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Light microscopic catalase histochemistry in mussel digestive gland tissue

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Summary. Different light microscopical procedures for the histochemical demonstration of catalase were tested in cryostat sections of mussel digestive gland tissue by using both benzidine and diaminobenzidine (DAB) as hydrogen donors. The selected procedure, which was also applied to mouse liver for comparative purposes, consisted of incubation in media containing 0.2% DAB and 0.3% H_2O_2 at pH 10.4 for 35 min at 42 °C. Addition of 0.01 M imidazole to the incubation medium increased the staining intensity of the histochemical procedure. The positive reaction product was localized in epithelial cells lining the digestive tubules and the ducts. The histochemical reaction was inhibited partially by aminotriazole or sodium azide and disappeared completely by omission of H_2O_2 from the incubation medium. On the other hand, heat resistant non-enzymatic reactions were observed in sites known to contain lipofuscins such as epithelial cells of the gastrointestinal tract and connective tissue brown cells.

Key words: Catalase, Histochemistry, Benzidine, Diaminobenzidine, Imidazole, Mussel, Digestive tissue, Light Microscopy

Introduction

The digestive gland of bivalve molluscs consists of primary ducts arising from the stomach, followed by thinner, branched secondary ducts opening into the digestive tubules (Morton, 1983). The digestive tubules are comprised of digestive cells, involved in the intracellular digestion of food material, and basophilic cells, whose functions are still not well understood (Owen, 1970, 1972b, 1973; Pal, 1971; Morton, 1983; Cajaraville et al., 1990a). In addition to duct cells, both digestive and basophilic cells contain peroxisomes identified by using the alkaline 3,3'-diaminobenzidine (DAB) technique (Fahimi, 1969; Novikoff and Goldfischer, 1969) in the bivalves *Nucula sulcata* (Owen, 1972a), *Ruditapes decussatus* (Henry, 1987) and *Mytilus sp* (Cajaraville et al., 1992b). The brief report of Yokota (1970) also indicates that DAB-positive peroxisomes are found in a number of marine bivalve molluscs. Within the gastropod molluscs, Dannen and Beard (1977) find peroxisomes, identified by morphological criteria, in the kidney of two terrestrial pulmonate gastropods, but while peroxisomes of *Arion ater* are reactive to DAB cytochemistry, peroxisomes of *Ariolimax columbianus* are not. Finally, peroxisomes have been detected cytochemically in the nervous system of the marine gastropod *Aplysia californica* (Beard and Holtzman, 1985).

The data quoted above illustrates that few investigations have been coducted on the cyto- and histochemical localization of catalase, the marker enzyme for peroxisomes, in molluscs. The aim of the present study was to apply the methodology of catalase histochemistry to the digestive gland tissue of mussels, *Mytilus galloprovincialis* Lmk. This seems particularly interesting in view of the world-wide use of mussels in pollution monitoring programmes (Bayne, 1989) and preliminary studies have already been conducted on the use of mussel catalase activity as an indicator of damage caused by petroleum hydrocarbon exposure (Di Giulio et al., 1989; Cajaraville et al., 1990b, 1992a; Livingstone et al., 1990).

Materials and methods

Animals

Mytilus galloprovincialis individuals were collected from Meñakoz, Biscay (43°24'N, 2°93'W) and transferred to the laboratory. Individuals (2.5-3.5 cm shell length) were maintained in 25-l polyethylene-covered tanks in aerated thermostatized (15-16 °C) semicontinuous water flow system with activated charcoal and glass-wool filtered natural seawater (Zierbena, Biscay). Mussels were maintained unfed for

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10 days, and afterwards they were fed with a commercial filter-feeder food (Marine Invertebrate Diet, Hawaiian Marine Imports Inc., distributed by Carolina Ltd.).

Male C57BL/6 mice weighing about 25 g were kept on a normal laboratory diet and water *ad libitum*.

Histochemistry

Small pieces of freshly excised mussel digestive gland were frozen using Bright Cryo-spray (dichlorodifluoromethane, -50/-55 °C) and stored at -26 °C. Samples were then placed on aluminium cryostat specimen holders and embedded in Bright Cryo-M-Bed. Sections (9-10 µm) were cut in a Bright cryostat (5030 microtome) at a cabinet temperature of -30 °C. Sections were then collected on glass slides brought from room temperature and stored at -70 °C until required for staining.

The histochemical reaction for catalase using benzidine (Panreac) was demonstrated at pH 7 using 4M H_2O_2 (Panreac) as described by Silveira and Hadler (1978). Briefly, cryostat sections were fixed in a 15% formalin solution containing 3% calcium chloride at 4 °C for 5 h, washed in distilled water and immersed in a 30% ammonium sulphate solution for 3 min. Sections were incubated under continuous agitation for 1 min in a medium containing equal parts of a 1% ethanolic benzidine solution and a 8M H_2O_2 solution in phosphate buffer 0.01M, pH 7. Sections were then washed in distilled water and mounted in glycerine jelly or dehydrated in ethanol and mounted in DPX or Eukitt. The omission of H_2O_2 from the incubation medium was used as control. Sodium azide (0.02M and 0.1M) and 3-amino-1,2,4- triazole (0.1M) were used as inhibitors.

Catalase detection using 3,3'-diaminobenzidine tetrahydrochloride (Sigma) was carried out on cryostat sections fixed either in Baker's formol calcium with 2.5% sodium chloride at 4 °C for 10 min, followed by washing in water with 2.5% sodium chloride for 2 min, or in 4% formaldehyde in 0.1 M phosphate buffer, pH 7.4 with 1% calcium chloride at 4 °C for 10 min, 2.5 h, 5 h or 24 h, followed by washing in the same buffer (Van Bogaert et al., 1981).

Sections were incubated in freshly prepared medium with DAB dissolved in 0.05M tris-HCl buffer, pH 9 or pH 10.4. The pH was checked and adjusted to the desired value with 0.1M NaOH after the addition in the dark of DAB to concentrations of 0.2% or 0.36% and H_2O_2 to concentrations of 0.3% or 1.5%. The DAB staining was intensified by the addition of 0.01M or 0.1M imidazole (Sigma) to the DAB medium. Incubation was performed in the dark at about 42 °C with preheated solutions and recipients and lasted for 35 or 60 min. Finally, slides were washed twice in distilled water, dehydrated in graded ethanol and mounted in Eukitt or DPX. Sections were photographed in a Nikon «Optiphot» microscope.

Mouse liver is known to be rich in peroxisomes (Miller, 1964; Goldfischer and Essner, 1970; Roels et al., 1975) and was used in some of the experimental protocols together with mussel digestive gland tissue. Liver sections (9 µm) were cut as described above and fixed in 4% formaldehyde in 0.1M phosphate buffer, pH 7.4 with 1% calcium chloride for 5 h. Liver sections were incubated together with mussel digestive gland sections in the DAB incubation medium containing 0.2% DAB, 0.3% or 1.5% H_2O_2 and 0.01M imidazole at pH 10.4.

Control reactions were carried out in incubation media lacking either H_2O_2 or DAB. Incubation media containing only imidazole (0.01 or 0.1M) and H_2O_2 (0.3%) or only imidazole (0.01 or 0.1M) were used as well. Sections were also incubated in the complete medium plus imidazole together with sodium azide (Merck) at concentrations of 0.02M or 0.1M (Silveira and Hadler, 1978) or 3-amino-1,2,4-triazole (Sigma) at concentrations of 0.01M or 0.1M (Silveira and Hadler, 1978; Beard and Holtzman, 1985). 0.005M or 0.05M KCN was added to the incubation medium to inhibit mitochondrial reactivity (Novikoff et al., 1972). As a control for nonenzymatic reactions, additional tissue sections were heated in water for 10 min at 95 °C to inhibit enzymatic reactions (Novikoff et al., 1972; Roels et al., 1975) and subsequently incubated in the complete DAB medium (with or without imidazole).

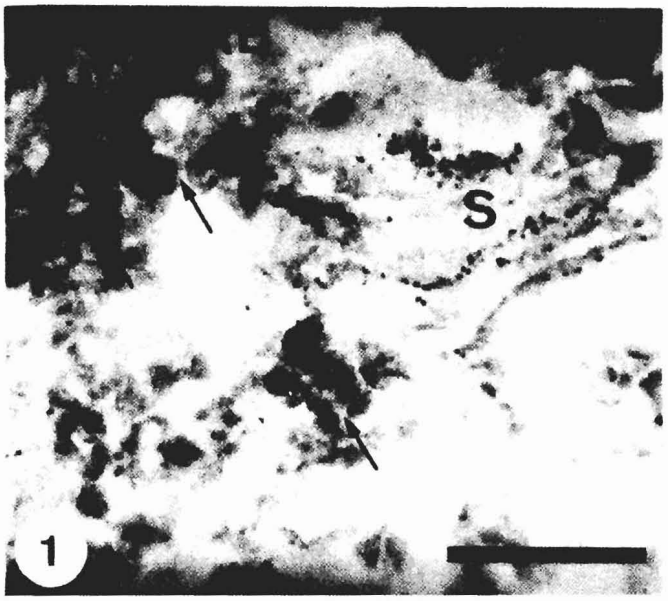
Results

Catalase detection with DAB

Fixation with Baker's formol calcium or formaldehyde for 10 min was not enough for the maintenance of the structural integrity of mussel tissue with the different protocols studied. Therefore, formaldehyde fixation for 2.5 or 5 h was used in subsequent experiments. The results obtained with 24 h fixation were similar to those with 5 h.

Concerning the process of incubation, similar results were obtained with the two concentrations of DAB used (0.2% and 0.36%) at both pH 9 and pH 10.4. In the same way, only slight differences were observed when comparing the different concentrations of H_2O_2 used (0.3% and 1.5%) apart from a less intense background staining when the lowest concentration was used. The lower DAB and H_2O_2 concentrations (0.2% and 0.3% respectively) were thus selected, since it also allowed an easier pH adjustment. Incubation for 35 min was enough to obtain a good contrast of stain and there was no difference between mounting the DAB-treated material in DPX or Eukitt.

Oxidized DAB resulting from this method was deposited within certain cell types of mussel digestive gland (Fig. 1). Great accumulations of strongly reactive bodies were located in the basal or mid-cytoplasm of cells comprising the stomach and the intestine (Fig. 1). The location and morphology of these positive bodies was similar to that of pigment granules or lipofuscins described in other bivalve molluscs (Zacks, 1955).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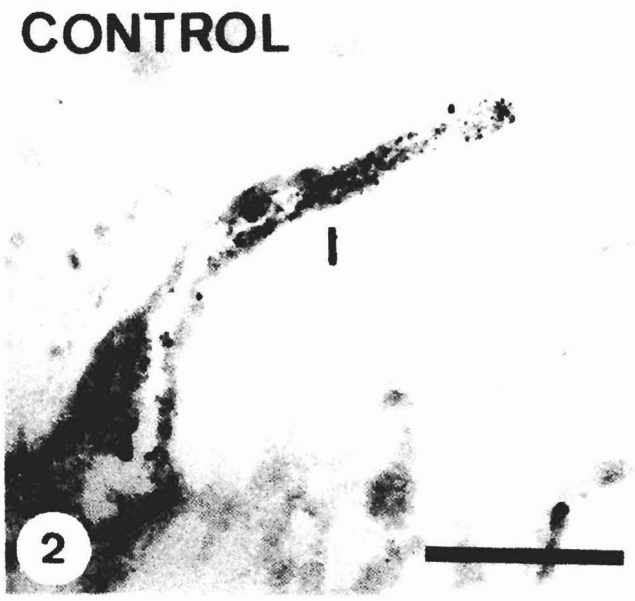
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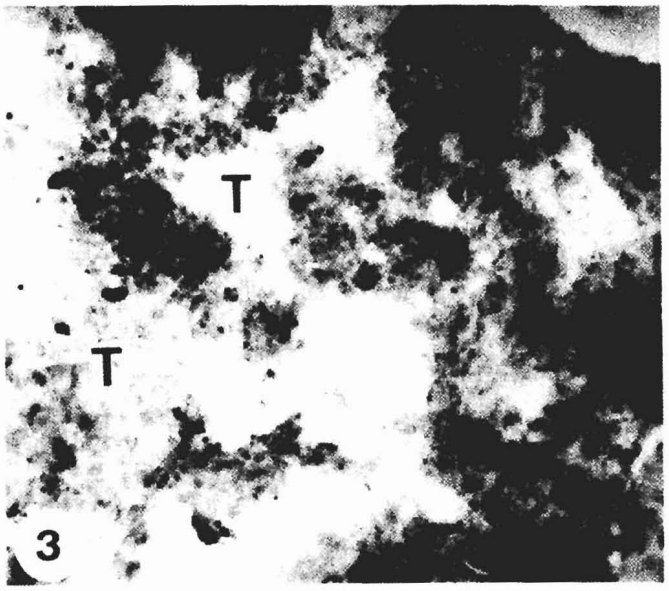
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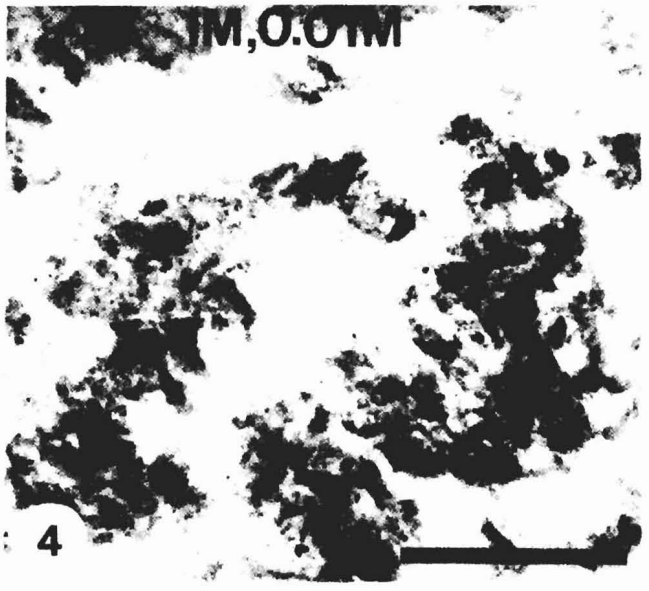
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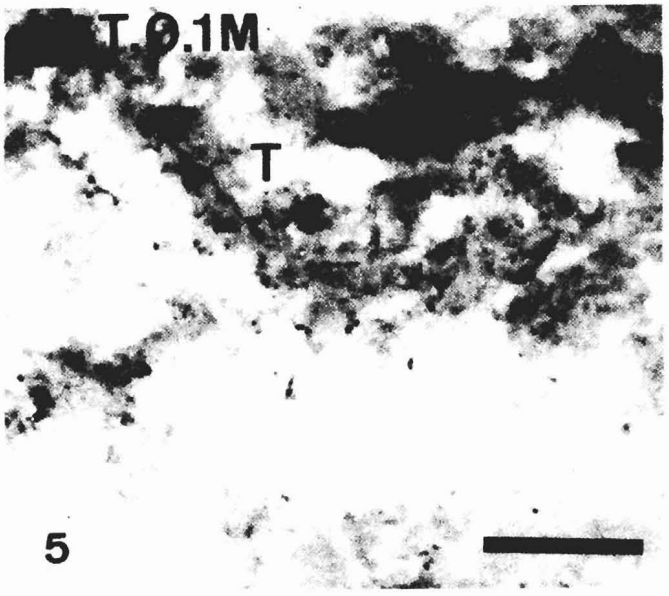
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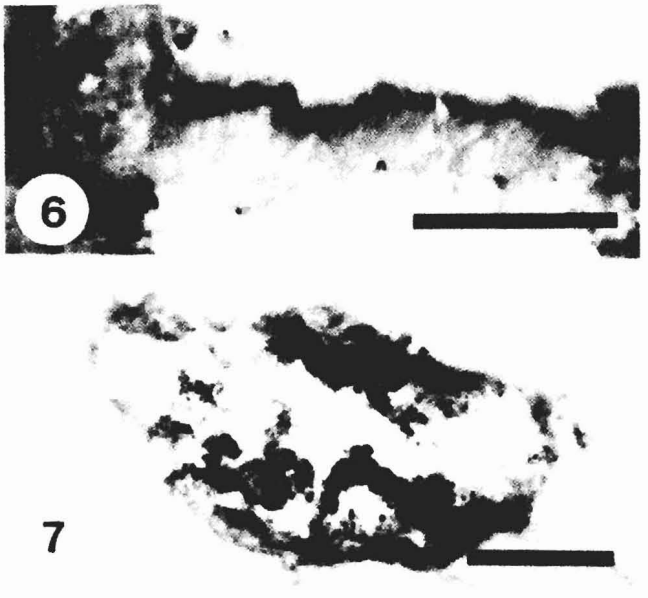
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Table 1. Fixation and incubation conditions selected for the histochemical demonstration of catalase in cryostat sections of freshly excised digestive gland tissue of mussels.

FIXATION CONDITIONS	
4% Formaldehyde in 0.1M phosphate buffer pH 7.4, with 1% $CaCl_2$ at 4 °C for 5 h.	
INCUBATION CONDITIONS	
pH	10.4
DAB	0.2% (5.55×10^{-3}M)
H_2O_2	0.3% (0.1M)
Imidazole	0.01M
T	42 °C
Time	35 min

Furthermore, this reaction was observed even in sections incubated without H_2O_2 (Fig. 2).

The epithelial cells of the digestive tubules showed a number of DAB-positive granules distributed through the whole cytoplasm (Fig. 3). Reaction product was also discernible in the apex of cells comprising the epithelium of the collecting ducts. Among the cells found in the connective tissue, brown cells showed strong DAB positive reactivity. Very small positive granules also appeared in association with collagen fibres in the connective tissue. Apart from these granular brown DAB precipitates, a yellowish diffuse reaction was observed in the different cell types (Figs. 1, 3, 4).

Addition of imidazole to the incubation medium increased both the reaction intensity and contrast of all DAB-positive sites (compare Figs. 3, 4). The lowest concentration of imidazole (0.01 M) was preferred since some large dark brown precipitates appeared when 0.1M imidazole was used.

When KCN (0.005M and 0.05M) was added to the incubation medium in order to inhibit mitochondrial cytochrome oxidase activity, the results did not differ from those described above. On the other hand, neither sodium azide nor aminotriazole produced a complete inhibition of the enzyme activity (Fig. 5). The addition of 0.1M aminotriazole to the incubation medium resulted in a less intense DAB reaction of cells comprising the digestive epithelium (Fig. 5). However, the positive bodies associated with lipofuscins in the stomach epithelium and in the brown cells were not responsive to the treatment with sodium azide or aminotriazol at any of the concentrations tested (Figs. 6, 7).

The omission of H_2O_2 from the incubation medium abolished the appearance of positive reaction product in both the digestive gland tubules and the ducts (Fig. 2). Reaction product remained in places where lipofuscins were present, such as within the cells of the gastrointestinal tract epithelium and within brown cells (Fig. 2). The small positive granules distributed through the connective tissue were negative. Results using the medium with DAB plus imidazole and without H_2O_2 were similar to those obtained with the DAB medium without H_2O_2. In addition, no reaction was seen in heat-treated sections except in lipofuscin granules of stomach cells and connective tissue brown cells. Imidazole did not stain the different tissues on its own, as demonstrated using incubation media containing either only imidazole or only imidazole and H_2O_2.

The procedure selected for catalase demonstration (Table 1) was tested in mouse liver cryostat sections with comparative purposes (Figs. 8, 9). At low magnifications, the staining was observed to be stronger in the perivenous region than in the periportal or midzone regions of the liver lobule (Fig. 8). At higher magnification, a number of brown discrete granules were present in the cytoplasm of hepatocytes (Fig. 9). The deposition of the reaction product was not completely inhibited by the addition of 0.1M aminotriazole (Figs. 10, 11). Reaction product disappeared when the incubation medium lacked H_2O_2 (Fig. 12).

Catalase detection with benzidine

Stomach cells showed greenish reaction product deposits mainly associated with lipofuscins (Fig. 13).

Plate I. Light micrographs of cryostat sections of mussel digestive gland tissue fixed with 4% formaldehyde in 0.1M phosphate buffer, pH 7.4, with 1% $CaCl_2$ for 5 h at 4 °C and incubated for catalase with the DAB procedure with 0.3% H_2O_2 at pH 10.4 for 35 min at 42 °C.

Fig. 1. Note the strong reactivity in the basal portion of the stomach (S) epithelial cells and in cells of the digestive tubules (arrows). Scale bar= 300 μm.

Fig. 2. Incubation in the DAB medium lacking H_2O_2. Reaction product remains within the intestinal epithelium (I), while no positivity is observed in digestive tubules. Scale bar= 300 μm.

Fig. 3. Note the small positive granules found within the epithelium of the digestive tubules (T). Apart from these brown granular DAB precipitates, a diffuse reaction is also observed in the cytoplasm of digestive cells. Scale bar= 40 μm.

Fig. 4. The DAB reaction has been intensified with the addition of 0.01M imidazole (IM) to the incubation medium. Note the increased reaction intensity of granules within the digestive tubules. Scale bar= 200 μm.

Fig. 5. 0.1 M aminotriazole (AT) has been added to the incubation medium. Positive granules are still evident in the epithelium of the digestive tubules (T). Scale bar= 100 μm.

Fig. 6. Incubation as in Fig. 5. Lipofuscins in the apex of duct cells remain reactive. Scale bar= 100 μm.

Fig. 7. Incubation as in Fig. 5. Note the strong reactivity of lipofuscins within the intestinal epithelium. Scale bar= 100 μm.

Green positive granules were also seen in brown cells of the connective tissue. A light blue diffuse reaction was observed in cells of the digestive tubule epithelium (Fig. 14) with reactive bodies being smaller and much more numerous than those stained with the DAB-catalase procedure.

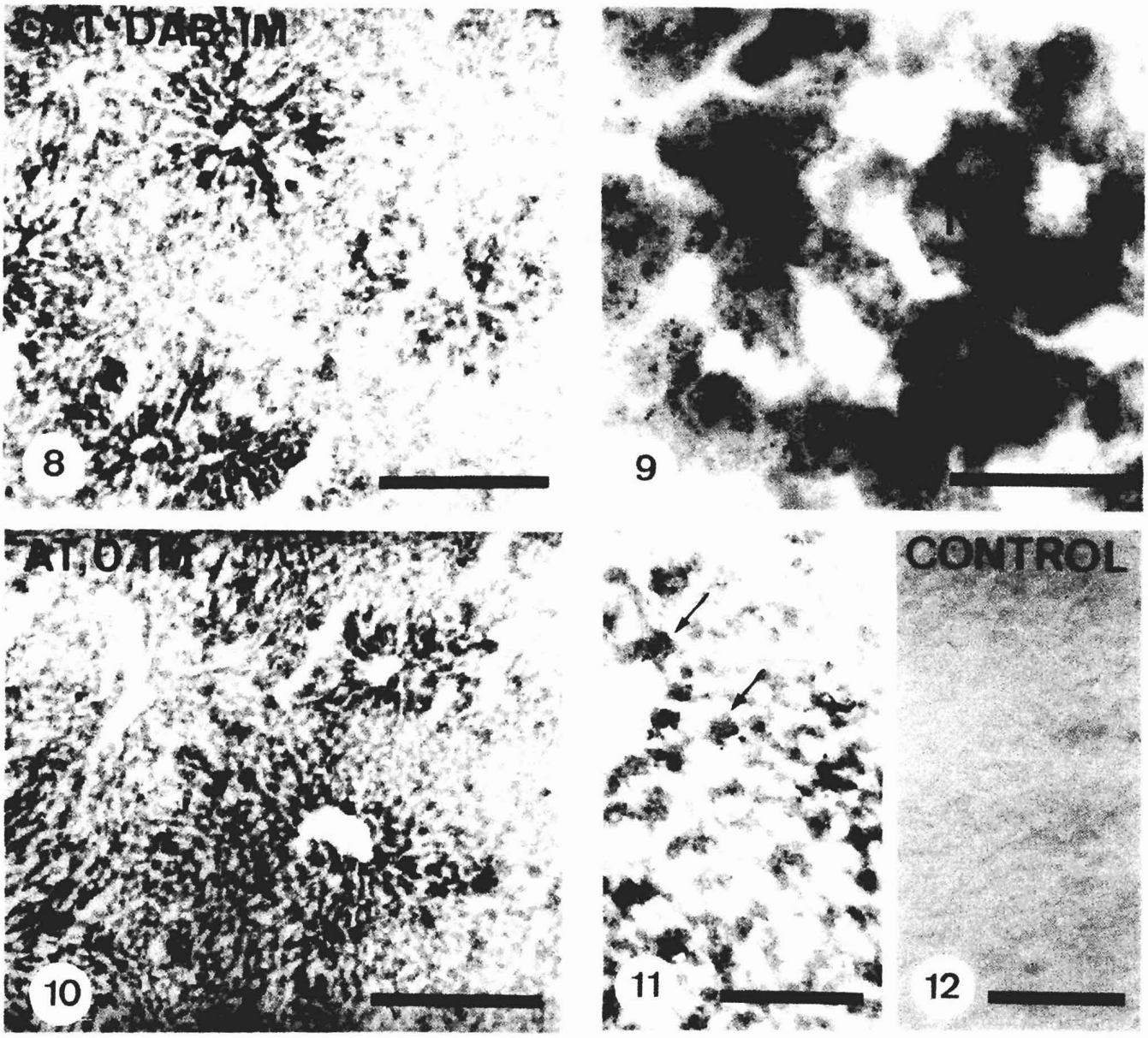

Plate II. Light micrographs of cryostat sections of mouse liver fixed as in Figs. 1-7 and stained for catalase with the DAB plus imidazole procedure.

Fig. 8. The staining is stronger in the perivenous region than in the periportal or midzone regions of the liver. Scale bar= 300 μm.

Fig. 9. Higher magnification micrograph showing the numerous positive granules present in the cytoplasm of hepatocytes. N: nucleus. Scale bar= 30 μm.

Fig. 10. Low power view of the liver showing the slightly decreased intensity of the reaction after addition of 0.1M aminotriazole (AT) to the incubation medium. Scale bar= 300 μm.

Fig. 11. Incubation as in Fig. 10. At higher power, some positive granules (arrows) are still observed within the hepatocytes. Scale bar= 100 μm.

Fig. 12. Incubation in the DAB medium lacking H_2O_2. Liver tissue appears completely negative and no reaction is observed within the hepatocytes. Scale bar= 100 μm.

There was a quick loss of reaction product which was clearly appreciable 3 h after mounting the slides. 12 h later reaction product only remained in sections mounted in DPX, and even in the DPX-mounted material only the stomach showed some reaction product after 2 days (Fig. 15).

When H_2O_2 was absent from the incubation medium no reaction product was seen, except in cells bearing lipofuscins. Similarly, both aminotriazole and sodium azide (0.1M) inhibited to some degree the formation of reaction product in all positive sites except in brown cells and stomach cells (Fig. 16).

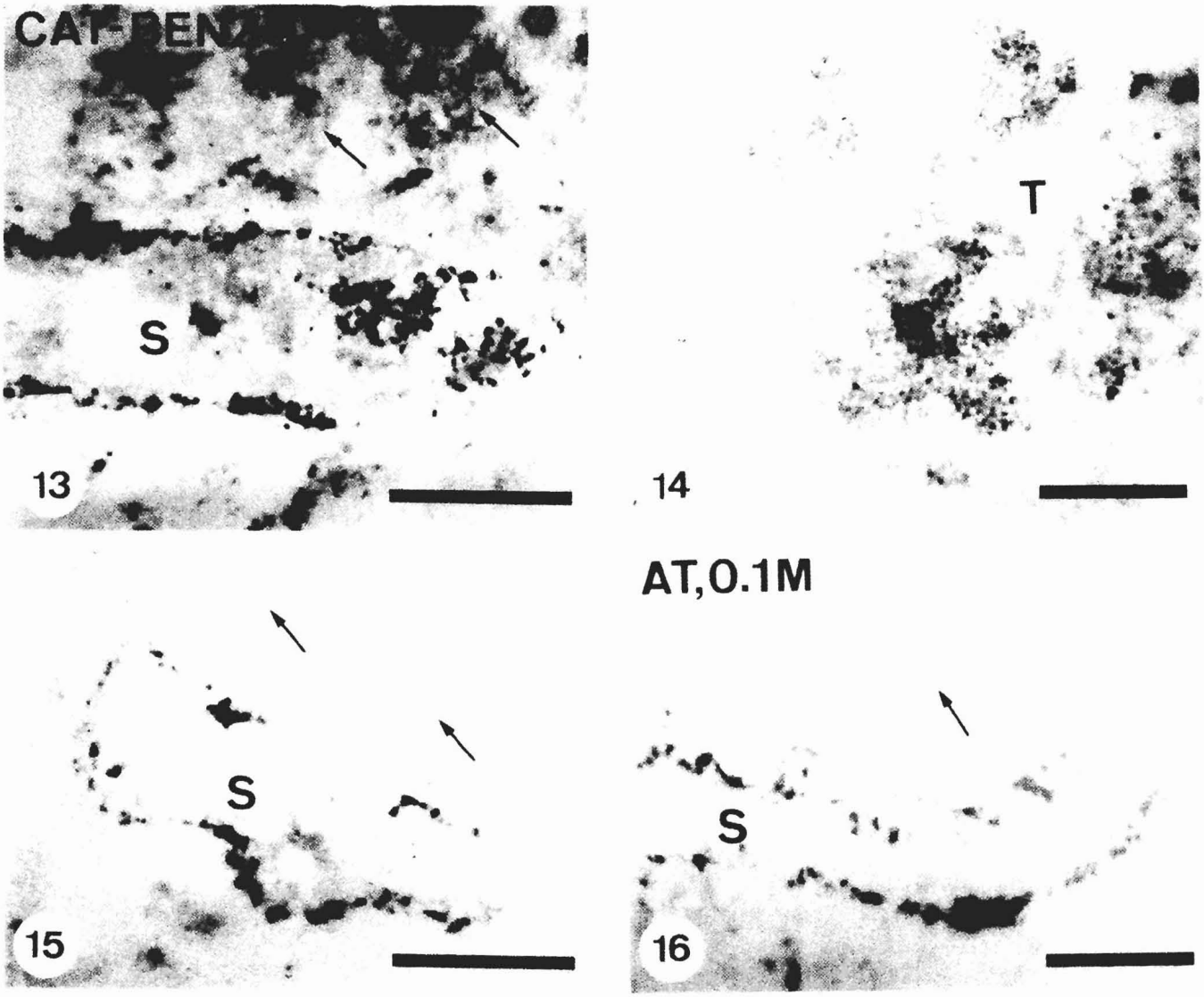

Plate III. Light micrographs of cryostat sections of mussel digestive gland tissue fixed as in Figs. 1-7 and stained for catalase with the benzidine procedure of Silveira and Hadler (1978).

Fig. 13. Photograph taken immediately after mounting the slides in glycerine. Stomach cells are strongly reactive in the basal portion and positive reaction product is also deposited in digestive tubules (arrows). S: stomach. Scale bar= 300 μm.

Fig. 14. Photograph taken immediately after mounting the slides in DPX. Numerous positive granules are observed in cells comprising the epithelium of the digestive tubules (T). Scale bar= 50 μm.

Fig. 15. Photograph taken two days after mounting the slides in DPX. Digestive tubules (arrows) appear unstained while the lipofuscins of the stomach cells (S) are still very reactive. Scale bar= 300 μm.

Fig. 16. 0.1M aminotriazole (AT) has been added to the incubation medium and the photograph has been taken immediately after mounting the slides in DPX. No reaction product is observed in digestive gland tubules (arrows). S: stomach. Scale bar= 200 μm.

Discussion

In the present study different light microscopical procedures for the histochemical demonstration of the peroxidatic activity of catalase have been tested in mussel digestive gland tissue. Catalase has been demonstrated by using both benzidine and DAB as hydrogen donors. Although the brown reaction product with DAB showed a lower colour contrast than the blue reaction product with benzidine, the procedure of Silveira and Hadler (1978) seems less useful since stability of the reaction product lasted only for few hours. In addition, some tissue damage was observed with this method, possibly due to the high concentration of H_2O_2 used (4M).

The staining pattern of digestive tubules and ducts was different with the two procedures, possibly indicating that different structures or cell organelles were stained. The blue reaction product of benzidine was diffuse and filled the whole cell, suggesting that the benzidine procedure, carried out at neutral pH (Silveira and Hadler, 1978), demonstrates other peroxidases in addition to catalase in mussel digestive gland tissue. On the other hand, the DAB reaction product was deposited in the form of discrete brown granules consistent with a peroxisomal localization of catalase in molluscan tissues (Yokota, 1970; Owen, 1972a; Dannen and Beard, 1977; Beard and Holtzman, 1985; Henry, 1987; Cajaraville et al., 1992b). An additional yellowish diffuse reaction was also observed in the different cell types stained with DAB. This possibly represents an artefactual cytoplasmic staining due to diffusion of catalase from peroxisomes. This may be related to the fact that frozen sections were obtained from unfixed tissues. Although extraperoxisomal catalase has been demonstrated in some mammalian species (Roels, 1976; Roels et al., 1977; Yamamoto et al., 1988), the possibility of an extraperoxisomal localization of catalase in mussel cells does not seem plausible since mouse liver cells, used here as positive controls and known to contain catalase only in peroxisomes, showed the same diffuse reaction pattern.

Since Graham and Karnovsky (1966) introduced the use of DAB as an organic hydrogen donor for peroxidase reactions, there have been several attempts to improve the sensitivity of the methods which use this reactive (De Bruijn et al., 1986; Scopsi and Larsson, 1986; Nemes, 1987; Halbhuber et al., 1988; Gossrau et al., 1989; Merchenthaler et al., 1989) and to allow the distinction between the different organelles and enzymes where peroxidase activities are located (Novikoff and Goldfischer, 1969; Fahimi, 1969; Roels et al., 1975; Herzog and Fahimi, 1976; Angermüller and Fahimi, 1981). Some of the conditions used by the authors mentioned above have been modified in the present work. We used an incubation temperature of 42 °C since both high temperature and pH seems to stimulate DAB staining of peroxisomes (Goldfischer and Essner, 1970; Roels et al., 1975). The DAB concentration was reduced from 0.36% to 0.2% to minimize background staining and to increase the contrast of the brown deposits of the reaction product. Different H_2O_2 concentrations have been used in the literature for the demonstration of catalase activity: 0.01% (Kerckaert et al., 1989), 0.02% (Novikoff and Goldfischer, 1969), 0.035-0.1% (Roels et al., 1975), 0.05% (Novikoff et al., 1972), 0.06% (Goldfischer and Essner, 1969; Geerts and Roels, 1981), 0.15% (Angermüller and Fahimi, 1981), 0.3% (Herzog and Fahimi, 1976), 12% (Silveira and Hadler, 1978). Since peroxidase activity is reported to be inhibited when concentrations of H_2O_2 higher than 0.03% (Herzog and Fahimi, 1976) or 0.15% (Silveira and Hadler, 1978) are used, a concentration of 0.3% was selected in the present work for catalase demonstration. The use of higher concentrations gives similar results, but tissue damage is also increased. Herzog and Fahimi (1976) also found that a 0.3% concentration of H_2O_2 was optimal for catalase demonstration in lacrimal gland of the rat when a 0.36% DAB concentration was used. No postosmication (Graham and Karnovsky, 1966) was carried out for catalase demonstration, since although the staining intensity is enhanced, sensitivity is not increased (Scopsi and Larsson, 1986). The omission of the osmication step renders a less time consuming procedure with considerable lower costs. This is particularly interesting in view of the huge numbers of specimens to be handled in marine pollution monitoring programmes.

The pH of 10.5 used for the incubations has been shown to be optimal for catalase in a wide spectrum of plant and animal tissues and prevents the simultaneous staining of peroxidase and of mitochondrial oxidases (see review by Deimann et al., 1991). However, although mitochondria stain optimally at pH 6, some reaction product is also found at alkaline pH (Novikoff et al., 1972) and different KCN concentrations are commonly used in order to inhibit mitochondrial reactivity. Our results showed that the distribution and intensity of DAB positive bodies were not changed when 0.005M or 0.05M KCN was added to the incubation medium, which indicates that mitochondrial cytochrome oxidases are not reactive under our experimental conditions. Therefore, the alkaline DAB method used in the present work seems adequate for the selective demonstration of catalase in mussel digestive gland tissue. However, we found no evidence of complete inhibition of the reaction when using the catalase-specific inhibitor aminotriazole. Total inhibition of histochemically demonstrable catalase by aminotriazole has been reported in tissues as different as rat liver (Novikoff and Goldfischer, 1969; Angermüller and Fahimi, 1981), amphibian pancreas (Trandaburu, 1980), digestive tissue of bivalve molluscs (Owen, 1972a) nervous tissue of a marine gastropod (Beard and Holtzman, 1985) and kidney of a terrestrial gastropod (Dannen and Beard, 1977). The negative results obtained in the present work may be related to the fixative used since the presence of substances able to be oxidized by the H_2O_2 -catalase complex (i.e., formaldehyde) prevent

the irreversible catalase inhibition by aminotriazole (Margoliash et al., 1960). In addition, the inhibitory effect of aminotriazole seems to occur only at low H_2O_2 concentrations (Margoliash and Novogrodsky, 1958).

Although Halbhuber et al. (1988) have reported that addition of imidazole does not increase the amount of the final reaction product in the Ce-H_2O_2-DAB procedure, several studies have used imidazole to enhance the staining intensity and the sensitivity of the DAB reaction product at neutral pH (Simionescu et al., 1975; Malmgren and Olsson, 1978; Straus, 1979, 1982; Scopsi and Larsson, 1986; Cornelese-ten Velde et al., 1989). No increased staining intensity has been observed at acid pH (Straus, 1982). The present work demonstrates for the first time that addition of imidazole is useful for DAB histochemistry at alkaline pH. The addition of imidazole to the incubation medium yields darker reaction product precipitates thus improving the histochemical detection of the enzyme. Straus (1982) reports that addition of 0.01M imidazole is as effective as the addition of 0.1M imidazole for the histochemical demonstration of peroxidase at neutral pH. The lower concentration has been preferred in the present work since no additional contrast but higher background staining is obtained with the higher concentration. The molecular mechanism underlying the stimulation by imidazole is unknown at present but may involve the enhancement of catalase activity. Imidazole, in its unprotonated form, is able to bind cytochrome c probably by displacing the methionine-80 sulphur from iron coordination in reactions with 1:1 stoichiometry (Schejter and Aviram, 1969; Sutin and Yandell, 1972). This fact is suggested to explain the enhancement of the peroxidase activity of the cytochrome c-derived heme-peptides by imidazole (Simionescu et al., 1975). A second hypothesis could be that imidazole forms a complex with the oxidized DAB. The possibility that imidazole acts as a hydrogen donor for the peroxidase reaction should be discarded since imidazole is present in its unprotonated form under our experimental conditions (pK 6.95 for ionization of imidazole).

In conclusion, catalase is histochemically demonstrable in cryostat sections of mussel digestive gland tissue by a modification of the alkaline DAB procedure of Fahimi (1969). Of the modifications tested, incubation in media containing 0.01M imidazole, 0.2% DAB and 0.3% H_2O_2 at pH 10.4 for 35 min at 42 °C seems appropriate for future studies. The positive reaction product is localized in epithelial cells lining the digestive tubules and the collecting ducts. Sites known to contain lipofuscins, such as the cells lining the gastrointestinal tract and the brown cells of bivalves (Zacks, 1955; Ruddel and Wellings, 1971), stain very intensely with the different DAB procedures and also with the benzidine technique. These products are heat-resistant, insensible to inhibition and they even appear when there is no H_2O_2 in the incubation medium. We therefore suggest that this reaction is non-enzymatic. Dannen and Beard (1977) have also reported that oxidized DAB is deposited in residual lipid bodies of the kidney of two gastropod molluscs and that the reaction is unaffected by heating to 60 °C or by the presence of aminotriazole. Heat resistant non-enzymatic reactions are elicited by haemoglobin, myoglobin and lipofuscins in a variety of mammalian tissues stained with both DAB (Novikoff et al., 1972; Roels et al., 1975) and benzidine (Straus, 1964; Silveira and Hadler, 1978). These results have been associated with the peroxidase activity of haemoglobin and other iron-containing catalysts in the cells (Straus, 1964; Goldfischer and Schiller, 1971; Silveira and Hadler, 1978) which may also be positive without the addition of H_2O_2 to the incubation medium (Roels et al., 1975). In agreement with this notion, the lipofuscin-type pigment present in brown cells of bivalves is known to contain large amounts of iron (Ruddell and Wellings, 1971), and lysosome-derived lipofuscins present in the digestive gland and kidney of bivalves also contain a number of metals including iron (Brown, 1982).

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