

THE USE OF BEEF LIVER CATALASE AS A PROTEIN TRACER FOR ELECTRON MICROSCOPY

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ABSTRACT

Beef liver catalase was injected intravenously into mice, and its distribution in the kidney, myocardium, and liver was studied with the electron microscope. A specific and relatively sensitive method was developed for its ultrastructural localization, based on the peroxidatic activity of catalase and employing a modified Graham and Karnovsky incubation medium. The main features of the medium were a higher concentration of diaminobenzidine, barium peroxide as the source of peroxide, and pH of 8.5. Ultrastructurally, the enzyme was seen to permeate the endothelial fenestrations and basement membranes of tubular and glomerular capillaries of the kidney. The urinary space and tubular lumina contained no reaction product. In the myocardial capillaries, the tracer filled the pinocytotic vesicles but did not diffuse across the intercellular clefts of the endothelium. In liver, uptake of catalase was seen both in hepatocytes and in Kupffer cells.

Crystalline beef liver catalase, a heme protein with a molecular weight of 240,000 (26), is known to split hydrogen peroxide to water and oxygen. Under conditions of low peroxide concentration, it also takes part in the peroxidation of various hydrogen donors (4, 14, 19, 20). The biological significance of the peroxidatic activity of catalase has been emphasized by Keilin and Hartree (19), and de Duve and Baudhuin (6).

Peroxidatic activity has been recently localized ultrastructurally in hepatic microbodies by incubating tissue sections with 3,3' diaminobenzidine, and it has been suggested that microbody catalase is probably responsible for this reaction (7, 15, 24). Fahimi has demonstrated (8) that hepatic microbody staining with diaminobenzidine most probably is due to catalase.

These observations prompted us to explore the use of beef liver catalase as an exogenous protein

tracer which can be visualized at the ultrastructural level. Metallic and protein tracers which have been employed in ultrastructural biological research include horse-radish peroxidase (mol wt 40,000) (11), ferritin (mol wt 500,000) (10), lactoperoxidase (mol wt 82,000) (13), and myeloperoxidase (mol wt 160,000) (12).

The present communication discusses technical and biological considerations in the histochemical localization of catalase as a protein tracer, and briefly describes the distribution of intravenously injected enzyme in the kidney, myocardium, and liver of mice.

MATERIALS AND METHODS

White male mice of the Charles River strain weighing 25–30 g and fed on a standard diet were used. In studies of catalase localization in liver, the animals were starved 24 hr prior to the experiment (9).

Catalase Preparation

Twice crystallized beef liver catalase (Stock No. C-100, Sigma Chemical Co., St. Louis, Mo.) was used in all the experiments. According to the supplier, this preparation contains 26–27 mg per ml of catalase crystals in suspension, with 0.1% thymol as a preservative. Just before injection, the catalase crystals were dissolved by ultrasonic vibration at 35–40°C in a “Maxomatic” ultrasonic vibrator (L&R Manufacturing Co., Kearny, N.J.) for 10–30 min. It is important to maintain the temperature below 40°C to avoid denaturation of the protein. The catalase preparation was removed from the sonicator as soon as solution had been achieved, and kept at 37°C. The clear, dark green catalase solution was then slowly injected into the tail vein, in doses of 0.5–2 ml per 30 g mouse, over a period of 5 min.

Fixation

Animals were sacrificed by decapitation at 30 min after injection, and small slices of heart, liver, and kidney were immersed in a fixative containing 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4 with CaCl₂, for 3 hr at room temperature (16).

Histochemical Technique

After an overnight wash in 0.1 M cacodylate buffer pH 7.4, 40 μ sections were cut on a TC-2 (Sorvall), Smith-Farquhar tissue chopper (27) and collected in 0.05 M Tris-HCl buffer (pH as in the incubation medium). The sections were then incubated in a modified Graham and Karnovsky's medium (11) containing 10 mg of 3,3' diaminobenzidine tetrahydrochloride (DAB) (Sigma Chemical Co., St. Louis, Mo.), 10 ml of 0.05 M Tris-HCl buffer and, as a peroxide source, a) 0.1 ml of 1% H₂O₂ or b) a suspension of 25 mg of barium peroxide in 1 ml of Tris-HCl buffer sealed in a dialysis bag. The pH of the incubation medium was varied from 7.1 to 8.5. Sections were incubated at 37°C for 1, 2, and 4 hr with mild agitation in a rotator.

At the end of incubation, sections were washed in Tris HCl buffer, postfixed in 1.3% osmium tetroxide in s-collidine buffer for 90 min, rapidly dehydrated, and embedded in Epon 812 (22). For light microscopy, 1 μ thick sections were cut and viewed without further counterstain. For electron microscopy, silver and gold sections were cut on an LKB Ultratome III and viewed, either unstained or lightly stained with lead citrate (25), in a Philips EM 200 electron microscope.

RESULTS

General

By light microscopy, tissues from catalase-injected animals exhibited a brown reaction product in the lumens of capillaries in the myocardium and kidney, in hepatic sinusoids, and in the basement membranes of the renal glomeruli and tubules (Figs. 1 and 2). Lumens of renal tubules did not show a reaction product up to 30 min after injection. Renal and hepatic microbodies, red blood cells, and leucocyte granules were also stained.

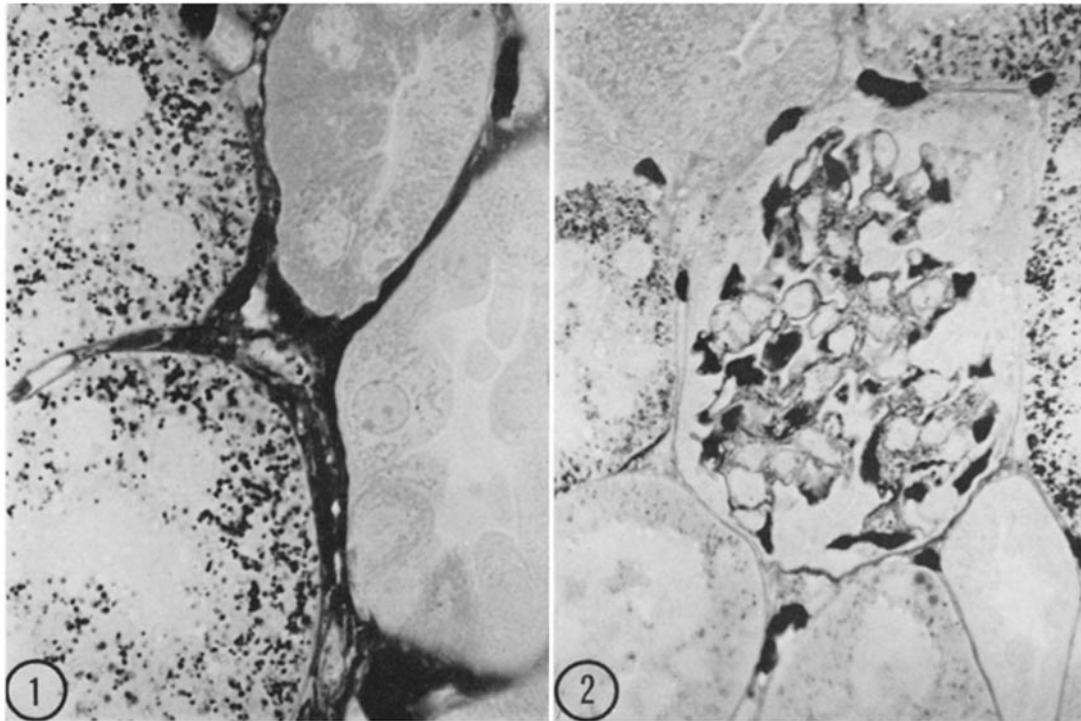
By electron microscopy, electron-dense, granular, clumpy material was present in the same locations as the brown reaction product seen by light microscopy. In addition to microbody and red cell staining, mitochondrial cristae were sometimes stained after prolonged incubation. Further details on the ultrastructural localization are given below.

Technical Considerations

DOSE AND TOXICITY: Because of limitations of solubility and the tendency of catalase to crystallize, a concentrated solution was made from a crystalline suspension by ultrasonic vibration at 35–40°C. Stronger staining of exogenous catalase was obtained by injecting higher doses (up to 2 ml) of this preparation; however, 0.5 ml was found adequate. The animals tolerated the catalase injections well and developed no untoward reactions up to 18 hr after injection. Histologic and ultrastructural observations on tissues of animals injected with various batches of the preparation revealed no cellular or vascular damage. However, we have been informed that one batch of this preparation was found to be lethal to mice on intravenous injection (M. J. Karnovsky, personal communication). Also, a few samples of the Sigma C-100 catalase preparation have been found to be difficult to solubilize by sonication at 37°C. It is therefore advisable to check each batch for possible toxic reactions and for complete solubility before large numbers of animals are injected with the preparation.

VARIABLES IN THE INCUBATION MEDIUM: The intensity of the reaction could be enhanced by increasing the pH of the incubation medium to 8.5, although the pH optimum for catalase is 7. This finding is in agreement with the observation on the staining of microbodies in liver (attributable to catalase) at pH 8.5, reported recently (24).

Incubation at 37°C for 1–2 hr gave adequate



Length of scale line for all figures is 1 μ .

FIGURE 1 Light micrograph of kidney showing reaction product in peritubular capillaries and basement membrane, but not in tubular lumina. Proximal tubules to the left show staining of microbodies. $\times 1000$.

FIGURE 2 Light micrograph of renal glomerulus showing staining of basement membrane. $\times 1000$.

staining, although stronger reactions were obtained with longer incubation. The use of 0.1% DAB in the incubation medium rather than 0.05% employed in the peroxidase reaction (11) appeared to increase the staining intensity.

Better tissue preservation was observed when BaO_2 in a dialysis bag was used as the peroxide source. Free H_2O_2 often resulted in focal swelling of endothelial cells with occasional disruptions. This could have been due to a violent catalytic decomposition of H_2O_2 in vascular lumens. In addition, staining intensity was often enhanced when BaO_2 was used.

SAMPLING: As in the peroxidase reaction, penetration of the substrates was sometimes limited to the superficial areas of tissue blocks. Sampling should therefore be confined to the more superficial areas when sections are being cut.

Controls

All controls were incubated at 37°C for 2 hr and compared with test sections incubated under the same conditions.

TO DEMONSTRATE THE SPECIFICITY OF THE REACTION FOR CATALASE: a. Sections of tissues from animals not injected with catalase were incubated in the complete medium. No staining was evident in vascular lumens or other tissue elements, except red cells, white cells, microbodies, and occasionally mitochondria.

b. Sections of tissues from catalase-injected animals were incubated in the complete medium containing 2×10^{-2} M of 3-amino, 1,2,4-triazole (Mann Research Lab., N.Y., N.Y.) which is a specific inhibitor of hepatic catalase (23). Staining of exogenous catalase and microbodies was abolished while red cell staining persisted.

c. Tissue sections from catalase-injected animals were incubated in the complete medium containing 10^{-1} , 10^{-2} , and 10^{-3} M KCN. 10^{-1} M KCN abolished completely and 10^{-2} M, partially, the staining of exogenous catalase and microbodies.

d. Sections from injected animals were pre-incubated in 1% H_2O_2 in Tris-HCl buffer for 30 min and incubated in the complete medium containing 0.1% H_2O_2 . Staining of exogenous catalase was markedly diminished with persistence of patchy, very faintly staining foci. Microbody staining was completely abolished, but red cell staining persisted. It is known that high concentrations of H_2O_2 inhibit the peroxidatic action of catalase (21).

TO EXCLUDE NONSPECIFIC ADSORPTION OF DAB TO CATALASE: When a peroxide source was omitted from the incubation medium, patchy and partial staining of exogenous catalase and microbodies persisted. Since this could be attributed to endogenous liberation of H_2O_2 from microbodies and mitochondria through various oxidase systems, sodium pyruvate, a known inhibitor of tissue oxidase systems (1, 2), was employed as an inhibitor. When sections from injected animals were incubated in media containing 2×10^{-3} M pyruvate without a peroxide source, all staining was abolished. When a peroxide source was added, staining of exogenous catalase, microbodies, and red cells was restored.

TO EXCLUDE NONSPECIFIC ADSORPTION OF OXIDIZED DAB TO CATALASE: Sections from injected animals were incubated in 9 ml of the Tris-DAB mixture without a peroxide source, followed by oxidation of the DAB by adding 1 ml of 0.03 M potassium ferricyanide (which readily oxidizes DAB) (11). In another experiment, sections were incubated in Tris-DAB medium containing 2×10^{-3} M pyruvate for 90 min, and were further incubated for 15 min after oxidation of the DAB by addition of 1 ml of 0.03 M potassium ferricyanide. There was no staining of exogenous catalase and microbodies after these procedures.

Localization of Injected Catalase

Although detailed experiments are in progress, observations which shed light upon catalase as a high molecular weight tracer are presented.

In the kidney, lumens of peritubular capillaries and endothelial fenestrae contained the reaction product (Fig. 3). In glomeruli, the reaction product was present in the capillary lumens, the

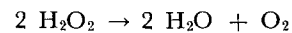
endothelial fenestrations, and in basement membrane (Fig. 4). Catalase did not seem to pass beyond the epithelial slits up to 30 min after injection. Tubular lumens did not show any reaction product. The findings here are similar to those described for myeloperoxidase (12).

In myocardium, catalase was visualized in capillary lumens and endothelial pinocytotic vesicles (Fig. 5). It did not seem to cross the foci of constriction between endothelial cells (Figs. 5 and 6), as opposed to the free passage of horse-radish peroxidase through these junctions into the perivascular space (17). In liver, the reaction product was visible in sinusoids outlining the hepatic microvilli, with uptake into the liver cells and Kupffer cells (Fig. 7).

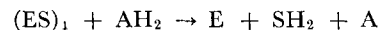
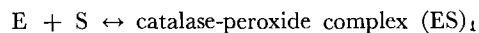
DISCUSSION

A specific, sensitive technique for the demonstration of exogenous beef liver catalase in tissues has been presented.

Classically, catalase acts as an oxidase by rapidly splitting hydrogen peroxide into water and oxygen:



In 1936, Keilin and Hartree discovered another action of catalase, namely its peroxidatic activity (18). Since then, a large number of oxidizable substances (including alcohols, pyrogallol, nitrites, and formate), which react with catalase in the presence of low peroxide concentrations, have been recognized (4, 14, 19, 20). Chance (5) has postulated that the reactions of catalase (E), with peroxide substrate (S), in the presence of a hydrogen donor (AH_2) take place in the following manner:



It is thus well established that catalase, in the presence of low concentrations of peroxide, is capable of behaving as a peroxidase.

To achieve slow liberation of peroxide in the incubation medium, Keilin and Hartree used BaO_2 as a peroxide source (19). Laser (21), using a system in which hydrogen peroxide was separated from the incubation medium by a cellophane membrane, showed that higher concentrations of enzyme and hydrogen donor and lower concentrations of peroxide promoted the peroxidatic action of catalase. These considerations were incorporated

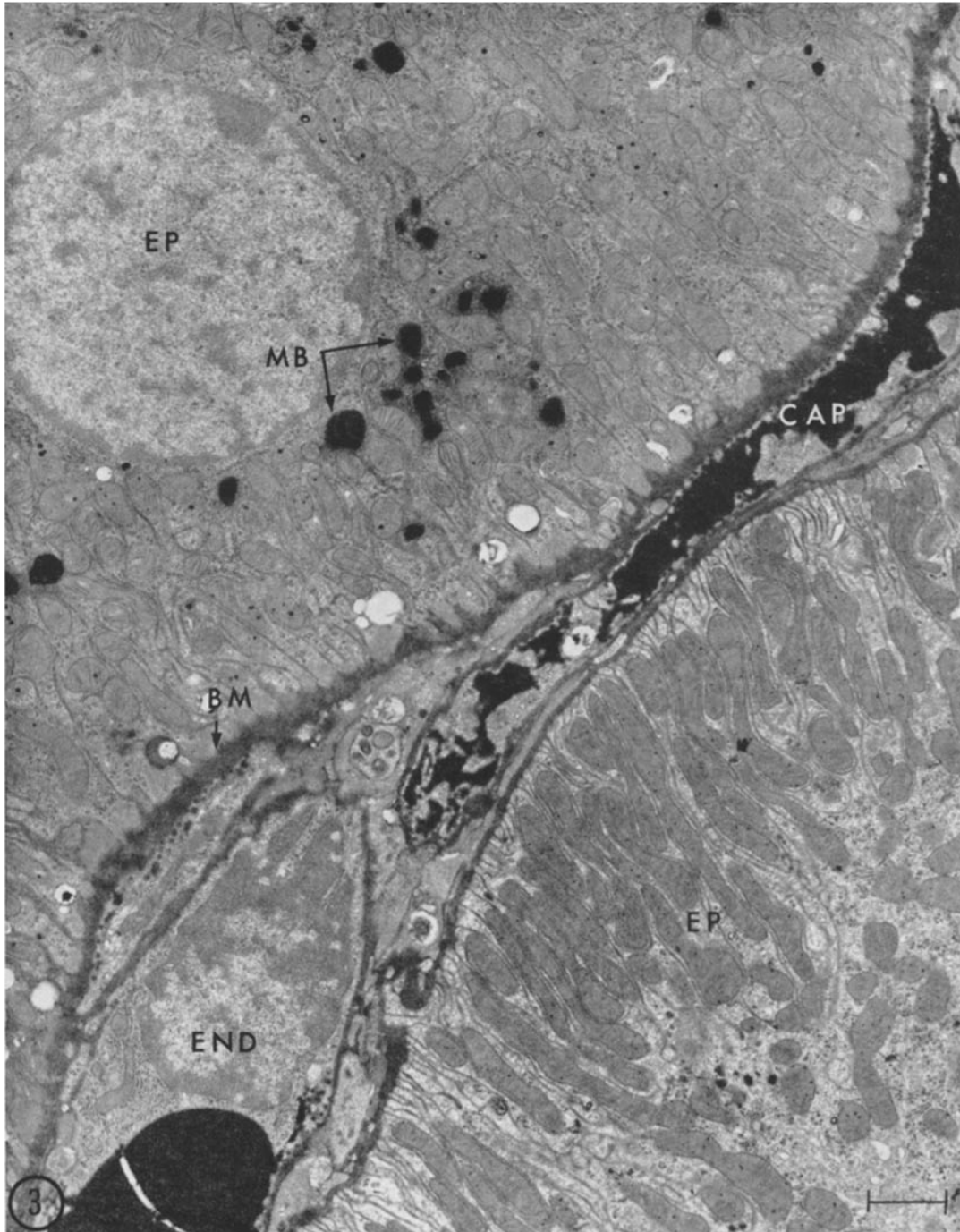


FIGURE 3 Electron micrograph of peritubular capillary in the kidney. Exogenous catalase is seen in the lumen of the capillary (*CAP*), endothelial fenestrations and basement membrane (*BM*). Renal microbodies (*MB*) are also stained. Lead Citrate. Endothelial cell (*END*), epithelial cell (*EP*). $\times 11,000$.



FIGURE 4 Part of renal glomerulus showing reaction product in vascular spaces (*VS*), endothelial fenestrations and basement membrane (*BM*). The tracer is present at the level of the slit pores (thin arrow). Lead Citrate. Urinary space (*US*), endothelial cell (*END*), epithelial cell (*EP*). $\times 23,000$.

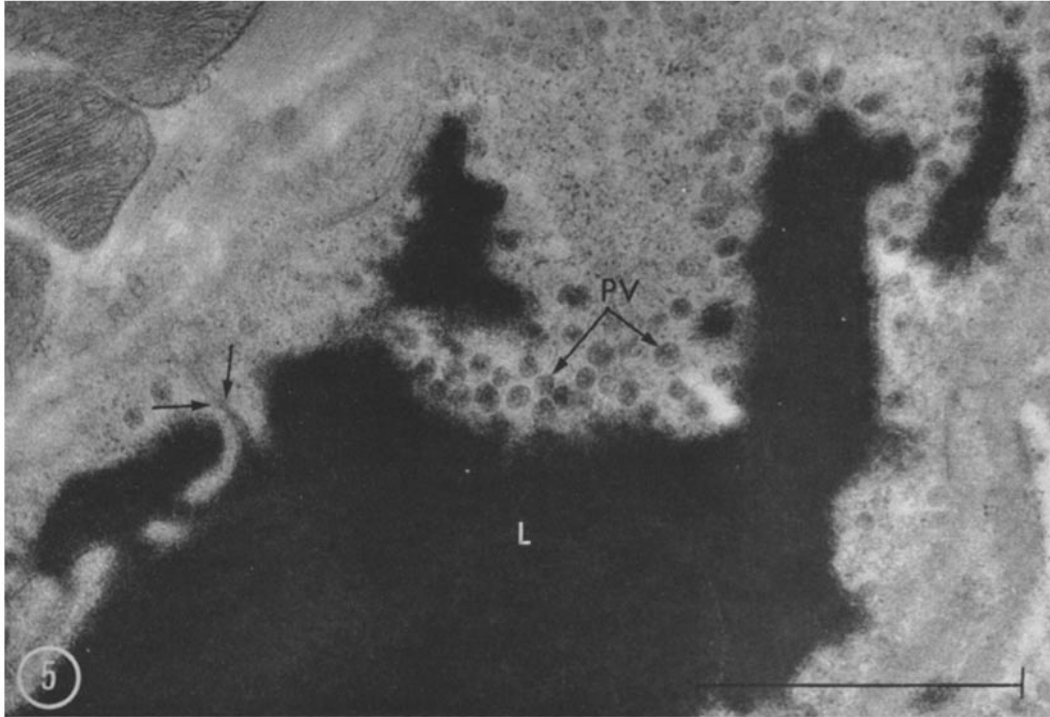


FIGURE 5 Myocardial capillary with reaction product in the lumen (*L*) and pinocytotic vesicles (*PV*). Catalase does not pass through the intercellular cleft (arrows). Lead Citrate. Approximately $\times 45,000$.

into the method presented here by the use of BaO_2 suspensions in dialysis bags as peroxide source and high concentrations (0.1%) of DAB in the incubation medium.

Beef liver catalase is commercially available in a pure form and can be injected intravenously in mice and rats without showing much evidence of toxic effects. Specifically, endothelial and parenchymal cellular damage are not present by electron microscopy. The histochemical technique for catalase is specific and the reaction can be suppressed by 3-amino, 1,2,4-triazole, an inhibitor of hepatic catalase (23); this inhibitor does not abolish the peroxidatic reaction of horse-radish peroxidase, so that, theoretically, both enzymes can be used concomitantly and identified selectively.

The method is sensitive. Catalase appears as an electron-opaque reaction product after injection of doses as low as 13 mg per 30 g mouse.

The chief merit of catalase as a tracer is the intermediate size of the molecule: between that of horse-radish peroxidase and that of ferritin. How-

ever, it has been shown that under some conditions, such as lyophilization and treatment with strong acids and alkalies, catalase may split into molecules of smaller size ($\pm 60,000$) (28). It is unlikely that significant splitting did occur in vivo in our experiments, because the localization of injected catalase was different from that of horse-radish peroxidase (12, 17) and similar to that of larger molecules such as ferritin (3, 10). Thus at 30 min after injection, in contrast to horse-radish peroxidase, the tracer had not crossed the "close junctions" of most capillary endothelia in the myocardium and was not found in renal tubules. However, it is possible that smaller amounts of split molecules had remained undetected with the present method.

The technique has some disadvantages. The method of preparation of the catalase solution is tedious, and denaturation of the protein may occur if the solution is heated above 40°C . If high doses of tracer are to be employed, its low solubility requires injection of large volumes intravenously into experimental animals. Lastly, the presence of

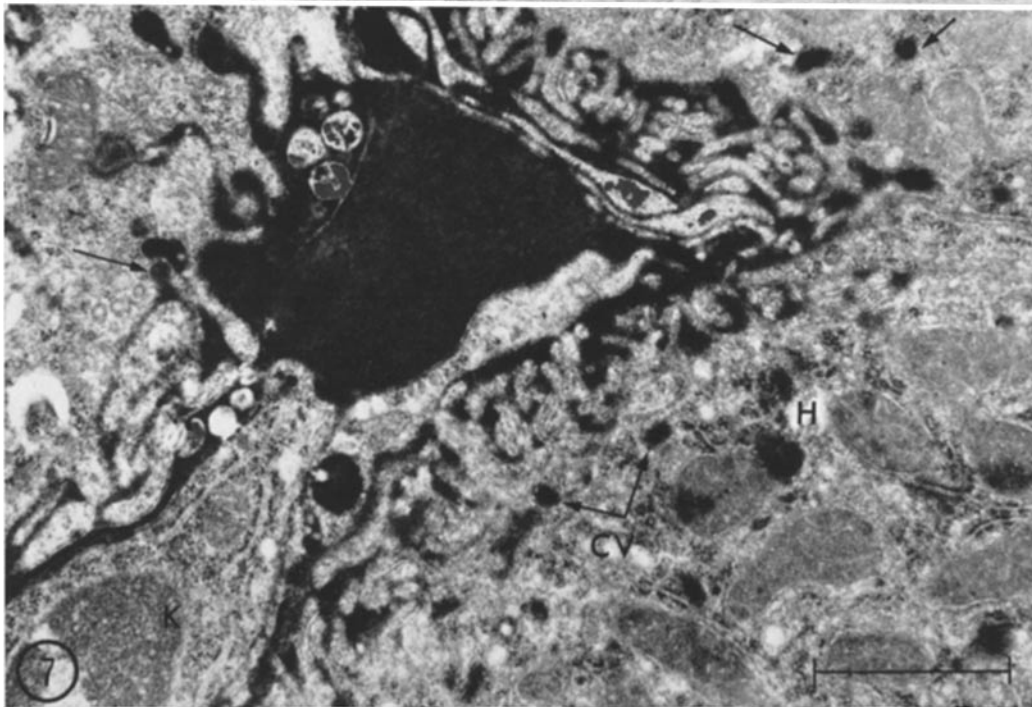
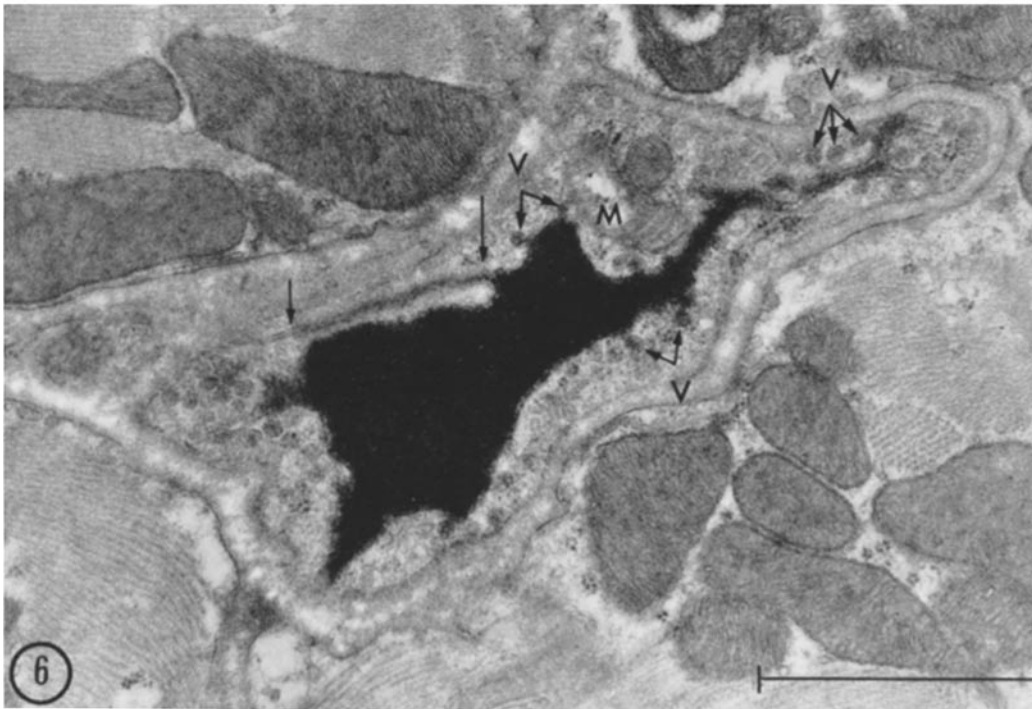


FIGURE 6 Myocardial capillary showing same features as Fig. 5 but with a better demonstration of the intercellular cleft (arrows). Pinocytotic vesicles (*V*), mitochondrion (*M*). Lead Citrate. $\times 36,000$.

FIGURE 7 Electron micrograph of liver showing reaction product in hepatic sinusoid and in coated phagocytic vesicles (*CV*) in hepatocytes (*H*). Uptake of catalase by Kupfer cells (*K*) is also shown. (arrow). Lead Citrate. $\times 25,000$.

endogenous catalase in microbodies may pose a problem in studies on cellular uptake of exogenous catalase.

Note Added in Proof: Since the submission of this manuscript we have noted that when tissues of normal rats (Charles River strain), uninjected with catalase, are stained for catalase activity, a moderately electron-opaque reaction product is visualized in the lumen of some capillaries. Since this staining is sensitive to amino-triazole it is most probably due to endogenous catalase in rat plasma. As indicated in the above manuscript

such a reaction has not been observed in mouse tissues.

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REFERENCES

1. AEBI, H., J. QUITT, and A. HASSAN. 1962. Uricase, Xanthinoxidase und Monoaminoxidase als H_2O_2 -Donoren peroxydatischer Umsetzungen. *Helv. Physiol. Pharmacol. Acta.* **20**:148.
2. AEBI, H., F. STOCKER, and M. EBERHARDT. 1963. Abhängigkeit der Monoaminoxidaseaktivität und peroxydatischer Umsetzungen von der Mitochondrienstruktur. *Biochem. Z.* **336**:526.
3. BRUNS, R. R., and G. E. PALADE. 1968. Studies on blood capillaries. II. Transport of ferritin molecules across the wall of muscle capillaries. *J. Cell Biol.* **37**:277.
4. CHANCE, B. 1950. On the reaction of catalase peroxides with acceptors. *J. Biol. Chem.* **182**: 649.
5. CHANCE, B. 1963. Enzyme kinetics in the transient state. In *Technique of organic chemistry*. Part II, Investigation of rates and mechanisms of reactions. S. L. Friess, E. S. Lewis, and A. Weissberger, editors. Interscience Pubs., Inc., John Wiley & Sons, Inc., New York. **8**:1318.
6. DE DUVE, C., and P. BAUDHUIN. 1966. Peroxisomes (Microbodies and related particles). *Physiol. Rev.* **46**:323.
7. FAHIMI, H. D. 1968. Cytochemical localization of peroxidase activity in rat hepatic microbodies (Peroxisomes). *J. Histochem. Cytochem.* **16**:547.
8. FAHIMI, H. D. 1968. Peroxidase activity of microbody catalase. *J. Cell Biol.* **39**:42 (Abstr.)
9. FAHIMI, H. D., and M. J. KARNOVSKY. 1968. Fine structural localization of exogenous peroxidase in rat liver. In *Third International Congress of Histochemistry and Cytochemistry*, Summary Reports. R. M. Rosenbaum, editor. Springer-Verlag New York, Inc. 55. (Abstr.)
10. FARQUHAR, M. G., S. L. WISSIG, and G. E. PALADE. 1961. Glomerular permeability. I. Ferritin transfer across the normal glomerular capillary wall. *J. Exp. Med.* **113**:47.
11. GRAHAM, R. C., and M. J. KARNOVSKY. 1966. The early stages of absorption of injected horse-radish peroxidase in the proximal tubule of the mouse kidney: Ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem.* **14**:291.
12. GRAHAM, R. C., and M. J. KARNOVSKY. 1966. Glomerular permeability. Ultrastructural cytochemical studies using peroxidases as protein tracers. *J. Exp. Med.* **124**:1123.
13. GRAHAM, R. C., and R. W. KELLERMAYER. 1968. Bovine lactoperoxidase as a cytochemical protein tracer for electron microscopy. *J. Histochem. Cytochem.* **16**:275.
14. HEPPEL, L. A., and V. T. PORTERFIELD. 1949. Metabolism of inorganic nitrite and nitrate esters. I. The coupled oxidation of nitrite by peroxide-forming systems and catalase. *J. Biol. Chem.* **178**:549.
15. HIRAI, K. 1968. Specific affinity of oxidized amine dye (Radical intermediater) for heme enzymes: Study in microscopy and spectrophotometry. *Acta Histochem.* **1**:43.
16. KARNOVSKY, M. J. 1965. A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. *J. Cell Biol.* **27**:137A.
17. KARNOVSKY, M. J. 1967. The ultrastructural basis of capillary permeability studied with peroxidase as a tracer. *J. Cell Biol.* **35**:213.
18. KEILIN, D., and E. F. HARTREE. 1936. Coupled oxidation of alcohol. *Proc. Roy. Soc. Ser. B. Biol. Sci.* **119**:141.
19. KEILIN, D., and E. F. HARTREE. 1945. Properties

- of catalase. Catalysis of coupled oxidation of alcohols. *Biochem. J.* **39**:293.
20. KEILIN, D., and E. F. HARTREE. 1955. Catalase, peroxidase, and metmyoglobin as catalysts of coupled peroxidatic reactions. *Biochem. J.* **63**:310.
 21. LASER, H. 1955. Peroxidatic activity of catalase. *Biochem. J.* **61**:122.
 22. LUFT, J. H. 1961. Improvements in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.* **9**:409.
 23. MARGOLIASH, E., and A. NOVOGRODSKY. 1958. A study of the inhibition of catalase by 3-amino 1:2:4 triazole. *Biochem. J.* **68**:468.
 24. NOVIKOFF, A. B., and S. GOLDFISCHER. 1968. Visualization of microbodies for light and electron microscopy. *J. Histochem. Cytochem.* (Abstr. and oral communication) **16**:507.
 25. REYNOLDS, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* **17**:208.
 26. SAMEJIMA, T., and J. T. YANG. 1963. Reconstitution of acid denatured catalase. *J. Biol. Chem.* **238**:3256.
 27. SMITH, R. E., and M. G. FARQUHAR. 1963. Preparation of thick sections for cytochemistry and electron microscopy by a nonfreezing technique. *Nature.* **200**:691.
 28. TANFORD, C., and L. LOVRIEN. 1962. Dissociation of catalase into subunits. *J. Am. Chem. Soc.* **84**:1892.