LACTATE ACCUMULATION IN THE SHELL OF THE TURTLE *CHRYSEMYS PICTA BELLII* DURING ANOXIA AT 3 °C AND 10 °C

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Summary

Lactate concentrations were measured in the shell and plasma of the turtle *Chrysemys picta bellii* after 3 months of submergence anoxia at 3 °C and during and after 9 days of submergence anoxia at 10 °C. Liver and skeletal muscle lactate levels were also measured in control and anoxic animals at each temperature. At 3 °C, mean shell lactate concentration (N=4) reached 133 mmol kg⁻¹ shell mass and plasma lactate levels were 144 mmol l⁻¹; at 10 °C, shell and plasma lactate concentrations (N=5) rose in parallel during anoxic exposure, to 70.8 mmol kg⁻¹ shell mass and 78.9 mmol l⁻¹, respectively, and returned in parallel to control levels during 9 days of recovery. At the end of the anoxic periods, an estimated 44 % of the total body lactate resided in the shell at 3 °C and 43 % at 10 °C, and indirect evidence suggests that the shell buffered these same fractions of the acid load. Because of the high lactate concentration per kilogram of shell water (416 mmol kg⁻¹ at 3 °C; 221 mmol kg⁻¹ at 10 °C) and the known formation of calcium lactate complexes, it is postulated that most of the lactate existed in the shell in combined form. I conclude that sequestration of lactate within the shell represents a potentially major adaptation to anoxic acidosis for this animal and, together with the previously described release of shell carbonates, may account for up to two-thirds of the total lactic acid buffering in this animal.

Key words: acid–base balance, bone, submergence, Western painted turtle, *Chrysemys picta bellii*.

Introduction

A major adaptation of freshwater turtles that permits them to cope with prolonged anoxia is their capacity to tolerate severe non-respiratory acidosis. Large accumulations of the anaerobic anionic end-product, lactate, occur in both intracellular and extracellular fluids. In extreme cases, at 3 °C, approximately the temperature at which these animals hibernate, plasma lactate levels reached 150–200 mmol l⁻¹ after 3–5 months of submergence anoxia (Ultsch and Jackson, 1982; Jackson and Heisler, 1982). Because the production of lactate is associated with an equivalent generation of protons (Hochachka and Mommsen, 1983), the rise in lactate concentration represents an enormous acid load on the animals and a serious threat to their acid–base balance.

Previous work has shown that the turtle defends its blood acid–base state and keeps its pH within a viable range primarily by a relatively high extracellular buffering capacity, principally in the form of bicarbonate (Smith, 1929), and by ionic exchanges with other compartments that serve to enhance extracellular buffering (Jackson and Heisler, 1982). Important plasma ionic changes that are apparently associated with acid–base-relevant transfers include decreases in levels of Cl⁻ and increases in K⁺, Ca²⁺ and Mg²⁺ levels. The increases in concentrations of the divalent cations are particularly striking and appear to be derived from the shell of the turtle (Warburton and Jackson, 1995). The distinctive anatomical structure of the turtle, its shell, therefore plays an important role in acid–base balance as a source of supplementary buffering for the extracellular fluid.

We have recently observed, in a study of lactate distribution during anoxia at 20 °C, that lactate can enter the shell and be stored there in significant quantities (Jackson et al. 1996). This has led to the hypothesis that this role of the shell may be of paramount importance in lactic acid buffering in these animals. The goal of the present study, therefore, was to investigate this aspect of shell function; the shell uptake of lactate at 3 °C and 10 °C was studied because of the high concentrations of circulating lactate that can be reached at these temperatures. The time course of the change in shell and plasma lactate levels was also examined at 10 °C in an attempt to understand the kinetics of this process.

Materials and methods

*Animals*

Western painted turtles, *Chrysemys picta bellii* Gray, of both sexes, weighing 250–736 g were obtained commercially.

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Those studied at 3 °C were kept for 1 month at 5–10 °C before being slowly acclimated at 2 °C per day for 2–4 days to 3 °C in early January. The 10 °C animals were held at room temperature (22 °C) before being cooled to 10 °C at 3 °C per day in November or December. They were not fed after the cooling process had begun.

**Experimental protocol: 3 °C**

Two groups of four animals each were studied: the first group (mean body mass 362 g) was kept in shallow water at 3 °C for 3 months with free access to the water surface for breathing; the second group (mean body mass 329 g) was submerged without access to the surface in water at 3 °C that was bubbled continuously with 100 % N₂. No animals in either group were surgically prepared in any way.

After 3 months, turtles in each group were killed by decapitation and blood was immediately sampled by cardiac puncture for determination of plasma lactate concentration. Tissue samples (liver and skeletal muscle) were collected, freeze-clamped, weighed and stored at –80 °C for later analysis of lactate levels. Samples of shell were taken from selected sites on the plastron (three sites) and carapace (three sites) and were stored frozen at –20 °C for later analysis of lactate levels. These sites were selected as representative on the basis of a thorough analysis of shell lactate concentrations of anoxic turtles at 20 °C (Jackson et al. 1996). Plasma, tissue and shell lactate concentrations were measured on deproteinized samples as described below. Additional analyses were performed on some of these samples (plasma osmolality, tissue glycogen concentration) to confirm concordance with earlier studies, but these data will not be reported here.

**Experimental protocol: 10 °C**

All turtles at this temperature had catheters (PE 90) surgically implanted into the subclavian artery under Brevalt anesthesia (10 mg kg⁻¹) as described previously (Jackson et al. 1974). Two series of experiments were carried out.

### Series 1

Turtles (N=5) were submerged in anoxic N₂-equilibrated water at 10 °C for 9 days and were then allowed to recover with access to air for 9 days. Blood and shell samples were collected on day 0 (control), at 1, 3, 6 and 9 days of submergence, and at 1, 3, 6 and 9 days after resumption of air-breathing. Blood plasma was analyzed for the concentration of lactate as described below. Disks of shell (0.7 cm diameter), sampled from the margins of the carapace using a hand punch (Roger Whitney of Rockford, Inc., model 5 Jr), were weighed promptly, dried to constant mass (2 days at 80 °C), powdered (see below) and then analyzed for lactate concentration (N=5). One disk was taken from each turtle at each sampling period, and a soft rubber stopper was pressed into the hole to prevent seepage of blood from exposed shell surface. In a previous study (Jackson et al. 1996), we found that the changes in lactate level in the shell margin were similar to those in the more central portions of the shell.

### Series 2

Two groups of turtles (N=6 in each group) were held in 10 °C water, the first group with access to air, and the second group submerged for 9 or 11 days in anoxic N₂-equilibrated water. Blood and tissue samples were collected from all animals at the end of the anoxic period. Blood was sampled from the catheters and the turtles were then killed using excess Brevalt, and samples of liver and skeletal muscle were collected and immediately freeze-clamped. Blood plasma and tissues were analyzed for lactate concentration. Inorganic ion concentrations were also analyzed in series 1 and 2 (data not shown), and the values conformed to those found in a previous study using the same experimental conditions (Herbert and Jackson, 1985a).

These procedures were approved by the Animal Use Committee of Brown University in accordance with US Government regulations for animal treatment. Long-term submergence in cold water is a normal occurrence in the life history of these animals.

**Analytical procedures**

Plasma lactate concentration was measured enzymatically (Test Kit 826 from Sigma Chemical Co., St Louis, MO, USA) on deproteinized samples (two parts 8% perchloric acid to one part plasma) with appropriate dilutions. Values are expressed as mmolL⁻¹.

Tissue lactate concentration was analyzed on samples that had been homogenized and deproteinated (5 ml g⁻¹ of 0.6 mol l⁻¹ perchloric acid) under liquid nitrogen. The supernatant of the thawed slurry was analyzed for lactate concentration (spectrophotometrically) using Sigma reagents and is expressed as mmol kg⁻¹ wet tissue mass.

Shell water content was determined by drying fresh samples at 80 °C to constant mass. Lactate concentration was determined on undried shell at 3 °C and on dried shell at 10 °C. The similarity of the results using dried and undried shell indicated that drying had no effect on shell lactate levels. Samples were pulverized to powder under liquid nitrogen using either a mortar and pestle (on 3 °C samples) or a freezer mill (SPEX Certiprep Inc., model SPEX 6700-117) (on 10 °C samples). Weighed samples (approximately 200 mg) of powder were incubated with five (3 °C samples) or seven (10 °C) parts of 8 % perchloric acid (PCA) at 25 °C for 24 h prior to analysis. The supernatant was analyzed for lactate concentration as above, and the values are expressed as mmol kg⁻¹ wet shell mass. Dilutions of samples incubated with the highest proportion of PCA were adjusted to return the ratio of PCA to sample to approximately 2:1. At this ratio, the buffer in the enzyme cocktail is able to neutralize the solution and prevent perchlorate precipitation.

**Statistics**

Values are expressed as means ± s.e.m. Differences between control and test groups were determined using t-tests. Changes occurring in a single group over time were tested using repeated-measures analysis of variance (ANOVA) and, if a
significant change was found, individual differences were determined using Bonferroni’s method. Differences were considered significant at $P<0.05$.

## Results

### 3°C data

Plasma and tissue lactate levels increased dramatically as a result of prolonged anoxia at 3°C (Table 1) to values similar to those observed previously (Jackson and Heisler, 1982, 1983). Shell lactate levels also increased substantially (Table 1) to values that were nearly as high (in mmol kg$^{-1}$) as plasma lactate levels (in mmol l$^{-1}$). The elevated lactate concentration was similar in all parts of the shell tested (Fig. 1), as was observed previously at 20°C.

### 10°C data

Repeated measurements of plasma and shell lactate levels on the same animals during and following 9 days of submergence anoxia (series 1) revealed a similar pattern of change in both compartments (Fig. 2). Shell values (in mmol kg$^{-1}$) increased more slowly but reached a mean peak lactate concentration that was 90% of the plasma value (in mmol l$^{-1}$) and not significantly different from it. The agreement of these values does not necessarily indicate equilibration between the two compartments, however,
because the units in which shell lactate is expressed (mmol kg\(^{-1}\) shell mass) are not the same as diffusional lactate activity (see Discussion). Tissue lactate levels (series 2) also increased, although not to the same extent as plasma and shell lactate levels (Table 1).

Shell water content changed significantly over time in the series 1 animals using repeated-measures ANOVA, but the only significantly different paired values, according to Bonferroni’s method, were between time 0 and 1 day of anoxia. Shell water content did not change significantly during the anoxic period, and water accounted for 31.8±0.6 % of shell mass during this time (N=5), close to the 10 °C anoxic value of 32.7±0.1 % reported by Warburton and Jackson (1995). A value of 32 % can therefore be taken as the shell water content of anoxic turtles.

Discussion

These data at 3 and 10 °C, together with previous results at 20°C (Jackson et al. 1996), demonstrate that the turtle shell is an important site for lactate storage during prolonged anoxia. At each temperature, the concentration of lactate within the shell (in mmol kg\(^{-1}\)) approached the concentration within the plasma (in mmol l\(^{-1}\)), although because of the small water volume of the shell, the concentration within the shell per unit volume of water is far higher than in the plasma (see below). The similarity between the time courses of the changes in plasma and shell lactate concentrations at 10 °C reveals the exchange process to be a relatively rapid one, suggesting a reasonable perfusion of the shell even under anoxic conditions. This is surprising in view of the suppression of cardiovascular function (Herbert and Jackson, 1985b) and the uncertain, but probably low, shell blood flow during anoxia.

The shell apparently contributes in two distinct ways to acid–base buffering in the anoxic turtle. First, as described previously (Jackson and Heisler, 1982; Warburton and Jackson, 1995), it serves as a source of calcium and magnesium carbonates for export to the extracellular fluid to supplement its buffering capacity. This is a well-known response of skeletal tissues in other organisms, including both vertebrates (Irving and Chute, 1932; Burnell, 1971; Poyart et al. 1975) and invertebrates (deFur et al. 1980), and appears to be due to a passive dissolution of bone carbonates (Bushinsky and Lechleider, 1987). Second, the shell, as demonstrated by the present study, acts as a temporary storage site for lactate, accumulating it during anoxia and releasing it during recovery as plasma lactate concentration falls. This has not been described previously in the literature.

The contribution of the shell lactate uptake mechanism to whole-body lactate distribution can be estimated readily from available data, but the physical state of lactate in the shell and the importance of shell lactate accumulation to acid–base balance are less certain and will be discussed below.

Lactate distribution at the end of anoxia

For the present purposes, whole-body lactate can be considered to reside in three distinct compartments: the shell and skeleton, the extracellular fluid not within the shell (ECF), and the intracellular fluid not within the shell (ICF). For a 1 kg turtle, the shell component (in mmol) is the product of shell lactate concentration (mmol kg\(^{-1}\)) and shell mass (0.32 kg; Jackson et al. 1996). The ECF component can be estimated as the product of plasma lactate concentration (mmol l\(^{-1}\)) and total non-shell ECF volume (0.240 l; Jackson et al. 1996). The ICF component can be estimated as the product of muscle lactate concentration (mmol kg\(^{-1}\) cell water) and non-shell ICF volume (0.304 l; Jackson and Heisler, 1983; Wasse et al. 1991; Jackson et al. 1996). For turtles that have been anoxic at 3 °C for 3 months, at 10 °C for 9 days, and at 20 °C for 6 h, this calculation yields total body lactate burdens, per kilogram body mass, of 96.8 mmol kg\(^{-1}\), 52.4 mmol kg\(^{-1}\) and 16.3 mmol kg\(^{-1}\), respectively. The shell, according to this analysis, contains 44 % of the total body lactate concentration at 3 °C, 43 % at 10 °C and 30 % at 20 °C (Table 2). Note that the skeleton per se was not studied, and this may contribute even further to the process of lactate storage. Skeletal elements of Chrysemys picta bellii, not including the shell and associated bone, constitute approximately 5.5 % of the body mass (D. C. Jackson, unpublished observations).

Physical state of lactate in shell

Although the physical state of lactate within the turtle shell is uncertain, some inferences can be made on the basis of reactions in the plasma. Under normoxic conditions in the extracellular fluid, lactic acid, at a pK of approximately 4 (Williams et al. 1978), is almost fully dissociated to the lactate anion. However, in the presence of high concentrations of Ca\(^{2+}\), an appreciable fraction of ECF lactate can combine with Ca\(^{2+}\) to form the cationic complex Ca–lactate\(^+\) (Jackson and Heisler, 1982). The association constant for this reaction is low (approximately 201 mol\(^{-1}\)), and significant concentrations of the complex are only formed when both lactate and Ca\(^{2+}\) concentrations are high, a condition that is uncommon in animals other than the anoxic turtle (Jackson and Heisler, 1982). In a previous study at 3 °C (Jackson and Heisler, 1982), after anoxic exposure similar to that employed in the present study, it was concluded that two-thirds of the plasma Ca\(^{2+}\) (total concentration 34.1 mmol l\(^{-1}\)) and one-fifth of the plasma lactate (total concentration 145.1 mmol l\(^{-1}\)) existed in this

### Table 2. Estimated distribution of lactate for a 1 kg anoxic turtle at 3, 10 and 20°C

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>ECF (non-shell)</th>
<th>ICF (non-shell)</th>
<th>Shell</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>34.6 (36 %)</td>
<td>19.6 (20 %)</td>
<td>42.6 (44 %)</td>
<td>96.8 (100 %)</td>
</tr>
<tr>
<td>10</td>
<td>18.9 (36 %)</td>
<td>11.0 (21 %)</td>
<td>22.5 (43 %)</td>
<td>52.4 (100 %)</td>
</tr>
<tr>
<td>20</td>
<td>7.3 (45 %)</td>
<td>4.1 (25 %)</td>
<td>4.9 (30 %)</td>
<td>16.3 (100 %)</td>
</tr>
</tbody>
</table>

*Values are mmol kg\(^{-1}\) body mass (% of total lactate concentration).*

ECF, extracellular fluid; ICF, intracellular fluid.

*Values at 20 °C are taken from Jackson et al. (1996).*
A similar reaction occurs between lactate and Mg$^{2+}$ (Cannan and Kibrick, 1938), but it is quantitatively less important because of the lower concentration of Mg$^{2+}$ in anoxic turtle plasma (Jackson and Heisler, 1982).

The present results suggest that an interaction between lactate and Ca$^{2+}$ may occur within the shell itself, although presently it is impossible to say what the reaction product(s) may be. The potentially large amounts of available Ca$^{2+}$ within the shell may explain why this structure can act as a sink for lactate and may mean that a major fraction of the lactate is in combined form. Furthermore, because of the small volume of shell water (only 32% of the shell mass), it is unlikely that much of the lactate could exist as the free anion in solution. In support of this, we can recalculate shell lactate concentration as mmol kg$^{-1}$-water and make the same calculation for the intracellular and extracellular compartments. Concentrations of lactate expressed per kilogram water are presented in Fig. 3. These calculations reveal that, even if the free lactate in the shell water at 3°C equalled the extracellular value, this would only represent 36% of the total shell lactate. The very high values for shell lactate concentration strongly support the hypothesis that a sizable fraction of the lactate must be present in combined form, but whether in solution as the complex or as a precipitate is uncertain. Whatever its chemical form, the lactate within the shell is readily released back into the blood after the anoxic period, as shown by the recovery data of the 10°C turtles (Fig. 2).

**Implication for lactic acid buffering**

It is also uncertain what contribution lactate sequestration in the shell makes to the acid–base balance of the anoxic turtle. The presence of lactate in the shell *per se* does not prove a buffering role for the shell, because the lactate may have entered as a neutral salt or in exchange for another strong anion. It is unlikely, however, that either of these events occurs because there is no obvious cation or anion that could be moving in the right quantities and in the right direction. For example, Ca$^{2+}$ and Mg$^{2+}$ leave the shell during anoxia (Warburton and Jackson, 1995), as apparently does Na$^+$ (D. C. Jackson, unpublished observations), and shell [K$^+$] does not change (D. C. Jackson, unpublished observations). A strong cation such as one of these would have to move into the shell along with lactate and its shell concentration would have to rise if neutral salt uptake were the mechanism by which lactate concentration increased. The obvious candidate for an anionic counterion is Cl$^-$, but the plasma concentration of this ion is significantly reduced during anoxia in the turtle (Herbert and Jackson, 1985a) and so it is unlikely to be released in significant amounts from the shell in exchange for lactate.

A reasonable assumption, therefore, is that lactate enters the shell with an accompanying proton or, alternatively, in exchange for carbonate. These are equivalent processes from an acid–base standpoint that would each serve to neutralize lactic acid and defend a positive extracellular strong ion difference (Stewart, 1983). As with the previously described release from the shell of calcium and magnesium carbonates (Jackson and Heisler, 1982), the proposed mechanism of lactic acid uptake exploits the large shell CO$_2$-buffering potential; however, the storage mechanism has the distinct advantage over the release mechanism that it sequesters lactate and avoids large increases in circulating levels of Ca$^{2+}$ and Mg$^{2+}$.

Assuming that lactate uptake does constitute an equivalent shell buffering of lactic acid, it follows that this is a major contributor to acid buffering in this situation. At 3°C and 10°C, over 40% of the lactic acid may be buffered by this mechanism. As previously described, the shell also contributes by exporting carbonates to the ECF, presumably accompanied chiefly by Ca$^{2+}$ and Mg$^{2+}$ (Jackson and Heisler, 1982). The magnitude of this latter response can be estimated from the increase in circulating levels of these divergent cations, because urinary excretion is minimal in the anoxic turtle (Warburton and Jackson, 1995; Jackson *et al.* 1996). At 3°C, of the 96.8 mmol kg$^{-1}$ body mass of lactate accumulated, approximately 25% is buffered by calcium carbonate and magnesium carbonate release, on the basis of measured plasma ion changes (Jackson and Heisler, 1982); at 10°C, approximately 19% of the 52.4 mmol kg$^{-1}$ body mass of lactate is buffered by carbonates released from the shell, on the basis of measurements of plasma cation values (D. C. Jackson, unpublished observations). Thus, the lactate uptake mechanism into the shell, estimated to represent over 40% of the total lactate at both temperatures, may be nearly twice as important quantitatively as the buffer release mechanism from the shell, and together these two shell mechanisms accomplish almost two-thirds of the total lactic acid buffering in the body. This new finding extends our understanding of how crucial the shell and its buffering power are to the ability of the turtle to tolerate anoxia and severe lactic acidosis. It is important to re-emphasize, however, that this calculation assumes that the

![Fig. 3. Lactate concentrations of anoxic turtles at three different temperatures in plasma, skeletal muscle and shell calculated as mmol kg$^{-1}$-water. Data at 20°C are from Jackson *et al.* (1996). Skeletal muscle water content was calculated from Jackson and Heisler (1983) and Wasser *et al.* (1991), and plasma water content from D. C. Jackson (unpublished observations).](image-url)
lactate storage in the shell represents an equivalent buffering of acid.

**Implications for shell ion and CO₂ exchanges**

The existence of a major ionic movement into the shell complicates the exchange stoichiometry between the shell and the ECF during prolonged anoxia. It has been established that Ca²⁺ and Mg²⁺ move out of the shell and that lactate moves in. Recent data (D. C. Jackson, unpublished observations) suggest that Na⁺ may also leave the shell during anoxia. The major weak ion balancing these various fluxes is probably carbonate, although bicarbonate participation cannot be excluded (Poyart et al. 1975). We have found previously that approximately 97% of the total body CO₂ resides in the shell and that prolonged anoxia at 3°C causes a significant reduction in the shell CO₂ level (Warburton and Jackson, 1995). The present study suggests that this reduction in shell CO₂ concentration is due both to efflux with divergent cations and to counter-movement with lactate, either as a carbonate ion or as molecular CO₂ following proton buffering within the shell. We are currently undertaking in vitro experiments to describe these exchanges quantitatively. In vivo, the CO₂ generated from acid buffering can diffuse from the ECF into the surrounding water and, at 3°C, this loss is adequate to prevent a significant rise in blood Pco₂ (Ueltsch and Jackson, 1982). Consequently, at this temperature, the lactic acid that enters the shell and is buffered and sequestered there may have no impact whatsoever on blood and non-shell tissue acid–base status.

**Implications for other animals**

It is possible that lactate storage may occur in the skeletal tissues of other animals and contribute to lactic acid buffering, but no reports of this could be found in the literature. *Chrysemys picta bellii* and related turtles are surely exceptional in this regard because of the high anoxic lactate levels achievable and because of their extraordinarily large skeletal mass. Because they represent extreme examples of this phenomenon, they may serve as useful animal models for the study of skeleton–lactate interactions. The difference between turtles and other animals that can accumulate lactate may, however, be quantitative and not qualitative. It is of interest that comparative studies show that turtles with reduced shell calcification, such as the soft-shelled turtle *Trionyx spinifer*, are far less tolerant to anoxia (Ueltsch et al. 1984) than *Chrysemys picta bellii*. The reduced effectiveness of shell acid-buffering could be an important limitation to this animal.

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**References**


