S.K. Krisans

Localization of Cholesterol Synthesis Enzymes in Peroxisomes

Abstract

Rat liver peroxisomes contain enzymes of the isoprenoid synthesis pathway which leads to cholesterol, dolichol, coenzyme Q, heme *a* and isoprenylated protein synthesis. Specifically, peroxisomes have been shown to contain acetoacetyl-CoA thiolase, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) synthase, HMG-CoA reductase and most recently, mevalonate kinase. In addition, rat liver peroxisomes in the presence of cytosol have been shown to convert mevalonic acid to cholesterol and dolichol. The largest concentration of cellular sterol carrier protein 2 (SCP-2), a protein thought to facilitate the transport of sterols, is also found in rat and human peroxisomes as well as significant levels of apolipoprotein E, a major constituent of several classes of plasma lipoproteins. Moreover, we have demonstrated that the cholesterol biosynthetic capacity is severely impaired in cultured skin fibroblasts obtained from patients with peroxisomal deficiency diseases. These findings support the likelihood that peroxisomes play an essential role in isoprenoid biosynthesis. However, the exact nature of the role of peroxisomes is far from being understood. We have recently shown that squalene synthase is exclusively found in the endoplasmic reticulum. Thus, the compartmentalization of cholesterol biosynthetic processes remains to be elucidated.

Introduction

The Pathway of Cholesterol Synthesis

The pathway of cholesterol synthesis involving cytosolic enzymes and enzymes localized in the endoplasmic reticulum (ER) has been studied in great detail. Figure 1 illustrates the compartmentilization of cholesterol synthesis in the cytosol and ER. The initial part of the cholesterol synthesis pathway is the two-step conversion of acetyl-CoA to HMG-CoA. The first step in the formation of HMG-CoA is the condensation reaction in which two molecules of acetyl-CoA are used to form acetoacetyl-CoA. This reaction is catalyzed by an acetoacetyl-CoA thiolase. This step is followed by the addition of another acetyl-CoA to the acetoacetyl-CoA to form HMG-CoA. This step is catalyzed by HMG-CoA synthase. These enzymes are present in the cytosol. In addition, the mitochondria

also contain a different set of these enzymes to produce HMG-CoA for ketogenesis. HMG-CoA produced in the cytosol is then converted to mevalonate in the ER by HMG-CoA reductase, the rate limiting enzyme of cholesterol biosynthesis. The further conversion of mevalonate to farnesyl diphosphate requires the five following enzymes: 1) mevalonate kinase; 2) phosphomevalonate kinase; 3) mevalonate diphosphate decarboxylase; 4) isopentenyl diphosphate isomerase, and 5) farnesyl diphosphate synthase. These enzymes are present in the cytosol. Farnesyl diphosphate is then converted to cholesterol by integral membrane-bound enzymes of the ER. These include

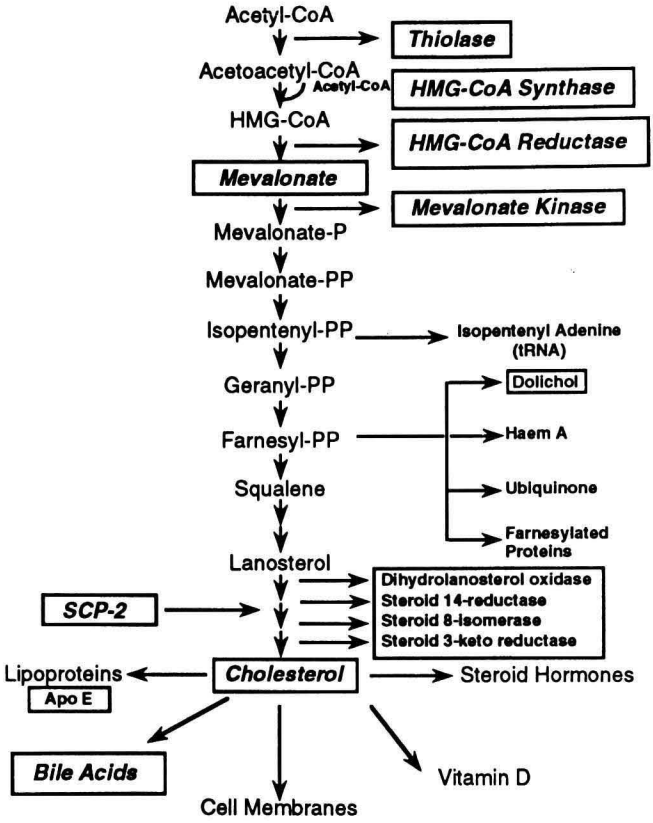

Fig. 1. The Pathway of Cholesterol Biosynthesis Involving Cytosolic Enzymes and Enzymes Localized in the Endoplasmic Reticulum. The initial part of the cholesterol synthesis pathway is the two-step conversion of acetyl-CoA to HMG-CoA. These enzymes are present in the cytosol. In addition, the mitochondria also contain a different set of these enzymes to produce HMG-CoA for ketogenesis. HMG-CoA produced in the cytosol is then converted to mevalonate in the ER by HMG-CoA reductase, the rate limiting enzyme of cholesterol biosynthesis. The further conversion of mevalonate to farnesyl diphosphate requires five enzymes which are present in the cytosol. Farnesyl diphosphate is then converted to cholesterol by integral membrane-bound enzymes of the ER. Farnesyl diphosphate is also utilized for biosynthesis of dolichol (which occurs in the peroxisomes and ER), for biosynthesis of heme A and ubiquinone in the mitochondria, and for biosynthesis of isoprenylated proteins in the cytosol.

squalene synthase to produce squalene, which then cyclizes to lanosterol. The final stages of sterogenesis involve the removal of three methyl groups from lanosterol and the migration and reduction of double bonds to produce cholesterol. At least nineteen discrete reactions are used to convert lanosterol to cholesterol. Farnesyl diphosphate is also utilized for biosynthesis of dolichol (which occurs in the peroxisomes and ER) [1], for biosynthesis of heme A and ubiquinone in the mitochondria, and for biosynthesis of isoprenylated proteins in the cytosol [9].

Cholesterol Synthesis in Peroxisomes

Localization of 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase (HMG-CoA reductase) in Rat Liver Peroxisomes.

HMG-CoA reductase in the ER is a transmembrane glycoprotein composed of two identical 97 kDa subunits. Keller et al. [17] was the first to demonstrate that the enzyme in liver cells is present not only in the ER but also within the peroxisomes. Immunoelectron labeling of ultrathin frozen sections of normal liver, using two monoclonal antibodies to purified rat liver microsomal HMG-CoA reductase, clearly demonstrated that the enzyme is concentrated in the matrix of liver peroxisomes as well as localized in the ER [17].

In another study by Keller et al. [18] experiments were designed to determine quantitatively what percentage of the total liver HMG-CoA reductase activity is attributable to the peroxisomal enzyme and to determine if the peroxisomal and the endoplasmic reticulum enzymes are independently regulated. Normal rat livers and livers obtained from animals in which the total HMG-CoA reductase activity was increased by cholestyramine treatment were fractionated by differential and density gradient centrifugation. Two completely independent analyses of the fractions were performed: 1) immunoelectron microscopy with quantitation of the antigenic sites of HMG-CoA reductase and 2) quantitative enzyme activity measurements with computer assisted analyses [18].

The results demonstrated that 30% of the total rat liver HMG-CoA reductase activity is located in the peroxisomes of cholestyramine treated animals. Cholestyramine treatment also produced a 6-7 fold increase in the specific activity of peroxisomal HMG-CoA reductase whereas the microsomal HMG-CoA reductase specific activity increased by 2 fold. Figure 2 illustrates the immunolabeling for HMG-CoA reductase in peroxisomes after cholestyramine-treatment.

The two studies reached different conclusions regarding the levels of reductase localized in normal peroxisomes. In the second study [18], it was determined that only about 5% of the reductase activity was present under control conditions. Whereas, Keller et al. [17] in the first study estimated that the peroxisomal contribution of total liver reductase in control animals to be 'a substantial fraction, but not more than 50%'. In addition, in the second study the

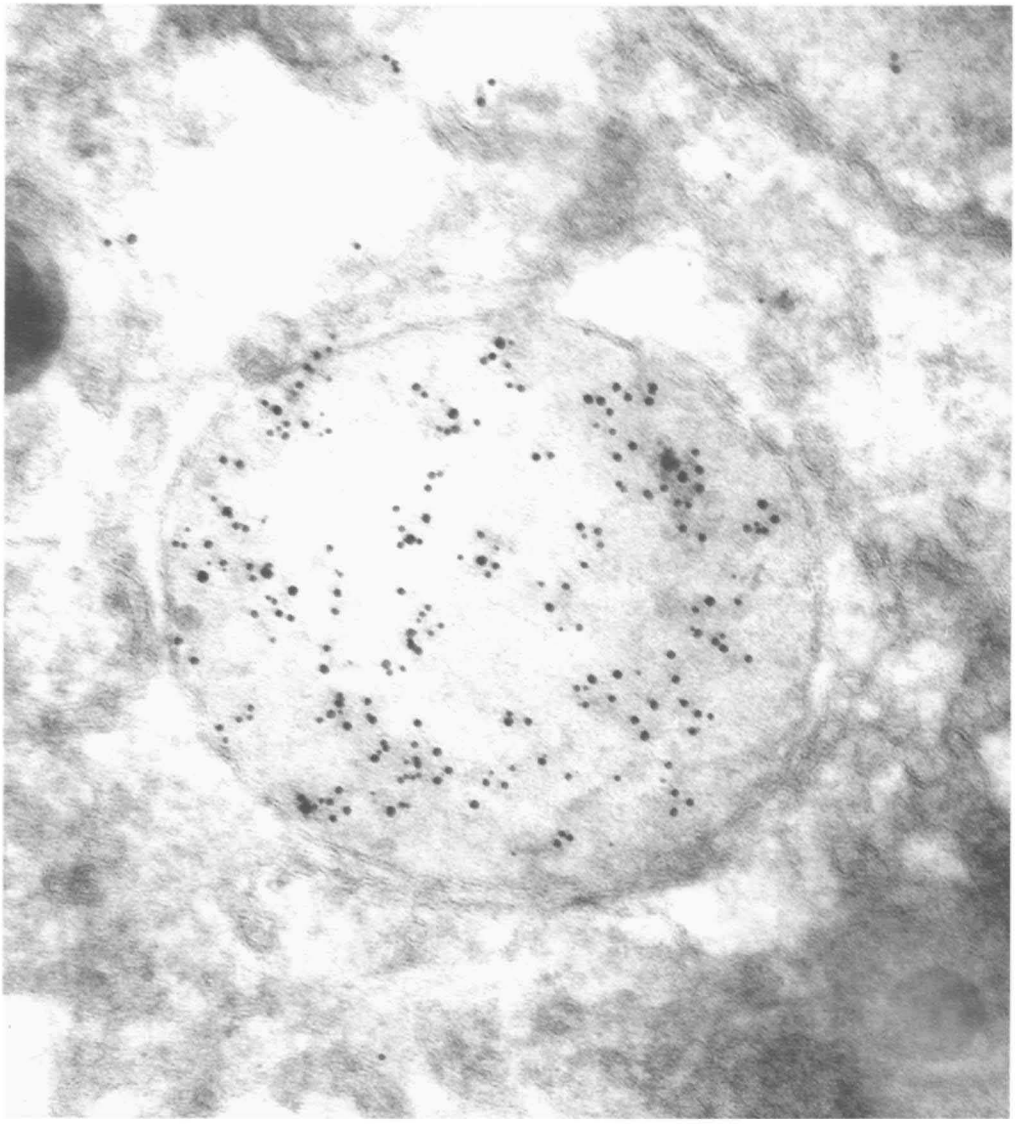

Fig. 2. Representative Electronmicrograph of Liver Cells from Cholestyramine-treated Animals. Cryosections were immunolabeled with monoclonal antibodies to HMG-CoA reductase. In addition to the immunolabeling of the peroxisomal matrix, gold particles can be detected in the area of the endoplasmic reticulum. Electronmicrograph provided by Dr. G. Keller.

density of immunolabeling in peroxisomes from normal animals was considerably lower than reported in the previous study by Keller et al. [17], even though the same reagents were employed. The explanation for this discrepancy was not obvious to us at the time of the study.

Subsequent to this work, we have demonstrated that peroxisomal reductase

activity has a distinctly different diurnal cycle to that of the ER reductase [31]. The highest specific activity of HMG-CoA reductase in the microsomes was at D-6 (6 hours in the dark cycle), consistent with previous reports. In contrast, the highest specific activity of the peroxisomal reductase was measured at 2 hours in the light cycle (L-2). The peroxisomal reductase activity measured at L-2 was 5 to 6 fold greater than the activity measured at D-6. In addition, the specific activity of the peroxisomal reductase at L-2 was greater than the microsomal specific activity at L-2.

These results provide a clear explanation why lower immunolabeling of reductase in peroxisomes was observed in the second study. The second study was performed at the middle of the dark period (peak activity for the ER reductase), whereas in the original study by Keller et al., [17] the electron microscopy was performed on liver samples obtained at the beginning of the light period, which we now recognize is the peak period for peroxisomal reductase. Thus, the percent peroxisomal contribution to total reductase activity depends upon time of measurement, and is considerably greater at 2 hours in the light cycle than at the middle of the dark period.

The localization of HMG-CoA reductase activity in normal rat liver peroxisomes has now been confirmed by other workers [1]. It is interesting to note that the publications report higher reductase specific activity values in peroxisomes as compared to ER, although the time of measurement is not given.

Conversion of mevalonic acid to cholesterol by rat liver peroxisomes.

In an effort to clarify the role of peroxisomal HMG-CoA reductase we next investigated if mevalonic acid could be converted to cholesterol *in vitro* by peroxisomes in the presence of cytosolic proteins [39]. A high resolution three-step procedure was employed to resolve the newly synthesized cholesterol from the complex mixture of sterol intermediates in cholesterol biosynthesis and confirmation of the identity of newly synthesized cholesterol was obtained by recrystallization with added non-radioactive cholestenyl acetate standard. The results showed that highly purified rat liver peroxisomes were able to convert mevalonic acid to cholesterol in the presence of cytosolic proteins *in vitro*. The peroxisomal rate of conversion was 87.12 pmol/mg/h, as compared to the microsomal rate of 135.25 pmol/mg/h. This observation has been confirmed, and further supported by a study which reported that peroxisomes in fact contain dihydrolanosterol oxidase, steroid-14-reductase, steroid-3-ketoreductase and steroid-8-isomerase activities, enzymes required for the conversion of lanosterol to cholesterol [2].

Subcellular Localization of Sterol Carrier Protein-2 in Rat Hepatocytes: Its Primary Localization to Peroxisomes.

We next investigated the subcellular localization of sterol carrier protein-2 (SCP-2), to which highly specific antibodies were available. As purified from rat liver cytosol [28], SCP-2 is a non-enzymatic protein of 13.5 kDa which has been

shown in *in vitro* experiments to be required for several stages in cholesterol utilization and biosynthesis. SCP-2 is presumed to be required in the enzymatic conversion of lanosterol to cholesterol by rat liver microsomal enzymes (28), in the utilization of cholesterol via esterification by acyl-CoA cholesterol acyltransferase [8], and in bile acid synthesis [25]. Using affinity-purified rabbit antibodies against pure SCP-2 from rat liver, we have demonstrated by immunoelectron microscopy that the largest concentration of SCP-2 is inside rat liver peroxisomes [19]. A representative micrograph is shown in Figure 3. These results were confirmed by immunoblotting experiments, utilizing highly purified cell fractions. The results are illustrated in Fig. 4. In the immunoblotting experiments we also observed a band at 55–60 kDa. In a previous study by Wirtz's group [40], a band corresponding to the 55–60 kDa protein was the only immunoreactive component reported in peroxisomal fractions, but the identity

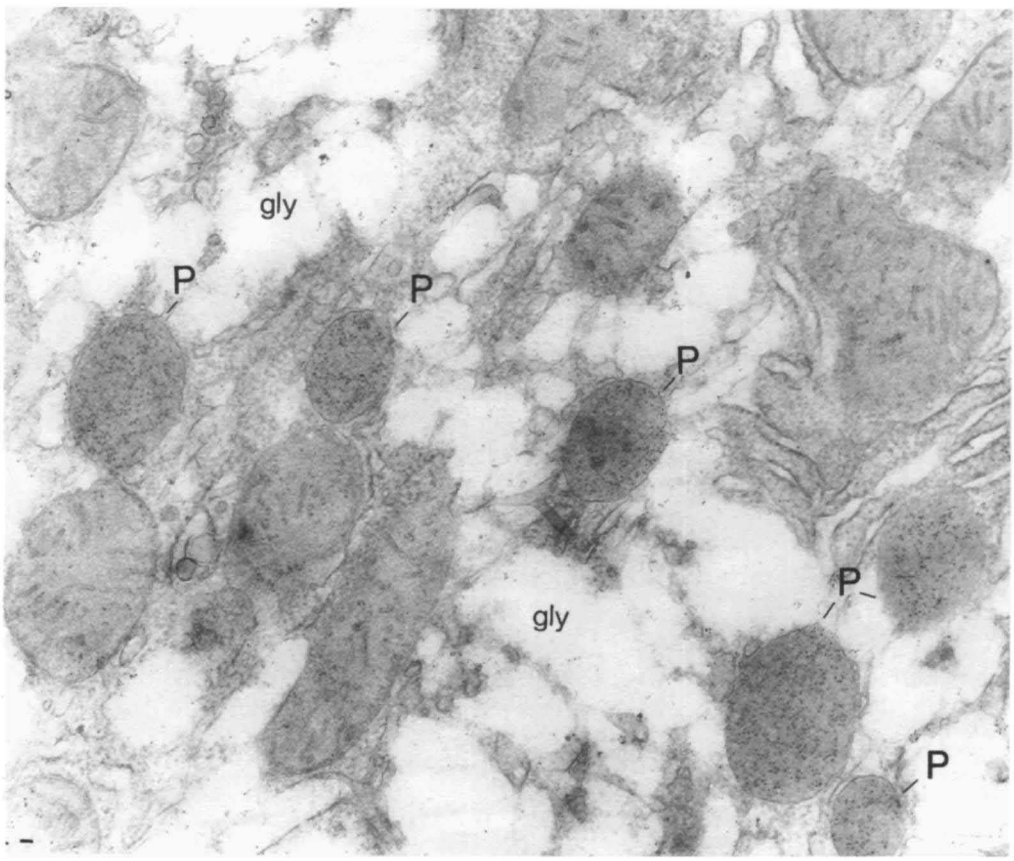

Fig. 3. Representative Electronmicrograph of Liver Cells from Control Animals. Cryosections were immunolabeled with polyclonal antibodies to sterol carrier protein-2. The peroxisomes (P) are intensely and uniformly immunolabeled. The mitochondria and cytosol exhibit a significantly lower level of labeling. Electronmicrograph provided by Dr. G. Keller.

of that component and its relationship to the 13.5 kDa protein was not established. The relationship of SCP-2 to the 55–60 kDa protein has recently been established. It has been demonstrated that the deduced primary sequence of a cDNA encoding the 55–60 kDa protein contains, at the C-terminal region, the full sequence of the 13.5 kDa SCP-2, including the 20 amino acid presequence [34]. Thus, it is not surprising that antibodies made against the 13.5 kDa protein were shown to cross react with the 55–60 kDa protein. The function of this high molecular mass protein is not known. It is significant to note, that both of these proteins contain the C-terminal peroxisome-targeting signal Ala-(Ser)-Lys-Leu

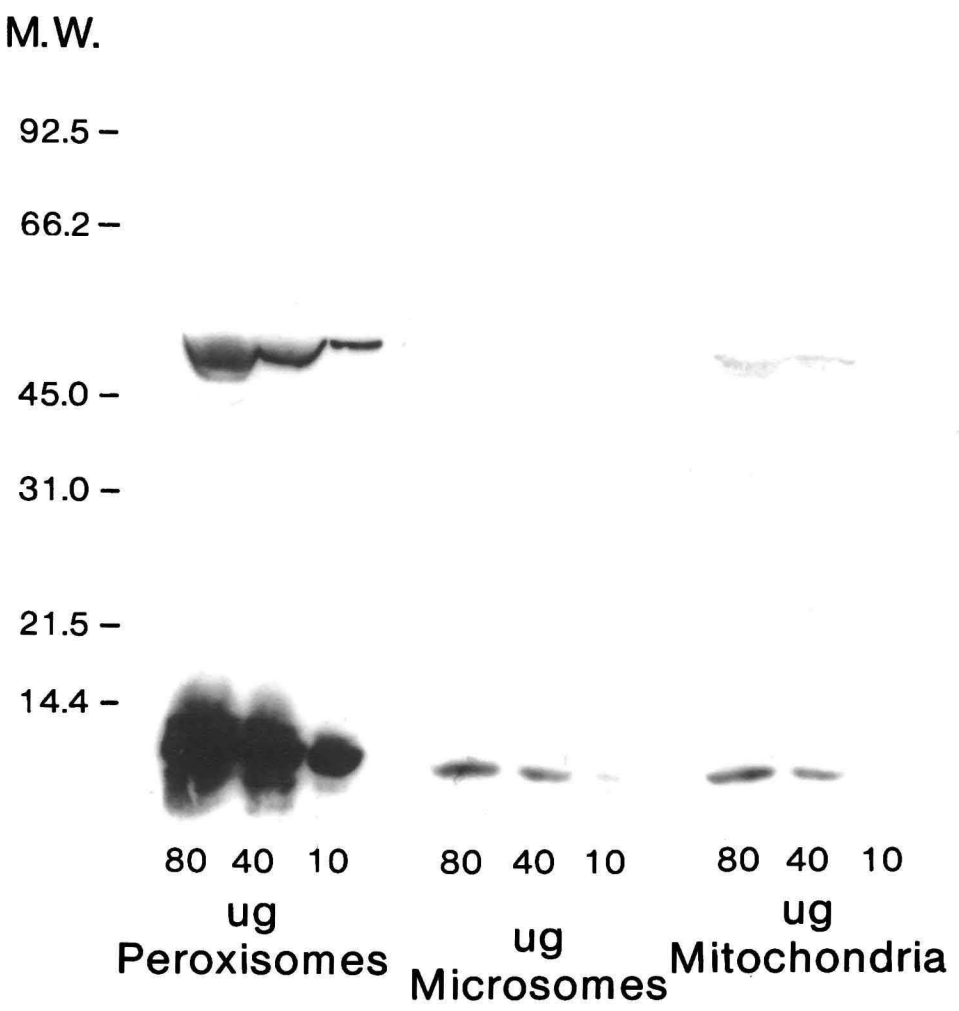

Fig. 4. Immunoblot of Proteins for Sterol Carrier Protein-2. Bands at 13.5 and 55 kDa are the only ones that are immunolabeled. The labeling of the peroxisomal fraction greatly exceeds that of the other fractions.

motif [10]. That peroxisomes contain the largest concentration of SCP-2 is also consistent with the recent findings [37] that SCP-2 is deficient in liver tissue as well as fibroblasts from patients with peroxisomal deficiency diseases.

Localization of Acetoacetyl-CoA Thiolase in Rat Liver Peroxisomes.

It has been well documented that peroxisomes contain at least one thiolase, the peroxisomal β-oxidation thiolase, that accepts long chain 3-ketoacyl CoAs [23]. Figure 5 illustrates the induction of thiolase activity in the peroxisomal, mitochondrial and cytosolic fractions after gemfibrozil-treatment of rats. The thiolase specific activity in the peroxisome fraction increased by 40 fold, and accounted for over 50% of the total after 3 weeks of gemfibrozil-treatment. We have partially purified a thiolase protein from peroxisomes of gemfibrozil-treated

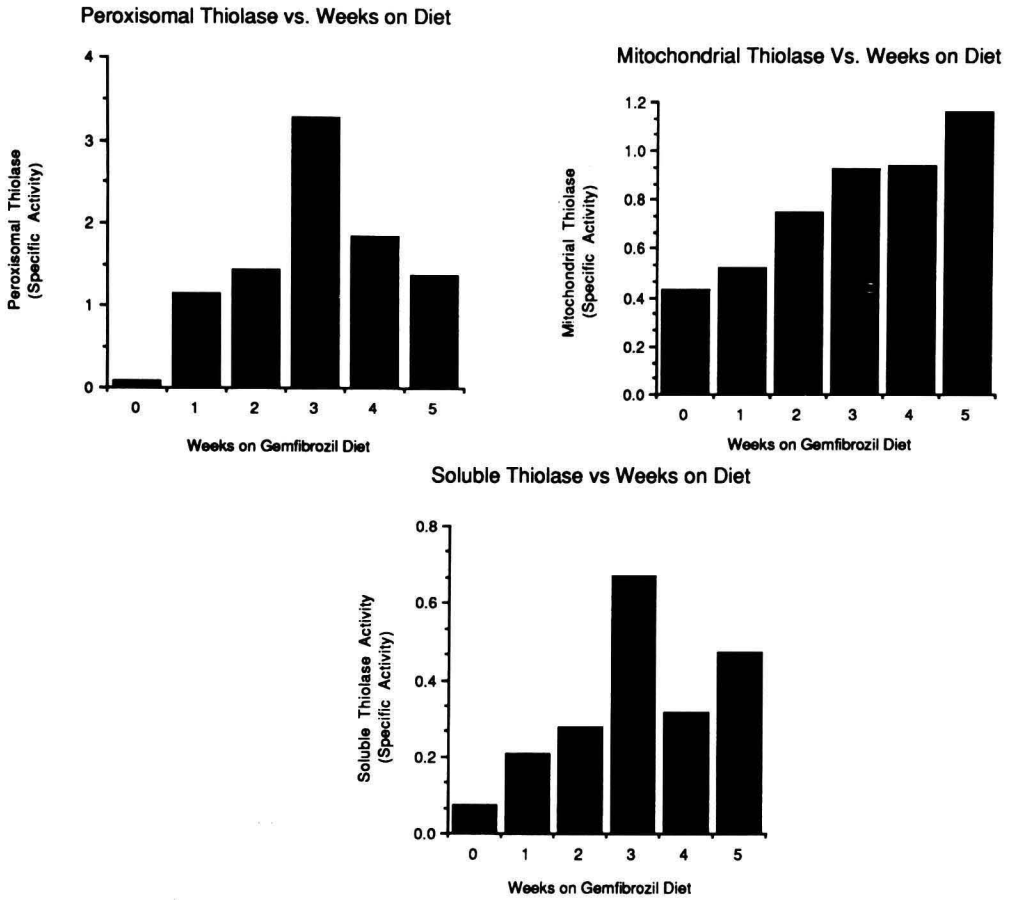

Fig. 5. Effect of Gemfibrozil on the Subcellular Distribution of Thiolase Specific Activity in Rat Liver. Thiolase activity was measured with acetoacetyl-CoA as substrate.

animals and were able to measure by a spectrophotometric assay the formation of acetoacetyl-CoA [38]. Thus, peroxisomes contain a thiolase capable of producing acetoacetyl-CoA [38]. Fig. 6 illustrates the results. This observation has been confirmed by a study in which it was demonstrated that peroxisomes incubated with [^{14}C]acetyl-CoA are able to produce [^{14}C]acetoacetyl-CoA [14].

Whether or not this activity represents another function of the well known β-oxidation thiolase or is a second thiolase is not yet known. The presence of a second thiolase in rat liver peroxisomes has been recently demonstrated [5].

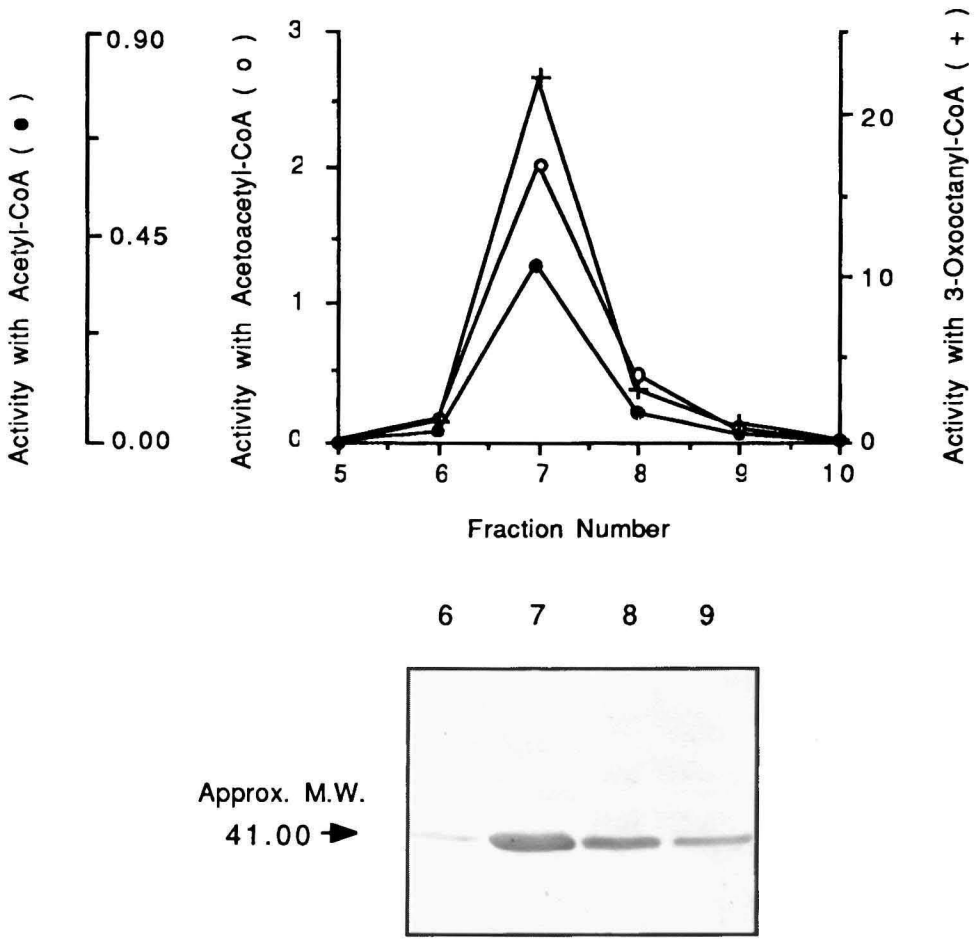

Fig. 6. Comparison of Thiolase Activity. Upper section, comparison of the column elution profiles for thiolase activity using acetoacetyl-CoA, 3-oxooctanoyl-CoA (cleavage activity) and acetyl-CoA (condensation reaction) as substrates. Lower section, immunoblot of peroxisomal 3-ketoacyl-CoA thiolase of the peak elution fractions.

Upon sequencing several cDNA clones, it was revealed that there are at least two distinct thiolase enzymes localized to rat liver peroxisomes, one identical to the previously published rat liver peroxisomal β-oxidation thiolase (thiolase 1) and a novel thiolase (thiolase 2). The mRNA encoding thiolase 2 is induced approximately twofold upon treatment of rats with the peroxisome-proliferating drug, clofibrate. In contrast, the peroxisomal 3-ketoacyl-CoA thiolase (thiolase 1) mRNA is induced more than tenfold under similar conditions. The function of the thiolase 2 in mammalian cells is not known. There is also a report of two thiolases in peroxisomes from yeast [22], one specific for 3-ketoacyl-CoA substrates and the other for acetoacetyl-CoA.

Localization of 3-hydroxy-3-methylglutaryl coenzyme A synthase (HMG-CoA synthase) in Rat Liver Peroxisomes.

We also have preliminary data that suggest that highly purified peroxisomal fractions prepared from gemfibrozil-treated rats contain HMG-CoA synthase activity [21]. Table I illustrates the results. The isolated peroxisomes do not contain any cytosol, as determined by the cytosolic enzyme marker phosphoglucose isomerase. Furthermore, it is unlikely that the measured activity in the peroxisomal fraction is due to the mitochondrial activity, since the mitochondrial synthase requires Triton X-100 for measurement of full activity, whereas, Triton X-100 was found to be inhibitory to the peroxisomal HMG-CoA synthase activity. Studies are in progress to purify and characterize the peroxisomal HMG-CoA synthase.

Localization of Mevalonate Kinase in Rat Liver Peroxisomes.

We recently reported that mevalonate kinase (42 kDa) that was isolated from rat liver and believed to be a cytosolic protein was localized in rat liver peroxisomes and cytosol [36]. In addition, we found that the mevalonate kinase monoclonal antibody used in this study cross reacted with a mitochondrial protein (46 kDa) and a lower molecular weight cytosolic protein (40 kDa).

Table I. Specific activity of HMG-CoA synthase in peroxisomal, mitochondrial and cytoplasmic fractions from liver of gemfibrozil-treated animals.

	nmol/min per mg
Peroxisomes (>95% pure)	1.3
Mitochondria[1] (85% pure)	18.0
Cytosol (>90% pure)	7.2

[1]Mitochondrial HMG-CoA synthase is measured in the presence of 0.2% Triton X-100. This concentration of Triton X-100 inhibits the peroxisomal synthase by 30–50%.

Mevalonate kinase activity could be measured in the peroxisomal and cytosolic fractions but not in the mitochondrial fraction. These data were thought to support either of the two following interpretations: 1) There are two distinct mevalonate kinase proteins, one localized in the peroxisomes (42 kDa) and one in the cytosol (40 kDa). The 42 kDa protein found in the cytosol after treatment with hypolipidemic drugs is due to rupture of peroxisomes. 2) There is only one mevalonate kinase protein which is localized in the peroxisomes. The lower molecular weight protein in the cytosol (40 kDa) is a proteolytic fragment of the peroxisome protein.

We now have data that supports the conclusion that there is only one mevalonate kinase protein that is predominantly localized to peroxisomes. In addition, we also demonstrate that mevalonate kinase is targeted to and imported into peroxisomes [4]. Our conclusion that mevalonate kinase is predominantly localized in peroxisomes is also supported by the data that in cells and tissues obtained from patients with peroxisome deficient diseases (Zellweger syndrome and neonatal adrenoleukodystrophy) mevalonate kinase protein and activity is severely reduced.

In order to determine if the 40 kDa protein observed in the cytosolic fraction is a proteolytic fragment of mevalonate kinase, we prepared and examined four different mevalonate kinase antibodies for cross reactivity with the 40 kDa protein. Three antibodies were raised to synthetic peptides of rat mevalonate kinase. The first peptide corresponded to the first 15 amino acids of the N-terminal portion of the protein (N-MVK, NH_2-M-L-S-E-V-L-L-V-S-A-P-G-K-V-I-COOH), the second peptide corresponded to the last 15 amino acids of the C-terminal portion of mevalonate kinase (C-MVK, NH_2-S-A-T-S-I-E-D-P-V-R-Q-A-L-G-L-COOH), and the third peptide corresponded to 19 amino acids located between amino acids 166 and 184 of the mevalonate kinase protein (M-MVK, NH_2-N-P-L-K-D-R-G-S-I-G-S-W-P-E-E-D-L-K-S-COOH). The fourth antibody was raised against purified mevalonate kinase protein obtained from rat liver of cholestyramine plus mevinolin treated rats (Polyclonal MVK). All four polyclonal mevalonate kinase antibodies (N-MVK, C-MVK, M-MVK and Polyclonal MVK) as well as the monoclonal antibody recognized the 42 kDa mevalonate kinase protein, as would be expected, whereas, the lower molecular weight protein is only recognized by the monoclonal antibody (4). If the 40 kDa protein was indeed a proteolytic fragment of mevalonate kinase we would expect that either the C-MVK or the N-MVK antibody would cross react with the 40 kDa protein as well as the M-MVK and the Polyclonal MVK antibodies. Since this was not observed, we conclude that the lower molecular weight protein is not a proteolytic fragment of mevalonate kinase [4]. Fig. 7 illustrates the localization of mevalonate kinase as determined by immunoblotting of individual gradient fractions. As shown, fractions 17–20 (the peak peroxisomal fractions) contain a substantial amount of immunoreactivity for mevalonate kinase, and fractions 1–8 (containing the cells cytosolic proteins and proteins released from ruptured peroxisomes) also contain significant amounts of mevalonate kinase.

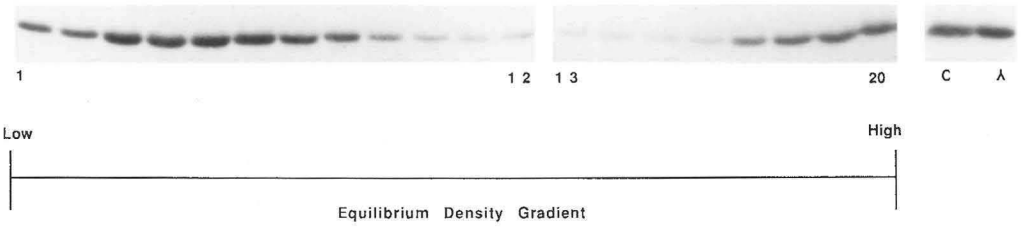

Fig. 7. Immunoblot of Proteins from the Gradient Fractions for Mevalonate Kinase. A peroxisome enriched fraction prepared by differential centrifugation of rat liver homogenates was further purified by equilibrium density centrifugation on a linear Nycodenz density gradient to separate peroxisomes from other cell organelles. Twenty fractions were collected. The gradient fractions were separated by SDS polyacrylamide gel electrophoresis (100 ug of protein), transferred to nitrocellulose and incubated with the C-terminal mevalonate kinase antibody. The highest purity peroxisomal fractions are located at the dense end of the gradient (fractions 17–20). However, a significant amount of the mevalonate kinase is also present at the light end of the gradient (fractions 1–8). 100 ug of a purified cytosol fraction (c) and semi-enriched peroxisomal fraction (l) was also included. Similar distribution profiles are obtained with the N-MVK, M-MVK and polyclonal MVK antibodies.

We have shown previously that mevalonate kinase is easily solubilized during cell homogenization [36]. Thus, cell fractionation studies are not a reliable means of unequivocally determining subcellular localization. Therefore, we investigated the subcellular localization of mevalonate kinase in CV-1 monkey kidney cells after transfection with a plasmid containing the mevalonate kinase cDNA [4]. The results indicate that mevalonate kinase is transported to the peroxisomes in the transfected CV-1 cells. In all transfected cells, the localization of mevalonate kinase was always peroxisomal. Fig. 8 illustrates the immunoflouresence pattern obtained in cultured cells with an antibody that recognizes peroxisomal proteins, with mevalonate kinase antibody and an antibody that recognizes cytosolic proteins. It is clear that the distribution pattern of mevalonate kinase resembles a punctate pattern similar to that of a peroxisomal distribution.

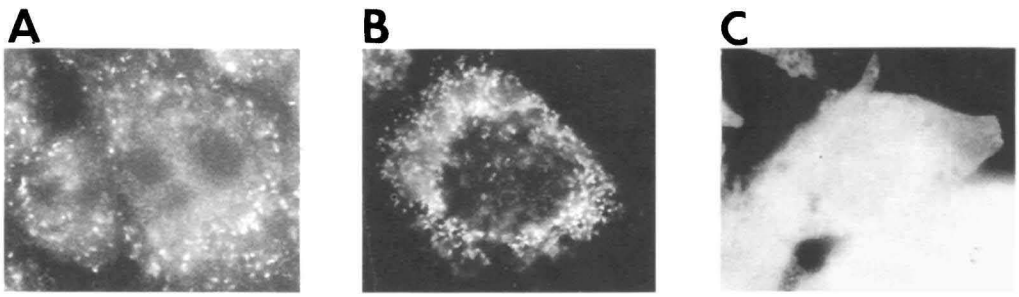

Fig. 8. Mevalonate Kinase is Localized to Peroxisomes in Mammalian Cells. (A) Immunolocalization of peroxisomal proteins (using an antibody made against the peroxisomal targeting signal), (B) the distribution of mevalonate kinase, and (C) the distribution of cytosolic proteins. The immunoflouresence of mevalonate kinase displays a punctate pattern.

To further confirm the localization of mevalonate kinase to peroxisomes, blocks of liver tissues from control and cholestyramine plus mevinolin treated animals were processed for immunoelectron microscopy. Specific immunolabeling for mevalonate kinase was detected in the matrix of the peroxisomes [4].

To determine if mevalonate kinase was defective in patients diagnosed with peroxisomal deficiency diseases, we immunoblotted liver homogenates for mevalonate kinase from four controls and from two patients diagnosed with Zellweger syndrome and one patient diagnosed with neonatal ALD. Mevalonate kinase levels were dramatically reduced (20–26% of control) in the peroxisomal deficient patients samples [4]. In addition, mevalonate kinase activity was also reduced in skin fibroblasts obtained from the peroxisome-deficient patients, further supporting the conclusion of mevalonate kinase localization to peroxisomes.

An important question regarding the presence of cholesterol synthesizing enzymes in peroxisomes is whether they make a significant contribution to total cholesterol biosynthesis. Thus, the finding that mevalonate kinase is predominately, if not exclusively, localized in peroxisomes would suggest that peroxisomes are not only essential for cholesterol biosynthesis but also for all other fundamental products of mevalonate metabolism, including dolichol, ubiquinone and prenylated proteins.

A mutation in the gene coding for mevalonate kinase is presumed to cause the genetic disease mevalonic aciduria, which is the first proposed inherited disorder of the cholesterol biosynthetic pathway in humans [32]. Mevalonic aciduria is transmitted as an autosomal recessive trait, and cells from these patients have less than 10% of the normal levels of mevalonate kinase activity. Given the data presented here, we propose that mevalonic aciduria be included in the growing number of peroxisomal deficiency diseases.

Leukotriene metabolism requires functional peroxisomal β-oxidation enzymes [27]. A recently published study reports elevated levels of these metabolites in mevalonic aciduria patients [26], suggesting that more than one peroxisomal enzyme maybe deficient in these cells. In addition, another study shows that the synthesis of coenzyme Q in skin fibroblasts from these patients is also defective [15]. Studies are in progress to evaluate if other peroxisomal enzymes are deficient in these cells.

Subcellular Localization of Squalene Synthase in Rat Hepatic Cells: Biochemical and Immunochemical Evidence.

Squalene synthase catalyzes the formation of squalene from trans-farnesyl diphosphate 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 has been purified and monospecific antibodies for squalene synthase have been produced [35].

This enabled us to study the subcellular localization of squalene synthase by three different methods; 1) analytical subcellular fractionation and

Table II. Specific activities of enzymes in peroxisomal and microsomal fractions from liver of gemfibrozil-treated animals

	Squalene Synthase[1]	Esterase[2]	Catalase[2]	HMG-CoA Reductase[1]
Peroxisomes	22.7	0.17	8.8	217.0
Microsomes	704.0	4.8	0.15	742.0
Ratio of sp. act[3]	0.032	0.035	58.7	0.292

[1]Specific activity in pmol/min per mg of organelle protein
[2]Specific activity in U/min per mg of organelle protein
[3]Specific activity in peroxisomes divided by specific activity in microsomes

measurements of enzyme activities; 2) immunodeterminations of squalene synthase in the isolated subcellular fractions with a monospecific antibody, and 3) by immunoelectron microscopy techniques.

All three methods gave consistent results [36]. The data clearly illustrate that squalene synthase enzymatic activity and squalene synthase are exclusively localized in the endoplasmic reticulum. The minor quantity of squalene synthase protein and activity found in the peroxisomal fraction could clearly be attributed to the slight contamination of the peroxisomal fractions by the microsomes. This is illustrated by the data in Table II and Fig. 9.

The specific activities of squalene synthase, HMG-CoA reductase, esterase (marker enzyme for microsomes) and catalase (marker enzyme for peroxisomes) were determined for the most highly purified peroxisomal fractions obtained by density gradient centrifugation 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 II shows the specific activities of squalene synthase, HMG-CoA reductase, esterase and catalase in peroxisomal and microsomal fractions obtained from liver of gemfibrozil-treated rats. As can be seen from the ratio of specific

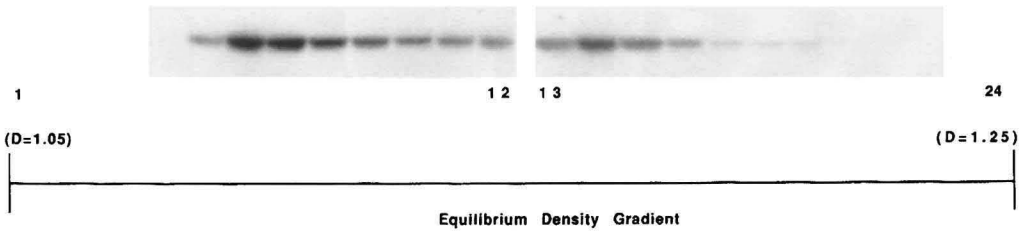

Fig. 9. Immunoblotting of Proteins from Gradient Fractions of Control Animals for Squalene Synthase. The gradient fractions were separated by SDS polyacrylamide gel electrophoresis (100 ug of protein), transferred to nitrocellulose and incubated with a squalene synthase antibody. Fractions in the middle of the gradient represent the ER proteins and exhibit a strong signal with the squalene synthase antibody. Fractions 18–22 contain the highly purified intact peroxisomes and do not exhibit a signal for squalene synthase.

activities, the presence of squalene synthase in peroxisomal fractions can be attributed to the microsomal contamination of the fraction as determined by esterase activity (marker enzyme for microsomes). The ratio of the specific activity of squalene synthase (.032) are in a similar range as the ratio of specific activity of esterase (.035). Whereas, the ratio of the specific activity of HMG-CoA reductase is 0.292, reflecting both a peroxisomal and microsomal localization.

We also determined the relative levels of squalene synthase protein found in the isolated gradient fractions (Fig. 9). As can be seen, there is very little immunoreactivity at the dense end of the gradient where the peroxisomes sedi-

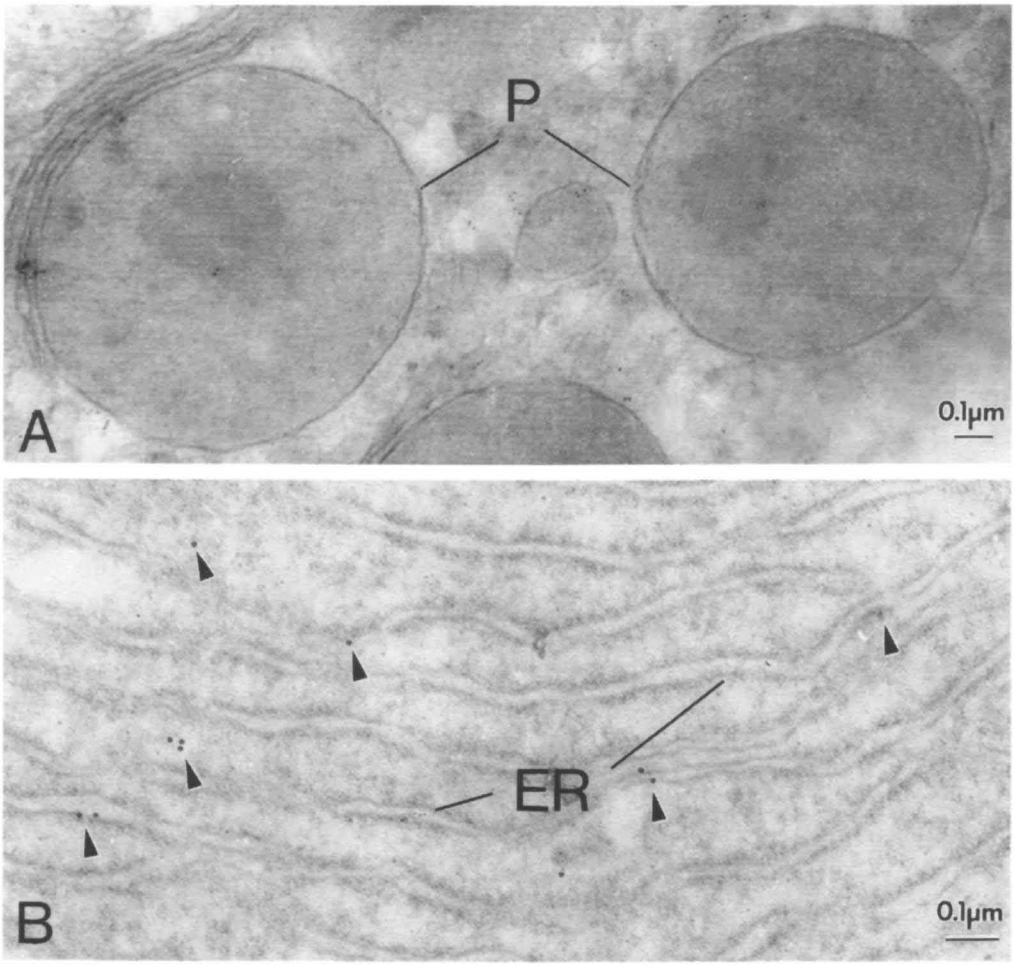

Fig. 10. Representative Electronmicrographs of Liver Cells from Normal Animals. Electron micrograph of cryosections labeled with rabbit antibody to squalene synthase followed by gold adducts of goat antibodies to rabbit IgG. A) Liver peroxisomes from control animals. No immunolabeling can be observed over the two peroxisomes (P) visible in the field. B) Endoplasmic reticulum from control animals. Arrowheads point to gold particles associated with the lamellae of the endoplasmic reticulum. Electronmicrograph provided by Dr. G. Keller.

ment (fractions 20–24) supporting the conclusion that squalene synthase is exclusively localized in the endoplasmic reticulum.

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 (Fig. 10).

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 [24], there are normal levels of squalene synthase [4]. In addition, in liver tissue obtained from these patients there are also normal levels of squalene synthase activity (unpublished data).

The observation that squalene synthase was exclusively in the ER was unexpected, since as mentioned previously, in a earlier publication we reported that highly purified rat liver peroxisomes are able to convert mevalonic acid to cholesterol in the presence of an added cytosolic fraction [39]. It is clear from the data presented above, that the squalene synthase activity required for this conversion was not originally peroxisomal. Rather, a more likely explanation is that the activity for squalene synthase came from the cytosol fraction. In fact, it was recently demonstrated that microsomal squalene synthase can be endogenously proteolytically cleaved, solubilized and retain enzymatic activity [35]. This solubilized portion of the protein retains catalytic activity and after subcellular fractionation is found in the cytosol fraction. This would explain the observation that peroxisomes plus cytosol were able to synthesize cholesterol. However, it does raise questions of how many of the other enzymes involved in the biosynthesis of cholesterol could potentially exhibit similar behavior and therefore be assigned the incorrect subcellular localization.

A recent study was published reporting the presence of squalene synthase activity in rat peroxisomes [7]. This conclusion was based solely on the measurement of squalene synthase activity in two fractions: a microsomal and a peroxisomal fraction.

Cholesterol Synthesis in Cultured Skin Fibroblasts from Patients with Peroxisome-deficiencies.

In humans the vital importance of peroxisomes for cellular and developmental processes has been clearly demonstrated by the recognition of a number of recessive, inherited diseases which cause defects in the biogenesis of the peroxisome [24]. Somatic cell fusion studies indicate that fibroblasts from these patients can be placed into a number of different complementation groups.

The relative importance of peroxisomes in cholesterol synthesis in human cells was determined by comparing cholesterol synthesis rates in cultured skin fibroblasts from two normal individuals, to sixteen patients with peroxisomal deficiencies and three patients diagnosed with rhizomelic chondrodysplasia punctata (RCDP) [13]. Though RCDP is characterized by reduced peroxisomal plasmalogen biosynthesis, most other peroxisomal activities do

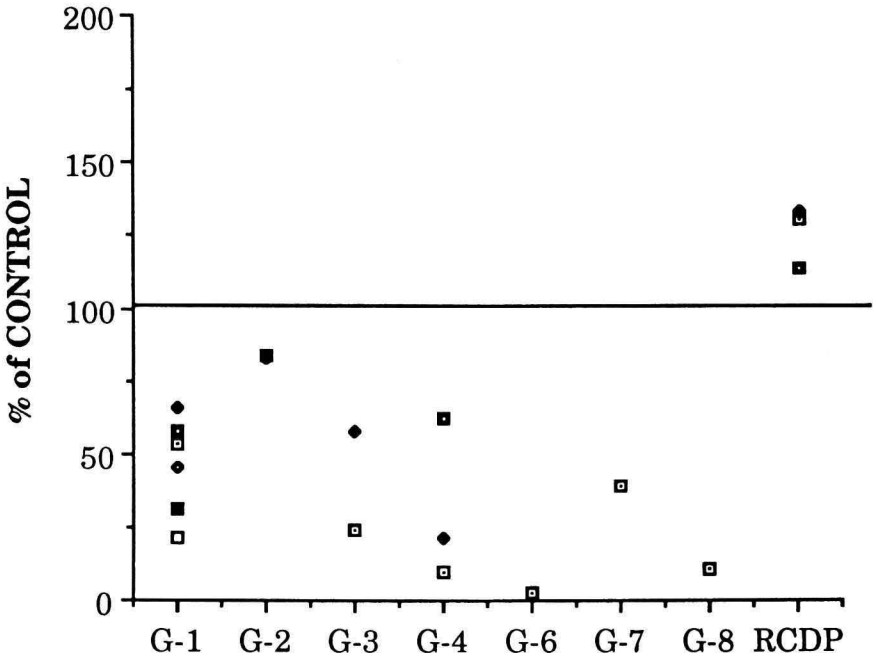

Fig. 11. Cholesterol Synthesis in Fibroblasts of Patients in each of the Seven Complementation Groups and in Fibroblasts of Patients with RCDP. The results are expressed as mean of % of control (taken as 100%) Values obtained from five of the 16 cell lines were not significantly different from control values. All other cell lines were different ($P < 0.05$). The cell lines in Groups 6, 8 and one cell line from Group 1 (45% of control) were diagnosed with the NALD phenotype. One cell line from Group 1 (66% of control), was assigned the hyperpipecolic acidemia phenotype and all the rest were Zellweger phenotypes. Cholesterol synthesis in the three RCDP cell cultures was not significantly different from control values.

not appear to be seriously affected, and peroxisome morphology seems normal [24].

Seven complementation groups were studied, consisting of one six member group, one three member group, three groups comprising single cases and two groups with two cases each. Fig. 11 illustrates the results. The average values for cholesterol synthesis were below control values in all the 16 peroxisome-deficient fibroblast cell cultures. The range of cholesterol synthesis in these cells was 2% to 84% of control values. In contrast, cholesterol synthesis in the three RCDP cell lines was not significantly different from control values [13].

The defect in cholesterol synthesis demonstrated for the peroxisome-deficient fibroblasts may be due to a deficiency in one or more of the cholesterol synthesizing enzymes that have been demonstrated to be localized in peroxisomes. A specifically attractive possibility would be HMG-CoA reductase since it catalyzes the rate limiting step in cholesterol biosynthesis. However, it is plausible that another peroxisomal enzyme or protein in the pathway (such as acetoacetyl-CoA thiolase, mevalonate kinase, or SCP-2) is so deficient in these cells that HMG-CoA reductase is no longer the rate limiting step.

A recent abstract has confirmed our finding that cholesterol synthesis is severely reduced in these patients and in addition data are presented that imply that the LDL receptor regulation may also be affected in these cell lines [6].

Other general metabolic functions such as protein synthesis, lysosomal acid lipase activity, and mitochondrial butyrate oxidation have been shown to be normal in the Zellweger fibroblasts [10].

Summary and Conclusions

In summary, we have demonstrated that peroxisomes contain acetoacetyl CoA thiolase, HMG-CoA synthase, HMG-CoA reductase, mevalonate kinase, and sterol carrier protein-2. In addition, peroxisomes contain significant levels of apolipoprotein E, a major constituent of several classes of plasma lipoproteins [12]. The pathway for cholesterol synthesis and the steps where peroxisomes have been shown to be involved (boxed area) by our group and others is illustrated in Fig. 12.

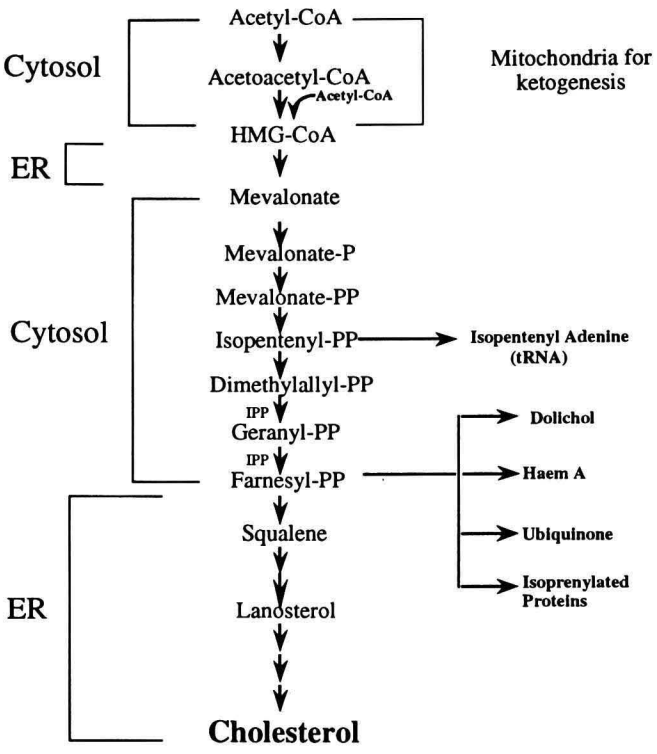

Fig. 12. The Cholesterol Synthesis Pathway in Animal Cells. The cholesterol synthesis enzymes that have been demonstrated to be localized in peroxisomes as well as other steps in cholesterol metabolism where peroxisomes have been shown to be involved are represented by the boxed areas.

Thus, the presence of cholesterol synthetic enzymes in peroxisomes [1,2,4,14,17,18,19,36,38], the low serum cholesterol levels in patients suffering from peroxisomal deficiency diseases [3,29,30,33] and the reduced cholesterol synthetic capacity of cells lacking peroxisomes [6,13] argue quite strongly that peroxisomes are essential for normal cholesterol synthesis.

As more highly specific antibodies to cholesterol synthesis enzymes become available, it will be interesting to see how many other proteins that are involved in cholesterol metabolism may actually be peroxisomal.

References

1. Appelkvist, E.L. and A. Kalen. Biosynthesis of dolichol by rat liver peroxisomes. *Eur. J. Biochem.* **185**, 503–509, 1989.
2. Appelkvist, E.L., M. Reinhart, R. Fischer, J. Billheimer and G. Dallner. Presence of individual enzymes of cholesterol biosynthesis in rat liver peroxisomes. *Arch. Biocheml. Biophys.* **282**, 318–325, 1990.
3. Barth, P.G., R.B.H. Schutgens, R.J.A. Wanders, H.S.A. Heymans, A.E. Moser, H.W. Moser, E.M. Bleeker-Wagemakers, K. Jansonius-Schultheiss, M. Derix and G. F. Nelck. A sibship with a mild variant of Zellwger syndrome. *J. Inher. Metab. Dis.* **10**, 253–259, 1987.
4. Biardi, L., A. Sreedhar, A. Zokaei, R. Bozeat, N.B. Vartak, J.E. Shackelford, G.A. Keller and S.K. Krisans. Mevalonate kinase is predominantly localized in peroxisomes and is defective in patients with peroxisome deficient disorders. *J. Biol. Chem.* **269**, 1–9, 1994.
5. Bodnar, A.G. and R.A. Rachubinski. Cloning and sequence determination of cDNA encoding a second rat liver peroxisomal 3-ketoacyl-CoA thiolase. *Gene* **91**, 193–199, 1990.
6. Collins, C.J., F. Keyserman, S.C. Rumsey and R.J. Deckelbaum. Peroxisomal disease patients have Hypocholesterolemia and abnormal cholesterol synthesis and LDL receptor regulation in vitro. *American Heart Association Annual Meeting.* Abstract.
7. Ericsson, J., E. Appelkvist, A. Thelin, T. Chojnacki and G. Dallner. Isoprenoid biosynthesis in rat liver peroxisomes. *J. Biol. Chem.* **267**, 18708–18714, 1992.
8. Gavey, K.L., B.L. Noland and T.J. Scallen. The participation of sterol carrier protein 2 in the conversion of cholesterol to cholesterol ester by rat liver microsomes. *J. Biol. Chem.* **256**, 2993–2999, 1981.
9. Goldstein, J.L. and M.S. Brown. Regulation of the mevalonate pathway. *Nature* **343**, 425–430, 1990.
10. Gordon, J.A., P.H. Figard and A.A. Spector. Hydroxyeicosatetraenoic acid metabolism in cultured human skin fibroblasts. *J. Clin. Invest.* **85**, 1173–1181, 1990.
11. Gould, S.J., G.A. Keller, N. Hosken, J. Wilkinson and S. Subramani. A conserved tripeptide sorts proteins to peroxisomes. *J. Cell Biol.* **108**, 1657–1664, 1989.

12. Hamilton, R.L., J.S. Wong, L.S. Guo, S. Krisans and R.J. Havel. Apolipoprotein E localization in rat hepatocytes by immunogold labeling of cryothin sections. *J. Lipid Res.* **31**, 1589–1604, 1990.
13. Hodge, V.J., S.J. Gould, S. Subramani, H.W. Moser and S.K. Krisans. Normal cholesterol synthesis in human cells requires functional peroxisomes. *Biochem. Biophys. Res. Commun.* **181**, 537–541, 1991.
14. Hovik, R., B. Brodal, K. Bartlett and H. Osmundsen. Metabolism of acetyl-CoA by isolated peroxisomal fraction: formation of acetate and acetoacetyl-CoA. *J. Lipid Res.* **32**, 993–999, 1991.
15. Hubner, C., G.F. Hoffmann, C. Charpentier, K.M. Gibson, B. Finckh, H. Puhl, H.A. Lehr and A. Kohlschutter. Decrased plasma ubiquinone-10 concentration in patients with mevalonate kinase deficiency. *Pediatric Research* **34**, 129–133, 1993.
16. K.D. Stamellos, J.E. Shackelford, R.D. Tanaka and S.K. Krisans. Mevalonate kinase is localized in rat liver peroxisomes. *J. Biol. Chem.* **267**, 5560–5568, 1992.
17. Keller, G.A., M.C. Barton, D.J. Shapiro and S.J. Singer. 3-Hydroxy-3-methylglutaryl-coenzyme A reductase is present in peroxisomes in normal rat liver cells. *Proc. Natl. Acad. Sci. USA* **82**, 770–774, 1985.
18. Keller, G.A., M. Pazirandeh and S. Krisans. 3-Hydroxy-3-methylglutaryl coenzyme A reductase localization in rat liver peroxisomes and microsomes of control and cholestyramine-treated animals: quantitative biochemical and immunoelectron microscopical analysis. *J. Cell Biol.* **103**, 875–886, 1986.
19. Keller, G.A., T.J. Scallen, D. Clarke, P.A. Maher, S.K. Krisans and S.J. Singer. Subcellular localization of sterol carrier protein-2 in rat hepatocytes: its primary localization to peroxisomes. *J. Cell Biol.* **108**, 1353–1361, 1989.
20. Krisans, S.K. The role of peroxisomes in cholesterol metabolism. *Amer. J. Resp. Cell Mol. Biol.* **7**, 358–364, 1992.
21. Krisans, S.K., N. Rusnak, G.A. Keller and P.A. Edwards. Localization of 3-Hydroxy-3-methylglutaryl coenzyme A synthase in rat liver peroxisomes. *J. Cell Biol.* **107**, 122, 1988.
22. Kurihara, T., M. Ueda and A. Tanaka. Occurrence and possible roles of acetoacetyl-CoA thiolase and 3-ketoacyl-CoA thiolase in peroxisomes of an n-alkane-grown yeast, Candida tropicalis. *FEBS Lett.* **229**, 215–218, 1988.
23. Lazarow, P.B. Compartmentation of beta-oxidation of fatty acids in peroxisomes. **In**: H. Sies, (ed.). *Metabolic Compartmentation.* Academic Press. New York. 317–329, 1982.
24. Lazarow, P.B. and H.W. Moser. Disorders of peroxisome biogenesis. **In**: C.R. Shriver, A.L. Beaudet, W.S. Sly and D. Valle (eds.). *The Metabolic Basis of Inherited Disease.* McGraw Hill. **sixth edition**, 1479–1509, 1989.
25. Lidstrom-Olsson, B. and K. Wikvall. The role of sterol carrier protein 2 and other hepatic lipid-binding proteins in bile-acid biosynthesis. *Biochem. J.* **238**, 879–884, 1986.

26. Mayatepek, E., G.F. Hoffmann and H.J. Bremer. Enhanced urinary excretion of leukotriene E4 in patients with mevalonate kinase deficiency. *J. Pediatr.* **123**, 96-98, 1993.
27. Mayatepek, E., W.D. Lehmann, J. Fauler, D. Tsikas, J.C. Frolich, R.B.H. Schutgens, R.J.A. Wanders and D. Keppler. Impaired degradation of leukotrienes in patients with peroxisome deficiency disorders. *J. Clin. Invest.* **91**, 881–888, 1993.
28. Noland, B.J., R.E. Arebalo, E. Hansbury and T.J. Scallen. Purification and properties of sterol carrier protein. *J. Biol. Chem.* **255**, 4282–4289, 1980.
29. Poll-The, B.T., J.M. Saudubray, H.A.M. Ogier, M. Odievre, J.M. Scotto, L. Monnens, L.C.P. Govaerts, F. Roels, A. Cornelis, R.B.H. Schutgens, R.J.A. Wanders, A.W. Schram and J.M. Tager. Infantile Refsum disease: an inherited peroxisomal disorder; comparison with Zellweger syndrome and neonatal adrenoleukodystrophy. *Eur. J. Pediatr.* **146**, 477–483, 1987.
30. Poulos, A. and P. Sharp. Plasma and skin fibroblast C_{26} fatty acids in infantile Refsum's disease. *Neurol.* **34**, 1606–1609, 1984.
31. Rusnak, N. and S.K. Krisans. Diurnal variation of HMG-CoA reductase activity in rat liver peroxisomes. *Biochem. Biophys. Res. Commun.* **148**, 890–895, 1987.
32. Schafer, B.L., R.W. Bishop, V.J. Kretuniis, S.S. Kalinowski, S.T. Mosley, K.M. Gibson and R.D. Tanaka. Molecular cloning of human mevalonate kinase and identification of a missense mutation in the genetic disease Mevalonic Aciduria. *J. Biol. Chem.* **267**, 13229–13238, 1992.
33. Scotto, J.M., M. Hadchouel, M. Odievre, M.H. Laudat, J.M. Saudubray, O. Dulac, I. Beucler and P. Beaune. Infantile phytanic acid storage disease, a possible variant of Refsum's disease: three cases, including ultrastructural studies of the liver. *J. Inher. Metab. Dis.* **5**, 83–89, 1982.
34. Seedorf, U. and G. Assmann. Cloning, expression, and nucleotide sequence of rat liver sterol carrier protein 2 cDNAs. *J. Biol. Chem.* **266**, 630–636, 1991.
35. Shechter, I., E. Klinger, L.M. Rucker, R.G. Engstrom, J.A. Sirito, M.A. Islam, B.R. Boettcher and B.D. Weinstein. Solubilization, purification, and characterization of a truncated form of rat hepatic squalene synthetase. *J. Biol. Chem.* **267**, 8628–8635, 1992.
36. Stamellos, K. D, J.E. Shackelford, I. Shechter, G. Jiang, D. Conrad, G.A. Keller and S.K. Krisans. Subcellular Localization of Squalene Synthase in Rat Hepatic Cells: Biochemical and Immunochemical Evidence. *J. Biol. Chem.* **268**, 12825–12836, 1993.
37. Suzuki, Y., S. Yamaguchi, T. Orii, M. Tsuneoka and Y. Tashiro. Nonspecific lipid transfer protein (sterol carrier protein-2) defective in patients with deficient peroxisomes. *Cell Struct. Funct.* **15**, 301–308, 1990.
38. Thompson, S.L. and S.K. Krisans. Rat liver peroxisomes catalyze the initial step in cholesterol synthesis: the condensation of acetyl-CoA units into acetoacetyl-CoA. *J. Biol. Chem.* **265**, 5731–5735, 1990.

39. Thompson, S.L., R. Burrows, R.J. Laub and S.K. Krisans. Cholesterol synthesis in rat liver peroxisomes: conversion of mevalonic acid to cholesterol. *J. Biol. Chem.* **262**, 17420–17425, 1987.
40. Van der Krift, T. P., J. Leunissen, T. Teerlink, G.P.H. van Heusden, A.J. Verkleij and K.W.A. Wirtz. Ultrastructural localization of a peroxisomal protein in rat liver using specific antibody against the non-specific lipid transfer protein (sterol carrier protein-2). *Biochim. Biophys. Acta.* **812**, 387–392, 1985.

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