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Cytochemical Studies on the Localization of Methanol Oxidase and Other Oxidases in Peroxisomes of Methanol-Grown *Hansenula polymorpha*

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Abstract. The localization of methanol oxidase activity in cells of methanol-limited chemostat cultures of the yeast *Hansenula polymorpha* has been studied with different cytochemical staining techniques. The methods were based on enzymatic or chemical trapping of the hydrogen peroxide produced by the enzyme during aerobic incubations of whole cells in methanol-containing media. The results showed that methanol-dependent hydrogen peroxide production in either fixed or unfixed cells exclusively occurred in peroxisomes, which characteristically develop during growth of this yeast on methanol. Apart from methanol oxidase and catalase, the typical peroxisomal enzymes D-aminoacid oxidase and L-α-hydroxyacid oxidase were also found to be located in the peroxisomes. Urate oxidase was not detected in these organelles. Phase-contrast microscopy of living cells revealed the occurrence of peroxisomes which were cubic of form. This unusual shape was also observed in thin sections examined by electron microscopy. The contents of the peroxisomes showed, after various fixation procedures, a completely crystalline or striated substructure. It is suggested that this substructure might represent the in vivo organization structure of the peroxisomal enzymes.

Key words: Cytochemical localization – Peroxisome – Catalase – Methanol oxidase – D-Amino acid oxidase – α-Hydroxyacid oxidase – Methanol-assimilating yeast – *Hansenula polymorpha.*

The recent discovery of microbodies, which develop during growth of a variety of yeasts on methanol (van Dijken et al., 1975b; Sahm et al., 1975; Fukui et al., 1975b; Hazeu et al., 1975) has generated a considerable interest in the physiological function of these organelles. The observation that microbodies occur abundantly in yeasts during growth on methanol has led to the suggestion (van Dijken et al., 1975b) that these organelles may contain enzymes involved in methanol metabolism. Recent biochemical studies have supported this suggestion. Roggenkamp et al. (1975) and Fukui et al. (1975a) have isolated microbodies from methanol-grown yeasts and showed that these organelles contain methanol oxidase, the enzyme which initiates the oxidation of methanol in yeasts (Ogata et al., 1975), and catalase. On the basis of these results and analogous to the situation in animal and plant microbodies which typically contain both oxidases and catalase (De Duve and Baudhuin, 1966), it has been suggested that the microbodies of methanol-grown yeasts are peroxisomes (Roggenkamp et al., 1975; Fukui et al., 1975a; van Dijken et al., 1975c).

In the experiments of Roggenkamp et al. (1975) and Fukui et al. (1975a) considerable amounts of catalase and methanol oxidase appeared outside the microbodies in non-sedimentable fractions. The extra-peroxisomal occurrence of these enzymes may be due to the high fragility of the peroxisomes (Avers, 1971), causing leakage of the enzymes from these organelles during the different procedures involved in the isolation. Alternatively, the results may indicate an in vivo localization of both methanol oxidase and catalase in the cytoplasm as well as in peroxisomes. Indications that the former possibility is most likely were obtained in cytochemical staining experiments which demonstrated that catalase activity is only found in the peroxisomes of methanol-grown yeast (van Dijken et al., 1975c) and not in the cytoplasm.

This paper describes the results of a cytochemical study of the localization of methanol oxidase and a number of other peroxisomal oxidases in methanol-grown *Hansenula polymorpha.* The unusual ultrastructure of the peroxisomes in cells of this organism, when grown in a methanol-limited chemostat, is also discussed.

MATERIALS AND METHODS

Microorganism and Cultivation

Hansenula polymorpha de Morais et Maya CBS 4732 was used in all experiments. The organism was grown in methanol-limited chemostat cultures at 37° C and a dilution rate of 0.1 h^{-1} on a mineral medium described by van Dijken et al. (1976).

Fixation Techniques

Yeast cells were harvested from the chemostat and washed once with distilled water. Two methods of fixation were employed: 1) 1.5% $KMnO_4$ for 20 min at room temperature and 2) prefixation in 6% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2, containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ for 18 h at 0° C followed by washing for 60 min in the cacodylate buffer and postfixation in a 1 : 1 mixture of 2% OsO_4 in cacodylate buffer and 5% $K_2Cr_2O_7$ in demineralized water (adjusted to pH 7.2 with 1 M KOH) for 60 min at room temperature.

Cytochemical Staining Techniques

1. Staining of Catalase with 3,3'-Diaminobenzidine (DAB) and Hydrogen Peroxide. Cells were fixed in 6% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2 for 30 min at 0°C, washed 5 times with buffer and resuspended in 5 ml incubation mixture, containing: a) 0.06% H_2O_2 and 10 mg DAB in 0.1 M bicarbonate buffer pH 10.5 for 4 h at 37° C (van Dijken et al., 1975b) or b) 0.06% H_2O_2 and 10 mg DAB in 0.1 M Tris-HCl buffer pH 8.5 for 3.5 h at 37° C. Before incubation sample b) was immersed in 0.1% $CuSO_4$ in 0.09% NaCl and washed once with 0.1 M Tris-HCl buffer (Legg and Wood, 1970). Control samples were preincubated for 30 min with 50 mM 3-amino-1,2,4-triazole, followed by incubation with the same concentration of this inhibitor of catalase in the incubation medium. Experiments with oxidized DAB were performed according to the method of Hirai (1971). Fixed cells were washed and resuspended in 5 ml incubation mixture containing 10 mg oxidized DAB in 0.1 M Tris-HCl buffer pH 8.5 for 4 h at 37° C.

2. Staining of Catalase with DAB and Methanol. Cells were fixed in 6% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2 for 30 min at 0° C and washed five times with 0.1 M Tris-HCl buffer pH 8.5. Also *unfixed* cells, washed twice with 0.1 M Tris-HCl buffer pH 8.5 were used. After washing, the cells were resuspended in 5 ml incubation buffer, containing 10 mg DAB and 50 mM methanol in 0.1 M Tris-HCl buffer pH 8.5, for 2 h at 37° C. During incubation the suspensions were continuously aerated. In control experiments, either unfixed or glutaraldehyde-fixed cells were preincubated during 30 min in 50 mM aminotriazole in 0.1 M Tris-HCl buffer pH 8.5 and then transferred to the incubated mixture containing the same concentration of inhibitor. Anaerobic incubations were performed by bubbling oxygen-free nitrogen through the solutions during incubation. In addition to the incubations described above, double incubations were also performed. In these experiments both unfixed or glutaraldehyde-fixed cells were incubated with DAB/methanol as described above. The reaction was then stopped by placing the tubes on ice. The cells were washed twice with 0.1 M bicarbonate buffer pH 10.5 and incubated for 4 h in 5 ml incubation medium containing 0.06% H_2O_2 and 10 mg DAB in 0.1 M bicarbonate buffer pH 10.5 at 37° C.

3. Staining of Peroxisomes with DAB and Exogenous Catalase with Methanol as a Substrate. For the preparation of the catalase medium, the method of Venkatachalam and Fahimi (1969) was modified as follows: 2 ml of a suspension of beef liver catalase crystals (containing 20 mg/ml catalase) was centrifuged and the pellet fixed in 6% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2 for 30 min at 0 °C. The sediment was then washed with 0.1 M Tris-HCl pH 8.5, resuspended in 50 ml of the Tris-HCl buffer and sonicated for 30 min until it was completely dissolved. In order to facilitate catalase impregnation in the yeast cells, their permeability was increased by freezing and thawing. Cells were first sprayed in liquid nitrogen, the nitrogen was then poured off and the frozen cell clumps were immersed in cold 6% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2. After thawing, the procedure was repeated twice. Fixation was then continued for 20 min in fresh glutaraldehyde solution. After two washings in 0.1 M Tris-HCl buffer pH 8.5, the cells were resuspended in 50 mM aminotriazole in the Tris buffer for 60 min at 37° C, washed three times with this buffer and incubated in 5 ml of the above catalase-Tris medium for 15 min, followed by incubation in 5 ml of the catalase medium supplemented with 10 mg DAB and 50 mM methanol for 2.5 h at 37° C. Controls were performed by omitting either methanol or catalase from this incubation medium. The impregnation of exogenous catalase in the cells was followed by preincubation in the catalase-Tris medium and subsequent incubation in 5 ml medium containing 10 mg DAB and 0.06% H_2O_2.

4. Staining of Peroxisomal Oxidases with Cerium Chloride. The method described by Briggs et al. (1975) was modified as follows: either glutaraldehyde-fixed cells or unfixed cells were incubated in 0.1 M Tris-maleate buffer pH 7.5, containing 50 mM amino triazole and 5 mM $CeCl_3$ at 37° C for 30 min, followed by incubation in the same medium supplemented with 50 mM methanol, 50 mM D-alanine or 50 mM sodium glycollate, for 3 h at 37° C under continuous aeration. The incubation media were freshly prepared before use and the small amount of cerium hydroxyde precipitate that usually forms at pH 7.5 was removed by filtration. After incubation, the cells were resuspended in 0.1 M cacodylate buffer pH 6.0 for 30 min to remove any additional cerium hydroxyde precipitate formed during the incubation. Controls were performed by bubbling oxygen-free nitrogen through the solutions during the incubation.

The incubation media were renewed every hour in all the above experiments.

Postfixation after Cytochemical Staining

Cells were postfixed in 1.5% $KMnO_4$ for 30 min or in a 1 : 1 mixture of 2% OsO_4 and 5% $K_2Cr_2O_7$ for 60 min, washed twice with water and embedded in 1.5% agar. After dehydration in a graded alcohol series, the agar blocks were embedded in Spurr's (1969) epoxy resin or Epon 812. Ultrathin sections were cut with a diamond knife, partly stained with leadcitrate and uranylacetate (Kölbel, 1970) and examined in a Philips EM 300 electron microscope.

Chemicals

Beef liver catalase was obtained from Sigma Chemical Company, St. Louis, Miss., U.S.A.

RESULTS

Effect of Fixation Techniques on the Ultrastructure of Peroxisomes

Cells of *Hansenula polymorpha*, grown in a methanol-limited chemostat contain many peroxisomes, which are easily recognized in the phase-contrast microscope by their typical rectangular shape (Fig. 2). In young buds peroxisomes cannot be seen by phase-contrast microscopy, but they are observed in the UV-microscope as small round organelles.

The ultrastructure of methanol-grown *Hansenula polymorpha* has been described previously (van Dijken et al., 1975b). A typical electron micrograph of the organism after thin sectioning of $KMnO_4$-fixed cells is shown in Figure 1. The profiles of the peroxisomes fill the larger central part of the cell, the nucleus and the mitochondria are found in close proximity to the cell wall. In the bud the peroxisomes are much smaller, they always appear clustered and show a round or oval shape. After $KMnO_4$-fixation the peroxisomes show no internal substructure. However, prefixation in glutaraldehyde, followed by postfixation in a mixture of OsO_4 and $K_2Cr_2O_7$ resulted in a much better preservation of peroxisome substructure. Using this technique the peroxisomes, including the small peroxisomes in developing buds, always showed a very regular, crystalline matrix (Fig. 3). The periodicity of these structures varied between 92–105 Å which is in agreement with the observations of Hazeu et al. (1975) on peroxisomes of methanol-grown *Pichia pastoris*.

Cytochemical staining of glutaraldehyde-fixed cells with DAB, followed by postfixation with OsO_4 according to the original method of Graham and Karnovsky (1966), showed that the preservation of cell structure of *Hansenula polymorpha* is rather poor. Similar results have been reported for other ascomycetous yeasts (May, 1974; Osumi et al., 1975; van Dijken et al., 1975c). OsO_4-$K_2Cr_2O_7$ fixation improved the results but $KMnO_4$ fixation was finally adopted as a general postfixative after cytochemical staining of *Hansenula polymorpha* because of optimal ultrastructural resolution. In the course of experiments in which the results of both fixation procedures were compared after staining with DAB, it was noted that the distribution of the oxidation product of this compound and the amount formed was not affected by the method of post-fixation. This indicated that the final stain observed in thin sections represents the DAB oxidation product after reaction with $KMnO_4$.

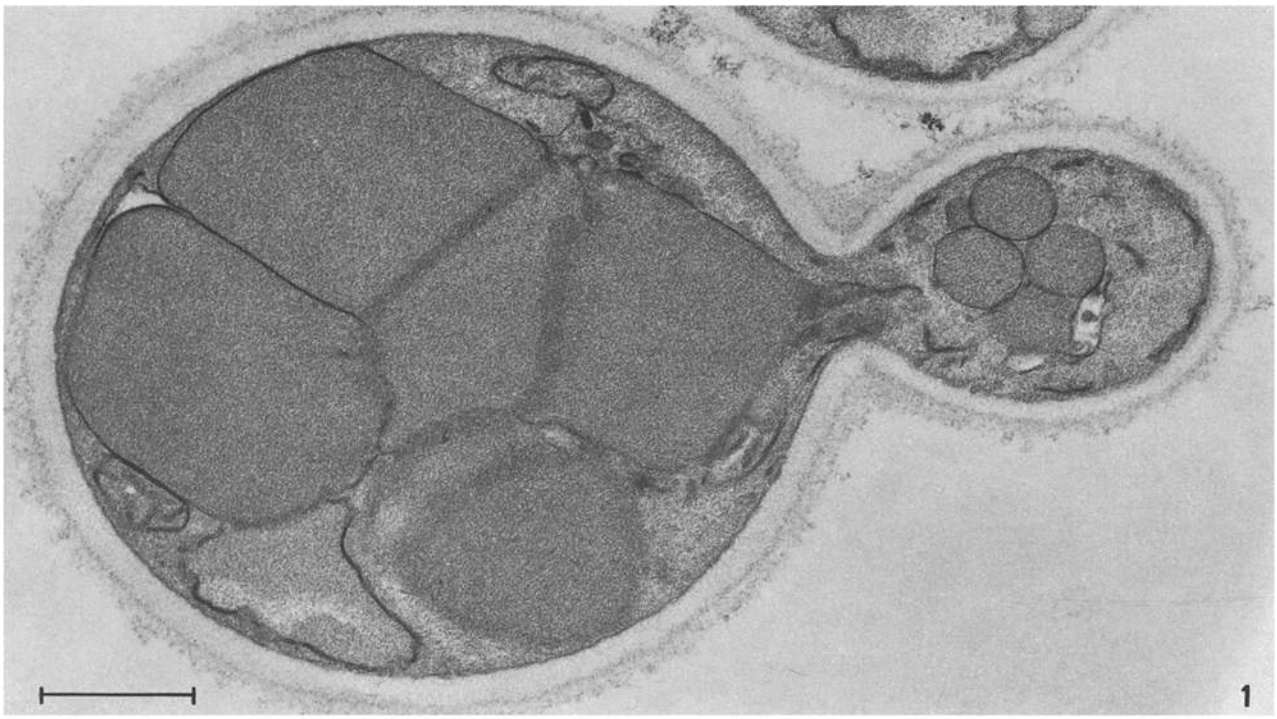
Staining of Catalase with DAB and Hydrogen Peroxide

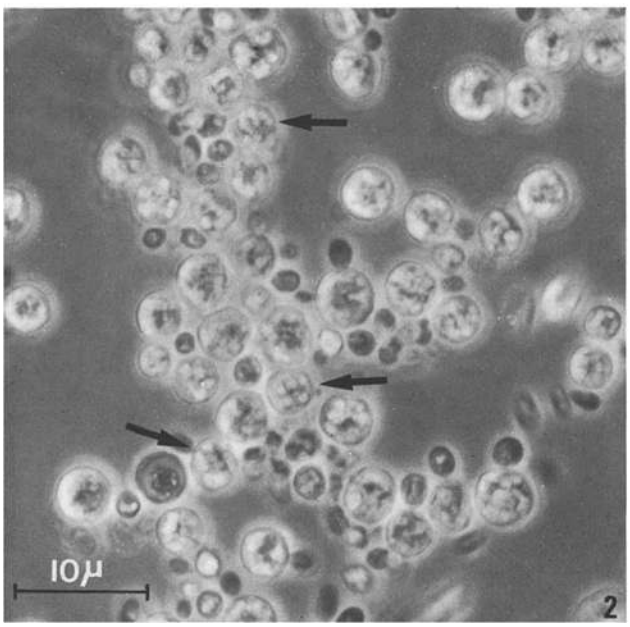
The cytochemical localization of catalase activity in methanol-grown cells of *Hansenula polymorpha* has been reported previously (van Dijken et al., 1975c). In these experiments glutaraldehyde-fixed cells were incubated at pH 10.5 with DAB and H_2O_2 and after post-fixation with $KMnO_4$ positively stained peroxisomes were observed without a visible intraperoxisomal substructure. The general preservation of the ultrastructure of the cell was rather poor in these experiments, which is probably due to the high pH and the long incubation time required during the incubations. We have now been able to obtain a positive result of the DAB reaction with H_2O_2 at lower pH values by including cupric ions as a catalyst in the reaction mixture (Legg and Wood, 1970). The ultrastructure of the cell after incubations at pH 8.5 is much better preserved (Fig. 7) and it is now possible to conclude that the pronounced accumulation of the stain at the periphery of the peroxisomes observed at pH 10.5 (van Dijken et al., 1975c) is exclusively confined to the peroxisomal membrane at the lower pH value. Incubations at pH 8.5 with oxidized DAB resulted in unstained peroxisomes, indicating that adsorption of oxidized DAB is not responsible for the staining (Fig. 5). In control experiments with aminotriazole, a known inhibitor of catalase activity, staining of the peroxisomes was not observed. However, in these experiments as well as in incubations with DAB and H_2O_2 or with oxidized DAB, accumulation of the stain in mitochondria was apparent. This may be due to staining of cytochrome *c* peroxidase, which is located in the mitochondria (Hoffman et al., 1970; Todd and Vigil, 1972; Erecińska et al., 1973). Incubations of unfixed cells with DAB and different concentrations of H_2O_2 always resulted in unstained peroxisomes. This is in agreement with the results of Roels et al. (1975) who showed that prefixation with glutaraldehyde is required to demonstrate peroxidative activity of catalase with DAB and H_2O_2.

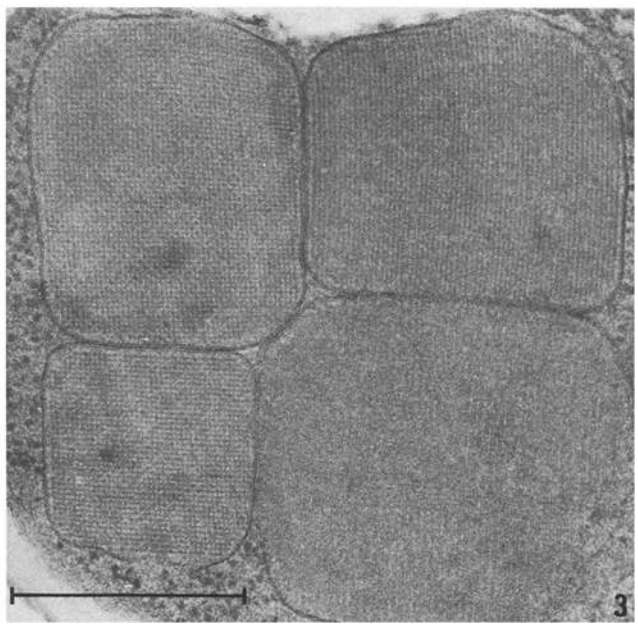
Staining of Catalase with DAB and Methanol

Incubations of *unfixed* cells with DAB and methanol in aerated suspensions at pH 8.5 resulted in the accumulation of a DAB reaction product in the peroxisomes of methanol-grown *Hansenula polymorpha* (Fig. 4). The peroxisomes were not always stained uniformly, especially those parts lying closest to the perifery of the cells sometimes were stained less intensively and occasionally not stained at all. The uniformly stained part of the peroxisome always showed a crystalline matrix. As in the incubations with DAB and H_2O_2, a pronounced accumulation of the stain on the peroxisomal membrane is found when methanol is used as a source of intracellular H_2O_2.

An unexpected result in these experiments was the finding that the peroxisomes in developing buds did not show an accumulation of a DAB-reaction product (Fig. 8). This indicates that methanol oxidase and/or catalase activity is absent in these peroxisomes. It is of interest to note that the peroxisomal membranes also remained unstained. When budding was complete, i.e. when the crosswall between mothercell and bud had been closed, a DAB reaction product was again found in the peroxisomes in the bud (Fig. 9a–c). As in the incubation with DAB/H_2O_2 (Fig. 7), the mitochondria were intensively stained in these experiments.







Abbreviations. M mitochondria; *N* nucleus; *P* peroxisome. The cells are fixed/postfixed with $KMnO_4$, unless otherwise mentioned. The marker represents 0.5 μ

Fig. 1. Section of budding cell of *Hansenula polymorpha* showing several peroxisomes in mothercell and bud

Fig. 2. Light micrograph of living cells: dependent on the plane of focus the rectangular shape of the peroxisomes can be observed (*arrows*)

Fig. 3. Detail of a glutaraldehyde-OsO_4/$K_2Cr_2O_7$ fixed cell showing peroxisomes with a crystalline matrix

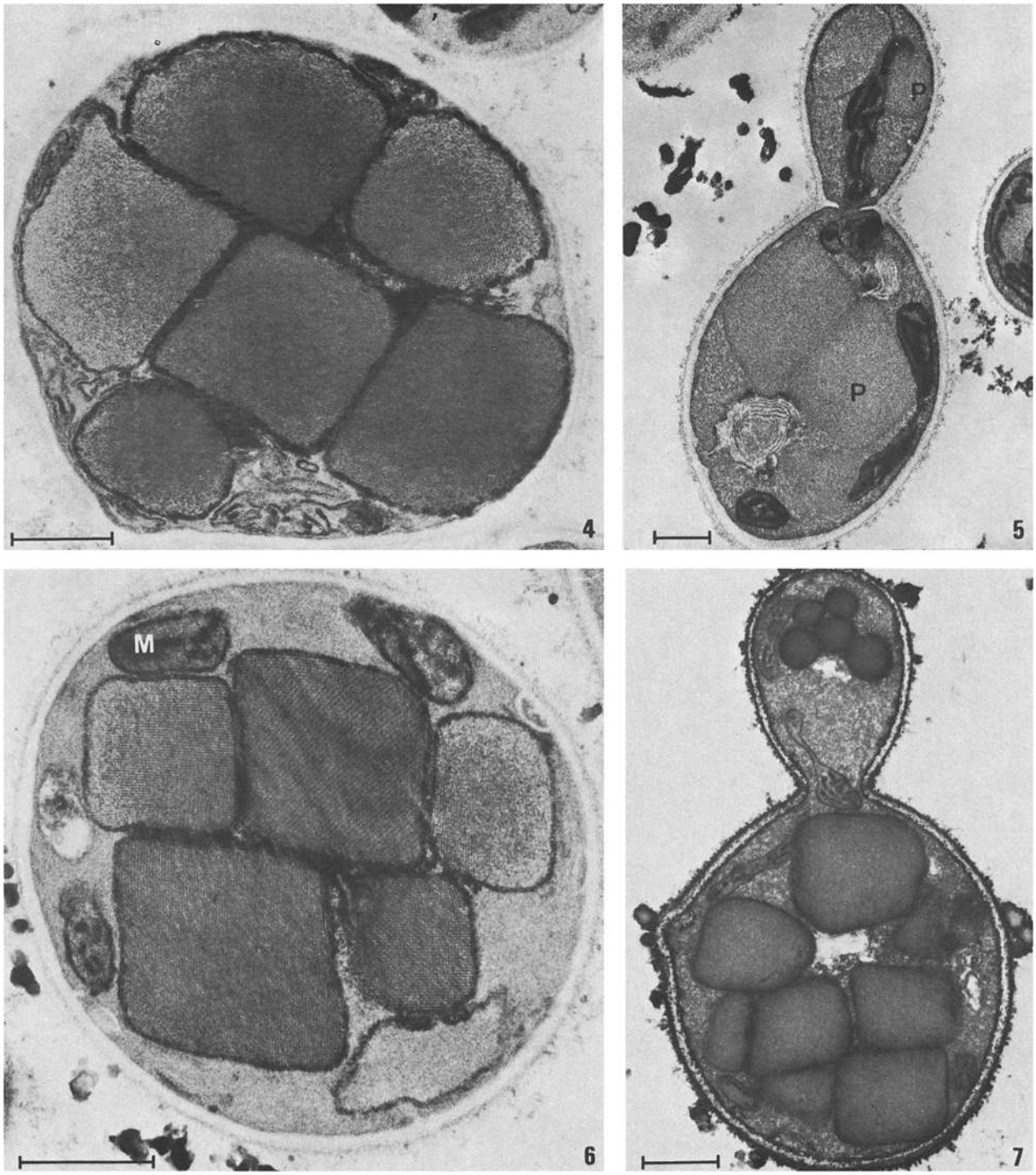
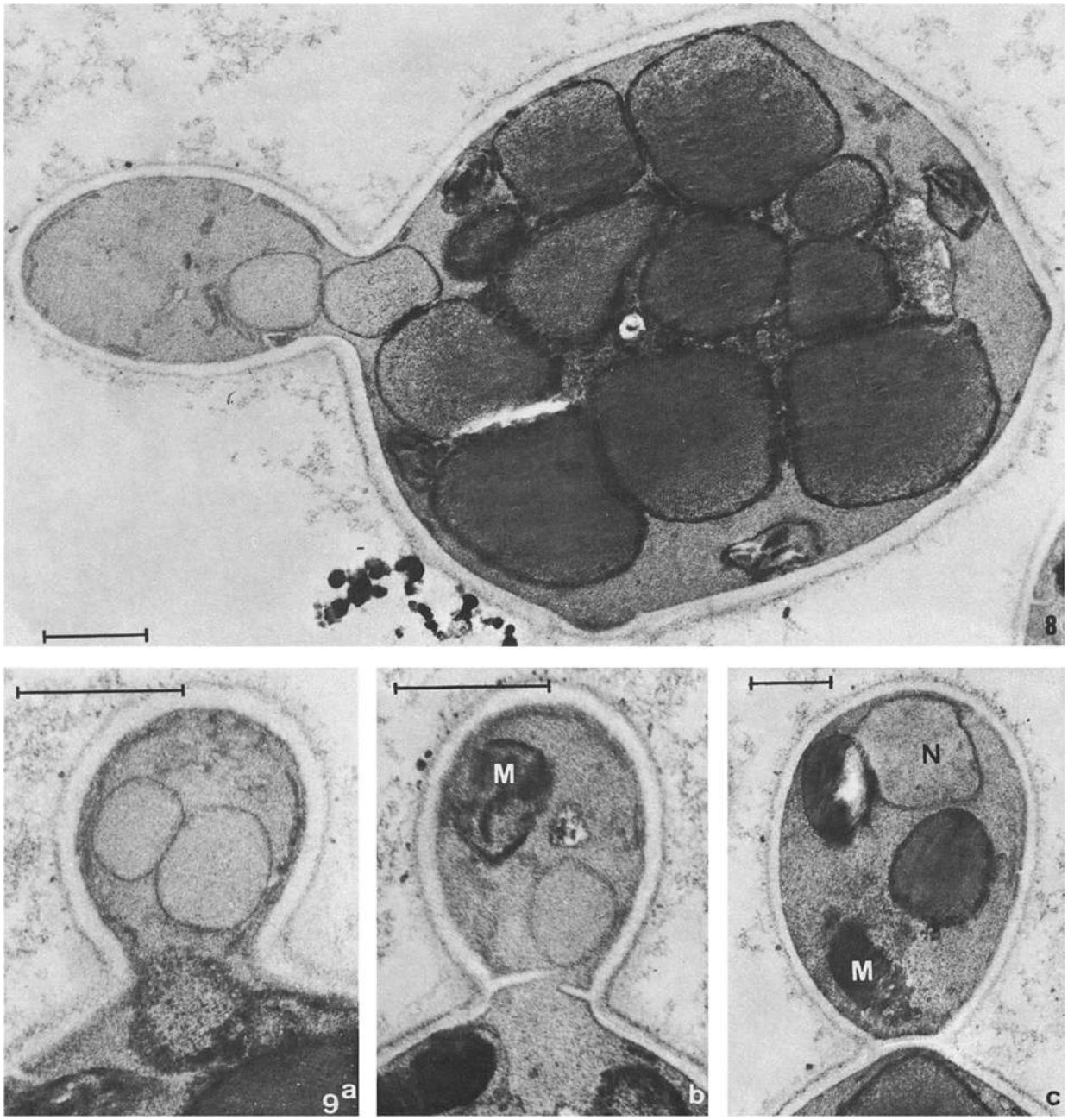


Fig. 4. Unfixed cell incubated with DAB and methanol showing stained peroxisomes. The unstained part of the organelles does not show a crystalline substructure

Fig. 5. Budding cell incubated with oxidized DAB; the mitochondria are heavily stained whereas the peroxisomes are not stained

Fig. 6. Glutaraldehyde-fixed cell incubated with DAB and methanol. Note that prefixation does not affect the result of a DAB/methanol incubation (compare with Fig. 4)

Fig. 7. Budding cell incubated with DAB and H_2O_2 at pH 8.5. The peroxisomes in mothercell and bud are stained and do not show a crystalline substructure



Figs. 8 and 9. Sections of unfixed cells incubated with DAB and methanol

Fig. 8. Budding cell with stained peroxisomes in the mothercell. The peroxisome in the bud is not stained whereas only a small deposit of reaction product can be observed in the peroxisome present in the neck between mothercell and bud. The cutting artefacts in the mothercell are illustrative for the hardness of the peroxisomes stained with this procedure

Fig. 9a–c. Different stages of the development of the bud. Peroxisomes, unstained when budding is not complete (a and b) are positively stained when the cross wall has closed (c)

Also positively stained endoplasmatic reticulum (ER) and nuclear membranes were frequently found.

Prefixation with glutaraldehyde showed a better general preservation of cell ultrastructure, but had no influence on the results of the DAB/methanol incubations (Fig. 6). The only difference observed was that in the latter case both ER and nuclear membranes remained unstained. Therefore, peroxidases and not catalase may be responsible for the staining of these membranes after incubations with unfixed cells (Roels et al., 1975). Control experiments with triazole showed unstained peroxisomes and positively stained mitochondria. Anaerobic incubations also resulted in unstained peroxisomes while 5–10% of the cells had positively stained mitochondria.

In order to determine whether loss of stainability of part of the peroxisomes as described above (Figs. 4, 6, 8, and 9) is due to leakage of enzymes involved in the formation of the stain, the different media and fixatives used were examined for catalase- and methanol oxidase activity. These experiments indicated that no leakage of these enzymes from the cells had occurred.

Double incubations on glutaraldehyde fixed cells, i.e. a DAB/methanol incubation followed by a DAB/H_2O_2 incubation, showed that the unstained parts of the peroxisomes after DAB/methanol incubation, including the unstained peroxisomes in the developing bud, became stained during a subsequent DAB/H_2O_2 incubation (Figs. 13 and 14). Controls, i.e. the same procedure applied to *unfixed* cells showed no additional staining of the peroxisomes after the DAB/H_2O_2 incubation. These results clearly indicate that catalase is present in the originally unstained parts. Therefore, the results of the DAB/methanol incubations, where methanol oxidase is supposedly responsible for the generation of H_2O_2, suggest that a DAB-oxidation product is only formed at sites where methanol oxidase activity is present. This would indicate that the DAB/methanol technique can be regarded as a method for the cytochemical localization of methanol oxidase. In order to investigate this in more detail, experiments were performed on the localization of methanol oxidase activity by use of exogenous catalase and of $CeCl_3$ as reagents for the cytochemical detection of H_2O_2 formation.

Staining of Peroxisomes with DAB and Exogenous Catalase with Methanol as a Substrate

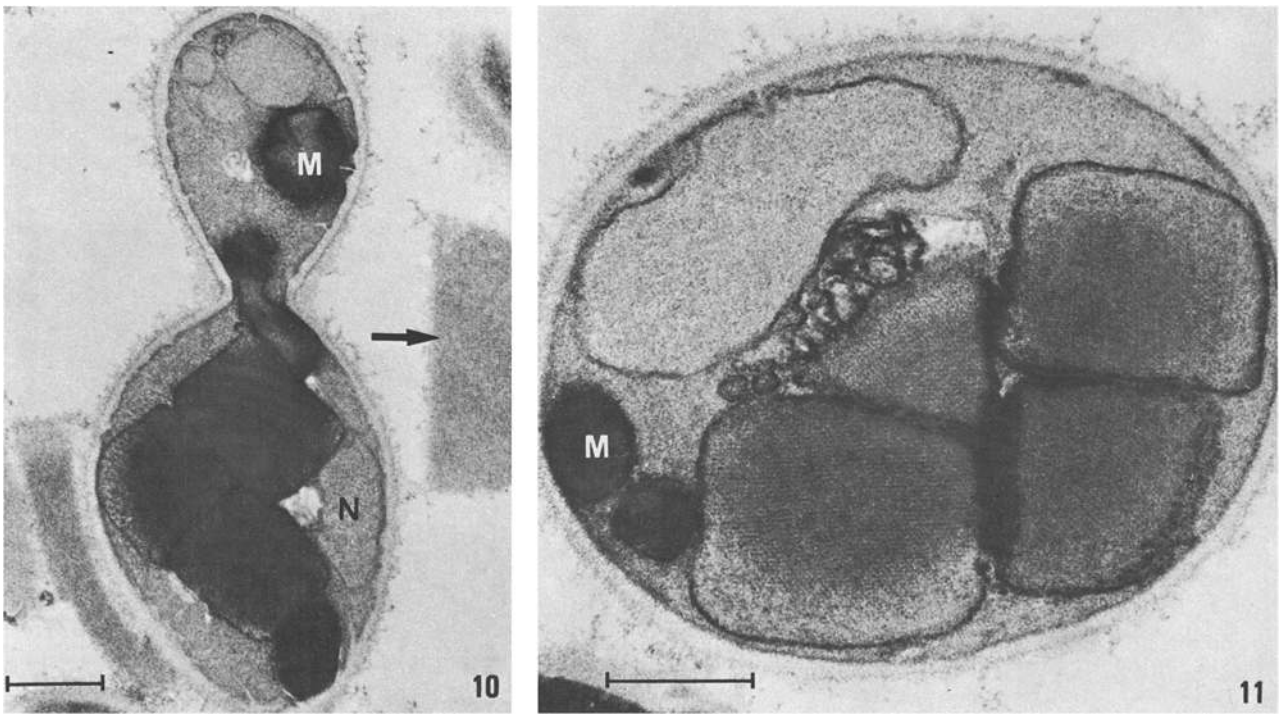
The impregnation of exogenous catalase in the cells was facilitated by spraying cell suspensions in liquid nitrogen followed by thawing in glutaraldehyde solution. This latter procedure was adopted because biochemical experiments showed that thawing in buffer resulted in the leakage of both methanol oxidase and catalase from the cells while, when the cells were thawed in glutaraldehyde, only a minimal leakage of catalase and no leakage of methanol oxidase was detected. Thus, we only used prefixed cells in the experiments discussed below. Incubation of cells subjected to the above freezing and thawing procedure in the catalase-DAB/methanol medium, under conditions where aminotriazole inhibited endogenous catalase, revealed in more than 90% of the cells a DAB-reaction product in the peroxisomes (Fig. 10). The distribution of this reaction product was comparable to that obtained after DAB/methanol incubations where endogenous catalase was responsible for the peroxidative oxidation of DAB (Fig. 6). In these experiments, the stained part of the peroxisomes showed, although somewhat masked, a crystalline substructure after $KMnO_4$-postfixation; the unstained part showed no substructure (Fig. 11). Furthermore, after these incubations the peroxisomal membrane also showed pronounced accumulation of stain. In addition the mitochondria were positively stained.

Although biochemical experiments showed no leakage of methanol oxidase into the medium during the above procedure, it became clear from electronmicroscopic observations that in several cells intracellular leakage of enzyme had occurred and in these cases the DAB-oxidation product was found in the cytoplasma and vacuoles. Cells, preincubated with the catalase-medium and postincubated with DAB/H_2O_2 showed positive stain in all parts of the cell. Since in these experiments endogenous catalase was inhibited with aminotriazole, these results indicate that exogenous catalase really had penetrated inside the cell organelles. In order to exclude the possibility that in the incubation with exogenous catalase the staining of the peroxisomes is due to an adsorption of oxidized DAB to this exogenous catalase, glutaraldehyde-fixed catalase crystals were mixed with the incubation medium. These crystals remained unstained (Fig. 10).

Control experiments in which methanol was omitted from the incubation medium, showed unstained peroxisomes and positively stained mitochondria. When catalase was omitted, part of the cells showed a slight accumulation of the DAB-reaction product in the peroxisomes despite the preincubation with aminotriazole (Fig. 12). However, the intensity of the stain in the peroxisomes was never as high as when catalase was present in the incubation media.

Staining of Peroxisomal Oxidases with Ceriumchloride

Since oxidation of methanol by methanol oxidase leads to the formation of H_2O_2, the site of H_2O_2-production



Figs. 10 and 11. Cells incubated with exogenous catalase, DAB and methanol

Fig. 10. Budding cell with stained peroxisomes in the mothercell. The peroxisomes in the bud are not stained. The catalase crystal (*arrow*) is not stained

Fig. 11. Magnification showing the crystalline substructure of the stained part of the peroxisomes

may also be located by means of $CeCl_3$. This compound reacts with H_2O_2 to form cerium perhydroxide. The method was originally described in combination with OsO_4 as a post-fixative (Briggs et al., 1975), but our results indicated that also $KMnO_4$ can successfully be used for postfixation.

Glutaraldehyde-fixed cells incubated with $CeCl_3$/methanol in the presence of aminotriazole, show a reaction product in the peroxisomes, revealing a crystalline substructure after $KMnO_4$-postfixation (Fig. 15). The cerium technique was also used to investigate the cytochemical localization of other peroxisomal enzymes, namely D-amino acid oxidase and L-α-hydroxyacid oxidase. When glutaraldehyde-fixed cells were incubated in a medium, containing D-alanine instead of methanol, a reaction product was also observed in the peroxisomes (Fig. 16). Similar results were obtained when glycollate was used as a substrate (Fig. 17). In both experiments a crystalline structure was observed in the peroxisomes. However, when Na-urate or uric acid was used as a substrate in order to demonstrate urate oxidase activity, no reaction product was observed in the peroxisomes. After incubations with $CeCl_3$ a reaction product was also found in the cristae of the mitochondria (Figs. 15–17). However, this was also observed when the oxidase-specific substrate was omitted from the incubation medium. Anaerobic incubations with $CeCl_3$, in which the formation of H_2O_2 is prevented, resulted in complete lack of reaction product in the peroxisomes with either methanol, D-alanine or glycollate.

In order to obtain optimal information with respect to the localization of reaction product, it appeared to be essential to determine the incubation time carefully since accumulation of reaction product easily causes overstaining and masking of cell structures. Furthermore, it was found to be essential to preincubate the cells with $CeCl_3$ because of the low penetration rate of the cerium ions.

A general remark concerning incubations where methanol is used as a substrate for the generation of H_2O_2 has to be made. In our experiments with unfixed cells we observed a decrease of the pH of the incubation medium from 8.5–7.9 which occurred within 30 min. Similar incubations started at pH 7.5 showed a decrease to pH 6.5 in the same time interval. Since methanol oxidase activity is pH-dependent with an optimum at pH 8.5, it is essential for optimal results

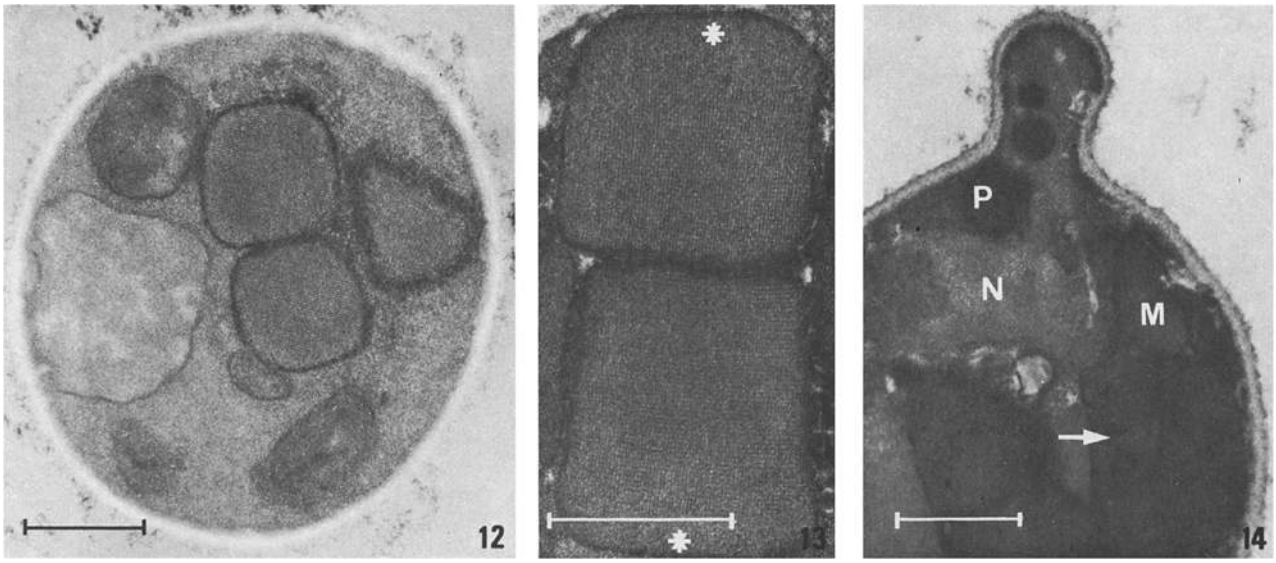


Fig. 12. Cell from a control experiment in which catalase was omitted from the incubation medium. Note that the peroxisomes are only weakly stained as compared to Figure 10

Figs. 13 and 14. Sections of glutaraldehyde fixed cells incubated with DAB and methanol and subsequently stained for catalase (DAB/H_2O_2 incubation)

Fig. 13. Detail showing 2 peroxisomes. The original unstained part of the peroxisomes after DAB/methanol incubation (*) has subsequently stained with DAB and H_2O_2 (compare with Fig. 6)

Fig. 14. Detail of a budding cell showing positively stained peroxisomes in the bud without internal substructure. The peroxisomes in the mothercell show a regular substructure (*arrow*)

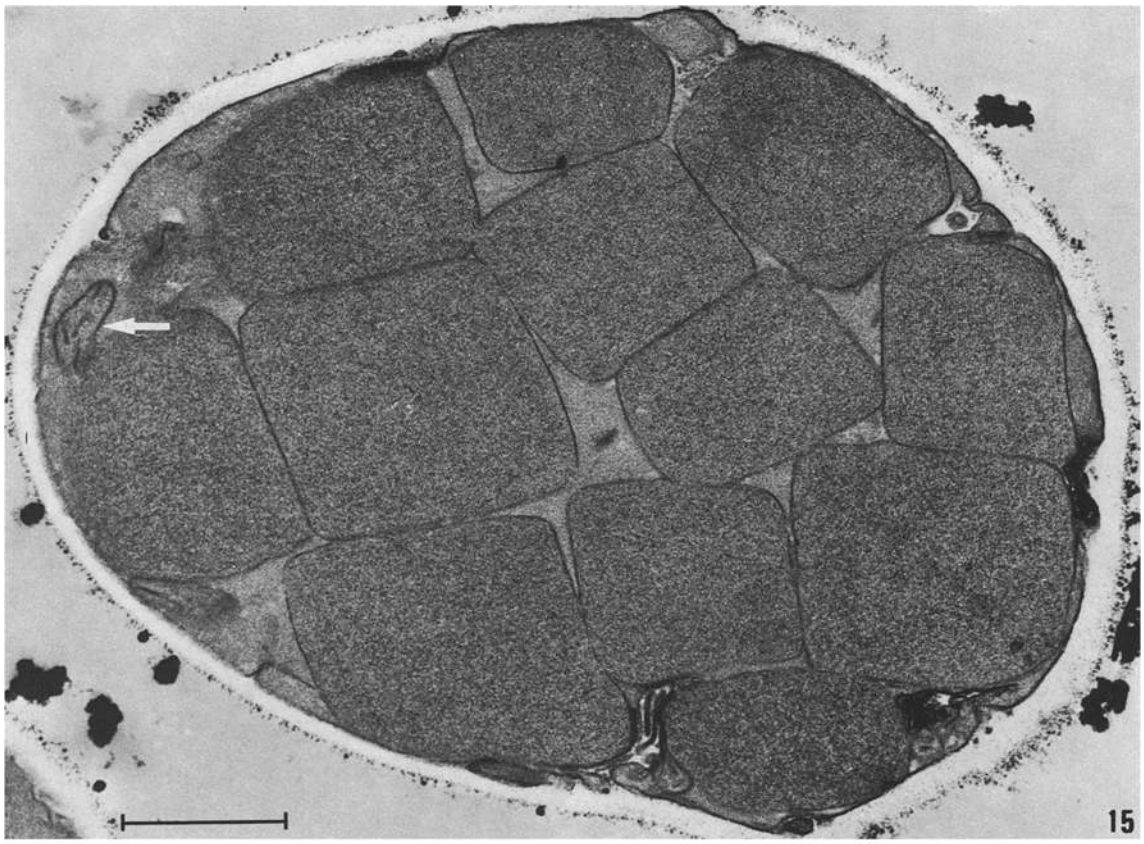
that during incubations in methanol-containing media, the pH is checked at regular intervals. The above decrease in pH during these incubations is likely to be due to the incomplete oxidation of methanol to formic acid, which was excreted in the medium.

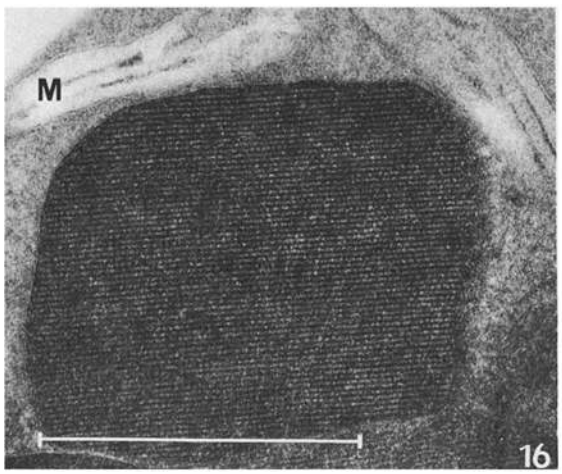
DISCUSSION

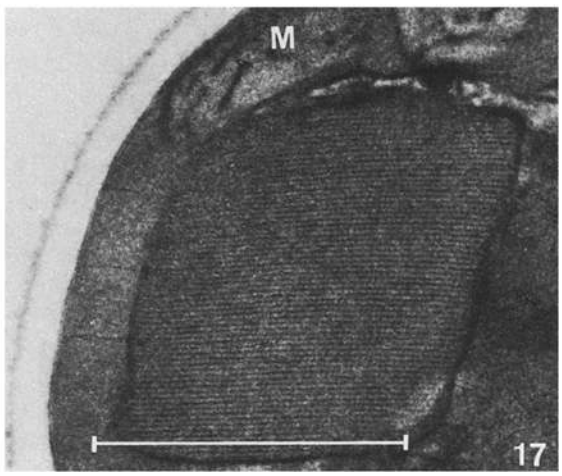
In previous papers we have shown that the yeast *Hansenula polymorpha*, when grown in continuous culture under methanol limitation, contained many microbodies which may be regarded as peroxisomes (van Dijken et al., 1975b, c). Crystalline structures were observed in thin sections of these peroxisomes and also in freeze-etched preparations; however, it remained unclear to which extent the observed crystallinity was induced by the preparation techniques used.

The crystalline matrix of the peroxisomes is not observed after $KMnO_4$-fixation (Fig. 1). However, in cytochemical staining experiments with methanol-containing aerated media on unfixed or glutaraldehyde-fixed cells and after postfixation with $KMnO_4$, a crystalline structure is present in the peroxisomes (Figs. 4 and 6). This cannot be due to a stabilization of peroxisomal substructure by formaldehyde formed from methanol by methanol oxidase during the incubation, since after incubation with $CeCl_3$/D-alanine, where no formaldehyde is generated, a crystalline structure is also observed. It is suggested that in the cytochemical staining procedures, described above, the reaction product is deposited at the edge of every single crystal unit, thus building a regular network, left intact after $KMnO_4$ fixation. The crystallinity of the peroxisomes was also nicely observed after glutaraldehyde-OsO_4-$K_2Cr_2O_7$ fixation and is furthermore indicated by their peculiar form; in freeze-etch preparations they appear almost cubic of form (van Dijken et al., 1975b) and their rectangular shape is also clearly visible by phase-contrast microscopy (Fig. 2). It is therefore tempting to suggest that the crystallinity of the peroxisomes represents an in vivo organizational structure; the absence of this structure after $KMnO_4$ fixation alone may be explained to result from the destruction of the protein crystal by the fixative.

Completely crystalline peroxisomes have also been observed in *Pichia pastoris* after formaldehyde-OsO_4 fixation (Hazeu et al., 1975). In methanol-grown *Can-*







Figs. 15–17. Sections of glutaraldehyde-fixed cells incubated with $CeCl_3$ and different substrates for the endogenous generation of H_2O_2 by the peroxisomal oxidases

Fig. 15. Cell incubated with $CeCl_3$ and methanol revealing methanol oxidase activity in the peroxisomes. The reaction product is present in all organelles. Note the crystalline substructure. The mitochondria are also stained (*arrow*)

Fig. 16. Stained peroxisome after incubation with $CeCl_3$ and D-alanine

Fig. 17. Stained peroxisome after incubation with $CeCl_3$ and glycollate

dida boidinii the peroxisomes contained crystalline inclusions which are thought to consist of methanol oxidase (Sahm et al., 1975). Fukui et al. (1975a), however, postulated on the basis of their results from cytochemical staining of isolated peroxisomes from a *Kloeckera* species, that the crystals in these organelles represent highly ordered catalase. Our results of the cytochemical staining experiments on whole cells of *Hansenula polymorpha* suggest that the different conclusions drawn by Sahm et al., and Fukui et al. with respect to the identity of the peroxisomal crystals may be compatible. We postulate that the crystalline structure in the peroxisomes of methanol-grown *Hansenula polymorpha* (and probably other methanol-assimilating yeasts) represents a highly ordered organization of typical peroxisomal enzymes, namely catalase and several oxidases. This hypothesis is further substantiated below.

Thin sections of *Hansenula polymorpha*, after cytochemical staining with DAB/H_2O_2, show that catalase is present throughout the peroxisomes (Fig. 7). Although in these experiments no crystalline structure was observed, experiments on protoplasts, using OsO_4 as a postfixative, revealed uniformly stained peroxisomes with a completely crystalline matrix (van Dijken et al., 1975c). It is therefore concluded that catalase is distributed throughout the peroxisomes of methanol-grown *Hansenula polymorpha*. It is of interest to note that the DAB-reaction product is insoluble and therefore not subject to diffusion (Essner, 1974). However, it is well-known that peroxisomes are very fragile and catalase itself may leak from peroxisomes when these are maltreated or not adequately fixed. The results of Fukui et al. (1975a) who found that the DAB-reaction product after incubation with H_2O_2 was exclusively located in the crystalline inclusions of isolated peroxisomes from a *Kloeckera* sp. may be explained by a leakage of catalase from these organelles during the isolation procedure. Our experiments on whole cells did not show any leakage of catalase from the peroxisomes. Redistribution of catalase within the peroxisomes before incubation, however, may not be excluded; experiments on cells subjected to freezing and thawing showed that catalase leaks more easily from the peroxisomes than does methanol oxidase; this result is supported by the work of Sahm et al. (1975) on sphaeroplast lysates and X-press extracts of methanol-grown *Candida boidinii*. The greater mobility of catalase as compared to the peroxisomal oxidases, resulting in a redistribution of catalase may be the explanation for the absence of any crystalline structure after prolonged DAB/H_2O_2 incubations and postfixation with $KMnO_4$.

It has been shown that cytochemical staining of peroxisomal catalase is also possible when methanol is used as an intracellular source of H_2O_2 for the DAB-oxidation (van Dijken et al., 1975c). Incubations of aerated cell suspensions of glutaraldehyde-fixed cells with methanol and DAB and postfixed with $KMnO_4$, show intensive staining of the peroxisomes. Accumulation of the stain does not occur during anaerobic conditions. It can therefore be concluded that methanol oxidase is responsible for the generation of H_2O_2, required for the DAB-reaction with catalase. It is of interest to note that the above conditions is thought to simulate the "in vivo" situation where methanol oxidase generates the H_2O_2 which can be used by catalase for both catalatic and peroxidative reactions. It may be argued, however, that the conditions in the experiment described above are artificial since the peroxidative activity of catalase is stimulated as a result of glutaraldehyde prefixation (Herzog and Fahimi, 1974a, b; Roels et al., 1975). The finding that staining of catalase in media containing DAB and methanol also occurs with unfixed cells, excludes this possibility and clearly disproves the hitherto postulated requirement of glutaraldehyde prefixation of catalase for a peroxidative reaction with DAB. However, the above reaction was only possible when the in vivo situation was simulated (i.e. generation of H_2O_2 by methanol oxidase). When unfixed cells were incubated with DAB and H_2O_2, the results were invariably negative over the pH range 8.5–10.5. These observations are therefore consistent with biochemical results which have shown that native catalase is only active in a peroxidative way when H_2O_2 is generated at a low rate by an oxidase (Keilin and Hartree, 1955; Oshino et al., 1973; van Dijken et al., 1975a).

The cytochemical experiments with DAB and methanol showed that methanol-dependent staining exclusively occurred in the peroxisomes. The lack of reaction product at other cellular sites can be explained by the absence of either methanol oxidase or catalase. In order to test the latter possibility the catalase present in the peroxisomes was inactivated with aminotriazole and the cells were subsequently impregnated with a sonicated catalase-suspension, followed by incubation with DAB and methanol. This experiment, in which a methanol-dependent DAB-reaction product can only be expected to occur at the sites of methanol oxidase activity, resulted in an identical distribution of electron dense product as compared to DAB/methanol incubations alone. On the basis of these experiments it can be concluded that incubation of whole cells with DAB and methanol results in the staining of peroxisomes primarily as a result of the localization of methanol oxidase in these organelles, which generates the H_2O_2 required for the staining of catalase.

The absence of methanol oxidase-and catalase activity outside the peroxisomes during cytochemical

staining does not exclude the possibility that these enzymes are present in concentrations too small to be detected by the techniques used. In biochemical fractionation studies it has been found that considerable amounts of methanol oxidase (up to 35%) and catalase (up to 60%) are non-sedimentable (Fukui et al., 1975a; Sahm et al., 1975). This would indicate a concentration of these enzymes in the cytoplasm which is in the same order of magnitude as their concentration in the peroxisomes, since the cytosol volume (minus nucleus, mitochondria and peroxisomes) is probably not much larger than the total peroxisomal volume. It is therefore tentative to conclude that our procedures should have revealed such high concentrations of soluble enzyme. Consequently it seems that at least the major part of the non sedimentable methanol oxidase and catalase in the experiments of Fukui et al. and Sahm et al. has its origin in leakage from peroxisomes.

The localization of methanol oxidase in the peroxisomes of methanol-grown *Hansenula polymorpha* was further confirmed by a more direct technique: using glutaraldehyde-fixed cells a very intensive accumulation of electron-dense material occurred in the peroxisomes after incubation with methanol and $CeCl_3$. In these experiments the catalase present in the peroxisomes was inhibited with aminotriazole allowing the $CeCl_3$ to trap the H_2O_2 generated by the oxidase. Preliminary experiments on animal tissue have shown that the cerium technique may be generally applicable for the cytochemical demonstration of oxidases (Veenhuis and Wendelaar Bonga, in preparation). Experiments on whole cells of *Hansenula polymorpha* showed that besides methanol oxidase, also the typical peroxisomal oxidases D-amino acid oxidase (using D-alanine as a substrate) and L-α-hydroxyacid oxidase (using glycollate as a substrate) were stained in the peroxisomes by the $CeCl_3$ technique (Figs. 16 and 17).

Cytochemical experiments with DAB and methanol on cells grown in continuous culture under methanol limitation showed that the peroxisomes were not always uniformly stained. Moreover, it was found that the small peroxisomes in the bud of a budding cell remained unstained until the crosswall between mothercell and bud had closed (Figs. 8 and 9a–c). These results can only be explained on the basis of the absence or inactivity of methanol oxidase in these young organelles. Cytochemical experiments with DAB/H_2O_2 clearly showed that catalase is present (Fig. 7). Furthermore, when incubations with DAB and methanol were continued with DAB/H_2O_2, the originally unstained parts after DAB/methanol treatment became stained (Figs. 13 and 14). It must therefore be concluded that in young peroxisomes methanol oxidase and not catalase is absent or inactive. This conclusion is supported by the observation that peroxisomes in young buds were also unstained after incubation with $CeCl_3$/methanol.

Unfortunately, our present results do not allow a discrimination between the possibility that methanol oxidase is either absent or inactive. However, it seems likely that the absence of staining at the perifery of mature peroxisomes and in young peroxisomes is due to the presence of inactive methanol oxidase rather than to the absence of the enzyme, since it was frequently observed that young peroxisomes in the mother cell were initially positive after division, but were always unstained after moving inside the bud until bud formation had been completed (Fig. 9c). Furthermore, $OsO_4/K_2Cr_2O_7$ fixation always revealed a uniform crystalline matrix of the whole peroxisome, both in mature cells and in the growing bud. When it is accepted that peroxisomes in methanol-limited cells of *Hansenula polymorpha* have a regular (crystalline) structure, it follows that the absence of methanol oxidase in parts of mature peroxisomes and in young peroxisomes would have resulted in a change of the crystalline structure, which is, as argued above, the result of an ordered organization of the peroxisomal enzymes. It remains unclear, however, in which way a variety of typical peroxisomal enzymes are ordered inside the peroxisome to form a highly regular crystalline matrix.

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