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EFFECTS OF ANOXIA ON THE EXTRA- AND INTRACELLULAR ACID–BASE STATUS IN THE LAND SNAIL *HELIX LUCORUM* (L.): LACK OF EVIDENCE FOR A RELATIONSHIP BETWEEN PYRUVATE KINASE DOWN-REGULATION AND ACID–BASE STATUS

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Summary

The aims of the present study were to describe a possible correlation between the regulation of the key glycolytic enzyme pyruvate kinase and the acid–base status in the haemolymph and in several other tissues of land snails during anoxia. To illustrate whether such a relationship exists, we determined (i) the acid–base variables in the haemolymph and tissues of the land snail *Helix lucorum*, (ii) the kinetic properties of pyruvate kinase from several tissues and (iii) the levels of the anaerobic end-products D-lactate and succinate in the haemolymph and tissues of aerobic and anoxic *Helix lucorum*. The results showed that the pH of haemolymph (pHe) decreased significantly over the first 20 h of anoxia and then recovered slowly towards control values. A similar pattern was observed for intracellular pH (pHi), which decreased significantly over the first 16 h of anoxia and slowly returned towards control levels. The reduction and recovery of pHi and pHe seem to reflect the rate of anaerobic metabolism. The main anaerobic end-products, D-lactate and succinate, accumulated rapidly during the initial stages of anoxia and more slowly as anoxia progressed. The decrease in the rate of accumulation of anaerobic end-products during prolonged anoxia was due to the conversion of tissue pyruvate kinase to a less active form. The results demonstrate a correlation between pyruvate kinase down-regulation and the recovery of acid–base status in the haemolymph and the tissues of land snails during anoxia.

Key words: land snail, *Helix lucorum*, anoxia, pyruvate kinase, acid–base status.

Introduction

Like marine molluscs, pulmonate land snails are able to survive long periods of anoxia during which they deplete tissue glycogen reserves for energy production (von Brand, 1944; Oudejans and van der Horst, 1974). However, Rees and Hand (1990) stated that anaerobic processes do not contribute to the energy metabolism of land snails during dormancy. Nevertheless, some evidence indicates that, at certain times during dormancy, energy production in land snails may be based on anaerobic processes. It has been reported that terrestrial snails may find themselves in oxygen-free conditions when they burrow deeply into the ground during hibernation (von Brand, 1944). Moreover, anaerobic metabolism seems to occur in dormant pulmonates because the rate of oxygen consumption falls below measurable limits for hours or even days (Schmidt-Nielsen et al., 1971; Nopp, 1974). Moreover, Wieser and Wright (1978) have shown that land snails possess a high glycolytic potential that may be used for anaerobic energy production even when the animal's environment appears to provide an adequate supply of oxygen.

The main metabolic end-products that accumulate in the tissues and haemolymph of land snails are D-lactate and succinate (Wieser, 1981; Churchill and Storey, 1989). However, it has been reported that, during prolonged anoxia, pyruvate is directed into fatty acid biosynthesis, resulting in an increase in fatty acid levels. According to Oudejans and van der Horst (1974) and van der Horst (1974), D-lactate is reoxidized during prolonged anaerobiosis to pyruvate and, despite the presence of a high D-lactate dehydrogenase activity in all tissues of snails, D-lactate is decarboxylated anaerobically to acetyl-CoA, which then is used for lipid synthesis.

The mechanisms that might control the flow of pyruvate into fatty acid biosynthesis in the tissues of land snails during anoxia are not known. According to published data, however, the key glycolytic enzyme pyruvate kinase may play an important role in the flow of pyruvate towards fatty acid biosynthesis. Pyruvate kinase from some tissues of the land snail *Otala lactea* is converted to a less active form during the first 12 h of anoxia (Whitwam and Storey, 1990). The main mechanism involved in the conversion of pyruvate kinase to a

less active form seems to be phosphorylation of the enzyme. The changes in kinetic properties of pyruvate kinase that produce a less active form of the enzyme lead to a decline in the glycolytic rate and a consequent reduction in the rate of accumulation of the anaerobic end-products D-lactate and succinate (Churchill and Storey, 1989).

The accumulation of D-lactate and succinate in the haemolymph and tissues of land snails during anoxia may result in a significant change in acid–base balance in the extracellular and intracellular fluids. It has been shown for the haemolymph of *Helix pomatia* that accumulation of these anaerobic end-products causes a significant reduction in extracellular pH (Wieser, 1981). However, a reduction in the pyruvate kinase activity in the tissues of land snails is correlated with a reduction in glycolytic rate and, consequently, with a reduction in the rate of accumulation of D-lactate and succinate in the tissues during prolonged anoxia. The down-regulation of pyruvate kinase activity and the possible redirection of pyruvate into fatty acid biosynthesis during prolonged anoxia may have a positive influence on the acid–base status of tissues. The extent of acidification of the intracellular fluids in marine molluscs during anaerobiosis is dependent on the specific pathways operating (Pörtner et al., 1984; Pörtner, 1987, 1989). However, cells can regulate their intracellular pH (pHi) both by physicochemical buffering and by exchange of acid–base equivalents across the plasma membrane (Walsh and Milligan, 1989). Some investigations have revealed that invertebrate tissues have some ability to regulate pHi (Thomas, 1974; Zange et al., 1990; Ellington, 1993). Nevertheless, we do not know how the acid–base status is affected in the tissues of land snails during short-term and long-term anoxia.

In the present study, we have examined whether the regulation of pyruvate kinase activity is correlated with changes in the acid–base status in the extra- and intracellular compartments of land snails during periods when energy production is based on anaerobic processes. Specifically, the acid–base status of the haemolymph and several other tissues (foot, heart, suboesophageal ganglia and mantle) of the land snail *Helix lucorum* was studied after 0, 2, 4, 8 16, 20, 24 and 48 h of anoxia. Moreover, we studied the kinetic properties of pyruvate kinase from all four tissues, and we determined the concentrations of the anaerobic end-products D-lactate and succinate in the tissues and haemolymph.

Materials and methods

Adult specimens of *Helix lucorum* (L.) were collected in the vicinity of Edessa, in northern Greece, and maintained in an active state in large glass boxes at 25 °C and with a 14 h:10 h L:D photoperiod. The snails were fed daily with lettuce leaves and supplied with water. Relative humidity was maintained at a high level (approximately 85 %) by sprinkling the interior of the boxes with water every day. Anoxia was imposed as described by Whitwam and Storey (1990). In brief, active snails were placed in a moist jar containing pieces of lettuce. The jar was then flushed with pure nitrogen gas (N_2) for 20 min and sealed with a rubber stopper. An oxygen electrode was fitted through the stopper to monitor O_2 levels in the jar. The P_{O_2} in the sealed jar was close to zero (approximately 1 mmHg; 1 mmHg = 0.1333 kPa), and the jar was flushed with N_2 at intervals to keep the P_{O_2} at this low level. The jar was maintained at 25 °C and on a 14 h:10 h L:D photoperiod for 48 h. After the appropriate period of exposure to anoxic conditions (at 2, 4, 8, 16, 20, 24 and 48 h), individuals were removed, and the haemolymph and tissues were collected and analyzed as described below.

Collection of haemolymph and determination of $[Ca^{2+}]$, pH, P_{CO_2}, P_{O_2} and levels of the anaerobic end-products D-lactate and succinate

Haemolymph collection was performed as described by Pedler et al. (1996). In brief, after removing a small section of shell to expose the pericardium of both active and anoxic snails, the heart was punctured with a needle fitted in a syringe previously filled with pure nitrogen, and haemolymph was collected. Haemolymph pH was immediately measured using a capillary pH electrode (G299 A, BMS Mk2, Radiometer, Copenhagen) calibrated at 25 °C with Radiometer precision buffers. Total CO_2 (C_{CO_2}) was determined according to the method of Cameron (1971) using a Radiometer P_{CO_2} electrode (E5037) calibrated with known concentrations of $NaHCO_3$. Haemolymph P_{CO_2} was then calculated using a modified version of the Henderson–Hasselbach equation:

$$P_{CO_2} = C_{CO_2}/[(10^{pH-pK} \times \alpha_{CO_2}) + \alpha_{CO_2}], \quad (1)$$

using a pK value of 6.189 and a solubility of CO_2 (α_{CO_2}) of 0.044 mmol l^{-1} $mmHg^{-1}$ at 25 °C (Barnhart, 1986). The concentration of bicarbonate in the haemolymph ($[HCO_3^-]_e$) was calculated according to the following equation:

$$[HCO_3^-]_e = C_{CO_2} - (\alpha_{CO_2} \times P_{CO_2}). \quad (2)$$

The P_{O_2} of the haemolymph was determined using an oxygen electrode (E5047, Radiometer, Copenhagen). Ca^{2+} levels in the haemolymph of active and anoxic snails were determined as described by Wieser (1981) using an absorption photometer. For the determination of D-lactate and succinate levels, haemolymph was collected in ice-cold 6 % perchloric acid containing 1 mmol l^{-1} EDTA. After centrifugation (15 000 ***g*** for 10 min) and neutralization with 5 mol l^{-1} KOH containing 50 mmol l^{-1} triethanolamine, D-lactate and succinate levels were determined enzymatically according to the methods of Lowry and Passonneau (1972) and Michal et al. (1976) respectively.

Determination of the non-bicarbonate buffer value of haemolymph

The non-bicarbonate buffer value ($\beta_{NB} = -\Delta[HCO_3^-]_e/\Delta pHe$) of haemolymph from active and from 20 h and 40 h anoxic snails was determined by CO_2 equilibration in an intermittently rotating cuvette (tonometer model 273; Instrumentation Laboratory, Paderno Dugano, Italy) flushed with humidified

CO_2 (range 1–5 %) in air. The samples of haemolymph were tonometered for 25 min to allow equilibration and analysed for pH and C_{CO_2} as described above.

Determination of intracellular pH (pHi)

pHi was determined using the homogenate method developed by Pörtner et al. (1990). Tissues samples were ground under liquid nitrogen. Approximately 100 mg of the tissue powder was placed into 500 µl of ice-cold medium (160 mmol l^{-1} potassium fluoride, 1 mmol l^{-1} nitrilotriacetic acid, pH 7.4). After filling up the vial with medium, the mixture was stirred with a needle to release air bubbles, mixed using a Vortex mixer, and centrifuged (15 000 ***g***) for 30 s. Within 3 min after thawing of the tissue powder in the medium, the pH of the supernatant was measured using a Radiometer capillary electrode (G299A).

Preparation of tissue homogenates for D*-lactate and succinate assays*

D-Lactate and succinate levels were determined in neutralized perchloric acid extracts of the tissues after 0, 2, 4, 8, 16, 24 and 48 h of anoxia. Frozen tissues were homogenized in 9 vols of ice-cold 6 % (w/v) perchloric acid containing 1 mmol l^{-1} EDTA using a Polytron homogenizer. The homogenization tube and probe were maintained at 4 °C in an ice bath. The homogenates were then centrifuged at 10 000 ***g*** for 20 min at 5 °C, and the supernatant was neutralized with 5 mol l^{-1} KOH containing 50 mmol l^{-1} triethanolamine. The neutralized extracts were allowed to stand on ice for 15 min and centrifuged as described above, and D-lactate and succinate levels were then determined.

Preparation of tissue homogenates for the determination of the kinetic constants of pyruvate kinase

For studies of enzyme kinetic constants, the tissues (foot, heart, oesophageal ganglia and mantle) collected from active snails and snails exposed to anoxia for 2, 4, 8, 16, 24 and 48 h were homogenized 1:5 (w/v) in homogenization buffer containing 100 mmol l^{-1} sodium fluoride, 5 mmol l^{-1} EDTA, 5 mmol l^{-1} EGTA, 0.1 mmol l^{-1} phenylmethylsulphonyl fluoride, 30 mmol l^{-1} 2-mercaptoethanol and 40 % glycerol (v/v) in 50 mmol l^{-1} imidazole-HCl (pH 7.0). After centrifugation at 25 000 ***g*** for 20 min at 4 °C, the supernatant was removed and passed through a 5 ml column of Sephadex G-25 equilibrated in 40 mmol l^{-1} imidazole-HCl buffer (pH 7.0) containing 5 mmol l^{-1} EDTA, 15 mmol l^{-1} 2-mercaptoethanol and 20 % (v/v) glycerol to remove metabolites of low molecular mass (Helmerhost and Stokes, 1980). The column was centrifuged in a desktop centrifuge at maximum speed (2000 ***g***) for 1 min, and the supernatant was used for studies of enzyme kinetic constants.

Enzyme assays and kinetics

Enzyme activity in four tissues (foot, heart, mantle and oesophageal ganglia) was monitored at 340 nm using a Hitachi 150-20 recording spectophotometer with a water-jacketed cell holder for temperature control. Standard assay conditions for pyruvate kinase were 50 mmol l^{-1} imidazole-HCl buffer, 2 mmol l^{-1} ADP, 0.15 mmol l^{-1} NADH, 50 mmol l^{-1} KCl, 5 mmol l^{-1} $MgCl_2$, 2 units of dialyzed lactate dehydrogenase and 2 mmol l^{-1} phosphoenolpyruvate (PEP). Assays for pyruvate kinase from these tissues were performed at 25 °C and at pH values that correspond to the measured intracellular pH of the tissue at each period of anoxia. The effector constant ($S_{0.5}$ PEP) was determined at saturating ADP concentration and calculated from Hill plots. IC_{50} values (the concentration of inhibitor producing a 50 % reduction in enzyme activity) for the inhibitors ATP and alanine for enzyme preparations from all four tissues were determined at 0.5 mmol l^{-1} PEP. To determine the IC_{50} value for Mg-ATP, a 1:1 molar mixture of $MgCl_2$ and ATP was used as the source of ATP; Mg^{2+} in this determination was in addition to the 5 mmol l^{-1} Mg^{2+} in the standard assay.

Statistical analyses

The results are presented as means ± S.E.M. Significance of differences was tested using the Bonferroni correction, which takes into consideration multiple comparisons. The level of significance was set at P=0.05.

Results

The effects of anoxia on the acid–base variables and P_{O_2} of haemolymph in the snail *Helix lucorum* are shown in Fig. 1. Exposure of the snails to anoxic conditions caused a significant reduction in extracellular pH (pHe) within the first 20 h of anoxia. However, acidification of the haemolymph occurred after after 4 h of anoxia; no significant change in pHe took place within the first 4 h (Fig. 1A). In the period of anoxia between 4 and 20 h, pHe fell from 7.79±0.025 to 7.23±0.021 (N=15). After 20 h of anoxia, pHe slowly began to recover. Significant reductions in $[HCO_3^-]_e$ and P_{CO_2} were observed in the haemolymph within the first 8 h of anoxia (Fig. 1B,C, respectively). However, statistically significant changes in the levels of these two variables were observed only after 2 h of anoxia. The concentration of bicarbonate decreased from 20.79±0.5 to 4.1±0.31 mmol l^{-1} (N=15), and P_{CO_2} fell from 13.95±0.33 to 4.01±0.22 mmHg (N=15). During the subsequent stages of anoxia, both the level of bicarbonate and P_{CO_2} increased gradually. After 48 h of anoxia, the bicarbonate level was 8.18±0.52 mmol l^{-1} and P_{CO_2} was 10.27±0.62 mmHg. P_{O_2} in the haemolymph fell sharply within the first 2 h of anoxia and thereafter remained at a low level (Fig. 1D). During the first 2 h of anoxia, P_{O_2} fell from 68.34±5.6 to 30.12±3.8 mmHg (N=15) and, after this, it gradually decreased to 12.35±1.75 mmHg by 48 h of anoxia.

The level of Ca^{2+} in haemolymph increased significantly during anoxia. This increase was approximately linear for the first 8 h, and thereafter $[Ca^{2+}]$ increased at a slower rate (Fig. 2). $[Ca^{2+}]$ increased from 7.04±1.12 mmol l^{-1} in the active snails to 30.83±2.55 mmol l^{-1} in the 8 h anoxic snails (N=10). After 48 h of anoxia, $[Ca^{2+}]$ reached 52.78±2.10 mmol l^{-1}.

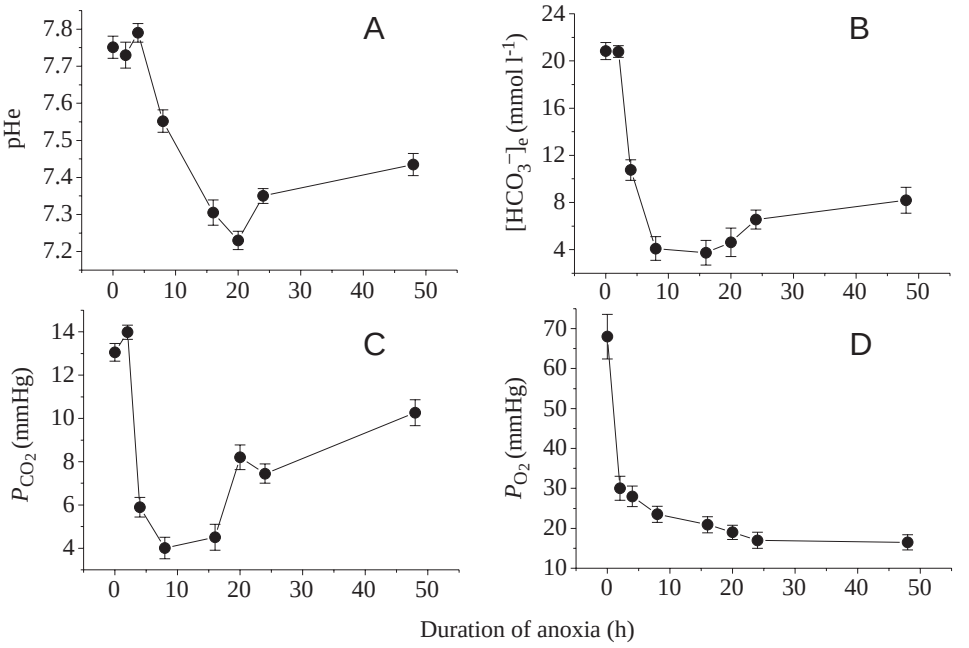

Fig. 1. Changes in the acid–base variables (pHe, $[HCO_3^-]$ and P_{CO_2}) and the partial pressure of oxygen (P_{O_2}) in the haemolymph of the land snail *Helix lucorum* during anoxia. Values are given as means ± S.E.M., *N*=15 determinations on separate preparations from different animals. 1 mmHg = 0.1333 kPa.

The non-bicarbonate buffer value of haemolymph (β_{NB}) was determined in active snails and in snails kept under anoxic conditions for 20 h and 48 h. The non-bicarbonate buffer value of haemolymph was unaffected by anoxia. It was 1.85±0.11 slykes (*N*=6) in the haemolymph of active snails and 1.94±0.21 slykes (*N*=6) and 1.89±2.12 slykes (*N*=6) after 20 h and 40 h of anoxia, respectively. A mean buffer value was used to draw the buffer line in the pHe/$[HCO_3^-]_e$ diagram (Fig. 3).

The pHi values of tissues (foot, heart, ganglia and mantle) from active and anoxic snails are shown in Fig. 4. The pattern of changes in pHi during anoxia was similar in all four tissues. pHi decreased significantly during the first 16 h of anoxia and thereafter increased until 48 h of anoxia. Among the tissues examined, however, the mantle showed the largest decrease in pHi. Within the first 16 h of anoxia, pHi decreased in the foot from 7.45±0.02 to 7.18±0.02, in the heart from 7.11±0.02 to 6.85±0.02, in the oesophageal ganglia from 7.42±0.03 to 6.98±0.02 and in the mantle from 7.65±0.03 to 6.83±0.02 (*N*=15). After 16 h of anoxia, pHi gradually returned towards control levels. Specifically, pHi was 7.33±0.02 in the foot, 7.08±0.01 in the heart, 7.38±0.03 in the ganglia and 7.51±0.03 in the mantle after 48 h of anoxia.

The levels of the anaerobic end-products D-lactate and succinate in the tissues and haemolymph of active and anoxic snails are given in the Table 1. The rate of accumulation of D-

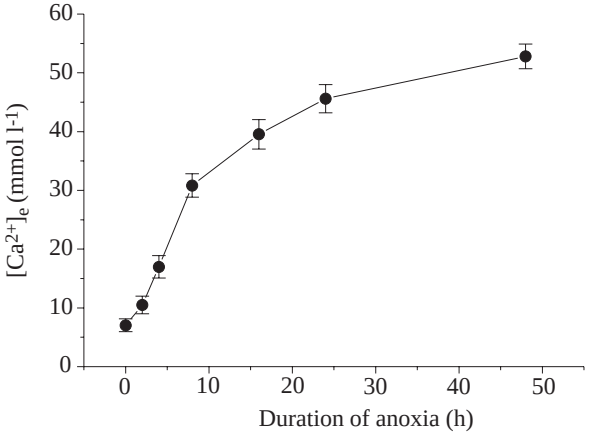

Fig. 2. Changes in the level of Ca^{2+} in the haemolymph of the land snail *Helix lucorum* during anoxia. Values are given as means ± S.E.M., *N*=10 determinations on separate preparations from different animals.

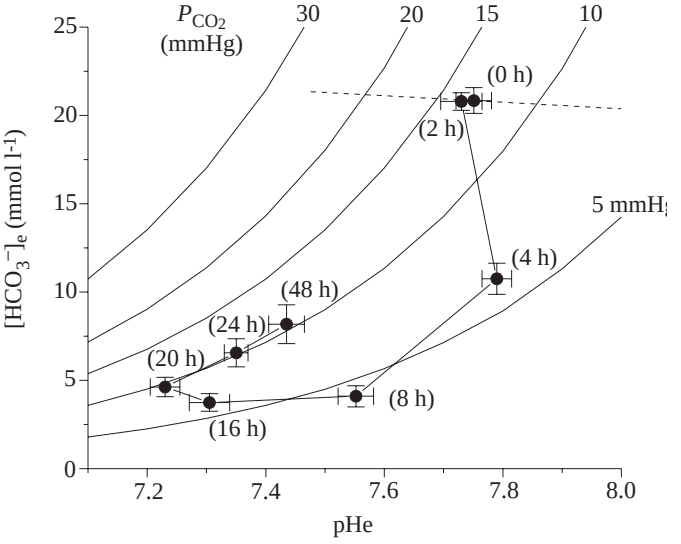

Fig. 3. Changes in the extracellular (haemolymph) acid–base status of the land snail *Helix lucorum* during anoxia presented as a pHe/$[HCO_3^-]$ diagram with the buffer line (dashed line) measured *in vitro*. Values are given as means ± S.E.M., *N*=8 determinations on separate preparations from different animals. 1 mmHg = 0.1333 kPa.

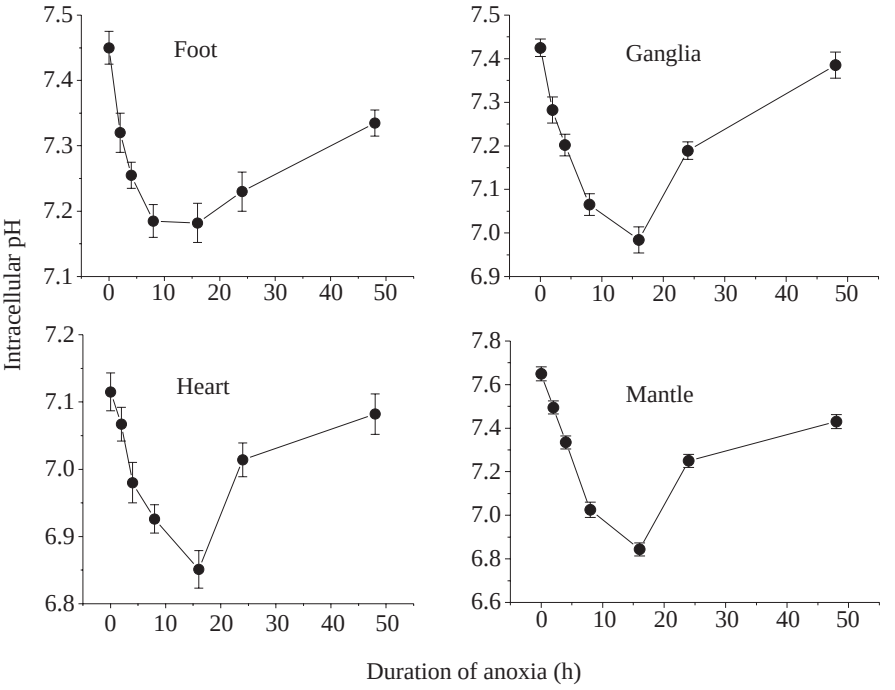

Fig. 4. Changes in the intracellular pH of several tissues of the land snail *Helix lucorum* during anoxia. Values are given as means ± S.E.M., *N*=15 determinations on separate preparations from different animals.

lactate and succinate was high during the first 8 h of anoxia, and thereafter the rate of accumulation was significantly reduced. The foot accumulated the largest amounts of D-lactate. The mean rate of accumulation of D-lactate in the foot was approximately 1.16 μmol g^{-1} h^{-1} during the first 8 h of anoxia and approximately 0.19 μmol g^{-1} h^{-1} over the subsequent 40 h. In the mantle, the mean rate of D-lactate accumulation was 0.76 μmol g^{-1} h^{-1} during the first 8 h and approximately 0.080 μmol g^{-1} h^{-1} over the next 40 h. In the heart, the rates were 0.72 μmol g^{-1} h^{-1} and 0.094 μmol g^{-1} h^{-1}, respectively; in the ganglia, the rates were 0.94 μmol g^{-1} h^{-1} and 0.14 μmol g^{-1} h^{-1}, respectively.

The haemolymph seems to serve as a sink where D-lactate excreted from the cells is accumulated. As shown in Table 1, D-lactate appeared in the haemolymph during the initial stages of anoxia and reached a concentration of 38.35±4.56 mmol l^{-1} (*N*=3) at 8 h of anoxia. This corresponds to a mean rate of accumulation of D-lactate of 4.76 mmol l^{-1} h^{-1}. The rate of accumulation of D-lactate decreased over the next 40 h of

Table 1. *Concentrations of the anaerobic end-products D-lactate and succinate in the tissues and haemolymph of* Helix lucorum *during anoxia*

	Duration of anoxia (h)						
	0	2	4	8	16	24	48
Foot							
Lactate (μmol g^{-1} wet mass)	0.25±0.03^{a}	4.75±0.52^{b}	8.45±0.64^{b}	9.53±1.11^{b}	12.02±1.25^{c}	15.78±1.23^{c}	17.02±2.1^{c}
Succinate (μmol g^{-1} wet mass)	0.56±0.04^{a}	1.85±0.11^{a}	2.45±0.34^{b,c}	3.67±0.24^{c}	4.52±0.56^{c}	5.68±0.78^{c}	6.45±0.51^{c}
Mantle							
Lactate (μmol g^{-1} wet mass)	0.26±0.019^{a}	0.87±0.1^{a}	4.51±0.35^{b,c}	6.33±0.41^{c}	7.49±0.81^{c}	8.13±0.55^{c}	9.55±1.45^{c}
Succinate (μmol g^{-1} wet mass)	0.95±0.042^{a}	1.06±0.15^{a}	1.92±0.21^{b}	3.55±0.3^{c}	3.05±0.41^{c}	3.75±0.25^{c}	4.28±0.45^{c}
Heart							
Lactate (μmol g^{-1} wet mass)	0.16±0.02^{a}	1.44±0.095^{b,c}	4.3±0.42^{c,d}	5.92±0.62^{c,d}	6.57±0.71^{c,d}	8.06±0.9^{d}	9.67±0.11^{d}
Succinate (μmol g^{-1} wet mass)	0.15±0.012^{a}	0.82±0.07^{a}	1.56±0.13^{b,c}	3.43±0.28^{b,c}	4.65±0.38^{c}	6.05±0.75^{c}	7.62±0.68^{c}
Oesophageal ganglia							
Lactate (μmol g^{-1} wet mass)	0.13±0.015^{a}	1.41±0.13^{b}	3.78±0.41^{c,d}	7.66±0.62^{c,d}	9.01±0.73^{d}	11.65±1.56^{d}	13.45±1.45^{d}
Succinate (μmol g^{-1} wet mass)	0.63±0.07^{a}	0.75±0.09^{a}	0.92±0.11^{a}	1.13±0.13^{a}	1.35±0.15^{a}	1.48±0.95^{a}	1.85±0.25^{a}
Haemolymph							
Lactate (mmol l^{-1})	0.25±0.03^{a}	1.55±0.17^{a}	16.41±0.75^{b}	38.35±4.56^{b}	49.23±3.58^{b}	52.97±4.85^{b}	56.46±7.5^{b}
Succinate (mmol l^{-1})	0.03±0.004^{a}	1.43±0.11^{b}	5.23±0.41^{b}	9.13±0.72^{b}	14.83±1.1^{c}	18.11±2.1^{c}	20.43±3.11^{c}

Values are means ± S.E.M., *N*=3 determinations on separate preparations of enzyme from different animals.
Different superscript letters indicate significant differences within the same row as determined using the Bonferroni correction (P<0.05).

Table 2. *Effects of anoxia on the kinetic properties of pyruvate kinase from the suboesophageal ganglia of* Helix lucorum

Duration of anoxia (h)	$S_{0.5}$ PEP (mmol l^{-1})	V_{max} (μmol $min\ g^{-1}$ wet mass)	IC_{50} ATP (mmol l^{-1})	IC_{50} Alanine (mmol l^{-1})
0 (pH 7.4)*	0.058±0.004[a]	21.34±1.17[a]	3.02±0.25[a]	4.08±0.35[a]
2 (pH 7.3)	0.051±0.002[a]	19.55±1.25[a]	3.29±0.35[a]	3.68±0.28[a]
4 (pH 7.2)	0.051±0.005[a]	22.08±1.75[a]	3.50±0.41[a]	4.05±0.35[a]
8 (pH 7.0)	0.100±0.009[b,c]	13.13±1.42[b,c]	1.95±0.08[a]	1.54±0.08[b]
16 (pH 6.9)	0.110±0.008[b,c]	13.22±1.40[b,c]	2.05±0.17[a]	1.61±0.01[b]
24 (pH 7.2)	0.150±0.011[c]	12.15±1.08[c]	1.95±0.15[a]	1.49±0.11[b]
48 (pH 7.4)	0.140±0.012[c]	12.05±0.95[c]	2.02±0.18[a]	1.55±0.12[b]

Values are means ± S.E.M., N=3 determinations on separate preparations of enzyme from different animals.

Effector constants ($S_{0.5}$) were determined at 0.5 mmol l^{-1} phosphoenol pyruvate (PEP) for IC_{50} values for all enzyme preparations.

Different superscript letters indicate significant differences within the same column, as determined by applying the Bonferroni correction (P<0.05).

IC_{50} is the inhibitor concentration producing a 50 % reduction in activity.

*Assays, at each period of anoxia, were performed at the pH value indicated in parentheses, which corresponds approximately to the intracellular pH measured for ganglia at the same period of anoxia.

Table 3. *Effects of anoxia on the kinetic properties of pyruvate kinase from the foot of* Helix lucorum

Duration of anoxia (h)	$S_{0.5}$ PEP (mmol l^{-1})	V_{max} (μmol $min\ g^{-1}$ wet mass)	IC_{50} ATP (mmol l^{-1})	IC_{50} Alanine (mmol l^{-1})
0 (pH 7.5)*	0.068±0.004[a]	25.24±1.17[a]	5.02±0.45[a]	3.08±0.25[a]
2 (pH 7.3)	0.061±0.006[a]	26.55±2.05[a]	5.29±0.35[a]	3.25±0.28[a]
4 (pH 7.2)	0.061±0.007[a]	27.08±2.75[a]	4.95±0.50[a]	3.05±0.26[a]
8 (pH 7.1)	0.120±0.009[b]	15.13±1.01[b,c]	2.05±0.08[b]	1.02±0.08[b]
16 (pH 7.1)	0.180±0.008[b]	14.22±1.60[c]	2.05±0.27[b]	1.11±0.01[b]
24 (pH 7.2)	0.170±0.011[c]	14.15±1.38[c]	1.85±0.20[b]	0.95±0.11[b]
48 (pH 7.4)	0.190±0.012[c]	15.05±1.15[c]	1.92±0.18[b]	1.04±1.13[b]

Values are means ± S.E.M., N=3 determinations on separate preparations of enzyme from different animals.

Effector constants ($S_{0.5}$) were determined at 0.5 mmol l^{-1} phosphoenol pyruvate (PEP) for IC_{50} values for all enzyme preparations.

Different superscript letters indicate significant differences within the same column, as determined by applying the Bonferroni correction (P<0.05).

IC_{50} is the inhibitor concentration producing a 50 % reduction in activity.

*Assays, at each period of anoxia, were performed at the pH value indicated in parentheses, which corresponds approximately to the intracellular pH measured for the foot at the same period of anoxia.

anoxia (to 0.45 mmol l^{-1} h^{-1}); after 48 h, the concentration was 56.46±7.5 mmol l^{-1} (N=3).

In contrast to D-lactate, a lower rate of succinate accumulation was observed in the tissues of *H. lucorum* during anoxia (Table 1). Amongst the tissues examined, the foot had the highest rate of accumulation of succinate and the oesophageal ganglia the lowest rate. Like D-lactate, succinate accumulated in the haemolymph from the early stages of anoxia and its concentration reached 9.13±0.72 mmol l^{-1} (N=3) after 8 h of anoxia, which corresponds to a mean rate of accumulation of 1.13 mmol l^{-1} h^{-1}. The rate of accumulation of succinate decreased over the next 40 h of anoxia (to 0.28 mmol l^{-1} h^{-1}), and after 48 h the level of succinate was 20.43±3.11 mmol l^{-1} (N=3) in the haemolymph.

The kinetic constants of pyruvate kinase from the ganglia, foot, heart and mantle of active and anoxic snails *H. lucorum* are shown in Table 2–5. Overall, pyruvate kinase from the tissues of active snails (0 h of anoxia) exhibited the same affinity ($S_{0.5}$) for its substrate (PEP) and the same IC_{50} value for the inhibitors Mg-ATP and alanine. However, a higher V_{max} was measured in the heart (Table 2) and a lower V_{max} in the mantle (Table 4). During anoxia, the kinetic properties of pyruvate kinase from all four tissues seemed to be similarly affected. By 8 h of anoxia, pyruvate kinase was converted to a less active form as judged by the increases in the $S_{0.5}$ value for the PEP and decreases in the IC_{50} values for the inhibitors alanine and ATP. In all cases, at 48 h, $S_{0.5}$ for PEP increased approximately threefold and IC_{50} values decreased significantly, indicating a higher sensitivity to inhibition by Mg-ATP and alanine.

Discussion

Like other pulmonates, the land snail *H. lucorum* is able to survive anoxia for at least for 3 days at 25 °C and high relative humitidy (80 %). When exposed to anoxic conditions, hypoxia develops rapidly and anaerobic metabolism seems to be up-

Table 4. *Effects of anoxia on the kinetic properties of pyruvate kinase from the heart of* Helix lucorum

Duration of anoxia (h)	$S_{0.5}$ PEP (mmol l^{-1})	V_{max} (μmol min mg^{-1} wet mass)	IC_{50} ATP (mmol l^{-1})	IC_{50} Alanine (mmol l^{-1})
0 (pH 7.1)*	0.065±0.004^{a,b}	33.44±2.11^{a}	2.85±0.21^{a}	4.51±0.31^{a}
2 (pH 7.0)	0.069±0.002^{a,b}	35.55±1.83^{a}	3.01±0.25^{a}	4.58±0.42^{a}
4 (pH 6.9)	0.062±0.005^{a,b}	32.08±2.71^{a}	3.10±0.31^{a}	4.01±0.48^{a}
8 (pH 6.9)	0.090±0.009^{b,c}	18.26±1.02^{b}	1.11±0.07^{b}	2.63±0.12^{b,c,d}
16 (pH 6.8)	0.110±0.008^{c}	15.22±1.32^{b}	0.92±0.11^{b}	2.68±0.23^{b,c,d}
24 (pH 7.0)	0.180±0.011^{d}	17.15±1.01^{b}	0.81±0.09^{b}	2.41±0.19^{c,d}
48 (pH 7.1)	0.220±0.012^{d}	17.05±1.90^{b}	0.82±0.11^{b}	1.65±0.19^{d}

Values are means ± S.E.M., N=3 determinations on separate preparations of enzyme from different animals.

Effector constants ($S_{0.5}$) were determined at 0.5 mmol l^{-1} phosphoenol pyruvate (PEP) for IC_{50} values for all enzyme preparations.

Different superscript letters indicate significant differences within the same column, as determined by applying the Bonferroni correction (P<0.05).

IC_{50} is the inhibitor concentration producing a 50 % reduction in activity.

*Assays, at each period of anoxia, were performed at the pH value indicated in parentheses, which corresponds approximately to the intracellular pH measured for the heart at the same period of anoxia.

Table 5. *Effects of anoxia on the kinetic properties of pyruvate kinase from the mantle of* Helix lucorum

Duration of anoxia (h)	$S_{0.5}$ PEP (mmol l^{-1})	V_{max} (μmol min mg^{-1} wet mass)	IC_{50} ATP (mmol l^{-1})	IC_{50} Alanine (mmol l^{-1})
0 (pH 7.7)*	0.078±0.008^{a}	11.02±1.23^{a}	3.02±0.25^{a}	4.65±0.52^{a}
2 (pH 7.5)	0.075±0.007^{a}	10.55±1.25^{a}	3.29±0.35^{a}	4.79±0.45^{a}
4 (pH 7.3)	0.071±0.008^{a}	12.08±1.75^{a}	3.50±0.41^{a}	4.19±0.32^{a}
8 (pH 7.0)	0.180±0.009^{b}	9.110±0.49^{a}	1.95±0.08^{a}	2.34±0.18^{b}
16 (pH 6.9)	0.220±0.008^{b}	7.710±0.88^{b}	2.05±0.17^{a}	2.11±0.29^{b}
24 (pH 7.2)	0.250±0.011^{b}	8.050±1.01^{b}	1.95±0.15^{a}	1.98±0.15^{b}
48 (pH 7.4)	0.260±0.012^{b}	7.720±0.95^{b}	2.02±0.18^{a}	2.06±0.22^{b}

Values are means ± S.E.M., N=3 determinations on separate preparations of enzyme from different animals.

Effector constants ($S_{0.5}$) were determined at 0.5 mmol l^{-1} phosphoenol pyruvate (PEP) for IC_{50} values for all enzyme preparations.

Different superscript letters indicate significant differences within the same column, as determined by applying the Bonferroni correction (P<0.05).

IC_{50} is the inhibitor concentration producing a 50 % reduction in activity.

*Assays, at each period of anoxia, were performed at the pH value indicated in parentheses, which corresponds approximately to the intracellular pH measured for mantle at the same period of anoxia.

regulated very quickly in *H. lucorum*, as judged by the changes in haemolymph P_{O_2} and in the levels of anaerobic end-products. Within the first 2 h of anoxia, P_{O_2} was significantly reduced in the haemolymph (Fig. 1D), and at 4 h the levels of the anaerobic end-products D-lactate and succinate had increased significantly in the tissues and haemolymph (Table 1). As far as haemolymph P_{O_2} is concerned, although the P_{O_2} in the jar where the snails were kept during anoxia was almost zero, the P_{O_2} in haemolymph remained at higher levels up to 48 h. We do not know what affects the retention of oxygen in the haemolymph. However, Wieser (1981) reported that the haemocyanin of the snail *H. pomatia* binds oxygen more tightly under conditions resulting from prolonged anaerobic metabolism in the tissues.

One of the responses to anoxia was a significant reduction in extracellular pH (pHe), which was significant after 4 h. The decline in pHe continued until 20 h, and thereafter pHe recovered slowly. According to our results, the decreased pHe was due to a metabolic rather than a respiratory acidosis. Metabolic acidosis is depicted on the pHe/$[HCO_3^-]_e$ diagram (Fig. 3) as a decrease in $[HCO_3^-]_e$ below the non-HCO_3^- buffer line. This decrease is due to titration of HCO_3^- by H^+ according to the following equation:

$$H^+ + HCO_3^- \rightarrow CO_2 + H_2O\,. \quad (3)$$

The acid comes from the anaerobic end-products D-lactate and succinate (Table 1), which accumulated at high rates in the haemolymph over the first 8 h of anoxia. Although both anaerobic end-products appeared in the haemolymph during the early stages of anoxia, pHe started to decline only after 4 h (Fig. 1A). Within the first 4 h of anoxia, the sum of D-lactate and succinate concentrations in the haemolymph increased to approximately 21 mmol l^{-1} (Table 1). During the same period, $[HCO_3^-]_e$ decreased by approximately 10 mmol l^{-1} and the level of Ca^{2+} increased by approximately 10 mmol l^{-1}, indicating a mobilization of $CaCO_3$ probably derived from stores in the midgut gland or even in the foot (Burton, 1976). These data

indicate that protons produced in the haemolymph after the accumulation of anaerobic end-products are buffered by HCO_3^-, thus maintaining pHe at the same level during the first 4 h of anoxia (Fig. 3). After 4 h, however, pHe was significantly reduced until 20 h. During the same period, $CaCO_3$ dissolved in haemolymph but at a lower rate, as indicated by the accumulation of Ca^{2+} in the haemolymph (Fig. 2). In conjuction with the continued accumulation of D-lactate and succinate in the haemolymph, the accumulation of Ca^{2+} is probably the main cause of the acidification of the haemolymph.

Although equation 1 predicts an accumulation of CO_2 in the haemolymph, this does not occur (Fig. 1C), probably because of an increased ventilatory rate during the first hours of anoxia. When exposed to a nitrogen atmosphere, snails expose their body and keep their pneumostome open for a long time. After 16 h, however, CO_2 had begun to accumulate in the haemolymph, and P_{CO_2} reached a value of 10.27±0.6 mmHg at 48 h. However, pHe and $[HCO_3^-]_e$ were increasing slowly over this period (Fig. 1A,B, respectively). According to the results presented here, this shift in acid–base variables towards a recovery of pHe may be due both to a slower rate of accumulation of the anaerobic end-products D-lactate and succinate in the haemolymph and to the continuous buffering of these end-products by $CaCO_3$. Between 16 h and 48 h of anoxia, D-lactate and succinate were accumulating in sum in the haemolymph at a mean rate of 0.39 mmol l^{-1} h^{-1} (Table 1), while the level of Ca^{2+} was increasing at a mean rate 0.84 mmol l^{-1} h^{-1} (Fig. 2).

The present results are in accordance with those reported for other pulmonates and they reveal a high glycolytic potential in the tissues of land snails during anoxia. Anoxia caused a significant increase in both D-lactate and succinate levels in the haemolymph of *H. pomatia* (Weiser, 1981) and a significant reduction in extracellular pH. However, Weiser (1981) measured a lower pH value for haemolymph after 40 h of anoxia (pH 6.9), which may reflect higher levels of D-lactate (60 mmol l^{-1}) and succinate (40 mmol l^{-1}) than we have determined (Table 1).

The acid–base status of the haemolymph of *H. lucorum* seems to reflect the rate of anaerobic metabolism in its tissues. Both D-lactate and succinate accumulated in all the tissues examined during anoxia (Table 1). However, their rate of accumulation decreased significantly after 8 h. Similarly, Churchill and Storey (1989) reported that the rate of accumulation of D-lactate and succinate in anoxic tissues of *Otala lactea* decreased significantly during prolonged anoxia. According to our results, the decreased rate of accumulation of anaerobic end-products in the tissues of land snails after 8 h of anoxia coincides with the recovery of intracellular pH. The mechanisms involved in pHi regulation in molluscan tissues are not well understood. Recent investigations have shown that molluscan tissues regulate pHi either by ion exchange of acid–base equivalents or by physicochemical buffering (Thomas, 1977; Ellington, 1993; Kisney and Ellington, 1995; Zange et al., 1990). Thomas (1977) has reported that recovery of pHi in snail neurones during hypercapnia is due to activation of a Na^+/H^+ exchange coupled to a Cl^-/HCO_3^- exchanger. The same mechanism may be involved in the recovery of pHi in *H. lucorum* hearts under hypercapnic conditions (Rofalikou and B. Michaelidis, unpublished data). This ion exchanger is electroneutral (Thomas, 1984) and it is activated when pHi falls below a certain threshold (Boron, 1985). The transport of D-lactate out of the cells, either *via* a specific carrier or by passive diffusion, can play an important role in the regulation of pHi. Pörtner (1993) has suggested that lactate transport may serve as an additional mechanism of regulating pHi, which he termed non-ionic regulation. The contributions of the above mechanisms to the regulation of pHi in the tissues of *H. lucorum* during anoxia are not known and are now under investigation. Our results suggest that the recovery of pHi after prolonged anoxia seems to be related to the decreased rate of accumulation of D-lactate and succinate in the tissues.

Reductions in the rate of accumulation of D-lactate and succinate in the tissues of *H. lucorum* indicate a marked depression of glycolytic rate. However, the mechanisms involved in the depression of glycolytic rate in the tissues of land snails during anoxia are not well understood. The results presented suggest that metabolic rate depression is related to changes in the kinetic properties of the key glycolytic enzyme pyruvate kinase rather than to the fall in pHi. As shown in Tables 2–5, the kinetics of pyruvate kinase changed within the first 8 h of anoxia, resulting in a lower enzyme activity. However, these changes did not correlate with the changes in pHi since they occurred irrespective of the fall in pHi. In addition, pyruvate kinase activity was not restored during recovery of pHi. This correlation is similar to that reported by Brooks and Storey (1989), who showed that pyruvate kinase from *Busycon canaliculatum* was converted to a less active form irrespective of the extra- and intracellular pH. Data from previous investigations (Whitwam and Storey, 1990; Wieser and Lanckner, 1977, 1982) indicate that the molecular basis of the modification of the kinetics of pyruvate kinase in the tissues of land snails during anoxia may be reversible enzyme phosphorylation. On the basis of the results presented above, we suggest that anoxia-related tissue acidosis in *H. lucorum* is not the primary stimulus triggering metabolic rate depression. This suggestion is in accordance with earlier studies on the land snail *Oreohelix*, which show that pHi alone does not regulate metabolic suppression during dormancy (Rees and Hand, 1991). Similarly, recent investigations using land snails and marine worms have questioned the key role of pHi in metabolic depression and suggested that a low pHi promotes changes leading to hypometabolism (Barnhart, 1992; Reipschlanger and Pörtner, 1996; Pedler et al., 1996; Pörtner et al., 1998; Scholnick et al., 1994).

References

Barnhart, M. C. (1986). Control of acid–base status in active and dormant snails, *Otala lactea* (Pulmonata, Helicidae). *J. Comp. Physiol.* **156**B, 347–354.

Barnhart, M. C. (1992). Acid–base regulation in pulmonate molluscs. *J. Exp. Zool.* **263**, 120–126.

Boron, W. F. (1986). Intracellular pH regulation in epithelial cells. *Annu. Rev. Physiol.* **48**, 377–388.

Brooks, S. P. J. and Storey, K. B. (1989). Influence of hormones, second messengers and pH on the expression of metabolic responses to anoxia in a marine whelk. *J. Exp. Biol*. **145**, 31–43.

Burton, R. F. (1976). Calcium metabolism and acid–base balance in *Helix pomatia*. In *Perspectives in Experimental Biology*, vol. 1 (ed. P. S. Davies), pp. 7–16. Oxford: Pergamon Press.

Cameron, J. N. (1971). Rapid method for determination of total carbon dioxide in small blood samples. *J. Appl. Physiol.* **31**, 632–634.

Churchill, T. A. and Storey, K. B. (1989). Intermediary energy metabolism during dormancy and anoxia in the land snail *Otala lactea*. *Physiol. Zool.* **62**, 1015–1030.

Ellington, W. R. (1993). Studies of intracellular pH regulation in cardiac myocytes from the marine bivalve mollusk, *Mercenaria campechiensis*. *Biol. Bull.* **184**, 209–215.

Helmerhost, E. and Stokes, G. B. (1980). Microcentrifuge desalting: a rapid, quantitative method for desalting small amounts of protein. *Analyt. Biochem.* **104**, 130–135.

Kisney, S. A. and Ellington, W. R. (1995). Interspecific comparisons of capacity for regulation of intracellular pH in molluscan muscle. *Physiol. Zool.* **68**, 26–42.

Lowry, O. H. and Passonneau, V. J. (1972). *A Flexible System of Enzymatic Analysis*. New York: Academic Press.

Michal, G., Beutler, H. O., Lang, G. and Guenter, U. (1976). Enzymatic determination of succinic acid in foodstuffs. *Z. Analyt. Chem.* **279**, 137–138.

Nopp, H. (1974). Physiologische Aspekte des Trokckenschlafs der Landschnecken. *Sitzungsber. Oesterr. Akad. Wiss. Math.-Naturwiss. Klasse Abt. 1* **182**, 1–75.

Oudejans, R. C. H. M. and van der Horst, D. J. (1974). Aerobic–anaerobic biosynthesis of fatty acids and other lipids from glycolytic intermediates in the pulmonate land snail *Cepea nemoralis* (L.). *Comp. Biochem. Physiol.* **47**B, 139–147.

Pedler, S., Fuery, J. C., Withers, C. P., Flanigan, J. and Guppy, M. (1996). Effectors of metabolic depression in an estivating pulmonate snail (*Helix aspersa*): whole animal and *in vitro* tissue studies. *J. Comp. Physiol.* **166**B, 374–381.

Pörtner, H. O. (1987). Contributions of anaerobic metabolism to pH regulation in animals tissues – theory. *J. Exp. Biol.* **131**, 69–87.

Pörtner, H. O. (1989). The importance of metabolism in acid–base regulation and acid–base methodology. *Can. J. Zool.* **67**, 3005–3017.

Pörtner, H. O. (1993). Multicompartmental analyses of acid–base and metabolic homeostasis during anaerobiosis: invertebrate and lower vertebrate examples. In *Surviving Hypoxia: Mechanisms of Control and Adaptation* (ed. P. W. Hochachka. P. L. Lutz, T. Sick, M. Rosenthal and G. van der Thillart), pp. 139–146. Boca Raton, FL: CRC Press.

Pörtner, H. O., Boutilier, R. G., Tang, Y. and Toews, D. P. (1990). Determination of intracellular pH and P_{CO_2} after metabolic inhibition by fluorite and nitrilotriacetic acid. *Respir. Physiol.* **81**, 255–274.

Pörtner, H. O., Heisler, N. and Grieshaber, K. M. (1984). Anaerobiosis and acid–base status in marine invertebrates: a theoretical analysis of proton generation by anaerobic metabolism. *J. Comp. Physiol.* **155**B, 1–12.

Pörtner, H. O., Reipschlager, A. and Heisler, N. (1998). Acid–base regulation, metabolism and energetics in *Sipunculus nudus* as a function of ambient carbon dioxide level. *J. Exp. Biol*. **201**, 43–55.

Rees, B. B. and Hand, C. S. (1990). Heat dissipation, gas exchange and acid–base status in the land snail *Oreohelix* during short-term estivation. *J. Exp. Biol.* **152**, 77–92.

Rees, B. B. and Hand, C. S. (1991). Regulation of glycolysis in the land snail *Oreohelix* during estivation and artificial hypercapnia. *J. Comp. Physiol.* **161**B, 237–246.

Reipschlager, A. and Pörtner, H. O. (1996). Metabolic depression during environmental stress: the role of extra- *versus* intracellular pH in *Sipunculus nudus*. *J. Exp. Biol.* **199**, 1801–1807.

Schmidt-Nielsen, K., Taylor, C. R. and Shkolnik, A. (1971). Desert snails: problems of heat, water and food. *J. Exp. Biol.* **55**, 385–398.

Scholnick, D. A., Snyder, G. K. and Spell, A. R. (1994). Acid–base status of a pulmonate land snail (*Helix aspersa*) and a prosobranch amphibious snail (*Pomacea bridgesi*) during dormancy. *J. Exp. Zool.* **268**, 293–298.

Thomas, R. C. (1977). The role of bicarbonate, chloride and sodium ion in the regulation of intracellular pH in snail neurones. *J. Physiol., Lond.* **273**, 317–338.

Thomas, R. C. (1984). Experimental displacement of intracellular pH and the mechanism of its subsequent recovery. *J. Physiol., Lond.* **354**, 3P–22P.

van der Horst, D. J. (1974). *In vivo* biosynthesis of fatty acids in the pulmonate land snail *Cepea nemoralis* under anoxic conditions. *Comp. Biochem. Physiol.* **47**B, 181–187.

von Brant, T. (1944). Occurrence of anaerobiosis among invertebrates. *Biodynamica* **4**, 185–328.

Walsh, P. J. and Milligan, L. C. (1989). Coordination of metabolism and intracellular acid–base status: ionic regulation and metabolic consequences. *Can. J. Zool.* **62**, 2994–3004.

Whitwam, E. R. and Storey, B. K. (1990). Pyruvate kinase from the land snail *Otala lactea*: regulation by reversible phosphorylation during aestivation and anoxia. *J. Exp. Biol.* **154**, 321–337.

Wieser, W. (1981). Responses of *Helix pomatia* to anoxia: changes of solute activity and other properties of the haemolymph. *J. Comp. Physiol.* **141**B, 503–509.

Wieser, W. and Lancker, R. (1977). Inhibition of the pyruvate kinase of *Helix pomatia* L. by phospho-L-arginine. Phosphorylation or a novel mechanism? *FEBS Lett.* **80**, 299–302.

Wieser, W. and Lancker, R. (1982). Multiple control of *Helix pomatia* pyruvate kinase by high-M_r modulators. *FEBS Lett.* **138**, 299–302.

Wieser, W. and Wright, E. (1978). D-Lactate formation, D-LDH activity and glycolytic potential of *Helix pomatia* L. *J. Comp. Physiol.* **126**B, 249–255.

Zange, J., Grieshaber, K. M. and Jans, H. W. A. (1990). The regulation of intracellular pH estimated by ^{31}P-NMR spectroscopy in the anterior byssus retractor muscle of *Mytilus edulis* L. *J. Exp. Biol.* **150**, 95–109.