# Biogenesis of Peroxisomes: Immunocytochemical Investigation of Peroxisomal Membrane Proteins in Proliferating Rat Liver Peroxisomes and in Catalase-Negative Membrane Loops

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*Abstract*. Treatment of rats with a new hypocholesterolemic drug BM 15766 induces proliferation of peroxisomes in pericentral regions of the liver lobule with distinct alterations of the peroxisomal membrane (Baumgart, E., K. Stegmeier, F. H. Schmidt, and H. D. Fahimi. 1987. *Lab. Invest.* 56:554–564). We have used ultrastructural cytochemistry in conjunction with immunoblotting and immunoelectron microscopy to investigate the effects of this drug on peroxisomal membranes. Highly purified peroxisomal fractions were obtained by Metrizamide gradient centrifugation from control and treated rats. Immunoblots prepared from such peroxisomal fractions incubated with antibodies to 22-, 26-, and 70-kD peroxisomal membrane proteins revealed that the treatment with BM 15766 induced only the 70-kD protein. In sections of normal liver embedded in Lowicryl K4M, all three membrane proteins of peroxisomes could be localized by the postembedding technique. The strongest labeling was obtained with the 22-kD antibody followed by the 70-kD and 26-kD antibodies. In treated animals, double-membraned loops with negative catalase reaction in their lumen, resembling smooth endoplasmic reticulum segments as well as myelin-like figures, were noted in the proximity of some peroxisomes. Serial sectioning revealed that the loops seen at some distance from peroxisomes in the cytoplasm were always continuous with the peroxisomal membranes. The double-membraned loops were consistently negative for glucose-6-phosphatase, a marker for endoplasmic reticulum, but were distinctly labeled with antibodies to peroxisomal membrane proteins. Our observations indicate that these membranous structures are part of the peroxisomal membrane system. They could provide a membrane reservoir for the proliferation of peroxisomes and the expansion of this intracellular compartment.

The biogenesis of peroxisomes (POs)[1] has been the subject of intensive research in recent years (for review see references 14, 21, 41). All PO proteins, including PO membrane proteins (PMP), are synthesized on free ribosomes and probably transported posttranslationally into the organelle (24, 25, 40, 47, 53, 55, 64).

Recently, it has been shown that the targeting signal for the import of proteins into POs may be located not only at the carboxy-terminal end of the PO proteins (28, 29) but also in the amino-terminal part or even in the midportion (63), and it has been suggested that the proteins may be bound to a receptor before the ATP-dependent translocation over the PO membrane (38). Nevertheless, it remains unclear whether there are specialized regions in the PO membrane which preferentially import PO proteins from the cytoplasm.

1. *Abbreviations used in this paper*: DAB, 3,3′-diaminobenzidine; PAG, protein A-gold; PMP, peroxisomal membrane protein; POs, peroxisomes; SER, smooth endoplasmic reticulum.

The early observations of the close morphological association of POs with the smooth ER (SER) (51) led to the suggestion that POs may arise by budding from the ER (19, 52). Although this concept has been abandoned in the mean time, the exact nature and function of membranous structures seen occasionally attached to or associated with the PO membranes remains to be elucidated. For example, in proliferating POs, induced by partial hepatectomy or by treatment with hypolipidemic drugs, tail-like extensions as well as ring- or hook-shaped membranous structures have been described which were interpreted as connections between POs and SER (36, 37, 51, 56, 67, 68). Recent three-dimensional reconstruction studies of ultrathin sections have revealed that some of these structures are indeed interconnecting bridges between two or several spherical POs (27, 74). These findings are consistent with the concept of a distinct intracellular compartment containing the PO matrix proteins (54) referred to as "the PO reticulum concept" (42). The marked

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proliferation of POs induced by hypolipidemic drugs is a useful model for the investigation of peroxisomal biogenesis. In this study we have used a new hypocholesterolemic drug BM 15766, which has a well-defined target in the pathway of cholesterol biosynthesis (3, 4). We showed recently that this drug induces marked proliferation of POs in perivenous hepatocytes (5) causing distinct alterations of the PO membrane. From the several PMPs (23, 32, 33), we have used monospecific antibodies against those with $M_r$ of 22, 26, and 70 kD in conjunction with immunoblotting and immunoelectron microscopy, to characterize the membranous structures associated with proliferating POs. In addition, ultrastructural cytochemistry for marker enzymes of POs (catalase) and ER (glucose-6-phosphatase) have been applied to assess the relationship of the two organelles.

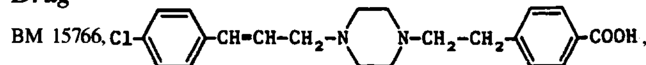
The results indicate that those membranous structures which are associated with proliferating POs are heavily labeled with the antibody against 70-kD PMP, indicating that they belong to the PO membrane system.

# Materials and Methods

## Animals

16 male Sprague–Dawley rats weighing 140–200 g and kept on a normal laboratory diet and water ad libitum were used. Half of them received 75 mg/kg body weight of BM 15766 suspended (75 mg/10 ml) in methylcellulose (tylose) for 14 d via a gastric tube. Corresponding controls were administered the same amount of tylose suspension. All animals were fasted for 16 h before death.

## Drug




was obtained through the courtesy of Boehringer-Mannheim GmbH (Mannheim, FRG). The drug inhibits 7-dehydrocholesterol-Δ7-reductase, the enzyme catalyzing the last step of the cholesterol synthesis (4).

## Isolation of Peroxisomes

Hepatic POs from controls and BM 15766-treated animals were isolated as described before (72). Briefly, crude PO fractions were obtained by differential centrifugation of the total homogenates prepared in a homogenization buffer (250 mM sucrose, 5 mM MOPS, 1 mM EDTA, 0.1% ethanol, pH 7.4) containing in addition 0.2 mM PMSF, 1 mM ε-amino-caproic acid, and 0.2 mM DTT. The crude PO preparations were subjected to density-gradient centrifugation on a continuous Metrizamide gradient (1.10–1.26 g $\times$ cm$^{-3}$) spun in a vertical rotor (VTi 50; Beckman Instruments, Inc., Fullerton, CA) at 20,000 rpm for ~1 h. The PO fractions banding in the density range of 1.24 g $\times$ cm$^{-3}$ were collected by means of a glass cannula and stored at −20°C. The purity of the PO fraction was assessed according to Leighton et al. (44) and by electron microscopy. Biochemical assays were performed according to standard procedures described before (72).

## Preparation of Antibodies

Monospecific antibodies against 22-, 26-, and 70-kD PMP were raised in rabbits as described previously (33). The specificity of the antibodies was assessed by immunoblotting. The antibodies (40 mg/ml) were diluted 1:1,000 or 1:2,000 before immunocytochemical incubation.

## Preparation of Protein A–Gold (PAG) Complex

Gold soles with a particle size of 12 nm were conjugated to protein A (Pharmacia Fine Chemicals, Uppsala, Sweden) at pH 5.9 using the method of Slot and Geuze (62) and the PAG complex was isolated by sucrose density-gradient centrifugation (61). The complex (diluted 1:50) exhibited an optical density of 0.45 at 528 nm and was stored in 25% glycerol at −20°C.

## Immunoblotting

SDS-PAGE was performed using a microslab electrophoresis apparatus (KS 8010 MSE; Marysol Industry Co., Ltd., Tokyo). Samples containing 4.2 μg/lane PO proteins were applied to gels (9 $\times$ 5 $\times$ 0.1 cm, 10–12.5% resolving, 3% stacking gel), stacked at 15 mA and resolved at 25 mA (total time ~1 h). Electrotransfer onto nitrocellulose sheets was accomplished at 30 V for 60 min.

For protein staining, the blots were washed for 1 h with 0.15 M PBS containing 0.05% Tween 20 and stained overnight with "Auro Dye" solution (49) according to the manufacturer's specifications (Janssen Pharmaceutica, Beerse, Belgium).

For immunoblots, the nonspecific binding sites were blocked with 0.15 M 10% newborn calf serum/PBS, pH 7.2, containing 0.05% Tween 20 (NCS/PBS/T) for 1 h with gentle rocking, changing the medium three times. Nitrocellulose sheets were incubated overnight with the appropriate antibodies diluted 200 times with NCS/PBS/T, followed by a fourfold wash. Antigen–antibody complexes were visualized by PAG (15) and the signals thus obtained were amplified by an anti-protein A step (8). The immunoblots were quantitated using a computer-controlled image analysis system (Texture Analysis System; E. Leitz, Inc., Wetzlar, FRG) as described recently (6). The optical extinction of each band was multiplied with its surface area and the results for each membrane protein from PO preparations of control and treated rats were compared.

## Morphology

The livers of all animals were fixed for 5 min by perfusion with 0.25% purified glutaraldehyde (Serva Feinbiochemica GmbH, Heidelberg, FRG) in 0.1 M Pipes buffer, pH 7.4, via the portal vein. Tissue sections, 50–100 μm thick, were cut with a "microslicer" (Dosaka Electron Microscopy Company, Kyoto, Japan), postfixed with the ferrocyanide-reduced $OsO_4$ (39), and embedded in Epon 812.

## Cytochemistry

For the cytochemical localization of catalase the tissue sections were incubated for 1 h with 5 mM 3,3'-diaminobenzidine (DAB) and 0.15% $H_2O_2$ in 0.01 M Teorell–Stenhagen buffer, pH 10.5, at 37°C (2). Glucose-6-phosphatase was localized using 2 mM disodium-glucose-6-phosphate as substrate dissolved in 0.1 M Tris-maleate buffer, pH 6.5, containing 3 mM $CeCl_3$ (57). The preincubation was carried out without substrate at 37°C for 30 min, followed by incubation with substrate for 1 h.

Acid phosphatase was localized with 3 mM disodium-CMP in 0.04 M sodium-acetate buffer, pH 5, and 5% sucrose containing 3 mM $CeCl_3$ (57). Preincubation and incubation conditions were similar to those for glucose-6-phosphatase.

## Immunoelectron Microscopy

For the immunocytochemical localization of the 22-, 26-, and 70-kD PMP, the tissue sections were embedded either in Lowicryl K4M (1, 7, 58) or in London Resin White (LR White) (50). Ultrathin sections were soaked for 1 h on drops of 1% BSA in 20 mM Tris-HCl, pH 7.4, and incubated overnight with the appropriate antibodies in 0.1% BSA in TBS. After washing several times on drops of TBS, the grids were incubated for 90 min with PAG complex diluted 1:50 or 1:60 in TBS. In some experiments Triton X-100 (0.05%) was added to the incubation and washing media. Subsequently, the grids were washed with distilled water and air dried, followed by contrasting with uranyl acetate and lead citrate.

# Results

## General Effects of Treatment with BM 15766

The serum sterols and triglycerides were reduced and the livers of treated rats were enlarged as described previously (5). This implies that the drug at the dose used (75 mg/kg body weight) exerted its hypolipidemic effect.

## Characterization of the Isolated PO Fractions from Treated and Control Rats

The specific activity of the reference enzyme, catalase, in PO

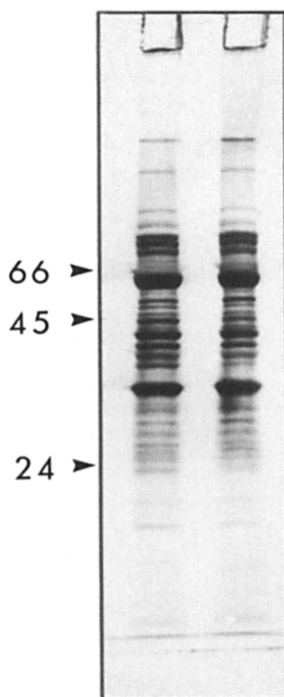



*Figure 1.* SDS-PAGE of highly purified PO fractions (4.2 μg protein/lane) from controls (*left lane*) and BM 15766-treated animals (*right lane*) blotted to nitrocellulose and stained with Auro Dye (49).

fractions from control and treated rats was 8.77 and 10.8 U/mg protein, respectively. This was ~38-fold higher than in the original homogenates, corresponding to our earlier observations (72). Furthermore, SDS-PAGE preparations of PO fractions, blotted onto nitrocellulose sheets and stained with Auro Dye (Fig. 1), revealed a pattern similar to that reported for highly purified POs (23). Although some differences between the blots of treated animals and controls were seen, the alterations of 22-, 26-, and 70-kD PMP in treated rats could not be readily detected in such preparations.

### *Localization of PMPs in Isolated Fractions by Immunoblotting*

The immunoblots prepared from isolated PO fractions and incubated with antibodies against 22-, 26-, and 70-kD PMP (Fig. 2) revealed for each protein a single band at the expected $M_r$ range, thus confirming the monospecificity of the antibodies used. In preparations incubated with 70-kD antibody, a very faint band with an $M_r$ of 68 kD was also observed, which has been suggested to be the product of proteolytic modification of the 70-kD protein (33). Quantification of the immunoblots from treated animals revealed an increase of the 70-kD PMP by 330%, while the 68-kD PMP remained unchanged. The 22-, and 26-kD PMP were only slightly reduced (10%) in comparison to controls.

### *Morphological and Cytochemical Observations*

The overall alterations of hepatic ultrastructure in rats treated with BM 15766 has been described in detail previously (5). In the present study, the main emphasis will be on the effects of treatment on POs with special attention on alterations of their membranes. In hepatocytes surrounding the terminal hepatic venules, marked proliferation of POs with formation of clusters was noted. These consisted of three to five POs which were separated only by a single cisterna of SER (Fig. 3 *a*). Membranous whorls resembling myelin-like figures were noted quite often in close proximity or in direct continuity with the limiting membranes of many POs (Figs. 3 *b* and 5 *a*). In addition, double-membraned loops, consisting of two parallel-running membranes separated by a distance of 40–60 nm, were seen in the close vicinity of many POs (Fig. 3 *d*). In contrast to the cisternae of SER, which are usually fenestrated in rat hepatocytes, the double-membraned loops did not exhibit any evidence of fenestration. Some double-membraned loops surrounding portions of the cytoplasm enclosed segments of SER, glycogen, and occasionally smaller POs (Figs. 3 *d*, 4, and 5 *c*). Some POs showed invaginations of their limiting membrane which appeared continuous with double-membraned loops extending into the cytoplasm. Some loops were also observed free in the cytoplasm at certain distance to POs (Fig. 5 *b*). Analysis of serial sections, however, revealed that such free loops were always continuous with the limiting membrane of a PO (Fig. 4). Some of the loops with cytoplasmic contents resembled autophagic vacuoles (Fig. 4, $S_3$), although never any evidence of autophagic degradation of their contents was observed. The nonlysosomal nature of such structures was confirmed by acid phosphatase cytochemistry (CMPase), which was negative, while lysosomes and the *trans*-Golgi cisternae were positive (Fig. 5 *d*). In sections stained with DAB for catalase, similar double-membranous channels (without DAB reaction product in their lumen) interconnected occasionally two positively stained POs (Fig. 3 *c*). The absence of catalase reaction in the lumen of the double-membraned loops is shown also in Fig. 5 *c*. In spite of the direct continuity of the loop with the PO membrane, the DAB reaction product is confined to the matrix of the PO and does not extend into the double-membraned loop (Fig. 5 *c*). In sections incubated for glucose-6-phosphatase, the

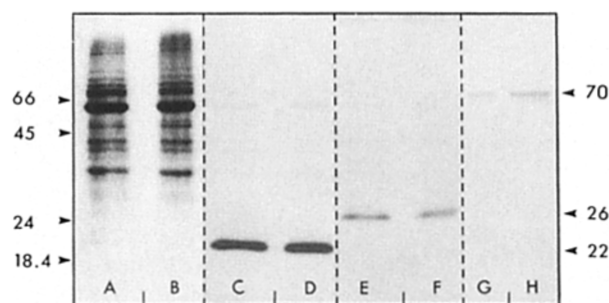
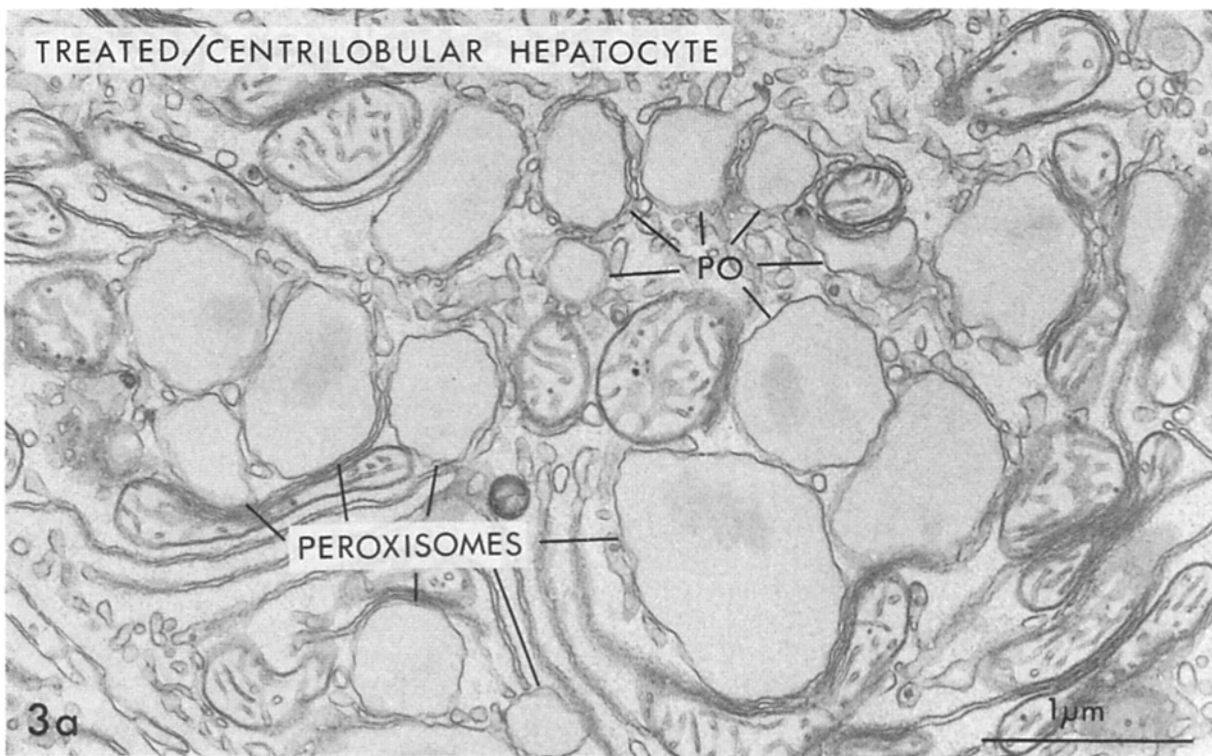
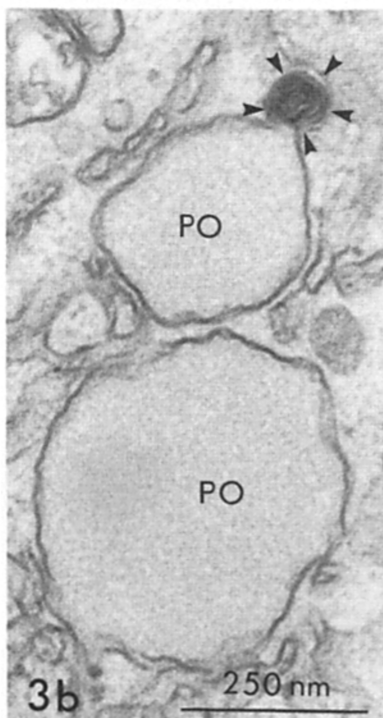
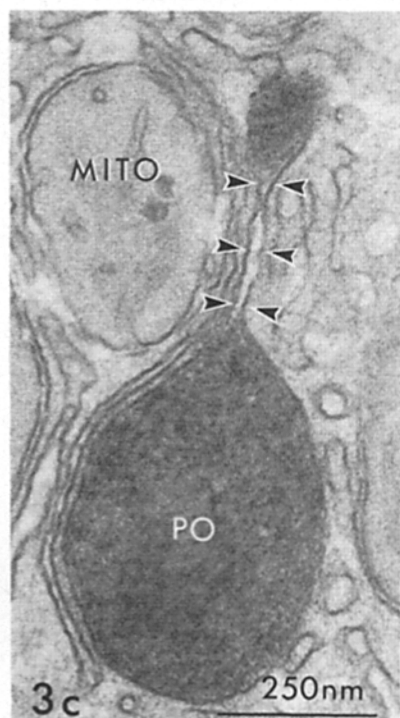



*Figure 2.* SDS-PAGE (lanes *A* and *B*) and immunoblots (lanes *C–H*) of highly purified PO fractions from control rats (lanes *A*, *C*, *E*, and *G*) and from BM 15766-treated animals (lanes *B*, *D*, *F*, and *H*). The gels were stained with Coomassie blue (lanes *A* and *B*). The blots were incubated with antibodies to 22- (lanes *C* and *D*), 26- (*E* and *F*), and 70-kD (*G* and *H*) PMP. All lanes contained 4.2 μg protein, except for lanes *G* and *H* (2.1 μg protein). The antigen–antibody complexes were visualized with the PAG complex. Note, the significant induction of 70-kD PMP with no alteration of 68-kD PMP by treatment with BM 15766 (lanes *G* and *H*). The 22- and 26-kD PMP are not significantly altered. The standards used were BSA, 66 kD; ovalbumin, 45 kD; trypsinogen, 24 kD; and bovine β-lactoglobulin, 18.4 kD (all obtained from Sigma Chemical GmbH, Munich, FRG).



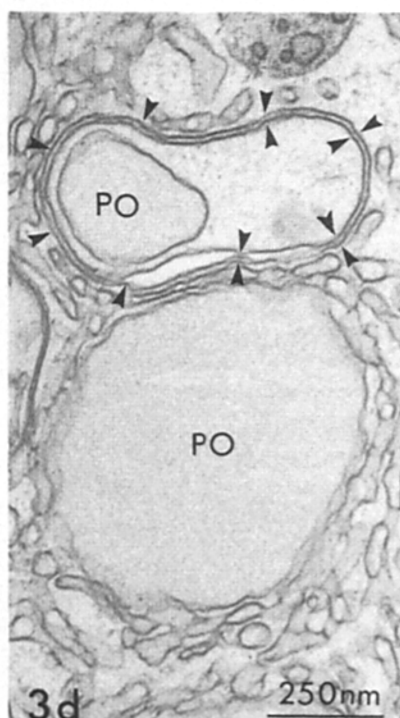




*Figure 3.* Electron micrographs of perivenous hepatocytes from treated animals. (*a*) Overview showing marked proliferation of POs (*PO*), which form large clusters consisting of three to five irregularly shaped particles. Note the close association of SER with the external surface of some POs. (*b*) A myelin-like figure (*arrowheads*) with apparent continuity with the PO membrane. (*c*) Section from material stained for catalase with DAB. A dumbbell-shaped PO (*PO*) is shown with two positively stained portions being interconnected by a catalase-negative double-membraned segment (*arrowheads*). (*d*) A distinct double-membraned loop (*arrowheads*) surrounding a small PO is shown next to a larger one (*PO*). The loop resembles somewhat the segments of SER, but in contrast to the latter, it is not fenestrated and shows a narrower lumen (40–60 nm in contrast to 70–90 nm for SER).

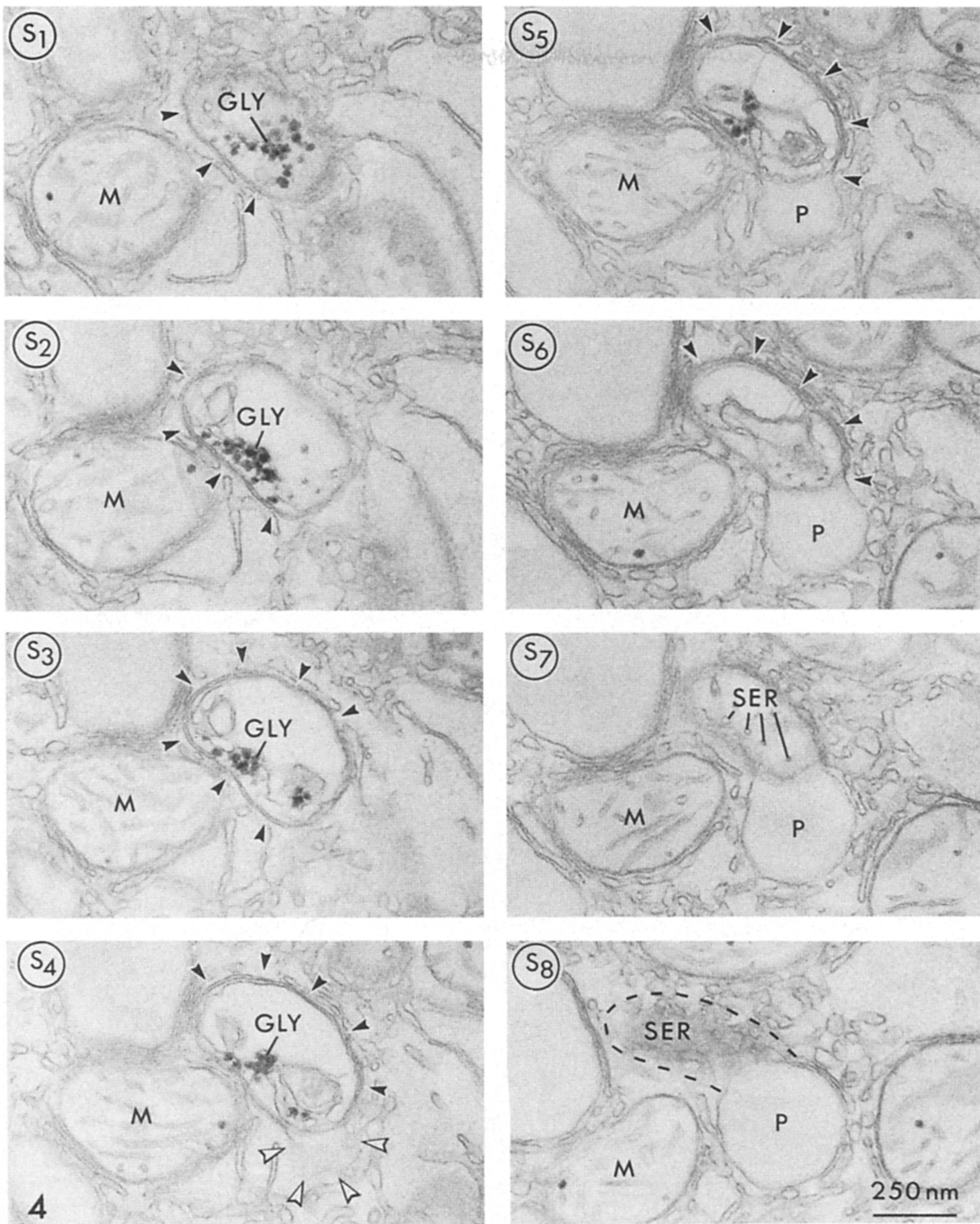



*Figure 4.* Serial ultrathin sections showing the relationship of a double-membraned loop (*black arrowheads*) to an adjacent PO (*P*) in the cytoplasm of a hepatocyte from an animal treated with BM 15766. In section $S_1$–$S_3$ only the double-membraned loop is seen in association with glycogen particles (*GLY*) and some SER segments. Starting with section $S_4$, a PO becomes visible (*white arrowheads*), which in subsequent sections ($S_5$ and $S_6$) shows membrane continuity with the loop. Note also the close relationship of the loop with fenestrated segments of SER (particularly in $S_6$–$S_8$). *M*, mitochondrion.

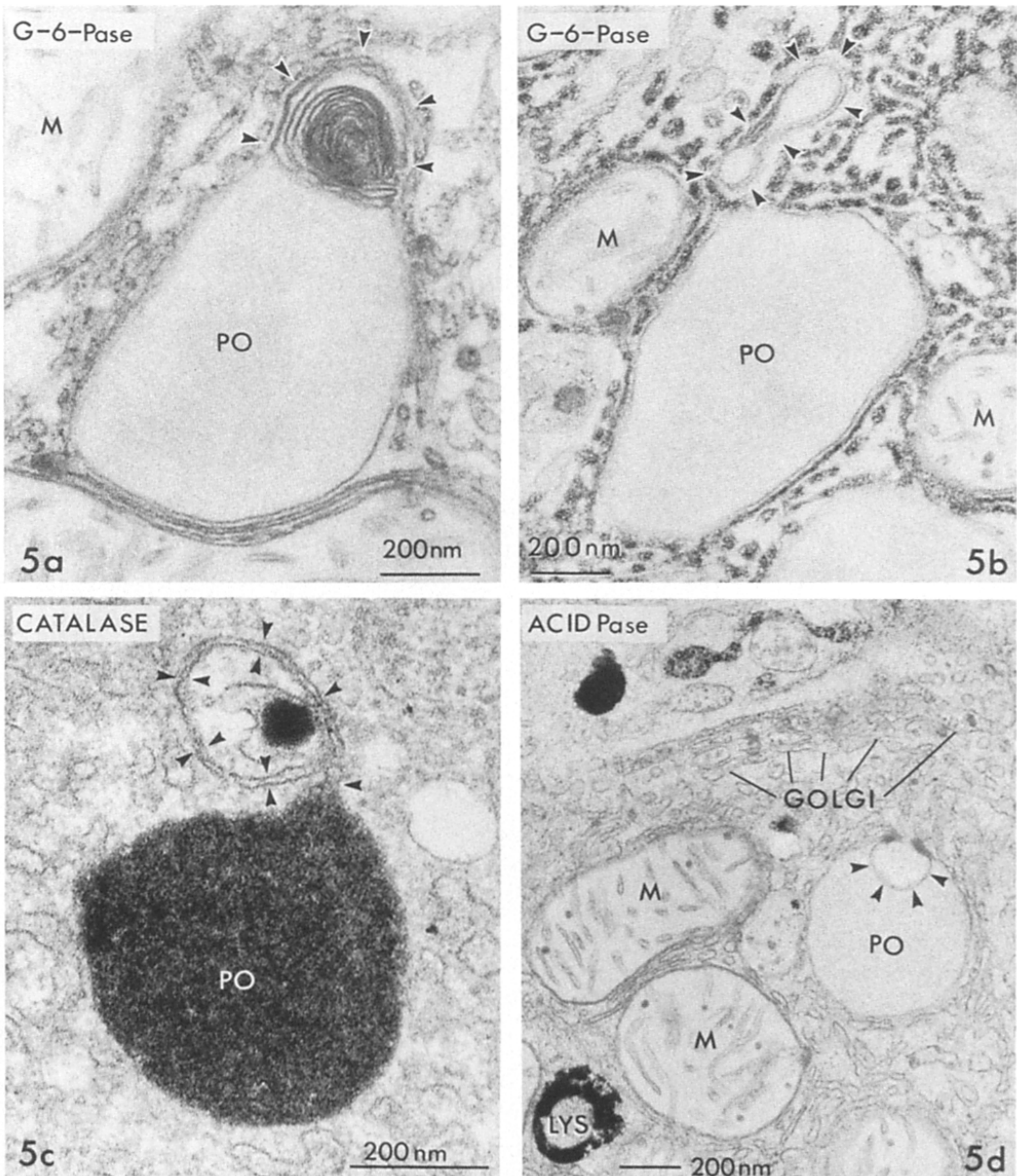



*Figure 5.* Electron micrographs from the livers of animals treated with BM 15766 showing alterations of POs. (*a*) Section incubated for glucose-6-phosphatase (*G-6-Pase*) showing a weak reaction in SER segments. A myelin-like figure, apparently surrounded by a double-membraned loop, is shown in close association with the membrane of a PO (*PO*). (*b*) Section incubated for the localization of glucose-6-phosphatase (*G-6-Pase*). Note the double-membraned loop (*arrowheads*) adjacent to a large PO (*PO*). Whereas, the loop is negative for glucose-6-phosphatase, all the SER cisternae are prominently stained. (*c*) Section incubated for catalase with DAB. Note the loop-like extension of the PO membrane (*arrowheads*). The catalase reaction product is confined to the matrix of the PO (*PO*) and does not extend into the loop. (*d*) Section incubated for acid phosphatase (*Acid Pase*) (CMPase). Note the positive staining in the *trans*-cisternae of the Golgi complex and in lysosomes (*LYS*). A PO (*PO*) with an altered limiting membrane (*arrowheads*) is free of reaction product. *M*, mitochondrion.

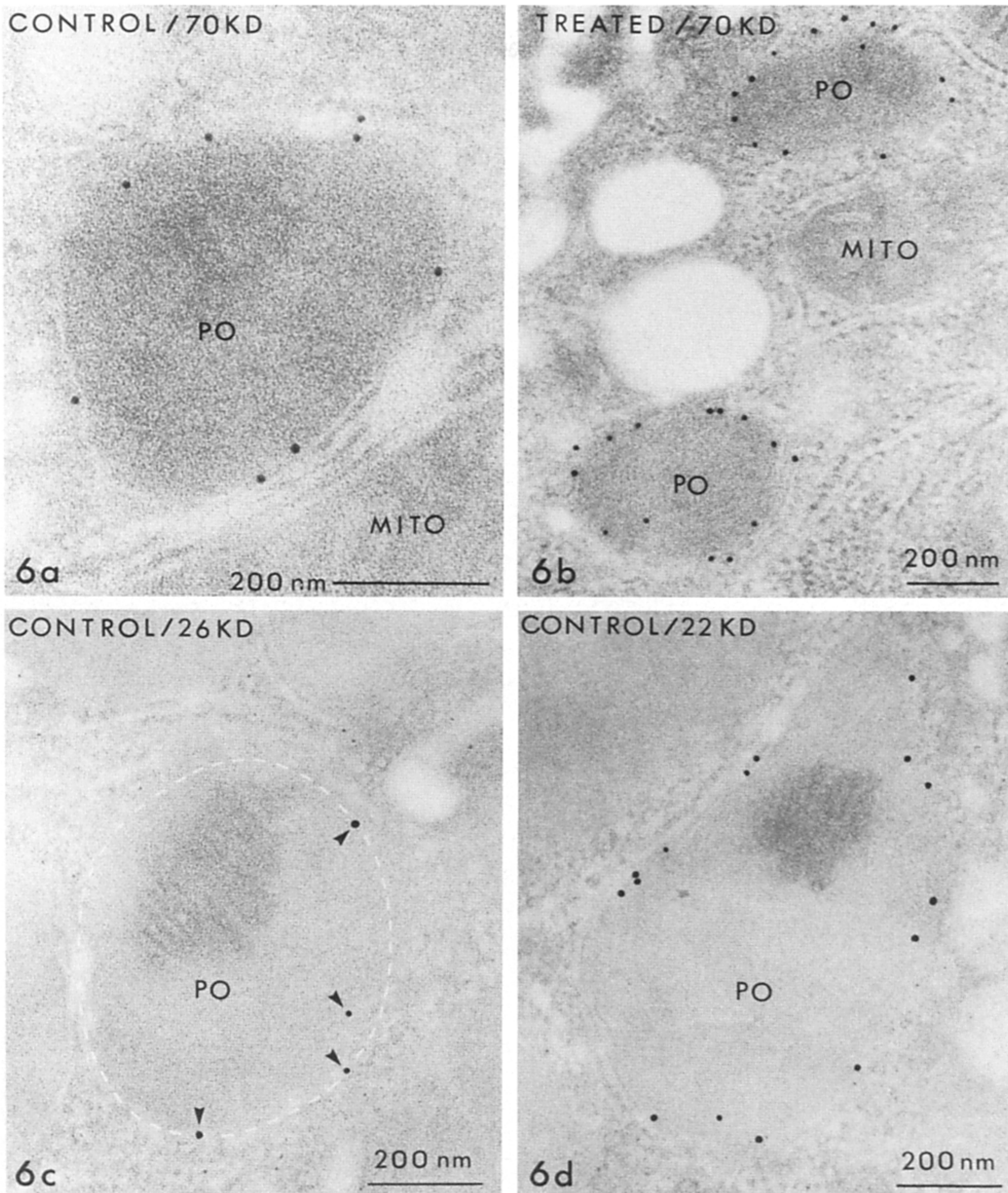



*Figure 6.* Sections of rat liver embedded in Lowicryl K4M and incubated with antibodies against PMP followed by PAG. (*a*) Localization of 70-kD PMP in a PO (*PO*) from a control animal. Note the distinct labeling of the PO membrane with gold particles, in contrast to a mitochondrion (*MITO*) and the SER membranes. (*b*) Localization of 70-kD PMP in the liver of a treated animal. The gold labeling is confined to the membranes of two POs (*PO*), while the mitochondria (*MITO*) and ER membranes are negative. The labeling density appears increased in comparison to the control rat (*a*). (*c*) Localization of 26-kD PMP in a control rat. The gold particles are noted on the limiting membrane of a PO (*PO*). This PMP showed the lowest labeling density in comparison to 22 and 70 kD. (*d*) Localization of 22-kD PMP in a control animal. Note the distinct labeling of the PO membrane. This PMP showed the highest labeling density in control rats in comparison to 26 and 70 kD.

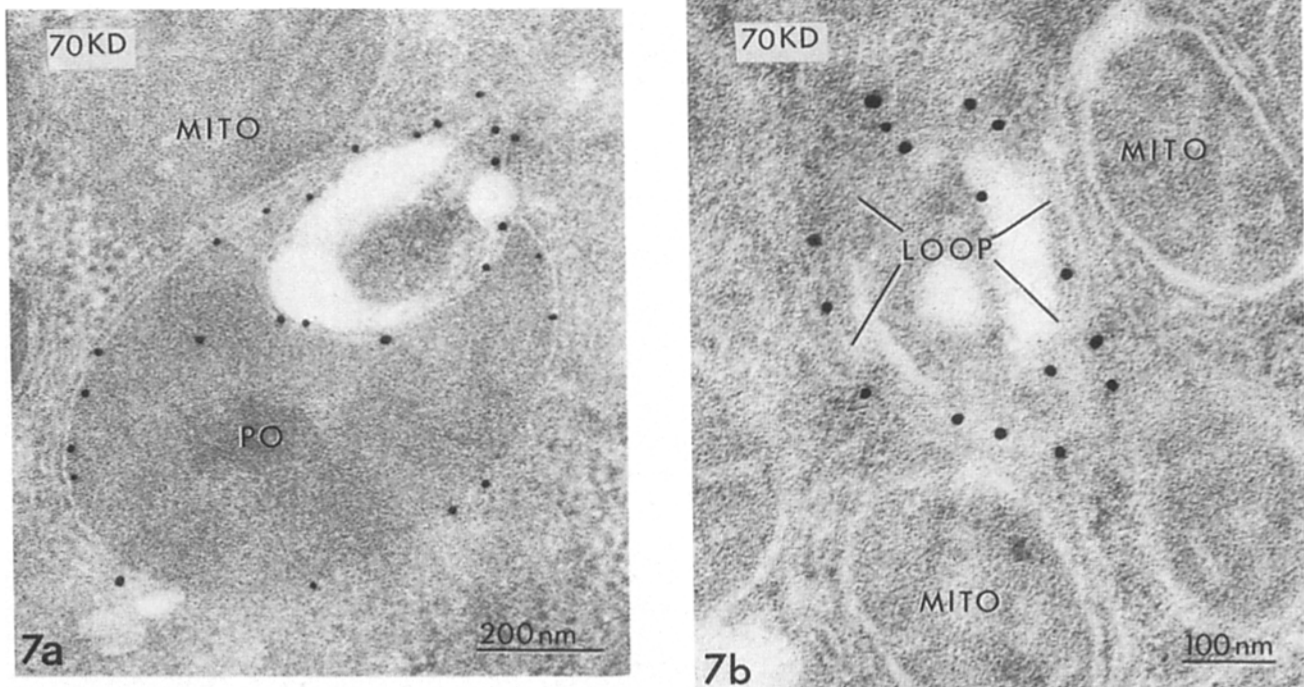



*Figure 7.* Sections of livers from rats treated with BM 15766, embedded in Lowicryl K4M (*a*) and in LR White (*b*), and incubated with the 70-kD PMP antibody. The membranes appear as negative images. (*a*) A PO (*PO*) with an invagination of its limiting membrane into the matrix showing heavy labeling for the 70-kD PMP. (*b*) A double-membraned loop labeled heavily with gold particles representing the antigenic sites for the 70-kD PMP. Note the absence of label in adjacent mitochondria (*MITO*) and ER membranes.

double-membraned loops were negative while the cisternae of ER contained the electron-dense reaction product (Fig. 5 *b*). Based on the above observations, the double-membraned loops could be easily distinguished from segments of SER by (*a*) negative reaction for glucose-6-phosphatase; (*b*) the narrower lumen (40–60 nm); and (*c*) lack of fenestrations.

### *Immunocytochemistry*

Incubation of either Lowicryl K4M- or LR White-embedded sections with the antibodies to 22-, 26-, and 70-kD PMP revealed exclusive labeling of the PO membranes with different intensities for various proteins (Fig. 6, *a–d*). In untreated control rats the highest labeling density was observed with the 22-kD protein, followed by the 70- and 26-kD PMP (Fig. 6, *a*, *c*, and *d*). Since immunoblotting of isolated POs indicated no significant alterations of 22- and 26-kD PMP in treated animals, we concentrated mainly on the distribution of 70-kD PMP in treated animals. The labeling density for this protein was conspicuously increased in livers of rats treated with BM 15766 (Fig. 6 *b*). Moreover, gold labeling for 70-kD PMP was observed in altered POs exhibiting invaginations of their limiting membranes (Fig. 7 *a*) and in adjacent cytoplasm where the double-membraned loops were usually observed (Fig. 7 *b*). Accumulation of gold particles was also seen adjacent to some POs, exhibiting a pattern similar to myelin-like figures.

### *Controls*

Immunocytochemical control preparations incubated with nonimmune serum showed no labeling of PO membranes. Parallel sections incubated with antibodies against catalase and uricase revealed exclusive labeling of either the matrix or the core region (73), while the double-membraned loops remained unlabeled (not shown).

## *Discussion*

### *Alterations of the PO Membrane*

The treatment of rats with the hypocholesterolemic drug BM 15766 induced marked proliferation of POs in centrilobular hepatocytes. Such POs displayed distinct membrane alterations including tail- or loop-like extensions into the cytoplasm, association with myelin-like figures, and invaginations into the matrix. Moreover, double-membraned loops composed of two distinct membranes, separated by a distance of 40–60 nm, which were observed frequently in the cytoplasm of hepatocytes, were found by serial sectioning to be always associated with the PO membranes. Such loops exhibited a negative reaction for glucose-6-phosphatase, were not fenestrated and thus could be easily distinguished from segments of SER. Moreover, by immunoelectron microscopy, positive labeling for the 70-kD PMP was obtained not only in such loops, but also in myelin-like figures adjacent to some POs. These observations suggest strongly that loops and myelin-like figures belong to the PO membrane system and thus may provide a membrane reservoir for the proliferation of POs and for the expansion of this intracellular compartment. The existence of a distinct compartment consisting of POs (PO reticulum) was proposed by Lazarow et al. (42)

and such an interconnected network has been demonstrated since by reconstruction of serial ultrathin sections in mouse and rat hepatocytes (27, 74).

It should be emphasized, however, that the loops and hook-shaped structures noted above are not unique features of the experimental model used in this study. Indeed, they have been observed in numerous previous reports and can be clearly identified in many published electron micrographs, although they were often mistaken as segments of SER. Thus, they were described in developing rat (70) and chicken hepatocytes (20), as well as in regenerating rat liver after partial hepatectomy (51). Furthermore, they are particularly frequent in experimental conditions associated with PO proliferation (22, 35–37, 43, 56, 66–68, 75). Hruban et al. (36) designated such loops as "gastruloid cisternae," which they considered as bridges between ER and POs. Finally, it should be mentioned that similar membranous structures in association with POs have also been described in cells other than mammalian hepatocytes (9, 10, 26, 30, 46, 69). Because of the negative DAB reaction for catalase in the lumen of double-membraned loops (Fig. 5 *c*) their identification as part of the PO membrane system has been extremely difficult. In this respect the immunocytochemical localization of PMP provides a unique approach for the proper identification of these membranous structures. Recently Santos et al. (59, 60), using mono- and polyspecific antibodies against the PMPs, detected by immunocytochemistry "ghost-like" structures in the cytoplasm of fibroblasts from Zellweger syndrome patients. These authors suggested that the ghosts represented PO membranes which could not import the matrical proteins because of defects in the translocation machinery. Moreover, long sinuous tubules with positive DAB content were described in the intestinal epithelial cells of a patient with neonatal adrenoleukodystrophy (11). The possible relationship of the ghost-like structures in fibroblasts and the sinuous tubules in intestinal epithelial cells with the double-membraned loops noted in the present study deserves further investigation.

Another important feature of proliferating POs in this study was their close association with myelin-like figures (Figs. 3 *b* and 5 *a*) which exhibited positive immunolabeling for the 70-kD PMP. Although myelin figures were considered for many years to be fixation artifacts, the systematic studies of Blanchette-Mackie and Scow (12, 13) have established that they represent the sites of accumulation of lipids, particularly ionized fatty acids and phospholipids, in the process of intracellular transport across cellular membranes. This is of particular interest for the biogenesis of POs since it is now generally accepted that the major phospholipids of organelle membranes are synthesized on the cytoplasmic face of the ER (16). The exact mode of transport of phospholipids to the organelles, however, is not yet clear. Recently Zaar et al. (76) described lateral membrane contacts between the SER and POs in bovine kidney cortex. Even after the isolation of POs by Metrizamide gradient centrifugation, small segments of SER remained attached to their external surface. The SER segments were clearly identified by the esterase reaction biochemically and by the positive glucose-6-phospatase staining cytochemically. The close membrane association between SER and POs was also observed in this study. In particular the double-membraned loops and some of the myelin-like figures in the vicinity of POs were completely surrounded by SER segments (Figs. 3 *d*, 4, and 5, *a* and *b*). This could provide the basis for the transfer of newly synthesized phospholipids from the SER by lateral diffusion to those specialized membranous structures which are continuous with POs.

In this respect it should be noted that treatment of rodents with hypolipidemic drugs induces also a marked perturbation in the phospholipid composition of the PO membrane (17). Moreover, a significant increase in the ratio of lysophosphatidyl choline to phosphatidyl choline observed in these animals could be responsible for the increased permeability (45), as well as fluidity (34), of the PO membrane.

### *PMPs*

Although a few proteins, such as NADH–cytochrome *c* (b5) reductase or acyl-coenzyme A synthetase, are present in the membranes of POs as well as SER (31, 48), the membranes of both compartments differ essentially in their protein compositions (23). All PMPs so far studied are synthesized on free ribosomes and probably transported posttranslationally to the organelle membrane (24, 40, 64).

The integral membrane proteins which are present exclusively in POs exhibit major bands of $M_r$ 70-, 68-, 26-, and 22-kD (23, 33, 40). The 68- and 70-kD proteins are closely related, the former being the product of proteolysis of the latter. Moreover, it has been suggested that PMPs of $M_r$ 42.5 and 28 kD are products of proteolysis of the 70-kD PMP (32). Treatment with PO-proliferating agents does not induce a uniform increase of all PMPs. Thus, the 70-kD integral membrane protein increases significantly after the treatment with clofibrate, thyroxine, Wy-14.643, and di(2-ethylhexyl)phthalate while the 68-kD (18) and the 22-kD PMP remain almost unchanged (18, 32, 33). Hashimoto et al. (33) and Crane et al. (18) additionally reported a slight induction of the 26-kD PMP.

In the present study a significant induction of 70-kD PMP with little alteration of the 68-, 26-, and 22-kD PMP was detected by immunoblotting. The augmentation of the 70-kD PMP was also noted in immunocytochemical preparations obtained by the postembedding technique (Fig. 6, *a* and *b*). Our observation of the localization of the 70-, 22-, and 26-kD PMP by the postembedding technique are essentially similar to those of Hashimoto et al. (33).

Little is known about the exact function of various PMPs. A pore-forming activity in the PO membrane has been described corresponding most closely to the presence of a protein with an $M_r$ between 22 and 28 kD, which may be responsible for the high permeability of POs (71). The fact that mainly the 70-kD PMP is reduced in the livers of patients with Zellweger syndrome (65), while it is significantly increased after the treatment with thyroxine and other PO-proliferating agents suggests that this protein could participate in the translocation of matrix proteins from the cytosol into the POs. Since the double-membraned loops were heavily labeled for the 70-kD PMP (Fig. 7 *b*), it would be tempting to speculate that those membranous structures are specialized regions of the PO membrane system which would preferentially import matrix proteins into the PO compartment.

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