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The ultrastructural localization of lysosomal acid hydrolases in developing oocytes of the common marine mussel *Mytilus edulis*

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Summary

Azo dye techniques were used to investigate the ultrastructural localization of lysosomal acid hydrolases in ovarian oocytes of the common marine mussel *Mytilus edulis*. The enzymes were arylsulphatase, β-glucuronidase, nonspecific esterase, *N*-acetyl-β-hexosaminidase and acid phosphatase. For arylsulphatase, the azo dye technique was compared with an alternative method using nitrocatechol sulphate as the substrate and barium as the capturing ion. Activity of all the enzymes was found to be associated with the yolk granules and with pinocytotic phenomena which were observed along the basal membrane of developing oocytes. Activity was also found to be associated with resorption of atretic oocytes.

Introduction

The cytochemical demonstration of lysosomal acid hydrolases, principally acid phosphatase, in yolk granules has been reported for a number of molluscs (Bluemink, 1967; Pasteels, 1973; Arni, 1974; Jong-Brink *et al.*, 1983; Pipe, 1985). It appears that yolk utilization is mediated by lysosomal enzymes in fertilized and atretic oocytes (Pasteels, 1973; Masuda & Dan, 1977; Decroly *et al.*, 1979). In molluscs that produce egg cells provided with perivitelline fluid, there are two populations of yolk granules, the first of which can function as primary lysosomes during embryonic development, fusing with phagosomes formed macropinocytotically from the perivitelline fluid. The second population of granules also contains hydrolytic enzymes which are presumed to be involved in the breakdown or transformation of the contents of the granules (Favard & Carasso, 1958; Bluemink, 1967; Bottke, 1973; Jong-Brink *et al.*, 1976). The percentage of yolk granules reacting positively for hydrolytic enzymes appears to be variable according to the degree of maturation of the oocytes (Arni, 1974; Jong-Brink *et al.*, 1983) and increases significantly in degenerating oocytes (Jong-Brink *et al.*, 1976).

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In the marine mussel *Mytilus edulis* the ovarian oocytes react positively for hydrolytic enzymes throughout the reproductive cycle. The levels of activity have been shown to vary according to the particular enzyme and the time of year sampled (Pipe, 1985). In the present study, an azo dye technique (Bowen, 1971) has been modified and used to investigate the ultrastructural localization of a number of acid hydrolases in ovarian oocytes of *M. edulis*. These were arylsulphatase, β-glucuronidase, nonspecific esterase, *N*-acetyl-β-hexosaminidase and acid phosphatase. For arylsulphatase, the azo dye technique has been compared with an alternative method, using nitrocatechol sulphate as the substrate and barium as the capturing ion (Hopsu-Havu *et al.*, 1967).

Materials and methods

Mantle tissue was dissected from mussels collected in April and June from Sharrow Point, Whitsand Bay, Cornwall. Tissue containing oocytes was cut into approximately 1 mm cubes and fixed for 30 min at 4° C in a 0.1 M PIPES-buffered mixture of paraformaldehyde (2%) and glutaraldehyde (2.5%), pH 7.2, containing 2.5% NaCl and 2 mM $CaCl_2$. Tissues were rinsed overnight in 0.1 M PIPES-buffered sucrose (0.75 M), pH 7.2, and cut into thinner slices, transferred to the incubation buffer for 30 min and then reacted for acid hydrolases as outlined in Table 1. Tissues for reaction controls were incubated without substrate. The samples were then rinsed in 0.1 M phosphate buffer, pH 7.4, and postfixed in 1% osmium tetroxide in phosphate buffer, for 1 h, followed by an overnight rinse in 0.1 M phosphate buffer, dehydration to 70% ethanol and overnight infiltration in several changes of LR white resin (London Resin Co. Ltd). Polymerization was carried out in gelatin capsules at 60° C for 24 h. Ultrathin sections were cut on a Reichert Ultracut E ultramicrotome, collected on carbon-coated grids and examined, without counterstaining, in a Philips 300 electron microscope with an accelerating voltage of 60 kV.

Results

Reaction product, which takes the form of very fine, electron dense particles, was found for all the enzymes tested, although not all yolk granules stained positively (Figs. 1–6). There was no apparent difference in localization of the reaction products for the different enzymes, although the density did vary slightly. Reaction product was generally found around the periphery of the yolk granules with very finely scattered particulate deposits throughout the matrix.

Tissues reacted for arylsulphatase using nitrocatechol sulphate as the substrate and

Fig. 1. Yolk granules from *M. edulis* ovarian oocyte reacted for arylsulphatase using nitrocatechol sulphate as the substrate and barium as the capturing ion. Scale bar, 0.5 μm.

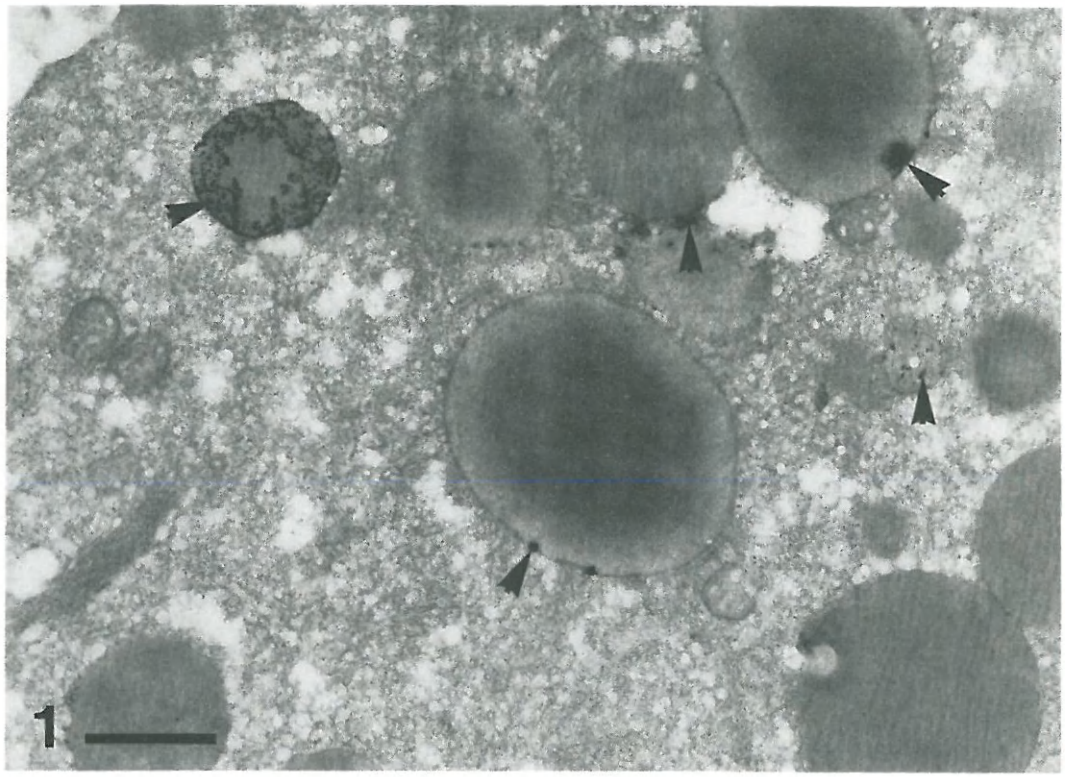
Fig. 2. Yolk granules from *M. edulis* ovarian oocyte reacted for arylsulphatase using naphthol-AS-BI-sulphate as the substrate and *p*-nitrobenzene diazonium tetrafluoroborate as the coupling agent. Scale bar, 0.5 μm.

Fig. 3. Yolk granules from *M. edulis* ovarian oocyte reacted for *N*-acetyl-β-hexosaminidase. Scale bar, 0.5 μm.

Fig. 4. Yolk granules from *M. edulis* ovarian oocyte reacted for β-glucuronidase. Scale bar, 0.5 μm.

Fig. 5. Yolk granules from *M. edulis* ovarian oocyte reacted for acid phosphatase. Scale bar, 0.5 μm.

Fig. 6. Yolk granules from *M. edulis* ovarian oocyte reacted for nonspecific esterase. Scale bar, 0.5 μm. Arrows indicate reaction product.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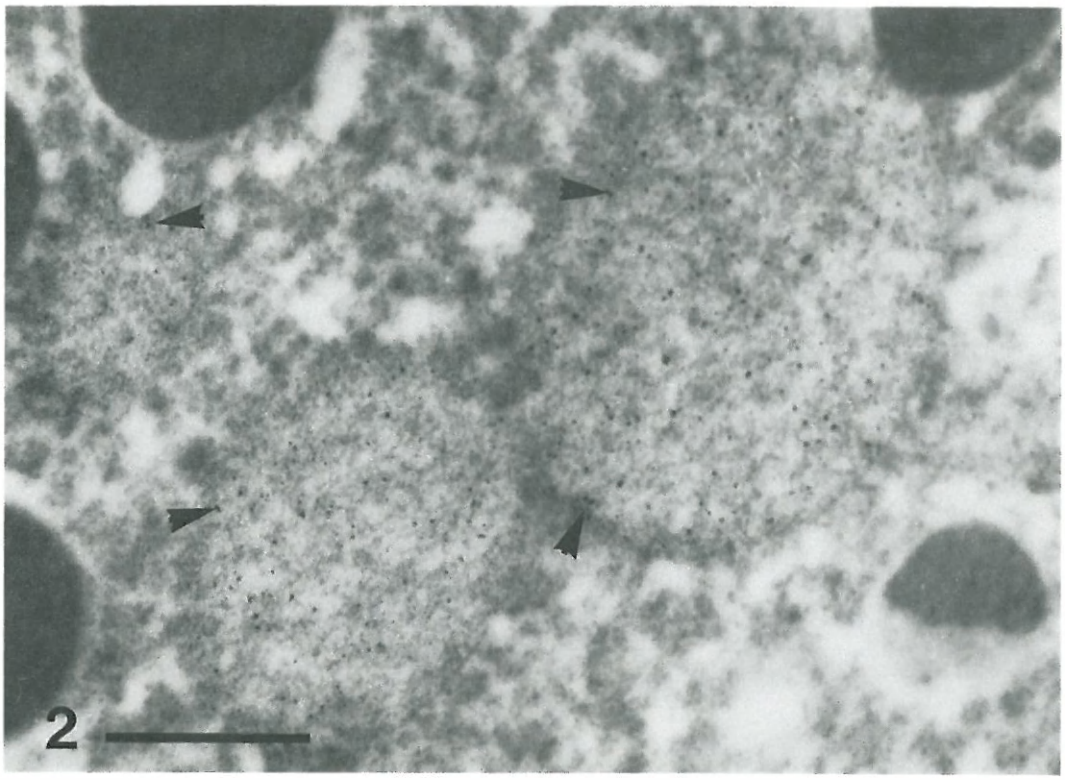
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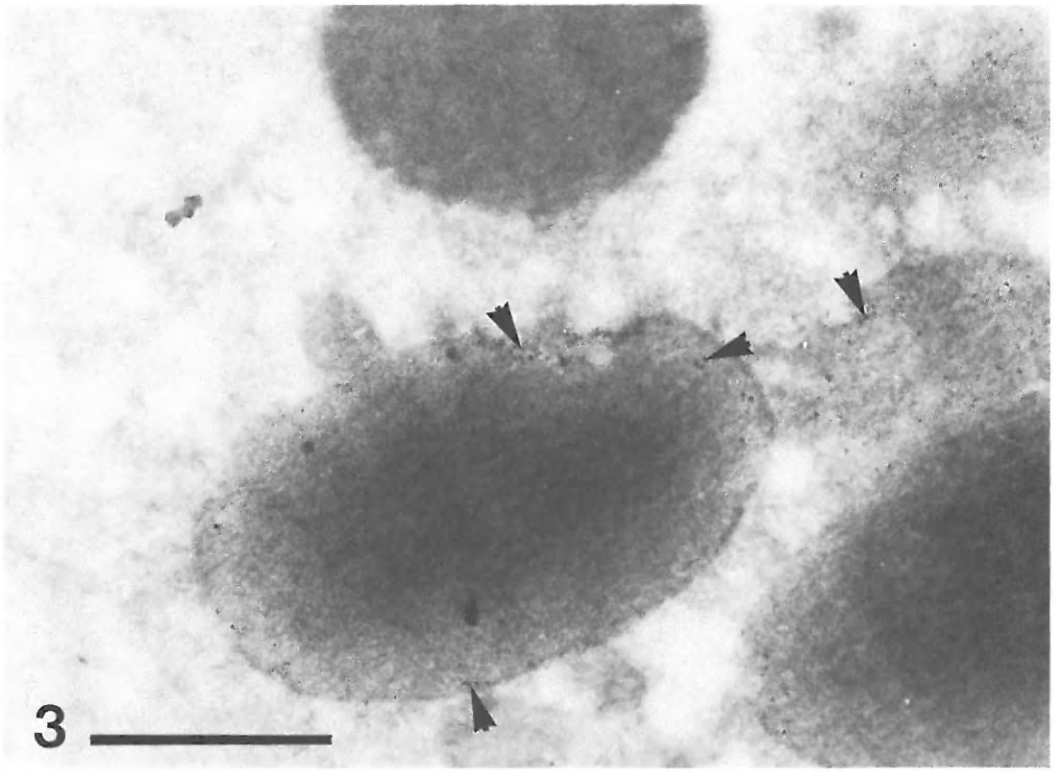
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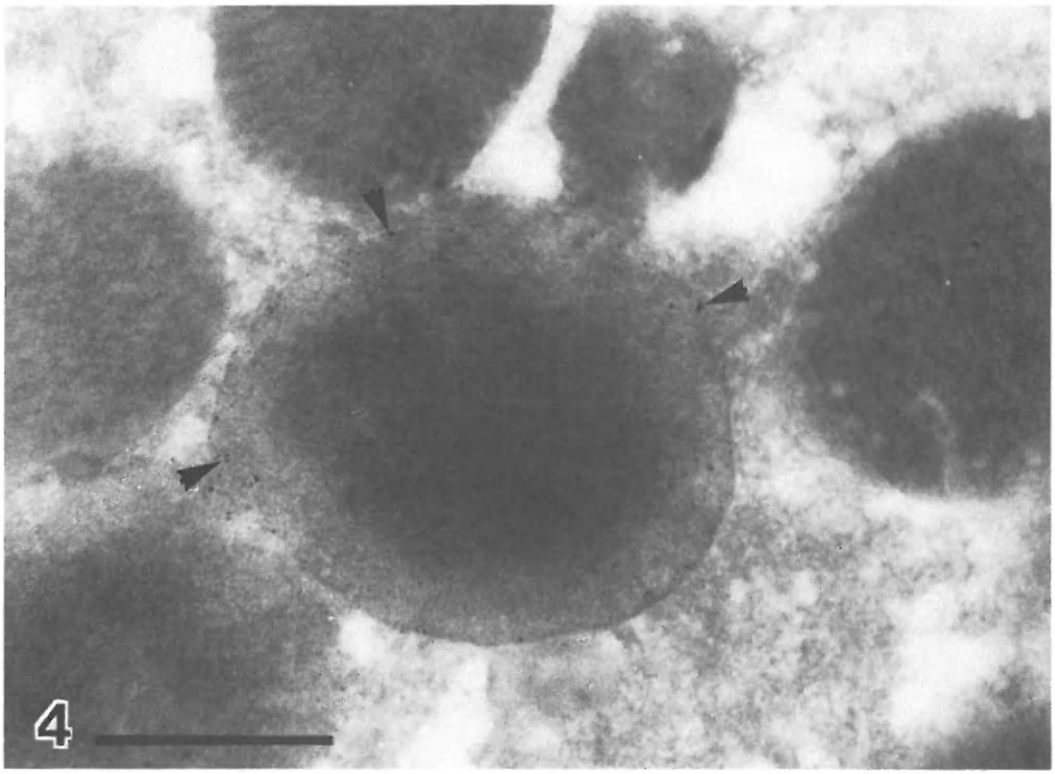
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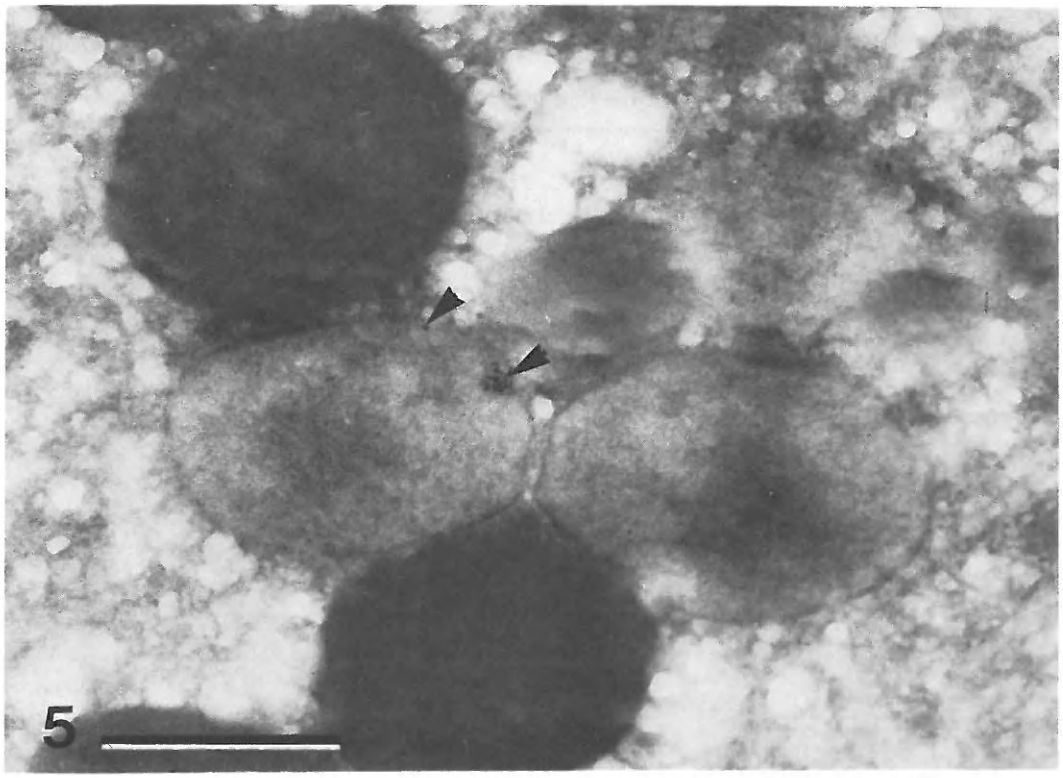
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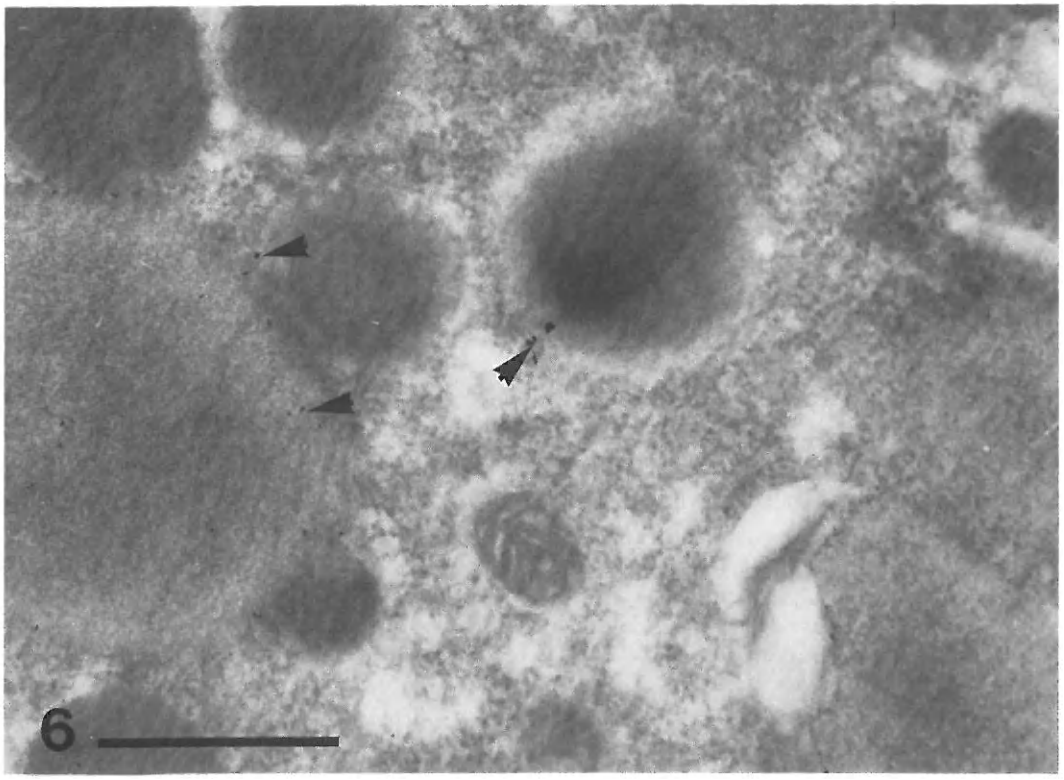
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Table 1. Details of procedures for reacting for lysosomal acid hydrolases.

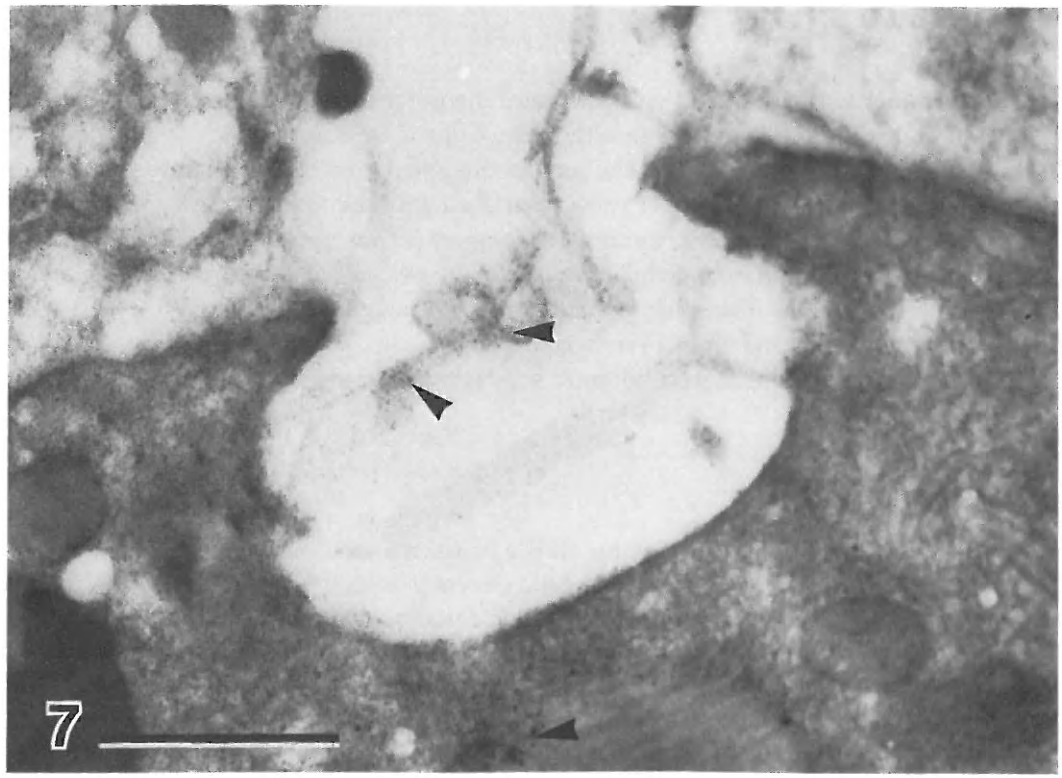
Enzyme	*Schedule*
Acid phosphatase	Incubate at 37° C for 1 h in: 4 mg naphthol-AS-BI-phosphate (Sigma) dissolved in 0.5 ml dimethylformamide, made up to 25 ml with 0.1 M acetate buffer (+ 2.5% NaCl), pH 4.5. Rinse in saline and postcouple in *p*-nitrobenzene diazonium tetrafluoroborate (Kodak) for 30 min at room temperature.
Nonspecific esterase	Incubate at 37° C for 1 h in: 10 mg naphthol-AS-D-acetate (Sigma) dissolved in 0.5 ml dimethylformamide, made up to 25 ml with 0.1 M citrate buffer (+ 2.5% NaCl), pH 5.0. Rinse and postcouple as for acid phosphatase.
N-Acetyl-*β*-hexosaminidase	Incubate at 37° C for 1 h in: 10 mg naphthol-AS-BI-glucosaminide (Sigma) dissolved in 1 ml 2-methoxyethanol, made up to 25 ml with 0.1 M citrate buffer (+ 2.5% NaCl), pH 4.5. Rinse and postcouple as for acid phosphatase.
β-Glucuronidase	Incubate at 37° C for 1 h in: 7 mg naphthol-AS-BI-glucuronide (Sigma) dissolved in 0.5 ml 50 mM $NaHCO_3$ made up to 25 ml with 0.1 M acetate buffer (+ 2.5% NaCl), pH 4.5. Rinse and postcouple as for acid phosphatase.
Arylsulphatase	Incubate at 37° C for 1 h in: 30 mg naphthol-AS-BI-sulphate (Sigma) dissolved in 1 ml 2-methoxyethanol, made up to 25 ml with 0.1 M acetate buffer (+ 2.5% NaCl), pH 5.5. Rinse and postcouple as for acid phosphatase.
Arylsulphatase	Incubate at 37° C for 45 min in: 120 mg *p*-nitrocatechol sulphate (Sigma) dissolved in 4 ml distilled water plus 12 ml 0.1 M acetate buffer pH 5.5, plus 4 ml 5% $BaCl_2$ solution. Rinse in 0.1 M acetate buffer, pH 5.5, for 1 min followed by 3% acetic acid for 2 min and a final rinse in 0.1 M acetate buffer.

barium as the capturing ion showed considerably heavier deposition of reaction product than those reacted using naphthol AS-BI substrate and *p*-nitrobenzene diazonium tetrafluoborate as the coupling agent (compare Figs. 1 and 2).

Pinocytotic phenomena were observed along the basal membrane of developing oocytes and tissues reacted for arylsulphatase using nitrocatechol sulphate–barium showed reaction product associated with the pinocytotic phenomena (Figs. 7 and 8). This

Fig. 7. Pinocytosis occurring along the basal membrane of a developing oocyte from *M. edulis* reacted for arylsulphatase using nitrocatechol sulphate as the substrate and barium as the capturing ion. Scale bar, 0.5 μm.

Fig. 8. Pinocytotic vesicle in developing oocyte of *M. edulis* reacted for arylsulphatase using nitrocatechol sulphate as the substrate and barium as the capturing ion. Arrows indicate reaction product. PV, pinocytotic vesicle. Scale bar, 0.5 μm.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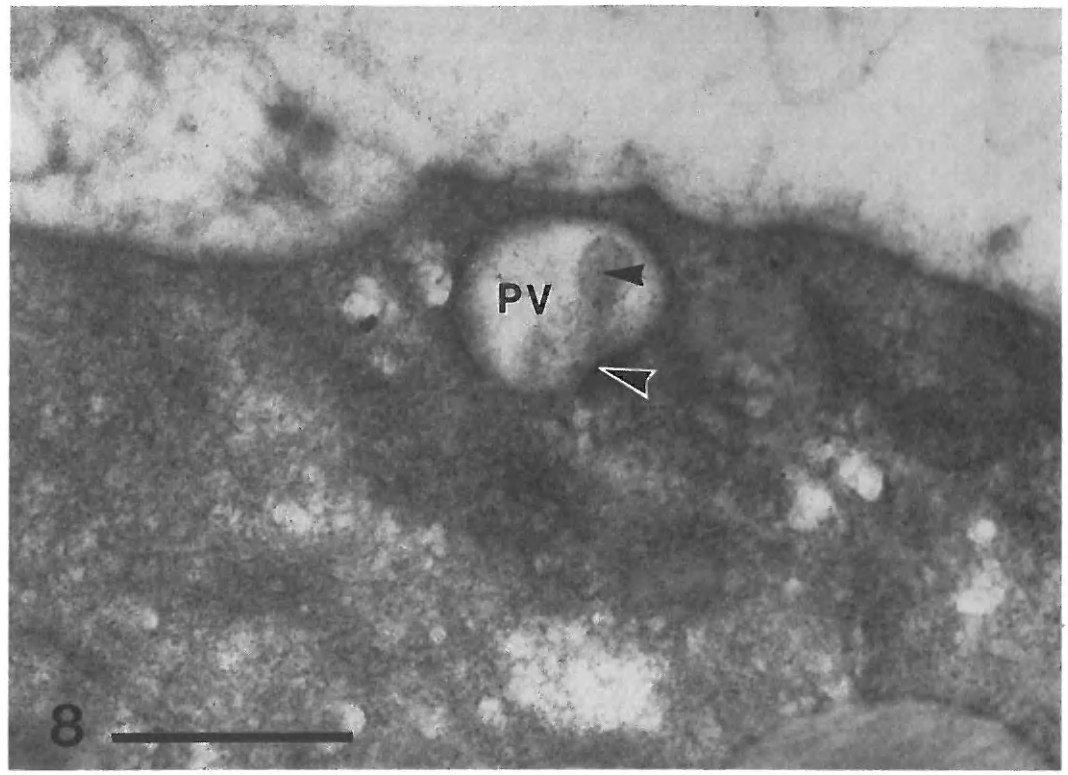
PV
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reaction product was associated with the basal membrane, with the particulate material being pinocytosed, and with the resultant pinocytotic vesicle.

Resorption of atretic oocytes by the surrounding acinar epithelial cells was observed. Heavy deposits of reaction product were associated with the resorption (Figs. 9 and 10). Dense reaction product for all enzymes was located within the lysosomes of the acinar epithelial cells (Fig. 9) and extracellular deposits were associated with the microvilli of the epithelial cells (Fig. 10). The yolk granules from the degenerating oocytes were also positively reactive around their periphery.

The control tissues, incubated without substrate, were found to be negative for all enzymes.

Discussion

The results show positive reactions for all the lysosomal enzymes tested. This uniform presence of a number of lysosomal hydrolytic enzymes associated with the yolk granules of *M. edulis* emphasizes that they can be considered as a form of lysosome.

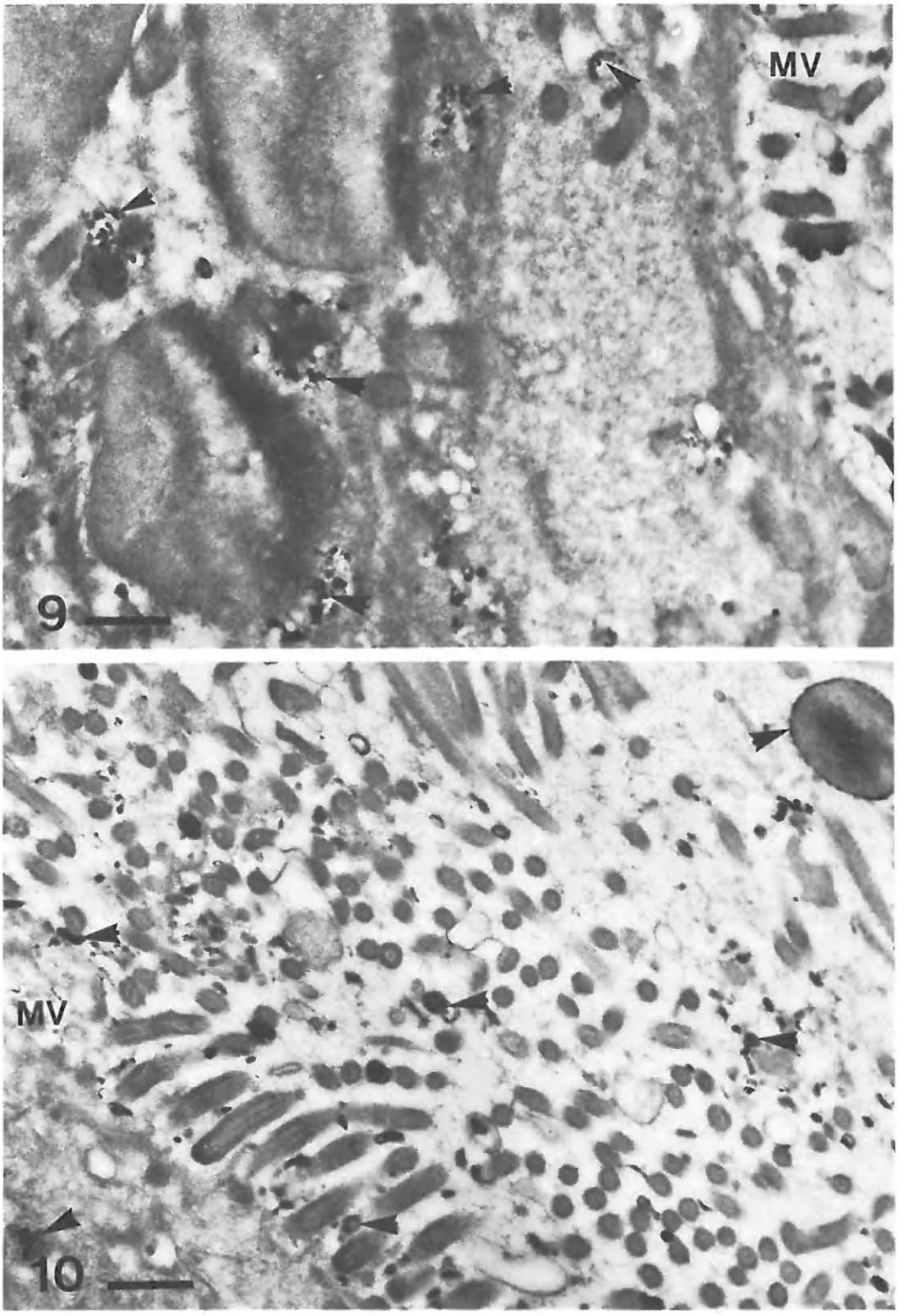
The main drawback to the use of the azo dye technique for ultrastructural localization of the activity of acid hydrolases in oocytes is that, due to the very fine nature of the deposited reaction product, low power observations are not possible. Where enzyme activities are very high, as in slug digestive gland and rat liver (Bowen, 1971), this does not appear to be a problem. Extending the incubation times would probably have resulted in increased azo dye deposition (Bowen, 1971).

The function of acid hydrolases in yolk granules during embryogenesis has been established for some species, in particular those with perivitelline fluid (see Jong-Brink *et al.*, 1983). The role, however, of acid hydrolases in ovarian oocyte yolk granules has not been established; the question has been posed as to whether the lysosomal enzymes in yolk granules are present throughout oogenesis in a latent form or whether their production in the cytoplasm and transfer into yolk granules is a part of the maturation process (Pasteels, 1973; Dohmen, 1983; Jong-Brink *et al.*, 1983). In the present study, acid hydrolase activity was found to be associated with pinocytotic phenomena observed in developing oocytes, indicating possible hydrolysis of macromolecules before and during incorporation into the pinocytotic vesicles. Pinocytotic vesicles give rise to yolk granules in developing oocytes of mosquitoes (Roth & Porter, 1964), while in the toad *Xenopus laevis* and some species of annelid worm there is fusion of pinocytotic vesicles with yolk granules during oogenesis (Olive & Clark, 1978; Wallace *et al.*, 1983). A similar situation

Fig. 9. Acinar epithelial cells from mantle tissue of *M. edulis* reacted for arylsulphatase using nitrocatechol sulphate as the substrate and barium as the capturing ion. Scale bar, 0.5 μm.

Fig. 10. Resorption of oocyte material by acinar epithelial cells in mantle tissue of *M. edulis* reacted for arylsulphatase using nitrocatechol sulphate as the substrate and barium as the capturing ion. Scale bar, 0.5 μm.

Arrows indicate reaction product. MV, microvilli.


MV
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MV
10

cannot be ruled out for *M. edulis* with the concomitant possibility of acid hydrolase involvement in yolk granule formation.

Unpublished observations (Pipe) indicate that pinocytosis along the basal membrane only occurs in the early stages of oogenesis and there is evidence for autosynthetic yolk formation during the later stages of oogenesis. This leads us to postulate tentatively the possibility that positively reacting yolk granules may be heterosynthetic, while negative granules may be autosynthetic.

Oocyte degeneration in molluscs is a frequent phenomenon, often resulting from environmental conditions such as extremes of temperature, desiccation or low levels of nutrition (Joosse *et al.*, 1968; Lucas, 1971; Jong-Brink, 1973). In *Biomphalaria glabrata* and *Lymnaea stagnalis*, oocyte degeneration appears to be a gradual process with breakdown of yolk granule membrane, disappearance of the oolemma and reduction in size concurrent with ingestion by surrounding follicle cells. In *M. edulis* there appears to be a breakdown of the vitelline membrane, releasing oocyte contents into the acinar lumen where they are resorbed by the surrounding epithelial cells. In the present study, heavy deposits of reaction products were located within lysosomes of the resorbing cells (Fig. 9). Strong extracellular activity was also found associated with the microvillous border of the resorbing cells and the degenerating yolk granules (Fig. 10). These observations indicate extracellular as well as intracellular breakdown of the oocyte contents by acid hydrolases and suggest this as another possible role for lysosomal enzymes in the yolk granules of ovarian oocytes.

These findings indicate that there is a need for further research to identify the functional role of lysosomal hydrolases in yolk granules, both in normal development of the eggs and in degenerative processes. The evidence for an involvement in the latter is particularly important in terms of understanding the mechanisms of cell injury induced by the action of environmental stressors, including toxic chemical pollutants, which are known to damage lysosomes in other molluscan cell types (Moore, 1985).

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