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Cytochemical localization of catalase and several hydrogen peroxide-producing oxidases in the nucleoids and matrix of rat liver peroxisomes

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Synopsis. The distribution of catalase, amino acid oxidase, α-hydroxy acid oxidase, urate oxidase and alcohol oxidase was studied cytochemically in rat hepatocytes. The presence of catalase was demonstrated with the conventional diaminobenzidine technique. Oxidase activities were visualized with methods based on the enzymatic or chemical trapping of the hydrogen peroxide produced by these enzymes during aerobic incubations.

All enzymes investigated were found to be present in peroxisomes. Catalase activity was found in the peroxisomal matrix, but also associated with the nucleoid. After staining for oxidase activities the stain deposits occurred invariably in the peroxisomal matrix as well as in the nucleoids. In all experiments the activity of both catalase and the oxidases was confined to the peroxisomes. The presence of a hydrogen peroxide-producing alcohol oxidase was demonstrated for the first time in peroxisomes in liver cells.

The results imply that the enzyme activity of the nucleoids of rat liver peroxisomes is not exclusively due to urate oxidase. The nucleoids obviously contain a variety of other enzymes that may be more or less loosely associated with the insoluble components of these structures.

Introduction

Peroxisomes are cell organelles characterized biochemically by the presence of catalase and one or more H_2O_2-producing oxidases. In kidney and liver cells of various vertebrate species, these organelles contain crystalline cores or nucleoids. The occurrence of such structures has been associated with the presence of urate oxidase. Although nucleoids have been described in peroxisomes of tissues without detectable urate oxidase activity, the association of this enzyme with the highly-ordered crystalline nucleoid of rat

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liver peroxisomes has been firmly established (Hruban & Rechcigl, 1969; Masters & Holmes, 1977; Antonenkov & Panchenko, 1978).

Investigations by Hruban & Swift (1964), Tsukada *et al.* (1966, 1971) and Lata *et al.* (1977) on isolated nucleoids from rat liver peroxisomes have shown urate oxidase to be the predominant enzyme in these structures. Huang & Beevers (1973) concluded that all of this enzyme present in the peroxisomes is likely to be located in the nucleoids. De Duve & Baudhuin (1966) calculated that urate oxidase represents only 10% of the total protein of the nucleoids. The studies of Hayashi *et al.* (1971, 1973, 1976) and Watanabe *et al.* (1977) indicated that the nucleoids consist of two highly-insoluble proteins, identified as urate oxidase and a framework protein, loosely associated with other more soluble proteins. One of these soluble proteins has been identified as D-amino acid oxidase. Other enzymes may be associated with the nucleoids, but the loose association of soluble proteins with the nucleoids possibly escapes biochemical analysis. For this reason, we studied the distribution of catalase and several oxidases in rat liver peroxisomes by cytochemical techniques.

The localization of catalase was established by the diaminobenzidine (DAB) procedure (Novikoff & Goldfischer, 1969). In addition, a modification of this technique was applied, which involved the replacement of exogenous H_2O_2 by a substrate specific for one of the peroxisomal oxidases. Oxidase activity results in the local production of the H_2O_2 required for the catalase-dependent oxidation of diaminobenzidine (Veenhuis *et al.*, 1976). By this technique the association of catalase with the oxidases can be studied more specifically. Data on the cytochemical localization of H_2O_2-producing oxidases are scarce, which may be due to the circumstance that reliable staining techniques became available only a few years ago. In earlier papers, we demonstrated that the cerium technique introduced by Briggs *et al.* (1975) for the submicroscopical localization of extracellular NADH-oxidase can be adapted for the demonstration of peroxisomal oxidases such as amino acid oxidase, α-hydroxy acid oxidase, urate oxidase and alcohol oxidase (Veenhuis *et al.*, 1976; Veenhuis & Wendelaar Bonga, 1977).

Materials and methods

Adult male and female rats (Wistar strain, approximately 300 g) were used. The liver was fixed by perfusion of the portal vein with ice-cold 3% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2, containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$.

For the demonstration of catalase activity, small blocks of liver tissue were cut and washed for 5 min in 0.1 M Tris-HCl buffer, pH 8.5, and incubated in 5 ml freshly-prepared medium containing 10 mg 3,3′-diaminobenzidine HCl (DAB) and 0.06% H_2O_2 in 0.1 M Tris-HCl, pH 8.5, for 1 h at 37°C.

For the alternative technique for the demonstration of catalase activity, with DAB and an oxidase-specific substrate for the production of endogenous H_2O_2, glutaraldehyde-fixed tissue blocks were washed for 5 min in 0.1 M Tris-HCl

buffer, pH 7.5. The tissues were incubated for 1–2 h under continuous aeration in 5 ml freshly-prepared and similarly-buffered medium containing 10 mg DAB and either DL-alanine, DL-lactate, methanol (all at 50 mM), sodium urate (dissolved to saturation). In control experiments, glutaraldehyde-fixed tissue was incubated with DAB but without oxidase substrate. Anaerobic incubations in the presence of DAB and oxidase substrate were performed by bubbling oxygen-free nitrogen through the incubation mixture.

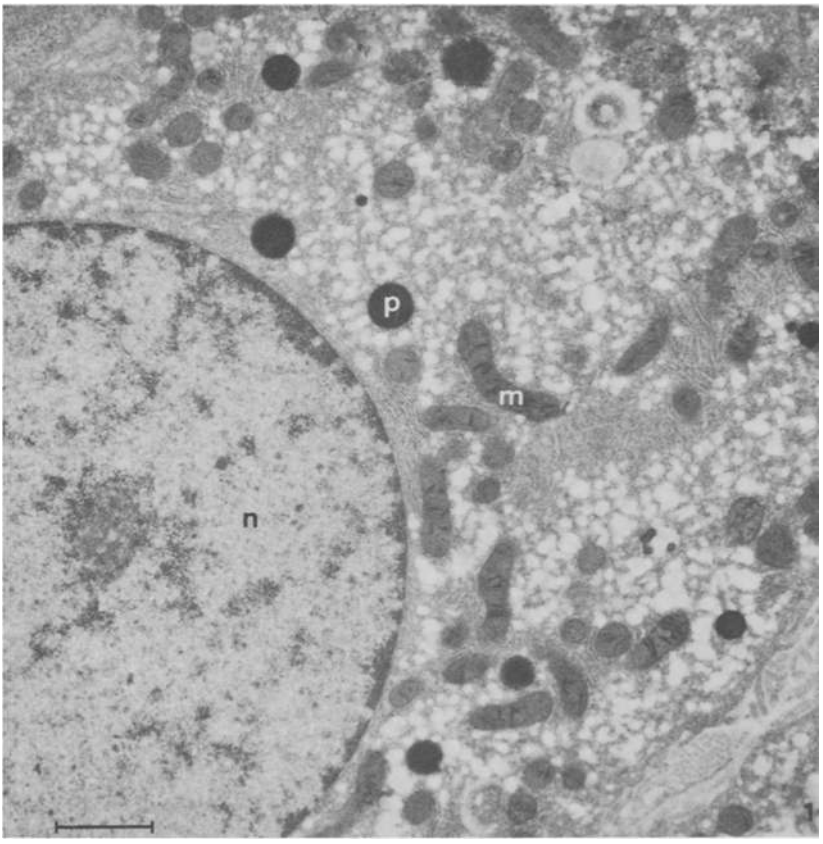
For the localization of oxidase activities, glutaraldehyde-fixed or unfixed tissue was used. The tissue blocks were washed twice in 0.1 M Tris-maleate buffer pH 7.5, pre-incubated for 60 min at 25°C in 5 ml of the same buffer containing 5 mM cerium chloride ($CeCl_3$) and 50 mM aminotriazole, and subsequently incubated for 1–2 h at 37°C under continuous aeration in the same medium containing, in addition, DL-alanine, DL-lactate, methanol (all at 50 mM) or sodium urate (dissolved to saturation). Afterwards the blocks were washed in 0.1 M cacodylate buffer, pH 6.0, to remove any cerium hydroxide formed during incubation. Control blocks were incubated anaerobically or in the absence of substrate.

After the cytochemical staining procedures, the tissue blocks were washed in distilled water, post-fixed for 30 min in a mixture of 1% osmium tetroxide and 2.5% potassium dichromate in 0.1 M cacodylate buffer, pH 7.2, at room temperature, and post-stained for 30 min in 1% aqueous uranyl acetate. The tissues were subsequently dehydrated in an ethanol series and embedded in either Epon 812 or Spurr's epoxy resin. Ultrathin sections were cut on a LKB-Ultrotome with a diamond knife, not post-stained and examined in a Philips EM300 electron microscope.

Results

Staining of catalase with DAB

The cytochemical staining experiments for the demonstration of catalase activity with DAB and various substrates were performed with glutaraldehyde-fixed cells, since it is well known that the peroxidatic activity of catalase is highly stimulated by this fixative (Roels & Wisse, 1973; Herzog & Fahimi, 1974; Roels *et al.*, 1975). The actual presence of catalase activity in the peroxisomes in the cells was demonstrated with the conventional technique, involving incubation with DAB and exogenous H_2O_2. Aerobic incubations of these cells with DAB in the presence of urate resulted in positively stained peroxisomes (Fig. 1). Similar results were obtained with the other substrates used namely methanol, DL-alanine and DL-lactate (Figs. 2–6). After control incubations in the absence of the substrate for an oxidase or after anaerobic incubations, staining of the peroxisomes was absent or very weak (Figs. 7 & 8), while staining of other structures, as after incubation with DAB and an oxidase substrate, was limited to the mitochondria. However, after all incubations with DAB in the presence of a substrate for the endogenous production of H_2O_2, the nucleoid still showed a higher electron density than the peroxisomal matrix (Figs. 3 & 4), and in high magnifications of very thin



Figures 1–6 show liver cells fixed with glutaraldehyde and incubated with DAB and different substrates for the endogenous generation of H_2O_2. m = mitochondria; n = nucleus; p = peroxisome; scale bars = 1 μm, unless otherwise indicated.
Figure 1. Section of cells after incubation with DAB and urate. The peroxisomes are intensely stained.

sections, the DAB reaction product could be observed within these structures (inset Fig. 4).

Staining of oxidases with $CeCl_3$

For the localization of oxidase activities in peroxisomes, rat liver cells were incubated with $CeCl_3$ and the substrates mentioned previously, and in the presence of aminotriazole to inhibit catalase activity. After incubation of glutaraldehyde-fixed tissue with $CeCl_3$ and urate, the peroxisomes were intensely stained (Fig. 9). Staining appeared to be restricted to the outer cell layers of the tissue block, probably because of the slow penetration rate of the cerous ions. Similar results were obtained with unfixed cells. In spite of a rather poor cell preservation, the peroxisomes were positively stained. In time-dependent incubations with $CeCl_3$ and urate, we observed positively-

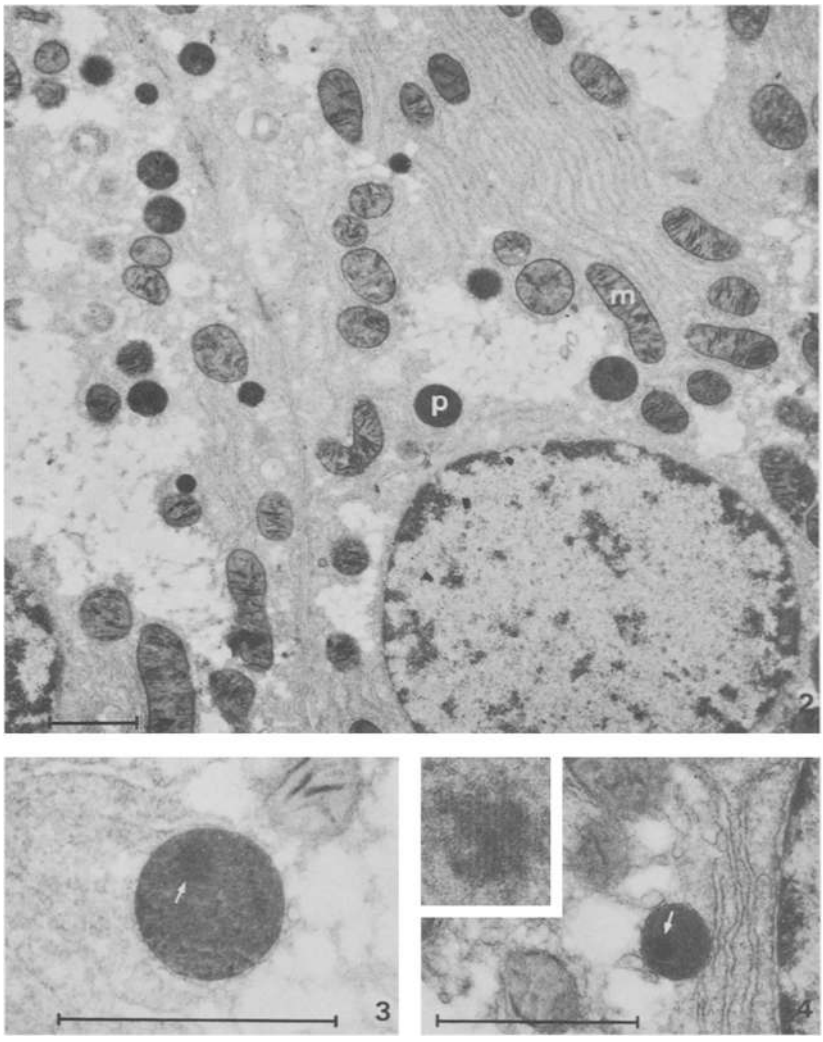
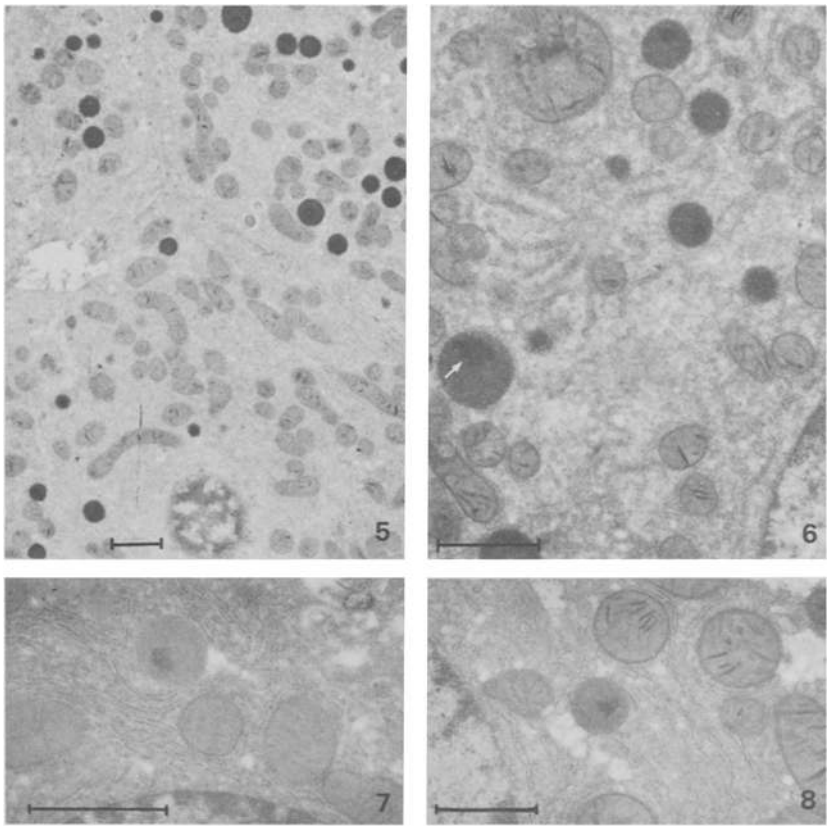


Figure 2. Section of cells after incubation with DAB and methanol, showing positively-stained peroxisomes.
Figures 3 & 4. Details of cells incubated with DAB and urate (Fig. 3) or DAB and methanol (Fig. 4) to demonstrate the difference in contrast between the nucleoids and the peroxisomal matrix. Inset: detail of a section through a peroxisome to demonstrate the DAB reaction product, present in the nucleoid after incubation with DAB and methanol.

stained nucleoides after 30 min incubation, while at the same time, the matrix showed little reaction product (Figs. 10 & 11). Prolonged incubations resulted in more intense staining of the matrix and the staining pattern was comparable to glutaraldehyde-fixed cells (Figs. 9 & 12). The visibility of the parallel dense lines of the nucleoids appeared to be enhanced after incubation with



Figures 5 & 6. Sections of cells after incubation with DAB and DL-alanine (Fig. 5) and DAB and DL-lactate (Fig. 6), showing positively-stained peroxisomes; arrow: nucleoid.

Figures 7 & 8. Sections of cells after routine fixation procedures with glutaraldehyde and osmium tetroxide–potassium dichromate (Fig. 7) or after incubation with DAB in the absence of any substrate (Fig. 8).

$CeCl_3$, which suggests that the reaction product is concentrated in the osmiophilic parts of the nucleoids (Figs. 11 & 12).

The staining pattern after incubation with $CeCl_3$ and methanol, DL-lactate or DL-alanine was similar to that observed after incubation with $CeCl_3$ and urate (Figs. 13 & 14). However, after the latter incubation the staining of the matrix was generally less intense than after incubation with urate. In addition, differences in staining density between individual peroxisomes in one cell were observed, which probably indicate differences in enzyme activities (Figs. 9 & 14). Apart from peroxisomal staining, small dense precipitates were occasionally found in the mitochondria and the ribosomes also showed enhanced contrast (Fig. 9). The staining of the mitochondria and ribosomes was apparently not due to activities of the oxidases under study, since after either substrate-free or anaerobic incubation with cerous ions, these structures were

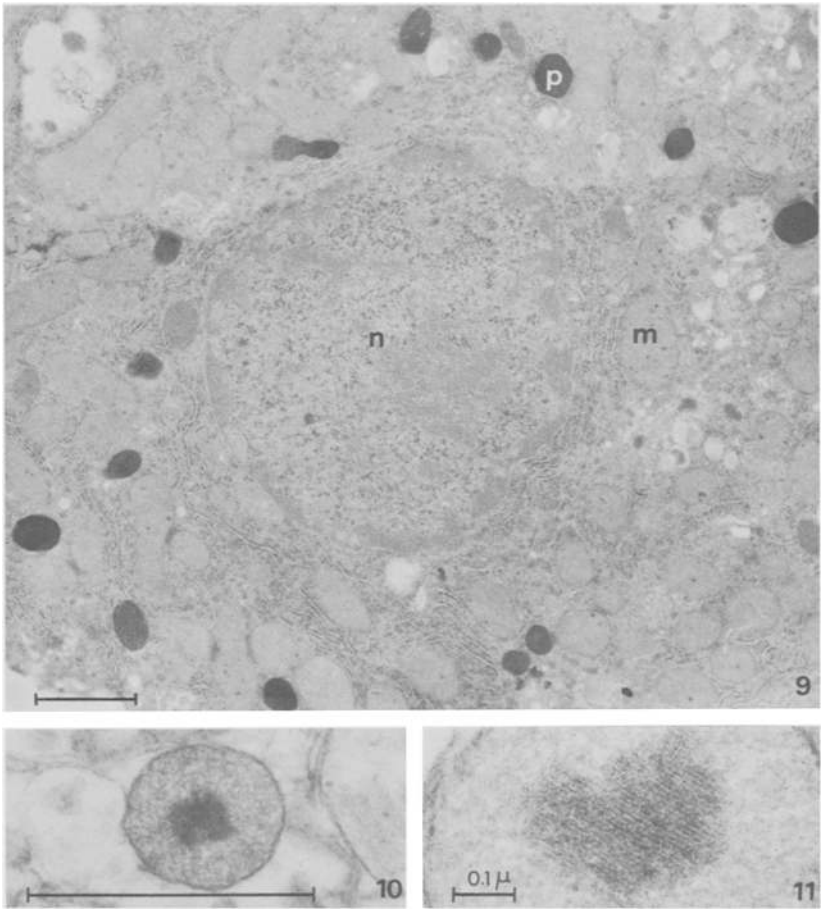


Figure 9. Section of cells incubated with $CeCl_3$ and urate showing intensely-stained peroxisomes.
Figures 10 & 11. Details of unfixed cells, incubated for 30 min with $CeCl_3$ and urate. The reaction product is mainly located in the nucleoids. Fig. 11, scale bar: 0.1 μm.

still equally well stained, while the reaction of the peroxisomes was extremely weak (Fig. 15).

Discussion

The presence of staining deposits in the peroxisomes observed after application of the procedures for catalase as well as for the oxidases implies that the enzymes investigated are located in the same organelle. In all experiments, catalase activity in the hepatocytes was confined to the peroxisomes, which is in accordance with the observations of Fahimi *et al.* (1976) on the same cell type. Indications of the presence of catalase activity in the cytoplasm, as reported for sheep, monkeys and guinea-pig liver by Roels (1976) and Roels *et*

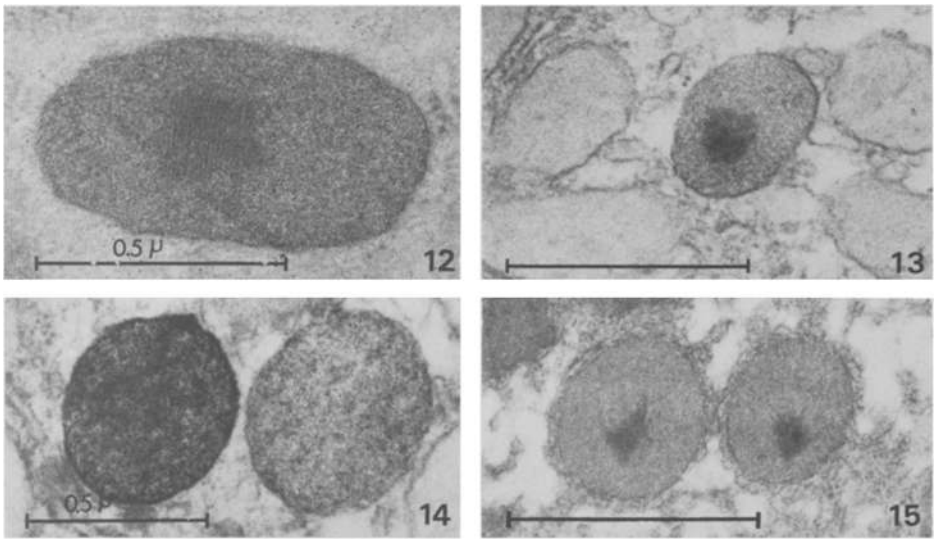


Figure 12. Detail of a glutaraldehyde-fixed cell, incubated with $CeCl_3$ and urate to demonstrate the pattern of stain in matrix and nucleoid. Scale bar: 0.5 μm.
Figure 13. Detail of a cell incubated with $CeCl_3$ and methanol. The reaction product is mainly located in the nucleoid.
Figure 14. Detail of a glutaraldehyde-fixed cell incubated with $CeCl_3$ and DL-alanine, showing differences in staining intensity of the matrix between two individual peroxisomes. Scale bar: 0.5 μm.
Figure 15. Detail of a cell, incubated with $CeCl_3$ in the absence of any substrate.

al. (1977), were not obtained. However, these authors used a different technique. The staining deposits we found on the mitochondrial membranes in the incubations with DAB were not dependent on the presence of substrate and may, therefore, be ascribed to the action of the cytochrome *c*–cytochrome oxidase complex (Roels, 1974). The distribution of the DAB-reaction product in the peroxisomes after incubation with DAB and an oxidase substrate indicates that catalase activity is present in both the matrix and the nucleoids. Hayashi *et al.* (1973, 1976) suggested that catalase is located exclusively in the peroxisomal matrix. Their suggestion was based on the high solubility of the enzyme. On the basis of our results, it seems likely that *in vivo* catalase is also present in the nucleoids, probably loosely bound or in a freely diffusable form. Nucleoid-associated catalase, often in crystalline form, is well known in plant microbodies (Frederick & Newcomb, 1969; Vigil, 1973; Frederick *et al.*, 1975). Although in mammals the enzyme usually occurs in a soluble or freely diffusable form, catalase-positive rodlike structures have been described in rat kidney peroxisomes (Barrett & Heidger, 1975) and in the peroxisomes of mouse salivary gland cells (Hanker *et al.*, 1977), while in mouse kidney and liver peroxisomes catalase activity has been localized on concentric lamellae in the nucleoids (Jones, 1975) and on multi-membranous structures present in the matrix (Masters & Holmes, 1977).

After the cerium procedure, performed on glutaraldehyde-fixed cells in the presence of urate, reaction products were observed in the peroxisomal matrix as well as in the nucleoid. The nucleoid invariably showed a higher electron density than the matrix. This staining pattern was even more pronounced after short incubations with unfixed cells. The observed difference in contrast between the nucleoid and the surrounding matrix greatly exceeded the difference in contrast found after incubation with cerium ions in the absence of substrate. Therefore, association of the oxidases with the nucleoids is likely. The possibility exists that the differences observed in staining intensity between nucleoid and matrix have been enhanced by leakage of enzymes out of the matrix into the cytoplasm. However, in unfixed as well as in glutaraldehyde-fixed cells we have never observed any sign of leakage of enzyme. All reaction products were confined to the peroxisomes. The possibility that in glutaraldehyde-fixed cells the staining of the matrix was due to diffusion of H_2O_2 generated by nucleoid-bound enzyme, or to diffusion of nucleoid-bound enzymes into the peroxisomal matrix, cannot be excluded but is unlikely because the results of incubation with $CeCl_3$ and urate were similar to those of incubation with DAB and urate. The DAB-reaction product is known not to be subject to diffusion (Essner, 1974).

The enzyme contents of nucleoids and matrix in rat liver cells have been extensively studied by biochemical techniques. Our finding of urate oxidase in the nucleoids is in agreement with conclusions based on fractionation experiments (Hayashi *et al.*, 1973, 1976; Lata *et al.*, 1977; Watanabe *et al.*, 1977) in which it was shown that this enzyme is largely confined to the highly insoluble fraction consisting of purified nucleoids. However, our cytochemical experiments indicate that this enzyme is present in the matrix as well as in the nucleoids.

Apart from urate oxidase activity, we have demonstrated activities of three other H_2O_2-producing oxidases in rat liver peroxisomes. The occurrence of two of these enzymes, amino acid oxidase and α-hydroxy acid oxidase, has been well documented for rat liver peroxisomes biochemically (Hayashi *et al.*, 1971, 1973; McGroarty *et al.*, 1974) and, as far as the latter enzyme is concerned, also cytochemically at the light microscope (Shnitka *et al.*, 1969) and electron microscope levels (Hand, 1974). Both enzymes have been assumed to occur in the peroxisomal matrix as they were easily solubilized from peroxisome preparations by freezing and thawing (Leighton *et al.*, 1969) but Hayashi *et al.* (1971, 1973), using more refined solubilization treatments, were able to show that they were at least partly loosely bound to the nucleoids. Our results support the latter finding but show that both enzymes occur in the matrix as well. Furthermore, we may conclude that the other enzyme studied, alcohol oxidase, has a similar distribution. Thus, the nucleoids in rat liver microbodies probably contain several enzymes, similar to the nucleoid inclusions in rat kidney microbodies, plant microbodies (Masters & Holmes, 1977) and the peroxisomes in methanol-grown yeast (Veenhuis *et al.*, 1976).

A H_2O_2-producing oxidase using methanol as substrate has not been

described so far in rat liver peroxisomes. It may represent an until now unknown hepatic pathway of alcohol breakdown. Methanol-oxidizing alcohol oxidase has been found before in the peroxisomes which develop in yeasts grown on methanol (Fukui *et al.*, 1975; Roggenkamp *et al.*, 1975). In cytochemical studies of the yeast *Hansenula polymorpha* the enzyme proved to be associated with the large crystalline cores typical for the peroxisomes of methanol-grown cells of this species (Veenhuis *et al.*, 1976, 1978).

The present results once more show that the cerium technique for the demonstration of H_2O_2 is a useful tool for the sub-microscopic localization of oxidase effectively. It seems to us the only reasonable and specific technical offering at present. The other direct methods in use, based on the capacity of some oxidases to reduce ferricyanide or tetrazolium salts (Boadle & Bloom, 1969; Shnitka & Talibi, 1971; Hand, 1974) are less specific since these compounds can be reduced by dehydrogenases and some oxidases (Hanker, 1975).

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