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VIE MILIEU, 2001, 51 (4) : 189-194

GLUTAMATE DEHYDROGENASE ACTIVITY IN *MYTILUS EDULIS*: THE EFFECT OF HYPERAMMONIA

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EXPOSURE
HYPERAMMONIA
GDH ACTIVITY
ADDUCTOR MUSCLE
MYTILUS EDULIS

ABSTRACT. – The effect of high levels of ammonia on glutamate dehydrogenase activity (GDH) in *Mytilus edulis* was evaluated. Adductor muscle GDH activity of 3.629 + 0.011 µmoles min^{-1} was found to be similar to that published for other species of bivalves and crustaceans. GDH activity showed a complete dependence on its co-factor (NADH) because no detectable activity was measured in the absence of NADH. The observed excitatory effect of ADP on the reductive activities of adductor muscle GDH indicated a possible regulatory link between amino acid production (for energy metabolism) and protein synthesis (for growth). The insignificant change ($P > 0.05$) in haemolymph protein levels of *M. edulis* exposed to hyperammonia found in this study, together with increased haemolymph free amino acids (FAA) levels found under the same conditions in an earlier investigation, suggest the involvement of GDH in the assimilation of exogenous ammonia into amino acids. It is concluded that enhanced GDH activity measured under hyperammonia, allows mussels to utilise transient increases in ammonia availability by assimilating most of the nitrogen present. Persistently high ammonia levels, however, may involve alternative metabolic pathways because GDH activity remains constant under prolonged exposure to hyperammonia. It would appear that a complex set of biochemical strategies have evolved to enable mussels to resist high ambient ammonia levels.

EXPOSITION
HYPERAMMONIA
ACTIVITÉ GDH
MUSCLE ADDUCTEUR
MYTILUS EDULIS

RÉSUMÉ. – L'effet des taux élevés de l'ammoniaque sur l'activité du glutamate déhydrogénase (GDH) de *Mytilus edulis* a été évalué. L'activité du GDH du muscle adducteur est égale à 3,629 ± 0,011 µmoles min^{-1}, celle-ci s'avérant semblable à celles trouvées pour d'autres espèces de Bivalves et de Crustacés. L'activité du GDH a montré une dépendance complète à l'égard de son cofacteur (NADH) parce qu'aucune activité discernable n'a été mesurée en l'absence du NADH. L'effet excitatoire observé de l'ADP sur les activités réductrices du GDH du muscle adducteur a indiqué un lien régulateur possible entre la production d'acide aminé (pour le métabolisme énergétique) et la synthèse de protéine (pour la croissance). Le changement non significatif ($P > 0.05$) des taux de protéine de l'hémolymphe de *M. edulis* exposé à un milieu hyper-ammoniacal (trouvé dans cette étude), ainsi que les taux accrus des acides aminés libres de l'hémolymphe (FAA) trouvés dans les mêmes conditions lors d'une première recherche, suggèrent la participation du GDH dans l'assimilation de l'ammoniaque exogène dans des acides aminés. On conclut que l'activité accrue du GDH mesurée sous hyperammonia, permet aux Moules d'utiliser des augmentations passagères d'ammoniaque en assimilant la majeure partie de l'azote présent. Un taux élevé d'ammoniaque peut cependant impliquer des voies métaboliques alternatives car l'activité du GDH est restée constante au cours de l'exposition prolongée des Moules à un milieu hyper-ammoniacal. Il semble qu'un ensemble complexe de stratégies biochimiques est impliqué pour permettre aux Moules de résister aux taux ambiants élevés d'ammoniaque.

INTRODUCTION

Glutamate dehydrogenase (GDH) is a key enzyme in carbohydrate and amino acid metabolism in animal cells which catalyses the amination of carbon skeletons and the deamination of amino acids (Lehninger 1976). GDH is specific to glutamate, 2-oxoglutarate (α-ketoglutarate), and NH_4^+ and at physiological pH 7.4, the activity of GDH with other substances is relatively low (Goldin & Frieden 1971). GDH is localised in mitochondria and has physiological significance in terms of free amino acid (FAA) metabolism despite its relatively low activity (Burcham *et al.* 1983, Moyes *et al.* 1985).

Several roles for the GDH system have been established, particularly in marine, euryhaline invertebrate species subjected to environmental changes. For example, GDH probably plays a major role in cell volume regulation by controlling the level of the intracellular FAA pool which, in bivalves, contributes 20-60% of the intracellular osmotic pressure (Hayashi 1987, Chew *et al.* 1994).

Changes in the kinetic properties of GDH under anoxia have also been reported. Ip *et al.* (1994) found a significant increase in glutamate affinity of GDH in the sipunculid *Phascolosoma arcuatum* under anoxia, which resulted in increased deaminating activity at physiological concentrations of glutamate. However, other species exposed to anoxic conditions such as *M. edulis* (Zurburg & De Zwaan 1981) and the mudskipper, *Periothalmodon schlosseri* (Peng *et al.* 1998) anoxia showed a significant increase in GDH aminating activities which led to enhanced tissue FAA levels which presumably detoxified excess ammonia accumulated during anaerobiosis.

It was shown earlier (Sadok *et al.* 1995) that *M. edulis* has the ability to take ammonia up from the external medium but, apart some marine invertebrate-alga associations (Rees *et al.* 1994, Roberts *et al.* 1999) no data are available on *M. edulis* metabolism under hyperammonia, although these animals have been used as biofilters in water with high ammonia levels (Enander & Hasselstrom 1994).

The present investigation was directed towards an attempt to elucidate better the results of hyperammonia on *M. edulis* described in an earlier study (Sadok *et al.* 1995) and, since mussels are able to withstand relatively high external ammonia levels, to investigate the potential role of GDH in ammonia detoxification. Consequently, only the reductive properties of GDH (i.e., glutamate formation) have been considered here.

MATERIAL AND METHODS

Collection and maintenance of M. edulis: Mussels were obtained from the intertidal zone at Filey, N. Yorkshire, UK. In the laboratory, shells were scrubbed clean of all epibiota and stock mussels were kept for two weeks in an aerated, circulating natural, seawater system (10 °C, salinity 32-34‰) equipped with a biofilter. Mussels were initially fed *Phaeodactylum tricornutum,* but 1 week prior to the experiments the mussels were not fed. For experiments in which mussels were exposed to hyperammonia, they were maintained in oxygenated and ammonia-enriched (3 mM) seawater which was prepared by making appropriate dilutions of a stock solution of ammonium sulphate. Groups of mussels (n = 6 each) from this medium were removed after 2h, 24h, and 4 d for protein and GDH activity determinations. The ammonia-enriched seawater was replaced each day to ensure its constancy, and dissolved ammonia levels monitored at regular intervals using flow injection analysis (FIA) (Hunter & Uglow 1993). No significant ammonia changes were found up to 24 h of exposure. On each sampling occasion, six mussels from the control tank were taken in addition to the six treated mussels.

Enzyme extract preparation: Enzyme extracts were prepared by homogenising a weighed adductor muscle sample in the presence of aluminium oxide (to disrupt the cells) in 50 mM Tris maleate, pH 6.5. The resultant homogenate was clarified by centrifugation at 16,000 g for 30 min, and the supernatant was filtered through Sephadex G-25 to eliminate the endogenous ammonium ion and some metabolites.

Enzyme assays: GDH was estimated using the methods of Ruis Ruano *et al.* (1985) by measuring the rate of decrease (as only the GDH-NADH reaction was considered in this study) at an absorbance of 340 nm using Unicam 86 25 UV/VIS spectrophotometer connected to a Chessel chart recorder. GDH activity in the reductive step was estimated from the oxidation of NADH to NAD^+ (ε = 6.22 cm^{-1} / μmole at 340 nm).

The reaction mixture, in a final volume of 3 ml contained 100 mM potassium phosphate pH 8, 100 mM ammonium chloride, 5 mM ADP, 6.5 mM α-ketoglutarate (αKG), and 0.04 mM NADH. The reaction was initiated by the addition of the muscle extract and the GDH activity was determined from the slope of the initial velocity (Trevor 1985) recorded at 340 nm at 25° C. Results were expressed as international units (I.U. = μmole NADH oxidized min^{-1}).

Protein determination: Following denaturation, total protein was measured using the Biuret method. The copper ions in the Biuret reagent react with the peptides to form a purple colour with an absorbency maximum at 540 nm. The intensity of the colour is proportional to the total protein concentration. The protein in the supernatant was denatured using the method of Gallagher *et al.* (1984), by adding 50 μl of a 4 N NaOH solution to 1 ml to the aliquot sample, this mixture was left for 1 h then centrifuged at 3000 g for 6 min. Following this extraction, the protein was analysed with a SIGMA diagnostics kit (Procedure No. 541). The enzymatic activity

was expressed as μmole min^{-1} mg^{-1} protein (Bergmeyer 1974).

Normally-distributed data ($P > 0.05$, K-S Lilliefors test) of GDH activity were subjected to analysis of variance (one way ANOVA) using SPSS (Subprogram of the statistical Package for the Social Sciences) on a PC. The level of significance for the F test used in conjunction with ANOVA variance data was at the 95% level of confidence.

RESULTS

Although the method of Ruis Ruano *et al.* (1985) was used in these studies, it was necessary to determine the validity of its use with the mollusc adductor muscle assays made here, as compared with the hepatopancreatic tissue used by Ruis Ruano and co-workers. Figure 1A shows the relationship between pH and GDH activity assayed in the reductive direction. The pH optimum of the reaction in glutamate formation was in the range 7.3 – 8.1. The effect of ADP, a GDH activator, on the glutamate-forming activities was found (Fig. 1B) to exhibit a hyperbolic kinetic pattern, with maximal activity occurring in the presence of 5mM ADP.

Optimal conditions of the extract assay for the NADH-dependent GDH activity were obtained using phosphate buffer. Enzyme saturation was obtained with 40 μM NADH but enzyme activity decreased at NADH concentrations > 200 μM (Fig. 2A). Figure 2B gives a graphical Michaelis-Menten plot for α-KG. The enzyme saturation by α-KG was obtained with 6.5 mM concentration of substrate. From the results described above, standard assay conditions were determined as those described the Methods section.

The mean reductive activity of GDH measured in extracts of adductor muscles in the glutamate-forming direction of the control group was 3.62 ± 0.011 μmoles $min^{-1}mg^{-1}$. protein under the extract assay conditions (n = 6). A summary of the enzyme activity of the control, 2 h, 24 h and 4 d ammonia-treated groups is given in Table I. A significant ($P < 0.05$) increase in the glutamate-forming GDH activity was found after 2 h of the hyperammonia treatment. The 24 h-treated group showed a slight, non-significant ($P > 0.05$) change in GDH activity. The GDH activities of the control groups showed no changes ($P < 0.05$) over the 4 d period. The initial induced, elevated GDH activities of the treated groups (*ca* 43.1% higher than those of the control group) were maintained for 4 days and may reflect acclimation of the animals to the contamination of their environment.

Although the hyperammonia-treated groups showed significant alteration of the muscle reductive GDH activities, their muscle protein concentrations (13.84 ± 1.92% wet wt) did not show

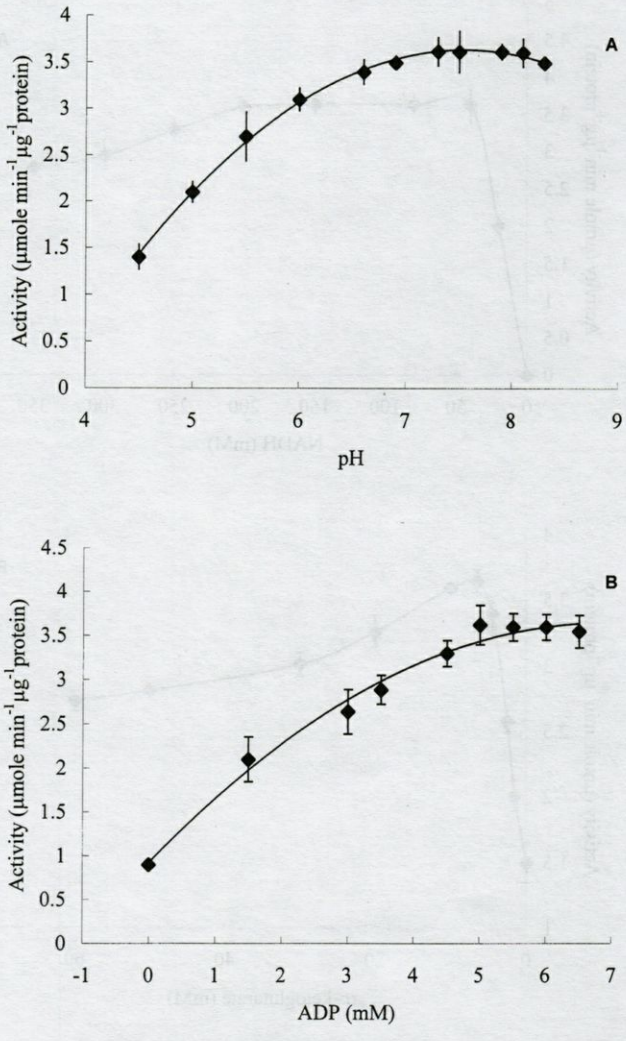

Fig. 1 – *Mytilus edulis*. A, pH and, B, ADP concentration effects on the reductive animation of α-ketoglutarate catalysed par GDH for the adductor muscle (vertical bars = SE, n = 6, each).

Table I. – *Mytilus edulis*. Glutamate dehydrogenase (GDH) activity in the adductor muscle of control and hyperammonia-exposed mussels. Values in column 3 are given ± SE (similar; a / different; b letters comparisons = $P > 0.05$ / $P < 0.05$, One Way ANOVA).

Exposure time to Ammonia enriched Seawater (3 mM)	GDH activity μmole min. $^{-1}mg^{-1}$ pr	SE (n=6)
(Control) 0h	3.629[a]	0.011
2h	6.890[b]	0.415
24h	7.102[b]	0.095
4 days	7.352[b]	0.162

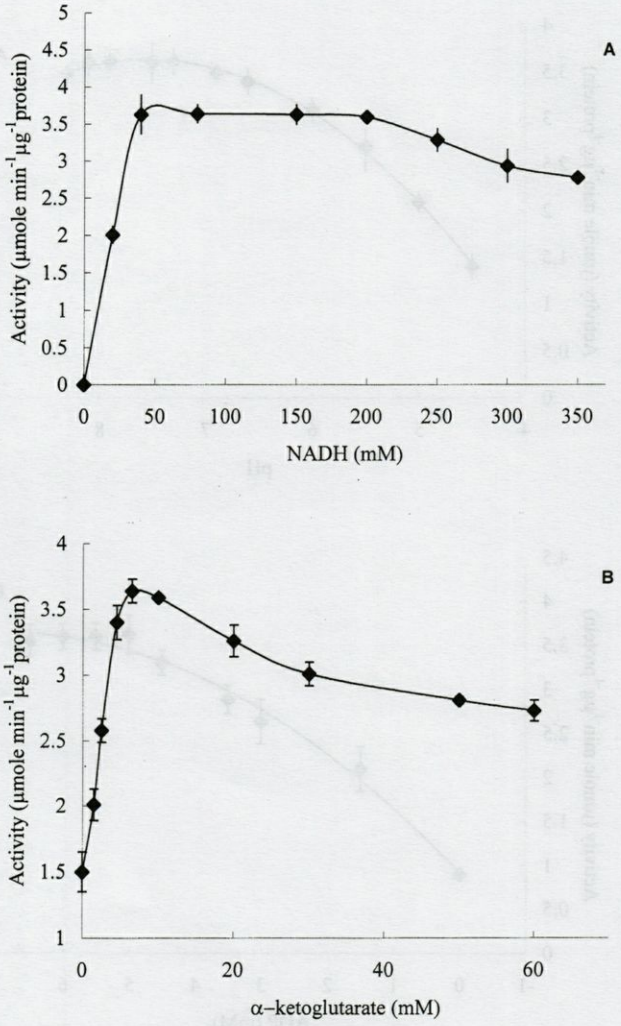

Fig. 2 – *Mytilus edulis*. A, Effects of NADH and, B , α-ketoglutarate concentrations on GDH activity in the adductor muscle (vertical bars = SE, n = 6, each).

significant ($P > 0.05$) changes over the experimental period, despite a lower mean protein concentration measured in the 4 d-exposed group (12.03 ± 0.87% wet wt).

DISCUSSION

The levels of GDH in *Mytilus edulis* adductor muscles measured here (Table I), and those measured in other bivalves, are low in comparison with those of cephalopod tissues (Bishop *et al.* 1983).

Although GDH is considered to be a key enzyme associated with nitrogen metabolism (Batrel & Le Gal 1983) and ammonia represents the bulk of nitrogen excreted (Hawkins & Bayne 1992), few data are available concerning the enzymes associated with nitrogen metabolism in *M. edulis*. Abundant information about GDH activities in other invertebrates is available and a significant relationship exists between ammonia excretion and GDH activity in some crustacean (e.g. *Praunus flexuosus*, Bidigare & King 1981) and annelids (e.g. *Arenicola marina*, Batrel & Le Gal 1983). The paucity of information on *M. edulis* GDH may be related to the relatively low levels of this enzyme in the tissues of that species.

The regulation of GDH is complex (Frieden 1976). The enzymes of some invertebrates utilise either NAD^+ or $NADP^+$ as co-factors (Male & Storey 1983) and others are either $NADP^+$- (Male & Storey 1983) or NAD^+-specific (Reiss *et al.* 1977). Ruis Ruano *et al.* (1985) used different buffers, nucleotides and metabolic ions to demonstrate that *in vitro* preparations of *M edulis* hepatopancreas showed GDH-NAD^+/H activity but no detectable GDH-$NADP^+$ activity. The present findings on the effects of NADH on *M. edulis* adductor muscle GDH activity reveal a total dependence of this enzyme on its co-factor, as no activity was detectable in the absence of NADH (Fig. 2A).

The present study also reveals that ADP exerts an activity effect on the reductive reaction of GDH. The concentrations used lie within the physiological range of this nucleotide and, therefore, are possible physiological regulators. This agrees with the results found for the hepatopancreas of *M. edulis* (Ruis Ruano *et al.* 1985) and with the data found for some crustaceans (Regnault 1989). The role of nucleotides such as ADP on GDH activity has been interpreted as a regulatory link between amino acid production and protein synthesis (Bidigare & King 1981, Dieter *et al* 1981). Hence an activity effect of ADP could be related to energy metabolism, because low energy levels correspond to high ADP concentrations which would stimulate GDH activity and amino acids could be used for energy metabolism. Conversely as energy levels increase, GDH would be inhibited and protein synthesis stimulated (Dieter *et al.* 1981). This implies a role for GDH in the regulation of energy production and growth (Bidigare & King 1981)

Inhibition of GDH by the substrate α-KG occurred at relatively high (> 7.8 mM) concentrations of the substrate (Fig. 2B), which suggests initially high substrate levels in the tissues. These levels in turn may be related to the high activities of various transaminases such as glutamate-oxalacteate and glutamate-pyruvate transaminases as has been shown in *Mya arenaria* mantle tissue (Moyes *et al.* 1985). Under normal conditions, high tissue levels of α-KG would thus reflect periods of GDH inhibition and intense amino acid synthesis.

In the present study, *M. edulis* adductor muscle GDH activity was maximal in the pH range shown

in fig. 1A and these results are in accordance with those of Ruano *et al.* (1995). The *in vitro* pH found for *M. edulis* adductor muscle is close to the internal physiological pH (Zange *et al.* 1990) indicating that GDH is likely to be of functional significance in these bivalves. It was previously shown that some acidification occured in the tissues of mussels under hyperammonia (Sadok *et al.* 1995) which would probably be accompanied by a drop in intracellular pH. The decreased pH would favour ammonia fixation because the optimum *in vitro* pH values for the aminative activities of GDH were lower than those for its oxidative deamination activities (Ruis Ruano *et al.* 1985).

The lack of studies on the effect of hyperammonia on bivalve physiology (Sadok *et al.* 1995), has meant that the activities of enzymes involved in nitrogen metabolism have been largely overlooked. Controversy, evoked by the relative contributions of host tissues to measured ammonia uptake by some bivalve-symbiont associations (Rees 1991), has been largely resolved, because recent work has demonstrated the ability of host tissue to take up ammonia (Fitt *et al.* 1993) and the involvement of enzymes in the regulation of the metabolism of enhanced ammonia in host tissues (Rees *et al.* 1994, Lee *et al.* 1999). These last authors showed that the assimilation of exogenous, inorganic nitrogen by the host tissues, which showed significant activities of the ammonia-assimilating enzyme, glutamine synthetase (Rees *et al.* 1994). Rees *et al.* (1994) showed that, under ammonia enrichment, host tissue had a significantly lower glutamine synthetase activity than the control group in normal seawater, and concluded that ammonia played a role in regulating glutamine synthetase activity. On the other hand, these authors did not detect any GDH activity in the host tissue. The present study revealed a relatively low GDH activity in the adductor muscles of *M. edulis* kept in normal seawater, and the involvement of this enzyme in the assimilation of exogenous ammonia, because significantly higher GDH aminating activities were found in the tissues of mussels exposed to hyperammonia (Table I). In some pelagic and benthic bacteria, hyperammonia also induces a significant increase in the aminating activities of GDH (Hoch 1992). However, Anmuth *et al.* (1982) found a lowered GDH activity in the wood-boring and mud-burrowing bivalves, *Lyrodus pedicellatus* and *Solemya velum*, following long-term incubation in 50 µmole l^{-1} NH_4–N medium.

The reaction involving the α-KG, the NADH and NH_4^+ is also catalysed by inorganic ions (Hochachka & Somero 1973). This reaction could increase the production of glutamate in mussels exposed to ambient ammonia and, *via* the transaminase reactions, glutamate could give rise to other amino acids (such as alanine and glycine) because the requisite sets of aminotransferases have been found in *M. edulis* (Bishop *et al.* 1983). This may explain the increase in haemolymph amino acids (measured as ninhydrin positive substances, NPS) levels found in hyperammonia-treated mussels (Sadok *et al.* 1995). Moreover, the insignificant ($P > 0.05$) drop in protein content measured here during the ammonia treatment period (5.89 ± 0.52% wet wt, after 4 d), emphasises this assumption as, in this case, catabolism of protein did not contribute to the observed rise in NPS levels.

Given the higher GDH activity found under hyperammonia it is likely that, in the event of a short-term increase in ammonia availability, mussels would have the potential to assimilate most of the nitrogen present. However, in the persistent presence of high ammonia levels, other metabolic pathways may be used, as GDH activity remained constant under hyperammonia. Furthermore, several mechanisms may operate simultaneously within an individual in order to maintain physiological homeostasis. Such diversity emphasises the complex nature of strategies which have evolved in mussels in order to enable them to withstand high levels of ammonia in their environment.

ACKNOWLEDGEMENTS. – We wish to thank the British Council and the University of Hull (U.K.) for supporting part of this work.

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Reçu le 6 juin 2001; received June 6,2001
Accepté le 31 juillet 2001; accepted July 31,2001