Cristina Nasci, Luisa Da Ros, David R. Livingstone

STUDIES ON ALDEHYDE DEHYDROGENASE IN MUSSEL, *MYTILUS GALLOPROVINCIALIS,* OF VENICE LAGOON AS A BIOMARKER OF ORGANIC POLLUTION

Riassunto. Le aldeidi sono molecole molto reattive derivate da numerose sorgenti, inclusi gli inquinanti organici.La principale via di detossificazione all'interno dell'organismo è l'ossidazione ad acido carbossilico catalizzata dall'aldeide deidrogenasi (ADH: EC 1.2.l.3). Questo enzima è presente nel fegato dei mammiferi in molte forme tra cui benzaldeide deidrogenasi (BDH) ma non propionaldeide deidrogenasi (PDH) essendo indotto dal Benzo(a)pirene ed altri idrocarburi aromatici.
L'attività di BPH e PDH e la risposta alla presenza di xenobiotici è stata studiata in tessuti di *Mytilus galloprovincialis* campionati in aree pulite ed inquinate di Venezia. L'attività enzimatica è stata misurata spettro fuori metricamente dalla riduzione del $NAD(P)^+$ a 25°C. Poiché la maggiore attività BDH e PDH è stata vista nel citosol della ghiandola digestiva tale frazione è stata usata per tutte le determinazioni. L'attività PDH è risultata sensibile al calore (viene soppressa dall'esposizione a 100°C per 15 min) e mostra linearità con la misura del campione (0. 25-l mg protein) in contrasto con quanto riscontrato per l'attività BDH. Esemplari di *M. galloprovincialis* sono stati esposti in laboratorio ad una concentrazione di 5 .µg/g p.u. di BaP e Menadione, separatamente, mediante iniezione nella cavità del mantello. L'attività BDH non ha evidenziato alcun cambiamento mentre l'attività PDH è aumentata di 2-3 volte negli organismi trattati rispetto agli animali controllo dopo 108 ore.
Nell'indagine in ambiente l'attività PDH è stata determinata in mitili, *M. galloprovincialis,* campionati da gennaio a luglio in due stazioni della laguna di Venezia a diverso grado di contaminazione. I risultati preliminari non hanno mostrato differenze significative tra le due aree ma suggeriscono un'andamento stagionale legato al ciclo fisiologico degli animali.

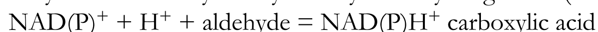
Summary. Aldehydes are higly reactive molecules produced from a variety of sources, including organic pollutants. The latter can occur either directly via metabolism, or as a consequence of oxidative processes such as lipid peroxidation. A major detoxication pathway of aldehydes is oxidation to carboxylic acid catalysed by aldehyde deydrogenase (ADH: EC 1.2.1.3.). The enzyme is present in multiple forms in mammalian liver, benzaldehyde dehydrogenase activity (BDH), but not propionaldehyde dehydrogenase activity (PDH), being induced by benzo(a)pyrene (BaP) and other aromatic hydrocarbons.
BDH and PDH activities and responses to xenobiotics were studied in tissues of *Mytilus galloprovincialis* collected from clean and polluted sites from Venice. Enzyme activities were measured spectrophotometrically by the reduction of $NAD(P)^+$ at 25° C. Highest PDH and BDH activities were present in digestive gland cytosol and this fraction was used for all further studies. PDH activity was heat-killable (100° C for 15 min) and showed linearity with sample size over a 4 fold range of sample concentration (0.25 - I mg protein). In contrast, a part of the BDH activity was not heat-killable and little linearity with sample size was seen, indicating some doubt over the enzymic nature of the measurements. PDH activity was similar with NAD' or $NADP^+$ as coenzyme, with optimal substrate concentrations of 2.5 mM NAD^+ and 0.25 mM propionaldehyde. *M. galloprovincialis* were exposed to BaP, the redox cycling quinone menadione (both dissolved in dimethylsulphoxide-DMSO), or DMSO (vehicle control), or no addition (control) by injection into the mantle cavity (xenobiotics dosage: 5µg/g wet wt.). No changes were seen in BDH activity, whereas PDH activity increased 2 to 3 - fold over control and vehicle control with 108 hours esposure. Elevation of PDH activity was seen with 48 hours esposure to BaP or menadione, but the same elevation was also seen for the vehicle control. In field studies PDH activity was deter-

mined in M. *galloprovincialis* collected from January to July 1994 in two different polluted areas of Venice Lagoon and different patterns of seasonal change were seen..

Key words: aldehyde dehydrogenase, mussel, biomarker, menadione, benzo[a]pyrene

INTRODUCTION

Aldehydes are highly reactive molecules produced from a variety of sources, including foreign compounds (xenobiotics) such as pollutants (LINDAHL, 1992). The formation from pollutants can occur directly via metabolism, or as a consequence of oxidative processes such as lipid peroxidation. The deleterious effects of aldehydes are many, including cytotoxicity, mutagenicity and carcinogenicity. A major pathway of detoxication of aldehydes is oxidation to carboxylic acids catalysed by aldehyde dehydrogenase (ADH; EC 1.2.1.3):



$NAD(P)^{+} + H^{+} + \text{aldehyde} = NAD(P)H^{+}$ carboxylic acid

ADH exists in most tissues and subcellular compartments. It is present in multiple forms in mammalian liver, benzaldehyde dehydrogenase (BDH) activity, but not propionaldehyde hydrogenase (PDH) activity, being induced by benzo(a)pyrene (BaP) and other polynuclear aromatic hydrocarbons (PAHS). In mammals, the enzyme is part of the xenobiotic-metabolising inducible [Ah]-gene battery Which also includes cytochrome P4501A1 (NEBERT et al., 1990). Nothing is known of the existence of ADH in mussels, although lipid peroxidation and other pro-oxidant processes occur, indicating a requirement for the enzyme (LIVINGSTONE, 1991). Lipophilic organic xenobiotics, such as pollutants, are readily taken up into the tissues of *Mytilus* sp. and other molluscs (LIVINGSTONE, 1991) Where they can be concentrated in lysosomes of digestive gland and other tissues leading to deleterious effects, including destabilisation of the lysosomal membrane (MOORE, 1988). Understanding the responses to, and effects of, pollutants at the molecular level is important in relation to the design of molecular biomarkers of pollutant impact (LIVINGSTONE, 1993).

OBJECTIVES

1. To characterize the presence and properties of ADH activity in mussel.
2. To determine the response of ADH activity to exposure to a model xeno biotic inducer, BaP, and model redox cycling oxidative stressor, 2-methyl

   l,4-naphthoquinone (menadione).
3. To investigate the ADH activity in mussel collected in two different polluted stations of Venice Lagoon.
4. To compare the response of ADH activity With lysosomal membrane damage (lysosomal stability) and oxidative damage (lipofuscin formation).
5. To examine the results of ADH as a possible biomarker of exposure to organic pollution.

6. To compare the responses for ADH With another potential biomarker of environmental stress-neutral red retention in blood cells (haemocytes).

MATERIALS AND METHODS

1. Mussels (4-5 cm length), *Mytilus galloprovincialis,* were collected from clean sites around Venice, Italy.
2. Exposure experiments were carried out on *M. galloprovincialis* in static, aerated seawater (half-water changed daily).

Expt. I (November 1993) - exposure to BaP or menadione in dimethyl-sulphoxide (DMSO) carrier.

Expt. II (March 1994) - exposure to menadione in ethanol carrier.

Mussels received a single injection of xenobiotic (dosage: 5 μg/g wet wt.) in 10 μl carrier, or carrier alone, into the mantle cavity. They Were then left dry and closed for 2 hours before being returned to the seawater. Mussels were sampled at different times up to 120 hours, and digestive glands removed and assayed for ADH activities, lipofuscin and lysosomal stability.

3. Field study Was carried out on *M. galloprovincialis* collected over one year (1994) from well-characterised clean (P. Lido) and polluted (P Marghera) sites in Venice Lagoon. Mussels were sampled bimonthly and digestive glands removed and assayed for ADH activities. Lysosomal stability was assessed in haemocytes using an *in vitro* test (neutral red retention time)
4. Subcellular fractions for enzyme studies were prepared by standard procedures by homogenisation in 10 mM Tris-HCl containing 0.5 M sucrose and 0.15 M KCl (Livingstone and Farrar, 1984). BDH and PDH activities were measured spectrophotometrically at 25°C by the reduction of NAD(P)+ at 340 nm (Förlin *et al.).* Standard assay conditions were: 53 mM KH_2P0_4/ K2HPO$_4$ pH 8.5, 2.5 mM $NAD(P)^+$, 10 mM propionaldehy de (in buffer) or 1 mM benzaldehyde (in triton X-100). Protein was measured by the method of LOWRY et al. (1951).
5. Histochemical studies were carried out on frozen digestive gland. Lipofuscin content of tertiary lysosomes was detected using the Schmorl reaction (PEARSE, 1972). Lysosomal stability was measured in digestive cells by the labilisation characteristics of latent f -N-acetylhexosaminidase activity (MOORE, 1988). Cytochemical studies Were carried out on fresh haemocytes preparations using the retention time of the cationic probe neutral red as a determinant of effect (LOWE et al., 1993; LOWS et al., 1995).
6. Values were compared by Mann-Whitney's U-test ($P< 0.05$) and ANOVA.

RESULTS

Enzyme characterization

1. Highest PDH and BDH activities were found in postmitochondrial

(9000g) fraction of the digestive gland. This fraction was used for all subsequent enzyme studies.
2. PDH activity was heat-killable, whereas a significant fraction of BDH activity was not. Linearity between sample concentration and enzyme activity was observed for PDH over a 4-fold range of sample concentration (0.25- 1 mg protein) but not BDH activity, indicating doubt over the enzymic nature of part or whole of the BDH activity.
3. Optimization studies were carried out on PDH activity only. Activity was similar with the same concentration of NAD^+ or $NADP^+$. Saturating substrate concentrations were 2.5 mM propionaldehyde and 2.5mM NAD^+. Michaelis-Menten kinetics, With apparent K_m values of 1.25 mM (NAD^+) and less than 0.1 mM (propionaldehyde), were indicated.

Exposure studies:
Expt. I - exposure to BaP or menadione (in DMSO)
1. No changes were observed in BDH activity (Table 1).
2. PDH activity increased about 2 to 3-fold over control in all treated condi tions, including DMSO alone, after 48 hours, but remained high with menadione exposure after 108 hours (Table 1).
3. Lipofuscin production was higher with menadione than DMSO alone, but decreased markedly With BaP exposure (Table 2).

Expt. II - exposure to menadione (in ethanol)
l. PDH activity increased with both menadione and ethanol alone exposure, but Was markedly higher in the farmer than the latter after 96 hours (Table 3).
2. Lysosomal stability Was markedly reduced by menadione exposure, but not by ethanol alone (Table 4).

Table 1. Propionaldehyde dehydrogenase (PDH) and benzaldehyde dehydrogenase (BDH) activities (in nmol $min^{-1}g^{-1}$ wet wt) in digestive gland supernatant (9000g) of mussel.

Activity	Time	Control	DMSO	BaP	Menadione
PDH	48 h	38.8±5.3	88.2±22.5	67.7±19.5	83.5±9.9*
PDH	108 h	39.5±2.0	27.4±4.1*	34.7±4.5	98.6±9.0*°
BDH	48 h	12.9±7.5	19.4±12.4	22.5±6.2	27.9±8.7
BDH	108 h	9.7±6.2	6.5±3.7	12.9±7.5	16.0±3.2

n=4.
Control activity at 48 h in nmoi min-1mg-1 protein range = 0.65±0.06 (PDH) and 0.24±0.1 (BDH) . P<0.05; * DMSO, BaP or Menadione compared to the Control at the same time, ° BaP or Menadione compared to DMSO at the same time.

Table 2. Lipofuscin content in digestive gland lysosomes of mussel (volume fraction %).

Time	Control	DMSO	BaP	Menadione
48 h	25.7±1.6	18.1±2.2	19.2±5.6	31.9±3.5°
96 h	ND	ND	ND	15.2±2.2
120 h	16.4±2.4	11.7±3.3*	1.74±0.4*	ND

n=5.
ND = not determined.
P<0.05; * DMSO, BaP or Menadione compared to the Control at the same time, ° BaP or Menadione compared to DMSO at the same time.

Table 3. Propionaldehyde dehydrogenase (PDH) activity (in nmol min^{-1} g ·ı wet wt.) in digestive gland supernatant (9000g) of mussel.

Time	Control	Ethanol	Menadione
12 h	43.3±4.9	52.1±7.8	56.5±12.4
24h	20±5.9	51,9±14.6	53.5±4.7*
48 h	39.2±4.9	40.8±4.1	30.6±4.1
72 h	23.1±5.2	23.3±4.5	28.3±6.6
96 h	26±3.4	20.7±3.9°	60,3±6.3*°
120 h	27.2±2.1	17.4±3.9	20.0±3.5

n=10.
Control activities in nmol mind protein-' wet wt. range from 0.31±0.09 to 0.85±0.12.
P<0.05 ; * Ethanol or Menadione compared to the control at the same time, ° Menadione compared to Ethanol at the same time.

Table 4. Labilization period of lysosomal ß-N-Acetylhexosaminidase of mussel digestive gland as % of controls (range from 25' to 29').

Time	Control	Ethanol	Menadione
24h	100±1.58	92.6±3.53	51.8±4.30*
48h	100±3.31	ND	55.5±0.10*
72 h	100±1.0	ND	43.5±3.25*
96h	100±2.55	100±1.22	48.1±2.00*

n= 5;
ND = not determined.
* P<0.05 Menadione compared to the controls at the same time.

Field study:
1. PDH activities were determined in samples from January to July 1994 (Figure 1). The data showed a similar trend in both stations with low values in winter and summer and a marked increase in spring.

PDH activity Was higher in P.Marghera than in P Lido in January and March but lower in May and July
2. The neutral red retention test indicated that the shortest dye retention times (i. e. increased lysosomal membrane damage) were found in the haemocytes of mussels from P.to Marghera, the most polluted site (Figure 2). No seasonal trend was apparent for both stations.

DISCUSSION

ADH:-like proteins have been detected in specialized light organs of cephalopods (WEISS et al., 1993), but this is the first study to indicate the existence of the enzyme in marine bivalves. The lack of apparent BDH activity may due to competition fur the benzaldehyde substrate by benzaldehyde oxidase Which has been detected in the digestive gland of several terrestrial gastropods (LARGE & CONNOCK, 1993). The increase in PDH activity With exposure to menadione indicates a response to pro-oxidant conditions, i.e. the production of reactive oxygen species and secondary oxidants. The increase in PDH activity with DMSO and ethanol exposure could be in response to radical or aldehyde formation. The increase in PDH but not BDH activities With exposure to xenobiotics is the reverse of that seen for mammals (LINDAHL, 1994), and indicates a phylogenetic difference in the regulation and functioning of the enzyme. The lack of response of BDH activity to PAHs (BaP) is consistent with previous studies on the apparent absence of the [Ah]-gene receptor in marine molluscs (HAHN et al., 1994).

Lipofuscin contains both oxidised lipid and protein, and can be formed as a result of lipid peroxidation. According to Table 2 lipofuscin content Was higher in menadione than vehicle control (DMSO) animals, Which indicates a pro-oxidant effect by menadione. The surprising marked decrease in lipofuscin with BaP exposure could be due to the preferential use of lipid hydroperoxide as a co-substrate for BaP metabolism (LEMAIRE et al., 1993). The decrease in lysosomal stability with menadione exposure is indicative of the uptake of the xenobiotic, or its metabolic products, into the lysosomes, and effects on the membrane (MoORE, 1988).

The field results are indicative of the regulation of PDH activity in mussels of Venice Lagoon. The overall data do not show a significant difference between the two stations (ANOVA) but suggest a relationship With seasonal physiological cycle of the animals. In fact, the enzymatic activity is significant (Mann-Whitney, $p<0.05$) lower in P Lido than in P. Marghera in Winter and in both sites the highest values Were reached in spring. This could be presumably linked to the changing metabolic status of the organism, itself dependent on such factors as gonad ripening, filtration rate, food availability and water temperature. The in vitro assay used in this study to assess the stability of the lysosomal membrane, has been originally developed in mussel digesti-

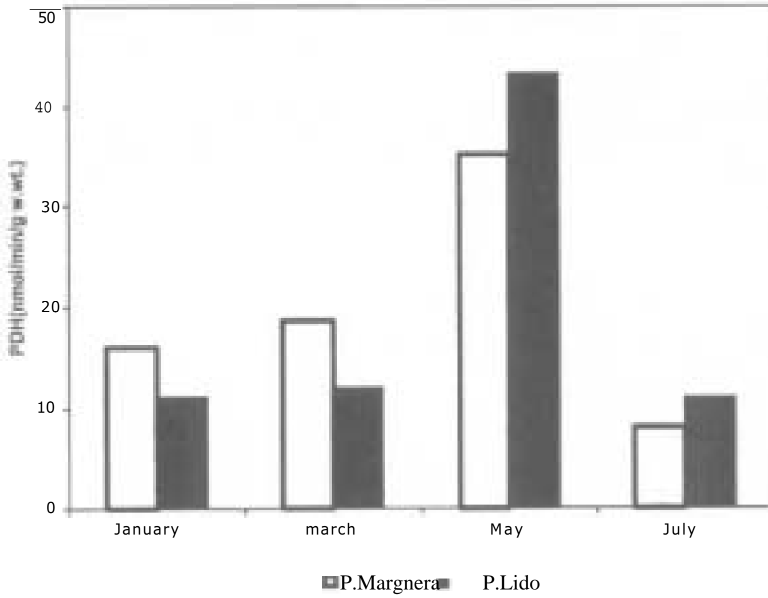


Figure 1. PDH activity (mean values) in digestive gland of mussels from polluted (P. Marghera) and clean (P Lido) sites.

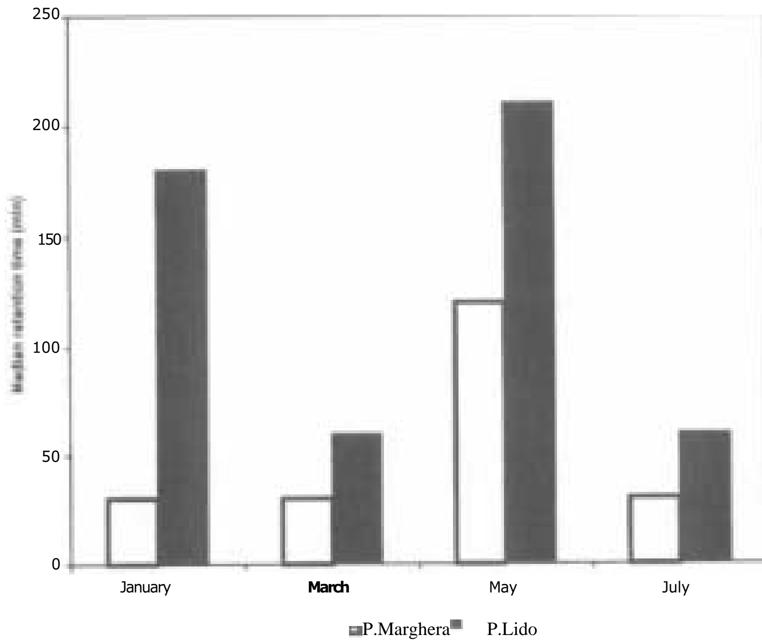


Figure 2. Neutral red retention time in haemocytes of mussels from polluted (P. Marghera) and clean (P. Lido) sites.

ve cells (LOWE et al., 1994). More recently, it has been modified and applied in mussel haemocytes (LOWE, pers. com.). The first results obtained here shows always higher values of neutral red retention time in the clean site with significant differences (Mann-Whitey, $p < 0.05$) between the tWo station except in March.

CONCLUSIONS

L ADH activity is present in the digestive gland of mussels and is indicated to be increased by pro-oxidant conditions.
2. The presence and regulation of ADH in mussels are indicated to be diffe rent from mammals.
3. ADITI activity is present in mussels of Venice Lagoon but its possible use as biomarker of environmental stress has to be investigated in relation with physiological cycle of animals.
4. The neutral red retention test in mussel hemocytes gives promising indications for its future use as a sensitive tool in field pollution effects monitoring.

ACKNOWLEDGEMENTS

This work has been supported in part by a grant of NRC/British Council (Italy) to C.Nasci and to L.Da Ros.

References

FÖRLIN L., LEMAIRE P, LIVINGSTONE D. R., 1995 - Comparative studies of hepatic xenobiotic metabolizing and antioxidant enzymes in different fish species. *Mar.Env: Res.* 39: 201-204.

LARGE A. T., CONNOCK M..J., 1993 - Aromatic alcohol oxidase and aldehyde oxidase activities in the digestive gland of three species of terrestrial gastropod *(Helix apersa, Anion ater, Limax flavus). Comp. Biochem. Physiol. l OB:* 489-491.

LINDAHL. R., 1992 - Aldehyde dehydrogenases and their role in carcinogenesis. *Crit. Rev. Biochem. Mol. Biol. 27:* 283-335.

LEMAIRE P., DEN BESTEN P J., O'HARA S. C. M., LIVINGSTONE D. R., 1993 - Comparative meta bolism of benzo(a)pyrene by microsomes of hepatopancreas of the shore crab *Carcinus moenas L.* and digestive gland of the common mussel *Mytilus edulis L.. Poly. Aromas. Comp. 3* (supplement): 1133-1140.

LIVINGSTONE D. R., GARCIA MARTINEZ P, WINSTON G. W., 1989 - Menadione-stimulated oxyradical formation in digestive gland microsomes of the common mussel, *Mytilus edulis L.. Aguat. Toxicol. 15:* 213-236.

LIVINGSTONE D. R., 1993 - Biotechnology and pollution monitoring: use of molecular biomarkers in the aquatic environment. *J Chem. Tech. Biotechnol. 57:* 195-211.

LIVINGSTONE D. R., 1991 - Organic xenobiotic metabolism in marine invertebrates. *Adv. Comp. Environ. Physiol.* 7: 45-185.

LIVINGSTONE D. R., FARRAR S. V., 1984- Tissue and subcellular distribution of enzyme activities of mixed function oxygenase and benzo(a)pyrene metabolism in the common mussel, *Mytilus edulis L.. Sci. Total Environ. 39: 209-235.*

LIVINGSTONE D. R., LIESE, MARTINEZ P. G., PIPE R. K., 1992 - Antioxidant enzymes in the digestive gland of the common mussel *Mytilus edulis. Mar. Biol.* 112: 265-276.

LOWE D. M., PIPE R. K., 1994 - Contaminant induced lysosomal membrane damage in marine mussel digestive cells: an *in vitro* study. *Aquat.Toxicol.* 30: 357-365.

LOWE D. M., SOVERCHIA C., MOORE M. N., 1995- Lysosomal membrane responses in the blood and digestive gland cells of mussels experimentally exposed to fluoranthene. *Aqua!. Toxicol.* 33:105-112.

LOWRY O. H., ROSESOURGH N. J., FARR A. L., RANDALL R. J., 1951 - Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193: 265-275.

MOORE M. N., 1988 - Cytochemical responses of the lysosomal system and NADPH-ferrihemoprotein reductase in molluscan digestive cells to environmental exposure to xenobiotics. *Mar. Ecol. Prog. Ser, 46:* 81-89.

NEBERT D.W., PETERSEN D.D., FORNACE A. J. Jr., 1990 - Cellular responses to oxidative stress: the [Ah] gene battery as a paradigm. *Environ. Health Perspect.* 88: 13-25.

PEARSE, AG. E., 1972 - Histochemistry, theoretical and applied. Vol. 2, ed. Churchill Livingstone, London, 757 pp.

WEIS V. M., MONTGOMERY M. K., MCFALL-NGAI, M. J., 1993 - Enhanced production of ALDH-like protein in the bacterial light organ of sepiolid squid *Euprymna scolopes. Biol. Bull.* 184: 309-321.

Author address:

C. Nasci, L. da Ros: Istituto di Biologia del Mare CNR, Castello 13641a, 30122 Venice, Italy D. R. Livingstone: NERC Plymouth Marine Laboratory, Citadel Hill, Plymouth PLI 2PB, UK