pure compound, it was nevertheless possible to obtain mixtures in which the major component had relative retention time values on the usual three columns that corresponded to those for authentic 7-dehydrocholesterol acetate, gave a single peak on each GLC column on co-chromatography with authentic material, and had the same \( R_F \) value (0.15) as authentic 7-dehydrocholesterol acetate when analyzed by TLC on Silica Gel H-silver nitrate. On this basis, we suggest that 7-dehydrocholesterol was probably present as a minor constituent of the unsaponifiable fraction from the cells incubated with added cholesterol.

**Identification of Cholesterol**—The identity of the 56-mlg sample of cholesteryl acetate obtained from the silicic acid-silver nitrate columns was established by comparisons of infrared spectra and GLC behavior (relative retention time values and co-chromatography on the usual three columns) of both the acetate and its saponification product with the spectra and GLC behavior of authentic cholesteryl acetate and cholesterol, respectively. The acetate was further characterized by its melting point of 109–111.5° (literature m.p. 116° (18)), its \( \varepsilon_24 \) value of -53° (litera-

---

**Table VI**

Liebermann-Burchard analyses of esters obtained by acetylation of sterols isolated from *T. pyriformis* incubated with added cholesterol

<table>
<thead>
<tr>
<th>Ester</th>
<th>Amounts</th>
<th>Liebermann-Burchard</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GLC analysis</td>
<td>Liebermann-Burchard</td>
</tr>
<tr>
<td>Cholesteryl acetate⁵</td>
<td>0.145</td>
<td>0.140</td>
</tr>
<tr>
<td>22-Dehydrocholesteryl acetate⁶</td>
<td>0.140</td>
<td>0.150</td>
</tr>
<tr>
<td>7,22-Bisdehydrocholesteryl acetate⁷</td>
<td>0.280</td>
<td>0.300</td>
</tr>
<tr>
<td></td>
<td>0.010</td>
<td>0.011</td>
</tr>
<tr>
<td></td>
<td>0.020</td>
<td>0.021</td>
</tr>
<tr>
<td></td>
<td>0.030</td>
<td>0.029</td>
</tr>
</tbody>
</table>

a These amounts were estimated by quantitative GLC with an SE-52 column.

b These amounts were estimated by comparisons of Liebermann-Burchard color intensities with those developed by known amounts of authentic cholesteryl acetate as a standard slow acting material and authentic 7-dehydrocholesterol acetate as a standard fast acting material.

⁵ Only slow acting material was detected.

⁶ Only fast acting material was detected.

⁷ Fortuitous overlap of peaks permitted the resolution of only four of the expected seven peaks for these four methyl groups.

⁸ The relative retention time for the \( \Delta^5,\Delta^{7},\Delta^{22} \)-triene acetate on the QF-1 column, for example, can be estimated by multiplying the value for the \( \Delta^{5},\Delta^{7} \)-diene acetate (5.42) by the ratio of the values for 22-dehydrocholesterol acetate (4.28) and cholesteryl acetate (4.73) to give a calculated relative retention time value of 5.49 (4.78/4.73 = 4.90).
tured, \(-47^\circ (18)\), its \(R_F\) value of 0.58 (authentic sample, 0.58) on a Silica Gel H-silver nitrate TLC plate, and its Liebermann-Burchard color reactions at two concentrations, which showed only slow acting material with a color intensity within experimental error of that of authentic cholesteryl acetate (see Table VI).

**Incubations with 26-\(^{14}\)C-Cholesterol—**Incubations of *T. pyriformis* were carried out in 10 flasks, each containing 500 ml of culture fluid and 5 mg of 26-\(^{14}\)C-cholesterol with a specific activity of 8.9 \(\mu\)Ci per mmole. The combined total lipid fraction isolated from the cells contained 69\% (1.76 \(\times\) 10\(^{6}\) dpm) of the \(^{14}\)C that was present in the starting 26-\(^{14}\)C-cholesterol.

The unsoaponifiable fraction was obtained in the usual way, and a small portion thereof was analyzed by TLC on a Silica Gel G plate that was developed with ethyl acetate-benzene (1:1, v/v); an autoradiogram showed two radioactive regions, one appropriate for sterols (\(R_F\) 0.72) and the other at the origin. The material eluted from the origin with methanol was not detectable by GLC on the SE-52, NGS, or QF-1 columns, presumably because of its high polarity and correspondingly long retention time. This highly polar material, which was observed in a previous study (15), is tentatively suggested to arise from air oxidation of 7,22-bisdehydrocholesterol, possibly during the isolation and purification procedures.

The unsoaponifiable fraction was acetylated and a small sample of the resulting material was analyzed by TLC on Silica Gel H-silver nitrate. Both phosphomolybdic acid staining and autoradiography revealed the same six well resolved spots; in order of decreasing intensity on the autoradiogram, these spots had the following \(R_F\) values: 0.10, origin, 0.31, 0.58, 0.48, and 0.15, corresponding to the \(R_F\) values for 7,22-bisdehydrocholesteryl acetate, polar material, an unidentified component, cholesteryl acetate, 22-dehydrocholesteryl acetate, and 7-dehydrocholesteryl acetate, respectively. Neither the material remaining at the origin nor the unidentified component with \(R_F\) 0.31 gave rise to detectable GLC peaks on the usual three columns; hence, materials of these types were not observed in the large scale incubation experiments described in an earlier section.

We suggest that the component with \(R_F\) 0.31 may be a thermally unstable transannular peroxide derived from 7,22-bisdehydrocholesteryl acetate by air oxidation. Consistent with this preliminary suggestion, we found by TLC analysis that a pure sample of 7,22-bisdehydrocholesteryl acetate that was allowed to stand for several weeks in benzene solution was partially converted to material with \(R_F\) 0.31 as well as to highly polar material remaining at the origin.

The mixture of acetates was separated into its components by column chromatography on silicic acid-silver nitrate with methods similar to those described in the legend to Table IV. The samples of cholesteryl acetate, 22-dehydrocholesteryl acetate, and 7,22-bisdehydrocholesteryl acetate obtained from this column were separately rechromatographed on silicic acid-silver nitrate columns, and the resulting acetates each showed a single spot of the appropriate \(R_F\) (0.58, 0.48, and 0.10, respectively) when analyzed by TLC on Silica Gel H-silver nitrate; GLC analysis of each of these three acetates on the usual three columns also showed a single peak of appropriate relative retention time, as well as a single peak on co-chromatography with authentic material in each case. The amounts of these samples were determined by quantitative GLC (SE-52 column) with the following results: cholesteryl acetate, 2.8 mg; 22-dehydrocholes-
teryl acetate, 1.5 mg; and 7,22-bisdehydrocholesteryl acetate, 7.1 mg. The \(^{14}\)C contents were measured and the specific activities were found to be 8.8, 9.2, and 8.7 \(\mu\)Ci per mmole for the cholesteryl, 22-dehydrocholesteryl, and 7,22-bisdehydrocholesteryl acetates, respectively; rechromatography did not alter these values. The agreement of these specific activities with that of the original 26-\(^{14}\)C-cholesterol (8.9 \(\mu\)Ci per mmole) is within experimental error, which indicates that both 22-dehydrocholesterol and 7,22-bisdehydrocholesterol are formed in *T. pyriformis* from cholesterol as a precursor.

Although the component of the mixture of acetates with \(R_F\) 0.15 that was suspected to be 7-dehydrocholesterol acetate (authentic sample, \(R_F\) 0.15) was not present in sufficient quantity to permit an accurate determination of its specific activity, the fact that \(^{14}\)C was found in this region of the chromatogram is qualitatively consistent with the tentative formulation of 7-dehydrocholesterol as a metabolic product of cholesterol.

Each of the three radioactive ester samples of known specific activity was subjected to periodate-permanganate oxidation with the subsequent addition of carrier isovaleric acid and preparation of \(p\)-bromophenacyl isovalerate by the methods described in “Experimental Procedure.” The resulting three samples of the \(p\)-bromophenacyl derivative were each recrystallized five times from ethanol-water (1:1, v/v), and the specific activities were determined for each crop of crystals; the results are given in Table VII. The observation that the \(^{14}\)C content of the derivative from the control experiment with the sample of 26-\(^{14}\)C-cholesterol acetate was small, and that it steadily decreased with further purification, clearly indicates that the saturated side chain in this ester is not converted to isovaleric acid by periodate-permanganate oxidation. The observation of constant specific activities in the experiments with 22-dehydrocholesteryl acetate and 7,22-bisdehydrocholesteryl acetate (Table VII) proves that the unsaturated side chain in each case is the source of the isovaleric acid produced by periodate-permanganate oxidation.

If the oxidations of the samples of 22-dehydrocholesteryl acetate and 7,22-bisdehydrocholesteryl acetate had each given isovaleric acid in 100\% yield, the specific activities of the resulting samples of \(p\)-bromophenacyl isovalerate would have been 222 and 219 dpm per mg, respectively; the observed average values of 67 and 151 dpm per mg (Table VII) imply isovaleric acid yields of 30 and 69\% from these two oxidations. The

**Table VI**

**Specific activities of samples of recrystallized \(p\)-bromophenacyl isovalerate obtained from isotopic dilution experiments involving periodate-permanganate oxidation of three 26-\(^{14}\)C-labeled sterol acetates isolated from *T. pyriformis***

<table>
<thead>
<tr>
<th>Source of sample</th>
<th>Specific activity</th>
<th>First recrystallization</th>
<th>Second recrystallization</th>
<th>Third recrystallization</th>
<th>Fourth recrystallization</th>
<th>Fifth recrystallization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesteryl acetate</td>
<td>10</td>
<td>9</td>
<td>7</td>
<td>5</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>22-Dehydrocholesteryl acetate</td>
<td>62</td>
<td>69</td>
<td>65</td>
<td>67</td>
<td>67</td>
<td></td>
</tr>
<tr>
<td>7,22-Bisdehydrocholesteryl acetate</td>
<td>115</td>
<td>140</td>
<td>150</td>
<td>155</td>
<td>148</td>
<td></td>
</tr>
</tbody>
</table>

Downloaded from http://www.jbc.org/ by guest on October 14, 2017
yields of isovaleric acid from experiments with unlabeled samples of these two  \( \Delta^{22} \)-acetates were consistently found by quantitative GLC analysis (DG 1 column) to be in the 70 to 80% range. Thus, the specific activity of the isovaleric acid from the oxidation of the radioactive 7,22-bisdehydrocholesterol acetate has the expected magnitude; the reasons for the observation of a lower value in the experiment with radioactive 22-dehydrocholesterol acetate are obscure.

**DISCUSSION**

These investigations show that cholesterol is metabolized by *T. pyriformis* with the introduction of \( \Delta^7 \) and \( \Delta^{22} \) double bonds, and that the addition of cholesterol to the growth medium results in the regulation of the biosynthesis by the protozoan of the squalene cyclization product, tetrahymanol. Although added cholesterol plays an important physiological role in this organism, there is no unequivocal evidence for the occurrence of sterols in cells grown in a sterol-free medium (9, 15, 43, 44). In experiments described elsewhere (39), the unsaponifiable fractions obtained from *T. pyriformis* incubated in culture fluid containing added \(^{14} \text{C}-\text{acetic acid, mevalonic acid, or squalene, but lacking added sterols, were examined by TLC; in each case, an autoradiogram revealed the presence of heavily labeled tetrahymanol. Moreover, the specific activity of the isovaleric acid from experiments with unlabeled samples of these two \( \Delta^{22} \)-acetates were consistently found by quantitative GLC analysis (DG 1 column) to be in the 70 to 80% range. Thus, the specific activity of the isovaleric acid from the oxidation of the radioactive 7,22-bisdehydrocholesterol acetate has the expected magnitude; the reasons for the observation of a lower value in the experiment with radioactive 22-dehydrocholesterol acetate are obscure.**

**REFERENCES**

6. **CONNER, R. L., Science, 126, 698 (1957).**
25. **CONNER, R. L., AND CLINE, B. S., J. Protozool., 11, 468 (1964).**


The Conversion of Cholesterol to $\Delta^{5,7,22}$-Cholestatrien-3$\beta$-ol by Tetrahymena pyriformis

R. L. Conner, F. B. Mallory, J. R. Landrey and C. W. L. Iyengar

J. Biol. Chem. 1969, 244:2325-2333.

Access the most updated version of this article at http://www.jbc.org/content/244/9/2325

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/244/9/2325.full.html#ref-list-1