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3-Hydroxy-3-methylglutaryl-coenzyme A reductase is present in peroxisomes in normal rat liver cells

(immunoelectron microscopy/subcellular fractionation/cholesterol metabolism)

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ABSTRACT The location inside rat liver parenchymal cells of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase; EC 1.1.1.34), the key regulatory enzyme in cholesterol biosynthesis, has been examined by immunoelectron microscopy and by subcellular fractionation. Although HMG-CoA reductase is generally thought to be exclusively a microsomal enzyme, we find that a substantial portion of cellular HMG-CoA reductase is localized in peroxisomes. Immunoelectron microscopic labeling of ultrathin frozen sections of normal rat liver, using two monoclonal antibodies to purified HMG-CoA reductase, showed that the enzyme is present in the peroxisomes at a higher concentration than at any other site inside the hepatocytes. Subcellular fractionation studies using Percoll and metrizamide gradients demonstrated a close correspondence of peaks of HMG-CoA reductase activity and of catalase activity, again revealing the presence of the reductase enzyme in peroxisomes. HMG-CoA reductase is therefore localized in peroxisomes in addition to being in the microsomal fraction.

As a key regulatory enzyme in biosynthesis of cholesterol, dolichol, and isopentenyl adenosine, 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase; EC 1.1.1.34) is the subject of intense study (1–3). The intracellular location of the enzyme should play an important role in its regulation. It is currently thought that the active enzyme is primarily, if not exclusively, localized to the endoplasmic reticulum. This view is based in part on early subcellular fractionation experiments (4), in which the enzyme activity was found in a postmitochondrial supernatant and was shown to be sedimentable at high *g* forces. More detailed studies, which involved fractionation of the endoplasmic reticulum (5), lent credence to the view that HMG-CoA reductase is localized in the microsomal fraction. Recent molecular biological studies on the primary structure (6) and the *in vitro* biosynthesis (7) of the enzyme provided additional support for this idea (see *Discussion*). The availability of specific antibodies directed to HMG-CoA reductase, both monoclonal (8) and polyclonal (9, 10), allows one, in principle at least, to carry out immunoelectron microscopic experiments to explore directly the intracellular localization of the enzyme. Heretofore, however, immunolabeling experiments have not been reported with normal cells such as hepatocytes; presumably the low total concentration of the enzyme in liver cells made it seem unlikely that adequate immunolabeling would be achieved. In UT-1 cells, a specially selected line of Chinese hamster ovary cells that produces over 100 times as much HMG-CoA reductase as the parental cells, immunoelectron microscopic localization of the enzyme has been investigated (11). In these cells, immunolabeling for the enzyme was reported to be associated with a unique intracellular membranous structure, termed the "crystalloid endoplasmic reticulum" (12), whose origin and composition are not known, and whose physiological relevance to normal cell structures is therefore unclear. In this paper, we report immunoelectron microscopic experiments on normal rat liver carried out by immunolabeling of ultrathin frozen sections of the tissue (13, 14) with two monoclonal antibodies (8) directed to purified rat liver microsomal HMG-CoA reductase. We obtained the unexpected result that a high concentration of the protein was detected in the peroxisomes of the liver cells. The subcellular localization of HMG-CoA reductase was therefore reexamined in cell fractionation studies. We found that, indeed, a significant amount of activity is associated with the peroxisomal fraction. These findings may have important implications both for studies of the regulation of HMG-CoA reductase and for models of peroxisome biogenesis.

A preliminary report of these results has appeared (15).

MATERIALS AND METHODS

Antibody Reagents. Mouse IgG monoclonal antibodies A and B (8), prepared against electrophoretically homogeneous HMG-CoA reductase isolated from rat liver microsomes, were used in the form of the 40% or 50% saturated ammonium sulfate fraction of mouse ascites fluid. The control monoclonal antibody JG-9 (16) is directed to a cell-surface antigen of chicken cells that is antigenically unrelated to HMG-CoA reductase. Polyclonal goat antibodies to rat liver catalase were the gift of Paul Lazarow (Rockefeller University) and were used in the form of the IgG fraction. Affinity-purified rabbit antibodies to mouse IgG and affinity-purified rabbit antibodies to goat IgG were adsorbed (17) with colloidal gold particles (18) of 6- to 8-nm diameter and used as the secondary labeling reagents for the mouse monoclonal antibodies and the goat anti-catalase antibodies, respectively.

Immunoelectron Microscopy. Female Wistar rats, 140–180 g body weight, maintained on normal diet, were obtained from Daniel Steinberg. The liver was fixed by portal perfusion with 4% (wt/vol) formaldehyde in 0.1 M sodium phosphate buffer, pH 7.2, containing 0.05 M lysine and 0.05 M sodium periodate (PLP fixative) (19). Blocks (1 mm^2) of the fixed liver were subsequently incubated in the same fixative for 4 hr. The blocks were then infused with 2.3 M sucrose, frozen, and ultrasectioned in the frozen state at −90°C in a Sorvall cryoultramicrotome as described (13, 14). The thawed sections, mounted on carbon-coated conditioned electron microscope grids, were then treated with one of the monoclonal antibodies: A (at 10 μg/ml), B (at 30 μg/ml), or JG-9 (at 30 μg/ml); or with the goat anti-catalase antibodies (at 65 μg/ml). After washing, the sections were then treated with the appropriate colloidal gold/antibody reagent. The immunolabeled sections were then either (*i*) positively

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Abbreviation: HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A.

stained by the absorption method of Tokuyasu (20) and embedded in Carbowax (Fig. 1 *A* and *B*) or (*ii*) treated with osmium tetroxide, dehydrated, and embedded in an ultrathin section of LR white acrylic resin by the procedure of Keller *et al.* (18) (Fig. 1 *C* and *D*). The sections were then examined in a Philips model 300 transmission electron microscope at 80 kV.

Subcellular Fractionation Studies. Female Sprague-Dawley rats (Murphy Breeding) fed a diet of 5% cholestyramine plus mevinolin [122 mg/100 g of feed (9)] were killed at the peak of the circadian rhythm of HMG-CoA reductase. The excised livers were homogenized in 10 vol of 250 mM sucrose/0.1% ethanol/1 mM dithiothreitol/0.1 mM leupeptin (Sigma), and a subcellular fraction L was obtained, essentially as described by Neat *et al.* (21). This preparation was then subjected to centrifugation on a 50% (vol/vol) gradient of Percoll (Pharmacia) (21), or on a 20-50% (wt/vol) gradient of metrizamide (Accurate Chemical and Scientific, Westbury, NY) (22). Percoll and metrizamide gradient centrifugations were performed in a VTi 50 rotor (Beckman) at 40,000 rpm for 1 hr at 4°C. Fractions were collected from the top of each gradient and assayed for HMG-CoA reductase (23) and for each of the following marker enzymes as described: catalase for peroxisomes (24); acid phosphatase for lysosomes (25); cytochrome *c* oxidase for mitochondria (26); and glucose-6-phosphatase for microsomes (27). The assay for HMG-CoA reductase involved a preincubation at 37°C for 20 min, conditions that inactivate the HMG-CoA lyase activity present in fractions containing mitochondria (28). The reaction product resulting from the action of HMG-CoA reductase on [^{14}C]HMG-CoA was identified as mevalonate by cochromatography with an authentic [^{3}H]mevalonic acid standard in three different thin-layer chromatographic systems (29).

RESULTS

Immunoelectron Microscopic Labeling of HMG-CoA Reductase in Rat Liver. Indirect colloidal gold immunolabeling of HMG-CoA reductase, using monoclonal antibody A as the primary antibody reagent, gave results represented by Fig. 1*A*. Intense labeling of all of the peroxisomes can be seen, mostly in their interior region but generally excluding the crystalloid core. Very little labeling is found elsewhere on the section. From a large number of such specimens, with at least 10 animals in studies carried out over a period of more than a year, we have found that the peroxisomes are always labeled and there is no detectable labeling over background of nuclei, mitochondria, lysosomes, glycogen particles, elements of the Golgi apparatus, or the rough endoplasmic reticulum. Occasionally, a small number of labels over the smooth endoplasmic reticulum (as is indicated by the open arrows in Fig. 1*A*) was observed. Similar results were obtained with monoclonal antibody B (Fig. 1*B*), except that the density of gold labeling of the peroxisomes was generally lower than with monoclonal antibody A. The control monoclonal antibody JG-9, used as a primary reagent under the same conditions, did not give any labeling of peroxisomes (Fig. 1*C*) or of any other structures in the liver cells. Furthermore, in unpublished immunolabeling studies with several polyclonal rabbit antibodies directed to secretory proteins of liver, we have never observed any significant peroxisomal labeling. With a polyclonal goat antibody to rat liver catalase, however, we obtained a high density of specific immunolabeling of peroxisomes (Fig. 1*D*), as expected.

Localization of HMG-CoA Reductase by Subcellular Fractionation. In a series of biochemical fractionation studies that complement the electron microscopy experiments, HMG-CoA reductase was also localized in peroxisomes. Metrizamide gradients were employed because they separate peroxisomes from other subcellular organelles without Triton WR1339 treatment (30). Gradient fractionation of cell homogenates in a 20-50% metrizamide gradient demonstrated that the peak of HMG-CoA reductase activity most closely corresponds to the peak of catalase activity (Fig. 2). In this gradient, fractions that contained 59% of the total catalase activity on the gradient, and 61% of the HMG-CoA reductase activity, were contaminated with only 8% of the activity of the endoplasmic reticulum marker enzyme, glucose-6-phosphatase.

To confirm these observations, we also employed an entirely different density gradient fractionation medium, Percoll (21). Resolution is somewhat lower with this procedure than with metrizamide gradients. Although significant leakage of both catalase and HMG-CoA reductase from peroxisomes occurs [Fig. 3, fractions 1-3 (22)], the HMG-CoA reductase and catalase activities again codistribute (Fig. 3, fractions 6 and 7). These data provide strong evidence for the peroxisomal localization of HMG-CoA reductase, as the sedimentation patterns of endoplasmic reticulum and the subcellular organelles are very different on Percoll and metrizamide gradients. Significant levels of HMG-CoA reductase were observed only in the fractions enriched in endoplasmic reticulum and peroxisomes. Mitochondrial and lysosomal fractions were essentially devoid of HMG-CoA reductase activity (Figs. 2 and 3).

DISCUSSION

The immunoelectron microscopic labeling experiments described in this report indicate that antigenic determinants (epitopes) recognized by the monoclonal antibodies A and B are present within peroxisomes in normal rat liver cells, at concentrations that are severalfold higher than in any other intracellular location. These two IgG antibodies, raised to electrophoretically pure microsomal HMG-CoA reductase, form specific complexes with the solubilized enzyme that can be immunoprecipitated by the addition of *Staphylococcus aureus* (8). The two monoclonal antibodies appear to be directed to two different epitopes on the HMG-CoA reductase molecule, since they act additively in the immunoprecipitation experiments. This last fact is important in establishing that it is indeed HMG-CoA reductase that is being immunolabeled in the present experiments. With any one monoclonal antibody, there is always the chance that the epitope recognized by the antibody is shared by HMG-CoA reductase and some otherwise entirely unrelated protein. However, with two monoclonal antibodies directed to different epitopes on the HMG-CoA reductase molecule, this possibility is virtually eliminated; it must be concluded that HMG-CoA reductase, or a protein that is antigenically very closely similar to it, is present at relatively high concentration in the peroxisomes. It should also be noted that the density of immunolabeling of the peroxisomes is higher with monoclonal antibody A than with antibody B (compare Fig. 1 *A* and *B*), despite the use of a 3-fold higher concentration of the latter in the labeling experiments. This is consistent with the fact that immunoprecipitation of HMG-CoA reductase was more effective with monoclonal antibody A than with B (8). The conclusion that it is peroxisomal HMG-CoA reductase that is labeled by the monoclonal antibodies is supported by our observation that, under appropriate conditions, polyclonal antibody to rat HMG-CoA reductase [a generous gift of J. L. Goldstein and M. S. Brown (10)] specifically immunolabels peroxisomes (data not shown).

The conclusion derived from the immunolabeling results, that significant amounts of HMG-CoA reductase are peroxisome associated, has been confirmed by the more classical methods of subcellular fractionation and gradient centrifugation, followed by comparisons with the gradient distributions of known enzyme markers (21, 22) (Figs. 2 and 3). Although prior treatment of rats with Triton WR1339 allows isolation of peroxisomes on relatively simple sucrose gradients (31),

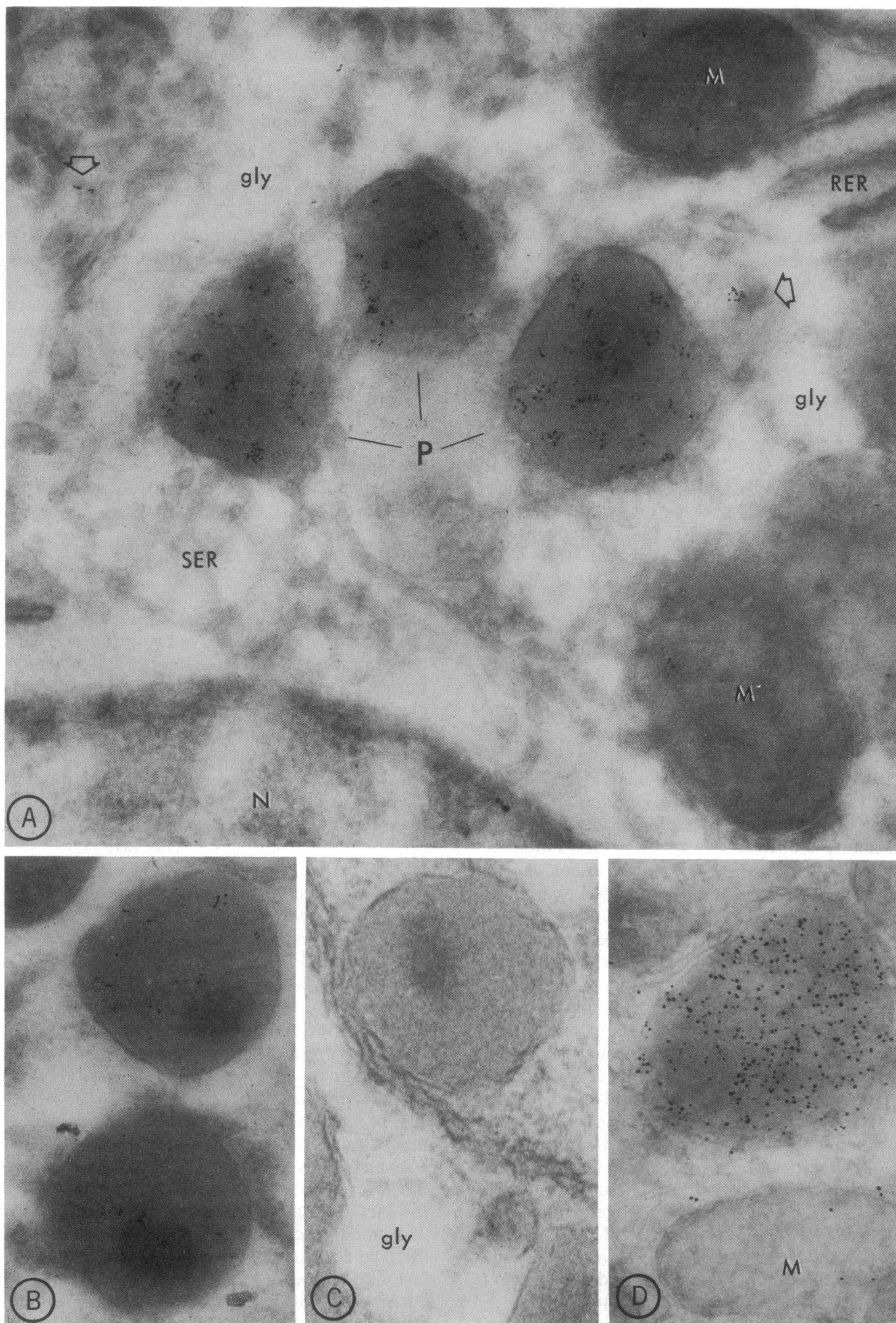

FIG. 1. (*A*) Ultrathin frozen section of normal rat liver, fixed by portal perfusion with PLP fixative (19) and immunolabeled for HMG-CoA reductase, using monoclonal antibody A as the primary antibody, followed by a colloidal gold adduct of rabbit antibodies to mouse IgG. The colloidal gold particles (6- to 8-nm diameter) are seen almost exclusively in the matrix of peroxisomes (P), with a few clusters (arrows) over elements of the smooth endoplasmic reticulum (SER). Note that the rough endoplasmic reticulum (RER), mitochondria (M), and nucleus (N) do not exhibit significant labeling. A glycogen field is indicated by gly. (*B*) Another ultrathin section of the same specimen as in *A*, immunolabeled by the same technique as in *A* except that the primary antibody was monoclonal antibody B. Two peroxisomes in the field are labeled, but at a density less than that in *A*. (*C*) Control specimen, prepared as in *A* except that, instead of a primary antibody to HMG-CoA reductase, the unrelated control monoclonal antibody JG-9 was used. The peroxisome in the upper part of the panel shows no labeling. (*D*) Ultrathin frozen section as in *A* treated with a primary polyclonal goat antibody (IgG fraction) to rat liver catalase, followed by a colloidal gold adduct of rabbit antibodies to goat IgG. (All panels ×63,000.)

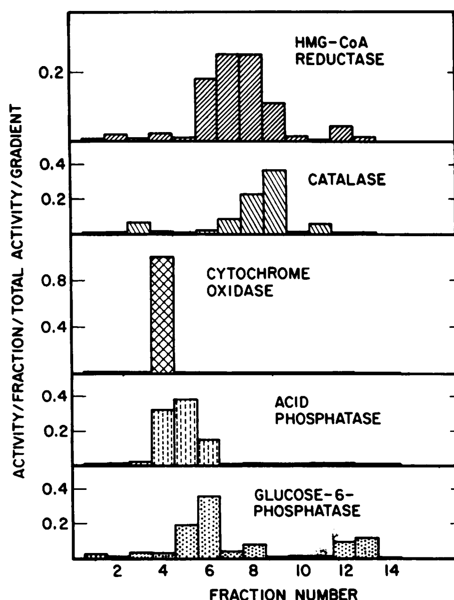

FIG. 2. Subcellular fractionation on a 20–50% metrizamide gradient. A low-speed pellet fraction of a rat liver homogenate that was substantially depleted in endoplasmic reticulum was used (see text for details); 80 mg of protein was layered on the gradient. Enzyme assays were carried out as described in the text.

we did not employ this approach because Triton stimulates membrane proliferation and alters HMG-CoA reductase activity (32). Instead we employed both metrizamide and Percoll gradients in a vertical rotor, because these media give improved separations relative to sucrose gradients. Although the sedimentation distribution of endoplasmic reticulum and subcellular organelles differs dramatically in the two media, a close correspondence of the distribution of HMG-CoA reductase activity with the peroxisomal marker enzyme, catalase, was observed (Figs. 2 and 3). Together, our immunolabeling and subcellular fractionation results provide two independent criteria, antigenic activity and enzymatic activity, for the peroxisome association of HMG-CoA reductase or a protein very closely similar to it.

The concentration of the enzyme in peroxisomes is substantial. This is indicated qualitatively by the fact that, at a maximum, the density of immunolabeling for HMG-CoA reductase in peroxisomes (Fig. 1*A*) was about $^1/_5$ that for catalase, the most abundant protein in the organelle (33). On the other hand, the absence of significant immunolabeling for HMG-CoA reductase over the endoplasmic reticulum does not imply that the enzyme is absent from this structure; it is certainly present there in normal liver (see below), but most likely at a concentration that is below the limits of detectability of our immunolabeling technique. If, for example, we were unable to detect a density of immunolabeling for HMG-CoA reductase over the endoplasmic reticulum that was $^1/_{10}$ or less the density over peroxisomes, then the fact that the total volume density of endoplasmic reticulum in a hepatocyte is some 10 times greater than that of peroxisomes (34) would still allow there to be as much total enzyme present in endoplasmic reticulum as in peroxisomes. A similar conclusion is reached from the subcellular fractionation studies. While losses of enzyme during fractionation and failure to obtain full HMG-CoA reductase activity after exposure to metrizamide render it difficult to quantitate the fraction of HMG-CoA reductase in peroxisomes, we estimate from our results that a substantial fraction, but not more than 50%, of cellular reductase is peroxisomal. Therefore, our present findings do not contradict the substantial evidence that HMG-CoA reductase is an enzyme of the endoplasmic reticulum; they conflict, however, with the inference, for which there is no supporting evidence, that the enzyme is *exclusively* located in the endoplasmic reticulum, or is at its highest concentration there in the normal liver cell. The reason that the peroxisomal content of HMG-CoA reductase was not detected in early subcellular fractionation studies was the inability to assay for the enzyme in fractions containing mitochondria because of their prominent HMG-CoA lyase activity (4). This problem has since been overcome by the finding that an appropriate 37°C preincubation of these fractions in buffers containing high levels of phosphate and EDTA inactivate the lyase activity (28).

In recent studies made possible by the selection of the UT-1 cell line that overproduces HMG-CoA reductase (12), the nucleotide sequences of the cDNAs corresponding to the mRNA and a deduced primary sequence of the enzyme have been determined (6). This sequence confirms that HMG-CoA reductase is an integral membrane protein, with a molecular weight of 97,100. The amino-terminal half of the sequence contains several stretches of hydrophobic amino acid residues; each such stretch probably spans the membrane bilayer. The carboxyl-terminal half is hydrophilic and protrudes from the membrane. The latter domain of the molecule is apparently readily proteolyzed from the cytoplasmic face of the microsomal membranes, yielding a soluble and enzymatically active fragment of ≈55,000 molecular weight. [This solubilized fragment was the antigen employed in the production of the monoclonal antibodies A and B used in this study (8)]. The whole enzyme contains at least one asparagine-linked high-mannose oligosaccharide chain (35), a structure known to become covalently attached to proteins in the endoplasmic reticulum. In other recent studies (7) of the biosynthesis of HMG-CoA reductase *in vitro*, it has been shown that the protein is inserted cotranslationally into membranes, and its insertion is dependent on the signal recognition particle, in a manner similar to that of proteins that are known to be intercalated into the membrane of the rough endoplasmic reticulum *in vivo*. Thus, all of this information is completely consistent with the view that UT-1 cell HMG-CoA reductase is a transmembrane integral protein of the endoplasmic reticulum.

How can one explain, therefore, the presence of the en-

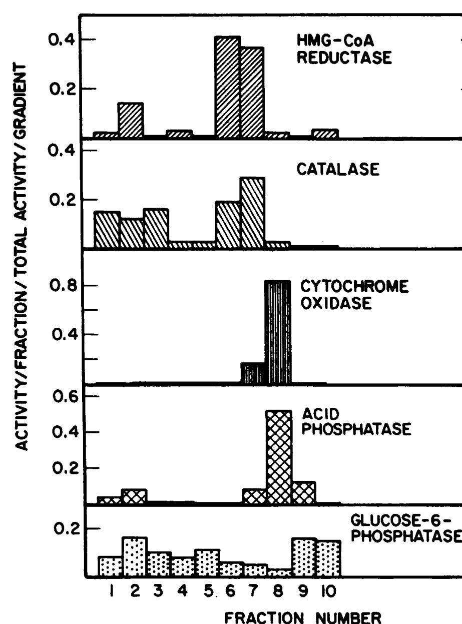

FIG. 3. Subcellular fractionation on a Percoll gradient. A rat liver homogenate prepared as described for Fig. 2 was fractionated by centrifugation in a Percoll gradient as described in the text; 30 mg of protein was layered on the Percoll.

zyme in peroxisomes? There are several conceivable explanations. One possibility is that there are at least two isoforms of HMG-CoA reductase in rat liver cells that share the antigenic epitopes recognized by monoclonal antibodies A and B, but whose structural differences result in one isoform being localized to the endoplasmic reticulum, the other to peroxisomes. The possibility that two different reductase proteins may exist is suggested, but not proven, by our finding that a nick-translated HMG-CoA reductase cDNA clone hybridized to two reductase mRNA bands on RNA blots of rat liver RNA (unpublished observations). Another possibility is that there is only one HMG-CoA reductase protein but that there exists a direct route of transfer of that protein from the endoplasmic reticulum to peroxisomes (36), although for many peroxisomal proteins this is apparently not the case (37). A difficulty with this suggestion is that the immunolabeling of HMG-CoA reductase was mainly associated with the soluble contents, rather than the membranes, of the peroxisomes. A third possibility is that the HMG-CoA reductase in the peroxisomes is an enzymatically active soluble fragment of the enzyme in the endoplasmic reticulum—i.e., that this fragment is released by proteolysis into the cytoplasm and then transferred across the peroxisomal membrane by a specific mechanism similar to that used for catalase and several other peroxisomal proteins (37). This possibility is consistent with the immunolabeling of the HMG-CoA reductase in the matrix of the peroxisomes. Clearly each of these different possible explanations carries important implications for cell biology, and the mechanism that accounts for the peroxisomal presence of HMG-CoA reductase is therefore worthy of investigation.

Aside from the question of how the enzyme gets into peroxisomes, however, its presence there may have significant consequences. Various regulatory studies suggest the existence of three basic control mechanisms for HMG-CoA reductase: (*i*) regulation of HMG-CoA reductase synthesis and degradation (6, 9, 10, 38), (*ii*) modulation of HMG-CoA reductase activity by phosphorylation and dephosphorylation (39), and (*iii*) control of HMG-CoA reductase activity or degradation through changes in the fluidity and structure of the microsomal membrane (3, 11). The existence of HMG-CoA reductase in two intracellular locations may permit separate regulation of the enzyme at the two sites. Furthermore, the product of the HMG-CoA reductase reaction, mevalonic acid, is a key intermediate in the biosynthesis of cholesterol, dolichol, and isopentenyladenosine (1–3). Sequestering the mevalonic acid produced by peroxisomal HMG-CoA reductase within the peroxisome compartment provides a plausible mechanism for the cell to earmark a portion of its mevalonic acid for discrete metabolic fates. The major liver-specific function of products derived from the HMG-CoA reductase reaction is synthesis of large amounts of cholesterol for conversion into bile acids and secretion in lipoproteins. Although there is abundant evidence for a role for peroxisomes in lipid metabolism (30) and cholesterol degradation (40), a potential role for a peroxisomal enzyme in sterol biosynthesis has not been suggested previously.

In summary, our demonstration that a substantial amount of the key biosynthetic regulatory enzyme HMG-CoA reductase is localized in peroxisomes opens a great many areas to exploration, both in the study of peroxisome function and biosynthesis and in the investigation of the regulation of HMG-CoA reductase and the biosynthetic pathways that it controls.

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