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A cytophotometric and electron-microscopical study on catalase activity in serial cryostat sections of rat liver

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Summary

The validity of the histochemical procedure for demonstrating catalase activity in cryostat sections of rat liver at the light- and electron-microscopical level was studied cytophotometrically. Incubations in the presence of 5 mM diaminobenzidine, 44 mM hydrogen peroxide and 2% polyvinyl alcohol performed on fixed cryostat sections resulted in the highest amounts of final reaction product precipitated in a fine granular form which was specific for catalase activity. Serial sections processed for electron microscopy indicated that the osmiophilic final reaction product was exclusively localized in the matrix and core of peroxisomes. The relationship between incubation time and the amounts of final reaction product generated by catalase activity as measured at 460 nm in mid-zonal areas of liver lobules showed non-linearity for the test-minus-control reaction because first-order inactivation of the enzyme occurred during incubation. Linearity of the test-minus-control reaction and section thickness was observed up to 8 μm. Catalase in rat liver showed a K_m value of 2.0 mM for its substrate hydrogen peroxide when the diaminobenzidine concentration was 5 mM. It is concluded that the procedure for demonstrating catalase activity in serial cryostat sections of rat liver at the light- and electron-microscopical level is specific and can be applied to quantitative purposes. This approach may be useful in pathology, when only small biopsies are available, when the tissue is heterogeneous, and when other histochemical markers also need to be studied in the same material.

Introduction

Catalase (E.C. 1.11.1.6) is one of the main enzymes playing a role in the catabolism of hydrogen peroxide (Chance *et al.*, 1979) and has been found in all aerobic microorganisms, in plants and animals. Catalase activity varies considerably in the different mammalian tissues, for example it is high in liver and kidney, but low in connective tissue. Moreover, catalase activity shows heterogeneous distribution patterns in tissues (Just *et al.*, 1989; Roels & Cornelis, 1989; Lindauer *et al.*, 1994). In most cells, catalase is for the most part bound to peroxisomes or microperoxisomes (De Duve & Baudhuin, 1966; Novikoff & Goldfischer, 1969). In erthrocytes, catalase is present in a soluble form in the cytoplasmic matrix. In the hepatocytes of most species, a small part of total catalase activity has been found in the cytoplasmic matrix, as determined with biochemical (Holmes & Masters, 1972; Fukami & Flatmark, 1986) and cytochemical (Roels, 1976; Roels *et al.*, 1977; Yamamoto *et al.*, 1988) techniques. A histochemical technique for the demonstration of peroxisomes was presented by Fahimi (1968). These organelles were visualized on the basis of their high catalase activity using the diaminobenzidine technique. Since then, the histochemical method for demonstrating catalase activity with diaminobenzidine has been optimized (Herzog & Fahimi, 1973, 1974, 1976; Roels *et al.*, 1975, 1986, 1987; Fahimi *et al.*, 1976; Le Hir *et al.*, 1979; Roels & Goldfischer, 1979; Angermüller & Fahimi, 1981; Deimann *et al.*, 1991). The method is based on a catalytic reaction in which catalase–Fe^{3+} reacts with hydrogen peroxide resulting in the formation of a catalase–H_2O_2 complex followed by a peroxidatic reaction in which this complex reacts with diaminobenzidine leading to the formation of catalase–Fe^{3+} and a diaminobenzidine polymer (Chance *et al.*, 1979).

For the selective demonstration of catalase activity in peroxisomes of rat liver, Angermüller & Fahimi

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(1981) recommended the fixation of tissues by perfusion with glutaraldehyde and incubation of the tissue in a solution of 5 mM diaminobenzidine in glycine–NaOH buffer at pH 10.5 in the presence of 0.15% hydrogen peroxide. Geerts & Roels (1981) showed that the amount of final product as measured cytophotometrically in Epon sections of artificial matrices containing liver catalase was proportional to enzyme activity after incubation with diaminobenzidine. As far as we know, *in situ* analysis of catalase activity in cryostat sections of rat liver has not been performed. Therefore, we studied the validity of the histochemical procedure for detecting catalase activity in cryostat sections of rat liver for quantitative purposes. Moreover, catalase activity was localized at the electron microscopical level using serial cryostat sections.

Materials and methods

Three male Wistar rats (200–250 g) were used. Animals were fasted for 24 h with water freely available. Livers were removed, cut into pieces up to 0.5 cm^3 and frozen in liquid nitrogen in closed vials. Tissue blocks were stored at −80°C until further used. Cryostat sections were cut at −25°C on a motor-driven Bright cryostat fitted with a retraction microtome, at a constant low speed to obtain sections of constant thickness (Van Noorden & Frederiks, 1992).

Light microscopy

Sections of 8 μm thickness were picked up onto clean glass slides and were stored in the cryostat cabinet until used. Then, sections were dried for 5 min at room temperature. The effects of fixation on the activity and localization of catalase were studied by incubating sections which were either unfixed or fixed with 0.1–0.5% (w/v) glutaraldehyde or 4% (w/v) formaldehyde dissolved in 100 mM sodium cacodylate buffer, pH 7.4, plus 1% $CaCl_2$, or in 1% (w/v) glutaraldehyde and 4% (w/v) paraformaldehyde in 100 mM sodium cacodylate buffer, pH 7.4, for 5 min at room temperature. After fixation, sections were washed in distilled water and dried at room temperature. The enzyme reaction was performed by incubating sections for 30 min at 37°C in a medium containing 0.1 M glycine–NaOH buffer, pH 10.5, 2% (w/v) polyvinyl alcohol (weight average M_r 70 000–100 000; Sigma, St Louis, MO), 5 mM 3,3′-diaminobenzidine (Sigma) and 44 mM H_2O_2 (Angermüller & Fahimi, 1981). Incubation media were freshly prepared, and H_2O_2 was added immediately before use. The final pH was adjusted to 10.5. After incubation, sections were washed in distilled water and mounted in glycerol jelly.

The highest activity and optimum localization of the final reaction product were found when sections were fixed with 0.3–0.5% glutaraldehyde or formaldehyde or a mixture of formaldehyde and glutaraldehyde. In all further experiments fixation with 0.3% glutaraldehyde was used.

The specificity of the reaction was studied by performing different control reactions as well as by incubating sections in the presence of enzyme inhibitors (Deimann *et al.*, 1991). The following control reactions were performed:

1 Incubation in a medium lacking H_2O_2
2 Heating sections in distilled water for 60 min at 100°C followed by incubation to demonstrate catalase activity
3 Pre-incubation with 0.3–3 M H_2O_2 in distilled water for 60 min at room temperature followed by incubation in standard medium.

The effects of different inhibitors on catalase activity were studied by incubating sections in media containing substrate and inhibitor or by pre-incubation with the inhibitor dissolved in distilled water for 30 min at 37°C prior to the enzyme incubation. The following inhibitors were used (Fahimi, 1969): 0.1–5 M sodium azide (Merck, Darmstadt, Germany), 1–200 mM 3-aminotriazole (Serva, Heidelberg, Germany). A combination of 50 mM 3-aminotriazole and 4.4–44 mM H_2O_2 in 0.1 M glycine–NaOH buffer, pH 10.5, was used in a pre-incubation step.

The validity of the histochemical reaction was investigated according to a number of criteria described by Stoward (1980):

1. Sections 8 μm thick were incubated for 0–60 min at 37°C
2. Sections with a thickness of 2–12 μm were incubated for 30 min at 37°C
3. The affinity of the enzyme for its substrate H_2O_2 (K_m-value) was determined by varying the H_2O_2 concentration (0–44 mM) in incubation media. Sections 8 μm thick were incubated for 30 min at 37°C.

Cytophotometry

Enzyme activity was analysed cytophotometrically with a Vickers M 85a scanning and integrating cytophotometer. Integrated absorbance values were determined at 460 nm in five mid-zonal areas (randomly chosen) of each of two consecutive sections for each incubation. The readings were taken using a 6.3 × Leitz planachromatic objective (NA 0.20) and mask with a diameter of 63 μm, thus providing an effective scanning area of 3119 cm^2. The band width was set at 65 and a scanning spot with an effective diameter of 3.2 μm was used. When a spot size of 0.5 μm was used, similar absorbance values were measured. The relative integrated absorbance readings as recorded by the instrument were converted into mean integrated absorbance values (MIA; Van Noorden & Butcher, 1986). Specific reaction rates of enzyme activity were determined by subtracting the control reaction (performed in the absence of substrate) from the test reaction (performed in the presence of substrate).

Student's *t*-test was employed for statistical analysis; differences were tested at the 0.05 level of significance. Regression analysis of the plots was performed when appropriate. The K_m-value of catalase was calculated from plots of substrate concentrations versus the ratio of substrate concentration and reaction velocity according to Wilkinson (1961).

Electron microscopy

Cryostat sections of 8 μm thickness were mounted on semipermeable membranes which were stretched over a gelled medium containing 3% (w/v) agar (Difco, Detroit, USA) in 0.9% NaCl (Schellens *et al.*, 1992). After adherence of the sections, the gelled medium was removed. Pieces of membrane with attached cryostat sections were cut out and fixed either in 0.3% (w/v) glutaraldehyde or in 1% (w/v) glutaraldehyde and 4% (w/v) paraformaldehyde in 100 mM sodium cacodylate buffer, pH 7.4, for 5 min at room temperature. Subsequently, sections were rinsed thrice with buffer and then incubated for catalase activity for 30 min at 37°C. After incubation, sections were post-fixed in 1% osmium tetroxide in 100 mM sodium cacodylate buffer, pH 7.4, for 1 h at 4°C and dehydrated according to routine procedures. Finally, sections were embedded in LX-112 epoxy resin on glass slides. This procedure allows the specimens to be embedded as flatly as possible which facilitates semi-thin and ultrathin sectioning. To assess the presence of tissue and the preservation of tissue morphology, 1–2 μm thick sections were studied light microscopically after staining with Toluidine Blue. Ultrathin sections (40–50 nm) were cut with an LKB Ultratome III and collected on single-hole copper grids carrying a Formvar film. Ultrathin sections were investigated with a Zeiss EM 100 transmission electron microscope either unstained or stained with uranyl acetate and lead citrate.

Results

Fixation of cryostat sections with at least 0.3% glutaraldehyde appeared to be a prerequisite for the demonstration of catalase activity in peroxisomes of rat liver. No staining of peroxisomes was found when fixation was omitted or when fixation was performed with lower concentrations of glutaraldehyde. Stronger fixatives such as 0.5% glutaraldehyde, 4% formaldehyde or a mixture of 1% glutaraldehyde and 4% formaldehyde did not further affect the staining intensity. Final reaction product, precipitated as a brown diaminobenzidine polymer, was found not only in a granular form in liver parenchymal cells, but also diffusely distributed in erythrocytes (Fig. 1). The amounts of granular final reaction product were similar in hepatocytes in periportal and pericentral zones (Fig. 1A). Moreover, final reaction product was rather homogeneously distributed within hepatocytes, although granules with different sizes and intensities were found (Fig. 1B).

At the electron-microscopical level, electron-dense reaction product was exclusively localized in peroxisomes of liver parenchymal cells after incubation of cryostat sections to demonstrate catalase activity (Fig. 2). Reaction product in hepatocytes was observed only in matrix and core peroxisomes and not in the cytoplasmic matrix or in other organelles such as mitochondria or endoplasmic reticulum. Peroxisomes of different shapes and sizes were observed, and the amounts of final reaction product in individual peroxisomes were slightly different (Fig. 2). The morphology of all subcellular structures was rather well-preserved, despite the fact that cryostat sections were used for incubation to demonstrate catalase activity (Fig. 2B). No distinct differences were observed in activity or localization patterns after fixation with 0.3% glutaraldehyde or with 1% glutaraldehyde and 4% formaldehyde.

The specificity of the reaction was studied by applying different control incubations as well as incubations in the presence of different inhibitors. Hardly any final reaction product was found when incubating in the absence of substrate. Heating in distilled water for 60 min at 100°C completely prevented the granular staining in hepatocytes as well as cytoplasmic staining in erthrocytes. Pre-incubations performed in the presence of high concentrations of H_2O_2 (1.8–3 M) resulted in a complete inhibition of the reaction. Granular final reaction product was not observed when incubations were performed in the presence of 2 M sodium azide, or higher, or when 0.5 M was used during pre-incubation. However, the use of azide induced cytoplasmic staining in hepatocytes which was more intense in pericentral than in periportal areas. Concentrations of 0.1 M aminotriazole or higher prevented the granular staining, but, again, a cytoplasmic staining appeared that was heterogeneously distributed over liver lobules. Granular staining was completely inhibited after a pre-incubation step with 50 mM aminotriazole and 44 mM H_2O_2 in glycine–NaOH buffer, pH 10.5. However, the cytoplasm was weakly stained, but this staining disappeared when reducing the H_2O_2 concentration to 4.4 mM.

The validity of the catalase reaction was investigated by varying incubation time and section thickness. Figure 3 shows the relationship between test, control and test-minus-control reactions and incubation time. Only a slight increase in absorbance in time was found for the control reaction. Non-linearity of test-minus-control reactions was followed by a levelling off. The relationship between log ($MIA_{max} - MIA_t$) for the specific (test-minus-control) reaction and incubation time is shown in Fig. 4. A linear relation with incubation time was found up to 30 min.

The amount of diaminobenzidine polymer specifically generated by catalase activity increased linearly with section thickness between 2 and 8 μm and levelled off with sections thicker than 8 μm (Fig. 5). The control reaction was linear at least up to 12 μm (Fig. 5). Figure 6 shows the relationship between substrate concentration (H_2O_2) and the amount of final reaction product generated. A fast increase is

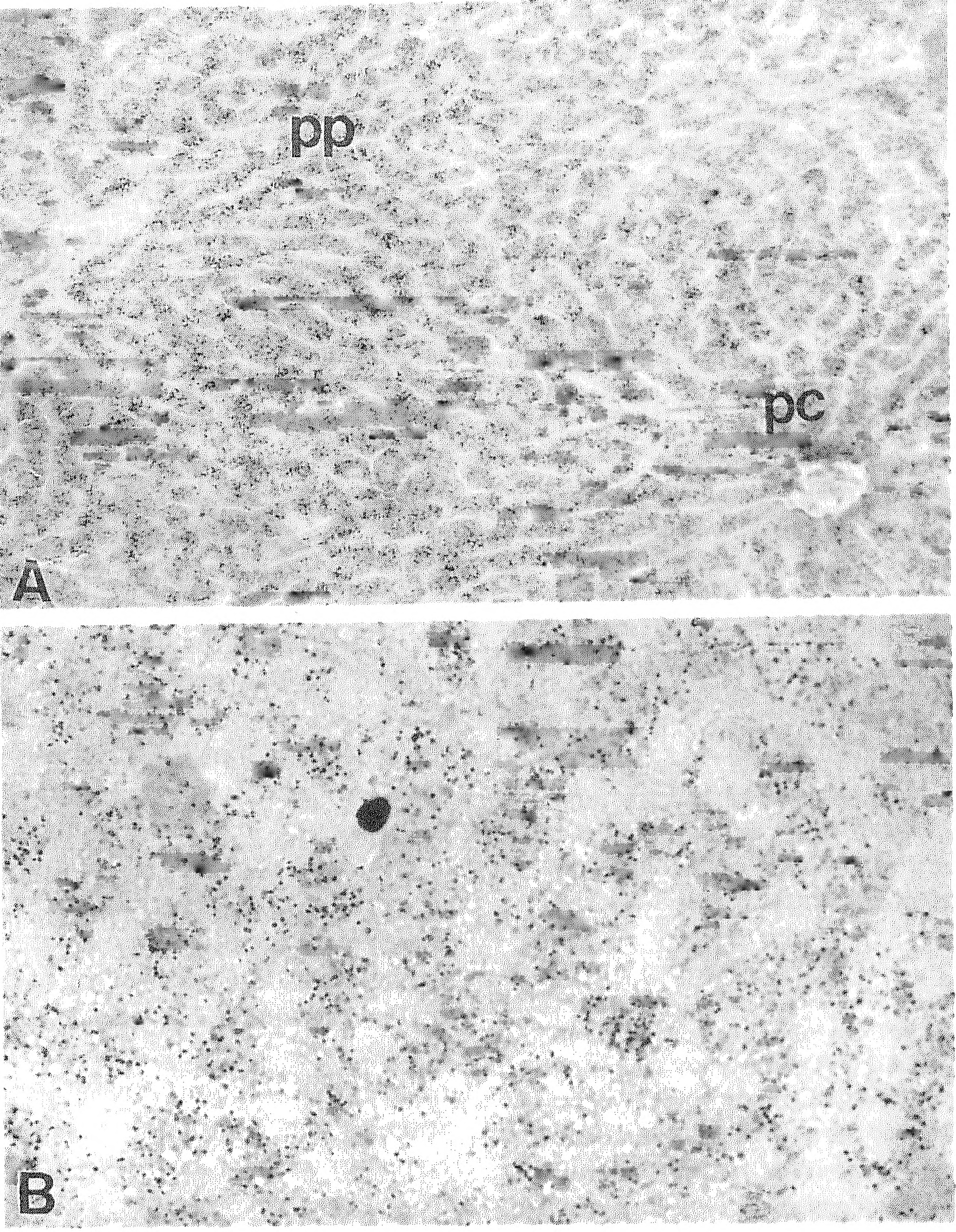

Fig. 1. Photomicrographs of cryostat sections of rat liver fixed with 0.3% glutaraldehyde and incubated for the demonstration of catalase activity. (A) Granular final reaction product is present in hepatocytes. No distinct heterogeneity in catalase activity was apparent in liver lobules. (B) Higher magnification to show that granules of different size and staining intensity are present in hepatocytes, whereas erthrocytes are stained homogeneously. A: × 150; B: × 600; pp = periportal area; pc = pericentral area.

followed by a levelling off. From these data, a K_m value of 2.0 mM was calculated.

Discussion

The present study demonstrates that cryostat sections can be used to detect catalase activity in peroxisomes in rat liver at the light- and electron-microscopical level (Figs 1 and 2). A prerequisite for optimal detection of catalase activity is fixation of cryostat sections with at least 0.3% glutaraldehyde. Usually, tissues or cells are immediately fixed (Angermüller & Fahimi, 1981; Roels *et al.*, 1986) either via perfusion or immersion followed by incubation of frozen or unfrozen sections. These materials were studied either immediately or embedded in plastic for the localization of catalase activity at the light- or electron-microscopical level (Angermüller & Fahimi, 1981; Roels & Goldfischer, 1979; Fahimi *et al.*, 1976). Another approach is the incubation of sections after embedding in plastic (Murray, 1992; Frederik & Bosch, 1993). A disadvantage of fixation and/or embedding of tissues or cells prior to incubation is that the same material cannot always be used for other enzyme histochemical or immunohistochemical procedures. Many enzymes are inactivated rapidly by chemical fixation or embedding (Chalmers & Edgerton, 1989; Frederiks & Bosch, 1993). The use of unfixed frozen material enables the detection of a large number of macromolecules in

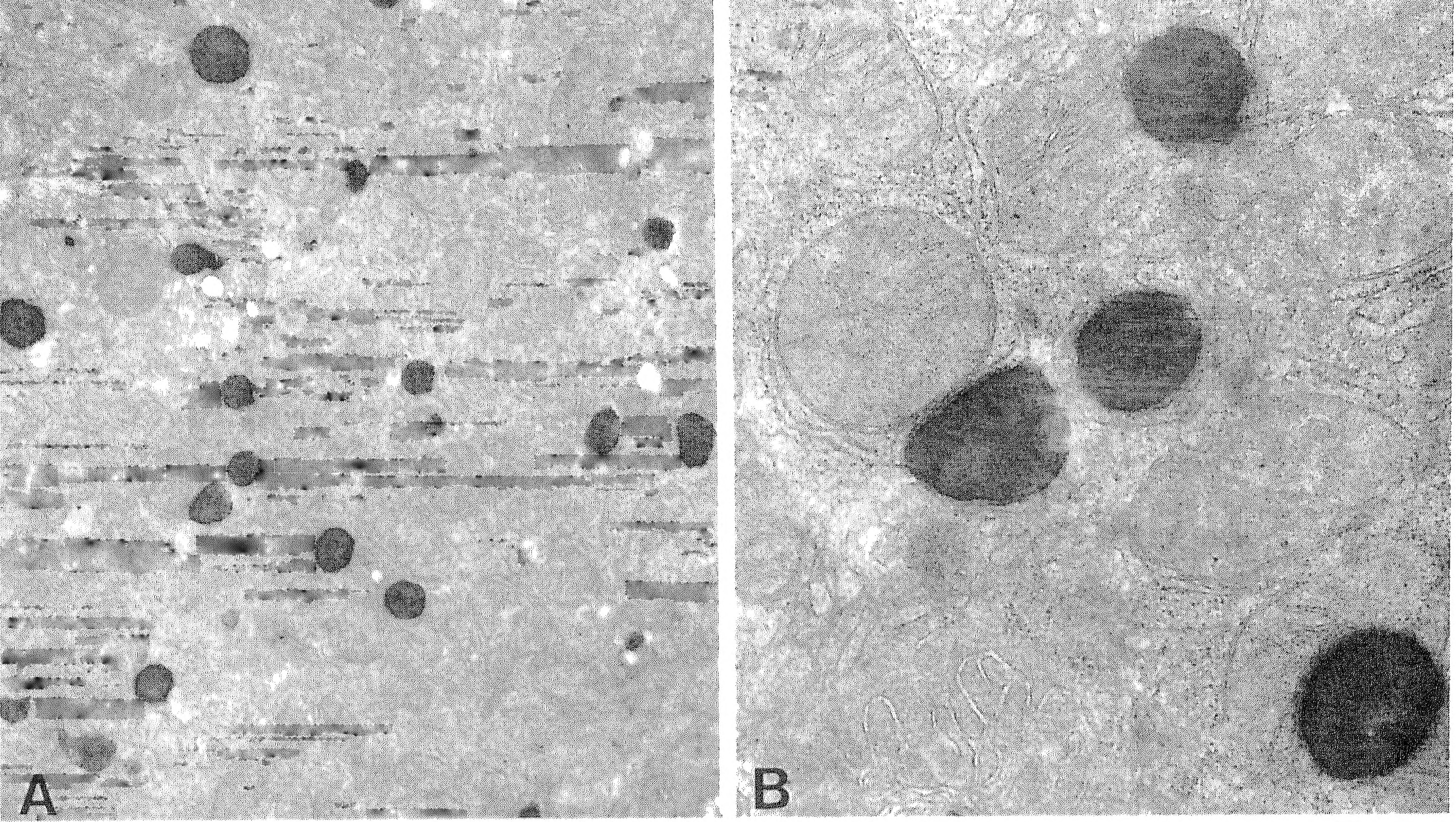

Fig. 2. Electron micrographs of cryostat sections of rat liver fixed with 0.3% glutaraldehyde and incubated for catalase activity. (A) Electron-dense final reaction product is present exclusively in the matrix and core of peroxisomes in liver parenchymal cells. Mitochondria and endoplasmic reticulum are devoid of electron-dense precipitate. Peroxisomes of different size and staining intensity are present. (B) Subcellular structures such as mitochondria and endoplasmic reticulum are well-preserved. A: × 9000; B: × 36 000.

serial sections by applying the most appropriate incubation conditions. In some cases, mainly biopsies from patients, the localization of catalase had already been previously investigated in frozen and then fixed materials, resulting in a rather bad morphology (De Craemer *et al.*, 1989, 1990). It has been demonstrated recently that reliable electron-microscopical studies can be performed on cryostat sections that had been incubated for the demonstration of enzyme activities

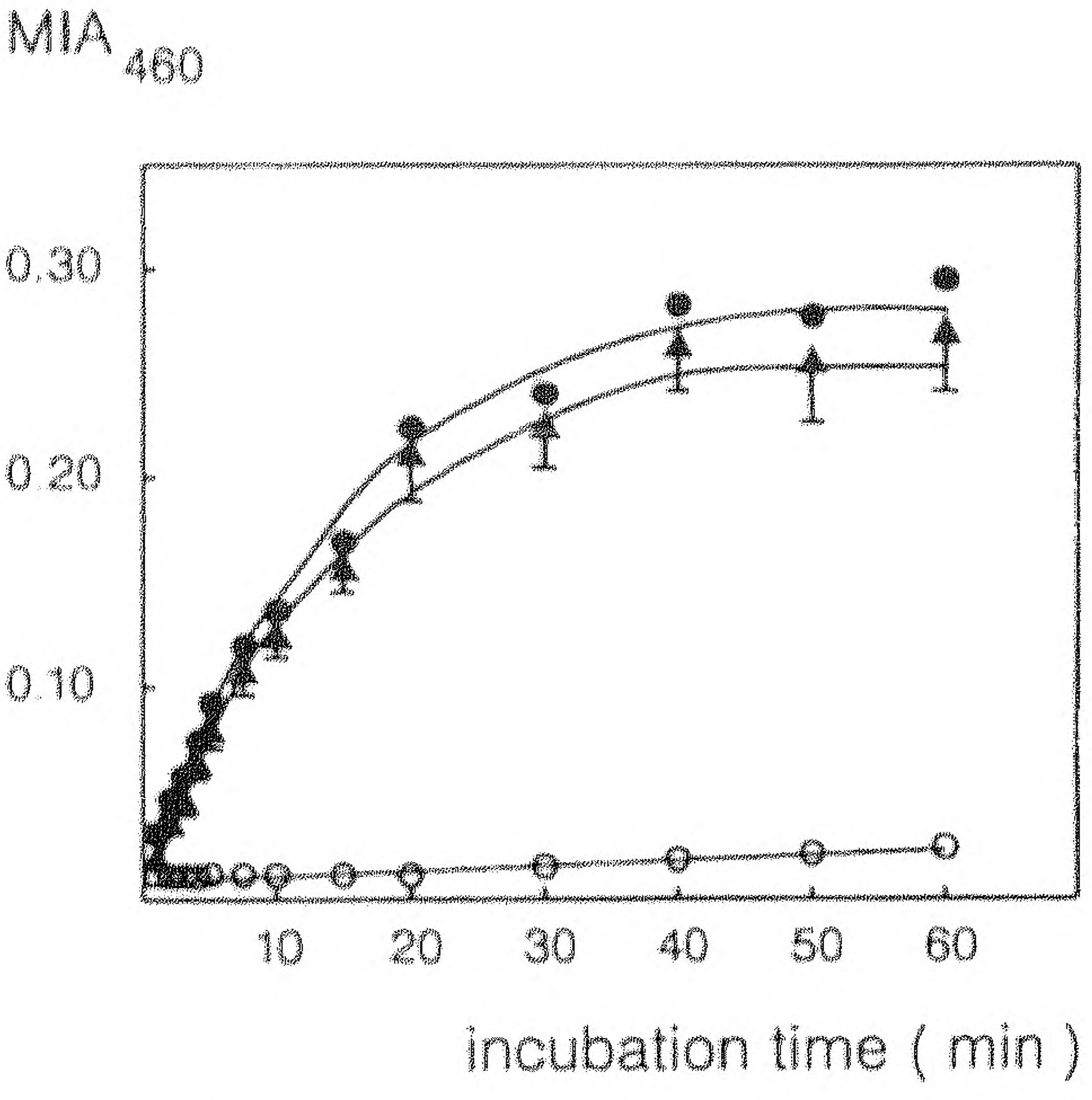

Fig. 3. Relationship between the amount of final reaction product generated and expressed as mean integrated absorbance at 460 nm (MIA_{460}) as measured in mid-zonal areas in rat liver sections (8 μm thick) against time of incubation to demonstrate catalase activity. Data for test (●), control (○) and test-minus-control (▲) reactions are shown.

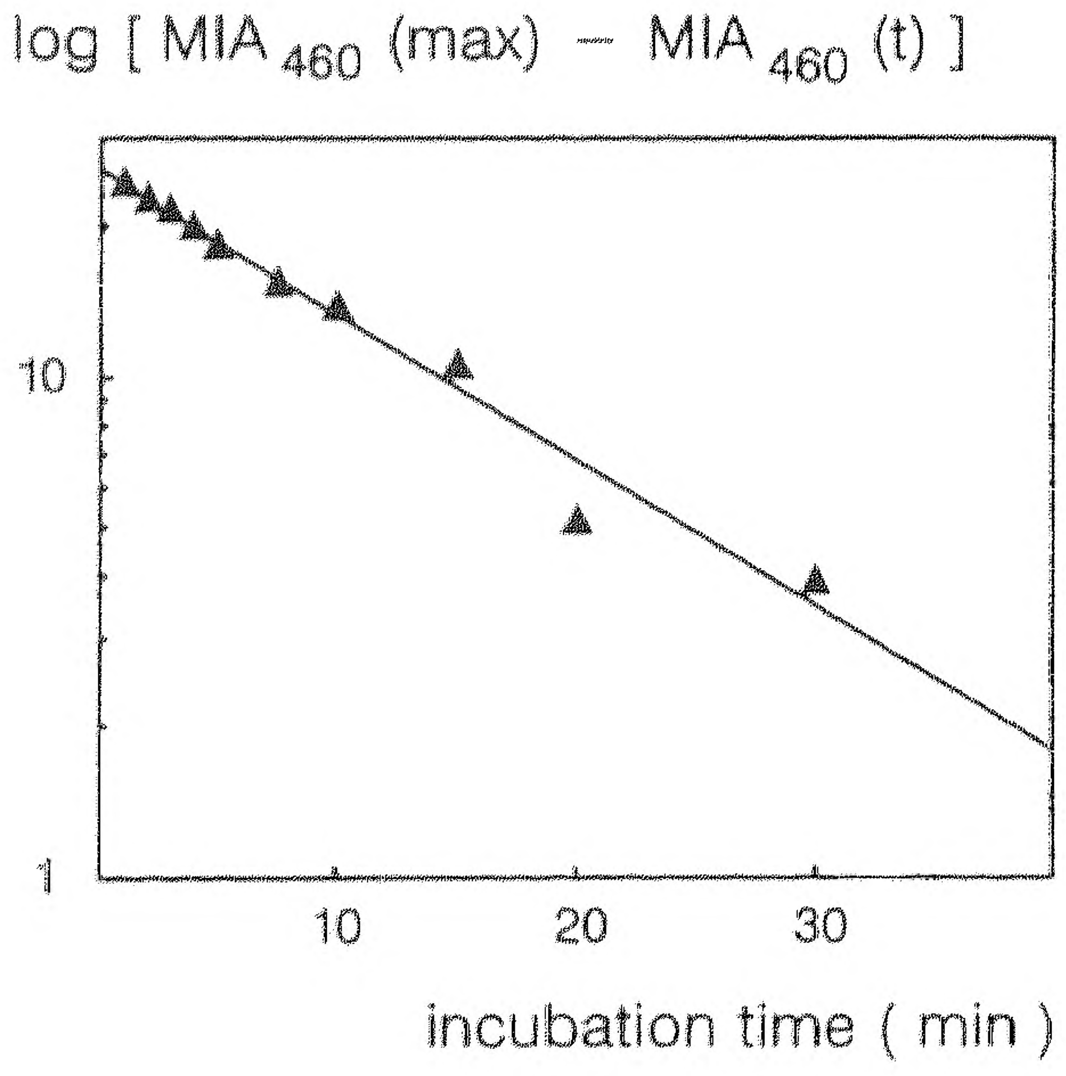

Fig. 4. Relationship between the specific amount of final reaction product expressed as the log (maximum absorbance minus absorbance at time t) against time of incubation for catalase activity. The correlation coefficient was − 0.984.

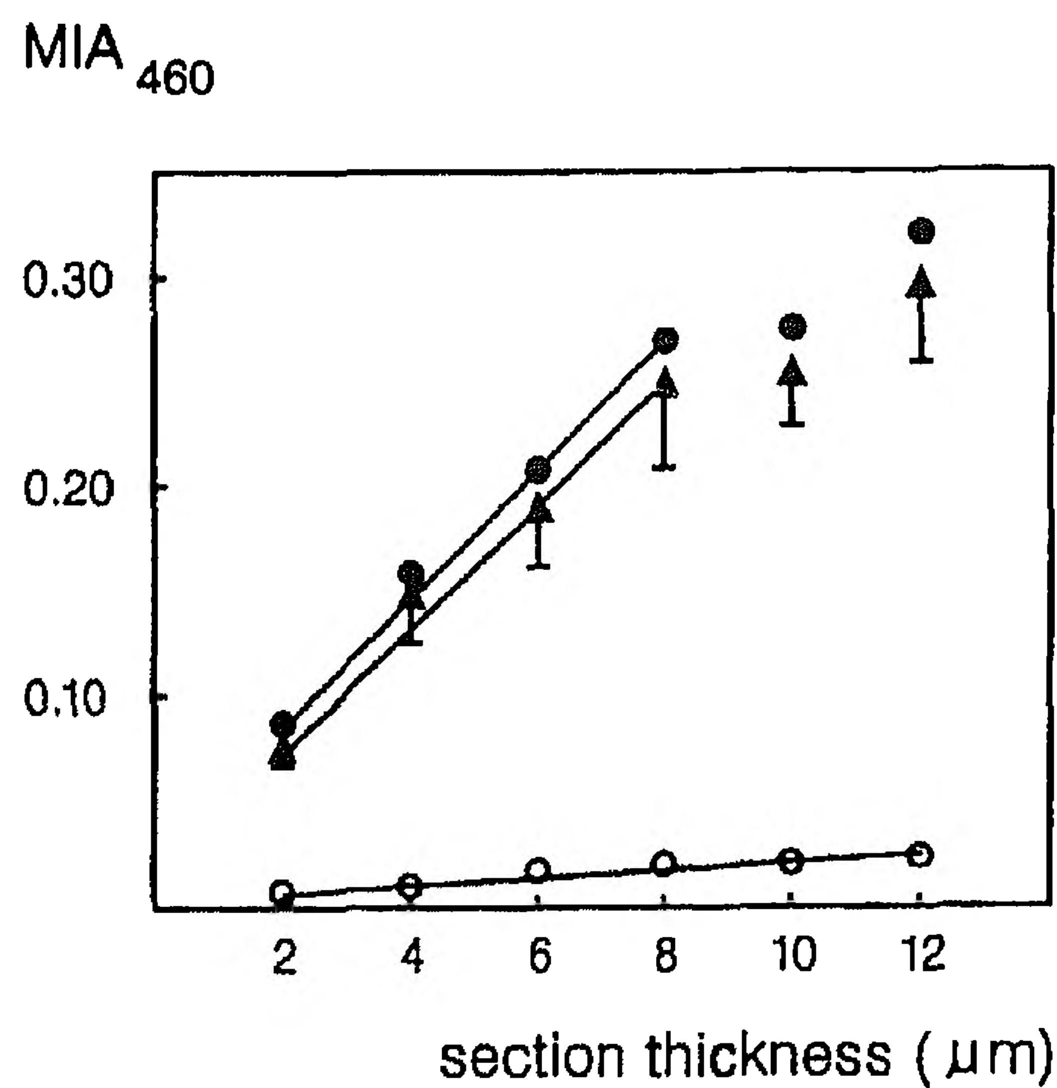

Fig. 5. Relationship between the amount of final reaction product expressed as mean integrated absorbance at 460 nm (MIA_{460}) measured in mid-zonal areas in rat liver sections against section thickness after incubation for 30 min for catalase activity. Data for test (●), control (O) and test-minus-control (▲) reactions are shown. A linear relationship was found between test-minus-control reaction and section thickness up to 8 μm; $r = 0.996$.

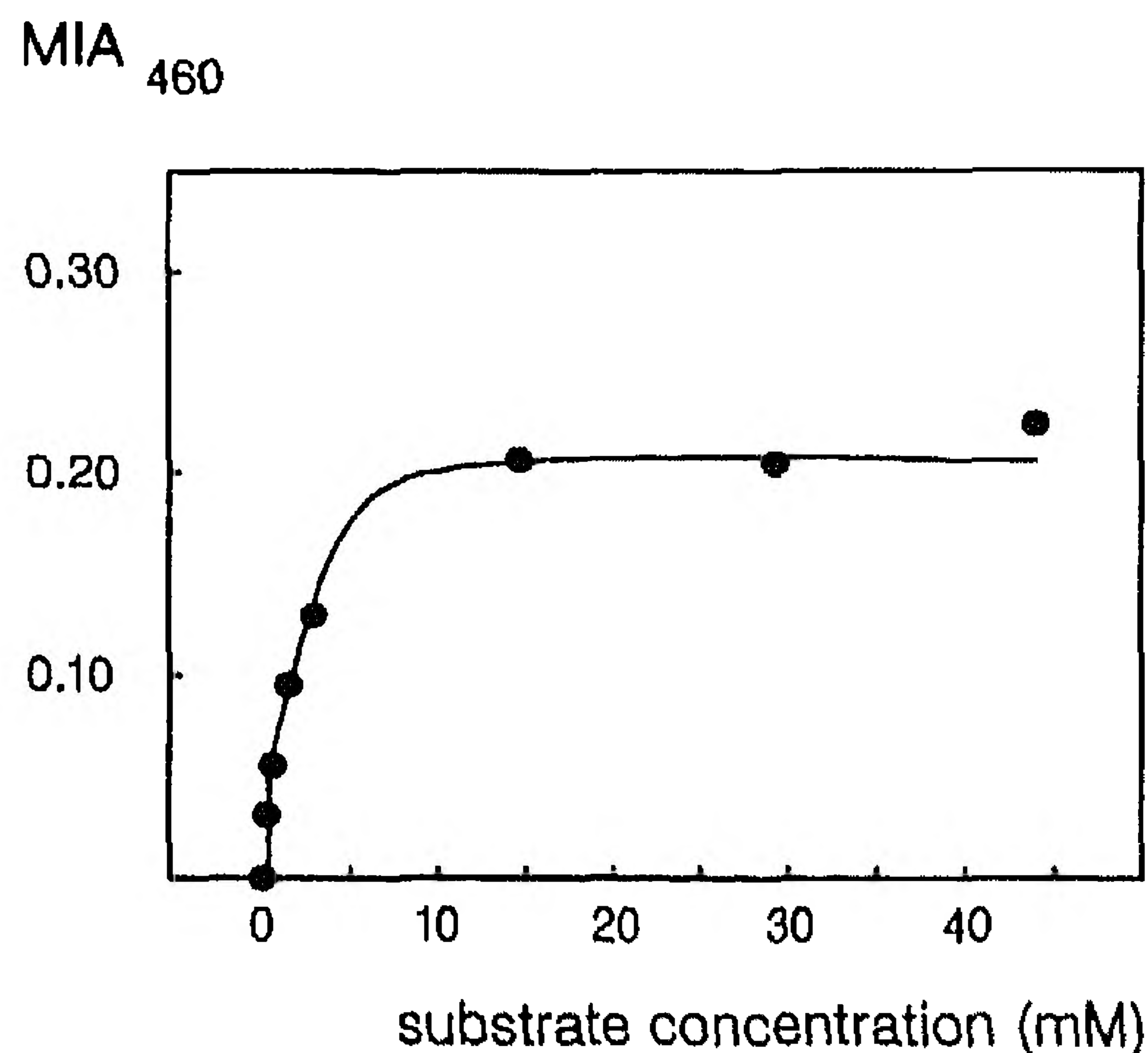

Fig. 6. Relationship between the amount of final reaction product expressed as mean integrated absorbance at 460 nm (MIA_{460}) as measured in mid-zonal areas in rat liver sections (8 μm thick) against hydrogen peroxide concentration after incubation for 30 min to demonstrate catalase activity. A K_m value of 2.0 mM was calculated.

even before chemical fixation (Schellens *et al.*, 1992; Van den Munckhof *et al.*, 1994; Schellens *et al.*, 1995; Song *et al.*, 1995).

The present study shows that subcellular structures are also well-preserved when mildly fixed cryostat sections attached to semipermeable membranes are incubated for catalase activity and then processed for electron microscopy.

The specificity of the reaction was assessed by testing a large number of inhibitors. Pretreatment with high concentrations of hydrogen peroxide ($\geq$ 1.8 M H_2O_2) destroyed catalase activity completely as was demonstrated before (Deimann *et al.*, 1991). This was also found when sections were heated for 60 min to 100°C. Other inhibitors of catalase activity such as sodium azide and aminotriazole prevented the formation of granular reaction product but induced cytoplasmic staining. Generation of final reaction product in the cytoplasm of liver parenchymal cells may have been caused by glutathione peroxidase activity. Pretreatment of the sections with 50 mM aminotriazole and 4.4 mM H_2O_2 prevented granular staining, without final reaction product to be generated in the cytoplasm.

The kinetics of catalase activity in rat liver were studied cytophotometrically. The reaction shows first-order inactivation (Fig. 3) which is in agreement with studies of Geerts & Roels (1981) using model systems containing catalase from liver. It has been assumed that the enzyme is inactivated at high pH, high H_2O_2 concentrations and high temperature (Geerts & Roels, 1981). The linearity of the relationship between the amount of final reaction product and section thickness from 2 to 8 μm indicates that compounds of the incubation medium, such as diaminobenzidine, penetrate sufficiently into cryostat sections up to 8 μm. This finding implies that sections of, at most, 8 μm thickness, have to be used for analysis. On the basis of all these findings it can be concluded that the method is valid for quantitative purposes.

A K_m value of 2.0 mM for catalase was found in rat liver. It had been shown before that optimum concentrations of hydrogen peroxide for detecting catalase activity are related to concentrations of the other substrate, diaminobenzidine (Le Hir *et al.*, 1979; Roels & Cornelis, 1989). Moreover, it has been demonstrated that both compounds can exert inhibiting effects on catalase activity (Geerts & Roels, 1981), and catalase activity is affected by prefixation (Le Hir *et al.*, 1979; Angermüller & Fahimi, 1981; Roels *et al.*, 1987). The K_m value of 2.0 mM for H_2O_2 applies to rat liver tissue after fixation with 0.3% (w/v) glutaraldehyde, with 5 mM diaminobenzidine present in the incubation medium. Because of the complexity of enzyme inactivation by both compounds, the value of this quantitative parameter is only limited.

In conclusion, the procedure described to demonstrate catalase activity in cryostat sections of rat liver at the light- and electron-microscopical level is specific and valid. This method enables combined light- and electron-microscopical studies of catalase activity in serial cryostat sections, which may be useful for pathology, when only small biopsies are available, when the tissue is heterogeneous, and when other histochemical markers also need to be studied in the same material.

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