CYTOCHEMICAL LOCALIZATION OF PEROXIDATIC ACTIVITY OF CATALASE IN RAT HEPATIC MICROBODIES (PEROXISOMES)

H. DARIUSH FAHIMI

From the Harvard Pathology Unit, Mallory Institute of Pathology, Boston City Hospital, Boston, Massachusetts 02118

ABSTRACT

Prominent staining of rat hepatic microbodies was obtained by incubating sections of aldehyde-fixed rat liver in a modified Graham and Karnovsky's medium for ultrastructural demonstration of peroxidase activity. The electron-opaque reaction product was deposited uniformly over the matrix of the microbodies. The microbodies were identified by their size, shape, presence of tubular nucleoids, and other morphologic characteristics, and by their relative numerical counts. The staining reaction was inhibited by the catalase inhibitor, aminotriazole, and by KCN, azide, high concentrations of H_2O_2, and by boiling of sections. These inhibition studies suggest that the peroxidatic activity of microbody catalase is responsible for the staining reaction. In the absence of exogenous H_2O_2 appreciable staining of microbodies was noted only after prolonged incubation. Addition of sodium pyruvate, which inhibits endogenous generation of H_2O_2 by tissue oxidases, or of crystalline catalase, which decomposes such tissue-generated H_2O_2, completely abolished microbody staining in the absence of H_2O_2. Neither diaminobenzidine nor the product of its oxidation had any affinity to bind nonenzymatically to microbody catalase and thus stain these organelles. The staining of microbodies was optimal at alkaline pH of 8.5. The biological significance of this alkaline pH in relation to the similar pH optima of several microbody oxidases is discussed. In addition to staining of microbodies, a heat-resistant peroxidase activity is seen in some of the peribiliary dense bodies. The relation of this reaction to the peroxidase activity of lipofuscin pigment granules is discussed.

In 1954 Rhodin introduced the term "microbody" to designate an organelle in the proximal convoluted tubules of mouse kidney, characterized by a single limiting membrane and a finely granular matrix (1). Later Rouiller and coworkers (2, 3) described a somewhat similar structure in the rat liver, which was also designated as microbody and which in addition contained an electron-opaque crystalloid core. Since then, numerous fine structural studies on hepatic microbodies of normal rats (4, 5), rats with hepatoma (6, 7), and rats treated with different chemical agents have been published (8, 9).

On the basis of biochemical analysis, de Duve and coworkers suggested the possibility that enzyme uricase in rat liver may be found in a distinct group of granules, separate from mitochondria, lysosomes, and microsomes (10, 11). Later, combined biochemical and electron microscopic studies conclusively established that this "distinct group of granules" consists in fact of microbodies which contain a variety of oxidases, in addition to a high concentration of catalase (12, 13). Because of the important role of microbodies in the metabolism of H_2O_2, de Duve suggested the term "peroxisome" for their designation (14), and in a

recent review de Duve and Baudhuin (15) discussed the possible biological significance of peroxisome enzymes in intermediary metabolism.

Graham and Karnovsky (16), reported a cytochemical staining procedure for the light microscopic localization of uricase in rat hepatic microbodies, and Allen and Beard (17) described a method for localization of α-hydroxy acid oxidase in renal microbodies. A procedure for staining microbodies at the electron microscope level, based on the oxidation of Karnovsky's 3,3′-diamino-benzidine (DAB) substrate (18), was reported independently by Novikoff and coworkers (19, 20), Hirai (21), and from this laboratory (22). Because of the inhibition of microbody staining by 3-amino-1,2,4-triazole (AT) which is an inhibitor of catalase (23), it was suggested that the staining with DAB was related to the enzyme catalase (19–22). Subsequent work in our laboratory has confirmed this notion and indeed we have since used crystalline beef liver catalase as a protein tracer and have visualized it in tissue sections by a cytochemical procedure similar to that used for staining of microbodies (24). In this paper a detailed description of our experiments on localization of peroxidatic activity of catalase in rat hepatic microbodies, with pertinent controls proving the enzymatic nature of this staining reaction, is given, and the possible biological significance of these findings is discussed. A preliminary report of these findings has been presented previously (25).

MATERIALS AND METHODS

Male adult albino rats of the Charles River strain (CDR), weighing from 250 to 300 g and kept on a normal laboratory diet, were used. The animals were fasted for 24 hr prior to sacrifice in order to decrease the content of hepatic glycogen, which interferes with the cytochemical procedure and the subsequent embedding (26).

Fixation

PERFUSION: Under light ether anesthesia the liver was fixed *in situ* by combined perfusion through the portal and hepatic veins as detailed previously (27). In the course of these experiments, however, it was noted that complete and satisfactory fixation of liver could be obtained by simple perfusion through the portal vein if a flow rate of 18–20 ml of fixative per minute was maintained for 10 min. The fixative was 1.25% distilled glutaraldehyde (28) in 0.15 M phosphate buffer, pH 7.2, with a final osmolality of 450 milliosmols (29).

IMMERSION: Animals were sacrificed by exsanguination, and small pieces of liver were quickly removed and cut, under a few drops of fixative, into thin strips 0.5 mm in thickness and fixed by immersion for 3 hr at room temperature. The fixative contained 4% formaldehyde (prepared from paraformaldehyde powder) and 1.25% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2 (30).

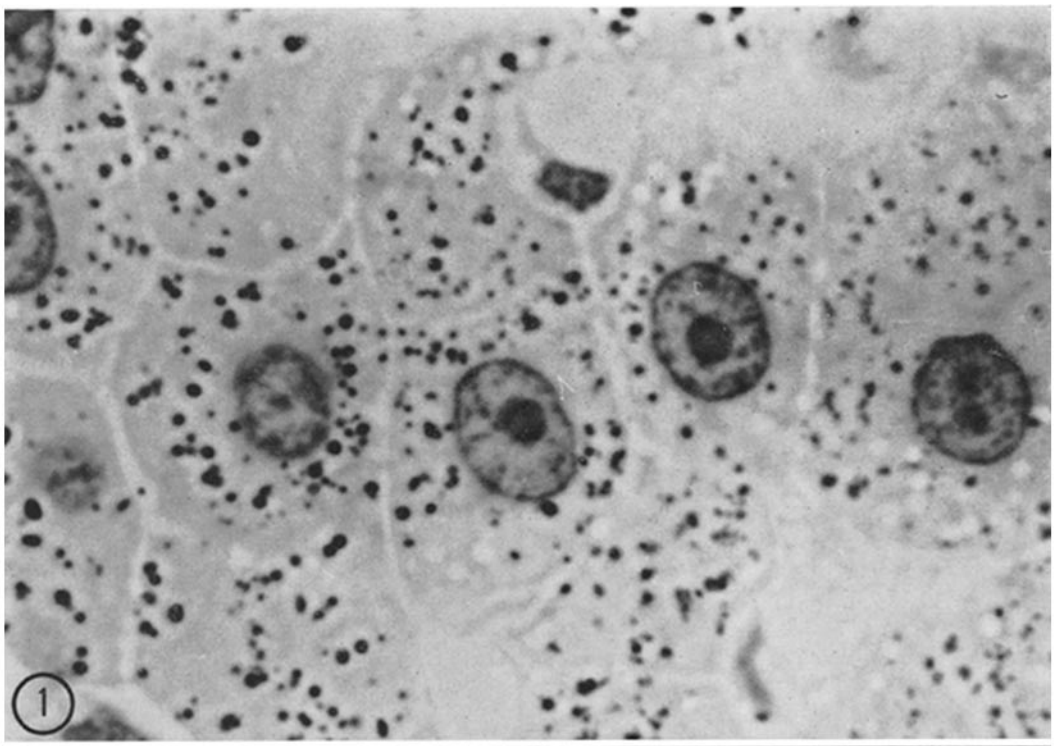
Cytochemical Technique

After an overnight wash in corresponding buffer solutions (phosphate buffer after perfusion and cacodylate buffer after immersion fixation), 50-μ sections were cut on a Sorvall TC-2 "Smith & Farquhar" tissue sectioner (Ivan Sorvall Inc., Norwalk, Conn.) (31) and incubated at 37°C for 30–120 min. The incubation medium contained 5 mg of 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, Mo.) in 10 ml of 0.1 M Tris-HCl buffer, and 0.02% H_2O_2. The pH of the medium was varied from 7.1 to 8.5 in different experiments. After incubation, sections were rinsed in buffer and postfixed for 2 hr in 2% osmium tetroxide in 0.2 M collidine buffer pH 7.2, dehydrated rapidly in graded ethanol solutions, and embedded in Epon 812 (32) or Araldite. 1-μ thick sections were examined with the light microscope, either without counterstain or after brief staining with 1% toluidine blue. Thin sections with silver and gold interference colors were cut on an LKB Ultrotome III and viewed, either unstained or counterstained lightly with uranyl acetate and lead citrate (33), in a Philips EM 200 electron microscope.

RESULTS

General Distribution of the Staining

Examination of sections with the light microscope revealed fine brown granules distributed evenly over the entire cytoplasm of hepatocytes (Fig. 1). This staining pattern differed from the usual peribiliary distribution of lysosomes (34) and resembled closely the pattern obtained after the staining of fresh frozen sections for uricase, which is a marker enzyme for the hepatic microbodies (16). The intensity and distribution of the stained granules did not vary substantially in different parts of the hepatic lobule. Examination of thin sections with the electron microscope revealed an electron-opaque reaction product in round and oval structures measuring 0.2–0.8 μ in diameter and distributed over the entire cytoplasm, forming occasional clusters (Fig. 3). The reaction product completely filled the matrix of the stained organelles and thus frequently obscured the limiting membrane around them. At higher



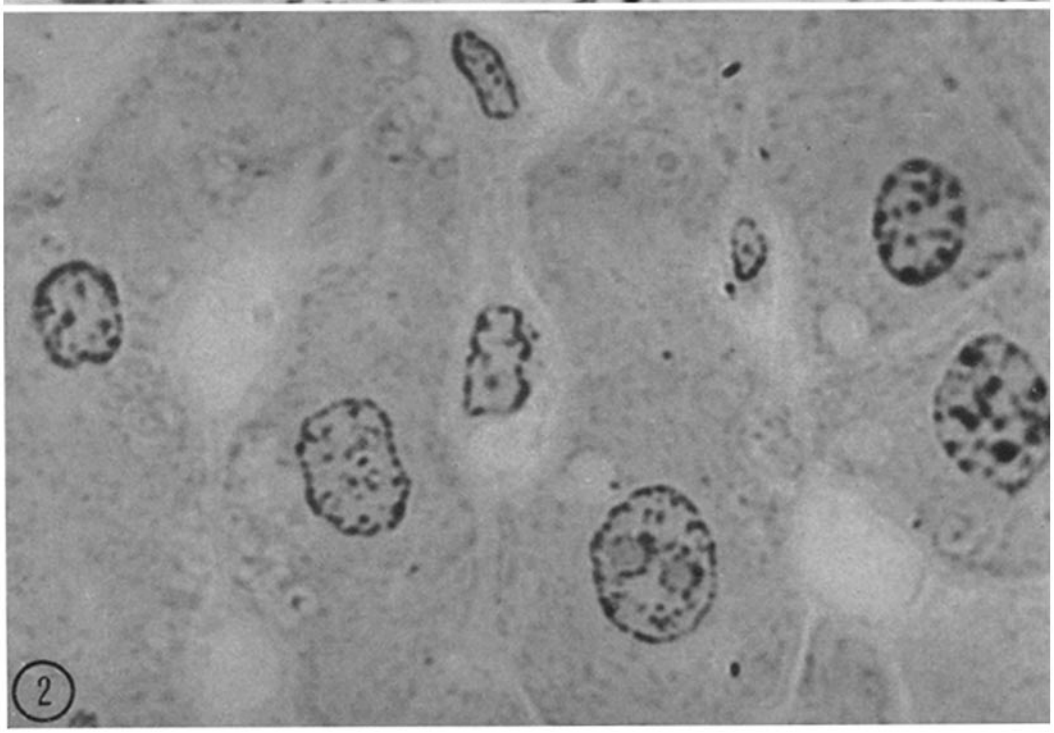


FIGURE 1 1-μ Epon section of rat liver incubated for 30 min at pH 8.5 for demonstration of peroxidase activity. Note the distribution of stained granules over the entire cytoplasm, which resembles the pattern of staining of uricase (16), a marker enzyme for rat hepatic microbodies. (Nuclei lightly counterstained with toluidine blue.) Light micrograph. × 2,200.

FIGURE 2 1-μ Epon section of rat liver incubated for 30 min in a medium containing Tris-HCl buffer pH 8.5 and DAB, but containing no H_2O_2. There is no evidence of staining of microbodies. A few stained granules are noted in liver cells and in Kupffer cells. By electron microscopy such granules are recognizable as dense bodies. (Nuclei lightly counterstained with toluidine blue.) Light micrograph. × 2,200.

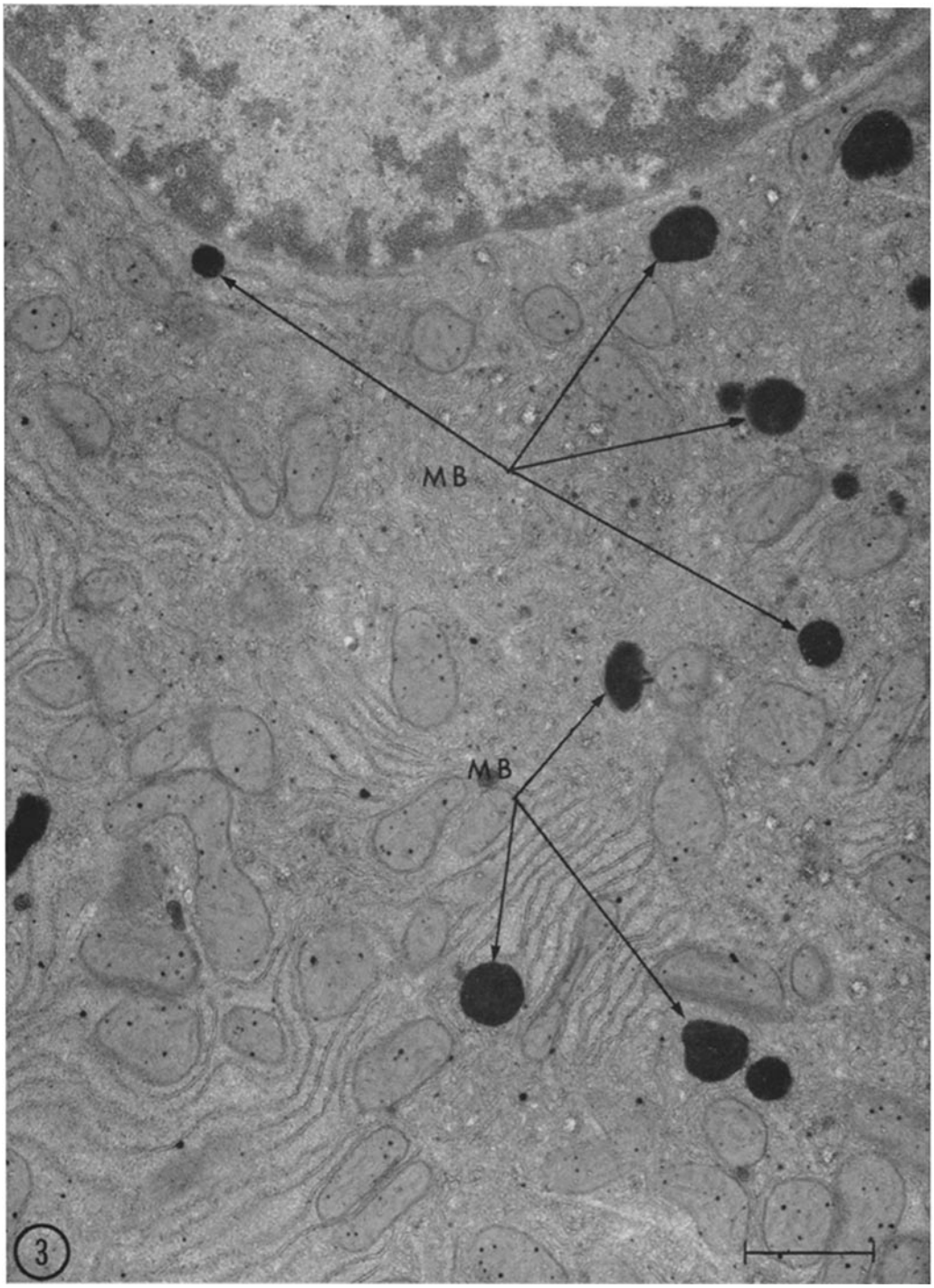


FIGURE 3 Part of a liver cell incubated for 60 min at pH 7.6 for peroxidase activity. There is uniform staining of the matrix of all microbodies (*MB*). The electron-opaque reaction product obscures the limiting membranes of most of the stained organelles. Most of the microbodies are spherical, but occasional ones with angular shapes are also noted (left border of the figure). (Section stained with uranyl acetate and lead.) × 19,000.

magnification some of the organelles with the reaction product contained crystalline nucleoids (Figs. 4 and 5). Whereas the majority of such nucleoids appeared tubular (Fig. 4), in favorable transverse sections a honeycomb pattern was also observed (Fig. 5). Some of the organelles with the reaction product showed small tail-like extensions into the adjacent smooth endoplasmic reticulum (Fig. 6). In addition to the above described group of organelles that were identified as microbodies, a few peribiliary dense bodies also showed an electron-opaque, globular deposit over a portion of their slightly electron-opaque matrix (Fig. 7). In contrast to microbodies, which all reacted positively, only a small number of dense bodies contained electron-opaque deposits. In mitochondria a coarse granular precipitate resembling the matrical granules was occasionally observed (Figs. 3 and 6). In addition to this granular pattern, a more diffuse staining of cristae and the outer membrane similar to that observed by Novikoff and Goldfischer (19) and Hirai (21) was seen when sections were incubated for several hours at pH 7.6.

Counts of Microbodies

The organelles which showed a diffuse reaction product over their entire matrix, and thus were considered as microbodies, were counted in 25 electron micrographs prepared at a final magnification of 15,000–20,000, and this count was compared with the total count of mitochondria on the same plates. A ratio of 3.9 mitochondria for each stained microbody was found.

Technical Considerations

FIXATION: Best staining results were obtained with the material fixed by perfusion with glutaraldehyde. Satisfactory fixation and staining were also achieved by immersion fixation of 0.5-mm thick tissue blocks fixed in the modified glutaraldehyde-formaldehyde fixative of Karnovsky (30). When thicker blocks were used, staining appeared patchy and was confined to well-fixed areas on the surface of the blocks.

VARIABLES IN THE INCUBATION MEDIUM: By raising the pH from 7.1 to 8.5 the intensity of the staining of microbodies increased. Thus, staining for 60 min at pH 7.6 gave the same intensity of reaction as 30 min at pH 8.5. In contrast to staining of microbodies, that of dense bodies was not affected by such changes in the pH. By raising the concentration of DAB from 0.05 to 0.1% the staining of microbodies was not affected, but when the concentration of H_2O_2 was increased from 0.01%, as used for localization of peroxidase (35), to 0.02%, stronger staining of microbodies was achieved.

INCUBATION TIME AND TEMPERATURE: The intensity of staining of microbodies increased with time of incubation. Furthermore, the staining was stronger when incubation was carried out at 37°C than at the room temperature (22°C). In general, at pH 8.5 and at 37°C, an incubation time of 30 min was sufficient for adequate staining of microbodies.

CONTROLS

To explore the nature of the staining reaction in microbodies and its specificity for the enzyme catalase a variety of controls were done. In these experiments 50-μ sections of livers fixed by perfusion with glutaraldehyde were incubated in control media at 37°C, pH 8.5, for 30 min–4 hr and were compared with test sections incubated in the regular incubation mixture for the same length of time.

1. The Role of H_2O_2 in the Staining of Microbodies

Staining of microbodies was abolished in sections incubated for 30 min in the absence of H_2O_2 (Fig. 2). After incubation for 2–3 hr, however, staining of microbodies developed, and this was comparable in its intensity to staining in regularly reacted test sections incubated for 30 min.

Endogenous generation of H_2O_2 by various tissue oxidases can substitute for H_2O_2 in the incubation medium and thus be responsible for the staining of microbodies in media lacking H_2O_2. To test this possibility, sodium pyruvate, which is known to stop the endogenous generation of H_2O_2 (36, 37), was added to the incubation medium. Sodium pyruvate (0.002 M) completely abolished the staining of microbodies in the absence of H_2O_2 (Fig. 8), even after incubations up to 4 hr. When 0.02% H_2O_2 was added to incubation medium, however, 0.002 M of pyruvate had no effect on the staining of the microbodies.

In another experiment, endogenously generated H_2O_2 was destroyed by addition of 5 mg crystalline beef liver catalase (Sigma Chemical

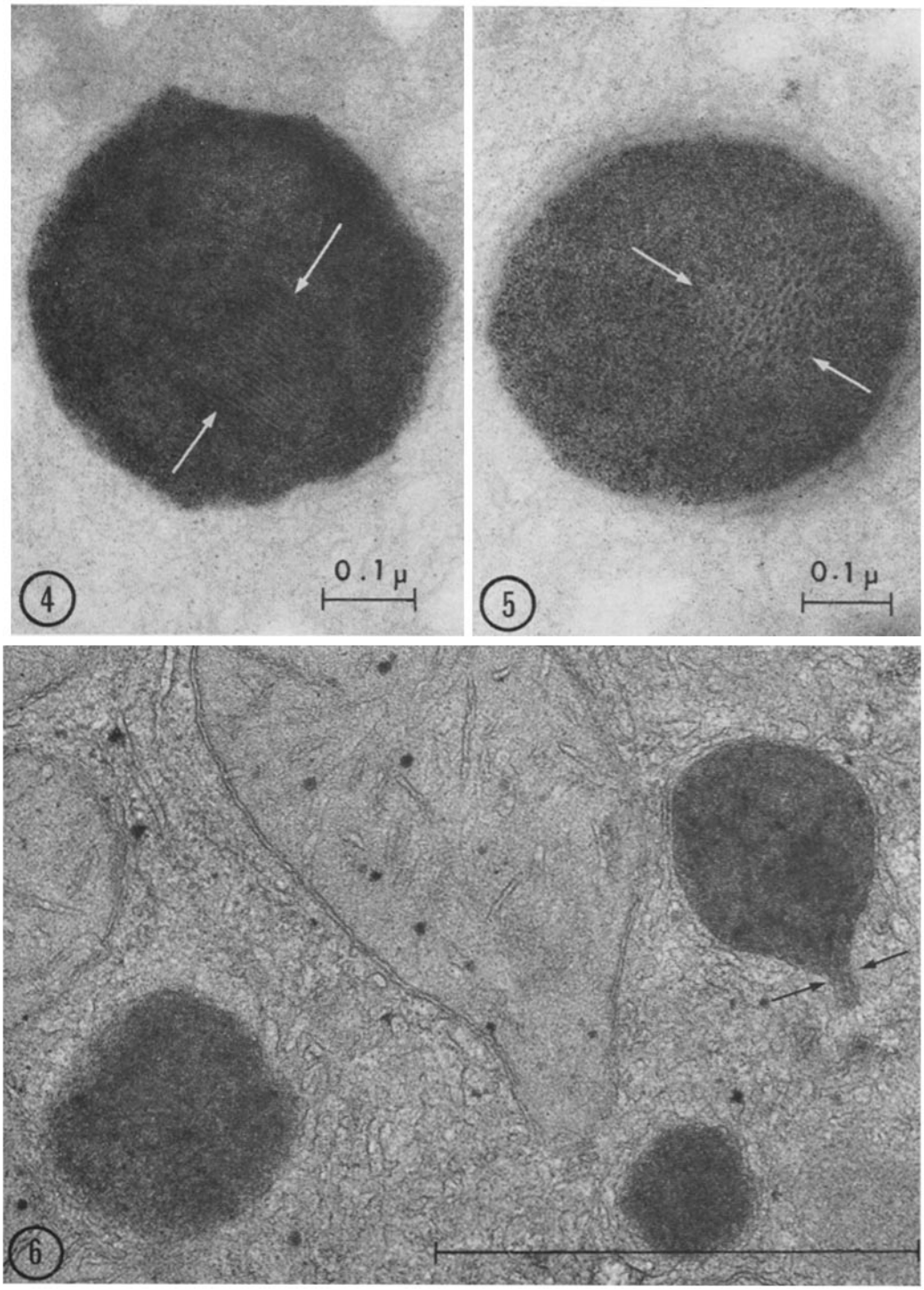
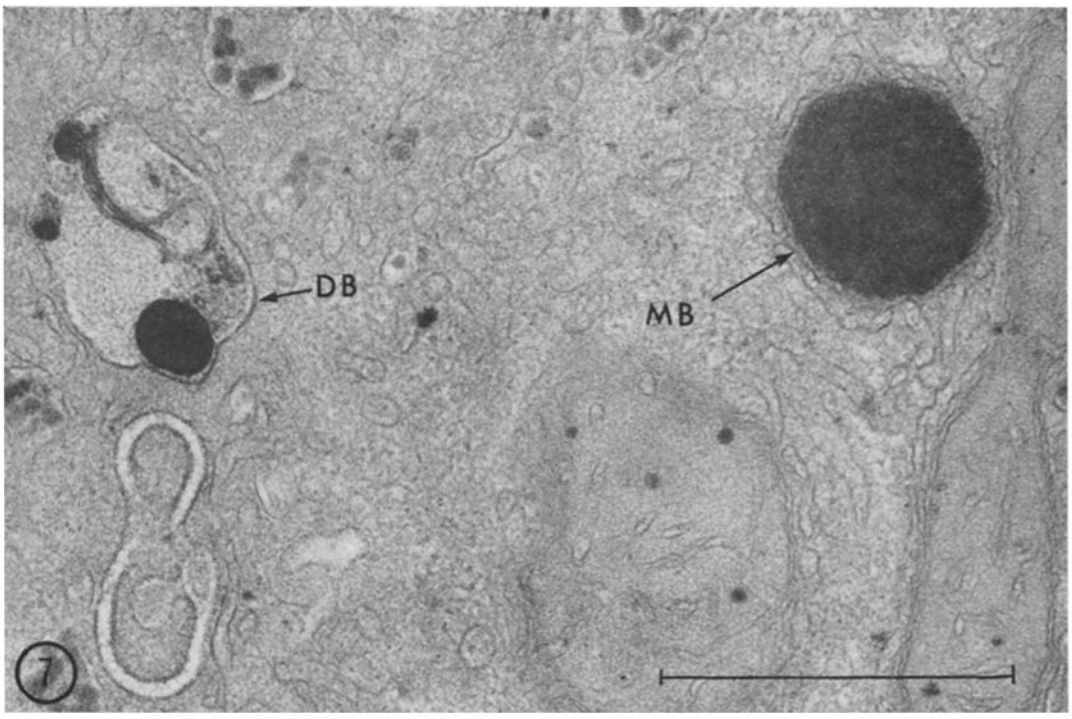


FIGURE 4 Microbody stained for peroxidase activity. The arrows point to a tubular nucleoid. In this figure the microbody has been sectioned parallel to the longitudinal axis of the crystalline nucleoid. (Section contrasted with lead citrate.) × 106,000.

FIGURE 5 Microbody containing a nucleoid (arrows) stained for peroxidase activity. In this figure the microbody has been sectioned perpendicular to the longitudinal axis of the crystalline nucleoid. Note the honeycomb appearance, which is due to the presence of the reaction product in the lumen of the tubules of the nucleoids (61). (Section contrasted with lead citrate.) × 110,000.

FIGURE 6 Three microbodies stained for peroxidase activity. Note the tail-like extension of one stained organelle into the adjacent smooth endoplasmic reticulum (arrows). (Section contrasted with uranyl acetate and lead.) × 70,000.



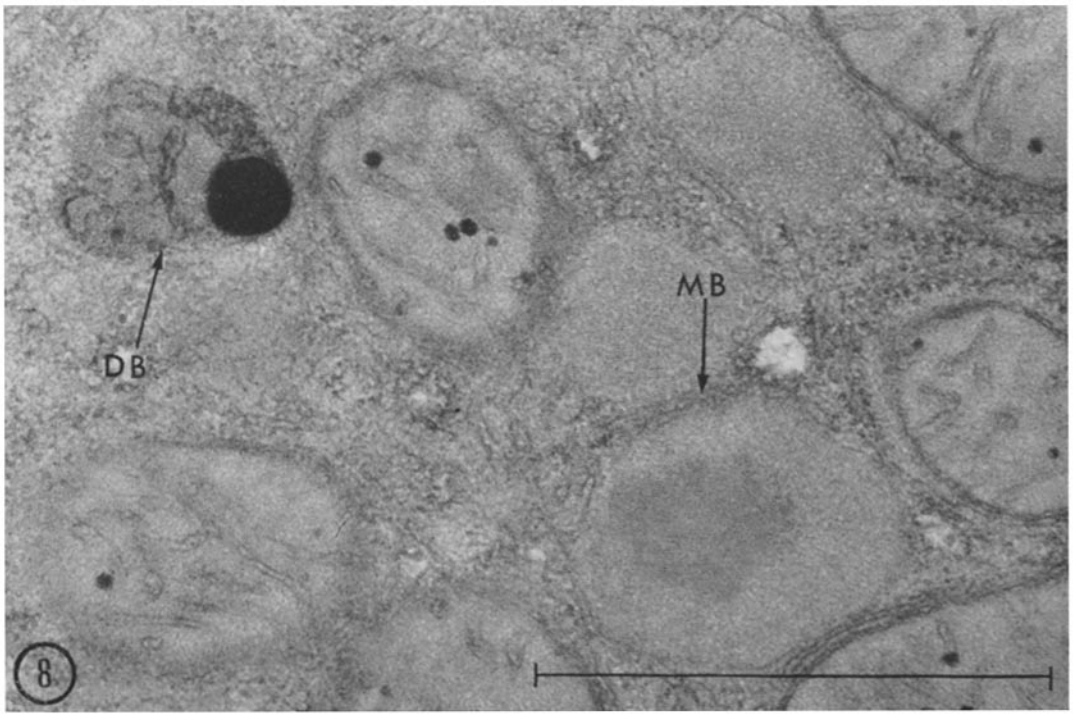
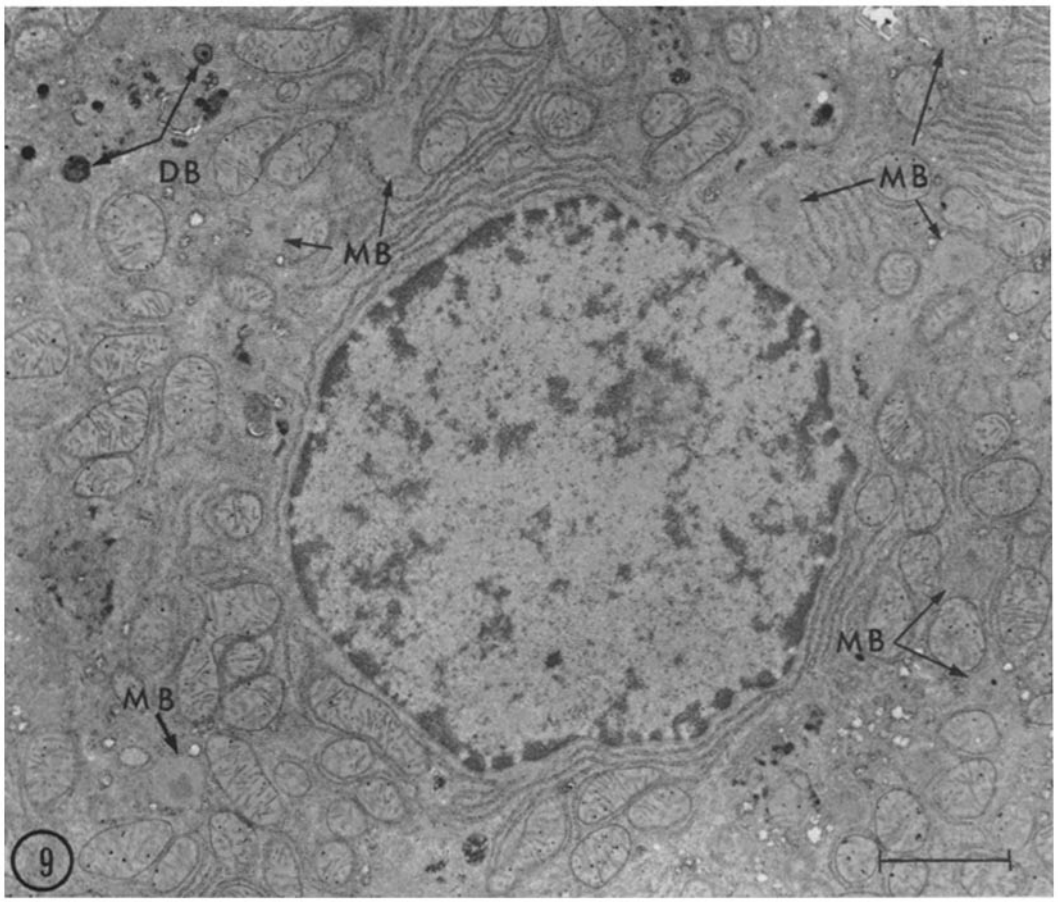


FIGURE 7 Portion of the cytoplasm of a liver cell reacted for peroxidase activity. The section shows a microbody (*MB*) with a uniform staining of the matrix and a dense body (*DB*) containing an electron-opaque globular precipitate and other membranous debris. (Section contrasted with lead citrate.) × 48,000.

FIGURE 8 Portion of the cytoplasm of a liver cell incubated for 60 min in an incubation medium containing Tris-HCl buffer pH 8.5, DAB, 0.002 M of sodium pyruvate, but containing no H_2O_2. Staining of the microbody (*MB*) is abolished but the reaction in the dense body (*DB*) persists. (Contrasted with lead citrate.) × 70,000.



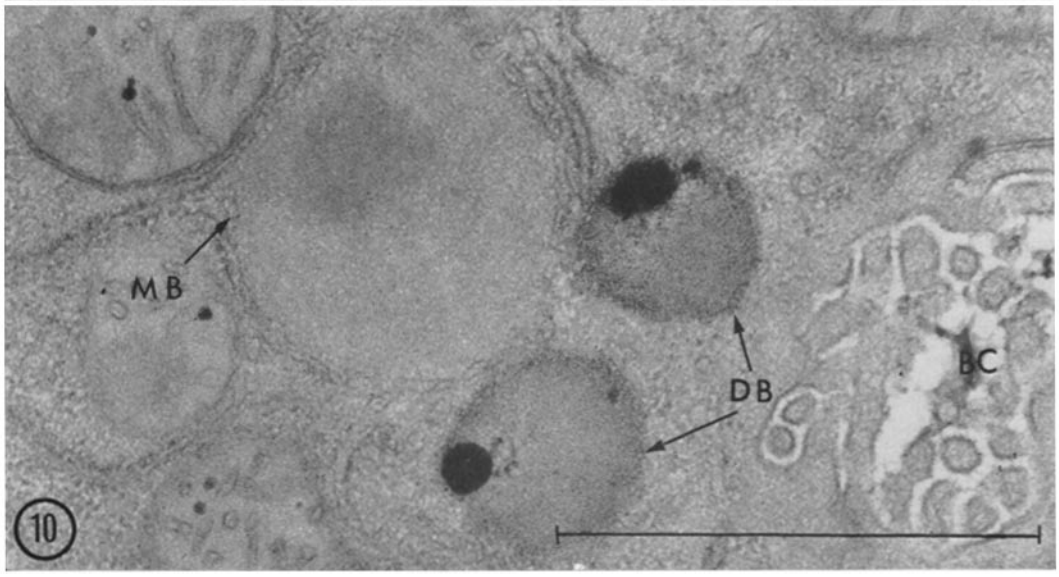


FIGURE 9 Liver cell incubated for 60 min for peroxidase activity in the presence of 0.02 M of aminotriazole. There is no staining of microbodies (*MB*), but some of the peribiliary dense bodies (*DB*) contain electron-opaque deposits. (Section contrasted with lead citrate.) × 16,500.

FIGURE 10 Peribiliary region of a liver cell incubated for peroxidase activity in the presence of 0.1 M of sodium azide. Staining of microbodies is abolished, but electron-opaque deposits are noted in the peribiliary dense bodies (*DB*). Bile canaliculus (*BC*). (Section contrasted with lead citrate.) × 64,000.

Co.) to the Tris-DAB medium. The staining of microbodies was completely abolished even in sections incubated up to 4 hr. It is also noteworthy that crystalline catalase did not react with DAB in the absence of H_2O_2 in the incubation medium.

2. Inhibitors

A known inhibitor of hepatic catalase, 3-amino-1,2,4-triazole (Mann Research Labs Inc., New York) (23, 38, 39), in the final concentration of 0.02 M, abolished completely the staining of microbodies (Fig. 9).

Potassium cyanide (0.01 M) inhibited partially, and sodium azide (0.1 M) completely, the staining reaction in microbodies (Fig. 10).

High concentrations of H_2O_2 also inhibited the staining of microbodies. For this purpose sections were pretreated for 30 min with 2% H_2O_2 in Tris buffer before incubation in a Tris-DAB medium containing 2% H_2O_2.

Boiling of sections for 5 min in 1.25% glutaraldehyde completely abolished the staining of microbodies.

3. Controls to Exclude Adsorption of DAB, or its Oxidation Product, to Microbodies

(*a*) Sections were incubated for 60 min in Tris-DAB medium containing 0.002 M of sodium pyruvate; no staining of microbodies occurred. Potassium ferricyanide (0.003 M) was then added to the incubation medium; the DAB was oxidized readily (35), causing a brown precipitate both in the medium and over the surface of sections. The microbodies, however, remained unstained after this procedure.

(*b*) In another experiment, DAB was first oxidized with potassium ferricyanide (0.003 M) and then tissue sections were incubated for 60 min in the medium. No staining of microbodies occurred. These controls (*a* and *b*) indicate that neither DAB nor its oxidation product have any affinity to bind to microbodies. In all the controls (groups *1*, *2*, and *3*) in which the staining of microbodies was inhibited, the electron-opaque deposits in a few dense bodies persisted (Figs. 8–10).

Other Observations

Occasionally, some of the material which was incubated in the complete incubation mixture showed evidence of poor preservation or absence of all cytomembranes, resembling the aspects of tissues fixed only in glutaraldehyde without postfixation in osmium tetroxide. These sections with poor membranes showed great affinity to stain with uranyl acetate and little affinity to lead citrate. When uranyl acetate treatment was followed by lead citrate, however, the matrix of most cytoplasmic particles became strongly electron opaque. Thus, the counterstain with uranyl and lead in these sections could be confused with the reaction product of DAB oxidation in microbodies and dense bodies, and it was therefore necessary to examine such material without counterstaining. Even though we have no exact explanation for this occasional observation, unpublished findings suggest that it is probably related to postosmication and dehydration procedures.

DISCUSSION

The findings presented in this paper indicate that in rat liver cells the microbodies are the intracellular sites of cytochemically demonstrable peroxidase activity.

Identification of Microbodies

The following morphologic criteria were used to identify as microbodies the organelles that exhibit a diffuse reaction product over their matrix: (*a*) their size, which varied from 0.2 to 0.8 μ, the majority measuring 0.5 μ in diameter; (*b*) their spherical shape, even though occasionally in livers fixed by perfusion organelles with angular shapes were also seen; (*c*) the presence of crystalline nucleoids in the matrix of some of the stained organelles; (*d*) their frequent occurrence in proximity to rough and smooth endoplasmic reticulum, with occasional tail-like extensions towards the smooth reticulum; and (*e*) their frequent grouping in clusters. These criteria conform to published morphologic data for the identification of microbodies in rat liver (2–9, 15). Furthermore, in all reacted material not a single normal-appearing, unstained microbody was observed, thus indirectly implying that the stained organelles must be the microbodies. In addition, counts of stained organelles revealed a ratio of 3.9 mitochondria for each stained organelle, which is consistent with the ratio of 4 mitochondria for each microbody reported by Baudhuin et al. (12) and by Loud et al. (40) for rat liver cells.

Mechanism of Staining of Microbodies

The results presented here strongly suggest that peroxidatic oxidation of DAB by the enzyme catalase is responsible for the staining reaction in microbodies. The sensitivity of this reaction to aminotriazole, an in vivo and in vitro inhibitor of hepatic catalase (23, 38, 39), and the inhibitory effect of high conentration of H_2O_2, which inhibits the peroxidatic activity of catalase (41), as well as the inhibition of the staining by KCN and azide, two classic inhibitors of catalase and other heme proteins (42), are findings all in line with this suggestion. Catalase is hitherto the only heme protein known to be present in microbodies, and it makes up about 40% of the total microbody protein (15). Furthermore, the observations of Keilin and Hartree (42–44) and Chance (45–47) have clearly established that catalase in the presence of a peroxide source can behave as a peroxidase and oxidize a variety of substrates. The biological significance of peroxidatic activity of catalase has been discussed by Keilin and Hartree (44), Aebi (48), and de Duve and Baudhuin (15). The latter authors suggested that microbody catalase functions mainly in its peroxidatic capacity, with the catalatic function serving only as a safety device to stem the overflow of H_2O_2 generated by microbody oxidases (15). The cytochemical studies presented here are in line with the suggestion that microbody catalase can indeed react peroxidatically, and they emphasize the central role of microbodies in the metabolism of H_2O_2 (14).

Since catalase is an indispensable component of the peroxisomes (15), the method of staining for catalase by the use of proper inhibitors, as described herein, can be useful in distinguishing peroxisomes, with catalase activity, from other organelles, which on purely morphological grounds are called microbody-like structures. In fact, in a recent study Frederick and Newcomb have used the catalase method with proper inhibitors and have identified certain microbody-like structures in tobacco leaves as true peroxisomes (64).

Wachstein and Meisel (49) investigated the histochemical localization of peroxidase activity in rat tissues, by using a somewhat different technique, but did not see any activity in parenchymal cells of the liver. Nishimura et al. (50), and recently Morikawa and Harada (51), using the fluorescent antibody technique, have demonstrated catalase activity in the cytoplasm of liver cells. Cytologically, Nishimura et al. (50) noted that catalase gave a densely packed, granular pattern in the cytoplasm of hepatocytes, a pattern which was also observed by Morikawa and Harada (51) when formalin-fixed frozen sections were used. This cytoplasmic granular pattern resembles the pattern of staining of microbodies in our material, and this finding is in agreement with the results of tissue fractionation studies on the localization of catalase in hepatic microbodies (15).

The fact that DAB alone, in the absence of exogenous H_2O_2, can stain microbodies has been considered as an indication that other nonenzymatic mechanisms may also be involved in the staining of microbodies (19, 21, 52). The control experiments presented here clearly demonstrate that the staining of microbodies with DAB alone is completely abolished in the presence of pyruvate, which suppresses the generation of H_2O_2 by tissue oxidase systems (36, 37). Thus it appears that endogenous generation of H_2O_2 by tissue oxidases is most probably responsible for the "nonspecific" staining of microbodies with DAB. Furthermore, the addition of crystalline beef liver catalase to the incubation medium also abolishes completely the staining of microbodies in the absence of H_2O_2. It is not clear whether exogenous catalase competes with the microbody catalase for the same substrate (DAB), or whether it inhibits the staining reaction by decomposing the H_2O_2 which is generated by the tissue oxidases.

In addition, our data indicate that neither DAB nor the product of its oxidation by potassium ferricyanide (35) has any affinity to bind to microbodies. The latter finding, as well as the inhibition of staining by boiling, is in conflict with the observations of Hirai (21, 52). That author noted that DAB, after oxidation in "air and light," showed a great affinity to bind to microbody catalase and that this affinity was not affected by exposure of sections to dry heat. The discrepancy between our findings and those of Hirai may be due to methodological differences in oxidizing the DAB and in inactivating the catalase in sections by heat. Hirai concluded that the nonenzymatic affinity of DAB to catalase must be responsible for the staining of microbodies (52), but our findings suggest that a heat-sensitive enzymatic process underlies the staining reaction.

Our method of boiling of tissue sections in glutaraldehyde, rather than in water or buffer, has given surprisingly good preservation of fine structure but at the same time has apparently denatured the enzyme protein, since no staining of microbodies could be observed.

The Alkaline pH Optimum of the Staining Reaction and its Possible Biological Significance

Novikoff and Goldfischer (19) have emphasized the importance of alkaline pH in the staining of microbodies with DAB. Our observations are in agreement with their findings since in our experiments the intensity of microbody staining increased by raising the pH from 7.1 to 8.5. Similarly, when crystalline beef liver catalase was used as a protein tracer and localized by a method similar to the one used here (24), it was noted that the intensity of the staining of catalase was stronger at pH 8.5. Goodman and Tephly (53) noted that the alkaline pH of 8.3 is most suitable for the determination of the peroxidatic oxidation of methanol by rat hepatic microbody catalase. Chance (46) indicated that the binding of catalase to H_2O_2, which is the first step in the peroxidatic oxidation of catalase substrates, is not affected by the variation of the pH over a wide range, but the reaction of catalase - H_2O_2 complex to different substrates may be affected by the pH, depending on the oxidizable substrate employed. Our observations on the oxidation of DAB and the findings of Goodman and Tephly with methanol oxidation (53) suggest that catalase oxidizes some substrates faster in an alkaline environment than at the neutral pH. This is of interest since most other enzymes associated with microbodies also have pH optima in the alkaline range. Thus, the urate oxidase has a pH optimum at pH 9 (54), the D-amino acid oxidase at about pH 8.5 (55), the L-α-hydroxy acid oxidase at about pH 8 (56), and the glycolate oxidase at pH 8.8 (57). Thus, one could reasonably speculate that probably such an alkaline pH prevails inside the microbodies, to further not only the production of H_2O_2 by the oxidases, but also to enhance the peroxidatic oxidation of certain still unknown "natural" substrates of the microbody catalase.

The Permeability of the Microbody Membrane

De Duve and coworkers have emphasized that the microbody membrane is distinctly more permeable to water and small molecules than the membranes of mitochondria or lysosomes (12, 15, 58). The diffuse and uniform deposition of a reaction product over the entire matrix of the microbodies may be closely related to the high permeability of the microbody membrane to small molecules such as H_2O_2 and DAB. In addition, the nondroplet and diffuse appearance of the reaction product once again confirms the suitability of DAB (18, 35, 59) as a substrate for fine structural cytochemistry.

The Relation of the Reaction Product to the Nucleoids of Microbodies

The fine structure and enzyme content of the nucleoids of rat hepatic microbodies have been studied extensively (5, 60, 61). Morphologically the nucleoids consist of parallel bundles of hollow tubules, which in cross-section have a honeycomb appearance and in longitudinal plane show a parallel packed, tubular structure (61). The only enzyme known to be associated with the nucleoids is urate oxidase. In our observations the nucleoids of microbodies did not stain for peroxidase. From a comparison of our electron micrographs with the model of microbody nucleoids proposed by Tsukada et al. (61), it appears that the substance of the nucleoids did not show any staining but that the lumen of the hollow tubules contained the reaction product. This finding emphasizes the intimate association of the nucleoids containing urate oxidase with the catalase which surrounds them.

Peroxidase Activity in Dense Bodies

Goldfischer et al. (62) have described a heat-stable and formaldehde-resistant peroxidase activity in lipofuscin pigment granules of human liver. In addition, these granules contain acid phosphatase activity and are, therefore, identified as lysosomes (62, 63). In our work with the rat liver, we have observed peroxidase activity in only a few of the peribiliary dense bodies. Although it may be difficult to distinguish, by electron microscopy, between the inherent electron opacity of some lysosomes and the electron opacity due to the reaction product of the DAB oxidation (Figs. 7, 8, and 10), by light microscopy the golden yellow color of the DAB oxidation product is easily identified in the peribiliary region. The reaction in dense bodies is not affected by inhibitors or by boiling, thus indicating

that nonenzymatic mechanisms are responsible for the staining of dense bodies with DAB. Goldfischer et al. (62) suggested that peroxidase activity in dense bodies reflected the presence of heme compounds that accumulate as the result of breakdown of cytoplasmic constituents in autophagic vacuoles. In unpublished observations, we have seen several autophagic vacuoles containing peroxidase activity. However, further studies are required to establish whether all autophagic vacuoles have the heat-resistant peroxidase activity. Finally, it should be emphasized that the peroxidase reaction in dense bodies should not be confused with the staining of the microbodies. Such a confusion could possibly account for the discrepancy between our results and the findings of Hirai, who reported the localization of a heat-resistant peroxidase activity in the rat hepatic microbodies (21, 52).

I wish to thank Doctors Ramzi S. Cotran and Morris J. Karnovsky for helpful discussions and critical review of this manuscript. The excellent technical assistance of Mrs. Joyce Warren, Iris Hageman, and Christine Yaeger is gratefully acknowledged.

This study was supported in part by Grant No. 08533-01 from the National Institute of Neurological Diseases and Stroke, and by Grant No. 57-57 from the Boston City Hospital.

Received for publication 25 March 1969, and in revised form 13 June 1969.

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