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Cytochemical Demonstration of Latency of Lysosomal Hydrolases in Digestive Cells of the Common Mussel, *Mytilus edulis*, and Changes Induced by Thermal Stress

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Summary. Latent β-glucuronidase and glucosaminidase activities have been demonstrated in small cytoplasmic particles, which may possibly be primary lysosomes, as well as some larger granules of the digestive cells of the common mussel. Latency was indicated by increased staining of these structures following incubation in buffer at pH 4.5 at 37° C. The exposure of mussels to temperatures of 25–28°C over a period of four days induced a significant decrease in the latency of lysosomal glucosaminidase. Thermal death produced labilization of lysosomes although selective release of hydrolase activity was indicated by the differential latency of glucosaminidase and glucuronidase. The injection of hydrocortisone induced a significant increase in latency in stressed animals, indicating that the stress response involved changes in structure and function of membranes.

Key words: Lysosomal latency – Digestive cells – *Mytilus edulis* – Thermal stress – Cytochemistry.

Introduction

Reduced stability of lysosomes has been associated with inflammatory responses (Chayen and Bitensky, 1971), particularly in rheumatoid arthritis. In addition many other conditions such as lysosomal sequestration of metal ions (Goldfischer, 1965) and various forms of stress (Gabrielescu, 1970) have been found to labilise lysosomes, thus increasing the levels of free hydrolases.

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Lysosomal acid hydrolases have been demonstrated in the digestive cells of several molluscs (Rosenbaum and Ditzion, 1963; Sumner, 1969; Moore, 1971; Moore and Halton, 1973). Using an electron cytochemical technique, Owen (1970) detected acid phosphatase within the lysosomal components of the digestive cells of the cockle, *Cardium edule*. The ultrastructure of the digestive cells of *Mytilus edulis* has been shown to be similar to that of *Cardium* by Owen (1972) and Thompson, Ratcliffe and Bayne (1974).

Latency of lysosomal hydrolase activity has been demonstrated in homogenates of the sterile septa of *Cerianthus lloydi* by Tiffon (1973). Biochemical determinations of latent activity may be misleading because of homogenization trauma and tissue heterogeneity. This latter factor is particularly relevant to many invertebrate tissues where cellular heterogeneity is often very marked. By employing a cytochemical technique it was hoped to overcome this problem and to examine latency in the lysosomes of digestive cells only, and not in the other cell types present. The lysosomes of some of these other cell types may well have different latency properties which could lead to an erroneous biochemical determination when they are considered as a whole.

Cytochemical methods of studying latency in mammalian cells have been developed for naphthylamidase (McCabe and Chayen, 1965), acid phosphatase (E.C. 3.1.3.2) and β-glucuronidase (E.C. 3.2.1.31) (Bitensky, Butcher and Chayen, 1973). These techniques permit quantitative cytochemical investigation of small numbers of cells and the alteration of lysosomal function induced by environmental and disease conditions.

The present study involved the development of a cytochemical test for latency and its application to our understanding of cellular stress responses and pathological changes in the common mussel, *Mytilus*. Modulation of lysosomal membrane stability was investigated using hydrocortisone which is known to stabilise lysosomes in stressed organisms thus reducing the free hydrolase activity (Weissmann, 1969).

Materials and Methods

Mytilus edulis (shell length 60–65 mm) were collected from the Lynher River (Tamar Estuary, Plymouth). Experimental animals were acclimated in a system of recirculating seawater for at least fourteen days. Mussels were fed with *Phaeodactylum tricornutum* (5 l of algal culture/20 l tank/24 h) by continuous administration.

Animals acclimated at 15° C (Summer, 1975) were subjected to a temperature of 28° C for 72 h in one experiment, 96 h in another. Samples of five animals were taken at 24 h intervals and the digestive glands excised for cytochemistry and cytology. A further set of experiments on thermal stress was conducted on animals acclimated at 11° C (Winter, 1976) and subjected to 25° C for 72 h. Samples of five animals were taken at 3, 6, 24, 48 and 72 h. In this second set of experiments, groups of five animals (controls at 11° C and experimental at 25° C) received injections into the adductor muscle of 0.25 ml of sterile saline ($NaCl$ – 33.7 g, KCl – 0.938 g, $CaCl_2 \cdot 6H_2O$ – 2.83 g, $MgCl_2 \cdot 6H_2O$ – 5.386 g, $Na_2HPO_4 \cdot 12H_2O$ – 0.193 g, Tris – 6.06 g, 1 M HCl 42.5 ml, distilled water to 1 litre and pH adjusted to 7.4) containing 0.1 mg/ml of hydrocortisone hemisuccinate (Sigma). Separate groups of five animals were injected with sterile saline only. Specimens were injected at 45.5 h and 69.5 h and sampled after 2.5 h (ie. 48 h and 72 h after the start of the experiment). A group of five mussels subjected to 25° C for 72 h was returned to the control temperature of 11° C for 2 h prior to sampling.

To examine the events following thermal death, mussels (acclimated at 10° C) were exposed to a temperature of 30° C and sampled when they started to gape (dead). Gaping specimens (five in each sample) were removed after 23, 47 and 70 h.

For cytochemical examination, small pieces (3–4 mm^3) of freshly excised digestive gland were dropped into Analar hexane at −70° C for 45 s and stored sealed at −70° C until required for sectioning. Cryostat sections (10 μm) were cut at a cabinet temperature of −26° C in a Bright's Cryostat. The haft of the knife was packed with crushed 'dry ice' and the sections were collected on glass slides at room temperature. The sections were stored in the cryostat for 30 min–2 h before staining.

The activities of β-glucuronidase (E.C.3.2.1.31) and glucosaminidase (E.C.3.2.1.29) were demonstrated in unfixed frozen sections using naphthol AS-BI glucuronide (Pearse, 1972) and naphthol AS-BI glucosaminide (Pearse, 1972) as substrates and fast red violet LB as simultaneous diazonium coupler. For the examination of latency of these enzymes, post-coupling methods were employed to prevent enzyme inhibition by the diazonium coupler. For β-glucuronidase the incubation medium contained 14 mg naphthol AS-BI glucuronide (Sigma) dissolved in 0.6 ml 50 mM Na HCO_3 which was made up to 50 ml with 0.1 M acetate buffer pH 4.5 containing 2.5% NaCl (w:v). To this medium was added 5 g of a low-viscosity collagen-derived polypeptide (Sigma P5115) to act as a colloid stabiliser (Bitensky, Butcher and Chayen, 1973). Incubation time for sections in this medium was 20 min at 37° C in a shaking water bath. Slides were then rinsed in saline (2.5% NaCl) for 2 min at 37° C before being transferred to 0.1 M phosphate buffer pH 7.4 (+2.5% NaCl) at 15° C containing fast red violet LB (1 mg/ml) (Sigma) as diazonium coupler for 10 min. Sections were then fixed for 10 m in Baker's formol calcium (+2.5% NaCl), rinsed in distilled water and mounted in glycerol gelatin.

For glucosaminidase, the incubation medium contained 15 mg naphthol AS-BI glucosaminide (Sigma) dissolved in 2.5 ml 2-methoxyethanol which was made up to 50 ml with 0.1 M citrate buffer pH 4.5 containing 2.5% NaCl, and 5 g polypeptide (Moore and Stebbing, 1976). Incubation time was 15 min and post-coupling was achieved by the method described above for β-glucuronidase. Serial sections for the same animals were preincubated in 0.1 M acetate buffer (+2.5% NaCl) pH 4.5 for β-glucuronidase and 0.1 M citrate buffer (+2.5% NaCl) pH 4.5 for glucosaminidase. Preincubation periods of 0–30 min were examined. Various concentrations of polypeptide (0–40% w:v), and different incubation times with substrates (2–25 min) at two incubation temperatures (20° C and 37° C) were also examined.

Quantitative densitometric measurements were carried out on glucosaminidase staining using a Zeiss Microscope Photometer equipped with a Zeiss diffraction-grating monochromator (Moore and Stebbing, 1976). The two wavelength method of Ornstein and Patau (Pollister, Swift and Rasch, 1969) was used; the selected wave-lengths (obtained from a spectral-absorption curve of a homogeneous distribution of the azo-dye) being 470 nm (λ_1) and 536 nm (λ_2). A measuring aperture of 4 μm diameter was used and section thickness was checked with the micrometer attachment on the fine focussing control. Ten areas per animal per preincubation period were read in the mid-cytoplasmic region of the digestive cells; this gave standard deviations of the mean relative absorbance in the range 3–10%.

For cytological examination, small pieces of digestive gland were fixed in Baker's formol-calcium (+2.5% NaCl) for 24 h at 4° C, changed to gum-sucrose at 0° C for 24 h prior to dehydration and embedding in wax. Sections were stained with haematoxylin and eosin.

Results

The digestive cell staining reactions for β-glucuronidase and N-acetyl-β-glucosaminidase were localised in small cytoplasmic granules (0.3–0.8 μm diameter) and larger granules (2–5 μm in diameter), and it is possible that these may be primary and secondary lysosomes respectively (Fig. 1 A, B). In many cells the small cytoplasmic granules were concentrated around the periphery of the larger vesicles and in certain instances also appeared to be associated with the interior of these vesicles. Similar staining distributions were obtained with simultaneous and post-coupling reactions.

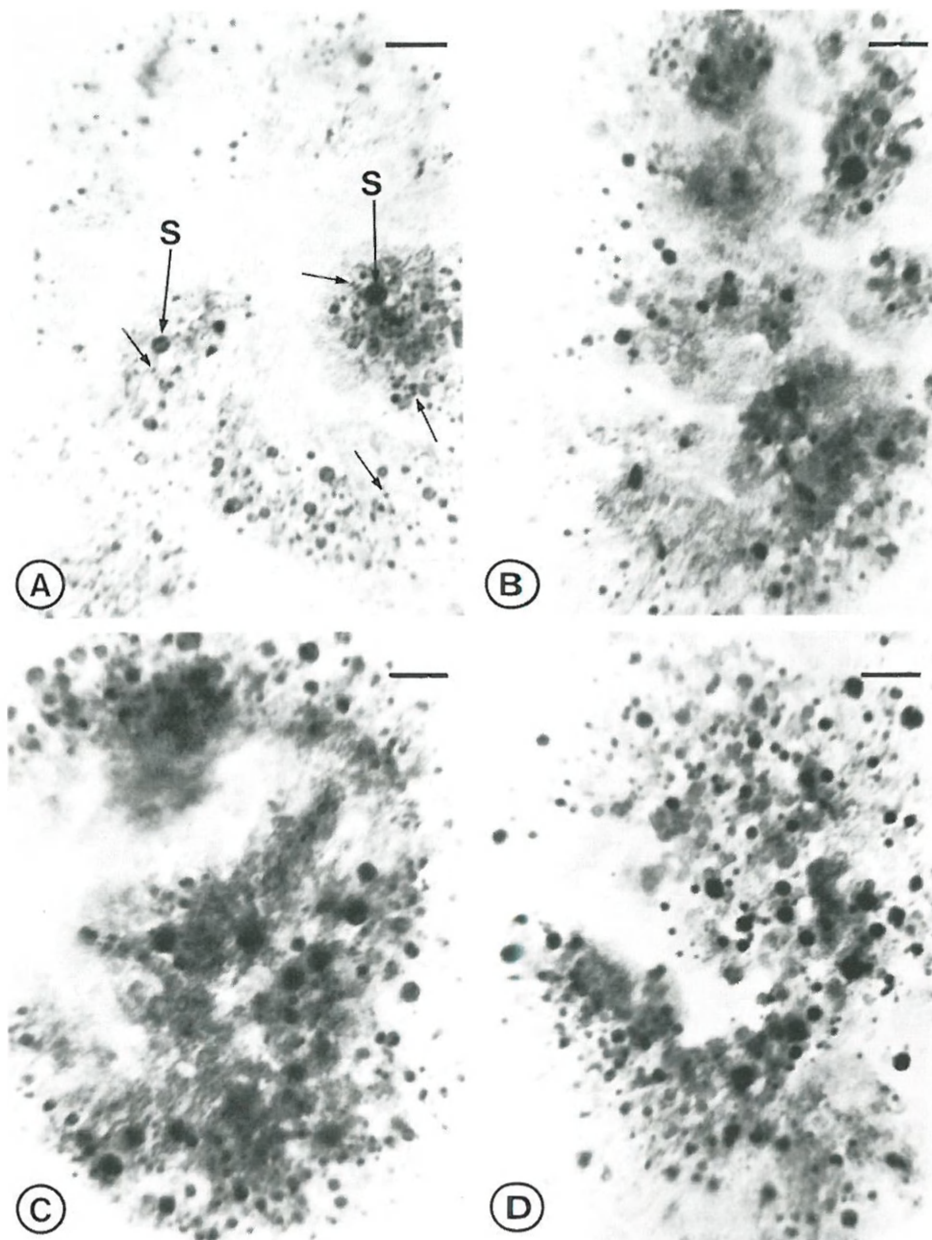

Fig. 1. A A digestive tubule stained to show glucosaminidase activity, with no preincubation at pH 4.5. Reaction product is associated with a few large granules (*S*) and small cytoplasmic granules which may possibly be primary lysosomes (arrowed). **B** A digestive tubule as in A preincubated for 5 min at pH 4.5. There is an increase in the numbers of lysosomes showing reaction product. **C** A digestive tubule as in A preincubated for 15 min at pH 4.5. There is a further increase in staining intensity and numbers of lysosomes. **D** A digestive tubule as in A preincubated for 20 min at pH 4.5. There is a slight decrease in staining intensity from C indicating a probable loss of labilised enzyme by diffusion. Scale bar = 10 μm

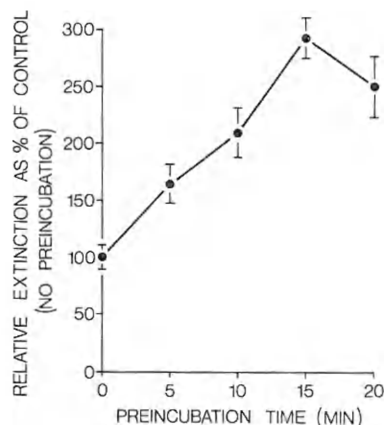

Fig. 2. The effect of preincubation in citrate at pH 4.5 on the cytochemical staining reaction for glucosaminidase in digestive cell lysosomes

Preincubation in 0.1 M acetate buffer (+2.5% NaCl) pH 4.5 for β-glucuronidase and 0.1 M citrate buffer (+2.5% NaCl) pH 4.5 for glucosaminidase produced a marked increase in staining intensity and numbers of small cytoplasmic granules (Fig. 1 B, C). This preincubation often produced a staining decrease in the larger granules within a period of 2–5 min. However, this response was frequently succeeded by an increased reaction with longer preincubation. Increased reaction product was associated, in some instances, with the small granules enclosed within these structures, while in other examples it occurred in the matrix of the larger granules. The optimal conditions for demonstration of latency were found to be an incubation and preincubation temperature of 37° C with 7–10% polypeptide, and an incubation period of 15 min for glucosaminidase and 20 min for β-glucuronidase. For mussels acclimated in the laboratory and, with sublittoral mussels, the optimum preincubation times required to produce maximum staining intensity (the labilization period) were found to be 18 min for glucosaminidase and 15 min for β-glucuronidase. Longer preincubation times produced a gradual decrease in staining intensity and numbers of granules (Fig. 1 D) probably due to diffusion of labilised enzyme. Cytophotometry of the staining reaction for glucosaminidase in the small granules showed that there was a significant increase in staining intensity over a period of 20 min preincubation (Fig. 2). With 30 min preincubation the staining intensity showed a marked decrease, falling to 74% of the non-labilised sections. This pattern is in agreement with the determination of the labilization period as described above.

In mussels acclimated at 15° C which were subjected to a temperature of 28° C, ($\Delta C = 13°$ C) the period of preincubation required to produce maximal staining (labilization period) was reduced to 20.0% of the control after 48 h at the increased temperature (($P < 0.001$) (Fig. 3). This was followed by a slight increase in labilization period at 72 and 96 h. Mussels collected in the winter prior to spawning and acclimated at 11° C and exposed to 25° C ($\Delta C = 14°$ C) showed a different response pattern to the increased temperature (Fig. 3). The labilization period decreased to approximately 70% of the controls within 24 h ($P < 0.05$). Latency

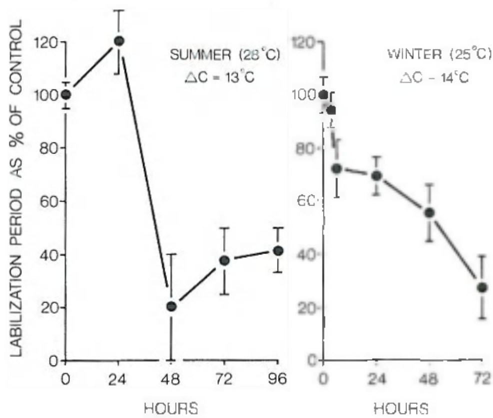

Fig. 3. *The effect of high temperature on the labilization period of lysosomal glucosaminidase, showing differences in seasonal response. (Pooled results of two experiments in each case)*

Table 1. Effects of hydrocortisone hemisuccinate on the latency of glucosaminidase in thermally stressed mussels

Treatment	Labilization period as % of control (±S.E.)	Significance (*t*-test)	
Controls 11° C	100 ± 6.8	NS[1]	NS
+ hydrocortisone	105.6 ± 5.6		
+ saline	94.4 ± 6.8		
Experimental 25° C (48 h)	55.6 ± 12.4	< 0.05	NS
+ hydrocortisone	88.9 ± 10.4		
+ saline	61.1 ± 5.6		
Experimental 25° C (72 h)	22.2 ± 13.6	< 0.05	NS
+ hydrocortisone	55.6 ± 5.0		
+ saline	13.9 ± 8.0		

[1] *NS* = not significant

of glucosaminidase was maintained at this level until 48 h, after which there was a marked decrease with a further 24 h at 25° C to 22.2% of the control ($P < 0.001$). This response was reproduced in a second experiment.

When thermally stressed animals in the winter ($\Delta C = 14°$ C) were injected with hydrocortisone the labilization period was greater (by 30% of the control, $P < 0.05$) than in the stressed, but non-injected and saline-injected, mussels (Table 1). Animals from the control group (11° C), injected with either hydrocortisone or saline, did not show any significant changes in latency properties (Table 1). Mussels subjected to 25° C for 72 h and returned to a temperature of 11° C for 2 h did not show any significant recovery of latency, having a labilization period of 20.8% ± 6.9 of the control.

The effects of thermal death on lysosomal latency are represented in Table 2. The labilization period of glucosaminidase was significantly decreased in all

Table 2. Effects of thermal death on the latency of glucosaminidase and β-glucuronidase in digestive cell lysosomes

Survival time (hours)	Labilization period as % of control (±S.E.)	
	β-Glucuronidase	Glucosaminidase
23	92.8 ± 14.3	47 ± 11.8
47	89.2 ± 30.7	29.4 ± 20.6
70	50 ± 8.6	17.6 ± 7.1

groups of dead animals. However, the labilization period of β-glucuronidase was significantly decreased only in those animals which died around 70 h at 30° C. This differed markedly from the pattern of glucosaminidase latency determined in the same animals indicating that there was selective activation of latent hydrolase activity. Animals which died at 47 h showed a large variance of the labilization period for both hydrolases (Table 2), and histological examination of animals which died around 47 h and 70 h showed disruption of cellular structure and increasing necrosis.

Discussion

The present study shows that cytochemical techniques can be used to investigate lysosomal function in the digestive cells of *Mytilus edulis*. The staining methods employed are standard for the demonstration of lysosomal hydrolases and the consistency of the staining pattern makes digestive cells ideal for studying the functional cytochemistry of lysosomes. The increases observed in particulate staining activity with preincubation at pH 4.5 are in broad agreement with those of Chayen and Bitensky (1971) and McCabe and Chayen (1965). The localisation of other lysosomal hydrolases such as acid phosphatase, arylsulphatase and non-specific esterase (Sumner, 1969) in *Mytilus* at the same cytoplasmic sites provides further evidence that the latent activity is probably lysosomal. Sumner (1969) has also shown a similar distribution pattern of lysosomal hydrolases in a number of lamellibranchs and gastropods, and pathological changes in localisation were demonstrated in littorinids, hydrobids and lymnaeids by Moore (1971) and Moore and Halton (1973).

The subjection of mussels to high temperature (25° C and 28° C) induced a significant decrease in the latency of lysosomal glucosaminidase. This effect is indicative of functional physico-chemical alterations in the lysosomes of thermally stressed mussels. The results show that the lysosomal responses in summer and winter are different. As the temperature increase over ambient (ΔC = 13 or 14° C) was almost the same in both sets of experiments, the inference may be that lysosomal and intracellular digestive regulatory processes differ from one another at these seasons. Alternatively, the summer animals may be more susceptible to the higher experimental temperature (28° C).

Hydrocortisone is known to stabilise lysosomes in stressed organisms thus reducing free hydrolase activity (Weissmann, 1969; Moore and Stebbing, 1976). In the present study, hydrocortisone was demonstrated to increase the labilization period of glucosaminidase in stressed mussels, but not in the controls. These findings support the evidence that the cytochemical demonstration of latency is a real effect and not an artefact. The absence of any significant effect in control mussels may result from selectivity in the mechanism of steroid-action for less stable lysosomes. However, this is speculative and cannot take account of other possible interacting physiological effects of hydrocortisone which may or may not alter its mode of action in stressed and unstressed mussels.

Animals subjected to a temperature of 30°C and examined close to physiological death showed a marked loss both of latent activity of glucosaminidase and of cellular integrity. These changes were undoubtedly associated with autolytic activity arising from a catastrophic breakdown of lysosomal regulation. This was in marked contrast to the animals which could survive at 28°C. It is interesting to note, however, that β-glucuronidase did not display the same dramatic decrease in labilization period, suggesting that labilization of structure-linked latency is a selective process. This selectivity may arise from multiple factors (Verity, 1973) including impermeability of the lysosomal membrane, internal inhibitors or cationic binding of the active site to the polyanionic lysosomal matrix (Koenig, 1969; Goldstone and Koenig, 1970).

The determination of lysosomal latency by cytochemical means provides a rapid and apparently sensitive method of studying the effects of stress at the cellular level in *Mytilus*, and further experimental work is in progress to examine the lysosomal responses to other environmental factors. However, the phenomenon of lysosomal latency is extremely complex and the present results indicate that there may be various populations of lysosomes with different latency properties and possibly enzymic content in a single cell.

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