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ION HOMEOSTASIS IN THE LEECH: CONTRIBUTION OF ORGANIC ANIONS

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Summary

Organic anion concentrations in the blood of *Hirudo medicinalis* were determined in samples from individual animals using reverse-phase high-pressure liquid chromatography (HPLC) and ultraviolet detection. Quantitatively important anions were malate, α-ketoglutarate, succinate, lactate and fumarate, constituting about 70% of total blood anions. Malate had the highest concentration, 34–68 mequiv l^{-1} blood, which could (depending on metabolic state) exceed the level of blood Cl^- (approx. 36 mequiv l^{-1}). Organic acid concentrations in the blood were considerably higher than in the tissue.

Blood organic acid concentrations changed more with P_{O_2} than with temperature. They were unaffected by short periods of aerobic exercise, but stress due to handling and prolonged restraint led to a drastic increase of blood lactate and succinate levels, while malate, fumarate and α-ketoglutarate levels decreased.

After feeding on hypertonic, Cl^--rich meals, the Cl^- concentration in leech blood increased far more than the cation concentrations. This was not compensated by a decrease of organic acid levels. The regulatory mechanisms for inorganic and organic ion homeostasis function independently.

Introduction

In the blood of the leech, *Hirudo medicinalis*, Cl^- balances only 30% of the cations (Boroffka, 1968; Zerbst-Boroffka, 1970). Quantitatively important anions are Krebs cycle intermediates and lactate (Zerbst-Boroffka, 1970; Zebe *et al.* 1981; Hildebrandt & Oeschger, 1987). However, the reported composition of the organic acid fraction and the individual concentrations of the acids vary considerably.

The present study investigates whether the observed variations are caused by different analytical procedures or physiological conditions of the animals and whether the determined organic acids represent the entire non-Cl^- anion equivalent needed for electroneutrality. On the basis of the results, the contribution of organic acids to ion homeostasis was investigated, in particular whether

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changes in inorganic ion concentrations observed after feeding on Cl^--rich, hypertonic blood (Zerbst-Boroffka, 1973; Wenning *et al.* 1980) lead to adjustments of organic acid levels.

Instead of using pooled material, as in all earlier studies, we analysed blood from individual animals using HPLC. Some of the results have appeared in abstract form (Wenning & Hoeger, 1987).

Materials and methods

Experimental conditions

Leeches (*Hirudo medicinalis*) were obtained through a commercial supplier (E. Nell, Blutegelimport, Recklinghausen) and kept in open containers with 0·5 l of Lake Constance water, changed weekly, containing (in mmol l^{-1}): KCl, 0·036; NaCl, 0·18; $CaCl_2$, 1·24; $MgSO_4$, 0·308 (Limnologisches Institut, Universität Konstanz). Ambient temperature was 16 °C.

Leeches were kept in groups with access to air ('non-aerated') or with constant aeration ('aerated'). 'Cooled' leeches were submerged in iced water for 10 min prior to blood sampling, which was carried out on a chilled dish. Sampling of 'non-cooled' leeches was done at room temperature.

For aerobic exercise, aerated leeches were forced to swim continuously for 15 min. They were cooled for blood sampling.

The effects of hypertonic, Cl^--rich meals were tested in two animal groups. Aerated leeches fed voluntarily on fresh rabbit blood (30–35 °C) or saline (in mmol l^{-1}: NaCl, 145; KCl, 5; L-arginine, 1; pH 6·8; Elliott, 1986) enclosed in sheep small intestine. Within 30 min they ingested 2–5 times their body weight. Blood samples were taken 45–60 min after termination of feeding (animals cooled). For crop-loading experiments, non-aerated animals were pinned in a wax dish and kept wet. Saline (in mmol l^{-1}: NaCl, 145; KCl, 5; pH 7·4) was infused *via* a flame-polished glass tube inserted into the crop (Zerbst-Boroffka, 1973). Blood was taken before and 1 h after crop loading from the same animal, which remained pinned and was not cooled. To detect regurgitation, Lissamine Green (SF, Chroma Gesellschaft) was added to all salines.

Fluid sampling and analysis

Leeches were pinned in a wax dish and kept wet. Blood (10–20 μl) was drawn from the dorsal vessel with glass capillaries (Zerbst-Boroffka, 1970). Operation and sampling were completed in 8–10 min. Haemocytes were removed by centrifugation (6000 *g*, 2 min; Hildebrandt, 1988). The supernatant was weighed and kept at −19 °C until analysis. Crop fluid, removed with a flame-polished glass tube inserted through the pharynx into the crop, was frozen immediately.

Samples (diluted 10-fold with distilled water) were purified on an anion-exchange column (SAX, Cl^- form, bed volume 100 μl) (Daish & Leonard, 1985). The organic acid fraction was eluted with 250 mmol l^{-1} H_2SO_4 and analysed by HPLC using 50–100 μl of the eluate. Organic acids were separated on a C18

reverse-phase column using 6 mmol l^{-1} phosphate buffer (pH 2·45) as the mobile phase (flow rate: 0·7 ml min^{-1}). Peaks were detected at 210 nm and, in some cases, also at 254 and 280 nm to exclude interfering compounds. The concentration of each acid was calculated either from the area (malate, lactate, fumarate, α-ketoglutarate) or the height (succinate, citrate) of the peak. Values were corrected for the recovery from the extraction column determined with external standards: (% ± S.D.; $N = 4–5$) 94 ± 3·5 (malate), 100 ± 4·7 (lactate), 98 ± 1·3 (α-ketoglutarate), 99 ± 1·6 (citrate), 97 ± 5·1 (succinate) and 104 ± 3 (fumarate). The methodical error was less than 6 %. Pyruvate was not determined since it eluted with the solvent front. Samples were processed and analysed within 48 h. To test the peak purity of malate, samples of the corresponding fraction were rechromatographed either on a reverse-phase or on an ion-exchange column and subjected to enzymatic analysis. Samples of the H_2SO_4 extracts were used to determine concentrations of inorganic phosphate, Pi (McKee & Fyfe, 1985).

For comparison, perchloric acid extracts of blood samples were prepared as described by Zebe *et al.* (1981) and analysed by HPLC.

Except for special purity H_2SO_4 and H_3PO_4 (Suprapur, Merck), all chemicals were reagent grade quality.

Oxygen in the water was measured with a Clarke-type electrode (Radiometer, Copenhagen, Denmark). In aerated containers, P_{O_2} was 20 kPa. It dropped, without aeration, to a stable value: at 16 °C to 5·3 ± 0·67 kPa ($N = 5$) and at 6 °C to 6·4 kPa within 48 h (5–13 animals).

For statistics, an unpaired, two-tailed *t*-test (pooled variances) was applied using SYSTAT on a personal computer.

The terms 'blood malate' (lactate, Cl^- etc.) are often used instead of 'malate (lactate, Cl^- etc.) concentration of the blood'.

Results

Handling and restraint

For immobilization, leeches are usually pinned through their suckers in a stretched position. The effects of the imposed stress (handling, prolonged restraint) on blood organic acids were examined by comparing cooled and non-cooled leeches (see Materials and methods). Cooling minimized muscular activity and mucus production, indicators of stress (Lent, 1973).

Duration of restraint had the greatest effect on blood lactate (Fig. 1). In uncooled leeches, the increase in lactate was five times that of cooled leeches (control) within the 10 min required for blood sampling and 13 times in 60 min of restraint. In contrast, it did not change in aerated and cooled animals after 15 min of muscular work (swimming; Table 1). A smaller increase in blood lactate was observed after feeding, probably owing to prolonged pharyngeal and body muscle peristalsis (Table 1). With prolonged restraint, concentrations of malate and fumarate decreased, while succinate levels increased (Fig. 1).

Inorganic ion concentrations have been determined previously in leeches (non-

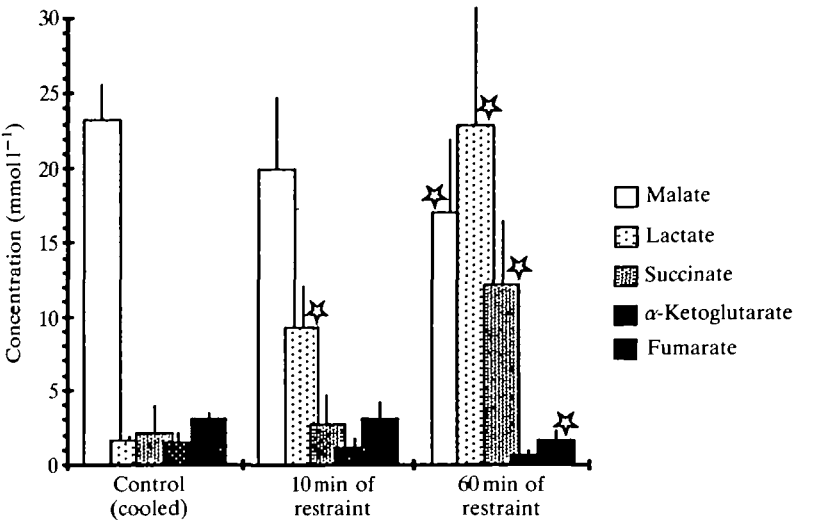

Fig. 1. Effects of prolonged handling and restraint on organic acid concentrations in the blood of *Hirudo medicinalis*. Control: animals were immobilized by cooling for blood sampling ($N = 7$). Leeches after 10 min ($N = 13$) and 60 min ($N = 10$) of restraint were not cooled for blood sampling. Leeches were not aerated. Values are given as mean ± S.D. Differences between control and treatments: (☆) $P < 0{\cdot}01$.

aerated, non-cooled) under prolonged restraint (Zerbst-Boroffka, 1970; Wenning *et al.* 1980). Blood $[Cl^-]$ was 36 ± 6 mequiv l^{-1} and blood cation concentration was 131 ± 13 mequiv l^{-1} ($Na^+ + K^+$). Under those conditions, total measured organic acid concentrations were between 70 and 95 mequiv l^{-1} and thus represented almost all non-Cl^- anions (Fig. 1).

Malate, whose concentration in leech blood was first measured by Zebe *et al.* (1981), was the most abundant anion (Table 1). In some physiological states, it accounted for half the total anion equivalents, thereby exceeding $[Cl^-]$. In blood samples treated with perchloric acid, as done previously (Zebe *et al.* 1981), or purified on the anion-exchange column, we detected blood malate levels 2–3 times higher than previously reported (Zebe *et al.* 1981: 9·39 mmol l^{-1}; Hildebrandt & Oeschger, 1987: 7–13 mmol l^{-1}). The same high malate concentrations were obtained by analysing the malate fraction enzymatically.

Assuming a pH of 7·4, Pi accounted for 3 mequiv l^{-1}. Citrate concentration was less than 1 mmol l^{-1} (see also Zebe *et al.* 1981; Hildebrandt & Oeschger, 1987). No significant amounts of isocitrate, oxaloacetate or acetoacetate were found.

Oxygen and temperature

Anoxia leads to changes in energy metabolism and affects organic acid concentrations in leech tissue (Zebe *et al.* 1981) and blood (Hildebrandt & Oeschger, 1987). In natural habitats, ponds or lakes, long-lasting changes in P_O and temperature occur (Hoffmann *et al.* 1986).

Table 1. *Effects of ambient temperature, oxygen, exercise and feeding on organic acid concentrations in the blood of* Hirudo medicinalis

Acids (mmol l^{-1})	6°C Aerated $N=6$	6°C Non-aerated $N=7$	16°C **Aerated Control** $N=6$	16°C Non-aerated $N=7$	25°C Aerated $N=6$	Exercise 15 min swimming Aerated $N=6$	1 h after feeding on rabbit blood Aerated $N=3$	1 h after feeding on saline Aerated $N=8$
Malate	28·7 ± 5·8	21·6 ± 5	**27·7 ± 5·5**	23·4 ± 2·2	22·1 ± 4·5	32·1 ± 3·1	34·1 ± 0·5	29·2 ± 8
Lactate	0·94 ± 0·67 ($N=5$) 6·2†	<0·13–2·7	**1·1 ± 1** ($N=5$) 8·6†	1·73 ± 0·37	<0·5	1·53 ± 1·2	2·3; 2·6; 10·6	6 ± 2·8
α-Ketoglutarate	3·87 ± 1·21	0·95 ± 0·66	**3·7 ± 1·15**	1·57 ± 0·66	3·98 ± 0·88	4·13 ± 0·3	5·87 ± 0·9	2·1 ± 0·6
Succinate	0·61 ± 0·25	3·22 ± 2·1	**0·9 ± 0·25**	1·48 ± 0·7 $N=6$ 6·4†	2·13 ± 0·44	1·01 ± 0·28	0·36 ± 0·02	0·34 ± 0·14
Fumarate	2·98 ± 0·44	2·58 ± 0·57	**3·5 ± 0·34**	3·15 ± 0·38	3·19 ± 0·63	4·16 ± 0·26	2·94 ± 0·39	1·87 ± 0·4
Total organic acids (mequiv l^{-1})	74 ± 13	57 ± 8	**74 ± 15**	63 ± 9	63 ± 12	84 ± 7·3	92 ± 6·7	73 ± 18
Pi (mmol l^{-1}): 1–2; Pi (mequiv l^{-1}): 3 (pH 7·4)								
Total measured acids (mequiv l^{-1})	≈77	≈60	**≈77**	≈66	≈66	≈87	≈95	≈76

All animals were cooled for blood sampling.
Values are given as mean ± S.D., differences: * $P < 0{\cdot}05$.
† Exceptional high value in one animal.

Variations in P_{O_2} affected organic blood ions more than variations in ambient temperature (Table 1). The concentration of succinate was higher and that of α-ketoglutarate was lower in non-aerated than in aerated leeches. Malate level decreased significantly only at 6°C in non-aerated leeches. Succinate concentration increased at 25°C even in aerated leeches. Total measured acid level varied between 60 (non-aerated, 6°C) and 77 (aerated, 6° and 16°C) mequiv l^{-1} (Table 1).

At 5·3 kPa (16°C), the pH of the water dropped from 8·1 to 7·6.

Feeding

To investigate whether the dramatic increase of blood [Cl^-] after feeding (from 36 to 95 mequiv l^{-1}) and the increase of blood volume due to fluid resorption (Wenning *et al.* 1980; Hildebrandt & Zerbst-Boroffka, 1988) would lead to a reduction in blood organic anion concentrations, results were compared after feeding on blood or on saline of similar ion composition. In both cases, succinate concentrations decreased (Table 1). Fumarate and α-ketoglutarate levels depended on the diet: both decreased after feeding on saline and α-ketoglutarate concentration increased after a blood meal.

Salt and water excretion by the nephridia have been investigated previously by measuring the time course of ionic and osmotic concentration changes in crop fluid, blood, primary urine and final urine after loading the crop with saline. The animals remained pinned while nephridial output was continuously measured by catheterization (Wenning *et al.* 1980; Zerbst-Boroffka *et al.* 1982). In this study, blood was analysed prior to and 1 h after crop-loading experiments, for comparison (data not shown). Levels of fumarate and malate decreased but, interestingly, lactate and succinate did not increase to the same extent as in the respective control group (60 min of restraint).

After feeding as well as after crop loading, total organic anion equivalents did not change significantly.

Crop fluid

With respect to inorganic ions, the crop fluid is iso-osmotic and iso-ionic to leech blood (Zerbst-Boroffka, 1973; Wenning *et al.* 1980). However, animals 1 h after feeding on saline and those starved for 7 months had only traces of organic acids in the crop fluid. The nature of other anions in the crop (e.g. amino acids) was not investigated.

Discussion

The high concentrations of organic acids in the blood of *Hirudo medicinalis* and the sensitivity of the HPLC method applied here allowed analysis of individual blood samples. As indicated by the low blood lactate level (Fig. 1, Table 1), immobilization by cooling and a short operation time yielded consistent organic acid levels minimally affected by the sampling procedure. Malate concentration

was considerably higher than reported previously (Zebe *et al.* 1981; Hildebrandt & Oeschger, 1987). Differences in blood malate levels apparently result from the physiological state of the animal and the blood sampling procedure rather than from the analytical method used: the level is 40% lower in non-aerated, non-cooled leeches ($17{\cdot}1\,mmol\,l^{-1}$, Fig. 1) than in aerated, cooled leeches ($27{\cdot}7\,mmol\,l^{-1}$, Table 1). The values of $14\,mmol\,l^{-1}$ lactate and $15\,mmol\,l^{-1}$ succinate (non-aerated, non-cooled leeches) given by Zerbst-Boroffka (1970) are consistent with our data (Fig. 1).

Considering individual variation, organic acids accounted for most of the anion 'deficit' between $131 \pm 13\,mequiv\,l^{-1}$ inorganic cations (Na^+, K^+) and $36 \pm 6\,mequiv\,l^{-1}$ inorganic anions (Cl^-) (Wenning *et al.* 1980, Table 1).

Organic acids and metabolism

Organic anions in body fluids have rarely been found in such large quantities as those reported here (70% of total anions). In some insect larvae, they account for up to 50% of total anions (Jeuniaux, 1971). Among other oligochaetes, some Lumbricidae and the gnathobdellid leech *Macrobdella decora* also have low blood [Cl^-] relative to cation concentrations (Oglesby, 1978; Wenning, 1987). It is not yet known whether organic acids serve as anions in these species.

As leeches live on a high-protein diet, organic acids derive from amino acid catabolism. After a meal, a rapid increase of NH_3 excretion is observed (Zebe *et al.* 1986). Transamination and deamination of amino acids feed the organic acid pool *via* α-ketoglutarate, as shown by its significant increase in leeches fed on blood compared to those fed on saline (Table 1). As leech blood contains negligible numbers of haemocytes (<1%; Hildebrandt, 1988), organic acids are produced in other tissues. Interestingly, the crop epithelium shows an abundance of succinate dehydrogenase activity after histochemical localization (M. Wadepuhl, U. Hoeger & A. Wenning, unpublished results).

Malate, α-ketoglutarate and fumarate concentrations are higher in the blood (this study) than in leech tissue (Zebe *et al.* 1981). The use of malate, the most abundant organic anion in tissue and blood, for energy storage under hypoxic conditions is obvious (Zebe *et al.* 1981; Zebe & Schöttler, 1986), but does not explain accumulation in the extracellular fluid at normal P_{O_2} values. Furthermore, whether the same concentration gradients between tissue and blood are maintained or whether they change according to the physiological situation is unknown.

Organic acids as anions

Salt and water balance in the leech is maintained by the nephridia (Zerbst-Boroffka, 1973). After feeding, the transcompartmental flow from crop to final urine increases. This is accompanied by a transient increase of blood [Na^+] and [K^+] (+ $10\,mequiv\,l^{-1}$) and, to a much greater extent, a rise in blood [Cl^-] (+ $50\,mequiv\,l^{-1}$) (Wenning *et al.* 1980). The latter seems to be a signal to increase nephridial salt output, since extracellular [Cl^-] is continuously monitored by a set of receptor neurones (Wenning, 1989). Since the nephridia excrete Na^+ and Cl^- in

equal amounts and no organic acids (Zerbst-Boroffka *et al.* 1982), an increase of blood [Cl^-] after feeding should be compensated by a decrease of organic anion levels. However, their concentrations did not change (Table 1) and, taking into account a blood volume increase of 25 % after feeding (Hildebrandt & Zerbst-Boroffka, 1988), could even increase. Thus, organic ions do not buffer transient inorganic ion changes. After ingestion of hypertonic, Cl^--rich saline, total anion levels ($\approx$161 mequiv l^{-1}) exceeded total cation levels ($\approx$141 mequiv l^{-1}) in the blood. The moderate increase of blood osmolality (from 190 to 261 mosmol kg^{-1} H_2O; Wenning *et al.* 1980) excludes additional univalent ions as a factor maintaining electroneutrality and suggests polyvalent cations.

In conclusion, Krebs cycle intermediates are the main anions in leech blood. Their individual concentrations change according to the metabolic state. To maintain electroneutrality, inorganic and organic ion homeostasis should be tightly coupled. After feeding, however, they are not down-regulated as would be expected from the dramatic increase of blood [Cl^-]. The mechanisms maintaining blood organic acid levels clearly function independently of the mechanisms readjusting inorganic ion levels.

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