

Three-dimensional Reconstruction of a Peroxisomal Reticulum in Regenerating Rat Liver: Evidence of Interconnections between Heterogeneous Segments

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Abstract. The three-dimensional (3-D) form and the interrelationship of peroxisomes (Po) in the model of regenerating rat liver after partial hepatectomy were studied by computer-assisted 3-D reconstruction of serial ultrathin sections. Po were labeled cytochemically for either catalase, which stains them all uniformly, or for D-amino acid oxidase (DAA-OX), which gives a heterogeneous reaction with lightly and darkly stained PO. In regenerating rat liver, Po exhibit marked pleomorphism with some budding forms and dumbbell-shaped ones. The 3-D reconstruction revealed many single spherical Po measuring 0.15–0.8 μm in diameter. In addition, two to five Po were found inter-

connected with each other via narrow 30–50-nm hourglass-shaped bridges forming a reticulum. Such aggregates of Po measured 1.5–2.5 μm across. Whereas all segments of this reticulum stained homogeneously for catalase, they exhibited a marked difference in the intensity of the DAA-OX reaction. These observations are consistent with the view of peroxisomal proliferation by budding or fragmentation from preexisting ones. Under such conditions of rapid growth as in regenerating liver, Po may be interconnected forming a reticulum. The interconnections between Po with differing DAA-OX activities suggest that they originate from the same parent organelle.

PEROXISOMES (Po)¹ in normal rat liver are single, membrane-bound, spherical organelles distributed randomly in the cytoplasm (7, 22, 48). Almost all proteins of peroxisomal matrix are synthesized on free ribosomes and are transported posttranslationally into the organelle (12, 26, 28, 33). The proteins of the peroxisomal membrane (19) follow essentially a similar pattern (10, 25). The exact route of transfer of these proteins and the ultrastructural basis of biogenesis of Po remain, however, still controversial. In most earlier studies, direct membrane continuities between the endoplasmic reticulum (ER) and Po were described, implicating the ER as the source of Po (9, 34, 35, 37). In 1970 Legg, Wood, and associates (30, 38) suggested the possibility of the formation of Po by budding from preexisting ones, and this view seems to have gained strong support recently (27). Furthermore, Lazarow et al. (29) suggested that Po may be interconnected permanently or transiently, forming a reticulum. Indeed, the existence of a peroxisomal reticulum has been shown by serial section studies in a few cell types including the mouse liver (13–15). There are, however, significant differences between Po of different cells and tissues (5), and even within the same cell the Po differ in their content of various oxidases. Thus only 40% of rat liver Po contain the crystalline cores associated with urate oxidase (3, 39), and hetero-

geneity in regard to D-amino acid oxidase (DAA-OX) (40, 45) and α -hydroxy acid oxidase (4) has been demonstrated by EM-cytochemical studies. The interrelationship of these different populations of Po and the question of their formation, whether they arise from the same precursor organelle, have to our knowledge not yet been studied. Using improved methods for staining of catalase (2) and DAA-OX (51) combined with computer-assisted three-dimensional (3-D) reconstruction of ultrathin sections, we have investigated the problem of the interrelationship of Po in the model of regenerating rat liver after partial hepatectomy. This approach has recently been shown to provide a powerful tool in the investigation of topography and the spatial relationship of cell organelles (32). The experimental model of regenerating rat liver after partial hepatectomy was chosen because the proliferation of Po in this system has been well documented (11, 34, 38, 41, 46). Since earlier reports and our own time-sequence studies (50) indicated that Po proliferation with pleomorphic forms was most pronounced at 24 h after hepatectomy, and decreasing thereafter, we have selected this time interval for 3-D reconstruction studies. The results indicate that in regenerating rat liver, in addition to the usual single, spherical Po, two to five Po are at times interconnected with each other, forming a "reticulum." Whereas all segments of the reticulum stain uniformly for catalase, they show marked heterogeneity with respect to DAA-OX.

1. *Abbreviations used in this paper:* DAA-OX, D-amino acid oxidase; ER, endoplasmic reticulum; Po, peroxisome(s); 3-D, three-dimensional.

Materials and Methods

Animals

Normal female Sprague-Dawley rats weighing ~180 g kept on a normal laboratory diet and water ad libitum were used. They were housed in a room with a 12-h light and dark cycle and were subjected to partial hepatectomy, removing approximately two-thirds (left and median lobes) of each liver (21). After the operation animals had access to 10% glucose (6) and were killed 24 h after the hepatectomy. All operations were performed between 8 and 10 a.m. to keep any interference by circadian changes of liver cell organelles (42) to a minimum.

Fixation

The livers were fixed for 5 min by portal vein perfusion with 0.25% purified glutaraldehyde (Serva, Heidelberg, Federal Republic of Germany) in 30 mM NaOH-Pipes buffer, pH 7.2, containing 8% sucrose. The livers were removed, cut into 1-mm-thick slices, and rinsed briefly in the same buffer containing 8% sucrose at 0–4°C. For cytochemical demonstration of catalase, the liver slices were fixed for an additional hour at 0–4°C in 1.5% glutaraldehyde in the same buffer. After a brief rinse, nonfrozen 50- μ m sections were cut with a microslicer (Dosaka EM Co, Kyoto, Japan).

Cytochemical Incubations

Catalase. Sections were incubated for 60 min at 37°C in the dark in a medium containing 5 mM 3,3'-diaminobenzidine tetrahydrochloride in 0.1 M glycine-NaOH buffer, pH 10.5, and 0.15% H₂O₂ (2). The control sections were incubated either in the same medium without H₂O₂ or in the complete medium containing 20 mM 3-amino-1,2,4-triazole.

D-Amino Acid Oxidase. The medium contained 50 mM D-proline (Sigma Chemical Co., Munich, Federal Republic of Germany), 3 mM cerium chloride, 50 mM sodium azide in 0.1 M Tris-maleate buffer, pH 7.8. Sections were preincubated for 30 min in the absence of substrate (D-proline) and for 60 min at 37°C in the complete medium (51). In addition to routinely used 50- μ m sections, incubations were also carried out with 25- μ m sections in order to assess the effect of section thickness upon the cytochemical result. Moreover, some sections were frozen in melting liquid nitrogen and thawed in order to improve the penetration of the incubation medium (23). Control sections were incubated either in the absence of substrate or in the complete medium containing 10 mM Kojic acid, an inhibitor of DAA-OX (5).

Processing for EM

After a brief rinse in the corresponding buffers used for the cytochemical incubation, sections were postfixated for 1 h at 0–4°C with 1% osmium tetroxide followed by reduction with potassium ferrocyanide (24). They were dehydrated in graded ethanol solutions and propylene oxide and embedded in Epon 812. Ultrathin sections were cut on an ultramicrotome, counterstained either briefly with lead citrate for catalase or with uranyl acetate for DAA-OX, and examined in a Philips EM-301 G electron microscope.

Serial Section Studies and 3-D Reconstruction of Po

Selected blocks incubated for catalase were sectioned serially and ribbons of consecutive sections with a thickness of 50–60 nm were collected on Formvar-coated single-slot grids. Photographs were taken without counterstaining at primary magnification of 18,000 in an electron microscope and were enlarged to 40,000 photographically for reconstruction studies. These were directly photocopied onto transparent acetate sheets for proper topographic alignment between consecutive sections. The exact thickness of sections was determined by measuring the thinnest fold in each section (49) and by prior calibration of the electron microscope using a carbon-grating replica (Ernest F. Fullam, Inc., Schenectady, NY). The profiles of individual Po on acetate sheets were traced by hand on an Apple graphic tablet and the coordinates were entered into the files of an Apple IIe microcomputer (Apple Computer, Inc., Cupertino, CA). The details of the computer-based 3-D reconstruction program NEUREC have been reported elsewhere (16, 17). The reconstructed 3-D images were viewed from different angles on the monitor of the microcomputer and were printed out using a printer (FX-80; Epson, Tokyo, Japan).

Results

The ultrastructural changes of hepatocytes in regenerating liver after partial hepatectomy were similar to those described previously (18, 34, 41, 46) and are not presented. In this study only the alterations of Po are discussed.

Localization of Catalase

The electron-dense reaction product of catalase was localized exclusively in the matrix of Po with no evidence of staining of any other organelles. Contrary to the previous report of Rigatuso et al. (38), the free and bound ribosomes adjacent to Po were unstained. The specificity of the reaction for catalase was confirmed in control experiments: omission of H₂O₂ or addition of aminotriazole.

Po in regenerating liver were pleomorphic with marked variation in size ranging between 0.1 and 0.8 μ m in diameter for spherical particles. They often formed clusters consisting of 2–5 and occasionally up to 10 Po. Interconnections between some spherical Po were occasionally observed which consisted of very short, narrow (30–50 nm) hourglass-shaped segments (Fig. 1, *a* and *c*). Some Po exhibited straight or sinuous taillike extensions measuring up to 1.5 μ m which also contained the catalase reaction product (Fig. 1 *b*). The intensity of the 3,3'-diaminobenzidine tetrahydrochloride reaction did not vary significantly in different Po but was somewhat weaker in such taillike extensions (Fig. 1 *b*). The membranes of ER were often associated with and surrounded the various forms of Po, but evidence of direct luminal continuity between the two organelles was never observed.

Analysis of Serial Sections and 3-D Reconstruction

The interrelationship of Po in regenerating liver was assessed by examination of consecutive serial sections stained cytochemically for catalase. Fig. 2, *a* and *b* show a series of 19 serial sections which pass through a total of six Po labeled *A–D* and *M–N*. The four Po labeled *A–D* are interconnected via very narrow hourglass-shaped bridges which are present only in sections S9 and S11 and the particles *M* and *N* are also connected as seen in section S11. The two sets of particles (*A–D*) and (*M–N*), however, are connected neither with each other nor with other Po within the cell. The 3-D reconstruction of these serial sections obtained by the computer program NEUREC is shown in Fig. 3 *a* and *b* revealing that particles *A*, *B*, *D*, and *N* are more or less spherical whereas particles *C* and *M* have an elongated shape. The rotation facility of NEUREC which permits the 3-D reconstructions to be viewed from different perspectives was particularly helpful in assessing the shapes of dilated portions and their interconnections. We have now examined 15 series of sections containing >200 Po. Based on these observations, it can be stated that in regenerating rat liver: (*a*) Many spherical Po with diameters ranging between 0.15 and 0.8 μ m are interconnected with each other. There are, however, also many single, spherical particles which show no connections to other Po, as described for normal rat liver (7, 48). (*b*) The number of spherical Po connected with each other ranges usually between two and five with a total length of ca. 1.5–2.5 μ m across. (*c*) The connections between Po consist either of narrow, short, hourglass-shaped bridges with a di-

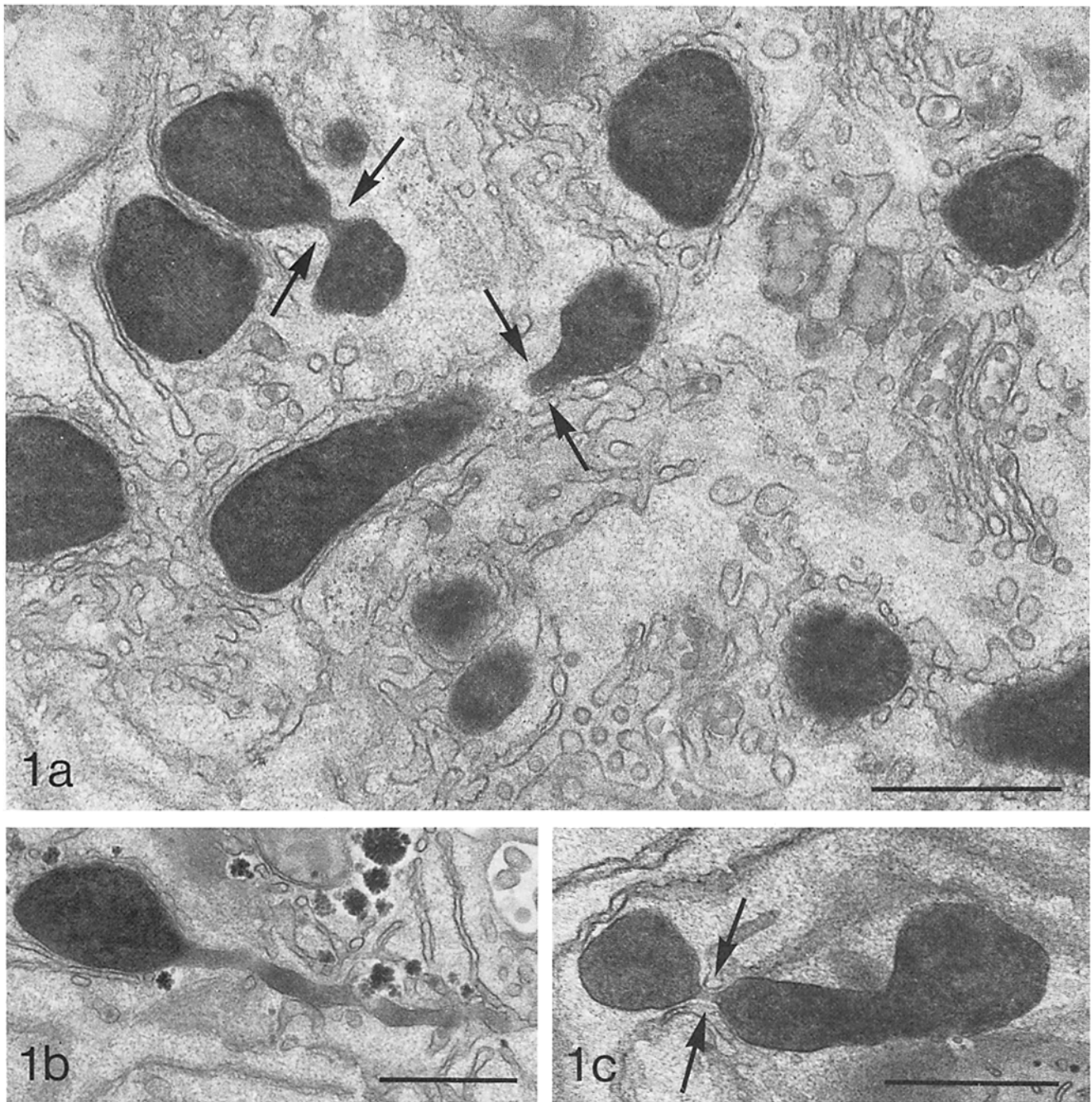


Figure 1. Cytochemical localization of catalase in Po at 24 h after partial hepatectomy. (a) Note the marked variation in shape and size of Po with some dumbbell-shaped forms (*arrows*). (b) A spherical Po with a taillike extension. (c) A pleomorphic Po connected via a narrow bridge (*arrows*) to a spherical Po. Bars, 500 nm.

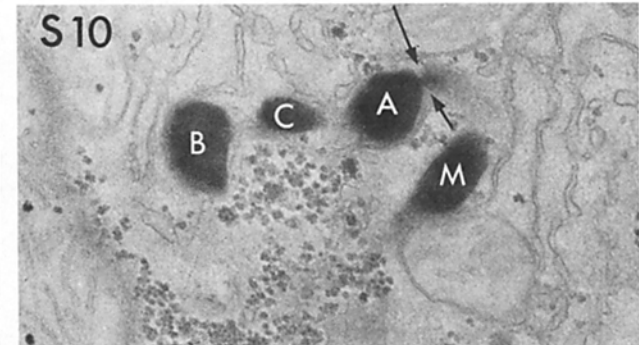
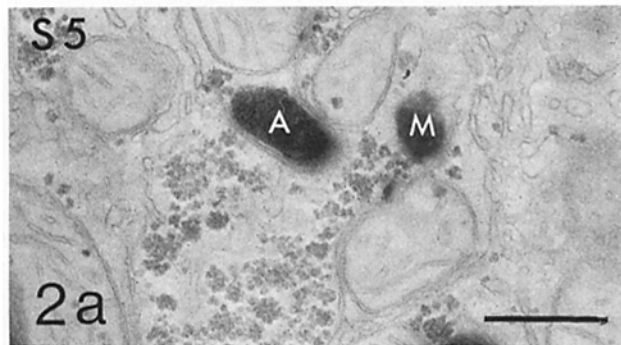
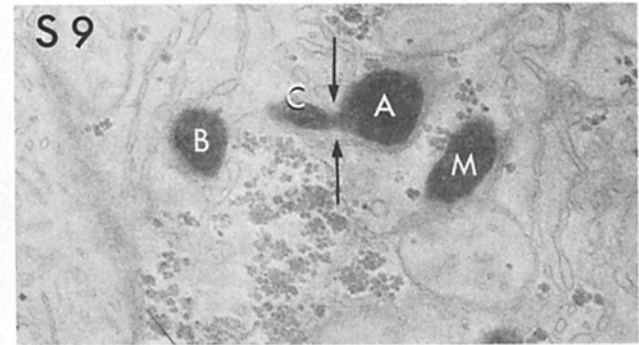
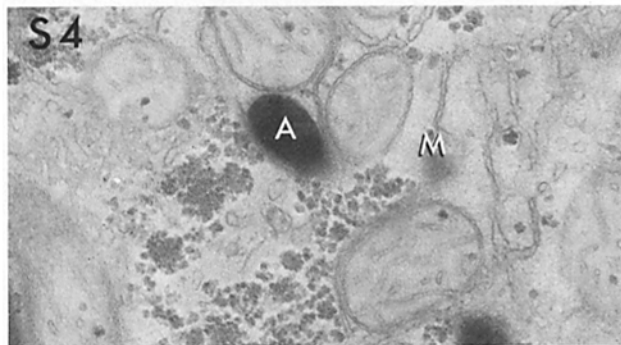
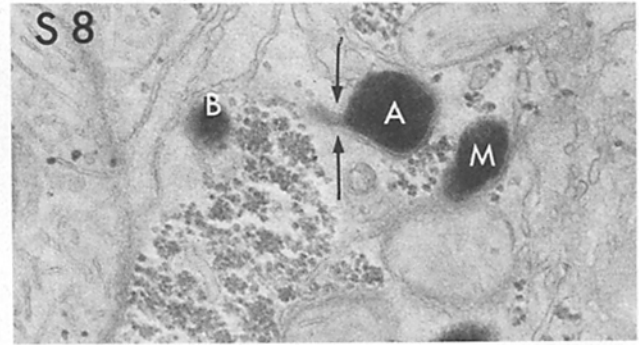
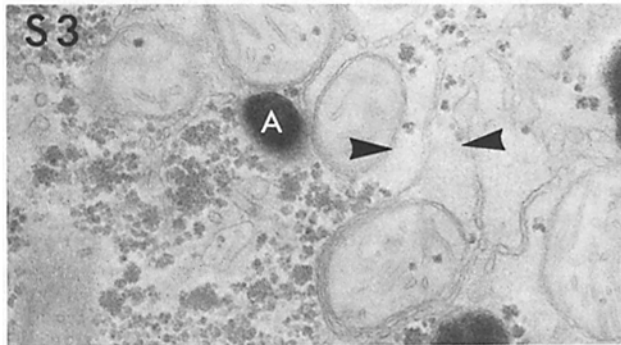
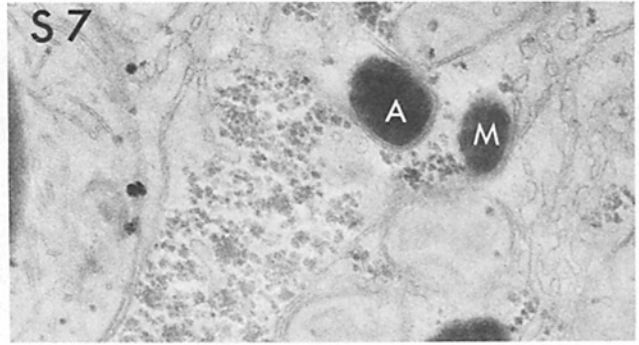
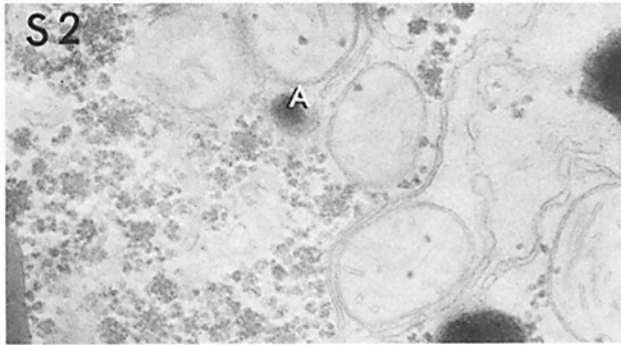
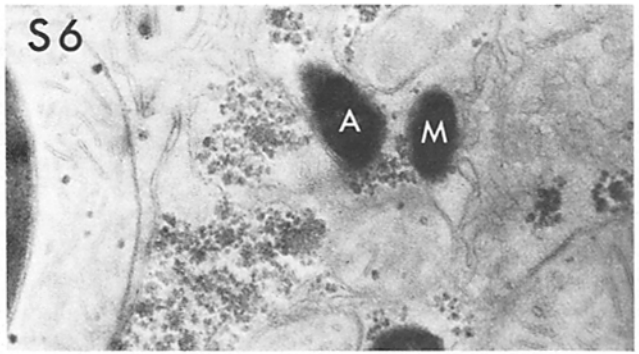
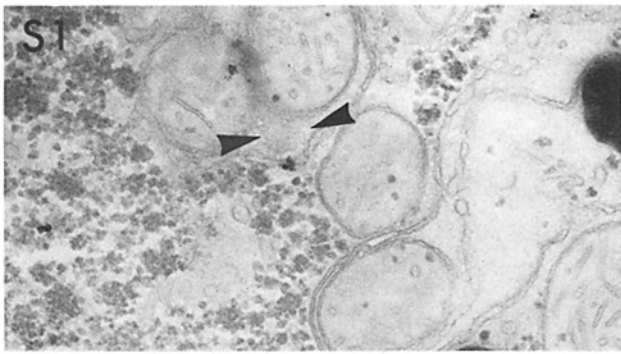
ameter of 30–50 nm, or of larger tubular segments with a diameter of 150–300 nm and a length of up to 2 μ m. Whereas the first type because of its small size is relatively rare in random sections, the latter type is more frequently observed. (d) In clusters of Po, not all individual Po are interconnected.

Localization of DAA-OX

In normal rat hepatocytes the reaction product of DAA-OX was localized in the matrix of Po with marked heterogeneity in the intensity of staining among individual Po of the same

cell (Fig. 4 a). This staining pattern was not related to the inadequate penetration of the incubation medium because similar results were obtained by incubation of either thinner (25 μ m) nonfrozen sections or after freezing and thawing (23). The specificity of the staining was confirmed by control experiments incubated either without D-proline or with an inhibitor of DAA-OX (51).

In regenerating rat liver the variation in the intensity of DAA-OX reaction in Po persisted (Fig. 4 b), with strongly positive Po being present next to moderately or weakly



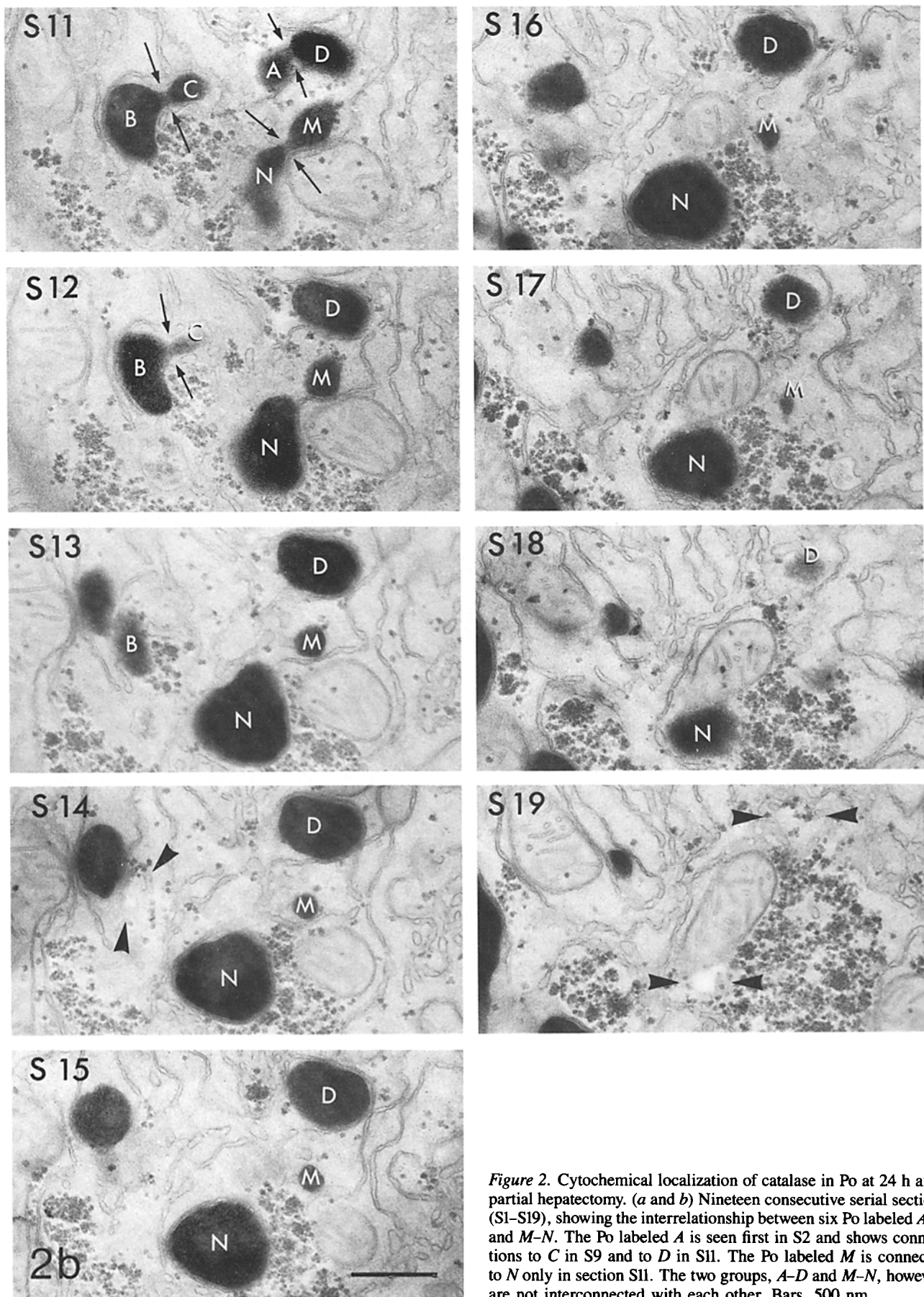


Figure 2. Cytochemical localization of catalase in Po at 24 h after partial hepatectomy. (a and b) Nineteen consecutive serial sections (S1-S19), showing the interrelationship between six Po labeled A-D and M-N. The Po labeled A is seen first in S2 and shows connections to C in S9 and to D in S11. The Po labeled M is connected to N only in section S11. The two groups, A-D and M-N, however, are not interconnected with each other. Bars, 500 nm.

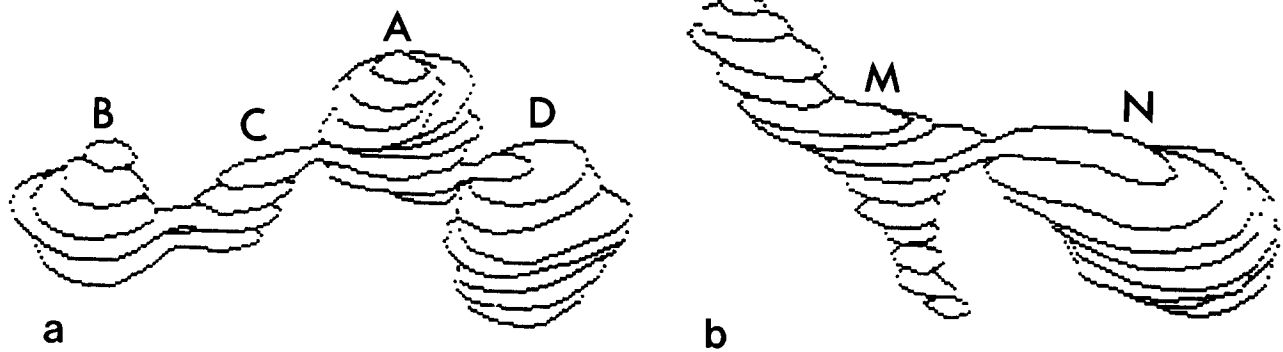


Figure 3. (a and b) 3-D reconstructions of Po labeled A-D and M-N in Fig. 2, a and b obtained by the computer program NEUREC. Note that particles A, B, D, and N are spherical in shape, whereas C and M have an elongated form. The interconnections consist usually of very narrow segments.

stained ones. This heterogeneity was observed also in elongated dumbbell-shaped Po, which showed heavy and light staining in different dialated portions (Fig. 4 b). Particular attention was paid to the pattern of DAA-OX staining in Po interconnected via narrow hourglass-shaped bridges. The most frequent interconnections were found between heavily and lightly stained Po (Fig. 4 c). However, in a few instances such bridges were also found between Po showing the same pattern of either light (Fig. 4 d) or dark reaction (Fig. 4 e). The intensity of the DAA-OX reaction in dumbbell-shaped Po was not related to the presence or absence of crystalline cores. Thus some core-containing segments exhibited a strong reaction (Fig. 4 f), while others were only moderately stained (Fig. 4 g).

Analysis of Serial Sections Stained for DAA-OX

The interrelationship of Po stained for DAA-OX was also analyzed in serial ultrathin sections (Fig. 5). This confirmed that: (a) Po with different intensities of staining were indeed interconnected; (b) single, spherical Po without interconnections showed different intensities of staining.

Discussion

The pleomorphic Po described in regenerating rat liver (34, 38) have been investigated using EM cytochemistry and computer-assisted 3-D reconstruction of ultrathin sections. The results indicate that two to five Po in regenerating liver are interconnected forming a reticulum. In contrast to earlier reports (30, 38), no evidence of staining of ribosomes adjacent to Po for any of the investigated enzymes was noted. Moreover, the localization of DAA-OX revealed marked heterogeneity in the distribution of this enzyme in various segments of the reticulum as well as in individual Po.

Biogenesis of Po and Po Reticulum

The old concept of biogenesis of Po based on biochemical studies of Higashi and Peters (20) and the ultrastructural observations of Novikoff and Shin (34) envisaged that peroxisomal proteins were synthesized in the ER, like secretory proteins, and would enter certain peripheral segments of ER, which would then pinch off becoming Po (for a review see de Duve [8]). This concept was challenged by subsequent

studies of Lazarow and associates (29), who proposed an alternative model involving the synthesis of peroxisomal proteins on free polysomes, and their posttranslational entry into preexisting Po. According to this concept, new Po form by fission from preexisting ones. Moreover, Lazarow et al. (29) suggested that the "tails" of Po seen in electron micrographs were interconnections between neighboring Po rather than connections between Po and ER as presumed earlier (9, 34, 37). Accordingly, the Po should be connected to each other, transiently or permanently, forming a so-called "Po reticulum." Several recent biochemical studies have provided strong support for the validity of this new concept (for a review, see Lazarow and Fujiki [27]). By EM the existence of an interconnected network representing the microbody or the Po compartment has been described in germinating spores of moss *Bryum capillare* (36) and in a mutant of *Neurospora crassa* (47), as well as in some sebaceous glands (13, 14) and recently also in mouse liver (15). In contrast to the pleomorphic Po of the mouse, serial ultrathin section studies of Wedel and Berger (48) have shown that Po of the normal rat liver are single, spherical organelles with an average diameter of 410 nm, distributed randomly in the cytoplasm (7). As demonstrated in the present study, this pattern changes dramatically in regenerating liver with the appearance of many pleomorphic Po (Fig. 1, a-c). Our computer-assisted 3-D reconstructions of ultrathin, serial sections revealed that such pleomorphic Po represent sections through an interconnected network with numerous spherical and tubular portions (Fig. 3, a and b). Since time-sequence studies indicate that pleomorphic Po and cluster formation are transient phenomena which are most prominent at 24-48 h after partial hepatectomy and disappear at later intervals (50), it seems quite likely that the interconnections between Po in regenerating rat liver are also transient corresponding to the phase of rapid growth of the Po compartment. The variation in the number of interconnected Po between two and five, and the observation of many single Po in our 3-D reconstructions underline the dynamic nature of the Po reticulum in regenerating rat liver. This implies also that Po must be breaking off from the "reticulum" to form new single Po. This resembles the budding phenomenon in Po of yeast *Hansenula polymorpha* (44), which under the condition of rapid growth forms small buds attached to larger Po. These break off later, moving into new yeast daughter cells giving rise to a new genera-

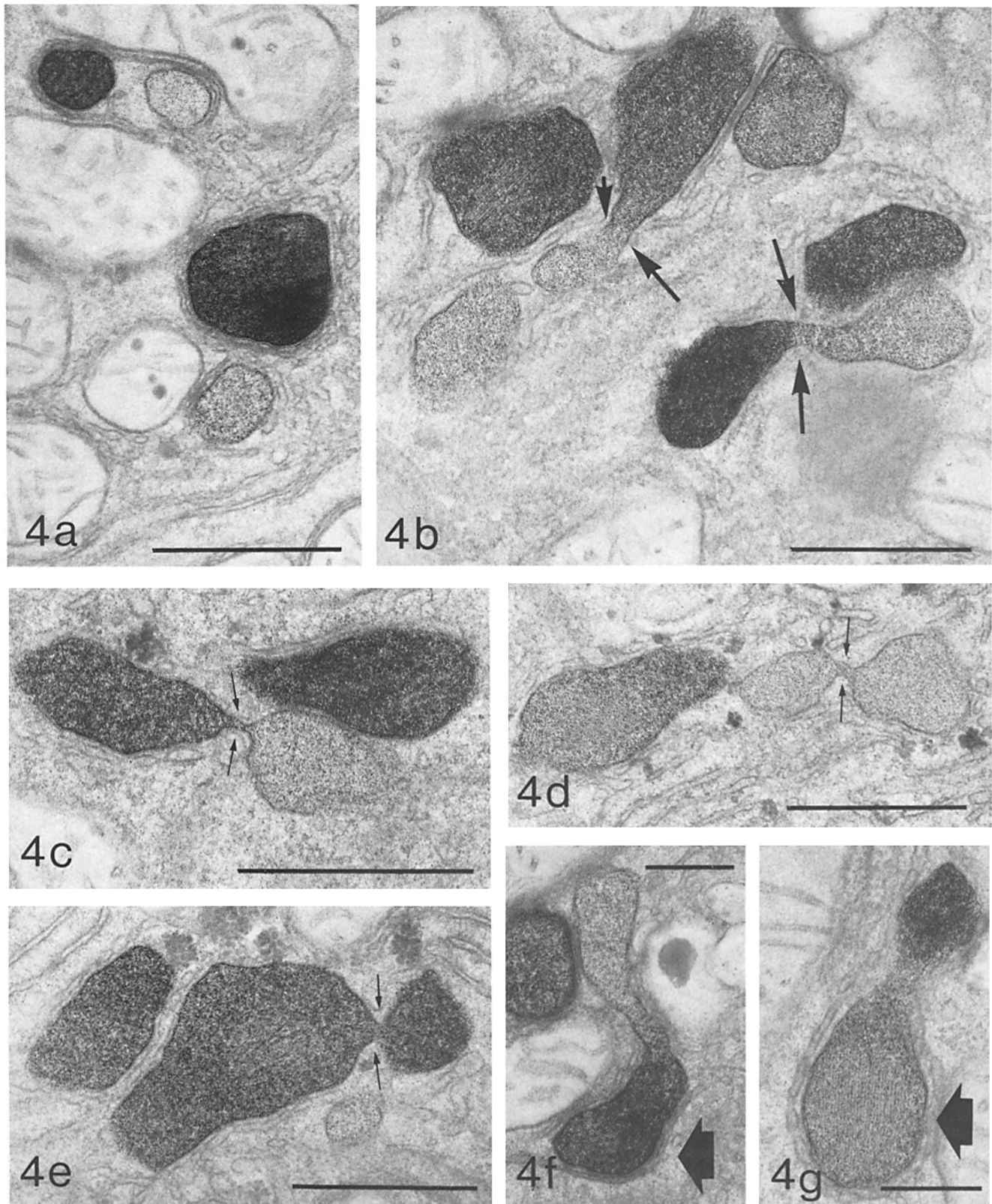


Figure 4. Cytochemical localization of DAA-OX. (a) Normal rat liver with several spherical Po showing different intensities of DAA-OX activity. Bar, 500 nm. (b) Regenerating liver: note the heterogeneous staining of pleomorphic Po. In one dumbbell-shaped Po, segments with heavy and light reaction are interconnected (arrows). Bar, 500 nm. (c) Two Po with different intensities of DAA-OX reaction are interconnected via a narrow bridge (arrows). This type of interconnection between heterogeneously stained Po was seen most frequently. Bar, 500 nm. (d and e) Interconnections between Po with the same type of reaction, stained either (d) weakly or (e) heavily for DAA-OX. Such interconnections between Po with the same reaction pattern were extremely rare. Bars, 500 nm. (f and g) Interconnections between Po with differing patterns of DAA-OX staining. The intensity of reaction for DAA-OX is not related to the presence or absence of crystalline cores (large arrows). Bars, 250 nm.

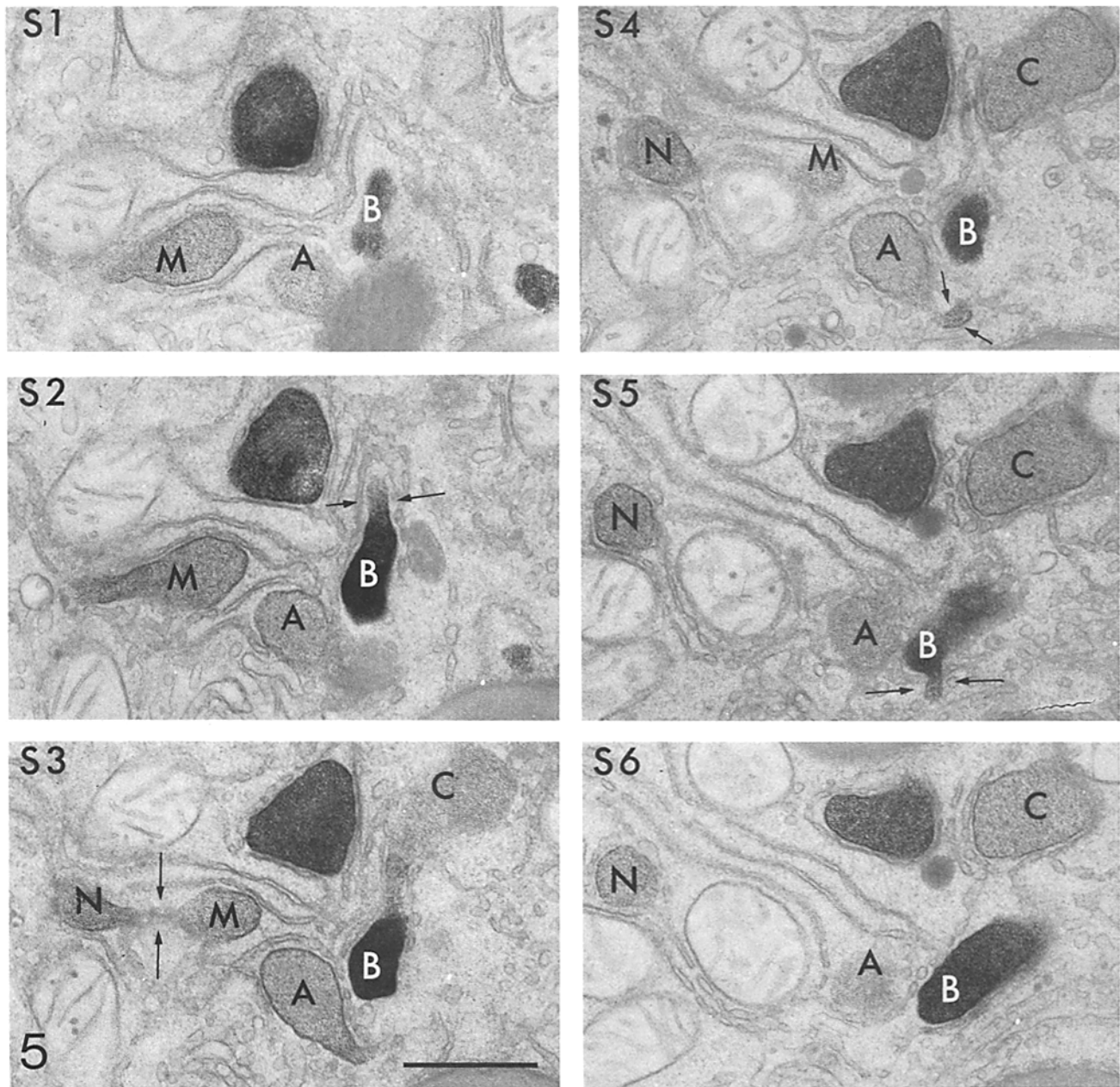


Figure 5. Cytochemical localization of DAA-OX. Six consecutive serial sections (S1-S6) showing interconnections between Po labeled A and B in S5, between B and C in S2 and S3, as well as between M and N in section S3. Note that Po with different intensities of staining are interconnected. Bar, 500 nm.

tion of Po. The possible site for breaking off of new Po from the reticulum could be the narrow, 30–50-nm, hourglass-shaped constrictions between spherical Po (Fig. 1, *a* and *c*). Because of their small size they would provide little resistance and could be sealed off immediately.

Heterogeneity of Po in Regenerating Rat Liver

In contrast to catalase which gave a uniform staining pattern in all Po, the reaction of DAA-OX was heterogeneous in various Po of the same cell (Fig. 4). This heterogeneity was not due to limited penetration of the incubation medium because it was not affected by the use of thinner (25 μ m) nonfrozen sections or by freezing and thawing in order to improve the penetration of cerium ions (23). The heterogeneous staining

of Po for various oxidases has been described before (4, 5, 40, 45) and microdissection studies combined with biochemical analysis have revealed differences in enzymatic activity of peroxisomal oxidases in periportal and perivenous hepatocytes (31). Our observations in regenerating rat liver show that, in large pleomorphic and dumbbell-shaped Po, segments with strong staining are directly connected to regions with light reaction (Fig. 4, *b* and *c*). The direct continuity between these regions was also confirmed by serial, ultrathin section analysis. This implies that the Po reticulum is composed of heterogeneous segments, suggesting that Po with differing DAA-OX activities originate from the same parent organelle. Moreover, the frequent observation of focal 30–50-nm, hourglass-shaped constrictions between seg-

ments stained strongly and weakly for DAA-OX (Fig. 4 c) would support the view that the fragmentation of Po from the reticulum occurs preferentially at these sites. Thus, the difference in enzymatic content within the reticulum could determine the sites of fragmentation. The presence of DAA-OX, however, does not seem to be the only determining factor, in that similar constrictions were also found, although very rarely, between spherical regions with the same type of DAA-OX activity (Fig. 4, d and e).

The heterogeneous distribution of staining for DAA-OX in the matrix of pleomorphic Po indicates that the enzyme must be oligomerized or possibly bound to a component of the matrix which would restrict its free movement. Indeed, recently Alexson et al. (1) showed that there are several poorly soluble matrix proteins within Po, which are difficult to extract, possibly owing to aggregation. These could correspond to the fibrillar or amorphous component of the matrix seen by EM (1). Using immunoelectron microscopy Usuda et al. (43) have shown that in rat kidney DAA-OX is restricted to the central region of the Po matrix whereas the peripheral regions of the matrix are free of labeling. These observations suggest that either oligomerization or the binding of DAA-OX to a component of the matrix (1) may restrict its movement giving rise to the focal heterogeneous staining in the matrix. In conclusion, our observations are consistent with the view of Po proliferation by budding or fragmentation from preexisting ones. Under such conditions of rapid growth as in regenerating liver, Po may be interconnected forming a reticulum. Whereas catalase is localized uniformly in all portions of the Po reticulum, DAA-OX shows a heterogeneous distribution. Single Po may form by breaking off at narrow 30–50-nm constrictions from the reticulum.

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