THE INITIAL STAGE OF ANAEROBIC METABOLISM IN THE SNAIL, *HELIX POMATIA* L

Wolfgang WIESER
Institut für Zoophysiologie der Universität Innsbruck, Innsbruck, Austria

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1. Introduction

In invertebrates two or three stages of metabolism may be distinguished when the animal is deprived of oxygen. During the initial stage lactate and/or alanine are rapidly formed. In the continued absence of oxygen processes leading to succinate, acetate or propionate become dominant [1–3]. The existence of such a sequence of metabolic events provides good opportunity for studying, on a comparative level, the regulation of carbohydrate degradation in animals.

I report here the results of a study of anaerobic metabolism in the pulmonate snail, *Helix pomatia*, known to be periodically anoxic in winter when buried in water-logged soil [4–6], but not when active in spring or summer. The time course of D-lactate formation and other metabolites was followed as well as that of the activity of one of the key enzymes of anaerobic metabolism, D-LDH, in the foot of the animal, during prolonged periods of anoxia.

2. Experimental

Winter and spring specimens of *H. pomatia*, collected in the vicinity of Innsbruck, were subjected, at room temperature, to an oligoxic stress for varying times. The animals were kept in a moist chamber which could be flushed continously with oxygen-free nitrogen. A vessel with alkaline pyrogallol was also put into the chamber. pO_2 was monitored with an oxygen electrode.

After a certain length of time under nitrogen one snail was removed, the foot excised, cut into two pieces, of which one was frozen immediately in liquid propane cooled in liquid nitrogen. The second piece was weighed and also frozen after exactly 5 min. D-Lactate, succinate and alanine concentrations were determined enzymatically [7]. The difference in D-lactate content between the two pieces is a measure of the activity of the D-LDH in the excised foot at room temperature.

Abbreviation: D-LDH, D-lactate dehydrogenase (EC 1.1.1.28)

3. Results

3.1. *Changes in the concentrations of metabolites. Seasonal aspects*

Figure 1 illustrates the changes in D-lactate, succinate and alanine concentrations in spring animals exposed to nitrogen for up to 32 h. In control animals kept in air no lactate was found ($n = 6$). Alanine concentration varied from 0–0.56 ($\bar{x} = 0.16$) μmol.g fresh weight (f.wt)$^{-1}$. Under nitrogen, alanine in the foot of the snail increased steadily up to a mean value of 8 μmol.g^{-1}. The concentration of succinate remained fairly stable at an even lower level (av. 5.1 μmol.g^{-1}). D-Lactate, however, showed a more complicated course. After about 14 h it had reached a first maximum of ~40 μmol.g f.wt^{-1}, then dropped to about half that value, whereupon it increased again to 60 μmol.g^{-1} and more. This is amongst the highest concentrations of lactate ever recorded in living tissue. None of the spring animals survived more than 31 h in nitrogen at room temperature. In winter snails, D-lactate concentration varied from 0–2 μmol.g f.wt^{-1} ($\bar{x} = 1.1$; $n = 9$). Under nitrogen, lactate increased rapidly after 4–6 h, reaching a peak between 6 and 11 h. However, after 14 h the concen-

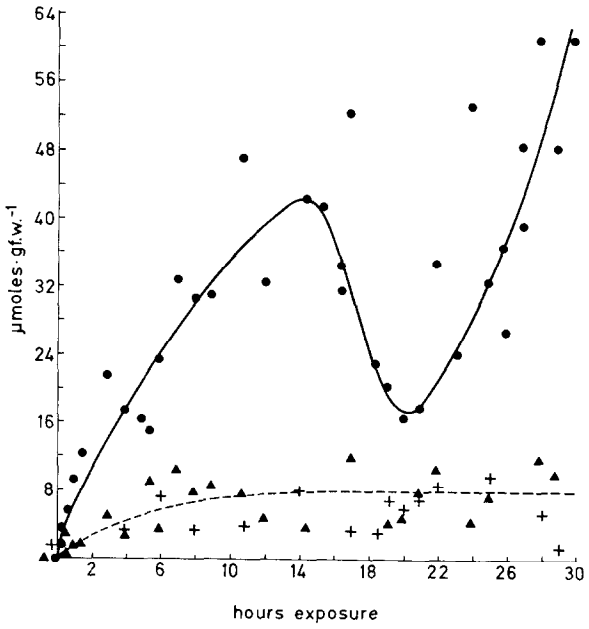

Fig.1. Concentration of D-lactate (circles), succinate (crosses) and L-alanine (triangles) in the foot of *Helix pomatia* (spring animals) after the animals had been exposed to oxygen-free nitrogen for varying lengths of time. The concentrations are in µmol. (g fresh tissue weight)$^{-1}$. The foot was severed from the snail, cut into two pieces, one of which was immediately frozen in liquid propane, the other was weighed and frozen after 5 min. The frozen pieces were homogenized in 6% (w/w) ice-cold perchloric acid with an Ultraturrax, the homogenate centrifuged at 17 000 rev./min for 10 min, the supernatant decanted, neutralized with K_2CO_3 and recentrifuged. The sediment was weighed and since the fresh weight of the second piece of the foot was also known, the fresh weight of the first piece could be calculated by extrapolation. D-Lactate was determined with D-LDH (Boehringer) in glycine-hydrazine buffer, pH 9.5, L-alanine with L-alanine-DH (Sigma) in Tris-hydrazine buffer (pH 10.0) and succinate in triethanolamine buffer (pH 7.4) with succinic thiokinase, pyruvate kinase and L-LDH (Boehringer) as in [4]. The lower regression curve shown is for alanine only. The upper curve was eye-fitted to the individual symbols. All determinations were carried out on freshly collected snails between April 24 and June 13, 1978.

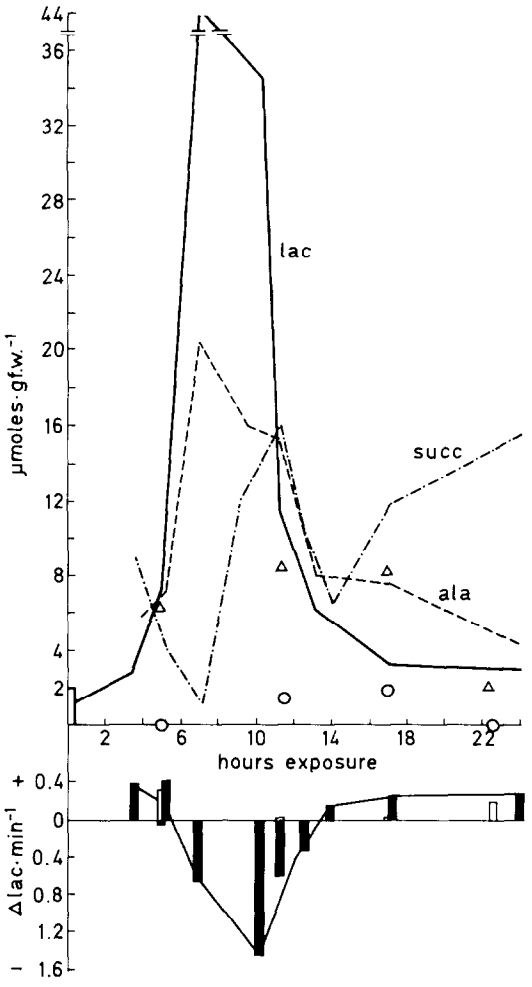

Fig.2. Upper portion: As fig.1 but with winter animals that had been kept in a cold room in the dark at 0–4°C. The animals were brought from the cold room to the lab and exposed to nitrogen. For clarity only the curves eye-fitted to the individual points are drawn. The scatter of the points was just as in fig.1. Open symbols refer to control animals kept at room temperature in air; open circles, D-lactate; triangles, alanine.

Lower portion: The activity of D-LDH in the excised foot at room temperature, expressed as µmol. (g fresh weight)$^{-1}$.min^{-1}. Changes in the concentration of D-lactate between the two pieces of the excised foot were either positive (gain) or negative (loss). Full bars, animals exposed to nitrogen; open bars, control animals kept in air.

tration of lactate returned to more or less the resting level (fig.2). The same is true for alanine which shows a peak synchronous with the lactate peak, increasing from a resting value of 6.4 ± 3 (range 2.1–8.6) µmol.g^{-1} and returning again to the resting level. The sequence of events recorded in fig.2 suggests that in

the winter snail the formation of lactate provides the energy for the first phase of anaerobic metabolism but is replaced, as the anoxic stress continues, by other anaerobic processes. As far as mollusks are concerned, two such processes have been discussed: the formation of succinate and/or propionate [1], and fatty acid synthesis [8,9]. The changes in the concentration of succinate in the foot of the winter snails represent, to some extent, a mirror image of the changes in the concentration of lactate and alanine. Thus it appears that the formation of succinate plays a role in the second phase of anaerobiosis in hibernating, but not in active, animals of this species.

3.2. *Activity of the D-LDH*

The activity of the D-LDH as illustrated in the lower part of fig.2 indicates that the excised foot of *H. pomatia* is capable of lactate oxidation when the concentration of this metabolite reaches a critical value. A more complete picture is revealed when winter and spring animals are combined and D-LDH activity in the excised foot is plotted against the initial value of lactate concentration in this tissue (fig.3). Lactate formation continues up to ~4 mM, which corresponds to the K_m-value of lactate oxidation in vitro [10]. Beyond this value lactate is oxidized to pyruvate with kinetics, at ≤36 mM, that closely resembles the in vitro kinetics of the reaction (inset, fig.3). However, at higher concentrations a second mechanism comes into effect and lactate disappearance is accelerated up to a value that is nearly 4-times higher than the maximum rate of the first phase of lactate oxidation in the tissue. This break can be explained only by an increase in the activity of LDH in the foot, either through recruitment of new LDH molecules from an inactive store, or by conformational changes of the already available molecules. The most attractive explanation of this phenomenon would be a variant of a mechanism suggested [11]: Normally LDH is partly inactive by being bound to cellular structures (see also [12]); the rise in lactate concentration leads to modifications of the intracellular environment which in turn cause more LDH molecules to be mobilized from their inactive state. A change in pH would appear to be the leading candidate as the effector which causes this transformation of the enzyme. A similarly crucial role of pH changes causing an increase in the excitability of crayfish slow muscle fibres under anoxia has recently been suggested [13].

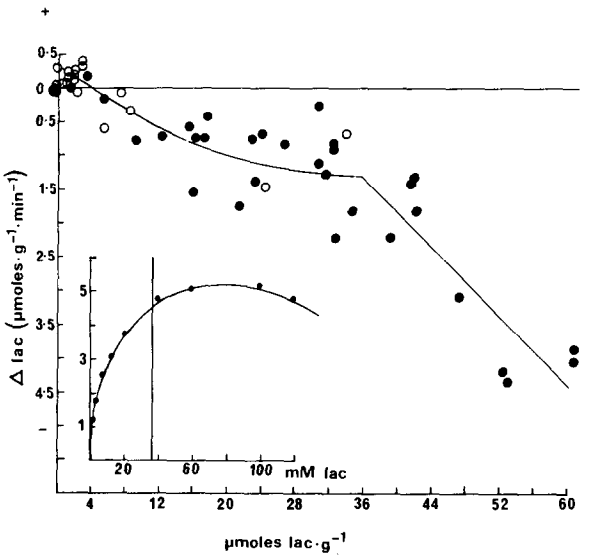

Fig.3. The activity of the D-LDH in the excised foot of *Helix pomatia* plotted against the concentration of D-lactate in this tissue. The activity of the enzyme is taken as the difference in the concentration of lactate between two pieces of the foot, the first one frozen immediately after excision, the second one 5 min later, during which time the second piece had been kept at room temperature. Open symbols, winter animals; full symbols, spring animals.

Inset: The rate of lactate oxidation in vitro plotted against substrate concentration. The foot of a snail was frozen and homogenized in 0.01 M glycine buffer (pH 8.6), the homogenate centrifuged at 17 000 rev./min for 25 min. The supernatant served as the source of the enzyme (see [10]). The vertical line indicates the concentration of lactate at which, in the in vivo experiments, a break in the kinetics of lactate oxidation was observed.

4. Discussion

The experiments with the foot of *H. pomatia* suggest the following course of events during the first phase of anaerobic metabolism in this species: Under anoxic conditions lactate is formed in all tissues and is distributed through the organism via the hemolymph. Despite the foot's ability to oxidize the metabolite the balance of the reaction catalyzed by LDH is such

that lactate accumulates. However, around at 36–40 mM changes in the intracellular environment become critical, leading to an activation of LDH and to an accelerated net removal of lactate from the foot (and probably from other tissues as well). This break in the rate of lactate oxidation depends on the presence of intact cellular structures since it is not observed in the in vitro assay (inset fig.3).

In winter animals the disappearance of lactate introduces a second phase of anaerobic metabolism, involving the formation of succinate and, perhaps, of other end products, whereas spring animals are unable to cope with conditions of prolonged anoxia and lactate begins to rise again. The disappearance of lactate must, of course, be paralleled by an equally rapid removal of pyruvate – either by the pathways leading to succinate [1] or by anaerobic decarboxylation to acetyl-CoA as suggested [9]. Acetyl-CoA could give rise to the synthesis of fatty acids, the energy required for this process being supplied by the first spurt of glycolysis.

It is clear that other mechanisms, too, must be involved in the switchover from one anaerobic phase to the next, but the accumulation of lactate may be the factor that starts the whole sequence of events.

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