Intracellular Distinction between Peroxidase and Catalase in Exocrine Cells of Rat Lacrimal Gland: A Biochemical and Cytochemical Study*

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Summary. The lacrimal gland (Glandula orbitalis externa) of rat contains both peroxidase and catalase and was used as a model for biochemical and cytochemical distinction between peroxidase and catalase. Both enzymes were isolated by ammonium sulfate precipitation from tissue homogenates, and the effects of fixation with glutaraldehyde and various conditions of incubation were investigated colorimetrically using DAB as hydrogen donor. The lacrimal gland peroxidase is strongly inhibited by glutaraldehyde treatment. In contrast, for catalase the fixation with glutaraldehyde is the prerequisite for demonstration of its peroxidatic activity. The maximal peroxidatic activity was obtained after treatment of catalase with 3% glutaraldehyde, higher concentrations being inhibitory. For lacrimal gland peroxidase, the maximal rate of oxidation of DAB is at pH 6.5, whereas for catalase it is at pH 10.5. The optimal concentration of H₂O₂ for lacrimal gland peroxidase is at 10⁻³ M and for peroxidatic activity of catalase at 10⁻¹ M. These optimal conditions obtained biochemically were applied to tissue sections of rat lacrimal gland. After the fixation of tissue with a low concentration of glutaraldehyde and incubation in the DAB medium at neutral pH containing 10⁻³ M H₂O₂ (Peroxidase medium), the reaction product was localized in the cisternae of the rough endoplasmic reticulum, in elements of the Golgi apparatus, and in secretory granules. After the fixation of tissue with 3% glutaraldehyde and incubation in the DAB-medium containing 10⁻¹ M H₂O₂ and at pH 10.5 (catalase medium), the staining in the endoplasmic reticulum, the Golgi-apparatus and in secretory granules was completely inhibited and reaction product was localized exclusively in small (0.2-0.5 μ) particles similar to small peroxisomes described in various other cell-types.

Introduction

The 3,3'-diaminobenzidine (DAB) reaction has been used for cytochemical localization of various enzymes with peroxidatic activity (Essner, 1974; Fahimi, 1975). A major handicap of DAB-methods has been their lack of specificity, which is especially disturbing when more than one enzyme with peroxidatic activity is localized within the same cell. A typical problem is that of intracellular distinction between catalase and peroxidase. Numerous attempts have been undertaken to distinguish between these two enzymes by the use of different fixation and incubation conditions and application of various inhibitors. Thus, alkaline DAB media have been used preferentially for localization of catalase (Novikoff and Goldfischer, 1969; Fahimi, 1969; Novikoff et al., 1972), whereas

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Abbreviations used this paper: DAB, 3,3'-diaminobenzidine tetrahydrochloride.
neutral pH has been applied for peroxidase (Graham and Karnovsky, 1966). Furthermore, whereas peroxidase is sensitive to prolonged fixation with glutaraldehyde (Fahimi, 1970; Hand, 1973; Fahimi et al., 1975 in press), catalase survives such fixation and indeed, as we have shown previously, its visualization with DAB requires prior treatment with glutaraldehyde (Herzog and Fahimi, 1974). The use of inhibitors such as aminotriazole (Heim et al., 1955) and DCPIP (Novikoff and Novikoff, 1972) for cytochemical distinction between the two enzymes has been of little help, since both enzymes are inhibited by these compounds (Herzog and Miller, 1972a; Roels, 1973; Fahimi, 1975). Recently, Roels et al. (1975) have reinvestigated these parameters in tissues which they presumed to contain exclusively either catalase (such as liver) or peroxidase (such as salivary glands), and proposed conditions for selective demonstration of each enzyme. The intracellular distinction between the two enzymes within the same cell, however, received little attention by these authors. Thus, although the occurrence of small catalase-positive particles (microperoxisomes) has been known in salivary glands (Hand, 1973) and Kupffer cells (Gray et al., 1973), the authors (Roels et al., 1975) made no mention of them and attributed all the DAB reaction product in these cells to peroxidase.

To our knowledge, there has been no systematic study dealing with isolation of the two enzymes from the same tissue and biochemical characterization of their reaction with DAB. Rat lacrimal gland is a suitable tissue for this purpose since it contains both catalase and peroxidase (Herzog and Miller, 1972b). In the present study we have a) isolated these enzymes from lacrimal gland (Glandula orbitalis externa) and separated them from each other; b) investigated the effects of glutaraldehyde fixation and various incubation conditions upon these enzymes, using the quantitative DAB-assay for peroxidase (Herzog and Fahimi, 1973); and c) applied the optimal conditions obtained biochemically for each enzyme to tissue sections for cytochemical distinction between catalase and peroxidase. The results demonstrate that the two enzymes are localized within different intracellular compartments.

Materials and Methods

Animals. Male albino rats (CDR, Charles River Strain, Charles River Laboratories, Wilmington, Mass.) weighing 150-180 grams and kept on a normal laboratory diet and water ad libitum were used.

Materials. 3,3'-diaminobenzidine tetrahydrochloride was obtained from Sigma Chemical Co., St. Louis, Mo. The purity and the degree of oxidation of DAB was checked colorimetrically prior to use as described recently (Fahimi and Herzog, 1973). Gelatin was from DIFCO-Laboratories, Detroit, Michigan; Hydrogen peroxide (30%) was purchased from Fisher, Scientific Co., Fairlawn, N.J., and glutaraldehyde was from Ladd Industries, Burlington, Vermont, or from Electron Microscopy Sciences (EMS), Fort Washington, PA. The Purity of the glutaraldehyde was checked prior to use by determination of the purification index (A 235/A 280) (Anderson, 1967). All other reagents used in this study were of analytical grade.

Isolation of Peroxidase and Catalase. Animals were perfused with saline for 3-5 min. in order to remove erythrocytes and leukocytes from blood vessels. This is an important preparative step, since both of these cell-types contain catalase and peroxidase. Lacrimal glands were homogenized in 0.25 M sucrose, tissue fraction were prepared by differential centrifugation, and catalase and peroxidase were isolated from pooled microsomal fractions and postmicrosomal supernatants using fractionated precipitation with ammonium sulfate.
Intracellular Distinction between Peroxidase and Catalase

Fig. 1. Absorption spectrum of a crude preparation of lacrimal gland peroxidase, isolated from microsomal fractions by precipitation with ammonium sulfate. Note the distinct peak at 415 nm (—) which is shifted to 430 nm upon addition of 5 mM \( \text{H}_2\text{O}_2 \) (—•—) (Formation of enzyme-substrate complex II) (Herzog and Miller, 1972b). Whereas catalase precipitated at 40–60% saturation with ammonium sulfate ('Catalase-Fraction'), peroxidase was obtained by treatment with 70–95% saturated ammonium sulfate ('Peroxidase-Fraction'). Precipitation of each fraction was repeated three times and was followed by 48 hours of dialysis against several changes of phosphate buffered saline. The peroxidase fraction had a light yellow brownish color with absorption maxima at 280 nm and 415 nm (Fig. 1). Upon addition of 5 mM \( \text{H}_2\text{O}_2 \), the absorption maximum at 415 nm shifted by 10–15 nm to 430 nm, which is typical for lacto-peroxidase (Chance, 1950). Peroxidase activity was determined with the colorimetric DAB-Assay (Herzog and Fahimi, 1973), catalase was estimated with the method of Lück (1965) and protein was measured according to the procedure of Lowry et al. (1951).

Treatment of Peroxidase and Catalase with Glutaraldehyde. Peroxidase and catalase-fractions from rat lacrimal glands were diluted to 1 to 1.5 mg protein per ml with 0.1 M sodium cacodylate buffer, pH 7.2, and were mixed at 25°C with various concentrations of glutaraldehyde, ranging from 0.1 to 12%, as described recently (Herzog and Fahimi, 1974). After 30 min. the mixture was dialyzed for 48 hr against several changes of saline buffered with 0.1 M sodium cacodylate. The effect of dialysis was monitored by checking the absorption at 280 nm, which indicates the presence of glutaraldehyde. Usually, after 24 hr of dialysis no trace of glutaraldehyde was detectable spectrophotometrically. No precipitation was observed during the treatment of enzyme fractions with glutaraldehyde or during dialysis. Since glutaraldehyde interferes with the Lowry-procedure catalase-protein determination was based on Soret band absorption as described previously (Herzog and Fahimi, 1974).

Peroxidase and glutaraldehyde-treated catalase were used for determination of optimal pH and optimal concentrations of \( \text{H}_2\text{O}_2 \) for oxidation of DAB, using the colorimetric DAB-assay (Herzog and Fahimi, 1973). Since the reaction rate of peroxidase increases with increasing concentrations of DAB, a final concentration of \( 10^{-2}\text{M} \) (which is a subsaturated solution of DAB) was used.
Cytochemical Studies. The optimal conditions of fixation and incubation as obtained by biochemical measurements (Table 2) were applied to sections of lacrimal gland. Rats were perfused through the left ventricle for two min. with saline, followed by 10 min. with 1.25% or 3% glutaraldehyde, buffered with 0.1 M sodium cacodylate at pH 7.2 and containing 0.05% CaCl₂, at room temperature. Lacrimal glands were excised and cut into thin strips which were postfixed by immersion in the same fixatives as indicated in Table 2. Thirty μ thick chopper sections (Smith and Farquhar, 1965) were preincubated for 1 hr without H₂O₂, followed by 2 hr of incubation in the complete medium. The composition of the incubation media is shown in Table 2. Incubation in the presence of H₂O₂ was carried out at room temperature (25°C) under constant agitation and in darkness. Since high concentrations of H₂O₂, as used for catalase, lower the pH of the incubation medium, this was readjusted after the addition of H₂O₂. After incubation, the tissue was postfixied in unbuffered 2% OsO₄, dehydrated, and embedded in Epon. One μ thick Epon sections were examined under a light microscope and photographed in a Zeiss Photomicroscope Ultraphot II. Ultrathin sections were cut with a diamond knife and examined unstained or stained with lead citrate (Reynolds, 1963) in a Philips EM 200 electron microscope.

Results

Effect of Glutaraldehyde Fixation

Fig. 2 demonstrates the effect of various concentrations of glutaraldehyde on the peroxidatic activity of catalase, and on peroxidase. Peroxidase is strongly inhibited by increasing concentrations of glutaraldehyde. This is in marked contrast to the peroxidatic activity of catalase which increases upon treatment with glutaraldehyde. The highest peroxidatic activity is obtained after treatment of catalase preparations with 3% glutaraldehyde.

The effect of glutaraldehyde fixation on the peroxidatic and catalatic activities of catalase is demonstrated in Table 1. Increasing concentrations of glutaraldehyde inhibit catalatic activity of catalase whereas the peroxidatic activity increases upon treatment with concentrations of up to 3% glutaraldehyde followed by inhibition with higher concentrations.

Table 1. Effect of Glutaraldehyde on the Peroxidatic and Catalatic Activity of Lacrimal Gland Catalase

<table>
<thead>
<tr>
<th>Glutaraldehyde concentration</th>
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<tr>
<td></td>
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<tr>
<td>Catalatic activity</td>
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<tr>
<td>Peroxidatic activity</td>
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</table>

Effect of pH

Fig. 3 demonstrates that optimal pH for peroxidase is at 6.5, whereas optimal pH for the peroxidatic activity of catalase is at 10.5.

Effect of H₂O₂-Concentration

The effect of various H₂O₂-concentrations was measured at optimal pH for each enzyme (Fig. 4). Under these conditions, peroxidase has its highest rate of
Fig. 2. Effect of glutaraldehyde on peroxidase (---) and peroxidatic activity of catalase (o--o) isolated from rat lacrimal gland. Peroxidase activity decreases with increasing concentrations of glutaraldehyde while the peroxidatic activity of catalase increases. Note that the highest peroxidatic activity is achieved after treatment of catalase with 3% glutaraldehyde and that peroxidase at this glutaraldehyde concentration is almost completely inhibited.

Fig. 3. Effect of pH on peroxidase (o--o) and on peroxidatic activity of catalase (---). Optimal pH for peroxidase is at 6.5 and for peroxidatic activity of catalase at 10.5.
reaction at $10^{-3}$ M $H_2O_2$, whereas the optimal $H_2O_2$-concentration for the peroxidatic activity of catalase is at $10^{-3}$ M $H_2O_2$. Recent observations indicate that increasing concentrations of DAB require increasingly higher concentrations of $H_2O_2$ (Fahimi, 1975). The optimal concentration of $H_2O_2$ obtained here corresponds to a DAB concentration of $10^{-2}$ M.

**Fig. 4.** Effect of various concentrations of $H_2O_2$ on peroxidase ($\circ\cdots\circ$) and peroxidatic activity of catalase ($\bullet\cdots\bullet$) at the optimal pH for each enzyme in the DAB assay. Optimal concentration of $H_2O_2$ for peroxidase is at $10^{-3}$ M $H_2O_2$ and higher concentrations inhibit markedly peroxidase activity. In contrast, the highest peroxidatic activity of catalase is reached at $10^{-1}$ M $H_2O_2$.

**Conditions for Cytochemical Visualization of Lacrimal Gland Peroxidase and Catalase**

In Table 2 the optimal conditions of incubation for peroxidase and catalase are summarized. Although glutaraldehyde has a strong inhibitory effect upon peroxidase, for proper ultrastructural preservation of tissue a brief fixation with

<table>
<thead>
<tr>
<th>Fixation</th>
<th>Peroxidase</th>
<th>Catalase</th>
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<tr>
<td>1.25% glutaraldehyde</td>
<td>10 minutes perfusion +</td>
<td>3% glutaraldehyde</td>
</tr>
<tr>
<td>10 minutes perfusion +</td>
<td>30 min immersion</td>
<td>10 minutes perfusion +</td>
</tr>
<tr>
<td>3% glutaraldehyde</td>
<td>2 hr immersion</td>
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**Table 2. Optimal Conditions for Cytochemical Distinction between Peroxidase and Catalase in Lacrimal Gland of the Rat**

<table>
<thead>
<tr>
<th>pH</th>
<th>Peroxidase</th>
<th>Catalase</th>
</tr>
</thead>
<tbody>
<tr>
<td>neutral (6.5-7.0)</td>
<td>10^{-2} M</td>
<td>alkaline (10.5)</td>
</tr>
<tr>
<td>DAB $10^{-2}$ M</td>
<td>10^{-2} M</td>
<td></td>
</tr>
<tr>
<td>$H_2O_2$ $10^{-4}$ M (0.003%)</td>
<td>10^{-2} M (0.3%)</td>
<td></td>
</tr>
</tbody>
</table>
Intracellular Distinction between Peroxidase and Catalase

Figs. 5a and b. Light micrographs of unstained sections from peroxidase- and catalase-incubated tissue of rat lacrimal gland. Reaction product of peroxidase (Fig. 5a) is visible in the cytoplasm, leaving the nucleus unstained, and in the secretory granules (SG) at the apex of acinar cells. In the catalase preparation (Fig. 5b) peroxidase activity is completely abolished and reaction product of catalase is visible in small particles (P) located in the basal portion of the cell. ×1,700

a low concentration of glutaraldehyde is needed. We therefore used 1.25% glutaraldehyde for fixation of tissues incubated for peroxidase, whereas for catalase we used the fixation with 3% glutaraldehyde, which is optimal for peroxidatic activity of catalase.

Cytochemical Results

In Figs. 5–8 the localization of peroxidase is compared with that of catalase in acinar cells of rat lacrimal gland.

By light microscopy, a strong reaction for peroxidase is seen in the cytoplasm and in all secretion granules (Fig. 5a). This is in contrast to the reaction of small particles in the catalase preparation, in which the staining of secretory granules is completely inhibited (Fig. 5b).

By electron microscopy, reaction product of peroxidase is present in the cisternae of the rough endoplasmic reticulum and in secretory granules (Fig. 6a), whereas catalase is localized in small (0.2–0.5 μ) particles located mainly at the basal portion of the cells (Fig. 6b). The endoplasmic reticulum does not react in catalase preparations. Furthermore, all Golgi cisternae as well as the condensing...
Fig. 6a and b
vacuoles stain prominently for peroxidase (Fig. 7a) but are negative for catalase (Fig. 7b). Occasionally some of the catalase-positive particles appear in close association with large lipofuscin-containing dense bodies (Fig. 7b). It should be noted that in peroxidase preparations a strong reaction is also observed over the intermembranous space of mitochondria (Figs. 6a, 7a). This reaction is due to cytochrome oxidase (Seligman et al., 1968; Anderson et al., 1975) and is not seen in the catalase preparations (Figs. 6b, 7b).

Figs. 8a-c show whorls of rough endoplasmic reticulum with centrally located single membrane-bound particles which stain prominently in the catalase medium and which are believed to represent peroxisomes. These figures emphasize that only through the combination of all parameters of fixation and incubation the intracellular distinction between catalase and peroxidase can be achieved. Thus, Fig. 8b demonstrates that sufficient peroxidase activity survives the fixation with 3% glutaraldehyde (Fig. 8b) and that in such preparations peroxisomes also stain after the incubation at neutral pH. It should be noted, however, that in Fig. 8a (1.25% glutaraldehyde) the intensity of staining in the peroxisomes is less than that in Fig. 8b (3% glutaraldehyde). In Fig. 8c the application of the optimal conditions for catalase results in exclusive staining of peroxisomes with complete inhibition of peroxidase in the endoplasmic reticulum.

Discussion

In this study the rat lacrimal gland was used as a model for the investigation of biochemical and cytochemical discrimination between peroxidase and catalase with the DAB-technique. Recently it was shown that this organ contains both peroxidase and catalase, which can be isolated by fractionated precipitation with ammonium sulfate (Herzog and Miller, 1972b). On the basis of quantitative oxidation of DAB (Herzog and Fahimi, 1973) the optimal conditions of fixation and incubation for each of these enzymes have been determined.

Fixation. Glutaraldehyde treatment inhibited markedly the activity of lacrimal gland peroxidase, while enhancing the peroxidatic activity of catalase. This effect of glutaraldehyde on the lacrimal gland catalase was similar to its influence on beef liver catalase, as we have recently demonstrated (Herzog and Fahimi, 1974). Whereas the maximal peroxidatic activity of beef liver catalase was obtained after treatment with 6% glutaraldehyde, the lacrimal gland catalase exhibited its highest peroxidatic activity after fixation with 3% glutaraldehyde. Therefore, a 3% solution of glutaraldehyde was used in this study for visualization of peroxidatic activity of lacrimal gland catalase. The prolongation of fixation time (by immersion for 2 hr, see Table 2) after the perfusion did not

Figs. 6a and b. Portions of secretory acinar cells. Tissue is prepared for visualization of peroxidase and catalase. Note the distinct reaction in the cisternae of the rough endoplasmic reticulum (ER) and in secretory granules (SG) in the peroxidase preparation (Fig. 6a). Peroxidase reaction is completely inhibited in the catalase preparation, in which reaction product is confined to small (0.2–0.5 μ) particles (P) mainly at the basal part of the cell (Fig. 6b). Fig. 6a: ×12,000. Fig. 6b: ×14000
Fig. 7a and b
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affect the peroxidatic activity of catalase but led to inhibition of endogenous peroxidase, thus influencing the discrimination between catalase and peroxidase. The inhibitory effect of glutaraldehyde upon endogenous peroxidase of various mammalian tissues was demonstrated in previous cytochemical studies (Fahimi, 1970; Hand, 1973; Roels, 1973; Fahimi et al., 1975 in press) and was confirmed biochemically in the present study. Our biochemical observations clearly indicate that fresh unfixed tissue should be most ideal for exclusive demonstration of peroxidases, since in such preparations peroxidase exhibits its highest activity, and catalase is not demonstrable with the DAB-technique (Fahimi et al., 1975; Roels et al., 1975). However, for ultrastructural studies a minimal preservation of tissue is required; therefore, we used 1.25% glutaraldehyde for demonstration of peroxidase. Although at this concentration of glutaraldehyde less than 25% of peroxidase survives the fixation, the remaining activity is sufficient for adequate cytochemical demonstration of the enzyme. One drawback of this fixation, however, is that in such preparations the peroxidatic activity of catalase becomes demonstrable, and thus the discrimination between the two enzymes is indistinct (Fig. 8a). Hence, proper discrimination between the two enzymes can be achieved only through the combination of optimal fixation and incubation conditions and by comparison of the results obtained for each enzyme.

Incubation. Marked differences were also noted between the two enzymes in respect to the optimal conditions of incubation. The optimal pH for peroxidatic activity of catalase was at pH 10.5, which is identical to that of beef liver catalase (Herzog and Fahimi, 1974). In contrast, optimal pH for lacrimal gland peroxidase is at pH 6.5 (Fig. 3). This is somewhat different from the pH optimum of horseradish peroxidase, which is at pH 4.3 (Herzog and Fahimi, 1973), but is close to the neutral pH used in most cytochemical studies for visualization of endogenous peroxidases in mammalian tissues (Venkatachalam et al., 1970; Fahimi, 1970; Herzog and Miller, 1970; Novikoff et al., 1971; Fahimi et al., 1975, in press; Roels et al., 1975). Under the optimal conditions for catalase no residual peroxidase activity was seen in the acinar cells of lacrimal gland (Fig. 8c). This is in contrast to the findings of Roels et al. (1975) who noted evidence of residual peroxidase activity in their catalase preparations. This is probably due to the lower pH of their catalase medium (pH 9.7) in which some peroxidase activity survives the incubation.

The optimal concentration of H₂O₂ for peroxidase was substantially lower (10⁻³M) than that needed for demonstration of catalase (10⁻¹M). These quantitative observations confirm the cytochemical findings of Novikoff (1970), who first reported marked differences in the substrate optimum of these enzymes.

Figs. 7a and b. Golgi region (G) of secretory acinar cells incubated for demonstration of peroxidase and catalase. Reaction product of peroxidase is visible in the cisternae of the rough endoplasmic reticulum and of the Golgi apparatus and in condensing vacuoles (Fig. 7a). In the catalase preparation reaction product is found in small particles (P) only (Fig. 7b). Note the close association of some of these particles with large lipofuscin containing dense bodies (DB). Fig. 7a: ×29,000. Fig. 7b: ×23,000
Fig. 8a--c
Figs. 8a–c. Whorls of rough endoplasmic reticulum in secretory acinar cells with centrally located particles (P) which stain prominently in the catalase preparation and which are believed to represent peroxisomes. Figs. 8a and b demonstrate that different concentrations of glutaraldehyde (Fig. 8a: 1.25%; Fig. 8b: 3%) do not discriminate between peroxidase and catalase. Tissue in Fig. 8c is fixed in 3% glutaraldehyde and incubated under optimal conditions for peroxidatic activity of catalase. Only peroxisomes react. Fig. 8a: ×49,000. Fig. 8b: ×26,500. Fig. 8c: ×36,500

It should be noted that high concentrations of H₂O₂ (10⁻¹⁰M), which are optimal for catalase, inhibit the activity of peroxidase and, in contrast, low concentrations of H₂O₂ (10⁻⁴M), which are optimal for peroxidase, are suboptimal for the demonstration of peroxidatic activity of catalase (Fig. 4).

The "optimal" conditions described in this report for discrimination between peroxidase and catalase were obtained primarily in biochemical studies and are not necessarily optimal for cytochemical localization of these enzymes. The application of these conditions to rat lacrimal gland revealed a distinct localization for each enzyme in a different cell compartment. Thus, peroxidase, a secretory protein (Herzog and Miller, 1972b), was localized in all cisternae of rough endoplasmic reticulum, in elements of the Golgi complex, and in all secretion granules. In contrast, the reaction product of catalase, a marker enzyme for peroxisomes, was found in small (0.2–0.5 μ) particles which resemble small peroxisomes described in a variety of other tissues (Novikoff and Novikoff, 1972; Hand, 1973; Hruban et al., 1972).

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