Conversion of Zymosterol-C\(^{14}\) and Zymostenol-H\(^{3}\) to Cholesterol by Rat Liver Homogenates and Intact Rats* 

GEORGE J. SCHROEPFER, JR.†

From the Departments of Physiological Chemistry and Medicine, Medical School, University of Minnesota, Minneapolis 14, Minnesota

(REceived for publication, December 29, 1960)

The conversion of lanosterol to cholesterol requires the removal of three methyl groups, reduction of the \(\Delta^{24}\) double bond, and "shift" of the nuclear double bond from position \(\Delta^{5}\) to position \(\Delta^{4}\). The mechanisms by which these changes occur and the sequence in which they occur are poorly understood at this time.

The finding that Waring Blender homogenates of rat liver are capable of efficiently converting zymosterol (\(\Delta^{5,24}\)-cholestadienol), but not zymostenol (\(\Delta^{5}\)-cholestenol), to cholesterol (2) suggests that reduction of the \(\Delta^{24}\) double bond of cholesterol precursors is probably a very late step in the formation of cholesterol (2-4). This view is strengthened by the isolation of desmosterol (\(\Delta^{5},24\)-cholesterol) from chick embryos and rat liver and skin (4-6) and the demonstration of the conversion of this sterol to cholesterol in the rat (5). It has also been suggested, however, that reduction of the \(\Delta^{24}\) double bond may occur relatively early in the reactions leading to the formation of cholesterol (7, 8).

The present study concerns some further observations on the convertibility of zymosterol and zymostenol to cholesterol in intact rats and rat liver homogenates. In both Bucher and Waring Blender homogenates of rat liver, zymostenol was found to be more readily convertible to cholesterol than was zymosterol, under the conditions studied. Observations made in this study suggest that zymosterol or other \(\Delta^{4}\)-sterols are not significant intermediates in the conversion of zymostenol to cholesterol.

The effect of triparanol, an inhibitor of cholesterol synthesis (9), on the conversions of zymosterol and zymostenol to cholesterol by the rat has also been studied.

**EXPERIMENTAL PROCEDURE**

**Animals**—Female rats of the Sprague-Dawley strain, weighing approximately 200 g, were used in this study. They were fed Purina laboratory chow. Triparanol, a gift from Dr. Thomas Blohm of the Wm. S. Merrell Company, was administered by subcutaneous injection in a daily dose of 5 mg, suspended in 0.1 ml of olive oil, for 11 days.

**Administration of Sterols by Intraperitoneal Injection**—In experiments in vivo the sterols were injected into the portal vein under ether anesthesia. The material to be injected was administered through a 25- or 27-gauge needle in the form of an alcohol-0.9% NaCl solution suspension (0.1 ml of alcohol, 0.2 ml of 0.9% NaCl solution). After removal of the needle, bleeding from the injection site was controlled by pressure with a cotton pledge moistened with a solution of thrombin. The abdominal wound was closed and the rat was placed in a cage with access to food and water. The animals were killed 20 hours later with ether. The liver sterols were isolated, after digestion in boiling 15% alcoholic KOH (5 ml per g of liver) for 3 hours, by adding an equal volume of water and extracting three to five times with 2 volumes of petroleum ether (b.p. 30-60°C).

**Preparation of Cell-free Homogenates of Liver**—Bucher homogenates were prepared as previously described (10). Waring Blender homogenates were prepared by homogenization in a Waring Blender for 30 seconds at 0°C. The homogenization medium in both cases was potassium phosphate buffer (0.1 M, pH 7.4, 2.5 ml per g of liver). Nicotinamide, DPN, and magnesium salts were not added. Whole cells and nuclei were removed by centrifugation (10).

Incubations were carried out in oxygen at 37°C in stopped vials for 1 hour in a Dubnoff metabolic shaker. The substrates were added in propylene glycol (25 or 50 μl/2.5 ml of homogenate). The sterols were isolated from the homogenates as previously described (10).

**Isolation and Purification of Radioactive Cholesterol**—The sterols were separated on silicic acid-Super-Cel columns with benzene as the eluting solvent (11). In most cases the columns were 1.2 cm in diameter and 100 cm in length. Fractions, 4 ml in volume, were collected. Purified (12) carrier cholesterol was added before chromatography of the products of the incubations.

After chromatography, the amount of radioactivity and cholesterol in each fraction tube were measured. The cholesterol was recovered from the counting fluid by isooxalation as the digi- nide and subsequent cleavage with pyridine-ether (10). The radioactivity associated chromatographically with cholesterol was further identified as cholesterol by passage through the dibromide (10), after the addition of purified (12) carrier cholesterol. The specific activity of the radioactive cholesterol was determined before and after passage through the dibromide.

The laborious chromatographic method was used because of the effectiveness of this procedure in the purification of cholesterol (10) and because it offered the possibility of detection of potential intermediates in the reactions studied.

**Measurement of Radioactivity and Sterols**—Radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer as previously described (10). Corrections for quenching...
were made with the use of an internal standard. Cholesterol and desmosterol were measured colorimetrically (10, 13).

Isolation of Radioactive Yeast Sterols—Fleischmann's baker's yeast was grown anaerobically for 60 hours according to the procedure of Klein (14) and subsequently oxygenated for 1.5 hours as outlined by Johnston and Bloch (2). The cells were collected by centrifugation at 0° and washed once with cold distilled water. Approximately 200 mg of the washed cells were incubated in 10 ml of medium of the following composition: potassium phosphate buffer (0.1 M, pH 7.4), MgCl$_2$ (0.004 M), DPN (0.0008 M), nicotinamide (0.03 M), and glucose (0.055 M). To this mixture were added 250 µg of sodium acetate-1-C$^{14}$ (specific activity, 13.5 c.p.m. per minute) purchased from Orlando Research, Inc., Orlando, Florida, in a volume of 25 µl. The incubation was carried out in an open 50-ml Erlenmeyer flask on a Thomas rotating table at room temperature for 34 hours. Distilled water was added periodically to maintain constant volume.

At the end of the incubation, the cells were collected by centrifugation and digested in boiling 15% alcoholic KOH (5 ml) for 3 hours. Water (5 ml) was added and the resulting mixture was extracted three times with 2 volumes of petroleum ether (b. p. 30-60°). The solvent was evaporated with a stream of nitrogen. The residue was applied to a silicic acid-Super-Cel column as described above, and the sterols were eluted with benzene. Aliquots were taken from selected tubes for measurement of radioactivity. The resulting chromatogram is shown in Fig. 1.

With the exception of the radioactivity in tubes 96 through 105, the chemical nature of the radioactive peaks was not studied. The chromatographic behavior of the first, third, and fourth radioactive peaks is very similar to that of squalene, lanosterol, and cholesterol, respectively. The radioactive compound corresponding to the second peak eluted from the column did not serve as an efficient precursor of cholesterol after intraportal injection into a rat.

Purification and Identity of Zymosterol-C$^{14}$—The contents of tubes 96 through 105 were combined, and the benzene was evaporated under nitrogen. The residue was rechromatographed on a 1.0 × 100-cm column. A single peak of radioactivity was observed. The contents of the tubes corresponding to the center of this peak were combined for use in the biological experiments.

Nonradioactive zymosterol isolated on a large scale from yeast, with essentially the same techniques as in the radioactive experiment, gave a melting point of 106-109° (literature, 108-109° (15)). The infrared spectrum$^1$ was identical with one of authentic zymosterol.$^2$

Evidence for the presence of a Δ$^{24}$ double bond was obtained by ozonolysis of the zymosterol-C$^{14}$. Of the theoretical amount of acetone-2, 4-dinitrophenylhydrazone C$^{14}$, 54% was recovered. (This yield is not corrected for any losses during the ozonolysis due to incomplete reaction or allylic rearrangement in the course of the reaction.)

When the zymosterol-C$^{14}$ was chromatographed with non-radioactive zymosterol, the radioactivity ran parallel to the carrier (with specific activities of 547, 560, 521, and 537 c.p.m. per mg on the contents of four successive fraction tubes). When the contents of tubes 81 through 90 were chromatographed with non-radioactive zymosterol, a clear separation of the radioactivity and zymosterol was observed.

In subsequent studies in vitro and in vivo with the zymosterol-C$^{14}$, several radioactive compounds were recovered which were more polar chromatographically than cholesterol. That these compounds were not formed from zymosterol during the digestion of the tissue preparation with the alcoholic KOH or during the chromatography is suggested by the following experiment. The liver of a normal rat was digested for 2 hours in boiling 15% alcoholic KOH. After cooling, zymosterol-C$^{14}$ was added to the mixture and the contents of the flask were heated under reflux for 3 hours. Recovery and chromatography of the sterols were carried out as described above. A single peak of radioactivity, clearly separated from the cholesterol of the liver, was recovered.

Preparation of Zymostenol-24, 25-$^{3}$—Zymosterol (1.5 mg, m.p. 106-109°) in purified (18) ethyl acetate (12 ml) was hydrogenated in the presence of 100 mc of tritium gas at room temperature over a Raney nickel catalyst (19). The reduction was carried out at atmospheric pressure for 8 hours. The solution was filtered to remove the catalyst, and the ethyl acetate was evaporated under nitrogen. To remove any readily exchangeable tritium, 5 ml of water and 5 ml of ethanol were added to the sterol and the mixture was allowed to stand at room temperature for 3 hours. The mixture was then heated gently on a steam bath for 15 minutes under nitrogen and, after cooling to room temperature, it was extracted three times with 20-ml portions of petroleum ether (b. p. 30-60°). The combined petroleum ether solutions were washed three times with 10-ml portions of water and evaporated to dryness under nitrogen. The yield was about 90%, based on colorimetric assay of the sterol. The changes with time in optical density at 630 mµ after treatment of the tritiated compound with the Liebermann-Burchard color reagent (20) were the same as those observed with an authentic sample of zymostenol (m.p. 120-130°). Zym-
Conversion of Zymosterol-C\textsuperscript{14} and Zymostenol-H\textsuperscript{3} to Cholesterol

**FIG. 2.** Chromatogram illustrating the separation of zymostenol-H\textsuperscript{3} and nonradioactive cholesterol. X-X-X, cholesterol measured colorimetrically; O-O-O, radioactivity.

**TABLE I**

Incorporation of zymosterol-C\textsuperscript{14} and zymostenol-H\textsuperscript{3} into cholesterol by rat liver homogenates

<table>
<thead>
<tr>
<th>Homogenate preparation*</th>
<th>Volume ml</th>
<th>Substrate</th>
<th>Amount of substrate added</th>
<th>Conversion to cholesterol\textsuperscript{b}</th>
<th>Specific activity of recovered cholesterol after addition of carrier\textsuperscript{c} before purification</th>
<th>Specific activity of recovered cholesterol after addition of carrier\textsuperscript{c} after purification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>c.p.m./mg</td>
<td>c.p.m./mg</td>
</tr>
<tr>
<td>Bucher 1</td>
<td>7.5</td>
<td>Zymosterol</td>
<td>&lt;1\textsuperscript{d}</td>
<td>30</td>
<td>23.3</td>
<td>33.4</td>
</tr>
<tr>
<td>Bucher 2</td>
<td>12.5</td>
<td>Zymosterol</td>
<td>&lt;1\textsuperscript{d}</td>
<td>31</td>
<td>22.2</td>
<td>18.9</td>
</tr>
<tr>
<td>Bucher 2</td>
<td>5.0</td>
<td>Zymostenol</td>
<td>3.0</td>
<td>51</td>
<td>143</td>
<td>145</td>
</tr>
<tr>
<td>Bucher 3</td>
<td>12.5</td>
<td>Zymosterol</td>
<td>7.5</td>
<td>26</td>
<td>11.8</td>
<td>12.5</td>
</tr>
<tr>
<td>Bucher 3</td>
<td>12.5</td>
<td>Zymostenol</td>
<td>7.5</td>
<td>41</td>
<td>231</td>
<td>225</td>
</tr>
<tr>
<td>Bucher 4</td>
<td>15.0</td>
<td>Zymosterol</td>
<td>&lt;1\textsuperscript{d}</td>
<td>27</td>
<td>39.3</td>
<td>36.5</td>
</tr>
<tr>
<td>Bucher 4</td>
<td>15.0</td>
<td>Zymostenol</td>
<td>9.0</td>
<td>41</td>
<td>392</td>
<td>409</td>
</tr>
<tr>
<td>Waring 1</td>
<td>10.0</td>
<td>Zymosterol</td>
<td>6.0</td>
<td>12</td>
<td>7.07</td>
<td>6.25</td>
</tr>
<tr>
<td>Waring 1</td>
<td>10.0</td>
<td>Zymostenol</td>
<td>6.0</td>
<td>25</td>
<td>161</td>
<td>150</td>
</tr>
<tr>
<td>Waring 2</td>
<td>5.0</td>
<td>Zymostenol</td>
<td>3.0</td>
<td>19</td>
<td>48.8</td>
<td>53.3</td>
</tr>
</tbody>
</table>

* Zymosterol and zymostenol were incubated together in the third and fourth Bucher homogenates and in the first Waring Blender homogenate.

b Percentage of the incubated radioactivity which was recovered in cholesterol after chromatography.

c After dilution with carrier cholesterol, the specific activity of the radioactive cholesterol isolated by chromatography was measured before and after purification through the dibromide.

d The specific activity of the zymosterol-C\textsuperscript{14} could not be accurately measured because of the very small amount of the radioactive compound. A minimal specific activity of 6000 c.p.m. per \(\mu\)g was estimated on the basis of the total amount of radioactivity isolated in the zymosterol-C\textsuperscript{14} and the estimated zymosterol content based upon analysis of a larger amount of the same yeast preparation from which the radioactive compound was isolated.

RESULTS

The results of the incubations of zymosterol and zymostenol with rat liver homogenates are shown in Table I. In all incubations, including those in which both substrates were incubated together with the same homogenate, a higher percentage of radioactivity derived from zymostenol was associated with cholesterol at the end of 1 hour than from zymosterol. In the incubations of the tritium-labeled zymostenol with the rat liver homogenates, most (over 85\%) of the added radioactivity was recovered in petroleum ether-soluble compounds at the end of 1 hour. The chromatogram of the sterols obtained after the incubation of zymostenol with the second Bucher homogenate is shown in Fig. 3.

The conversions of zymosterol and zymostenol to cholesterol in normal and triparanol-treated rats are shown in Table II. The chromatogram of the liver sterols of a triparanol-treated rat given zymosterol intraperitoneally is shown in Fig. 4. For comparison, the chromatogram of the liver sterols of a normal rat treated by injection of zymosterol is shown in Fig. 5. The chromatogram of the liver sterols of a triparanol-treated rat given zymostenol intraperitoneally is shown in Fig. 6.

**FIG. 3.** Chromatogram of the sterols obtained from a Bucher homogenate of rat liver which was incubated with zymostenol-H\textsuperscript{3} for 1 hour. Symbols are as in Fig. 2.
**TABLE II**

| Animal | Substrate | Amount of substrate injected | Injected radioactivity recovered from liver | Liver radioactivity in cholesterol | Specific activity of recovered cholesterol after addition of carrier
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mg</td>
<td>%</td>
<td>%</td>
<td>c.p.m./mg</td>
</tr>
<tr>
<td>Normal</td>
<td>Zymosterol</td>
<td>10</td>
<td>4.3</td>
<td>2</td>
<td>34.8</td>
</tr>
<tr>
<td>Treated</td>
<td>Zymosterol</td>
<td>10</td>
<td>3.4</td>
<td>2</td>
<td>34.8</td>
</tr>
<tr>
<td>Normal</td>
<td>Zymostenol</td>
<td>42</td>
<td>13.6</td>
<td>26</td>
<td>316</td>
</tr>
<tr>
<td>Treated</td>
<td>Zymostenol</td>
<td>42</td>
<td>14.4</td>
<td>27</td>
<td>302</td>
</tr>
</tbody>
</table>

* Twenty hours after the intraportal injection of the radioactive substrates.

* Percentage of radioactivity recovered from liver which was associated with cholesterol after chromatography.

* Same as Table I.

* Same as Table I.

The conversions of zymostenol and zymosterol to cholesterol have been shown to occur readily in both Bucher and Waring Blender homogenates of rat liver. Despite the shorter incubation time and the absence of added DPN, nicotinamide, and magnesium salts, the conversions observed in these experiments are roughly comparable in magnitude to those reported by Johnston and Bloch (2) in their carrier-free incubations in Bucher homogenates. In several experiments with Bucher homogenates, the extent of conversion of zymostenol to cholesterol was significantly greater than reported previously. No clear explanation can be offered for the different results obtained in this study and those reported previously (2) regarding the conversion of zymostenol to cholesterol in Waring Blender homogenates of rat liver, because different experimental techniques were used in the two studies.

**FIG. 4.** Chromatogram of the liver sterols of a triparanol-treated rat after the administration of zymosterol-\(C^{14}\). Symbols are as in Fig. 4.

**FIG. 5.** Chromatogram of the liver sterols of a normal rat after the administration of zymosterol-\(C^{14}\). Symbols are as in Fig. 4.

**FIG. 6.** Chromatogram of the liver sterols of a triparanol-treated rat after the administration of zymostenol-H\(^3\). Symbols are the same as in Fig. 4. The second radioactive peak is similar chromatographically to the substrate. This fraction was not studied further.
The possibility that zymosterol or other \( \Delta^4 \) sterols are significant intermediates in the conversion of zymostenol to cholesterol appears to be precluded on the basis of two observations made in this study. First, the conversion of zymosterol to cholesterol was greater than the conversion of zymosterol to cholesterol in the same homogenate preparation. Second, in the conversion of zymosterol to cholesterol, most of the radioactivity is retained in petroleum ether-soluble compounds. The tritium in the zymostenol was presumably located on carbon atoms 24 and 25. If, in the conversion to cholesterol, a \( \Delta^4 \) double bond were introduced, a considerable portion of the tritium would be removed. The recovery of 40 to 50% of the added radioactivity in cholesterol and a much greater percentage in petroleum ether-soluble compounds precludes such a possibility.

The finding that added nicotinamide, pyridine nucleotides, or magnesium salts are not needed to obtain effective conversion of zymostenol or zymosterol to cholesterol in liver homogenates represents an extension of previous findings reported by Olson et al. (22) and Frantz et al. (10, 23). The former workers found that DPN addition did not stimulate the conversion of lanosterol to cholesterol in Bucher homogenates (which contained added MgCl\(_2\) and nicotinamide). They also found that a TPNH-generating system could partially replace the soluble fraction of the liver homogenate in this conversion. Frantz et al. found that the addition of the combination of DPN, nicotinamide, and MgCl\(_2\) was not necessary for the conversion of \( \Delta^4 \) cholesterol to cholesterol. Under the same conditions, 4a-methyl-\( \Delta^4 \)-cholesterol was not converted to cholesterol. Efficient conversion of this sterol to cholesterol was observed in a homogenate when the combination of cofactors was added. These findings suggest that a pyridine nucleotide (probably TPNH) and Mg\(^{++}\) may be required cofactors in the removal of the 4, 4', and 14 methyl groups of cholesterol precursors. Precise delineation of the cofactor requirements for these reactions will await studies that use the combination of DPN, nicotinamide, diphosphopyridine nucleotide, or magnesium salts.

The accumulation of significant quantities of a sterol other than cholesterol has been noted in the tissues of triparanol-treated rats. The identification of this sterol as desmosterol was made by Avigan et al. (24) and confirmed by Frantz et al. (13). The conversion of lathosterol to cholesterol by rat liver homogenates is unaffected by pretreatment of the animals with triparanol (13). In the present study little or no conversion of zymosterol to cholesterol was observed in a rat treated with this drug. Most of the radioactivity recovered from the liver of this animal was associated chromatographically with desmosterol. As several other sterols behave very similarly to desmosterol in the chromatographic system used, sufficient evidence is not available to equate the radioactivity with desmosterol.

Both normal and triparanol-treated rats converted zymostenol to cholesterol in good yield. In the normal rat a higher conversion was observed.

The observations made in this study, along with those cited above, are compatible with the hypothesis that triparanol inhibits the reduction of the \( \Delta^5 \) double bond of cholesterol precursors.

**Summary**

1. The incorporation of zymosterol-\( \Delta^4 \) and zymostenol-24,25-\( \Delta^4 \) into cholesterol by both Bucher and Waring Blender homogenates of rat liver has been demonstrated.

2. These conversions did not require the presence of added nicotinamide, diphosphopyridine nucleotide, or magnesium salts.

3. On the basis of observations made in this study it is suggested that sterols with a \( \Delta^4 \) double bond are not significant intermediates in the conversion of zymostenol to cholesterol.

4. Zymostenol, but not zymosterol, was efficiently incorporated into the cholesterol of liver after intraperitoneal administration to rats treated with triparanol.

**Acknowledgments**—This work was carried out in the laboratory of Dr. Ivan Frantz, Jr. The author gratefully acknowledges his advice and encouragement. The ozonolysis experiment was performed by Dr. Frantz. Thanks are also due to Mr. T. Scallen and Mr. J. Emond for assistance in the isolation of the zymosterol.

**References**

Conversion of Zymosterol-C\textsuperscript{14} and Zymostenol-H\textsuperscript{3} to Cholesterol by Rat Liver Homogenates and Intact Rats

George J. Schroepfer, Jr.


Access the most updated version of this article at http://www.jbc.org/content/236/6/1668.citation

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/236/6/1668.citation.full.html#ref-list-1