THE EFFECT OF GLUTARALDEHYDE ON CATALASE

Biochemical and Cytochemical Studies with Beef Liver Catalase and Rat Liver Peroxisomes

VOLKER HERZOG and H. DARIUSH FAHIMI. From the Harvard Pathology Unit, Mallory Institute of Pathology, Boston City Hospital, Boston, Massachusetts 02118 and the Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115

INTRODUCTION

Peroxidatic activity of catalase (E.C.1.11.1.6) was discovered by Keilin and Hartree (1), and it has been firmly established that catalase oxidizes various substrates such as ethanol, methanol, nitrite, and formate (2–4). Crystalline beef liver catalase, used as an enzyme tracer (5), and the peroxisomal catalase (6, 7) have been visualized cytochemically by a modification of the 3,3′-diaminobenzidine tetrahydrochloride (DAB)[1] technique of Graham and Karnovsky (8), and it has been suggested that the peroxidatic activity of catalase is responsible for this reaction (9). The staining of peroxisomes with DAB, however, is seen only in aldehyde-fixed tissues (10, 11). In contrast, Schejter and Bar-Eli have shown that treatment of crystalline catalase with glutaraldehyde causes a marked inhibition of both the peroxidatic and the catalatic activities of the enzyme (12).

In this study, we have investigated the effect of fixation with glutaraldehyde on the catalatic and peroxidatic activity of crystalline beef liver catalase, using a variety of oxidizable substrates, including the DAB (13, 14). Furthermore, we have studied the reaction of freshly isolated rat liver peroxisomes with DAB before and after the fixation with glutaraldehyde. Our observations

[1] *Abbreviation used in this paper*: DAB, 3,3′-diaminobenzidine tetrahydrochloride.

indicate that glutaraldehyde inhibits the catalatic activity of catalase while apparently enhancing the oxidation of several hydrogen donors including the DAB.

MATERIALS AND METHODS

Reagents

DAB, *o*-dianisidine, pyrogallol, *p*-phenylenediamine, β-D(+)-glucose, β-NAD, crystalline beef liver catalase type C-100, and aldehyde-dehydrogenase grade II were obtained from Sigma Chemical Co., St. Louis, Mo., glucose oxidase from Worthington Biochemical Corp., Freehold, N. J.; and glutaraldehyde (70%) from Ladd Research Industries, Inc., Burlington, Vt. The purity of glutaraldehyde was determined by checking the purification index A 235/A 280 (15).

Treatment of Catalase with Glutaraldehyde

Crystalline beef liver catalase was diluted to 1 mg/ml with 0.1 M sodium phosphate buffer pH 7, and 2 ml of this clear catalase solution was mixed slowly under constant stirring with an equal volume of glutaraldehyde solution buffered to pH 7.2 with 0.1 M sodium cacodylate. The final concentration of glutaraldehyde in the mixture was varied between 0.1 and 24%. It was noted that when glutaraldehyde was added dropwise to the completely dissolved catalase solution, no precipitation occurred. After 30 min of treatment at room temperature (25°C), the mixture was dialyzed for 48 h against five changes of saline buffered to pH 7.2 with 0.01 M sodium cacodylate.

Biochemical Determinations

The catalatic activity was determined according to Lück (16) at 240 nm and 25°C. The peroxidatic activity was measured using DAB as hydrogen donor (13, 14) dissolved in 0.1 M "universal buffer" of Teorell and Stenhagen (17). The final pH was adjusted to 10.5, which is the pH optimum for oxidation of DAB by glutaraldehyde-treated catalase (18). For the assay, 2.85 ml of the DAB solution (5×10^{-4} M) and 0.1 ml of aldehyde-treated catalase preparation were mixed in a 3 ml cuvette and the reaction was started by the addition of 0.05 ml of 1.2% H_2O_2 solution. Alternatively, the exogenous H_2O_2 was replaced by a continuous hydrogen peroxide generating system (4) using 10 mM β-D(+)-glucose and 1 μg glucose oxidase in the final assay medium at pH 7.4. The optical density was recorded at 450 nm for pH 10.5 and at 460 nm for pH 7.4 assays (19) at room temperature (25°C) in double beam Beckman DBGT spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). The substrate solution with H_2O_2 but without catalase served as blank. The enzyme activity is expressed as ΔA per minute per milligram protein. In addition to the DAB, four other hydrogen donors were used: *p*-phenylenediamine (20); pyrogallol (21); *o*-dianisidine (22, 23); and ethanol (4).

The protein content of untreated catalase solutions was determined spectrophotometrically at 260 and 280 nm (24), and the results were confirmed with the method of Lowry et al. (25) using bovine serum albumin (Armour Pharmaceutical Co., Chicago, Ill.) as standard. Since glutaraldehyde has an absorption maximum at 280 nm (26) and, in addition, since its presence interferes with the results of the Lowry procedure, protein determinations for glutaraldehyde-treated catalase were based on the Soret band absorption as used previously by Schejter and Bar-Eli (12).

Staining for Peroxidase Activity of the Unfixed "Crude Peroxisomal Fraction"

The livers of male adult albino rats were homogenized in 0.25 M sucrose and a fraction consisting mainly of mitochondria and peroxisomes was obtained by differential centrifugation (27). The unfixed pellet was resuspended in a medium containing 0.1% DAB, dissolved in 0.15 M Tris-HCl buffer at pH 8.5 and 0.02% H_2O_2 for 1 h at 37°C (9). After the incubation, the resuspended particles were again centrifuged and the resulting pellet was fixed for 1 h in 3% glutaraldehyde, followed by postfixation in osmium tetroxide, and embedding in Epon (28).

In additional experiments, DAB-reacted pellets were fixed in glutaraldehyde and incubated for a second time in the DAB medium at pH 8.5 for 2 h at 37°C, followed by postosmication, and processing for electron microscopy.

Staining for Peroxidase Activity of the Fixed Crude Peroxisomal Fraction

The crude peroxisomal fraction was overlayed with 3% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.2 containing 0.025% calcium chloride. After 30 min of fixation at 4°C, the pellet was removed from the centrifuge tube and cut into 1 mm thin strips, which were rinsed briefly in 0.15 M cacodylate buffer pH 7.2 and were incubated for 2 h at 37°C in the alkaline DAB medium (9), followed by postosmication, and processing for electron microscopy.

RESULTS

Biochemical Findings

Glutaraldehyde treatment caused marked inhibition of the catalatic activity of catalase.

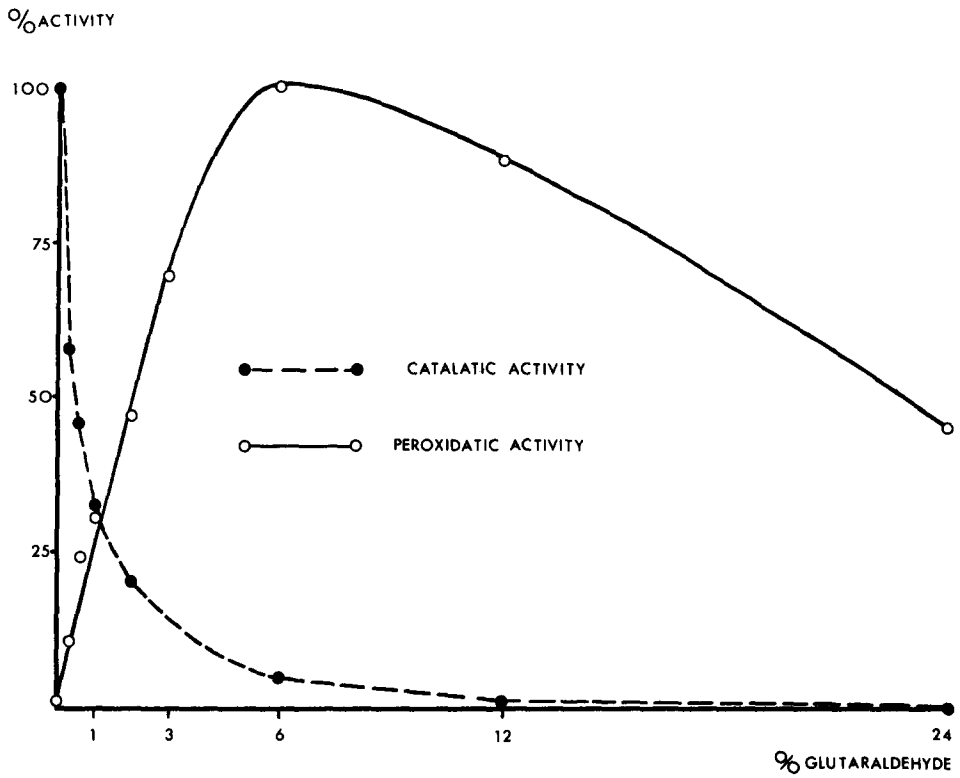


FIGURE 1 Effect of glutaraldehyde on the peroxidatic (○——○) and catalytic (●- - -●) activity of beef liver catalase. The peroxidatic activity was measured with DAB as hydrogen donor (13) and catalytic activity was determined according to the method of Lück (16). The catalytic activity decreases with increasing concentrations of glutaraldehyde while simultaneously the peroxidatic activity increases. Glutaraldehyde concentrations higher than 6%, however, also inhibit the peroxidatic activity.

Fig. 1 demonstrates that 1% glutaraldehyde inhibited 70%, and 6% glutaraldehyde about 90% of the catalatic activity. In contrast, the peroxidatic activity of catalase, as measured with DAB as hydrogen donor increased with increasing concentrations of glutaraldehyde, reaching a maximum with 6% glutaraldehyde, and decreasing with higher concentrations (Fig. 1). When the peroxidatic activity was assayed with *p*-phenylenediamine and *o*-dianisidine as hydrogen donors, the patterns of enhancement and inhibition of the peroxidatic activity by glutaraldehyde paralleled the results obtained with the DAB assay (Fig. 2). Similarly, when hydrogen peroxide was replaced by glucose, glucose-oxidase system in the DAB assay at pH 7.4, there was a gradual increase in the peroxidatic activity of catalase with increasing concentrations of glutaraldehyde (Table I). In contrast, when ethanol was used as an oxidizable substrate, the glutaraldehyde treatment caused a marked inhibition of the peroxidatic activity (Table I). Similar to ethanol, oxidation of pyrogallol was markedly inhibited after the treatment of catalase with glutaraldehyde (Fig. 2).

The addition of untreated catalase to the DAB-assay medium had an inhibitory effect upon the peroxidatic activity of glutaraldehyde-treated catalase (Table II).

Addition of glutaraldehyde to the DAB medium at pH 8.5 without H_2O_2 caused a slight increase of the optical density at 450 nm. There was, however, no evidence of such pseudoperoxidatic activity when catalase treated with glutaraldehyde and dialyzed for 48 h was added to the DAB medium, thus indicating that all free glutaraldehyde was removed by the dialysis procedure. In addition, no evidence of oxidation of DAB was noted when glutaraldehyde-treated bovine serum albumin was dialyzed for 48 h and added to the DAB medium, thus indicating that protein-bound

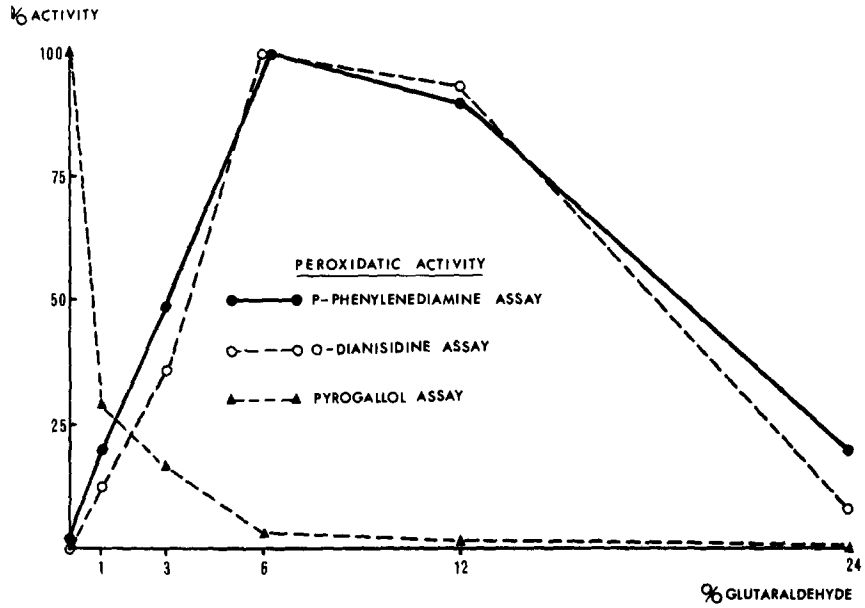


FIGURE 2 Effect of glutaraldehyde on the peroxidatic activity of beef liver catalase, as measured with three other hydrogen donors. The assays using *p*-phenylenediamine (●——●) and *o*-dianisidine (○- - -○) parallel the results obtained with diaminobenzidine in Fig. 1. When pyrogallol (▲- - -▲) is used as hydrogen donor, however, an inhibition of the peroxidatic activity of catalase by all concentrations of glutaraldehyde between 0.1 and 24% is noted.

TABLE I

Comparison between the Oxidation of Ethanol and DAB by Glutaraldehyde-Treated Catalase Using the Glucose, Glucose-Oxidase System as Hydrogen Peroxide Source at pH 7.4

	Glutaraldehyde concentration			
	0%	1%	3%	6%
Peroxidatic activity (ethanol oxidation)	100%	32%	26%	19%
Peroxidatic activity (DAB oxidation)	1.5%	5%	33%	100%

Note the apparent enhancement of the activity with increasing concentrations of glutaraldehyde when DAB is used as hydrogen donor, and in contrast, the inhibition of the peroxidatic activity when ethanol is used as substrate.
The activities are given in percent.

glutaraldehyde had no effect on the peroxidase assay. Similarly, glutaraldehyde-treated albumin had no influence on the method of Lück for measurement of catalatic activity (16).

TABLE II

Effect of Addition of Untreated Catalase on the Peroxidatic Activity of Glutaraldehyde-Treated Catalase, as Measured with the DAB Assay

	Amount of native catalase*			
	0	10 μg	25 μg	50 μg
Peroxidatic activity (DAB oxidation)	100%	80%	53%	15%

Note the inhibition of the peroxidatic activity by increasing amounts of untreated catalase in the assay system.
* In 3 ml cuvette.

Cytochemical Observations on Crude Peroxisomal Fraction

Incubation of the unfixed crude peroxisomal fractions in the DAB-H_2O_2 medium at pH 8.5 revealed reaction product in the intermembranous space of mitochondria with no or only minimal deposits in the matrix of peroxisomes (Figs. 3, 4). Some of these peroxisomes showed focal extractions of the matrix, which were probably due to

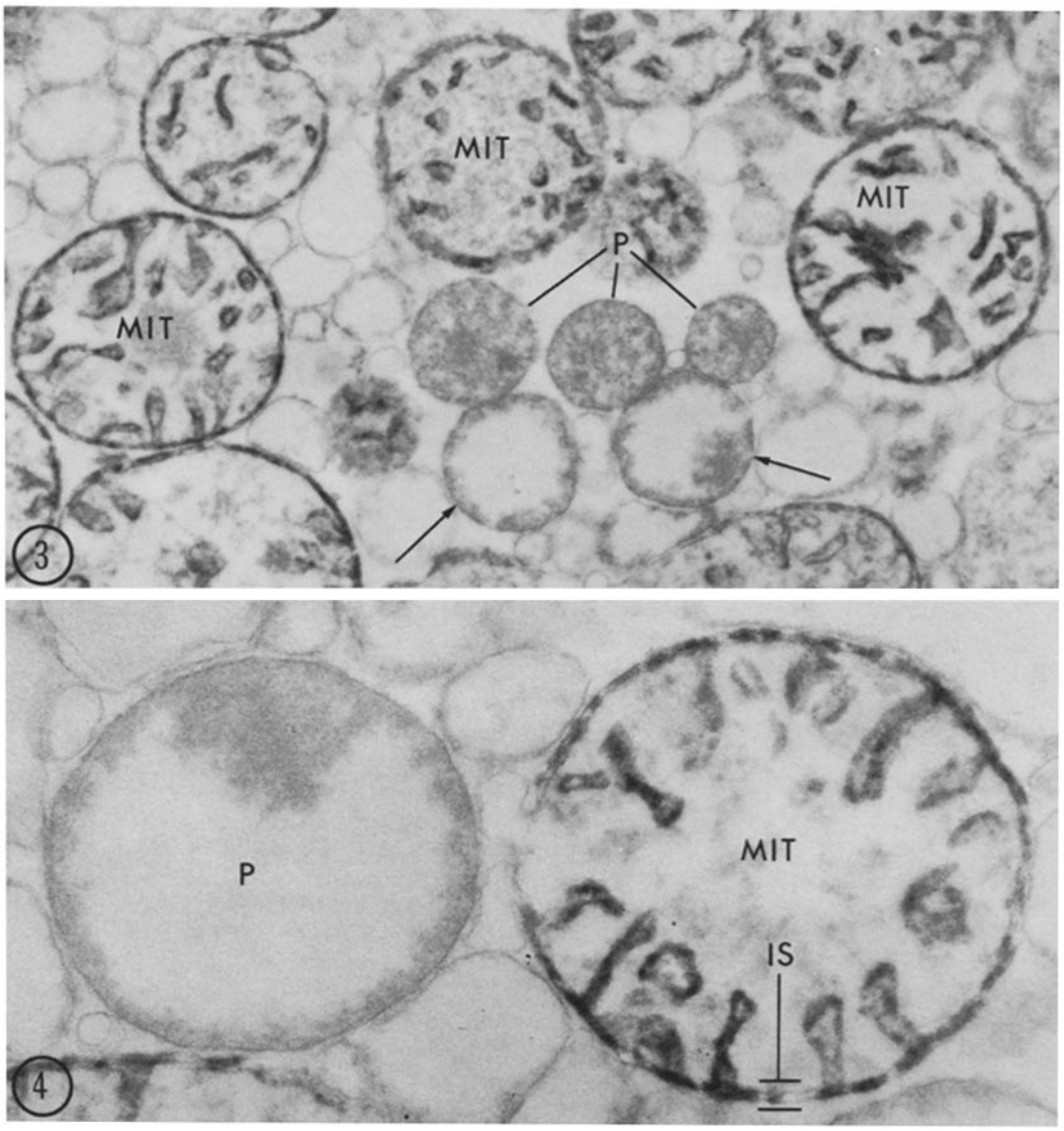


FIGURE 3 Unfixed crude peroxisomal fraction incubated in the DAB medium at pH 8.5. There is no or only minimal reaction product in peroxisomes (*P*), some of which show focal extraction (arrows). Mitochondria (*MIT*) in such unfixed fractions, however, stain prominently. × 19,000.

FIGURE 4 Unfixed crude peroxisomal fraction incubated in the DAB medium at pH 8.5. This higher power view shows the localization of the reaction product of oxidation of DAB in the intermembranous space (*IS*) of a mitochondrion (*MIT*). Note the partially extracted and unstained peroxisome (*P*). × 75,000.

prolonged incubation of unfixed pellets at 37°C. However even peroxisomes without extraction were lacking a DAB reaction product (Fig. 3).

Incubation of glutaraldehyde-fixed pellets in the alkaline DAB-H_2O_2 medium resulted in prominent electron-dense deposits in the matrix of peroxisomes with no reaction product in mitochondria (Fig. 5).

When primarily unfixed but reacted pellets were fixed subsequently in glutaraldehyde and then incubated for a second time in the alkaline DAB medium, evidence of DAB reaction product

was noted in both mitochondria and peroxisomes (Figs. 6, 7).

DISCUSSION

The observations presented here indicate that glutaraldehyde treatment apparently enhances the peroxidatic activity of catalase, as determined with DAB, *o*-dianisidine, and *p*-phenylenediamine assays. Similarly, freshly isolated peroxisomes from rat liver do not stain with DAB, but glutaraldehyde-fixed peroxisomes oxidize DAB readily. In contrast, glutaraldehyde treatment inhibits the catalatic, as well as the peroxidatic activity of catalase, when ethanol and pyrogallol are used as hydrogen donors.

Inhibition of the catalatic activity of the enzyme is in agreement with previous observations on the effect of glutaraldehyde upon the activity of several enzymes (29, 30), including catalase (12). It is generally believed that the loss of enzymatic activity is related to the reaction of glutaraldehyde with the lysine residues of enzyme proteins (30, 31). Shejter and Bar-Eli reported that glutaraldehyde cross-linked approximately 50% of the lysine molecules of catalase with an associated loss of 90% of the catalatic activity (12). These authors, as well as Ferrier et al. (32), however, were mainly interested in obtaining a water-insoluble catalase preparation, whereas in this study we have obtained a soluble preparation of cross-linked catalase. The advantage of this soluble preparation is that it can be subjected to various biochemical and spectrophotometric studies without further homogenization and disruption.

The most remarkable effect of glutaraldehyde on catalase appears to be the modification of the peroxidatic activity, which varies with different hydrogen donors. Thus, apparent enhancement of the peroxidatic activity is noted with DAB, *o*-dianisidine, and *p*-phenylenediamine, and inhibition with ethanol and pyrogallol. The loss of enzymatic activity is less severe with ethanol than with pyrogallol (compare Table I with Fig. 2). This observation is in agreement with findings of Schejter and Bar-Eli, who noted variable degrees of inhibition of peroxidatic activity of cross-linked catalase, using methanol and pyrogallol as hydrogen donors (12). These authors suggested that this could be due to steric alterations of the enzyme molecule which either caused diminished accessibility of the active site, or hindered the conformational changes of the enzyme necessary for the peroxidatic activity.

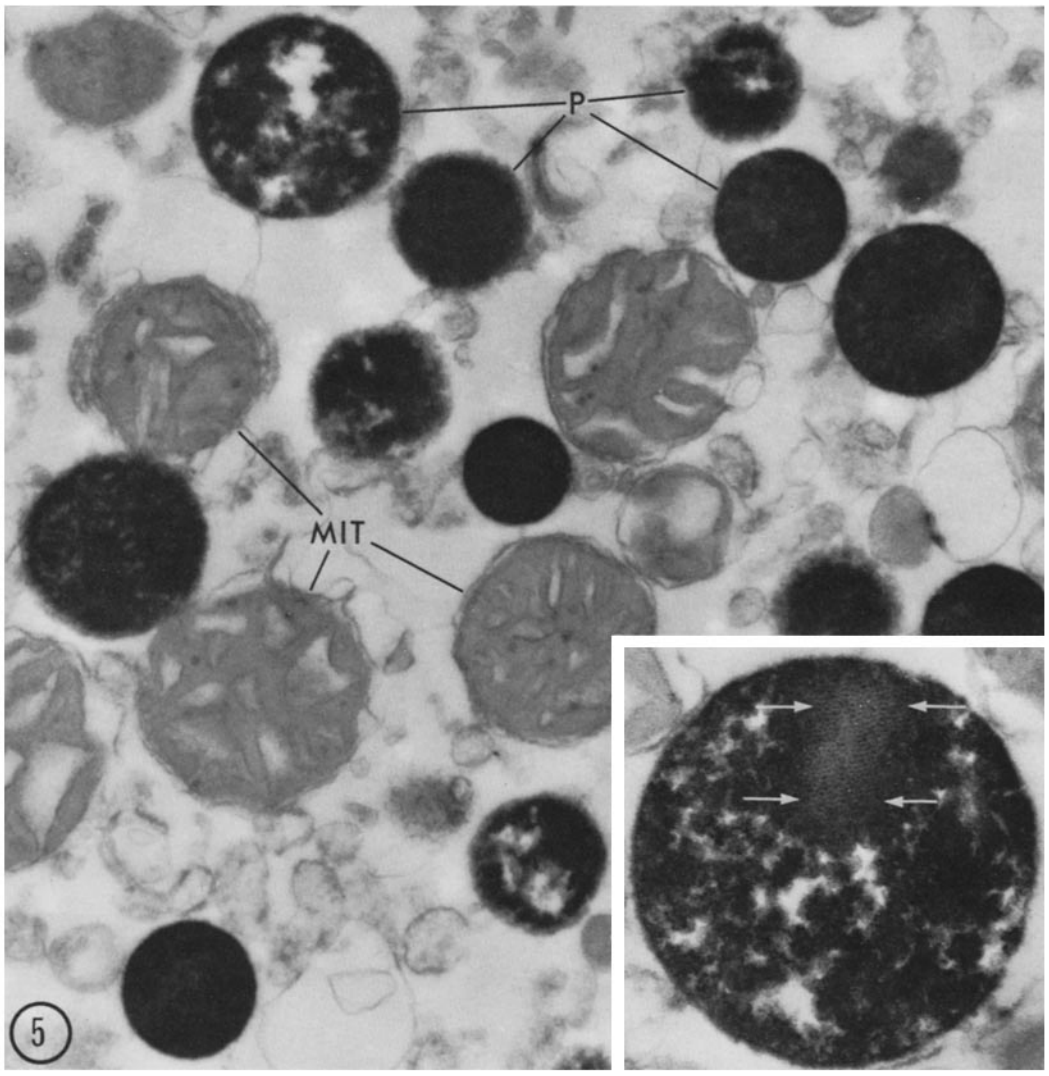
Another possible explanation for the modified peroxidatic activity of glutaraldehyde-treated catalase is provided by the observations of Feinstein et al. (33). These authors noted that treatment of catalase by denaturing agents such as urea, heat, and alkalinization caused marked inhibition of catalatic activity with simultaneous increase of peroxidatic activity. They postulated that peroxidase-active sites exist on the intact catalase molecule, but cannot be detected normally because of the extreme competition by catalatic active sites for available H_2O_2. Such catalatic active sites, however, are easily destroyed by a variety of reagents, thus permitting peroxidatic activity to become detectable. In agreement with this hypothesis, we noted that peroxidatic activity of glutaraldehyde-treated catalase, as determined with DAB assay, was markedly decreased when untreated catalase was added to the assay system (Table II).

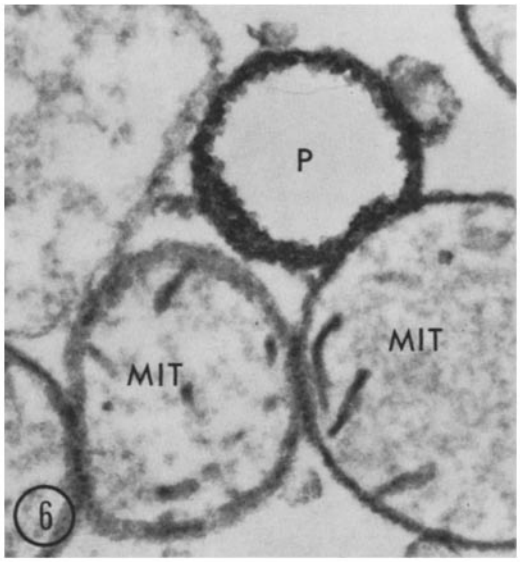
Finally, our findings provide further insight into the mechanism of staining of peroxisomes with the DAB reaction. In this respect, Goldfischer and

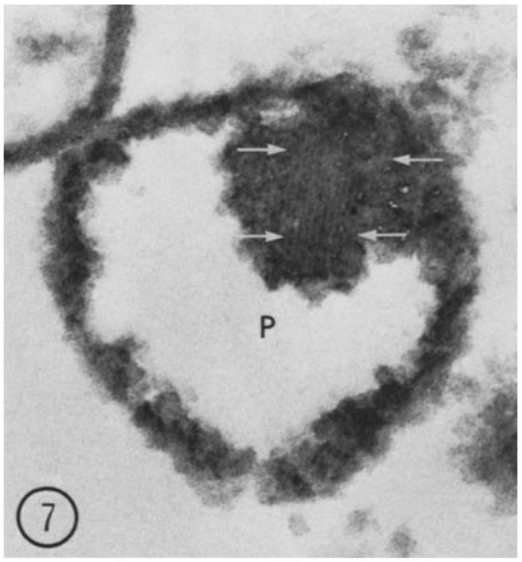
---

FIGURE 5 Glutaraldehyde-fixed crude peroxisomal fraction incubated in the DAB medium at pH 8.5. Reaction in mitochondria (*MIT*) is completely inhibited whereas the peroxisomes (*P*) stain prominently. × 52,000. *Inset:* At higher magnification, a crystalloid (arrows) is demonstrable in a peroxisome. × 120,000.

FIGURES 6 and 7 Crude peroxisomal fraction, freshly incubated in the DAB medium at pH 8.5, fixed in glutaraldehyde, and incubated a second time in the DAB medium at pH 8.5. In these preparations both mitochondria (*MIT*) and peroxisomes (*P*) contain reaction product. Although after this treatment most of the isolated particles show extraction, peroxisomes are still recognizable by the peroxidatic activity on part of their matrix adjacent to their membrane (Figs. 6, 7) and by the presence of crystalloids (arrows, Fig. 7). Fig. 6, × 57,000, Fig. 7, × 110,000.


P
MIT
5


P
MIT
MIT
6


P
7

Essner postulated that the staining of peroxisomes with benzidine compounds is probably due to splitting of catalase into subunits with peroxidatic activity during the incubation of sections at high alkaline pH and at 37°C (34). We have demonstrated, however, that incubation of unfixed peroxisomes at pH 8.5 and 37°C does not reveal peroxidatic activity[2] (Figs. 3, 4). This lack of staining in unfixed fractions is not due to loss of enzyme from the matrix of peroxisomes because a subsequent fixation and incubation reveal prominent staining in the matrix of the particles (Figs. 6, 7). Furthermore, the enhancement of the peroxidatic activity of catalase by glutaraldehyde treatment is seen not only at alkaline pH but also at neutral pH, with the DAB assay and glucose, glucose-oxidase system (Table I), as well as with *o*-dianisidine and *p*-phenylenediamine assays (Fig. 2). These observations demonstrate that fixation with glutaraldehyde is the most important prerequisite for staining of peroxisomes with DAB. However, since the oxidation of DAB by glutaraldehyde-treated catalase is optimal at pH 10.5 (18), the incubation at alkaline pH gives a stronger staining than that at neutral pH (6).

We thank Ms. A. Gray for technical assistance and Ms. D. Hickman for secretarial help.

This work was presented in part at the 12th Annual Meeting of The American Society for Cell Biology, St. Louis, Missouri 1972. *J. Cell Biol.* **55**(2,Pt.2):113 a.

This study was supported by National Institutes of Health grant NS 08533 from National Institute of Neurological Diseases and Stroke, and GRS-grant no. 57–182 from Boston City Hospital. Dr. Herzog is supported by Deutsche Forschungsgemeinschaft of Federal Republic of Germany, and Dr. Fahimi is the recipient of a Research Career Development Award from National Institutes of Health, Bethesda, Maryland.

Received for publication 25 May 1973, and in revised form 17 August 1973.

REFERENCES

1. KEILIN, D., and E. F. HARTREE. 1936. Coupled oxidation of alcohol. *Proc. R. Soc. Lond. B Biol. Sci.* **119**:141.
2. 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.
3. AEBI, H. 1960. Mécanisme et role biologique de l'action peroxidasique de la catalase. *Bull. Soc. Chim. Biol.* **42**:187.
4. OSHINO, N., R. OSHINO, and B. CHANCE. 1973. The characteristics of the "peroxidatic" reaction of catalase in ethanol oxidation. *Biochem. J.* **131**:555.
5. VENKATACHALAM, M. A., and H. D. FAHIMI. 1969. The use of beef liver catalase as a protein tracer for electron microscopy. *J. Cell Biol.* **42**:480.
6. FAHIMI, H. D. 1968. Cytochemical localization of peroxidase activity in rat hepatic microbodies (peroxisomes). *J. Histochem. Cytochem.* **16**:547.
7. NOVIKOFF, A., and S. GOLDFISCHER. 1969. Visualization of peroxisomes (microbodies) and mitochondria with diaminobenzidine. *J. Histochem. Cytochem.* **17**:675.
8. GRAHAM, R. C., and M. J. KARNOVSKY. 1966. The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney. Ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem.* **14**:291.
9. FAHIMI, H. D. 1969. Cytochemical localization of peroxidatic activity of catalase in rat hepatic microbodies. *J. Cell Biol.* **43**:275.
10. SIES, H., V. HERZOG, and F. MILLER. 1972. Electron microscopic and spectrophotometric studies on mitochondrial and peroxisomal reaction with diaminobenzidine in hemoglobin-free perfused rat liver. Proceedings of the Fifth European Congress on Electron Microscopy. University of Manchester, England. 274.
11. ROELS, F., and E. WISSE. 1973. Distinction cytochimique entre catalase et peroxydases. *C. R. Hebd. Seances Acad. Sci. Ser. D. Sci. Nat. (Paris).* **276**:391.
12. SCHEJTER, A. and A. BAR-ELI. 1970. Preparation and properties of cross-linked water-insoluble catalase. *Arch. Biochem. Biophys.* **136**:325.
13. FAHIMI, H. D., and V. HERZOG. 1972. A colorimetric assay for peroxidatic reactions based on the oxidation of 3,3′-diaminobenzidine (DAB). *J. Histochem. Cytochem.* **20**:840.
14. HERZOG, V., and H. D. FAHIMI. 1973. A new sensitive colorimetric assay for peroxidase using 3,3′-diaminobenzidine as hydrogen donor. *Anal. Biochem.* **55**:554.
15. ANDERSON, P. J. 1967. Purification and quantitation of glutaraldehyde and its effect on several enzyme activities in skeletal muscle. *J. Histochem. Cytochem.* **15**:652.

[2] Incubation of unfixed peroxisomes at pH 10.5 could not be performed because of their instability above pH 9.0.

16. LÜCK H. 1965. Catalase. *In* Methods in Enzyme Analysis. H. U. Bergmeyer, editor. Verlag-Chemie, Weinheim/Bergstrasse, Germany.
17. TEORELL, T., and E. STENHAGEN. 1938. Ein Universalpuffer fuer den pH-Bereich 2.0–12.0. *Biochem. Z.* **299**:416.
18. HERZOG, V., and H. D. FAHIMI. 1973. An improved cytochemical method for demonstration of the peroxidatic activity of beef liver catalase (BLC). *J. Histochem. Cytochem.* **21**:412.
19. FAHIMI, H. D., and V. HERZOG. 1973. A colorimetric method for measurement of the (peroxidase mediated) oxidation of 3,3′-diaminobenzidine. *J. Histochem. Cytochem.* **21**:499.
20. LÜCK, H. 1965. Peroxidase. *In* Methods in Enzyme Analysis. H. U. Bergmeyer, editor. Verlag-Chemie, Weinheim/Bergstrasse, Germany.
21. TAUBER, H. 1953. Oxidation of pyrogallol to purpurogallin by crystalline catalase. *J. Biol. Chem.* **205**:395.
22. PÜTTER, J. 1962. Ein Methode zur Bestimmung der Katalaseaktivität mit kleinen Substratkonzentrationen. *Z. Physiol. Chem.* (*Hoppe-Seyler's*). **329**:40.
23. Worthington Enzyme Manual, Peroxidase. Catalogues of the Worthington Biochemical Corporation, Freehold, N. J.
24. WARBURG, O., and W. CHRISTIAN. 1942. Isolierung und Kristallisation des Gaerungsfermentes Enolase. *Biochem. Z.* **310**:384.
25. LOWRY, O. L., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265.
26. FAHIMI, H. D., and P. DROCHMANS. 1965. Essais de standardisation de la fixation au glutaraldéhyde. I. Purification et détermination de la concentration du glutaraldéhyde. *J. Microsc.* (*Paris*). **4**:725.
27. HOGEBOOM, G. H. 1955. Fractionation of cell components of animal tissues. *Methods Enzymol.* **1**:16.
28. LUFT, J. H. 1961. Improvements in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.* **9**:409.
29. QUIOCHO, F. A., and F. M. RICHARDS. 1964. Intermolecular cross-linking of a protein in the crystalline state: carboxypeptidase A. *Proc. Natl. Acad. Sci. U. S. A.* **52**:833.
30. HOPWOOD, D. 1972. Theoretical and practical aspects of glutaraldehyde fixation. *Histochem. J.* **4**:267.
31. QUIOCHO, F. A., and F. M. RICHARDS. 1966. The enzymic behavior of carboxypeptidase-A in the solid state. *Biochemistry.* **5**:4062.
32. FERRIER, L. K., T. RICHARDSON, and N. F. OLSON. 1972. Crystalline catalase insolubilized with glutaraldehyde. *Enzymologia.* **42**:273.
33. FEINSTEIN, R. M., R. SAVOL, and J. B. HOWARD. 1971. Conversion of catalatic to peroxidatic activity in livers of normal and acatalasemic mice. *Enzymologia.* **41**:345.
34. GOLDFISCHER, S., and E. ESSNER. 1969. Further observations on the peroxidatic activities of microbodies (peroxisomes). *J. Histochem. Cytochem.* **17**:681.