Demonstration of cytochrome reductases in rat liver peroxisomes: biochemical and immunochemical analyses

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Abstract In this study we utilized the analytical cell fractionation approach in combination with immunoblotting techniques and immunoelectron microscopy to test for the presence of NADPH cytochrome P-450 reductase and NADH cytochrome c (b_5) reductase in rat liver peroxisomes. Highly purified peroxisomes from clofibrate-treated rats exhibited both NADPH cytochrome P-450 reductase activity and NADH cytochrome c reductase activity (using cytochrome c as an electron acceptor). These activities were inhibited by the respective reductase antibodies made against the endoplasmic reticulum (ER) enzymes. Immunoblot data in combination with immunoelectron microscopy indicated that the peroxisomal NADPH cytochrome P-450 reductase is localized in the matrix of the organelle and has a subunit of molecular weight similar to that of the ER enzyme, whereas the NADH cytochrome c (b_5) reductase is localized in the membranes of the peroxisomes. Again, the subunit molecular weight was similar to that of the ER enzyme. The presence of these reductases in peroxisomes further supports the role of this organelle in bile acid synthesis and cholesterol metabolism. —**Gutierrez, C., R. Okita, and S. Krisans.** Demonstration of cytochrome reductases in rat liver peroxisomes: biochemical and immunochemical analyses. *J. Lipid Res.* 1988. **29:** 613–628.

Supplementary key words immunoblotting • immunoelectron microscopy • NADPH cytochrome P-450 reductase • NADH cytochrome c reductase

Recent data from human peroxisomal disorders strongly suggest that peroxisomes may play an important role in bile acid synthesis (1). Furthermore, peroxisomal fractions obtained from rat liver have the ability to oxidize 3α,7α,12α-trihydroxy-5β-cholestanoic acid (THCA), an intermediate product of cholesterol metabolism, to cholic acid (2, 3), and to convert cholesterol to propionic acid (the expected three-carbon side-chain cleavage product) (4). Highly purified rat liver peroxisomes (95–98%) are also able to oxidize 26-hydroxycholesterol, the product of C-26 hydroxylation of cholesterol, to a C-24 bile acid (5). We have recently obtained evidence for the presence of hydroxylase activity in rat liver peroxisomes that catalyzes the conversion of 3α,7α,12α-trihydroxy-5β-cholestane (THC) to a cholestanetetrol (6). The hydroxylation of the C-26(27) position of the side chain of cholesterol and the hydroxylation of THC is known to be catalyzed in microsomes and/or mitochondria by a P-450 cytochrome system, involving either NADPH cytochrome P-450 reductase (microsomes) (7, 8) or a NADPH ferrodoxin reductase (mitochondria) (9–11). In the microsomal NADPH-supported system, cytochrome b_5 may also donate the second electron to cytochrome P-450 (12).

Whether or not the peroxisomal hydroxylase is a P-450 cytochrome has yet to be determined. This study was designed to investigate whether rat liver peroxisomes contain cytochrome reductases. A number of investigators have reported the presence of cytochrome b_5 and NADH cytochrome c (b_5) reductase in peroxisomes (13–17). However, at the time of these earlier studies, techniques were not readily available to obtain 98% pure peroxisomes, and optimal conditions for peroxisomal separation from lysosomes required treatment of the animals with Triton WR-1339, which is known to affect lipid metabolism and possibly the cytochrome system. In the present study we utilized the analytical cell fractionation approach for demonstrating the presence of NADPH cytochrome P-450 reductase and NADH cytochrome c (b_5) reductase in highly purified rat liver peroxisomes in combination with immunoblotting techniques and immunoelectron microscopy. Since microsomes are the only contaminant found in the purified peroxisomal fractions, the endoplasmic reticulum cytochrome reductases were analyzed in detail for comparison with those of the peroxisomes.

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MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (170–220 g) were used in this study. Standard lab chow or standard lab chow supplemented with 0.5% clofibrate w/w was provided ad libitum for at least 10 days. Animals were fasted overnight and killed by a guillotine.

Cell fractionation

Liver homogenates were fractionated into ν, λ, and ψ fractions as described by Leighton et al. (18) except that preinjection of rats with Triton WR-1339 was omitted and the λ fraction was washed only once. The ν fraction contains the nuclei and most of the mitochondria, the λ fraction is enriched in peroxisomes and lysosomes (similar to the L fraction of de Duve et al. (19)) and the ψ fraction contains the majority of microsomes and soluble components. The λ fraction was then further separated by centrifugation on a steep linear metrizamide (20–50% w/w) or Nycodenz (10–60% w/v) gradient (20, 21). Routinely, 6 ml of the λ fraction prepared from six rat livers was loaded on top of a 27-ml linear gradient. The gradient was centrifuged in a Sorvall OTD 75B centrifuge using a TV 850 ultra-vertical rotor at 40,000 rpm for 50 min at 8°C. A total of 20–25 fractions were collected from the bottom of the centrifuge tube with a two-way needle. Rat liver microsomes were prepared by separating the ψ fraction into a microsomal and a soluble fraction by centrifugation at 100,000 g for 60 min. All cell fractions were assayed for protein content, cytochrome reductase activities, and distribution of marker enzyme activities.

Assay of marker enzymes

Catalase and cytochrome oxidase activities were measured according to Leighton et al. (18), and Lazarow and de Duve (22), except that a molar absorptivity of 19 $mM^{-1}cm^{-1}$ for cytochrome c was used (23). Esterase was measured according to Beaufay et al. (24). Enzyme units are in μmol/min except for catalase which is expressed in the units used by Leighton et al. (18). Protein was determined by the method of Lowry et al. (25) using bovine serum albumin as a standard. Since metrizamide and Nycodenz interfere with the determination of protein, aliquots of the gradient samples were first precipitated in 10% TCA (trichloroacetic acid).

Assay of cytochrome reductases

NADPH cytochrome P-450 reductase and NADH cytochrome c reductase were measured according to Beaufay et al. (24). In both cases, the reduction of cytochrome c was monitored at 550 nm using the millimolar extinction coefficient of 19.6. NADPH cytochrome P-450 reductase was measured in the presence of 2.25 mM NADPH, while NADH cytochrome c reductase was measured with 2.25 mM NADH. NADH ferricyanide reductase (cytochrome b_5 reductase) was also determined according to Katsuyoshi and Sato (26), by following the reduction of ferricyanide. Measurements were done at 420 nm using the millimolar extinction coefficient of 1.02. Neither metrizamide nor Nycodenz was found to be inhibitory in the reductase assays using the microsomal fraction (prepared in 0.25 M sucrose) as a source of enzyme.

Isolation of membranes

Purified peroxisomal fractions (95–98% pure) prepared by equilibrium density gradient centrifugation and a purified microsomal fraction (>92% pure) were treated with sodium carbonate as described by Fujiki et al. (27). The membranes isolated by this technique retain integral membrane enzymes in active form and exhibit a normal trilaminar appearance.

Fixation of samples for immunoelectron microscopy

Small blocks of liver from clofibrate-fed animals were chopped in 3% formaldehyde, 0.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), and immersed in the same fixative for 1 hr. After washing in 0.1 M phosphate buffer (pH 7.2), the blocks were infused with 2.3 M sucrose and ultrathin sections were obtained as described below.

Cryoultramicrotomy

Ultrathin frozen sections were cut with a glass knife at −88°C, according to the method of Tokuyasu (28) in a Dupont-Sorval ultramicrotome MT-2 equipped with the LTC-2 cryoattachment. Ultrathin sections were transferred onto Formvar-coated copper grids and immunolabeled.

Immunolabeling

For immunolabeling experiments, the primary reagent was the antibody to rat liver ER NADPH cytochrome P-450 reductase or NADH cytochrome c (b_5) reductase. The secondary reagent was colloidal gold adducts of affinity-purified guinea pig antibodies to rabbit IgG. Colloidal gold particles of 6–8 nm diameter and the adduct were prepared as previously described (29). After immunolabeling, the grids were either treated with 2% osmium tetroxide, poststained in uranyl acetate and infused with LR white acrylic resin (London Resin Co. Ltd.) (29), or the grids were floated on 2% neutral uranyl acetate for 10 min, briefly washed in distilled water, and embedded in a solution containing 2% carbowax (mol wt 1540), 0.2% methyl cellulose (400 centiposes), and 0.02% aqueous uranyl acetate. After polymerization or drying, the grids were examined without poststaining in a Philips

model 300 transmission electron microscope at 80 kV equipped with an 11-μm diameter aperture.

Immunoblotting

Microsomal and peroxisomal proteins were separated on 12.5% polyacrylamide, 0.1% SDS slab gels (1.5 mm) according to Laemmeli (30). Electrophoresis was performed at 35 mA/gel constant current. Protein samples were prepared in solubilizing buffer (0.5 M Tris-HCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol, 5% glycerol, and 0.005% bromophenol blue) and incubated for 2-3 min in a boiling waterbath prior to loading. A mixture of molecular weight standards was routinely applied to the gel. This included: phosphorylase b, 92,500; bovine serum albumin, 66,200; ovalbumin, 45,000; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500; and lysozyme, 14,000.

These separated proteins were electrophoretically transferred to nitrocellulose paper in 20 mM Tris, 150 mM glycine, and 20% methanol. The nitrocellulose was incubated for 60 min at 37°C with 5% BSA Tris-saline, pH 7.4, in a gently shaking waterbath followed by overnight incubation with the specific antibody at 4°C. This was followed by 90 min incubation with ^{125}I-labeled protein A. The proteins of interest were visualized by exposing the nitrocellulose to Kodak XRP X-ray film at −70°C for 2 to 3 days.

Immunoprecipitation

Increasing concentrations of cytochrome P-450 reductase antibody were bound to 20 mg of Sepharose-A beads by incubating for 1 hr at room temperature in 0.2 ml of PBS (0.1 M Na_3PO_4, 0.15 M NaCl, pH 7.5). The antibody-Sepharose-A beads complex was washed and pelleted three times with 0.5 ml of PBS. The washes were discarded. Peroxisomal protein (1.6 mg) solubilized by freeze-thaw and microsomal protein (1.6 mg) were added to the beads and incubated in PBS for 2 hr at room temperature. All incubations were carried out with gentle continuous agitation. The beads were pelleted by a 5-sec centrifugation in a microcentrifuge, and the supernatants were removed and immediately assayed for NADPH cytochrome P-450 reductase activity. Under these conditions, only the beads with reacted antigen are pelleted. The beads were washed twice with 0.1% Triton X-100 in PBS, twice with 0.1% ovalbumin in PBS, and twice with PBS. SDS solubilizing buffer was added to the beads followed by 2 min boiling and immediate freezing. The Sepharose beads were then subjected to regular SDS-PAGE, immunoblotted, and the antigen-antibody complex was detected by the ^{125}I-labeled protein A method. Control samples consisted of Sepharose-A beads incubated with peroxisomal or microsomal protein in the absence of antibody.

Computer calculations

The distribution of cytochrome reductase activities in the various organelle fractions was evaluated quantitatively by means of a computer program that calculates optimal linear combinations of marker enzymes using a least squares criterion (31). This linear regression program was augmented to include the T-values and probabilities for the least-squares regression coefficients. With these additions we can state at the 95% confidence level which distributions of test enzymes are significant.

Materials

^{125}I-Labeled protein A was purchased from New England Nuclear. Autoradiography film and reagents were obtained from Merry X-Ray (San Diego, CA). Other chemicals were from Sigma. Polyclonal antibodies to rat NADPH cytochrome P-450 reductase and NADH cytochrome c (b_5) reductase were a generous gift from Dr. B. S. Masters (Medical College of Wisconsin, Milwaukee, WI).

RESULTS

Equilibrium density centrifugation

The peroxisome-enriched fraction prepared by differential centrifugation of liver homogenates from control animals was subjected to isopycnic centrifugation in order to separate the organelles on the basis of their different densities. All the fractions were assayed for protein, cytochrome oxidase activity (a mitochondrial marker), esterase activity (a microsomal marker), catalase activity (a peroxisomal marker), and for the three cytochrome reductase activities. NADPH cytochrome P-450 reductase was assayed using cytochrome c as an electron acceptor, and NADH cytochrome c reductase was assayed by use of cytochrome c and potassium ferricyanide as electron acceptors. The designation of NADH ferricyanide reductase was employed when potassium ferricyanide ($K_3Fe(CN)_6$) was the electron acceptor. **Fig. 1** illustrates the characteristics of one of three gradients. The mitochondria and microsomes were responsible for the major peak of protein, and the peroxisomes were coincident with the minor peak of protein (A). The intact peroxisomes (B) were located at the dense end of the gradient (to the right in Fig. 1) and were well separated from the microsomes (C) and the mitochondria (data not shown). The mitochondrial distribution in these gradients was similar to that reported previously (32). The minor peak of catalase activity (B) sedimenting at the light end of the gradient (to the left in Fig. 1) represents solubilized catalase activity due to breakage of peroxisomes. Lysosomes sediment at the far left of the gradient in this system (data not shown). The only minor contamination of the peak peroxisomal

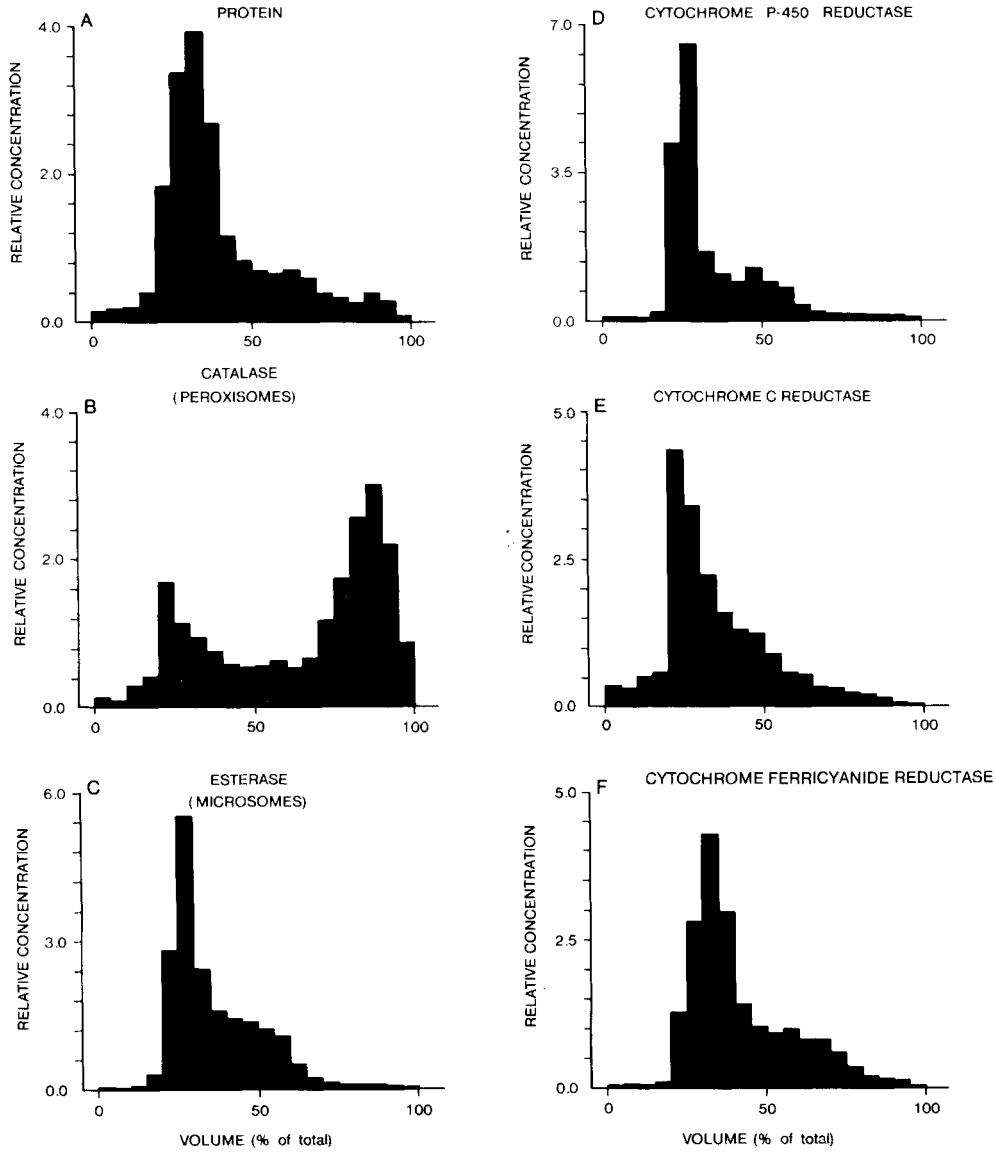

Fig. 1. Separation of peroxisomes from other cell organelles from normal rat liver. The ordinate, relative concentration, is derived by dividing the actual concentration of the enzyme in a particular fraction by the concentration of the enzyme that would be observed if the enzyme were homogeneously distributed throughout the gradient. The abscissa is normalized cumulative volume (total volume was 32 ml). The density of the gradient increases from left to right. Cytochrome P-450 reductase was measured in the presence of cytochrome c and NADPH. Cytochrome c reductase was measured in the presence of cytochrome c and NADH. Cytochrome ferricyanide reductase was measured in the presence of ferricyanide and NADH. The recoveries of all enzyme activities ranged from 70 to 97%.

fractions was due to a 4–6% microsomal contribution as determined by marker enzyme analysis (27). The distribution patterns of the three cytochrome reductase activities (D, E, F) showed very little activity coincident with the purified peroxisomal fraction area. All of this activity could be accounted for by the slight microsomal contamination present at this end of the gradient. The data indicate that normal rat liver peroxisomes do not contain any of the above three cytochrome reductase activities.

We next looked for the presence of cytochrome reductase activities in peroxisomes from liver obtained after clofibrate treatment of animals. Clofibrate is a known peroxisomal proliferator (33, 34). The results of a typical gradient are illustrated in **Fig. 2.** The protein distribution (A) showed a major peak coincident with the microsomes (C) and the mitochondria (data not shown), and a minor peak coincident with the peroxisomes (B). The peroxisomes were well separated from other cell organelles and sediment at the dense end of the gradient (to the right in Fig. 2), and showed very little damage as measured by solubilized catalase (the left end of the gradient). Purity calculations of the peroxisomes indicated 98% purity in the peak frac-

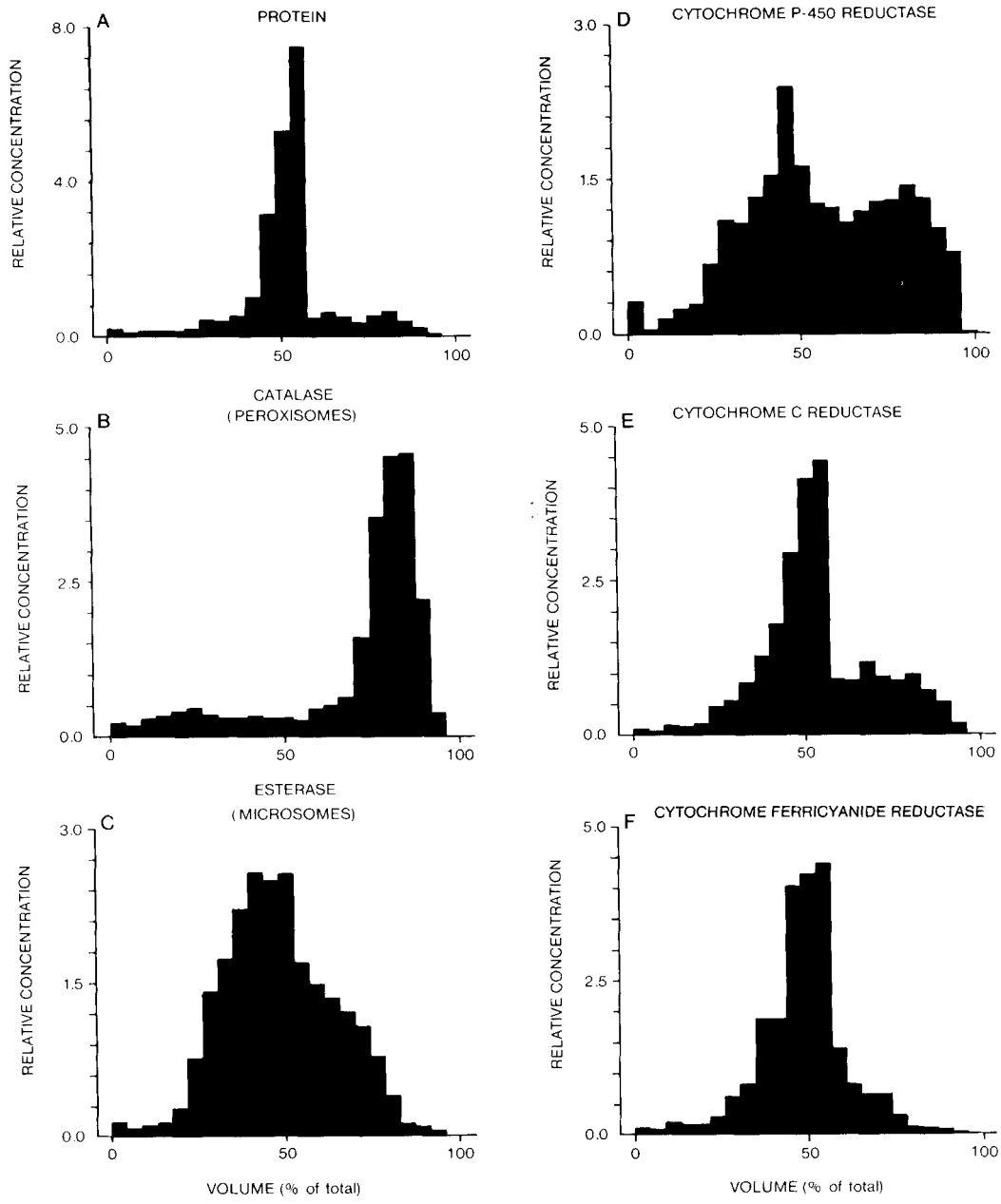

Fig. 2. Separation of peroxisomes from other cell organelles from liver of clofibrate-treated rats. The distribution of marker enzymes is plotted as relative concentration versus normalized cumulative volume (total volume was 32 ml). The density of the gradient increases from left to right. The recoveries of all enzyme activities ranged from 80 to 95%.

tions. The distribution patterns of NADPH cytochrome P-450 reductase and NADH cytochrome c reductase activities showed two peaks, a large peak cosedimenting with microsomes and a smaller peak cosedimenting with peroxisomes. The distribution pattern of NADH ferricyanide reductase showed only one peak of enzyme activity, coincident with microsomes. These results indicate that peroxisomes from clofibrate-treated rats contain NADPH cytochrome P-450 reductase activity and NADH cytochrome c reductase activity, but not NADH ferricyanide reductase activity.

Peroxisomal contribution to total liver cytochrome reductase activities

The percent of cytochrome reductase activities localized in the peroxisomes was determined from the differential centrifugation and density gradient data by applying the principle of calculating the linear combinations of marker enzyme distributions that would best fit the measured cytochrome reductase distributions. This method has been described in detail (31). This program makes use of all data from the fractionation procedure instead of merely

the few purest fractions. The method also requires good recoveries in the cell fractionations and assumes biochemical homogeneity. This linear regression program was augmented to include the T-values and probabilities for the least-squares regression coefficients. With these additions we can state at the 95% confidence level which distributions of cytochrome reductase enzymes are significant. **Table 1** shows the percent of peroxisomal contribution to total liver cytochrome reductase activities from clofibrate-treated rats. We calculated a 10% peroxisomal contribution of NADPH cytochrome P-450 reductase activity and a 5.5% peroxisomal contribution of NADH cytochrome c reductase activity. When NADH ferricyanide reductase was measured by $K_3Fe(CN)_6$ reduction, an average of less than 1% contribution was calculated. However, this level of activity is not significant at the 95% confidence level.

TABLE 1. Peroxisomal contribution to total liver cytochrome reductase activities from clofibrate-treated rats

Enzyme	Percent Activity[a] Mean	Range
NADPH cytochrome P-450 reductase	10.0	5.8–14.2
NADH cytochrome c reductase	5.5	5.5–5.6
NADH ferricyanide reductase	0.9[b]	0.1–1.6

[a] Mean of three density gradient centrifugations.
[b] Not significant at the 95% confidence level.

Specific activities of cytochrome reductases in peroxisomal and microsomal fractions

The specific activities of the cytochrome reductases were calculated for the most highly purified fractions obtained for each organelle. The purity of peroxisomes from clofibrate-treated animals was calculated to be between 96 and 99% based on the measurement of marker enzymes (27). The activities of marker enzymes for mitochondria and lysosomes in these fractions were below the level of detection. The only minor contaminant was the ER. The microsome fraction was calculated to be 92 to 94% pure. **Table 2** shows the specific activities of the cytochrome reductases in peroxisomal and microsomal fractions from liver of clofibrate-treated rats. Esterase activity (the marker enzyme for the ER) is also listed. As can be seen from the ratio of specific activities, the presence of NADPH cytochrome P-450 reductase activity and NADH cytochrome c reductase activity in peroxisomes cannot be attributed to the minor microsomal contamination (ratio of specific activities are greater than 0.03). However, the presence of NADH ferricyanide reductase activity in peroxisomes could be attributed to the microsomal contamination of peroxisomes. These data were in agreement with the linear combination calculations.

NADPH cytochrome P-450 reductase

Immunoprecipitation of cell fractions. Solubilized proteins (freeze–thaw) from a highly purified peroxisomal fraction (98%) obtained after density gradient centrifugation and a microsomal fraction obtained after differential centrifugation from clofibrate-treated animals were immunoprecipitated with increasing concentrations of NADPH cytochrome P-450 reductase antibody and the supernatant was assayed as described in Materials and Methods. **Fig. 3** illustrates that in both the peroxisomal and microsomal preparations the enzymatic activity decreases with the addition of antibody (IgG fraction). Approximately 90% of the peroxisomal activity is immunoprecipitated by 1.9 μg of antibody. About 70% of the microsomal activity is immunoprecipitated at this concentration. No inhibition of activity was observed with either peroxisomal or microsomal samples incubated with Sepharose-A beads in the absence of antibody.

Several of the immunoprecipitated fractions (pellets) were subjected to SDS-PAGE, electrophoretically transferred to nitrocellulose paper, and the antigen-antibody complex was detected by ^{125}I-labeled protein A. **Fig. 4** shows the results for the peroxisomal samples. Lane 1 was the control sample with Sepharose-A beads in the absence of antibody. Lanes 2 and 3 exhibited one protein band with an apparent M_r of 78 kDa. The microsomal samples also displayed one protein band with a similar M_r of 78

TABLE 2. Specific activities of enzymes in peroxisomal and microsomal fractions from liver of clofibrate-treated rats[a]

	NADPH-Cytochrome P-450 Reductase	NADH-Cytochrome c Reductase	NADH-Ferricyanide Reductase	Esterase
	μmol/min per mg			
Peroxisomes	(4)[b] 0.06 ± 0.04	(4) 0.10 ± 0.05	(4) 0.49 ± 0.22	(4) 0.15 ± 0.03
Microsomes	(3) 0.13 ± 0.03	(3) 0.50 ± 0.25	(3) 5.9 ± 2.6	(3) 5.2 ± 0.6
Ratio of sp act[c]	0.46	0.20	0.08	0.03

[a] All values given as mean and SD. Specific activity is defined as μmol of substrate utilized per minute per mg of organelle protein.
[b] The numbers in parentheses refer to number of samples analyzed.
[c] Specific activity in peroxisomes / Specific activity in microsomes

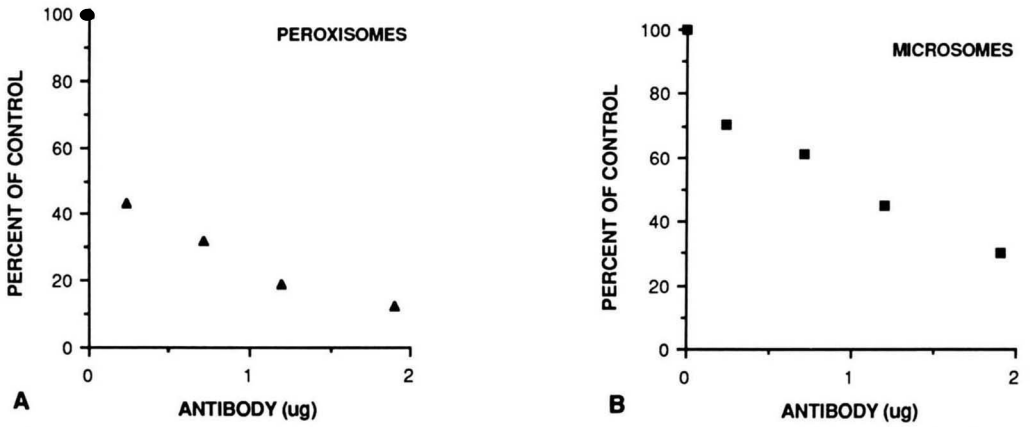

Fig. 3. Immunoprecipitation of (A) peroxisomal and (B) microsomal NADPH cytochrome P-450 reductase activity by antibody to the microsomal reductase. Ninety-eight percent pure peroxisomal fractions solubilized by freeze-thaw and 93% pure microsomal fractions from clofibrate-treated animals were immunoprecipitated with increasing concentrations of antiserum (IgG fraction) and the supernatant was assayed for enzyme activity as described in Materials and Methods.

kDa, consistent with previous reports (data not shown).

Immunoblot analysis of normal rat liver peroxisomes also demonstrated the presence of a 78 kDa protein, but in much lower quantity. This finding is in agreement with the inability to measure any NADPH cytochrome P-450 reductase activity in normal rat liver peroxisomes (Fig. 1).

Immunoblotting of peroxisomal and microsomal membranes. The native microsomal NADPH cytochrome P-450 reductase is known to be an integral membrane protein localized on the cytoplasmic side of the membrane (35–37). In order to determine the subcellular location of the peroxisomal protein, we prepared membranes from highly purified peroxisomes (98%) from clofibrate-treated animals. The membranes were prepared by the carbonate method (27). The membranes isolated by this technique retained integral membrane enzymes in active form and exhibited a normal trilaminar appearance (27). Since approximately 10% of total peroxisomal protein was membrane protein, membrane protein equivalent to this was used. **Fig. 5** clearly shows that the peroxisomal protein reacting with the NADPH cytochrome P-450 reductase antibody was located in the peroxisomal matrix (soluble fraction) (lane 2) and not in the membrane fraction (lane 1). Identical results are obtained when higher concentrations of peroxisomal membrane proteins are used. The same procedure was performed to prepare pure microsomal membranes from microsomes isolated from clofibrate-treated animals. Again, since approximately 30 to 50% of microsomal protein was membrane protein, membrane protein equivalent to this was used. **Fig. 6** confirmed the membrane localization of microsomal NADPH cytochrome P-450 reductase. Lane 2 (microsomal membrane protein) and lane 3 (microsomes) were the only lanes exhibiting the ^{125}I-labeled protein A antibody-antigen complex. These results demonstrate the presence of a protein in the

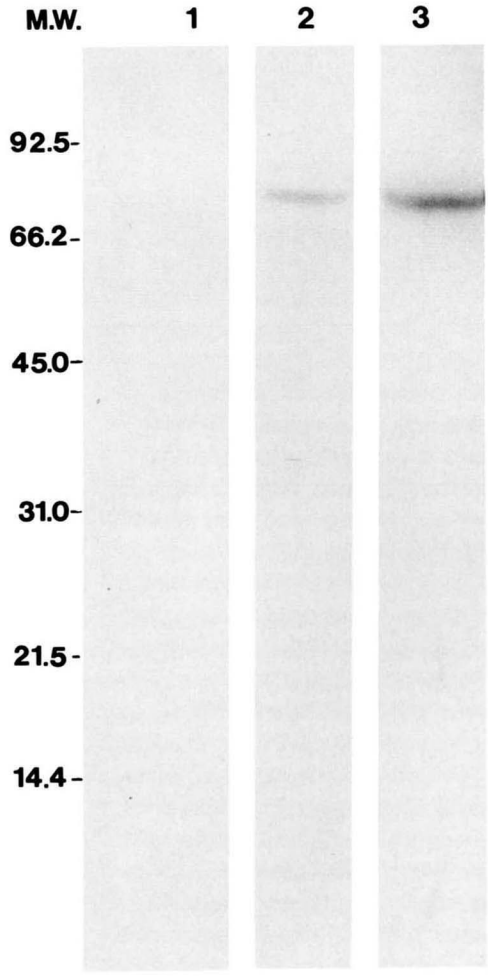

Fig. 4. Immunoblot of peroxisomal samples immunoprecipitated with NADPH cytochrome P-450 reductase antibody. Autoradiograph shows ^{125}I-labeled protein A antibody-antigen complex. Lane 1, control, Sepharose-A beads only; lane 2, immunoprecipitated with 0.24 μg of antibody; lane 3, immunoprecipitated with 0.72 μg of antibody.

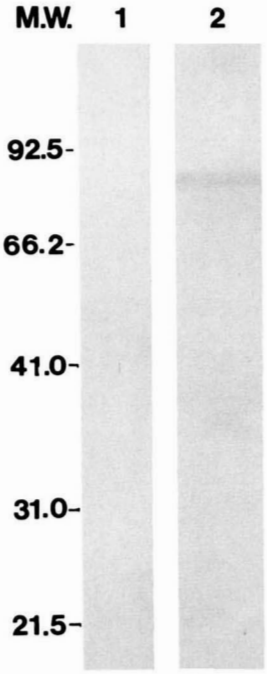

Fig. 5. Immunoblot of NADPH cytochrome P-450 reductase in peroxisomal fractions from clofibrate-treated animals. Lane 1, 20 μg of peroxisomal membranes, lane 2, 180 μg of soluble peroxisomal protein. The M_r of the protein is 78 kDa. Membranes were isolated by treatment with sodium carbonate.

matrix of rat liver peroxisomes that reacts with an antibody made against rat liver microsomal NADPH cytochrome P-450 reductase.

This peroxisomal protein also has a subunit M_r similar to the microsomal membrane protein.

Immunoelectron microscopy. To further confirm the presence of the protein in peroxisomes, indirect gold immunolabeling for NADPH cytochrome P-450 reductase was performed. Hepatic tissue from clofibrate-treated animals was prepared for immunoelectron microscopy as described in the Methods section. The results are represented in **Fig. 7** and **Fig. 8.** Gold particles are clearly visible in the peroxisomes (P), restricted mostly to their matrices. A large number of clustered gold particles are also detected in the lamellae of the ER. However, the matrices of the mitochondria (M) are almost totally devoid of gold particles. Since frozen sections were used, the ER appears diffuse and should not be mistaken for cytosol. These results confirm that the peroxisomal protein is primarily localized in the matrix of the organelle. Control sections prepared in the absence of antibody were completely devoid of gold particles.

NADH cytochrome c (b_5) reductase

Immunoblotting of peroxisomal and microsomal membranes. Immunoblotting of peroxisomal and microsomal fractions from clofibrate-treated animals was also performed with an antibody against rat microsomal NADH cytochrome c (b_5) reductase. The native microsomal NADH cytochrome c (b_5) reductase is also known to be an integral membrane protein localized on the cytoplasmic side of the membrane (35, 38). **Fig. 9** shows an immunoblot of highly purified peroxisomal membrane proteins (lane 1) and microsomal membrane proteins (lane 2) obtained from clofibrate-treated rats. The results clearly demonstrate the occurrence of a 35 kDa protein in peroxisomal membranes and confirm the presence of the microsomal membrane protein. No bands were observed in the soluble peroxisomal and microsomal fractions (data not shown).

Immunoblot analysis of normal rat liver peroxisomes also demonstrated the presence of a 35 kDa protein. The quantity of the microsomal protein from normal rat liver was considerably greater as compared to that from the clofibrate-treated liver (data not shown). This observation was also in agreement with the higher specific activity measurements of NADH cytochrome c reductase in normal microsomes.

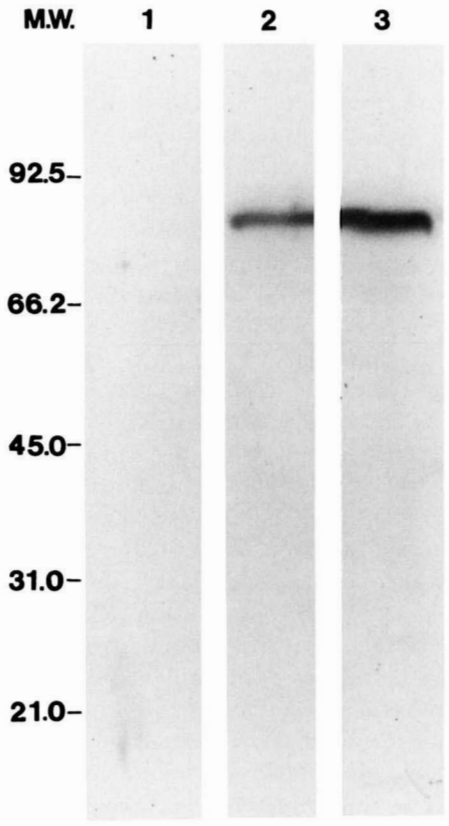

Fig. 6. Immunoblot of NADPH cytochrome P-450 reductase in microsomal fractions from clofibrate-treated animals. Lane 1, 140 μg of soluble microsomal protein; lane 2, 60 μg of microsomal membranes; lane 3, 200 μg of microsomal protein; 93% pure microsomes were used for the preparation of membranes. The M_r of the protein is 78 kDa. Membranes were isolated by treatment with sodium carbonate.

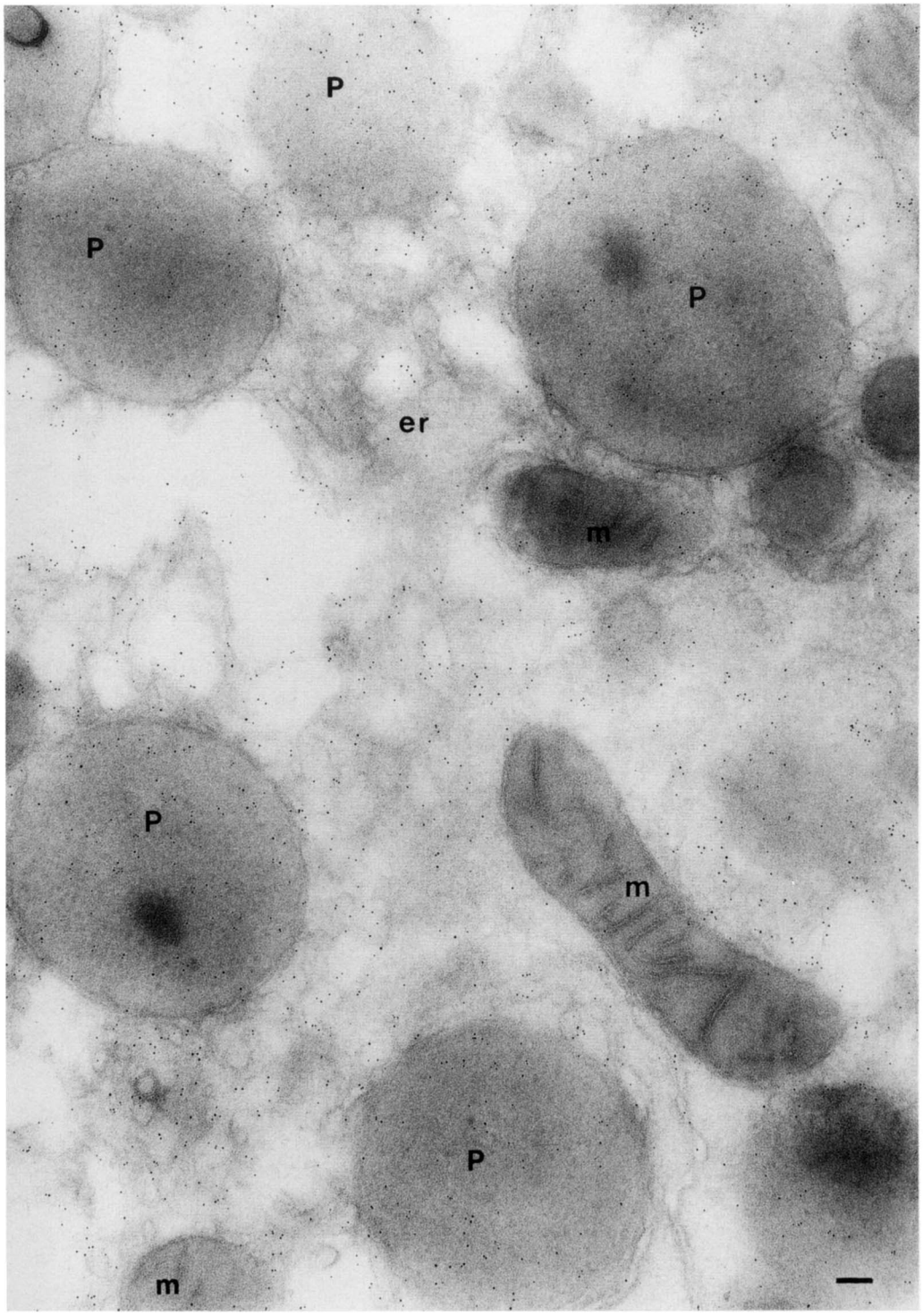

Fig. 7. Representative micrograph of liver cells from a clofibrate-treated rat that were immunolabeled with antibody against NADPH cytochrome P-450 reductase followed by a colloidal gold adduct of guinea pig antibodies to rabbit IgG. A high level of gold labeling is observed in the matrices of the peroxisomes (P). Numerous clusters of particles are also detected over the membranes of the ER. The low level of nonspecific labeling can be appreciated by viewing the matrices of the mitochondria (M) that are almost completely lacking of gold particles; mag. 45,500×; bar = 0.1 μm.

Immunoelectron microscopy. To further establish the localization of the protein in peroxisomes, indirect gold immunolabeling for NADH cytochrome c (b_5) reductase was also performed. Hepatic tissue from clofibrate-treated animals was used as described previously. These results support the conclusion that the peroxisomal protein is

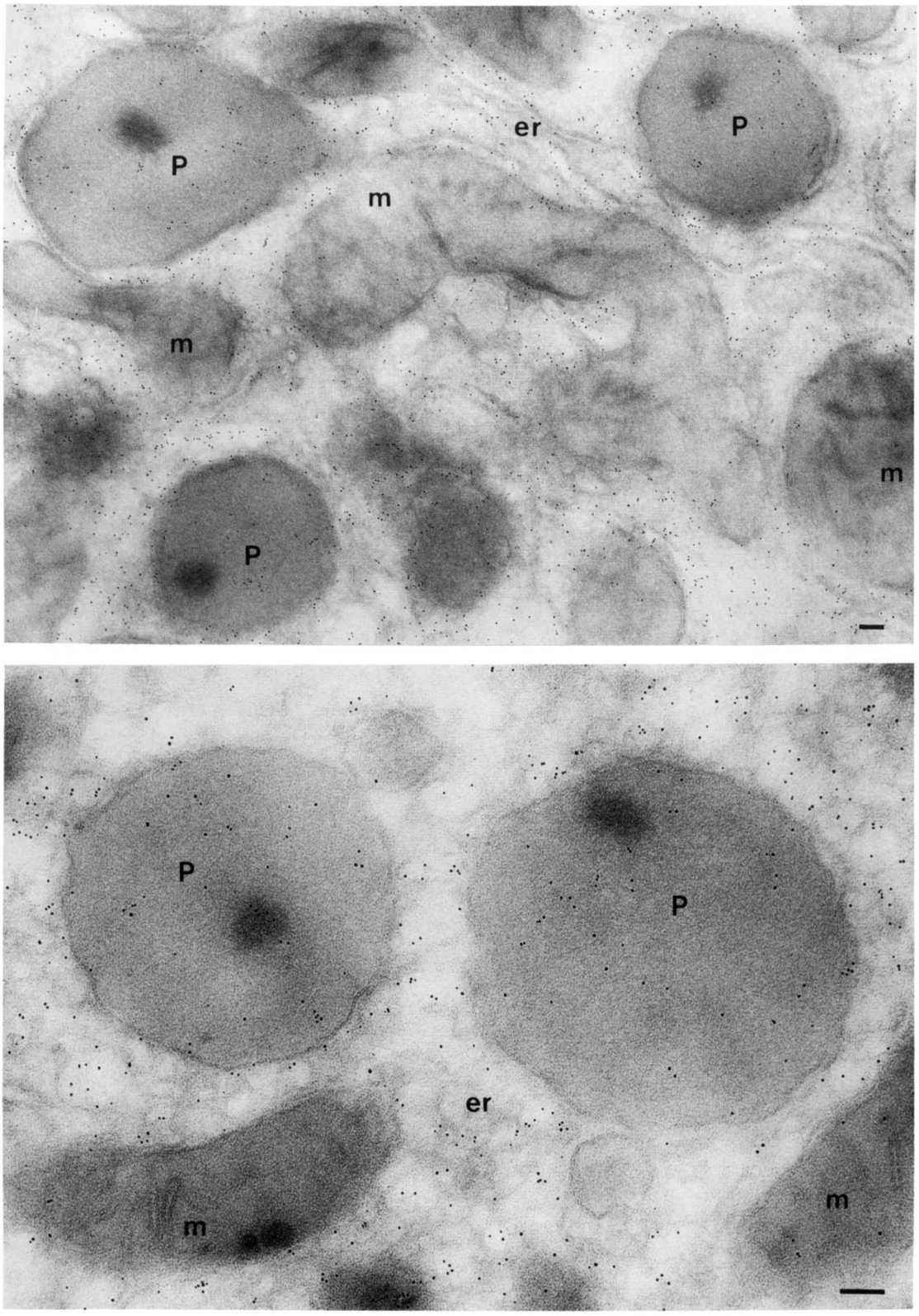

Fig. 8. Micrographs of liver cells from a clofibrate-treated rat that were immunolabeled with antibody against NADPH cytochrome P-450 reductase followed by a colloidal gold adduct of guinea pig antibodies to rabbit IgG. Top, numerous mitochondria (M) are shown to illustrate the low level of nonspecific labeling; mag. 41,400×; bar = 0.1 μm. Bottom, the labeling in the peroxisomes (P) is limited to the matrix; mag. 67,600×; bar = 0.1 μm.

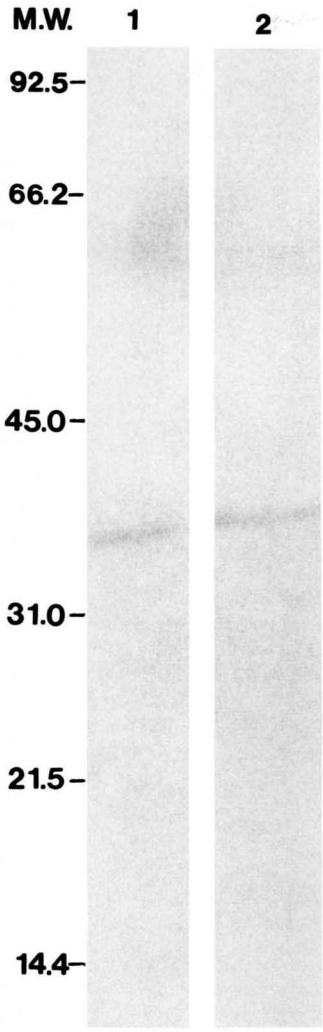

Fig. 9. Immunoblot of NADH cytochrome c (b_5) reductase in peroxisomal and microsomal membranes from clofibrate-treated animals. Lane 1, 50 μg of peroxisomal membranes; lane 2, 50 μg of microsomal membranes. The M_r of the protein is 35 kDa. Membranes were isolated by treatment with sodium carbonate.

localized in the membranes of the peroxisome and also associated with the membranes of the ER as well as the outer membranes of mitochondria, confirming earlier reports (15, 17, 39) (**Fig. 10**).

Inhibition of enzymatic activity by antibodies. Highly purified rat liver peroxisomal membranes and microsomal protein obtained from clofibrate-treated rats were titrated with increasing concentrations of antiserum to rat microsomal NADH cytochrome c (b_5) reductase. The antibody–cell fraction mixtures were preincubated for 10 min at room temperature before assaying for NADH cytochrome c reductase. The assays of the activities of both enzyme preparations were performed on approximately the same number of NADH cytochrome c reductase enzyme units in the cuvette. **Fig. 11** shows the results of these experiments. As can be seen, both the peroxisomal and microsomal preparations were inhibited by the addition of antiserum. Furthermore, the rate and absolute level of inhibition appeared to be very similar in both instances. The addition of Triton X-100 (0.25%) to the microsomal fractions had no effect on the enzyme activity. However, the addition of Triton X-100 to the peroxisomal membranes produced complete inhibition of activity. When control unrelated antiserum was used, no inhibition of activity was observed in either sample.

DISCUSSION

This study presents, for the first time, evidence of NADPH cytochrome P-450 reductase in rat liver peroxisomes. Data from the analytical cell fractionation studies of clofibrate-treated animals in combination with computer-assisted linear combination calculations demonstrate that peroxisomes contain about 10% of the total liver NADPH cytochrome P-450 reductase activity (using cytochrome c as an electron acceptor). Purity calculations of our fractions confirm that this reductase activity is truly peroxisomal and not due to microsomal contamination. Moreover, this activity is immunoprecipitated by NADPH cytochrome P-450 reductase antibody in a manner similar to the microsomal enzyme. However, immunoblot data from both normal and clofibrate-treated animals indicate that the peroxisomal enzyme has a location within the organelles different from the ER enzyme, but has a similar subunit molecular weight. In microsomes we find this protein is located in the membranes and has an apparent molecular weight of 78 kDa, which is in close agreement with previous reports (36, 37). Immunoblots of the peroxisomal fractions also show a protein band at 78 kDa, but it is present in the soluble fraction of the organelle. The localization of the soluble protein in peroxisomes is corroborated by immunoelectron microscopy, which revealed gold-labeled particles only in the matrix of the organelle.

The microsomal NADPH cytochrome P-450 reductase consists of a major hydrophilic portion, in which the catalytically active part resides, and a minor hydrophobic portion which is essential for attachment of the molecule to the membrane (35, 36). The hydrophilic portion of the enzyme molecule can be selectively split from its hydrophobic portion by digestion with a suitable protease, resulting in solubilization of the active fragment of the enzyme from the microsomes. The molecular weight of the native form is 78 kDa and the molecular weight of the hydrophilic, catalytically active part, is 68 kDa.

It is unlikely that the 78 kDa protein detected in the peroxisomes is a proteolytic cleavage product of the ER enzyme. However, it is possible that the peroxisomal pro-

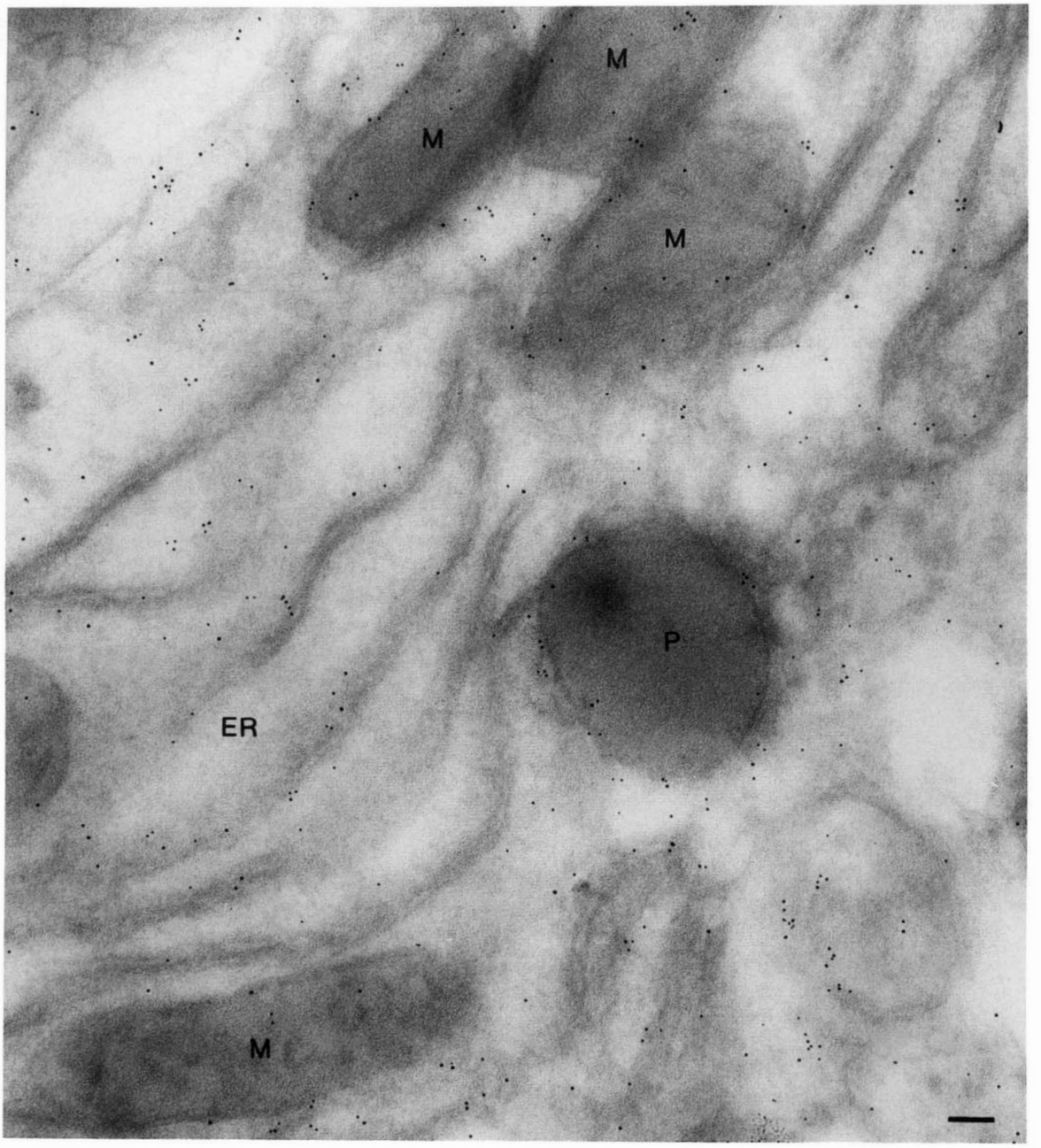

Fig. 10. Representative micrograph of liver cells from a clofibrate-treated rat that were immunolabeled with antibody against NADH cytochrome c (b_5) reductase followed by a colloidal gold adduct of guinea pig antibodies to rabbit IgG. Gold labeling is observed in the peroxisome (P), restricted mainly to the membrane. Numerous gold particles are also associated with the membranes of the ER and the outer membranes of the mitochondria. Mag. 68,000×; bar = 0.1 μm.

tein that reacts with the antibody is a proteolytic cleavage product or subunit of a larger native peroxisomal protein. This needs to be further investigated.

Table 3 presents a summary of some of the characteristics of the microsomal and peroxisomal NADPH cytochrome P-450 reductases. The main difference is, again, subcellular location. It is interesting to note that this is the third enzyme that is an integral membrane protein in the ER, and that is also found in the soluble compartment of the peroxisomes. 3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) is an integral membrane protein of the ER that was recently shown to be also localized in the matrix of the peroxisomes (32, 40). Carnitine acetyltransferase is also soluble

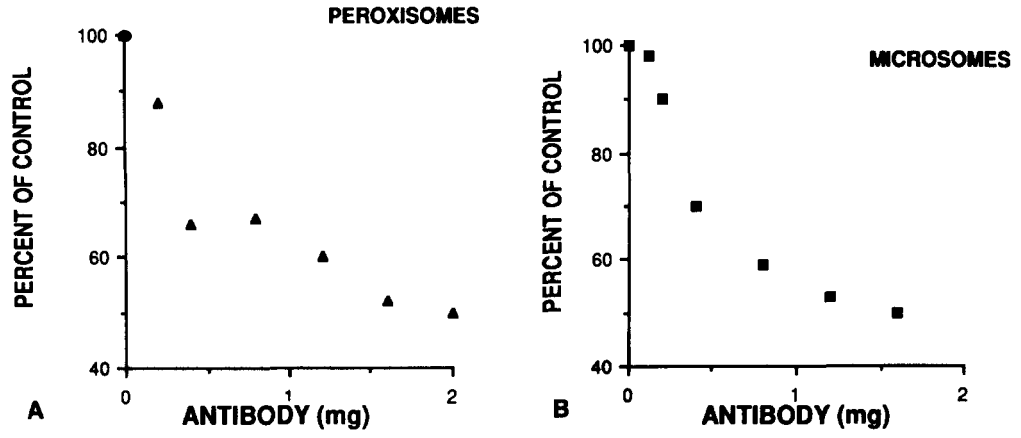

Fig. 11. Inhibition of (A) peroxisomal and (B) microsomal NADH cytochrome c (b_5) reductase activity by antibody to the microsomal reductase. Highly purified peroxisomal membrane fractions (8 μg) and 93% pure microsomal fractions (11 μg) from clofibrate-treated animals were preincubated with increasing concentrations of antiserum and assayed as described.

in peroxisomes and membrane-bound in microsomes. Due to the similarities in the properties of the carnitine acetyl transferase, it has been postulated that the same enzyme exists in both these organelles (41).

Several earlier studies have reported the presence of NADH cytochrome c reductase activity and NADH cytochrome b_5 reductase activity in peroxisomes (13–17). However, due to the significant contamination of some of these peroxisomal preparations, the pretreatment of animals with Triton WR-1339, and the variation in the reported absolute activities of the enzymes, the questions of their presence and level in peroxisomes needed to be clarified. In this study we utilized the analytical cell fractionation approach in combination with immunoblotting techniques and immunoelectron microscopy to better address this question. Our data show that peroxisomes from clofibrate-treated animals contain about 5–6% of the total liver NADH cytochrome c reductase activity, but little or no activity when assayed with $K_3Fe(CN)_6$ (i.e., cytochrome ferricyanide reductase). This cytochrome c reductase activity is also inhibited by NADH cytochrome c (b_5) reductase antibody in a manner similar to the microsomal enzyme. It is not clear whether the NADH cytochrome c reductase and NADH ferricyanide reductase in peroxisomes are the same enzyme. Immunoblot data from both normal and clofibrate-treated animals indicate that the peroxisomal enzyme has an M_r similar to that of the ER enzyme and is also associated with the peroxisomal membrane fraction. The membrane location of the protein in peroxisomes is further demonstrated by immunoelectron microscopy, which revealed gold-labeled particles mainly in the membrane of the organelle.

It is believed that NADH cytochrome c reductase and NADH cytochrome (b_5) reductase are the same enzyme in the ER that catalyzes the transfer of electrons from NADH to cytochrome b_5. This reductase is very similar

TABLE 3. Characteristics of hepatic microsomal and peroxisomal NADPH cytochrome P-450 reductases

	Microsomes	Peroxisomes
Apparent molecular weight	78,000	78,000
Subcellular location	membrane-bound	soluble
Effect of clofibrate treatment on enzyme activity	increases	measurable only in drug-treated animals
Specific activity (μmol/min per mg)	0.13	0.06
Effect of Triton X-100 solubilization on enzyme activity	no effect	no effect
Effect of NADPH cytochrome P-450 antibody on enzyme activity	inhibited	inhibited
Cytochrome P-450 content (nmol/mg)	1.0[a]	—[b]

[a] Rat microsomal cytochrome P-450 content (from ref. 57).
[b] Unknown.

TABLE 4. Characteristics of hepatic microsomal and peroxisomal NADH cytochrome c (b_5) reductases

	Microsomes	Peroxisomes
Apparent molecular weight	35,000	35,000
Subcellular location	membrane-bound	membrane-bound
Effect of clofibrate treatment on enzyme activity	decreases	measurable only in drug-treated animals
Specific activity reduction of cytochrome c (μmol/min per mg)	0.50	0.10
Specific activity reduction of $K_3Fe(CN)_6$ (μmol/min per mg)	5.9	not significant
Effect of Triton X-100 solubilization on enzyme activity	no effect	inhibited
Effect of NADH cytochrome c (b_5) antibody on enzyme activity	inhibited	inhibited
Cytochrome b_5 content (nmol/mg)	0.4[a]	0.04[a]

[a] An average value of rat microsomal and peroxisomal b_5 content was calculated from data in references 13, 17, and 42.

to the microsomal NADPH cytochrome P-450 reductase in that it also consists of a major hydrophilic portion, in which the catalytically active part resides, and a minor hydrophobic portion which is essential for attachment to the membrane (35). In this study, the apparent molecular weight of the peroxisomal and microsomal NADH cytochrome c (b_5) reductases was 35 kDa as estimated by SDS-PAGE. Since the peroxisomal reductase remained with the membrane fraction after carbonate treatment, it may also be an integral membrane protein. Whether it has the same molecular characteristics as the ER enzyme needs to be determined. **Table 4** summarizes the characteristics of the peroxisomal and microsomal NADH cytochrome c (b_5) reductases. The major differences are seen in substrate specificity and the effect of Triton X-100. As previously mentioned, several investigators have reported the presence of this reductase in peroxisomes (13, 14, 16). The reported values of specific activities for the cytochrome c reductase range from 0.07 to 0.41 μmol per min per mg of peroxisomal protein. Our data are in good agreement with the values reported by Appelkvist et al. (13) (0.07 μmol per min per mg). The estimated ER contamination in this study was also very low, 1.5%. There is also evidence for the presence of cytochrome b_5 in peroxisomes which comes from immunocytochemistry studies (15, 17) in combination with the functional assays showing b_5 reduction (17), and palmitoyl-CoA-dependent b_5 reduction in peroxisomes but not microsomes (42). An average value of peroxisomal b_5 content is listed in Table 4.

It is well known that the microsomal electron transport system mediates a large variety of reactions which include hydroxylation reactions in the synthesis of bile acids (7, 8, 43), lipid peroxidation (44), desaturation of fatty acids (45, 46), synthesis of plasmalogens (47), and cholesterol biosynthesis (48–51). The glyoxysomal membranes also contain an electron transport system quite similar to that of the ER. The glyoxysomal membrane is rich in flavin and contains cytochrome b_5, cytochrome P-420, NADH cytochrome b_5 reductase (as measured by $K_3Fe(CN)_6$ reduction), NADH cytochrome c reductase (as measured by cytochrome c reduction), and NADPH cytochrome P-450 reductase (14, 52, 53). The function of the glyoxysomal membrane electron transport system is not known. Since reducing equivalents in the form of NADH are produced during β-oxidation, it has been suggested that this electron transport system may participate in the reoxidation of intraglyoxysomal NADH (53).

It is clear from this study that peroxisomes from both normal and clofibrate-treated animals contain cytochrome reductases as demonstrated by immunoblotting. Whether the reductases in normal liver peroxisomes are inactive or are simply present in too low concentrations for biochemical measurements is not clear.

The presence of these enzymes in peroxisomes provides strong support to the growing number of studies demonstrating the role of this organelle in bile acid synthesis (2, 4–6). Specifically, it would be very significant if the hydroxylase activity reported in peroxisomes (6) is also a cytochrome P-450, as is the case for the ER and mitochondria (7, 9). More recently, the discovery of HMG-CoA reductase (the rate-limiting enzyme in cholesterol synthesis) in the peroxisomes (32, 40) raises the question of the possibility of cholesterol synthesis by peroxisomes. In fact, we have obtained evidence of cholesterol synthesis by peroxisomes in the presence of rat liver cytosol (54). The presence of the cytochrome reductases may be required for this synthesis, as is the case in microsomes (51). Finally, as in the glyoxysome, the presence of β-oxidation in peroxisomes is well established (55), but the mechanism of reoxidation of NADH in the peroxisome is not understood. The NADH cytochrome c reductase and cytochrome b_5 might participate in disposing of these reducing equivalents generated during β-oxidation as has been proposed (56).

The peroxisomal cytochrome reductase system appears to be unique. Moreover, since the physiological acceptors of the cytochrome reductases in peroxisomes are not known and the biochemical measurements were not optimized for peroxisomes, the actual capabilities of this electron transport system cannot yet be evaluated. Thus, the current rates obtained with artificial acceptors may, in fact, be minimal values. Only purifying the enzymes to homogeneity from the peroxisomes and comparing their properties will clearly establish whether or not the same enzyme is present in peroxisomes and microsomes.

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