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Use of microstereology and quantitative cytochemistry to determine the effects of crude oil-derived aromatic hydrocarbons on lysosomal structure and function in a marine bivalve mollusc, *Mytilus edulis*

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Summary

The marine bivalve mollusc, *Mytilus edulis* (blue mussel), is a noted accumulator of many environmental pollutants and is increasingly used for the chemical and biological assessment of environmental impact. The toxic effects of crude oil-derived aromatic hydrocarbons (30 μg/l total hydrocarbons) on the lysosomal–vacuolar system of the digestive cells have been investigated in cryostat sections of hexane-frozen digestive glands. Exposure to aromatic hydrocarbons reduced the cytochemically determined latency of lysosomal β-*N*-acetylhexosaminidase; lysosomal volume density and surface density increased while the numerical density decreased. Experimental exposure resulted in the formation of very large lysosomes which are believed to be largely autophagic in function and these results indicate a significant structural and functional disturbance of digestive cell lysosomes in response to hydrocarbons.

Introduction

The marine bivalve mollusc, *Mytilus edulis* (blue mussel), is a noted accumulator of many environmental pollutants and is increasingly used in the chemical and biological assessment of environmental impact (Bayne, 1976). The digestive cells of the mussel have a highly developed lysosomal–vacuolar system with large secondary lysosomes which are involved in the intracellular digestion of ingested food (Sumner, 1969; Owen, 1972; Moore, 1976, 1980a). These cells are of vital importance to the 'normal' functioning of the animal. Previous investigations have shown that the lysosomal–vacuolar system in the digestive cells of the digestive diverticula is highly responsive to environmental factors (Moore, 1980a). This responsiveness of the lysosomal system has involved changes in

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the cytochemically determined latency (latent activity = total activity − free activity) of *β-N*-acetylhexosaminidase, *β*-glucuronidase and arylsulphatase (Moore, 1976, 1980b; Moore *et al.*, 1978a, b, 1980a; Bayne *et al.*, 1981) and has been used to develop an index of cellular (lysosomal) response to environmental stressors (Bayne *et al.*, 1976; Moore 1980a). Latency of *β-N*-acetylhexosaminidase determined cytochemically in sections using naphthol AS-BI *N*-acetyl-*β*-D-glucosaminide has also shown good agreement with latency determined in cell-free lysosomal preparations from the digestive gland using *p*-nitrophenyl-*N*-acetyl-*β*-D-glucosaminide (Bayne *et al.*, 1981).

Additionally, structural alterations have been observed in the lysosomes of the digestive cells following a variety of experimental treatments including sex steroids (Moore *et al.*, 1978a), anthracene (Moore *et al.*, 1978b), temperature, starvation and salinity (Bayne *et al.*, 1978). These alterations, however, were not described in a quantitative manner.

The present investigation was designed to examine the toxic effects of the aromatic hydrocarbon rich water-accommodated fraction (WAF) of North Sea (Auk Field) crude oil on the latency of lysosomal *β-N*-acetylhexosaminidase and lysosomal structural parameters in the digestive cells.

Materials and methods

The concentration of total hydrocarbons in the experimental seawater was 30 μg/l and samples were taken after 34 and 103 days of exposure to WAF. Control samples were also taken at these times. Details of preparation and dosing of WAF have been described previously (Moore *et al.*, 1980b).

The latency of lysosomal *β-N*-acetylhexosaminidase was determined in 10 μm cryostat sections of hexane chilled (−70° C) digestive gland slices as described by Moore (1976). This involved pre-treatment of the sections in 0.1 M citrate buffer, pH 4.5, containing 2.5% NaCl at 37° C at intervals from 2 to 25 min in order to activate latent enzyme activity. This was followed by incubation (20 min) for *β-N*-acetylhexosaminidase using naphthol AS-BI-*N*-acetyl-*β*-D-glucosaminide (0.4 mg/ml) dissolved in 2-methoxyethanol at pH 4.5 and 37° C in 0.1 M citrate buffer containing 2.5% NaCl and 7% polypeptide (Polyep P5115) as a stabilizer (Bitensky *et al.*, 1973; Moore, 1976). The sections were rinsed in 3% saline and post-coupled using Fast Violet B (1 mg/ml) in 0.1 M phosphate, pH 7.4. Staining intensity was measured in the digestive cells using a Vickers M85 scanning microdensitometer at 560 nm as described by Moore *et al.* (1978a). Relative absorbance measured after 2 min pre-treatment was taken as free activity as there is often diffusible activity in sections stained without any pre-treatment. This diffusible activity may result from damage to some lysosomes during sectioning as well as from non-lysosomal enzyme. Total activity was taken as the maximum relative absorbance measured in sections from the pre-treatment series. Latent activity was expressed as a percentage of total activity as summarized below:

$$\text{Percentage latent activity} = \frac{\text{Total activity} - \text{Free activity}}{\text{Total activity}} \times 100$$

Free activity = staining intensity after 2 min pre-treatment

Total activity = maximal staining intensity obtained from measurements on pre-treated series of sections (2–25 min)

Microstereological analyses of secondary lysosomal structure within the digestive cells were performed on 10 μm cryostat sections stained for β-*N*-acetylhexosaminidase in which the latent enzyme had been activated. The methods for the microstereological analyses have been fully described by Lowe *et al.* (1981). Parameter estimators were determined for volume density (total volume of lysosomes/total volume of cytoplasm in the digestive cells sampled; $\hat{V}_L/\hat{V}_C$), surface density (total lysosomal surface area/total volume of cytoplasm in the digestive cells samples; $\hat{S}_L/\hat{V}_C$), numerical density (total number of lysosomes/total volume of cytoplasm in the digestive cells sampled; $\hat{N}_L/\hat{V}_C$) and the standard deviations (estimated) of these parameters.

Results and discussion

The results show that the percentage latency of β-*N*-acetylhexosaminidase was significantly reduced from the control values by treatment with WAF at both sampling times (Table 1). The decline in percentage latency of β-*N*-acetylhexosaminidase observed from 34 days to 103 days (Table 1) reflected a gradual decline in both physiological and cellular condition of the control animals during the course of this experiment (Widdows *et al.*, 1982). This deterioration was probably a long-term effect of maintaining the mussels in an aquarium system.

The structural changes in the secondary lysosomes induced by exposure to WAF can be clearly seen in Figs. 1 and 2; many of the secondary lysosomes have a greatly enlarged appearance and there is a thinning of the digestive tubule epithelium. The microstereological results show that there are significant increases in lysosomal volume per unit volume of digestive cell cytoplasm ($\hat{V}_L/\hat{V}_C$) at both sampling times (Table 1). This clearly supports the visual evidence in Figs. 1 and 2.

In addition to the volume increase there were significant increases in lysosomal surface density at both sampling times (Table 1) and a significant decrease in lysosomal numerical density after 103 days of exposure to WAF (Table 1).

Table 1. The effects of aromatic hydrocarbons in the water accommodated fraction of North Sea crude oil (30 μg/l) on latency of lysosomal β-*N*-hexosaminidase and lysosomal structural characteristics in the digestive cells.

*Experimental treatment**	*% latency of* β-N-*acetylhexosaminidase*	*Volume density*	*Surface density*	*Numerical density*
34 days control	34.16 ± 8.79	0.064 ± 0.030	0.147 ± 0.026	0.017 ± 0.006
34 days exposed	8.70 ± 10.29	0.127 ± 0.058	0.209 ± 0.019	0.015 ± 0.004
	P = 0.008, U-test	$P < 0.05$, t-test	$P < 0.05$, t-test	N.S.†, t-test
103 days control	16.70 ± 14.80	0.061 ± 0.024	0.148 ± 0.024	0.020 ± 0.014
103 days exposed	0	0.192 ± 0.062	0.190 ± 0.017	0.005 ± 0.003
	P = 0.048, U-test	$P < 0.05$, t-test	$P < 0.05$, t-test	$P < 0.05$, t-test

*Five mussels per sample.
†N.S., not significant.

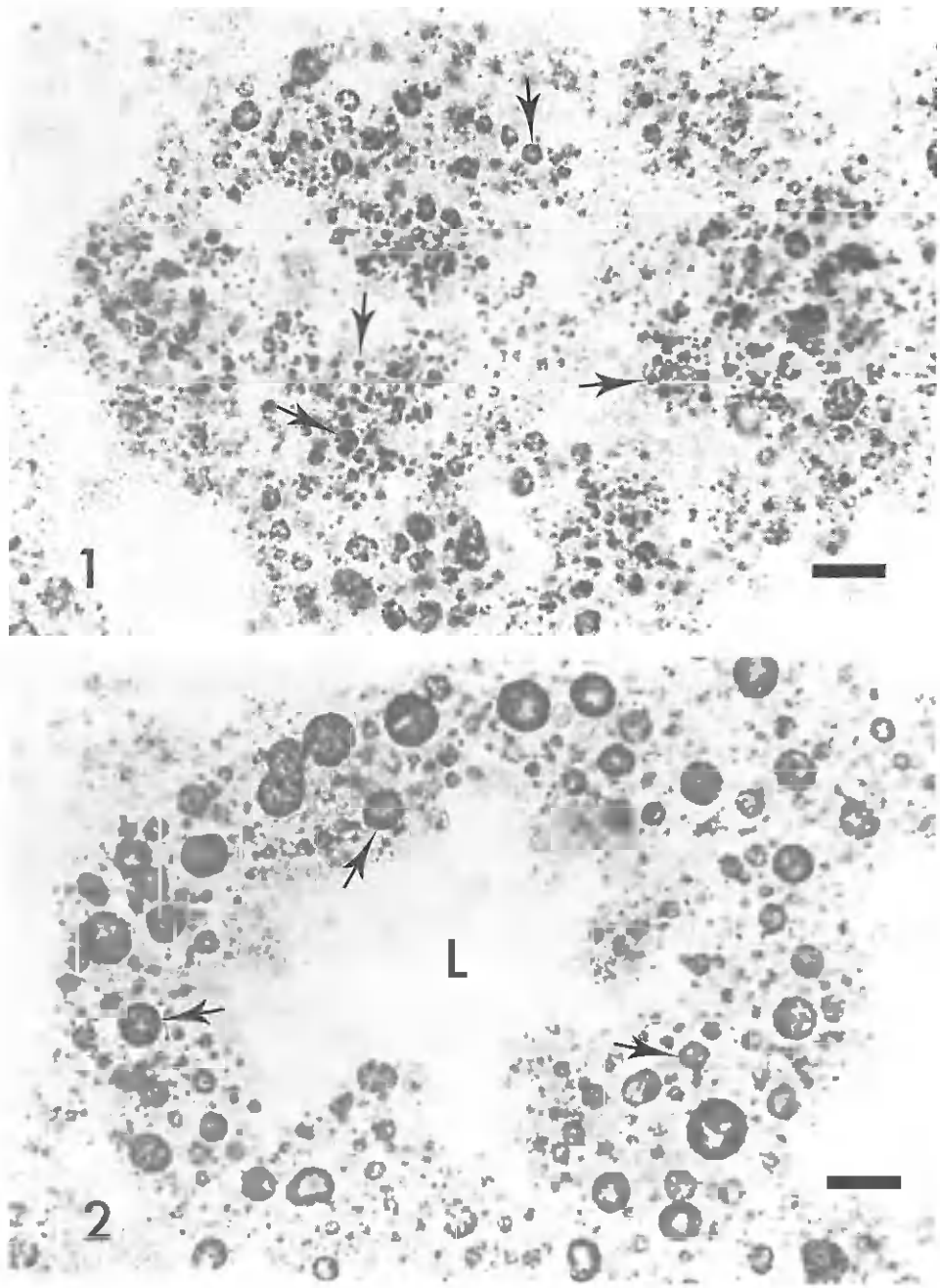

Fig. 1. A cryostat section through a digestive tubule from a control mussel sampled after 103 days showing lysosomes (arrowed) reacted for β-*N*-hexosaminidase activity in the digestive cells. Scale bar: 10 μm.

Fig. 2. A section as in Fig. 1 from a mussel exposed to aromatic hydrocarbons (30 μg/l) in WAF for 103 days. The secondary lysosomes are greatly enlarged (arrowed) and there is an apparent reduction in number. The digestive tubule epithelium displays a reduction in height and the tubule lumen (L) is enlarged in comparison with the control. Scale bar: 10 μm.

Exposure to aromatic hydrocarbons in WAF reduced the latency of lysosomal *β-N*-acetylhexosaminidase and resulted in the formation of large and presumably unstable lysosomes. These large lysosomes (Fig. 2) are believed to be largely autophagic in function and have been observed previously in starved mussels (Bayne *et al.*, 1978). That autophagy is occurring is further supported by the fact that the digestive cells from the same samples were significantly reduced in height by WAF as determined by image analysis (Lowe *et al.*, 1981). The mechanism by which these large lysosomes are formed may be indicated by the decrease in numerical density of the lysosomes, which could suggest a fusion of smaller lysosomes to form the large unstable type (based on latency data).

This alteration in lysosomal structure within the digestive cells represents a considerable disturbance of the normal (control) structure and can probably be interpreted as enhanced catabolism of cytoplasmic components by a lysosomal autophagic mechanism. The resulting increase in tissue catabolism is further supported by physiological data which shows a significant decrease in scope for growth (Widdows *et al.*, 1982).

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