Cholesterol Synthesis in Rat Liver Peroxisomes

CONVERSION OF MEVALONIC ACID TO ChOLESTEROL*

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The key regulatory enzyme of cholesterol, dolichol, and isopentenyl adenosine biosynthesis, 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase) is a 97-kilodalton transmembrane glycoprotein which was believed until recently to reside exclusively in the endoplasmic reticulum of mammalian cells. However, several recent publications have shown that the enzyme in liver cells is present not only in the endoplasmic reticulum but also within peroxisomes. In an effort to clarify the role of peroxisomal HMG-CoA reductase, highly purified (95%) rat liver peroxisomes from cholestyramine-treated rats were incubated with RS-[2-14C]mevalonic acid plus cytosolic proteins and then tested for the presence of newly synthesized cholesterol. For comparison, highly purified microsomes from the same liver preparation were incubated at several protein concentrations under the same conditions. A three-step procedure was employed to resolve the newly synthesized cholesterol from the complex mixture of sterol intermediates in cholesterol biosynthesis. After termination of the reaction and addition of a [3H]cholesterol standard, the incubation products were extracted and separated by thin layer chromatography into a number of fractions. The fraction containing C-27 sterols was further resolved by reverse-phase high pressure liquid chromatography. After acetylation, this fraction was then separated by silicic acid high pressure liquid chromatography. Confirmation of the identity of newly synthesized cholesterol was obtained by recrystallization with added non-radioactive cholesteryl acetate standard. The results indicate that highly purified rat liver peroxisomes are able to convert mevalonic acid to cholesterol in the presence of cytosolic fraction in vitro. An abstract of these results has been published (Krisans, S. K., Thompson, S. L., Burrows, R., and Laub, R. J. (1986) J. Cell Biol. 103, 525 (abstr.).)

The biosynthetic pathways of cholesterol, dolichol, and ubiquinone proceed through a common regulatory step, the reduction of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) to mevalonic acid by HMG-CoA reductase. This enzyme, previously considered to be exclusively microsomal, has recently been found to be localized in rat liver peroxisomes (2, 3).

The presence of HMG-CoA reductase in peroxisomes raises intriguing questions about the role of this organelle in lipid metabolism. It is well known that rat liver peroxisomes contain enzymes for the β-oxidation of long-chain fatty acids, a major pathway of lipid metabolism previously believed to be exclusively mitochondrial (4, 5). The key enzymes for the biosynthesis of glycerolipids are highly concentrated in the peroxisomes of rat brain and liver (6). And recent studies indicate that liver peroxisomes are also involved in the degradation of cholesterol to bile acids (7-10).

The fate of mevalonic acid in rat liver peroxisomes is unknown. This present study was undertaken to determine if mevalonic acid can be converted to cholesterol in vitro by peroxisomes in the presence of cytosolic proteins. Since microsomes are involved in cholesterol synthesis and are the only minor contaminant found in the purified peroxisomal samples, a comparison of microsomal cholesterol synthesis activity with that of the peroxisomes is presented.

EXPERIMENTAL PROCEDURES

Animals—Male Sprague-Dawley rats (150-200 g) were kept on a 12-h light-dark cycle for at least 2 weeks prior to each experiment. Standard lab chow was given ad libitum supplemented with 5% cholestyramine (w/w) for at least 4 days prior to killing. All rats were fasted for 12 and killed by decapitation 6 h into their dark cycle.

Preparation of Rat Liver Peroxisomes—Liver homogenates were first fractionated by differential centrifugation (11) to obtain a peroxisome-enriched fraction, a microsomal fraction, and a cytosolic fraction. All differential centrifugation fractions and subsequent dilutions were made in 0.25 M sucrose (1 mM Tris-HCl, pH 7.5, 5 mM EGTA, and 0.1% ethanol to protect catalase activity). The peroxisome-enriched fraction was then subjected to equilibrium density centrifugation in a TV-850 vertical rotor (40,000 rpm, 50 min) on a scroll-rotor with a 100,000 g gradient (8-10, 15). A total of 20-25 fractions were collected from the bottom of the tube with a two-way needle.

Preparation of Rat Liver Microsomes and the Cytosolic Fraction—Microsomes were prepared in two different ways. Rat liver homogenates were fractionated by differential centrifugation according to the method of de Duve et al. (12), resulting in a microsomal and a cytosolic fraction. In some experiments the microsomal fraction was further fractionated on a linear Nycodenz gradient (10-60%, w/v) which was centrifuged as described for the preparation of peroxisomes. The purity of the microsomal preparations as determined by marker enzyme analysis was 92-95%.

Assay of Marker Enzymes—All fractions were analyzed for marker enzyme activity and protein. The activities of catalase (a peroxisomal marker) and cytochrome oxidase (a mitochondrial marker) were measured according to Leighton et al. (11), and Lazarow and de Duve (13), except that a molar absorptivity of 19 mM cm⁻¹ for cytochrome c was used (14). Esterase (a microsomal marker) was measured according to Beaufay et al. (15). Acid phosphatase (a lysosomal marker) was measured according to Bergmeyer et al. (16). Protein...
Cholesterol Synthesis in Rat Liver Peroxisomes

was determined by the Lowry method (17), using bovine serum albumin as a standard. Since Nycodenz interferes with the determination of protein, aliquots of the gradient samples were first precipitated in 10% trichloroacetic acid.

**Cholesterol Synthesis Assay**—The cholesterol synthesis assay was adapted from Slakey et al. (18). Freshly isolated peroxisomal protein (0.8 mg) was incubated with cytosolic protein (1.0 mg) in a reaction mixture which contained the following: 55 mM Tris-HCl, 1 mM NADP, 5 mM glucose-6-phosphate, 1.2 units of glucose-6-phosphate dehydrogenase, 10 mM ATP, 10 mM MgCl₂, 1 mM NAD, 30 mM niacinamide, 5 mM glutathione, 5 mM potassium fluoride, 0.5% bovine serum albumin, and 2 μCi of DS-[2-¹⁴C]mevalonolactone (specific activity of 1.0 × 10⁶ dpm/mmol) in a final volume of 2 ml, pH 7.5. The samples were incubated at 37 °C in a shaking waterbath for 4 h under aerobic conditions. The reaction was terminated with 30 μl of 6 N KOH. An internal standard of [⁴H]cholesterol was then added for calculation of product recoveries.

Microsomal samples (0.08–0.8 mg) were incubated in an identical manner.

**Product Extraction**—After the addition of the [⁴H]cholesterol internal standard, the organic products were extracted with ether, evaporated under nitrogen, and hydrolyzed in 5% methanolic potassium hydroxide at room temperature overnight. After the addition of water, the organic compounds were re-extracted with ether, evaporated under nitrogen, and redisolved in methanol.

**Separation of Products**—The products were separated by three different chromatographic procedures based on the protocol of Hansbury and Scallen (19) (Fig. 1). The C-27 sterol fraction was obtained after thin layer chromatography using Silica Gel G plates and a hexane/ethyl acetate solvent system (70:30, v/v). The products were then further separated by reverse-phase high pressure liquid chromatography (HPLC). This step is capable of resolving C-27 sterols into mono-, di-, and triunsaturated compounds. Samples were injected on a Spherisorb ODS column (250 × 4 mm, 5-μm particles) and eluted with isopropyl alcohol/water (70:30, v/v) at 25 °C. Fractions containing the internal standard were pooled and acetylated. The sample was then injected onto a Lichrospher SI 100/2 column (250 × 4 mm, 5-μm particles) and eluted with isooctane/cyclohexane/toluene (8:2:9, v/v/v). Aliquots of the fractions were counted by a Beckman LS-3801 scintillation counter.

Final confirmation of this product as cholesteryl acetate was obtained by recrystallization with added non-radioactive cholesteryl acetate standard.

**Materials**—RS-[₂⁻¹⁴C]Mevalonolactone and [1,2-³H]cholesterol were purchased from Du Pont-New England Nuclear. Nycodenz was purchased from Accurate Chemical and Scientific Corp (Westbury, NY). Silica Gel G plates were obtained from Analtech (Newark, DE). All solvents (HPLC grade) were from Fisher. Other chemicals were from Sigma.

**RESULTS**

**Characterization of Purified Peroxisomes**—Cholesterylamine-treated animals, killed 6 h into their dark cycle, were employed. Under these conditions, the peroxisomes are responsible for 20–30% of total liver HMG-CoA reductase activity (3). Peroxisomes were prepared by equilibrium density centrifugation on a steep linear Nycodenz gradient. The purity of the peroxisomes was determined by the measurement of specific marker enzymes as previously described (9). The activities of acid phosphatase (a lysosomal marker) and cytochrome oxidase (a mitochondrial marker) were below the level of detection. The only measurable contaminant was esterase (a microsomal marker for both smooth and rough endoplasmic reticulum). Based on this biochemical measurements which are supported by morphological data, the peroxisomal fractions used in this study were between 90–95% pure.

**Separation of Cholesterol from Other Sterol Intermediates**—A high-resolution, three-step chromatographic procedure as described by Hansbury and Scallen (19) was employed for the separation of ¹⁴C-sterol intermediates. This rigorous procedure as demonstrated by the above authors is clearly capable of separating the complex mixtures of sterol intermediates in cholesterol biosynthesis. The first step involved the separation of the C-27 sterol fraction from the incubation extracts by thin layer chromatography. Two peaks of ¹⁴C radioactivity were obtained, one co-migrating with the [⁴H]cholesterol standard and the other with the solvent front, as shown in Fig. 2. This pattern was similar with both peroxisomal and microsomal samples. Next, the fraction containing the [⁴H] cholesterol standard (C-27 sterol fraction) was subjected to

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**TLC**

- 5,7,24 - Cholestatrienol: 0.38
- 5,24 - Cholestadienol: 0.49
- 8,24 - Cholestadienol: 0.50
- 5,7 - Cholestadienol: 0.54

**Reverse-Phase HPLC**

- 8(14) - Cholesterol: 0.69
- 8(9) - Cholesterol: 0.72
- 7 - Cholesterol: 0.72

**Silicic Acid HPLC**

- 5 - Cholesteryl Ac: 1.22
- 8(14) - Cholesteryl Ac: 1.36
- 8(9) - Cholesteryl Ac: 1.46
- 7 - Cholesteryl Ac: 1.55

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**Fig. 1.** Part of a diagram of a three-step chromatographic procedure illustrating the separation of sterol intermediates in cholesterol synthesis taken from Hansbury and Scallen (19). The C-27 sterol fraction was obtained after thin layer chromatography using Silica Gel G plates. The C-27 sterol fraction is then further separated into mono-, di-, and triunsaturated compounds by reverse-phase HPLC. The products are then acetylated and separated by silicic acid HPLC in order to resolve isomeric sterols. Relative retention times were based upon the retention time of 4,4,14α-trimethyl-Δ⁵-cholestene and 4,4,14α-trimethyl-Δ⁷-cholesteryl acetate.
Cholesterol Synthesis in Rat Liver Peroxisomes

There are two peaks of 14C radioactivity in both peroxisomal and microsomal samples, one co-migrating with the [3H]cholesterol standard and the other migrating with the solvent front. The first peak was further resolved on reverse-phase HPLC followed by silicic acid HPLC, resulting in two distinct peaks of 14C radioactivity, one co-migrating with the [3H]cholestenyl acetate standard. The second peak of 14C radioactivity was not further investigated, but may be Δ7-cholestenyl acetate as described by Hansbury and Scallen (19). Again, the radioactivity distribution pattern was similar between peroxisomal and microsomal samples. In contrast to reverse-phase HPLC, which does not resolve isomeric sterols with one carbon-carbon double bond, silicic acid HPLC of the sterol acetates successfully separates these compounds (19).

Confirmation of the Identity of Cholestenyl Acetate—In order to unequivocally identify the peak of 14C radioactivity co-migrating with the [3H]cholestenyl acetate standard after reverse-phase HPLC and silicic acid HPLC, we carried out four separate experiments, each experiment representing a fresh
of cholesterol from mevalonic acid (21). Also, peroxisomal and microsomal samples plus cystosol incubated without ATP and NADP showed no conversion.

**Microsomal Contribution to Cholesterol Synthesis in Peroxisomal Fractions**—Since the purified peroxisomal fractions used in this study contained 5–10% microsomal contamination, we wanted to verify that this microsomal contribution was not responsible for the rate of synthesis observed in the peroxisomal fractions. Microsomal protein (0.08 mg) equivalent to the maximum found in any of the peroxisomal samples was incubated under standard assay conditions in the presence of 1 mg of cytosolic protein as described, with the following modifications. In order to mimic the peroxisomal fraction as closely as possible, the incubation mixture also contained Nycodenz (4% w/v final concentration), the same concentration as found in the peroxisomal samples, plus added boiled protein, to bring the final protein concentration to 0.8 mg. The results are illustrated in Table III. Four experiments were carried out, using sample from the same microsomal preparations as in experiments 1–4 in Table II. As can be seen, in three of four experiments, no 14C radioactivity was detected. The recoveries of the [3H]cholesterol internal standard ranged between 5–10%, and care was taken to analyze and pool the same number of samples as in Table II. To analyze the same population of microsomes that co-sedimented with the purified peroxisomal samples during density gradient centrifugation, we performed the following experiment. The microosomal fraction, prepared by differential cen-

### Table I

**Identification of cholestenyl acetate in peroxisomal fractions by reverse isotope dilution.**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Specific activity</th>
<th>14C/3H ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholestenyl acetate</td>
<td>dpm/mg</td>
<td></td>
</tr>
<tr>
<td>First crystallization</td>
<td>280</td>
<td>0.33</td>
</tr>
<tr>
<td>Second crystallization</td>
<td>286</td>
<td>0.35</td>
</tr>
<tr>
<td>Third crystallization</td>
<td>233</td>
<td>0.37</td>
</tr>
<tr>
<td>Fourth crystallization</td>
<td>229</td>
<td>0.35</td>
</tr>
</tbody>
</table>

4 Cholestenyl acetate (50 mg) was added to 14C radioactivity obtained after silicic acid HPLC of peroxisomal fractions.

**Crystallization in methanol.**

**Crystallization in ethylacetate.**

**Crystallization in acetone.**

**Crystallization in ethylacetate.**

### Table II

**14C radioactivity co-migrating with cholesterol standard after HPLC separation of sterol intermediates in cholesterol biosynthesis.**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Peroxisomes</th>
<th>Microsomes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Reverse phase HPLC</td>
<td>Silicic acid HPLC</td>
</tr>
<tr>
<td></td>
<td>dpm/0.8 mg</td>
<td>dpm/0.8 mg</td>
</tr>
<tr>
<td>1</td>
<td>700,000</td>
<td>768,000</td>
</tr>
<tr>
<td>2</td>
<td>173,000</td>
<td>316,000</td>
</tr>
<tr>
<td>3</td>
<td>225,000</td>
<td>438,000</td>
</tr>
<tr>
<td>4</td>
<td>252,000</td>
<td>551,000</td>
</tr>
</tbody>
</table>

**0.8 mg of organelle protein plus 1 mg of cytosolic protein.**

**14C radioactivity corrected for recovery by use of [3H]cholesterol internal standard.**

**The samples containing the 14C radioactivity co-migrating with the cholesterol standard after reverse-phase HPLC were pooled, acetylated, and separated by silicic acid HPLC. The values given represent the total dpm divided by the number of samples pooled.**

### Table III

**Microsomal contribution to 14C radioactivity co-migrating with cholesterol standard in peroxisomal fractions.**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Silicic acid HPLC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dpm</td>
</tr>
<tr>
<td>1</td>
<td>None (3)</td>
</tr>
<tr>
<td>2</td>
<td>None (1)</td>
</tr>
<tr>
<td>3</td>
<td>48,000 (2)</td>
</tr>
<tr>
<td>4</td>
<td>None (2)</td>
</tr>
<tr>
<td>5</td>
<td>None (2)</td>
</tr>
</tbody>
</table>

**14C radioactivity corrected for recovery by use of [3H]cholesterol internal standard.**

**The number of samples pooled after reverse-phase HPLC separation.**

**Microsomal fraction equivalent in esterase activity to that normally found in peroxisomal fractions. The microsomal fraction was prepared on a Nycodenz gradient as described under "Experimental Procedures."**

### Table IV

**Specific activities of esterase and rate of cholesterol synthesis in peroxisomal and microsomal fractions.**

<table>
<thead>
<tr>
<th>All values given as mean and S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol synthesis</td>
</tr>
<tr>
<td>------------------------</td>
</tr>
<tr>
<td>Unit</td>
</tr>
<tr>
<td>Peroxisomes</td>
</tr>
<tr>
<td>(5)</td>
</tr>
<tr>
<td>87.12 ± 27.8</td>
</tr>
<tr>
<td>(5)</td>
</tr>
<tr>
<td>0.61 ± 0.2</td>
</tr>
</tbody>
</table>

**Assumes the final incorporation of five [14C]mevalonic acids, in cholesterol, when using [2-14C]mevalonate, with a 6th carbon lost as 14CO2 at the demethylation of lanosterol.**

**The numbers in parentheses refer to the number of samples analyzed.**

**Specific activity in peroxisomes.**

**Specific activity in microsomes.**
trifugation was loaded on a Nycodenz gradient as described under "Experimental Procedures." A sample of the microsomal fraction equivalent in esterase activity to that normally found in peroxisomal samples (sedimenting at a density of 1.23, the equilibrium density of peroxisomes) was then measured for cholesterol synthesis activity. The results are illustrated in Table III, experiment 5. Again, no 14C radioactivity was detected co-migrating with the [3H]cholesterol internal standard after silicic acid HPLC. From these results, we conclude that the microsomal contribution to cholesterol synthesis in the purified peroxisomal samples is negligible.

Specific Activities of Esterase and Rate of Cholesterol Synthesis in Peroxosomal and Microsomal Fractions—Table IV shows a comparison of the specific activities of esterase and the rate of cholesterol synthesis in peroxisomal and microsomal fractions. The rate of cholesterol synthesis in peroxisomes is 87.12 pmol/mg/h, compared to a rate of 135.23 pmol/mg/h for the microsomes. As can be seen from the ratio of esterase-specific activities, the peroxisomal rate of cholesterol synthesis cannot be due to the small microsomal contamination in the samples.

**DISCUSSION**

A rigorous, three-step chromatographic procedure was employed for the resolution of radioactive cholesterol from other sterol intermediates. In addition the identity of the product (cholesterol) was further confirmed by reverse isotope dilution studies. The results of this study show that rat liver peroxisomes in the presence of cytosolic proteins are capable of converting mevalonic acid to cholesterol. The peroxisomal rate of conversion was 87.12 pmol/mg/h, as compared to the microsomal rate of 135.25 pmol/mg/h. Since the peroxisomal fractions were slightly contaminated by microsomes, care was taken to demonstrate that this contamination was not responsible for the observed synthesis in the peroxisomal samples. In four of five experiments, (Table III) no 14C radioactivity co-migrating with the [3H]cholesterol internal standard could be detected after silicic acid HPLC. Since the 0.8-mg microsomal sample averaged 240,000 dpm (Table II), correcting for recoveries (5–10%), and assuming linearity, we would only expect a total of about 1,200 to 2,400 dpm of 14C radioactivity from the 0.08-mg microsomal sample after silicic acid HPLC. Furthermore, this activity is spread over a number of fractions, and only a portion of the fraction is counted. Thus, this low level of activity would not be detected. Therefore, we conclude from these results that the microsomal contamination does not account for nor probably does it contribute to the cholesterol synthesis activity measured in peroxisomes.

The pathway of cholesterol synthesis involving cytosolic and microsomal enzymes has been studied in great detail. The initial part of the pathway is the three-step conversion of acetyl-CoA to HMG-CoA. These enzymes are localized in the cytoplasm. The next step is the reduction of this molecule to mevalonate, considered to be the rate-controlling step in the biosynthesis of polyisoprenoids. This enzyme (HMG-CoA reductase) is a transmembrane glycoprotein found in the endoplasmic reticulum (22), and as recently reported, also in the matrix of rat liver peroxisomes (2, 3). Next, follow a series of phosphorylation reactions that both activate and decarboxylate mevalonate to isopentenyl pyrophosphate. This is followed by the synthesis of prenyl pyrophosphate, farnesyl pyrophosphate, and squalene, which then cyclizes to lanosterol. The final stages of sterologenesis involve the removal of three methyl groups from lanosterol and the migration and reduction of double bonds to give cholesterol. Nineteen discrete reactions are used to convert lanosterol to cholesterol. The enzymes responsible for the conversion of farnesyl pyrophosphate to squalene and lanosterol, are all integral membrane-bound proteins of the endoplasmic reticulum (28). Indeed, given the complexity and the number of reactions required for cholesterol synthesis, the results of this study are surprising. Future studies will have to address the many questions on the mechanism, enzymology, and function of these reactions in peroxisomes.

A full understanding of the metabolic role of the peroxisome has yet to be achieved. As mentioned in the Introduction, it is becoming clear that bile acid synthesis may be an important function of the organelle in rat hepatocytes (7–10). It is of significance to note that sterol carrier protein 2 (SCP-2), known to stimulate the conversion of lanosterol to cholesterol (24), as well as activate bile acid synthesis (25–27), and a number of other reactions involving the metabolism of cholesterol (28), has been recently localized in rat liver peroxisomes. Van der Krift et al. (29) were the first to show by immunoelectron microscopy the presence of SCP-2 or an SCP-2-like protein in rat liver peroxisomes. However, subsequent analysis of their isolated peroxisomes by immunoblotting indicated the absence of SCP-2 (13.5 kDa) from the peroxisomes and that a protein of 58 kDa was responsible for the immunological response. We have just recently confirmed the original observation by Van der Krift et al. (29) and also demonstrated the presence of the 13.5-kDa SCP-2 in the peroxisomes (1). In fact, the peroxisomes exhibited the highest degree of labeling per micromgram of organelle protein. Thus, these data and the present study suggest that peroxisomes may indeed play an important function in overall cholesterol metabolism.

Acknowledgments—We wish to thank Dr. S. J. Singer for stimulating discussions during the course of this study.

REFERENCES