THE INFLUENCE OF SEASONAL CHANGES ON ENERGY METABOLISM IN *MYTILUS EDULIS* (L).—III. ANAEROBIC ENERGY METABOLISM

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Abstract—1. Seasonal changes in the accumulation of end products after 48 hr of exposure to air and in the composition of the free amino acid pool were studied in *Mytilus edulis*.

2. The accumulation levels of succinate and acetate showed only weak seasonal changes.
3. Conversion of succinate to propionate was high in summer and virtually zero in winter.
4. Alanine and most other free amino acids were present in relatively high concentrations in summer and early autumn and reached minimal values in winter and early spring.
5. Exceptions were glutamate, aspartate and taurine, which showed hardly any season related changes and glycine, which changed inversely to the majority of the free amino acids.
6. The anaerobic formation of alanine was inversely proportional to the endogenous concentration.
7. The only other free amino acids affected by anaerobiosis were glutamate and aspartate, which respectively increased and decreased under these conditions.

INTRODUCTION

While many molluscs are well-adapted to survive hypoxic and anoxic conditions, there is only limited uniformity in the biochemical pathways of energy metabolism that are employed.

Although complete oxidative breakdown of energy substrates via the tricarboxylic acid cycle is not fully blocked during anaerobiosis (e.g. *Mytilus edulis*; De Zwaan, 1977), it is nevertheless severely restricted and necessarily accompanied by a simultaneous formation of succinate or a derivative, e.g. propionate (Kluytmans *et al.*, 1975). In all animals where anoxia leads to an activation of the phosphoenolpyruvate carboxy-kinase pathway these are therefore ubiquitous end products. However, other routes employed, either to maintain redox balance or to gain ATP by substrate level phosphorylations, may lead to accumulation of a variety of other intermediates such as lactate, alanine, acetate, octopine and *N*-(1-carboxyethyl)alanine, which are formed to a different extent in various species. Differences are observed not only between various molluscan species, but also intraspecific variations are frequently observed.

Experiments carried out by us with *Mytilus edulis* showed considerable differences in the relative amounts in which the various metabolic end products were formed, even when all variables were kept as identical as possible to those in experiments carried out before.

It therefore seems likely that some differences were due to changes within the animals and could be related to cyclic changes in environmental parameters and the gametogenic cycle.

To obtain more direct evidence for this assumed relationship, groups of mussels were exposed every 3

weeks during a 1.5 yr period and analysed for accumulation of anaerobic intermediates.

These data, in relation with many biochemical and environmental parameters which were determined simultaneously (Pieters *et al.*, 1979; Zurburg *et al.*, 1979) should give a better insight in the variability of end product accumulation.

MATERIALS AND METHODS

From February 1977 through June 1978 mussels were collected from the same locality in the Dutch Wadden Sea. The animals were cleaned, grouped according to shell length and kept in running seawater at ambient temperature. For further details is referred to Pieters *et al.* (1979). After a recovery period of 3–4 days, groups of 50 animals were subjected to anoxia by exposure to air for 48 hr just above the water level to keep their body temperature equal to the unexposed groups. Afterwards the animals were excised from the shells in a N₂-stream and after determination of the wet weight (including enclosed seawater, hemolymph and extrapallial fluid) homogenized with 2 vol of ice-cold bicarbonate buffer (0.02 M, pH 9.5) in a Sorvall Omnimixer for 3 min at maximum speed.

Succinate

For the determination of succinate, weighed aliquots of about 20 ml homogenate were deproteinized with 2 ml 70% perchloric acid and centrifuged for 10 min at 25,000 *g*. The supernatant was neutralized to pH 7.5 with 5 M K₂CO₃, stored overnight at –20°C, thawed and centrifuged for 10 min at 20,000 *g*. In the clear supernatant succinate was determined spectrophotometrically according to the method of Williamson (1974a).

Volatile fatty acids

Volatile fatty acids (VFA) were extracted by treating weighed aliquots of about 50 ml homogenate with

Fig. 1. Seasonal variations of the accumulation in total tissues of exposed (anaerobic) or immersed (aerobic) groups of 50 mussels of succinate (A), acetate (B), and propionate (C). Exposure time was 48 hr. In D the seasonal fluctuations of the anaerobic increase in succinate and acetate are plotted.

chloroform-methanol (2:1, v/v), according to Folch *et al.* (1957) after addition of 200 μ l 0.1 M valeric acid as an internal standard. After phase separation the methanol-water layer was isolated and the chloroform layer washed with 50 ml "upper phase" to which 0.3% NaCl had been added. The combined methanol-water fractions were adjusted to pH 9 with 1 N KOH and evaporated to dryness under reduced pressure. The residue was redissolved in 25 ml of redistilled water and transferred to a steam distillation flask.

The further isolation procedure and analysis were carried out as described before (Kluytmans *et al.*, 1975).

Free amino acids

For the determination of free amino acids (FAA) weighed aliquots of about 10 ml homogenate were acidified with 1 ml 70% perchloric acid. The precipitated proteins were removed by centrifugation during 10 min at 10,000 *g*. The pH was subsequently adjusted to pH 2-3 with 5 M K_2CO_3 and after 1 hr at 0°C centrifuged (10 min at 48,000 *g*) to remove $KClO_4$ crystals.

The supernatant was analysed for FAA by injecting 25 μ l samples into a LKB-3201 amino acid analyser, equipped with a column (length 30 cm, internal diameter 6 mm) filled with Durram DC-6A spherical cation exchanger.

Quantitative and qualitative determination of the amino acids was carried out by comparison with standard mixtures.

Alanine was also determined spectrophotometrically according to Williamson (1974b).

RESULTS

Succinate

The accumulation of succinate in mussels exposed for 48 hr at ambient temperature is shown in Fig. 1A. In all experiments a significant increase was observed with an average value of about 20 μ mol/g dry weight. While considerable fluctuations around this value were observed in the different experiments, the succinate accumulation showed no clear relation to seasonal changes.

Propionate

The above results were not however, true for the conversion of succinate to propionate (Fig. 1C). Aerobic values remained virtually zero, but there was a strong increase in anaerobic propionate accumulation during spring and early summer, a gradual decline during late summer and autumn and no synthesis during the winter period.

Acetate

No comparable changes were observed for acetate (Fig. 1B). While anaerobic values were generally slightly higher as aerobic values seasonal changes are not clear, with the exception perhaps of a tendency for somewhat higher levels (both aerobic and anaerobic) in early spring.

Alanine

Alanine levels were clearly related to seasonal changes (Fig. 2A). Both aerobic and anaerobic levels were minimal in early spring and reached a maximum in late summer. Since alanine is an anaerobic end product in *Mytilus edulis* the contribution of anoxia to the total accumulation levels during the seasons was obtained by plotting anaerobic minus aerobic values versus time in Fig. 2D. These data show that

while a net alanine formation occurred in winter and early spring, virtually no synthesis took place in summer and autumn.

Glutamate and glutamine

The observed levels of these amino acids are depicted in Fig. 2B. While glutamine changed in a way similar to alanine, glutamate showed no seasonal changes. On the contrary, while glutamine was unaffected by anaerobiosis, the level of glutamate was elevated after 48 hr of anoxia. This is more clearly seen in Fig. 2E.

Aspartate and asparagine

The seasonal fluctuations in asparagine were similar to those found for alanine and glutamine, albeit that the changes in asparagine were smaller than those observed in glutamine (Fig. 2C). On the other hand, anoxia decreased rather than increased the level of aspartate, as is evident from Fig. 2E. This figure also shows that neither glutamate nor aspartate accumulation decrease during anaerobiosis is strongly influenced by seasonal changes.

Other free amino acids

The changes observed in the other major free amino acids, including taurine, are depicted in Fig. 3. While taurine levels were high, they were apparently not affected by seasonal factors. As no reliable values for taurine were obtained in the first experiments these are omitted from Fig. 3.

Submitting groups of mussels to 48 hr of anoxia did not give much change in taurine levels, nor in the level of any of the other free amino acids shown in this figure.

Whereas serine, arginine, lysine, threonine and proline all showed some seasonal variations comparable to the changes discussed for alanine, asparagine and glutamine, the quantitatively most important amino acid glycine behaved in an opposite way. Glycine levels were high in winter and early spring and much lower in summer and autumn.

Temperature and salinity changes

Free amino acids are important expedients in adaptation to osmotic stress by many invertebrate osmoconformers. Salinity changes may therefore strongly influence the composition of the intracellular free amino acid pool.

Temperature and food availability are important parameters in energy metabolism.

For easy reference these data, which have already been published recently by Pieters *et al.* (1979) and Zurburg *et al.* (1979) are summarized in slightly modified form in Fig. 4.

DISCUSSION

Anaerobic metabolism

Of the common anaerobic end products encountered in *Mytilus edulis* propionate shows the strongest seasonal fluctuations in its accumulation rate (Fig. 1C). This is apparently not so for its precursor succinate (Fig. 1A), which shows a rather fixed accumulation level during all seasons, although of course total succinate synthesis, which is the sum of

Fig. 2. Seasonal changes in aerobic and anaerobic levels of alanine (A), glutamine (B), and aspartate (C) and aerobic levels of glutamine (B) and asparagine (C). Exposure time was 48 hr at ambient temperature. Anaerobic changes for alanine are shown in D and for glutamine and aspartate in E.

Fig. 3. Seasonal changes of some important free amino acids in total tissues.

succinate and propionate, will resemble the pattern found for propionate. From this data the conclusion can be drawn that succinate to propionate conversion is more affected by seasonal factors than is succinate synthesis *per se*. Moreover, it is clear that the absence of propionate synthesis is not due to a too low level of precursor. The form of the propionate accumulation curve strongly resembles the changes in temperature and salinity of that part of the Wadden Sea from which the mussels were obtained. This similarity is in

agreement with earlier experimental data showing that both the lag period between the onset of anoxia and the beginning of propionate synthesis and the rate of propionate synthesis are strongly temperature dependent (Kluytmans *et al.*, 1978). It was also found that succinate synthesis was less influenced by temperature changes than propionate formation, whereas an effect of salinity, which changes concomitantly with temperature, on propionate synthesis, cannot be excluded.

Fig. 4. Seasonal changes in some important environmental characteristics of the Dutch Wadden Sea during the period of experimentation.

Fig. 5. Statistical relation of alanine concentration with anaerobic accumulation (A) and salinity (B).

Seasonal effects on both aerobic and anaerobic acetate levels are inconspicuous, although there is a somewhat stronger anaerobic accumulation in spring and summer periods compared to the cold season (Fig. 1D). Most Δ acetate levels are above zero, confirming earlier results that some acetate is formed in anoxic mussels, but that it is not a major anaerobic end product. When sometimes high acetate levels are observed in groups of *Mytilus edulis*, they form a strong indication of the presence of dying animals.

In aerobic and anaerobic animals alanine levels show similar seasonal changes (Fig. 2A), which resemble to a certain extent the changes observed in propionate, and in the environmental parameters temperature and salinity (Fig. 4).

As alanine is a well-known contributor to intracellular osmolarity in osmoconforming molluscs (Pierce, 1971; Baginsky & Pierce, 1975; Livingstone *et al.*, 1979) salinity is probably a more important determining factor than temperature. However, as both parameters changed coincidentally in these experiments, a more or less strong temperature effect remains possible. Besides being a factor in osmoregulation, alanine is also involved in anaerobic metabolism in *Mytilus edulis* (De Zwaan, 1977; Kluysmans

et al., 1977). The contribution of anaerobiosis to the overall changes in alanine is shown in Fig. 2D. The changes in Δ alanine are more or less inverse to those in the total free alanine pool, suggesting a negative feedback effect of a high endogenous alanine level on its synthesis. It therefore seems that anaerobic alanine formation is in an indirect way influenced by seasonal changes, viz. via the total alanine pool. A more quantitative approach to these relations is given in Figs 5 and 6. A linear relationship is assumed between the plotted variables, which may not be correct in all cases, but is hard to validate or invalidate graphically due to the wide fluctuation of the individual data.

Figure 5B shows a moderately strong correlation of 0.64 between salinity and intracellular alanine concentration, which is in agreement with the data of Baginsky & Pierce (1975) and Livingstone *et al.* (1979). However, for a similar graph of alanine concentration versus temperature (not shown) a correlation coefficient of 0.7 was calculated. Although this could be mere coincidence, it could also mean that temperature is a regulating factor of the endogenous free alanine pool.

The statistical relation between the free alanine concentration and its anaerobic accumulation is depicted in Fig. 5A, which shows a strong correlation (0.86) between these variables. This makes it likely that feedback inhibition by intracellular alanine is a major regulating factor in anaerobic alanine formation. Environmental variables like temperature (Fig. 6A) and salinity (not shown) are only weakly correlated (0.31) with anaerobic alanine synthesis and are therefore probably only of minor importance.

This also applies to anaerobic acetate formation (Fig. 6C) and succinate accumulation (Fig. 6B), which show only weak, respectively no dependence on the environmental temperature.

However, the conversion of succinate towards propionate (Fig. 6D) appears to be very strongly temperature dependent, which confirms the more qualitative conclusion arrived at before.

In the calculation of the correlation coefficient values obtained for groups incubated at ambient temperatures below 5°C are omitted, because no propionate synthesis could be detected at these temperatures.

Two other compounds, not generally acknowledged in their relation to anaerobic metabolism should be mentioned here, viz. glutamate and aspartate. Figure 2E shows in all experiments an increase in glutamate and in virtually all experiments a decrease in aspartate after 48 hr of anoxia. Consequently glutamate should be considered as an anaerobic accumulation product, at least in *Mytilus edulis*, and contrary to earlier notions (Hochachka *et al.*, 1973; Hochachka, 1976) not as a substrate. Although an accumulation does not necessarily exclude its utilization, no simultaneous decrease in other free amino acids, e.g. proline, by which the glutamate pool could be replenished, was observed. Moreover, results from *in vitro* experiments with $\text{UL-}[^{14}\text{C}]$ glutamate are not in agreement with an active anaerobic glutamate conversion (De Zwaan *et al.*, 1975).

The decrease in aspartate level indicates a role as a substrate during anaerobiosis. Such a function has been proposed by Ebberink *et al.* (1979) and Zurburg

Fig. 6. Statistical relation of the anaerobic accumulation of alanine (A), succinate (B), acetate (C), and propionate (D) with the environmental temperature.

& Ebberink (1980), who assume that aspartate functions as an initial substrate during the first hours of anoxia, when the phosphoenolpyruvate carboxykinase pathway is not yet sufficiently activated to provide the oxaloacetate for succinate formation. As the average free aspartate pool in these experiments was 38 $\mu\text{mol/g}$ dry weight, whereas the rate of succinate synthesis varied between about 1 to 2 $\mu\text{mol/g}$ dry weight/hr, aspartate could support anaerobiosis in the blue mussel for about 1 day. Transamination with pyruvate or α -oxoglutarate could account for the observed initial increase in alanine (Kluytmans *et al.*, 1977) as well as for the increase in glutamate, while at the same time osmotic problems due to the utilization of aspartate are prevented, because the total free amino acid pool remains more or less constant.

However, the observed average decrease in aspartate after 48 hr was only 7.7 $\mu\text{mol/g}$ dry weight or about 20% of the total aspartate pool. This can possibly be explained in the following way: (1) Wijsman (1975) has shown that after 12 hr the pH in *Mytilus edulis* has fallen from 7.4 to about 6.6. As the pH is a major regulating factor of the phosphoenolpyruvate (PEP) branchpoint (De Zwaan, 1977), the PEP-CK activity is increased long before the aspartate pool is exhausted; (2) the initial increase in alanine concentration during anoxia is followed by a return to the starting level (Kluytmans *et al.*, 1977), which is reached after about 12 hr.

With a sufficient supply of oxaloacetate via the PEP-CK route the aspartate pool could be partly restored by transamination from alanine. This reversible shift of aspartate to alanine provides *Mytilus edulis* with an ample supply of substrate during the initiation of anaerobiosis, while the increased initial alanine concentration inhibits pyruvate kinase activity and promotes the switch of the PEP branchpoint.

Other free amino acids

In contrast to the free amino acids discussed so far, which are involved in anaerobic metabolism, the other compounds of the FAA pool show no or negligible changes during anaerobiosis. However, most of them show seasonal changes in concentration, more or less similar to those observed for alanine. Though, we observed a clear difference in behaviour between asparagine and glutamine on the one hand and aspartate and glutamate on the other hand. While the first change seasonally (Figs 2B and 2C), the latter remain more or less the same during the year. A near equilibrium of glutamate and aspartate with citric acid cycle intermediates can explain their rather constant values as well as, at least partly, account for the observed anaerobic changes.

More difficult to explain is the behaviour of the two major components of the free amino acid pool, viz. taurine and glycine. While both are generally considered as main contributing factors in osmotic adaptations (Baginski & Pierce, 1975), changes in seawater salinity during these experiments (Fig. 4) did not invoke significant changes in taurine, while glycine even decreased with increasing external salinity. The strong decrease in glycine is roughly equal to the combined increases in the other free amino acids, so that as a whole the free amino acid pool did not seem

to change in relation to environmental salinity changes. Whether this means that other compounds such as e.g. betaine are more important in osmoregulation by *Mytilus edulis* is at present unknown.

It is also unclear why glycine shows such a strong decrease during summer and autumn. While this amino acid can easily be converted to serine or pyruvate, the concomitant changes in serine are rather small, so this compound is probably in turn converted to pyruvate. As the glycine decrease starts at the moment that glycogen build-up begins (see Pieters *et al.*, 1979) a rather speculative explanation of the glycine changes could be that during the period of glycogenesis this compound provides pyruvate to fuel the tricarboxylic acid cycle. Some support for this idea is given by the fact that glycine decrease holds at the same moment as the glycogen build-up stops. However, no restoration of the glycine pool was observed until about 6 months afterwards, so evidently other factors are involved as well.

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