Studies of Cholesterol Biosynthesis

I. THE IDENTIFICATION OF DESMOSTEROL IN SERUM AND TISSUES OF ANIMALS AND MAN TREATED WITH MER-29

JOEL AVIGAN, DANIEL STEINBERG, HUGH E. VROMAN, MALCOLM J. THOMPSON, AND ERICH MOSETTIG

From the Section on Steroids, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland

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The compound (1-[p-(β-diethylaminoethoxy)-phenyl]-1-(p-tolyl)-2-(p-chlorophenyl)ethanol), known by its trade name as MER-29, has been shown by Blohm et al. to cause a depression in serum and tissue cholesterol and to inhibit cholesterol biosynthesis in animals (1, 2). These authors found that the incorporation of 1-C14-acetate into the total nonsaponifiable lipid fraction of rat liver and into the total digitonin-precipitable sterol fraction was not affected by the drug. However, these authors found that on purification of this fraction through the dibromide, most of the radioactivity proved to be in sterols other than cholesterol.

The present studies were undertaken in an attempt to elucidate the mechanism of action of MER-29 on cholesterol biosynthesis. The experiments reported here establish desmosterol (24-dehydrocholesterol) as a major sterol component in the serum, in the tissues of animals treated with MER-29. A calorimetric method for the determination of desmosterol in the serum and tissue cholesterol and to inhibit cholesterol biosynthesis in animals (1, 2). These authors found that the incorporation of 1-C14-acetate into the total nonsaponifiable lipid fraction of rat liver and into the total digitonin-precipitable sterol fraction was not affected by the drug. However, these authors found that on purification of this fraction through the dibromide, most of the radioactivity proved to be in sterols other than cholesterol.

The present studies were undertaken in an attempt to elucidate the mechanism of action of MER-29 on cholesterol biosynthesis. The experiments reported here establish desmosterol (24-dehydrocholesterol) as a major sterol component in the serum, in the liver, and in other tissues of animals treated with MER-29. A colorimetric method for the determination of desmosterol in the presence of cholesterol is described and tissue levels in treated animals are reported. Finally, some general observations of the effects of MER-29 on weight gain and on serum and liver lipid levels are presented. Preliminary reports of some of these results have appeared elsewhere (3).

EXPERIMENTAL PROCEDURE

Male rats of the Sprague-Dawley strain were maintained on a synthetic diet (4) to which MER-29 was added in various concentrations. The amount of the drug taken daily was estimated from the average daily consumption of the diet. For sterol analysis tissues were homogenized in 25 volumes of alcohol-acetone (1:1 volume for volume). Serum sterols were extracted with the same mixture. The digitonin-precipitable, Lieberman-Burchard-positive sterols were determined by the Sperry and Webb method (5). The concentrations of cholesterol and of desmosterol in a mixture were determined by the method described in detail below. Chromatographic separation of sterols was accomplished by a modification of the procedure of Idler and Baumann (6). The p-phenylazobenzoyl esters of the sterols were prepared by treatment with an excess of p-phenylazobenzoyl chloride in pyridine solution, subsequent precipitation of the product, and washing with a sodium bicarbonate solution and water. The esters (usually in the range of 40 to 70 mg) were chromatographed on a 40 × 1.8 cm column of silicic acid-Celite (2:1) by elution with a mixture of petroleum ether (b.p. 60–71°) and benzene (3:1 volume for volume). After development, the column was extruded and the yellow zones were mechanically separated and individually extracted. Optical rotations were determined in chloroform solutions of approximately 1% concentration. Infrared spectra were obtained with a Perkin-Elmer model 21 double beam spectrophotometer with sodium chloride prism and cells.

RESULTS

In Table I are shown the changes in body weight and changes in apparent serum cholesterol levels in small groups (3 to 6) of rats fed MER-29 at various dose levels. At dose levels of about 0.5 mg per day and higher there was definite inhibition of growth, the effect being more marked in younger rats. (Compare Experiment 2 with Experiment 3, Table I.) The depression of apparent serum cholesterol levels, however, was not attributable to the decreased food intake, as shown by Experiment 4, of Table I, in which one set of control animals was given only the same total amount of food as that consumed by the experimental animals. The effectiveness of the drug in lowering the apparent serum cholesterol level increased with dosage up to 5 mg per day but did not seem to be greater at 10 mg per day. These results are in essential agreement with those reported previously by Blohm et al. (2).

Analysis of serum and liver revealed that both the free and esterified cholesterol were depressed by the drug (Table II). There was also a reduction of phospholipids in serum but not in the liver. Triglycerides were elevated in both serum and liver.

Sterols isolated from the liver were precipitated with digitonin, the digitonides were washed with acetone-ether mixture (1:2 volume for volume) and ether and cleaved with pyridine. The sterols were then extracted with ether and recrystallized from methanol. When sterols from the livers of control animals were purified in this way, an essentially pure preparation of cholesterol was obtained (m.p. 143°, [α]D° −40°). However, when sterol from the livers of rats fed MER-29 for 2 weeks (10 mg per day) were treated in the same manner, the material isolated had a m.p. of 128–132° and [α]D° −39°. The melting point did not change.

The values shown in Tables I and II were obtained by the Sperry and Webb procedure. As discussed below, these values, because of the presence of 24-dehydrocholesterol, are intermediate between the true cholesterol level and the true total sterol level.

1 The Wm. S. Merrell Company, Cincinnati, Ohio.
TABLE I

Effect of MER-29 on rat weight and on serum total cholesterol

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>No. of days on diet</th>
<th>Approximate daily dose of MER-29 mg</th>
<th>Average initial weight g</th>
<th>Weight gain g</th>
<th>Serum total cholesterol mg/100 ml</th>
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<tbody>
<tr>
<td>1</td>
<td>50</td>
<td>0</td>
<td>148</td>
<td>251</td>
<td>71</td>
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<tr>
<td></td>
<td></td>
<td>0.1</td>
<td>148</td>
<td>271</td>
<td>01</td>
</tr>
<tr>
<td>2</td>
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<td>230</td>
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<tr>
<td></td>
<td></td>
<td>0.5</td>
<td>143</td>
<td>63</td>
<td>52</td>
</tr>
<tr>
<td>3</td>
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<td>184</td>
<td>162</td>
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<td></td>
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<td>220</td>
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<td>48</td>
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<tr>
<td>4</td>
<td>26</td>
<td>3.7</td>
<td>220</td>
<td>98</td>
<td>22</td>
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<td></td>
<td></td>
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<td>0</td>
<td>184</td>
<td>119</td>
</tr>
<tr>
<td>5</td>
<td>30</td>
<td>3.7</td>
<td>10</td>
<td>122</td>
<td>24</td>
</tr>
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</table>

* Pair fed control.

**TABLE II**

Effect of MER-29 on lipid content of rat serum and liver

<table>
<thead>
<tr>
<th></th>
<th>Serum</th>
<th>Liver</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>MER-29-fed†</td>
</tr>
<tr>
<td>Ester cholesterol</td>
<td>41</td>
<td>16</td>
</tr>
<tr>
<td>Free cholesterol</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>104</td>
<td>64</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>90</td>
<td>94</td>
</tr>
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</table>

* MER-29, 10 mg daily for 21 days.
† MER-29, 10 mg daily for 24 days.

Infrared spectra of desmosteryl benzoate (above) and of desmosteryl benzoate prepared from the sterol isolated from rat liver (below).

The authors are grateful to Dr. U. H. M. Fagerlund, who has kindly provided samples of desmosterol and its benzoate.
terol was found to be unsuitable for routine quantitative determinations, both because it is too time-consuming and because it requires relatively large samples. Scaling down of the chromatographic procedure did not yield satisfactory results. A colorimetric method for determination of cholesterol and of desmosterol in mixtures was developed. The method is based on the different absorption spectra given by the colored products formed by the two sterols in the Liebermann-Burchard reaction (Table III). Absorption was measured at 635 μm 35 minutes after development of the color, and then at 420 μm after 90 minutes. The precipitation and washing of the digitonides was carried out according to the Sperry and Webb method (5). The digitonides were cleaved with pyridine at 100° for 10 minutes, the sterols were extracted with ether, and the precipitated digitonin was centrifuged. It was essential to remove completely the digitonin, which gave a considerable blank at 420 μm. To do this, the solvent was evaporated and the residue dissolved again in dry ether. After evaporation of the ether, the Liebermann-Burchard color was developed as in the method of Sperry and Webb. The tubes were kept in the dark at 25° and light absorptions at the two wave lengths were measured as described above. Standards of pure cholesterol and desmosterol were analyzed at the beginning and end of each series of determinations. Typical analytical recoveries of the two sterols in a mixture are presented in Table IV.

The concentrations of 24-dehydrocholesterol and of cholesterol in several tissues of a rat fed MER-29 (0.1 % for 3 months) were determined by the colorimetric method (Table V). All of the tissues, including brain, contained a major sterol fraction that behaved like desmosterol. In the case of serum and liver, the new sterol revealed by the colorimetric method has been also positively identified as desmosterol. In the case of the other tissues we have not yet attempted direct isolation, but it seems reasonable to attribute the observed shifts in Liebermann-Burchard color to the presence of this same sterol.

The control samples show apparent small amounts of desmosterol. These low levels in normal tissues may only reflect the limited accuracy of the method or the presence of some other sterols related to cholesterol. Attempts to demonstrate desmosterol in normal human serum by extracting a large volume and applying the chromatographic method to the p-phenylazobenzoyl derivatives were unsuccessful.

**DISCUSSION**

The fall in true serum cholesterol level after prolonged treatment of rats at a high dosage of MER-20 (from 60 mg per 100 ml down to 6 mg per 100 ml) is striking.

Whereas there is partial replacement by desmosterol (22 mg in total sterols) in liver, lung, and skeletal muscle (2). In the light of the present findings, their results can probably be in part attributed to replacement of cholesterol by desmosterol, the latter giving a low color yield in their analytical procedure. Apparent depressions of cholesterol levels reported in some clinical studies (9) must likewise be reevaluated in view of the lower color yield given by desmosterol and the fact that desmosterol has been shown to accumulate in the serum of MER-29-treated patients (10).

Desmosterol has been found by Stokes et al. (8) to be a minor...
component of rat skin sterols. It has been also isolated by Fagerlund and Idler (11) from the barnacle. There is no evidence, however, that this sterol is present under normal conditions in mammalian tissues other than skin. The analytical methods used by us are not accurate enough to determine desmosterol in the presence of a very large excess of cholesterol and the results apparently indicating low levels of the former in control rat tissues may not be significant.

Stokes et al. (8) have demonstrated that isotopically labeled desmosterol is rapidly converted in vivo into cholesterol and have suggested the likelihood that the former represents a natural precursor of cholesterol. The accumulation under the influence of MER-29 of sizeable proportions of desmosterol in rat tissues furnishes additional evidence to this effect. Isotopic studies described in the accompanying paper (12) show that desmosterol accumulating in the livers of the drug-fed rats bears a precursor relationship to cholesterol, confirming that the drug acts by inhibiting the reduction of desmosterol.

SUMMARY

1. It has been demonstrated that treatment of rats with MER-29 (1-[p-(β-diethylaminoethoxy)-phenyl]-1-(p-tolyl)-2-(p-chlorophenyl)ethanol) leads to an accumulation of desmosterol (24-dehydrocholesterol) in the serum and tissues to the extent of 27 to 79% of the total sterols present. The identity of desmosterol was proven by several independent methods.

2. Desmosterol gives a lower color yield than cholesterol in the Liebermann-Burchard reaction, when measured at 635 μm, and a colorimetric method based on this observation has been developed for determination of desmosterol and cholesterol in mixtures of the two.

3. MER-29 causes an appreciable reduction in the level of rat serum sterols. The effect on tissue sterol concentrations, however, is generally very small.

Note Added in Proof—Since this manuscript was submitted W. M. Stokes and W. A. Fish (J. Biol. Chem., 235, 2604 (1960)) have reported the isolation of radioactive desmosterol from the livers of normal rats after injection of sodium acetate-1-C\textsuperscript{14}. By indirect methods they estimate the desmosterol concentration to be approximately 0.2 μg per g of liver.

REFERENCES

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