In the present study we investigated the subcellular localization of squalene synthase (farnesyl-diphosphate: farnesyl-diphosphate farnesyltransferase, EC 2.5.1.21). Squalene synthase catalyzes the formation of squalene from trans-farnesyl diposphate in two distinct steps and is the first committed enzyme for the biosynthesis of cholesterol. Recently, a truncated form of the enzyme from rat hepatocytes was purified, and monospecific antibodies for squalene synthase were produced. This enabled the subcellular localization of squalene synthase by three different methods: (i) analytical subcellular fractionation and measurements of enzyme activities; (ii) immunodeterminations of squalene synthase in the isolated subcellular fractions with a monospecific antibody; and (iii) immunoelectron microscopy. All three methods gave consistent results. The data clearly illustrate that squalene synthase enzymatic activity and squalene synthase are exclusively localized in the endoplasmic reticulum. In rat hepatic peroxisomes we were not able to detect any squalene synthase. In addition, we also demonstrated that squalene synthase in the microsomal fraction is dramatically regulated by a number of hypolipidemic drugs and dietary treatments.

There is considerable evidence that peroxisomes in mammalian cells not only have a role in oxidation of cholesterol to bile acids but also in cholesterol biosynthesis (1). Specifically, we have shown that peroxisomes contain the following cholesterol synthetic enzymes: (i) acetoacetyl-CoA thiolase, the enzyme required for the initial step in cholesterol synthesis (2, 3); (ii) 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase), the rate-limiting enzyme in cholesterol synthesis (4, 5); and (iii) mevalonate kinase (6). The localization of these enzymes to peroxisomes was demonstrated by a combination of immunoelectron microscopy and Western blotting as well as determination of enzymatic activity. In addition, biochemical activities of dihydrolanosterol oxidase, steroid 14-reductase, steroid 3-ketoreductase, and steroid 8-isomerase activities, enzymes involved in the conversion of lanosterol to cholesterol, have been reported to be present in peroxisomes (7). The largest concentration of cellular sterol carrier protein 2 is also found in rat and human peroxisomes (8, 9) as well as significant levels of apolipoprotein E, a major constituent of several classes of plasma lipoproteins (10). Moreover, the cholesterol biosynthetic capacity is impaired in cultured skin fibroblasts obtained from patients with peroxisomal deficiency diseases (11), suggesting that peroxisomes are essential for normal cholesterol synthesis in human cell fibroblasts.

We have reported previously that mevalonic acid could be converted to cholesterol in vitro by peroxisomes in the presence of a cytosolic fraction (12). The conversion of mevalonic acid to cholesterol requires a number of cytosolic enzymes to produce farnesyl pyrophosphate, which is then converted to cholesterol by integral membrane-bound enzymes of the ER.

To clarify the role of peroxisomes in cholesterol biosynthesis, it is necessary to know which of the enzymes of the cholesterol biosynthetic pathway are present in these organelles. Accordingly, in the present study we have investigated the subcellular localization of squalene synthase (farnesyl-diphosphate: farnesyl-diphosphate farnesyltransferase, EC 2.5.1.21). Squalene synthase catalyzes the formation of squalene from trans-farnesyl diposphate in two distinct steps (13) and is the first committed enzyme for the biosynthesis of cholesterol (14). Recently, a truncated form of the enzyme from rat hepatocytes was purified, and monospecific antibodies for squalene synthase were produced (15).

This enabled the subcellular localization of squalene synthase by three different methods: (i) analytical subcellular fractionation and measurements of enzyme activities; (ii) immunodeterminations of squalene synthase in the isolated subcellular fractions with a monospecific antibody; and (iii) immunoelectron microscopy.

EXPERIMENTAL PROCEDURES

Materials—Biochemicals were purchased from Sigma. Electrophoresis supplies were purchased from Bio-Rad. [3H]Farnesyl pyrophosphate and farnesylpyrophosphatase were purchased from American Radiolabeled Chemicals, Inc. Cholestyramine (Questran) was purchased from Bristol Laboratories, and mevinolin (Mevacor) was from Merck Sharp and Dohme Laboratories. Gemfibrozil and fenofibrate were the generous gifts of Warner-Lambert, Inc.

Animals—Male Sprague-Dawley rats (100-180 g) were maintained on a 12-h light-dark cycle. Standard Laboratory chow and water were given ad libitum. Rats were also fed the standard laboratory diet supplemented with either gemfibrozil (Lopid; Warner-Lambert) or fenofibrate at a concentration of 0.2%, w/w, for 2-5 weeks. Rats were also treated for 4-6 days with a diet containing 5% cholestyramine.
plus 0.1% mevinolin (lovastatin) or treated for 19–21 days with a diet containing 2.0% cholesterol or treated for 5–7 days with a diet containing 0.1% mevinolin or 5% cholestyramine. In most experiments the rats were fasted overnight and killed by decapitation 2 h into their light cycle.

**Cell Fractionation and Preparation of Rat Liver Peroxisomes**—The buffer for homogenization and differential centrifugation contained the following: 0.25 M sucrose, 5 mM Tris, 1 mM EDTA, and 0.1% ethanol at pH 7.5.

**Differential Centrifugation**—Rat liver was fractionated by differential centrifugation according to de Duve et al. (16) into five fractions: nuclear (N), mitochondrial (M), light mitochondrial (L), microsomal (P), and supernatant (S). The intact peroxisomes are mainly in the M and L fractions.

**Equilibrium Density Centrifugation**—Liver homogenates were first fractionated by differential centrifugation to obtain a λ fraction, containing peroxisomes, smaller mitochondria, and microsomes (similar to de Duve’s light mitochondrial fraction), a cytosolic fraction, a microsomal fraction, and nuclear fraction (S). The peroxisome-enriched fraction was then purified further by equilibrium density centrifugation in a TV-850 vertical rotor (40,000 rpm and w^2 set at 8 x 10^4) on a linear metrizamide or Nycodex (20–45% (w/w)) gradient. The buffer for the metrizamide gradient was 3 mM imidazole, pH 7.5, and for the Nycodex gradient was the 0.25 M sucrose homogenization buffer. Several of the isolations with metrizamide gradients were performed with the addition of 10 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 1 μM pepstatin A to the homogenization buffer and gradient. A 3.3% final concentration of sucrose was added to the 20% metrizamide solution at the time of gradient formation. The addition of sucrose creates an essentially iso-osmolar environment throughout the gradient. A total of 20–25 fractions were collected from the bottom of the tube with a two-way needle.

**Assay of Marker Enzymes**—All fractions were analyzed for marker enzyme activity and protein concentration. The activity of catalase (peroxisomal marker) was measured according to Leighton et al. (17). The activity of glutamate dehydrogenase (mitochondrial marker) was measured according to Schmidt (18). Phosphoglucone isomerase was used as a marker for the cytosolic fraction and measured according to the method of Noltmann (19). Esterase was used to measure microsomal activity according to the method of Beaufay et al. (20). Acid phosphatase, a lysosomal marker, was measured according to Bergmeyer et al. (21). Protein was determined by the BCA method (Pierce Chemical Co.), using bovine serum albumin as a standard. Samples containing metrizamide were first precipitated with 10% trichloroacetic acid since metrizamide interferes with the protein determination.

In all experiments, the isolated peroxisomes were at least 93% pure as determined by marker enzyme distribution. The isolated peroxisomes contained 0–1% mitochondrial contamination as estimated by glutamate dehydrogenase activity, and microsomal protein was the only other contaminant, at about 3–7%. There was no measurable cytosolic or lysosomal contamination as measured by phosphorylase isomerase and acid phosphatase, respectively.

**Squalene Synthase Assay**—Squalene synthase was assayed as described (15). Briefly, the assay mixture contained 100 mM potassium phosphate buffer, pH 7.4, 5 mM MgCl₂, 5 mM CHAPS, 10 mM dithiothreitol, 2 mM NADPH, and 10 μM [3H]farnesyl pyrophosphate (145 μCi/μmol) in a total volume of 100 μL. The reaction was stopped with 10 μL of 1 M EDTA, pH 9.2, and the product isolated by thin layer chromatography.

**Preparation of Monospecific Antibodies to Squalene Synthase Protein**—Squalene synthase was solubilized and purified, and antibodies were prepared as described (15).

**SDS-Gel Electrophoresis and Immunoblotting Techniques**—Proteins were separated on 9 or 10% polyacrylamide slab gels (1.5 mm) containing 0.1% SDS according to Laemmli (22), followed by the Bio-Rad Western blot protocol. Samples used for immunoblots of squalene synthase were run fresh on the day of preparation or the following day after overnight storage at −70 °C. Protein samples were prepared in solubilization buffer (0.0625 M Tris/HCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol, and 5% glycerol) and boiled for 5 min before loading onto the gel. The separated proteins were electrophoretically transferred to nitrocellulose paper in 25 mM Tris, 0.7 mM glycine using 150 mA constant current for 15 h (23). Blocking and rinsing of the nitrocellulose were performed as described by Parker et al. (24). Blots were incubated for 2 h at room temperature with the squalene synthase antisera (1:1,500). Visualization of squalene synthase was with affinity-purified 125I-protein A. The autoradiograms were scanned with an LKB laser densitometer to determine relative squalene synthase levels.

The peroxisomal targeting signal (PTS) anti-peptide and catalase antibodies were used, the nitrocellulose was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG for 60 min.

**Cryoultramicrotomy and Immunolabeling**—Small blocks of liver from normal animals were fixed in 3% formaldehyde and 0.5% gluteraldehyde in 0.1 M cacodylate buffer, pH 7.2. Cryoultramicrotomy was performed as described (25). The liver tissues were infused with 2.3 M sucrose before being frozen in liquid nitrogen for thin sectioning. Immunolabeling and thin plastic embedding of the cryosections were performed as described (26). Briefly, thin cryosections were immunolabeled with the anti-squalene synthase antibody at a concentration of 50 μg/ml, or with an IgG fraction of rabbit anti-catalase antibody used at 25 μg/ml, or with a monoclonal antibody against HMG-CoA reductase used at 18 μg/ml for 10 min. The characteristics of the catalase and HMG-CoA reductase antibodies have been described previously (4, 5). After washing in phosphate-buffered saline, the sections were treated with 10-nm colloidal gold adducts of affinity-purified goat antibodies to rabbit IgG or 6–8-nm colloidal gold adducts of affinity-purified goat antibodies to mouse IgG depending on the primary antibody used (26). The immunolabeled sections were treated by floating the grids on droplets of 1% reduced osmium, poststained in 2% ethanolic uranyl acetate, and infused with LR white acrylic resin (London Resin Co., London) (26). After polymerization in a vacuum oven, the sections were observed.

<table>
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<tr>
<th>Enzyme</th>
<th>Absolute activities*</th>
<th>N</th>
<th>M</th>
<th>L</th>
<th>P</th>
<th>S</th>
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* Units/g of liver (H).

* Results expressed as a percentage of the sum of the activities in the five fractions.

* Percent of H.

* nmol/g of liver.
Squalene Synthase in Rat Hepatic Cells

TABLE II

<table>
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* Units/g of liver (H).
* Results expressed as a percentage of the sum of the activities in the five fractions.
* Percent of H.
* nmol/g of liver.

Fig. 2. Differential centrifugation of normal rat liver. Nuclear, mitochondrial, light mitochondrial, microsomal, and supernatant fractions are represented from left to right in the order of their isolation and plotted according to de Duve et al. (16). Relative specific activity (RSA) is the percent of total activity in a given fraction divided by the percent of total protein in that fraction.

RESULTS

Induction of Rat Hepatic Microsomal Squalene Synthase—Rat liver microsomal fractions were prepared from control animals and animals treated with mevinolin, cholestyramine, cholestyramine plus mevinolin, cholesterol, gemfibrozil, or fenofibrate. Western blotting for squalene synthase of these microsomal fractions is shown in Fig. 1. The enzyme was greatly induced in the microsomal fraction after feeding rats with a diet containing either mevinolin or cholestyramine plus mevinolin and was induced to a slightly lesser degree after feeding rats with a diet containing cholestyramine or gemfibrozil. In contrast, microsomes obtained from animals fed with diets containing either cholesterol or fenofibrate showed decreased amounts (after fenofibrate treatment) or complete disappearance (after cholesterol feeding) of squalene synthase as compared with microsomes of animals fed a control diet.

To assess the role of peroxisomes in cellular cholesterol biosynthesis, we investigated the presence of squalene synthase in isolated peroxisomal fractions obtained from livers of control animals as well as animals treated with mevinolin, fenofibrate, and cholestyramine plus mevinolin.

Distribution of Squalene Synthase after Differential Centrifugation
Squalene Synthase in Rat Hepatic Cells

FIG. 3. Differential centrifugation of liver from a mevinolin-treated rat. Nuclear, mitochondrial, light mitochondrial, microsomal, and supernatant fractions are represented from left to right in the order of their isolation and plotted according to de Duve et al. (16). RSA is the relative specific activity, calculated as described in the legend to Fig. 2.

Isolation of Normal Rat Liver and Liver Obtained from Mevinolin-treated Animals—The liver was fractionated into five fractions: nuclear, mitochondrial, light mitochondrial, microsomal, and supernatant, as described (15). The enzyme distributions are given in Table I (normal liver) and Table II (mevinolin-treated). The mean relative specific activities are plotted versus cumulative protein according to de Duve et al. (16) in Fig. 2 (normal liver) and Fig. 3 (mevinolin-treated). The liver content of protein and of the marker enzymes (Tables I and II) is in the normal range, and the separation of nuclei, mitochondria, microsomes, and cell cytosol is similar to what has been described previously (27). The recoveries in the fractionations were also satisfactory. The majority of the glutamate dehydrogenase activity (marker for mitochondria) is located in the M fraction, and the greatest percentage of the esterase activity (marker for microsomes) is found in the P fraction. The intact peroxisomes, as determined by catalase activity, are primarily in the M and L fractions. A substantial amount of catalase activity is also found in the S fraction. This represents solubilized catalase leaked from ruptured peroxisomes produced by the isolation procedure.

The distribution of squalene synthase activity in normal (Table I) and treated liver (Table II) is very similar to the distribution of esterase activity, the marker for the microsomal fraction. The only major difference noted between the two fractionations is the increase in the absolute activity of squalene synthase in the liver of rats treated with mevinolin, as reported previously (15). These results imply that the squalene synthase activity is located principally in the microsomes. In no case is there an enrichment of squalene synthase activity above that of esterase activity in the M or L fraction, in which the intact peroxisomes are located. However, these results do not exclude the possibility that a very small portion of the squalene synthase activity could be associated with the peroxisomes. The differential centrifugation method does not adequately separate peroxisomes from microsomes to detect this.

Isolation of Peroxisomes from Mevinolin-treated Animals by Equilibrium Density Centrifugation—To separate the peroxisomes further from the microsomes, the M fraction (consisting primarily of peroxisomes, light mitochondria, and microsomes) obtained from mevinolin-treated animals was fractionated as described under "Experimental Procedures." Twenty-one fractions were collected, and the protein concentration and the following enzymes were determined in each fraction: glutamate dehydrogenase (mitochondrial marker), phosphoglucose isomerase (cytosolic fraction marker), esterase (microsomal marker), catalase (peroxisomal marker), and squalene synthase. Fig. 4 illustrates the distribution of these enzymes on the gradient (relative concentration versus percent cumulative volume). The mitochondria, as measured by glutamate dehydrogenase, are located close to the center of the gradient and are responsible for the major peak of protein. The intact peroxisomes (as measured by catalase activity) are well separated from the microsomal fraction (as determined by esterase) and are located at higher densities (to the right in Fig. 4). In agreement with earlier reports (6), a portion of the catalase activity is solubilized, as a result of rupture of the peroxisomes during the isolation procedure, and migrates at the light end of the gradient. The distribution of squalene
Fig. 4. Isolation of peroxisomes by equilibrium density centrifugation from liver of mevinolin-treated animals. A peroxisome-enriched fraction prepared by differential centrifugation of liver homogenates from mevinolin-treated animals was further purified by equilibrium density centrifugation on a linear metrizamide density gradient to separate peroxisomes from other cell organelles. The distribution of protein, glutamate dehydrogenase (marker enzyme for mitochondria), isomerase (marker enzyme for the cytosol fraction), esterase (marker enzyme for ER proteins), catalase (marker enzyme for peroxisomes), and squalene synthase is plotted as relative concentration versus cumulative volume. Relative concentration (c/c0) is derived by dividing the actual concentration of the enzyme in a particular fraction by the concentration of the enzyme that would be observed if the enzyme was homogeneously distributed throughout the gradient. The density of the gradient increases from left to right. The absolute composition of the starting material (λ fraction) is shown in Table III, together with the recoveries.

Squalene synthase activity coincides with the distribution of the esterase activity on the gradient, substantiating a microsomal localization of squalene synthase. The minor amount of squalene synthase activity located in the peroxisome area (80–100% cumulative volume) of the gradient is a result of microsomal contamination as calculated from the esterase distribution. If indeed any squalene synthase activity were localized in the peroxisomes, one would expect to see a peak of squalene synthase activity in the peroxisome area, coincident with the marker enzyme catalase. This was never observed.

Additional data demonstrating the localization of peroxisomal proteins on a representative gradient are illustrated in Fig. 5. The gradient fractions were immunoblotted with an antibody against a synthetic peptide that contained a PTS (serine-lysine-leucine) at its COOH terminus (28). It was shown earlier by immunofluorescence, immunocryoelectron microscopy, and immunoblots of proteins from subcellular fractions that the antipeptide antibodies specifically detect peroxisomes and peroxisomal proteins in mammalian cells (29). This antibody recognizes more than 20 different perox-
Fig. 5. **Immunoblot of proteins from the gradient fractions using the anti-PTS antibody.** The gradient fractions were separated by SDS-polyacrylamide gel electrophoresis (50 μg of protein), transferred to nitrocellulose, and incubated with the PTS antibodies. The highest purity peroxisomal fractions are located at the dense end of the gradient. However, a significant amount of the peroxisomal proteins is also spread out over the rest of the gradient.

Fig. 6. **Immunoblotting of proteins from gradient fractions of mevinolin-treated animals for squalene synthase.** The gradient fractions from mevinolin-treated animals (illustrated in Fig. 4) were separated by SDS-polyacrylamide gel electrophoresis (100 μg of protein), transferred to nitrocellulose, and incubated with the squalene synthase antibody. Fractions 5–7 represent the localization of the majority of the ER proteins contained in the starting material and exhibit a strong signal with the squalene synthase antibody. Fractions 16–21 (containing the peroxisomal proteins, illustrated in Fig. 5) contain only minor amounts. Fig. 6 also illustrates the specificity of this antibody for squalene synthase. There is no cross-reactivity with any other proteins in any fraction. Fig. 7 compares the relative immunoblot density.
Squalene Synthase in Rat Hepatic Cells

The relative density of the immunoblot end of the gradient (fractions 1 and 2). We believe the measured specific activity of squalene synthase in these fractions is overestimated. The overestimate is caused by the inability to measure accurately squalene synthase activity in fractions containing background levels of activity in combination with severely low protein levels. This interpretation is substantiated by the fact that the relative immunoblot density scan (equivalent to the specific activity of squalene synthase) is in excellent agreement with the specific activity of esterase (Fig. 7), as would be expected if the two enzymes were localized in the same organelle. In addition, the relative activity distributions (Fig. 4) of esterase and squalene synthase (which represent the relative activity of each fraction, illustrating the localization of organelles on the gradient) are also in excellent agreement. Thus, the residual amount of squalene synthase activity and protein detected in other areas on the gradient can clearly be explained by the minor microsomal contamination found in these fractions as determined by marker enzyme analysis. In addition, there is no correlation of the specific activity of catalase compared with the relative immunoblot density of squalene synthase in the peroxisome area (Fig. 7, fractions 16-21). Table III presents a summary of data complementary to Figs. 4, 6, and 7. Less than 7% of the total esterase and squalene synthase activity contained in the original homogenate was loaded on the gradient, and less than 0.13% of the total esterase activity and 0.26% of the total squalene synthase activity is found in the peak peroxisomal fractions. This is not a significant difference. Thus, all of the data presented above imply that squalene synthase from mevinolin-treated animals resides exclusively in the microsomal fraction.

We also investigated if squalene synthase was present in peroxisomes from animals fed a normal chow diet and a diet supplemented with fenofibrate.

Isolation of Peroxisomes from Control and Fenofibrate-treated Animals by Equilibrium Density Centrifugation—The peroxisome-enriched fractions prepared by differential centrifugation of liver homogenates from control and fenofibrate-treated animals were also subjected to equilibrium density centrifugation as described under "Experimental Procedures." All of the fractions were assayed for protein concentration and the following enzymes: glutamate dehydrogenase, phosphoglucose isomerase, esterase, catalase, and squalene synthase. Figs. 8 (normal) and 9 (fenofibrate-treated) illustrate the distribution of these enzymes on the gradients. The distribution of squalene synthase activity is coincident with the distribution of esterase activity in both gradients. In addition, the minor amount of squalene synthase activity found in the peroxisome area can again be accounted for by the slight microsomal contamination present at this end of the gradient as calculated from the esterase distribution. The calculated activities (percent of homogenate present in the purified peroxisomes) for control animals were 0.05 for esterase and 0.07 for squalene synthase. For the fenofibrate-treated animals the values were 0.43 for esterase and 0.45 for squalene synthase. These results support the conclusion that squalene synthase activity is not detectable in peroxisomes.

Immunoblotting of Squalene Synthase for Subcellular Localization from Control and Fenofibrate-treated Animals—The proteins in the gradient fractions obtained from control and fenofibrate-treated animals were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. The nitrocellulose was cut in half and the lower half probed for squalene synthase and the upper half for catalase, as a supplementary marker for peroxisomes. Fig. 10 illustrates the immunoblots obtained from control (panels I and II) and fenofibrate-treated animals (panels III and IV) using squalene synthase and catalase antibodies. As is shown, there is no detectable squalene synthase reactivity in the peak peroxi-

Fig. 7. Specific activity of squalene synthase, esterase, and catalase versus relative immunoblot density. Comparison of the specific activity of squalene synthase, esterase, and catalase (solid circles) in the gradient fractions from mevinolin-treated animals with the relative density of the immunoblot (open circles) illustrated in Fig. 6.

(obtained from Fig. 6) with the specific activities of squalene synthase, esterase, and catalase. The relative immunoblot density of squalene synthase is in good agreement with the measured specific activity of squalene synthase in all gradient fractions with the exception of several fractions from the light end of the gradient (fractions 1 and 2). We believe the measured specific activity of squalene synthase in these fractions is overestimated. The overestimate is caused by the inability to measure accurately squalene synthase activity in fractions containing background levels of activity in combination with severely low protein levels. This interpretation is substantiated by the fact that the relative immunoblot density scan (equivalent to the specific activity of squalene synthase) is in excellent agreement with the specific activity of esterase (Fig. 7), as would be expected if the two enzymes were localized in the same organelle. In addition, the relative activity distributions (Fig. 4) of esterase and squalene synthase (which represent the relative activity of each fraction, illustrating the localization of organelles on the gradient) are also in excellent agreement. Thus, the residual amount of squalene synthase activity and protein detected in other areas on the gradient can clearly be explained by the minor microsomal contamination found in these fractions as determined by marker enzyme analysis. In addition, there is no correlation of the specific activity of catalase compared with the relative immunoblot density of squalene synthase in the peroxisome area (Fig. 7, fractions 16-21). Table III presents a summary of data complementary to Figs. 4, 6, and 7. Less than 7% of the total esterase and squalene synthase activity contained in the original homogenate was loaded on the gradient, and less than 0.13% of the total esterase activity and 0.26% of the total squalene synthase activity is found in the peak peroxisomal fractions. This is not a significant difference. Thus, all of the data presented above imply that squalene synthase from mevinolin-treated animals resides exclusively in the microsomal fraction.

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Data complementary to Fig. 4.

**TABLE III**

Equilibrium density centrifugation of liver from mevinolin-treated rats

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<th>Constituent</th>
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<th>λ* fraction</th>
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* The λ fraction was loaded on the gradient of Fig. 4.

**FIG. 8.** Isolation of peroxisomes by equilibrium density centrifugation from normal liver. A linear metrizamide density gradient was used to separate peroxisomes. The density of the gradient increases from left to right. The recoveries of all enzyme activities and protein ranged from 80 to 105%. c/ci is the relative concentration, determined as described in the legend to Fig. 4.
some fractions (fractions 20–24, as illustrated by catalase) in either gradient. Since equal volumes of each sample were loaded on the gel, this represents relative activity measurements of squalene synthase and catalase, and not specific activity. Since equal quantities of protein were used in all fractions from the mevinolin-treated animals (Fig. 6), the distribution of squalene synthase represents specific activity. However, in all three treatment groups, there is no detectable squalene synthase in the peroxisome fractions which cannot be attributed to the minor microsomal contamination found in this area.

Specific Activities of Enzymes in Peroxisomal and Microsomal Fractions from Liver of Control, Mevinolin-, and Fenofibrate-treated Animals—The specific activities of squalene synthase and esterase were calculated for the most highly purified peroxisomal fractions obtained from the gradients and for the microsomal fractions obtained by differential centrifugation. The purity of the peroxisomes was calculated to be about 93%, and the microsome fraction was calculated to be around 94% pure. Table IV shows the specific activities of squalene synthase and esterase in peroxisomal and microsomal fractions obtained from liver of control, mevinolin-, and fenofibrate-treated animals. As can be seen from the ratio of specific activities, the presence of squalene synthase in peroxisomes can be attributed to the microsomal contamination found in this area. The ratio of the specific activities of squalene synthase (0.053–0.089) is in a range similar to the ratio of specific activities of esterase (0.068–0.096), whereas for HMG-CoA reductase, an enzyme localized to both organ-
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1 Squalene Synthase

II Catalase

III Squalene Synthase

IV Catalase

Equilibrium Density Gradient

**FIG. 10. Immunoblotting of proteins from gradient fractions of control and fenofibrate-treated animals for squalene synthase and catalase.** The gradient fractions were separated by SDS-polyacrylamide gel electrophoresis (50 μl) and transferred to nitrocellulose. The nitrocellulose was cut in half, and the upper half was treated with a catalase antibody and the lower half incubated with a squalene synthase antibody. Panels I and II represent fractions from control animals, and panels III and IV represent fractions from the fenofibrate-treated animals.

**TABLE IV**

Specific activities of enzymes in peroxisomal and microsomal fractions from liver of control (N), mevinolin (M)-, and fenofibrate (F)-treated animals

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>M</th>
<th>F</th>
<th>N</th>
<th>M</th>
<th>F</th>
<th>N</th>
<th>M</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Squalene synthase</td>
<td>pmol/min/mg</td>
<td>units/min/mg</td>
<td>pmol/min/mg</td>
<td>units/min/mg</td>
<td>pmol/min/mg</td>
<td>units/min/mg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peroxisomes</td>
<td>1.8</td>
<td>27.0</td>
<td>1.6</td>
<td>0.35</td>
<td>0.28</td>
<td>0.32</td>
<td>0.072</td>
<td>0.089</td>
<td>0.053</td>
</tr>
<tr>
<td>Microsomes</td>
<td>25.0</td>
<td>302.5</td>
<td>30.0</td>
<td>4.8</td>
<td>2.7</td>
<td>4.7</td>
<td>0.073</td>
<td>0.096</td>
<td>0.068</td>
</tr>
<tr>
<td>Ratio of specific activity</td>
<td>0.072</td>
<td>0.089</td>
<td>0.053</td>
<td>0.073</td>
<td>0.096</td>
<td>0.068</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Specific activity in pmol/min/mg of organelle protein.
* Specific activity in units/min/mg of organelle protein.
* Specific activity in peroxisomes divided by specific activity in microsomes.

The specific activity of squalene synthase in microsomes from mevinolin-treated rats is considerably greater than in control or fenofibrate-treated animals. These data are in agreement with the previous results shown in Fig. 1 illustrating the induction of squalene synthase protein in microsomes isolated from mevinolin-treated animals.

We also determined the levels of squalene synthase protein found in the purified microsomal fractions (obtained by differential centrifugation and calculated to be 94% pure) relative to those found in the purified peroxisomes. Fig. 11 shows an immunoblot of the purified fractions from two different treatments, fenofibrate and cholestyramine plus mevinolin. For comparison, we also included a 10-μg sample of microsomal protein which represents an overestimate of the calculated microsomal protein found in the 100-μg peroxisomal protein sample. As can be seen, the minor reactivity observed in the peroxisomal sample from the cholestyramine plus mevin-
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In this study, the subcellular localization of squalene synthase was investigated by three different methods: (i) analytical subcellular fractionation and measurements of enzyme activities; (ii) immunodeterminations of squalene synthase in the isolated subcellular fractions with a monospecific antibody; and (iii) immunoelectron microscopy. All three methods gave consistent results. The data clearly illustrate that squalene synthase activity and squalene synthase are exclusively localized in the endoplasmic reticulum. In rat hepatic peroxisomes obtained from control animals or animals treated with mevinolin, fenofibrate, or cholestyramine plus mevinolin we were not able to detect any squalene synthase activity or protein.

This conclusion is also corroborated by immunoelectron microscopy, which revealed labeling for squalene synthase only in the membranes of the ER cisternae but not in the peroxisomes. Whereas a cryosection treated with antibodies raised against HMG-CoA reductase clearly labeled the peroxisomes. The immunolabeling for HMG-CoA reductase in the peroxisomes was restricted to the matrix of the organelle. The rough endoplasmic reticulum compartment was also labeled, albeit with a lower density of gold particles (arrowhead). Fig. 12, panel D, illustrates the immunolabeling obtained with antibodies against catalase. As expected, the peroxisomes are intensely and uniformly labeled. The few gold particles that can be seen in the cytoplasm most likely represent molecules of catalase en route to the peroxisomal compartment.

DISCUSSION

inolin-treated rats is entirely due to the microsomal contamination. In the purified fractions obtained from fenofibrate-treated animals, only the 100-µg microsomal protein fraction shows reactivity. These data are in excellent agreement with the calculated specific activity ratios illustrated in Table IV and support the conclusion that squalene synthase is exclusively localized in the endoplasmic reticulum.

Computer Calculations—To interpret the gradient distribution data quantitatively, the amount of squalene synthase activity in each organelle was determined by applying the principle of calculating the linear combinations of marker enzyme distributions which would best fit the measured squalene synthase distributions. This method has been described in detail (27). This program makes use of all data from the fractionation procedure instead of merely the few purest fractions. This method also requires good recoveries in all of the cell fractionations. The program was augmented to include the T values and probabilities for the least squares coefficients. Utilizing this program (mean of three gradients) we calculate that 99.92% of the total squalene synthase is localized in the endoplasmic reticulum and 0.075% is localized in the peroxisomes. This level of activity in the peroxisomes is not significant at the 99% confidence level.

Immunoelectron Microscopy—To confirm further the exclusive localization of squalene synthase to the endoplasmic reticulum, blocks of control liver tissues were processed for immunoelectron microscopy as described under "Experimental Procedures." Indirect gold immunolabeling for squalene synthase in control animals is illustrated in Fig. 12, panels A and B. No specific immunolabeling could be detected in the peroxisomes of normal animals (panel A). However, the rough endoplasmic reticulum region of normal animals showed labeling (panel B). Gold particles were mostly detected associated with the membranes of the ER cisternae. The other organelles, in particular the nucleus, the lysosomes, and the Golgi apparatus, displayed no specific labeling for squalene synthase. For comparison with these results and to demonstrate the specificity of the immunolabeling for squalene synthase, we also treated the cryosections with antibodies made against HMG-CoA reductase and catalase. Fig. 12, panel C, shows a cryosection of normal rat liver immunolabeled with HMG-CoA reductase antibodies. The immunolabeling for HMG-CoA reductase in the peroxisomes was restricted to the matrix of the organelle. The rough endoplasmic reticulum compartment was also labeled, albeit with a lower density of gold particles (arrowhead). Fig. 12, panel D, illustrates the immunolabeling obtained with antibodies against catalase. As expected, the peroxisomes are intensely and uniformly labeled. The few gold particles that can be seen in the cytoplasm most likely represent molecules of catalase en route to the peroxisomal compartment.
by feeding rats a diet containing either mevinolin or cholesterylamine plus mevinolin, and to a slightly lesser degree by feeding rats a diet containing cholesterylamine or gemfibrozil (Fig. 1), whereas a diet containing either cholesterol or fenofibrate reduced squalene synthase in the microsomal fraction either to nondetectable (after cholesterol feeding) or to decreased levels (after fenofibrate treatment) as compared with animals receiving a control diet.

These observations are consistent with the previously reported results that the enzymatic activity of squalene synthase in the microsomal fraction is induced by mevinolin and other HMG-CoA reductase inhibitors and down-regulated by cholesterol feeding (15). Our data are also consistent with the reported finding that in the human hepatoma cell line HepG2, squalene synthase activity was observed exclusively in the microsomal fractions (30).

While this manuscript was in preparation, a study reporting the presence of squalene synthase activity in rat hepatic peroxisomes from control animals was published (31). This conclusion was based solely on the measurement of squalene synthase activity in only two fractions: a microsomal and a peroxisomal fraction. We repeated some of our studies on control animals using nonfasted rats and isolation and assay conditions similar to those described (31). However, we again could not detect any squalene synthase activity or protein in the pure peroxisomes which was not accounted for by the background microsomal contamination found in the peroxisomes.

Our conclusion that peroxisomes do not contain squalene synthase is also in agreement with the observation that in primary fibroblast cell cultures obtained from patients diagnosed with peroxisomal deficiency diseases, which display decreased levels of a large number of peroxisomal matrix proteins (32), there are normal levels of squalene synthase, whereas levels of HMG-CoA reductase (a protein shown to be localized in the peroxisomes) were present (19). Rather, a more likely explanation is that the activity for the synthesis of cholesterol was not originally peroxisomal. Rather, the activity (15) required for this conversion was not originally peroxisomal.

A number of metabolic pathways require coparticipation of enzymes located in both peroxisomes as well as enzymes found in other intracellular compartments (33). For example, the first steps of the mevalonate synthesis occur in the peroxisomes, whereas the terminal reactions are completed in the endoplasmic reticulum. Similarly, the oxidation of cholesterol to bile acids requires the participation of enzymes localized in the endoplasmic reticulum as well as peroxisomes. Little is known about the regulation of such pathways or about the shuttling of intermediates between compartments. The physiological importance of peroxisomal enzymes in the regulation of sterol metabolism remains to be clarified. However, the finding that in peroxisomal deficiency diseases there is a clear aberration in sterol metabolism suggests that peroxisomal metabolism plays at least a permissive role (11).

REFERENCES


* S. K. Krisans, unpublished data.