J. exp. Biol. (1982), 96, 29–43
With 7 figures
Printed in Great Britain

LONG-TERM SUBMERGENCE AT 3 °C OF THE TURTLE, *CHRYSEMYS PICTA BELLII*, IN NORMOXIC AND SEVERELY HYPOXIC WATER:

II. EXTRACELLULAR IONIC RESPONSES TO EXTREME LACTIC ACIDOSIS

BY DONALD C. JACKSON AND GORDON R. ULTSCH*

Division of Biology and Medicine, Brown University, Providence, Rhode Island 02912, *U.S.A.*

(*Received* 19 *January* 1981)

SUMMARY

Plasma [Na^+], [K^+], and [Cl^-] were measured on blood sampled periodically from turtles submerged in N_2-equilibrated (low-O_2) or air-equilibrated (high-O_2) water. Plasma osmolality and total [Ca^{2+}] and [Mg^{2+}] were measured on selected samples. Previously we reported that a severe metabolic acidosis developed under these conditions; in low-O_2 turtles, plasma [lactate$^-$], rising to over 200 m-equiv/l, was only partially buffered by [HCO_3^-], which fell from 40 to 5 m-equiv/l.

The balance of the buffering was accomplished by changes in strong ions which included (in low-O_2) a fall in [Cl^-] from 79·5 ± 1·4 m-equiv/l ($\bar{X}$ ± S.E.) to below 60 m-equiv/l, a rise in [Ca^{2+}] from 3 to over 100 m-equiv/l, and a rise in [Mg^{2+}] from 3 to over 30 m-equiv/l. Together, all ionic changes balanced the increase in [lactate$^-$], but this analysis is tentative because we do not know the distribution of Ca^{2+} and Mg^{2+} between free and bound forms *in vivo*. Plasma osmolality rose from 233 ± 4·1 to 382 ± 12·4 mosmol after 162 days in low-O_2, even though the turtles showed a gain in body weight. The ionic changes that occurred in plasma also occurred in the pericardial and peritoneal fluids, suggesting general extracellular fluid participation in lactate buffering. Bladder urine contained significant concentrations of both lactate and calcium, indicating that some acid excretion may have occurred during the submergence.

INTRODUCTION

Freshwater turtles are remarkable for their ability to survive for extended periods in the absence of oxygen (Belkin, 1963), and for their tolerance to the extreme acid-base disturbances that result therefrom. During prolonged breath-hold diving, the natural activity possibly leading to anoxia in turtles, a combined respiratory and metabolic acidosis generally occurs when the dives are sufficiently prolonged (Jackson & Silverblatt, 1974). At temperatures over 15 °C, in the range at which most previous

* Usual address: Department of Biology, University of Alabama, University, AL 35486, U.S.A.

studies have been performed, lactic acid levels in the blood from 20 to 55 mM have been reported following experimental dives lasting from several hours (Jackson & Silverblatt, 1974; Penney, 1974) to several days (Robin *et al.* 1964). Blood P_{CO_2} increased markedly at these temperatures, reaching values over 100 mmHg (Jackson & Silverblatt, 1974; Robin *et al.* 1964). Together these changes caused blood pH to fall to values as low as 6·6.

In our study of the western painted turtle, *Chrysemys picta bellii*, at 3 °C, however, we have observed a far more severe lactic acidosis, but without a persistent respiratory acidosis, during experimental dives lasting up to 6 months (Ultsch & Jackson, 1981). Plasma lactic acid concentration exceeded 200 mM, which represents an acid load of enormous magnitude because of the nearly complete dissociation (99·9%) of lactic acid to lactate ion under these conditions. Generally, the elevation of lactate in the blood or plasma causes a reduction of similar magnitude in the concentration of weak acid anions. In this extreme case of the cold anoxic turtle, however, the rise in plasma [lactate$^-$] was some 4 times greater than the pre-dive concentrations of HCO_3^- and net protein negative charge (the latter estimated from unmeasured anions of Smith, 1929) which together total only about 50 m-equiv/l. Most of this is HCO_3^- at a concentration of about 40 m-equiv/l, and while this is high relative to that of other vertebrates (Smith, 1929), it is still small compared to the observed lactate elevation.

In the present paper we describe the extracellular ionic adjustments associated with this acid load in the turtles. We report that in addition to the fall in weak acid anion concentrations, apparent transport of strong ions occurred between the plasma and other body compartments, with a net entry into the plasma of strong cations and a net loss of strong anions.

METHODS

Experimental protocol. The detailed description of animal preparation, analytical procedures and experimental plan are presented elsewhere (Ultsch & Jackson, 1981). In brief, turtles, *Chrysemys picta bellii* Gray, both catheterized and non-catheterized, were slowly acclimated to 3 °C, before being submerged at this temperature in either aerated (high-O_2) or N_2-equilibrated (low-O_2) water. Five groups of turtles were studied, each with 10–11 members:

Group 1: catheterized, apnoeic in high-O_2 water, with periodic blood samples taken from surviving animals.

Group 2: non-catheterized, apnoeic in high-O_2 water. These turtles were intact controls for group 1, with surviving members (N = 6) killed and sampled after 189 days of submergence.

Group 3: catheterized, apnoeic in low-O_2 water, with periodic blood samples taken from surviving animals.

Group 4: non-catheterized, apnoeic in low-O_2 water. These turtles were intact controls for group 3, with surviving members (N = 4) killed and sampled after 159–165 days of submergence.

Group 5: non-catheterized, access-to-air in high-O_2 water. These turtles were intact controls for long-term submergence in 3 °C water but without enforced apnoea, with surviving members (N = 5) killed and sampled after 189 days at 3 °C. A sixth survivor in this group was in poor condition and its body fluid data were not included.

Blood sampling and analysis. Blood samples (periodic in Groups 1 and 3; terminal in Groups 2, 4 and 5) were analysed promptly for haematocrit, pH, P_{O_2} and P_{CO_2}. Derived plasma was analysed in 2–4 days for the concentrations of lactic acid, glucose, Na^+, K^+ and Cl^-. The results for pH, P_{O_2}, P_{CO_2}, lactic acid and glucose are presented in the previous paper (Ultsch & Jackson, 1981); the results for Na^+, K^+ and Cl^- will be presented in this paper. Details regarding the sampling and analytical procedures are also described in the other paper.

Excess plasma remaining after the above analyses was stored in capped Eppendorf plastic vials at −12 °C and was available for subsequent measurements. These were measurements for which equipment was not readily available to us at the time of sampling or for which the importance was not apparent to us until later. The following measurements were thus made on selected plasma samples.

Ammonia was determined with a microdiffusion-colorimetric technique (Chaney & Marbach, 1962). Amino acids were measured with a Dionex Amino Acid Analyzer, Model D-556, using a lithium buffer system. Plasma osmolality was measured on 0·2 ml samples with a freezing-point osmometer (Osmette A, Precision Systems, Inc.). Calcium and magnesium concentrations were measured on plasma samples using a Perkin Elmer Model 303 Atomic Absorption Spectrophotometer. Samples were diluted with distilled water (calcium- and magnesium-free) to give a final concentration near 1 mM for each element. Standard solutions between 0·1 and 2·0 mM were run at the beginning and end of the analysis series to provide a calibration curve. Additional measurements of total calcium concentration in pericardial and peritoneal fluid samples were made using the o-cresolphthalein complexone colorimetric method (Sigma Chemical Co., Kit No. 585). This method can be used on 0·025 ml samples and results correlate closely with the atomic absorption technique which is the accepted standard. Plasma samples previously measured for calcium by atomic absorption in this study gave similar values when determined colorimetrically. Absorbance was read at 575 nM on a Beckman Model DUR Spectrophotometer.

All grouped data in this paper will be expressed as the mean ± S.E.

RESULTS

Plasma ion concentrations. Plasma $[Na^+]$ remained relatively unchanged until the final stages of diving in both groups (Fig. 1). Plasma $[Cl^-]$, on the other hand, fell significantly during apnoea in both high and low-O_2 groups (Fig. 2), while plasma $[K^+]$ rose steadily in low-O_2 animals, but not consistently in the high-O_2 animals (Fig. 3).

Ionic balance analysis. These ionic changes, together with the measurements of plasma $[lactate^-]$ and $[HCO_3^-]$ reported in the previous paper, permit us to construct an ionic balance sheet for the plasma. According to the requirement of electrical neutrality, the total cation concentration in plasma must be equal to the total anion concentration (all concentrations expressed as mequiv/l). Any discrepancy with this equality among our measured ions, termed an 'ion gap', would indicate unmeasured ionic species. The ion gap in our study is described as follows:

$$\text{ion gap} = ([Na^+]+[K^+])-([Cl^-]+[HCO_3^-]+[\text{lactate}^-]).$$

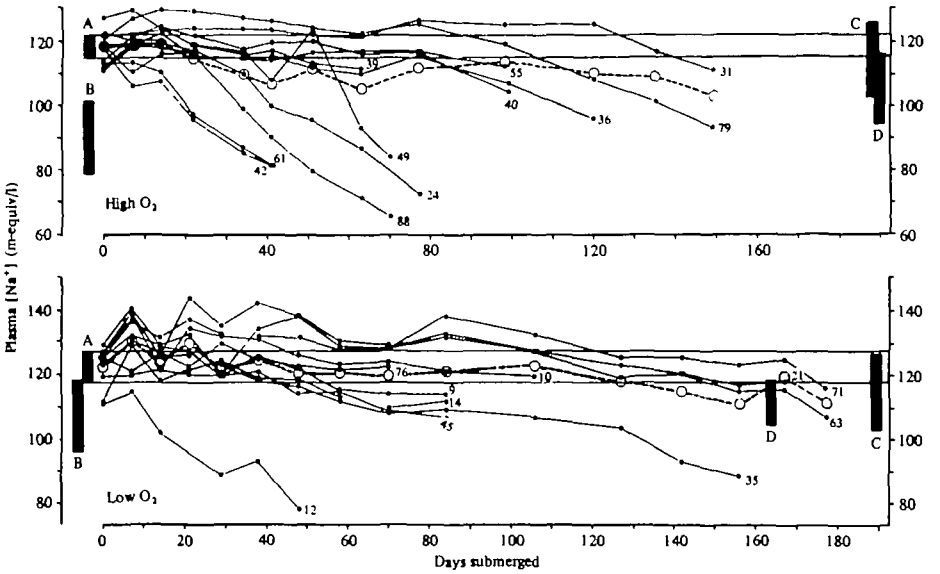

Fig. 1. Plasma [Na^+] of turtles at 3 °C submerged in either aerated water (upper panel) or N_2-equilibrated water (lower panel). In this figure and in Figs. 2, 3, 4 and 7, individual values for all catheterized turtles are shown and each animal is identified by its number at its final sampling period. The open circles, connected by the dotted line, are the mean values for surviving animals. The vertical black bars represent the 95 % confidence intervals for the following: (A) Pre-dive control values of the catheterized turtles; (B) the last values before death of the catheterized turtles; (C) values of five control turtles after 189 days at 3 °C with access to air; this bar is the same in each panel; (D) values for control turtles after prolonged apnoeic submergence; six high-O_2 turtles were sampled after 189 days, four low-O_2 turtles were sampled between 155 and 168 days.

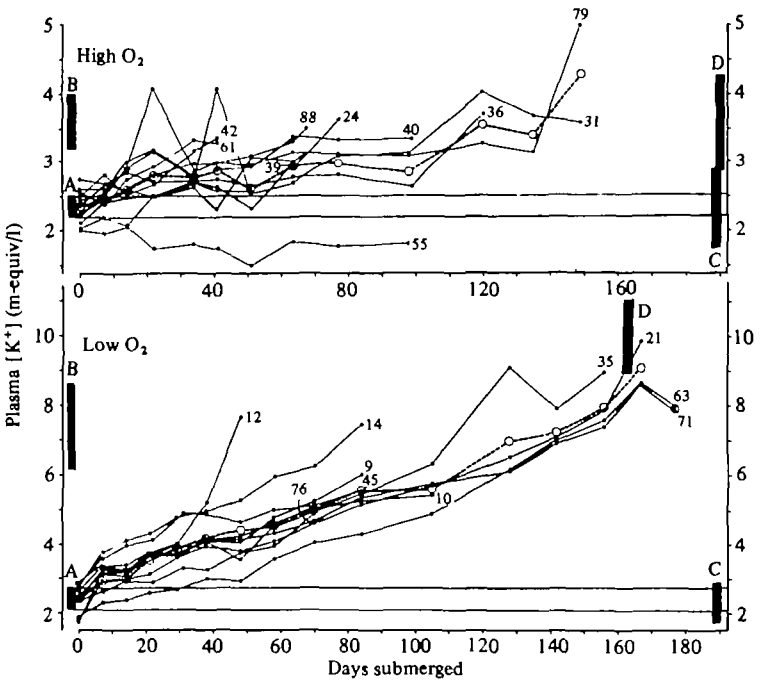

Fig. 2. Plasma [K^+] of turtles at 3 °C. For explanation of graph format, see legend for Fig. 1.

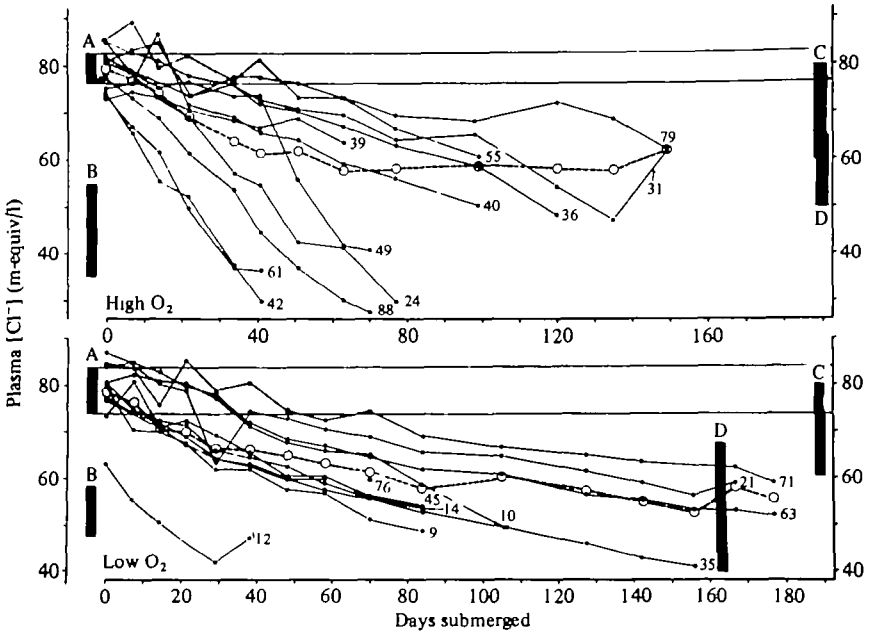

Fig. 3. Plasma [Cl^-] of turtles at 3 °C. For explanation of graph format, see legend for Fig. 1.

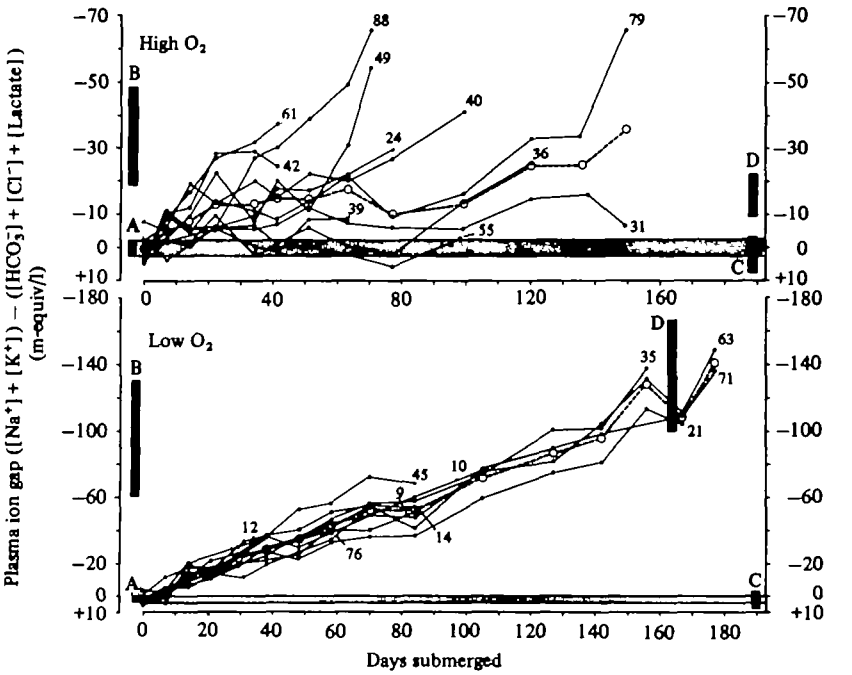

Fig. 4. Plasma ion gap of submerged turtles. The ion gap is defined as the sum of the measured cations minus the sum of the measured anions (all concentrations expressed as m-equiv/l). For explanation of graph format, see legend for Fig. 1.

We found that an ion gap with negative sign developed early in the dive and increased progressively particularly in the low-O_2 group in which the changes were greater and more uniform (Fig. 4). A change in the ion gap in this direction indicates unmeasured positive charge, and is a 'cation gap'.

We next sought the identity of the missing cation or cations in our ionic equation. These measurements were made on plasma samples that had been stored in a frozen state since the original measurements were made. Selected samples with large ion gaps were tested for ammonia and for amino acids. We hypothesized that the missing cation was either NH_4^+ or a positively charged amino acid. However, total amino acid concentrations were less than 1 mM in the samples tested, and ammonia values averaged 0·13 mM in three samples. Thus neither substance could account for the charge discrepancy.

We did find large and consistent changes in both calcium and magnesium in the plasma that increased progressively during diving (Fig. 5). The data depicted are from six turtles, three from each of the catheterized groups. The three high-O_2 animals (numbers 31, 55 and 79) had either low or average increases in lactic acid and were average to long-term survivors within this group. The three low-O_2 animals (numbers 35, 63 and 71) were among the longest surviving members of their group and had the highest final lactate concentrations and ion gaps. With respect to their final acid-base status, both selected subgroups closely resembled the non-catheterized controls for their respective treatments (Ultsch & Jackson, 1981). In this sense we consider the selected animals to be representative. We found that the sum of the concentrations of calcium and magnesium (expressed as m-equiv/l) was closely correlated with the associated lactate concentrations of the same set of plasma samples (Fig. 6).

Plasma osmolality. Osmolality increased significantly during submergence in the low-O_2 turtles (Table 1) and was nearly 150 mosmol/l above the pre-dive value in five animals tested at 162–165 days. In the pre-dive samples, the osmolality was 92% of the concentration (in mM) of the measured solutes. The solutes include Na^+, K^+ and Cl^- as reported in this paper, plus HCO_3^-, lactate$^-$ and glucose reported previously (Ultsch & Jackson, 1981); calcium and magnesium are not included in this analysis because of the uncertainty regarding their *in vivo* state (See Discussion). In the samples obtained after prolonged submergence, the osmolality exceeded the total measured solute concentration, indicating the presence of unmeasured solutes. The additional solute that would be required to match the 92% figure of the pre-dive samples is 47 mM at 126 days and 44 mM at 167 days. If the measured solute concentration were corrected for protein concentration and expressed in molal terms, the numbers in Table 1 would change but the relative discrepancy would persist.

Body weight. Turtles in both catheterized groups (1 and 3) increased in weight over the course of the submergence; the high-O_2 turtles (initial weight 577 ± 17 g) gained 68 ± 5·9 g, or 11·9% of their original weight, while the low-O_2 turtles (initial weight 680 ± 51 g) gained 37·2 ± 5·8 g, or only 5·4% of their original weight. No precaution was taken at either the initial or the final weighing times to prevent urine loss from the bladder; however, we believe that the changes we observed were too large to be accounted for by this effect. It is probable that the fungus infection that selectively

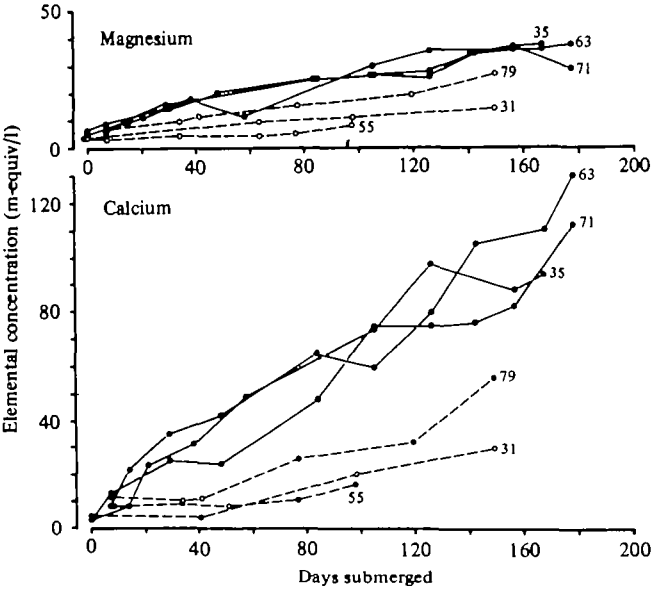

Fig. 5. Plasma [Mg^{2+}] and [Ca^{2+}] of six turtles submerged at 3 °C, three in air-equilibrated water (high-O_2) and three in N_2-equilibrated water (low-O_2).

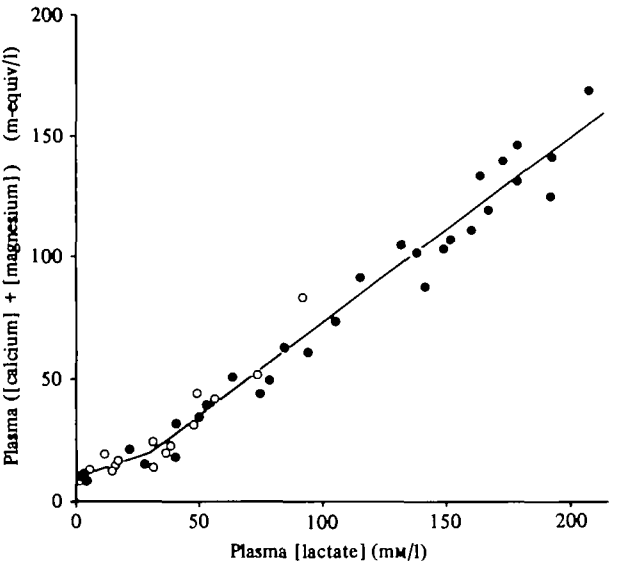

Fig. 6. Relationship between [lactate$^-$] and the sum of the [Ca^{2+}] and [Mg^{2+}] of plasma sampled from turtles submerged at 3 °C. Data are from same six turtles as in Fig. 5. The break in the curve was drawn by eye and occurs at the point where [lactate$^-$] is approximately equal to initial [HCO_3^-].

Table 1. *Plasma osmolality and measured solute concentration of turtles before and during anoxic submergence*

Days submerged	*N*	Osmolality (mosmol)	[Measured solutes] (mM)	Ratio (mosmol/mM)
0	8	233 ± 4·1	253 ± 3·3	0·92 ± 0·01
126	4	360 ± 17·8	344 ± 6·3	1·06 ± 0·05
162–167	5	382 ± 12·4	371 ± 11·2	1·03 ± 0·02

Values are mean ± S.E.

affected the high-O_2 turtles (Ultsch & Jackson, 1981) disturbed the osmoregulatory ability of these animals and permitted excess water influx. Because the final body weights were measured after the animals had died, we suspected, as the study proceeded, that the weight gains we were seeing were occurring *post-mortem* and were thus of doubtful significance. However, when we measured three of the surviving low-O_2 turtles at 167 days (with precautions to prevent breathing) we found that their weights were no different (on the average) than body weights subsequently measured on these turtles after their death, 10–11 days later.

It is of interest that the plasma osmolality of the low-O_2 turtles increased despite this weight gain. Presumably the additional weight was water that would be expected to have a diluting effect on the body fluids.

Blood haematocrit. Whole blood haematocrit values increased significantly during diving in the turtles in aerated water, but were highly variable in the anoxic animals (Fig. 7). Eventually, haematocrits tended to decline in both groups but this trend was particularly pronounced in the anoxic group. The fall in haematocrit could be attributed in part to the periodic blood sampling, which amounted to 10–15 ml per turtle over the course of the study; however, because the same volume was removed from the turtles in each group, the sampling loss can only partially account for the decline in the anoxic turtles. The sampling effect is revealed by comparing the catheterized turtles in each condition to their unoperated controls (Bar D, top and bottom, in Fig. 7).

On several occasions we observed a precipitous fall in haematocrit in anoxic turtles, which then reverted back to a higher level in subsequent sampling periods. We were able to draw several ml of anaemic blood under these conditions without mobilizing additional cells. We postulate that cells were selectively sequestered or trapped and then later released to the circulating blood to account for this anomalous behaviour. We never observed this phenomenon in a turtle in aerated water.

Our observation of increased haematocrit in the high-O_2 turtles is consistent with previous observations of cold-induced decreases in plasma volume in *Chelydra serpentina* and *Graptemys geographica* under both field (Semple, Sigsworth & Stitt, 1970) and laboratory (Stitt, Semple & Sigsworth, 1971) conditions. It was concluded that the effect of low temperature was to cause plasma sequestration and haemoconcentration (Stitt *et al.* 1971). This is in contrast to the occasional haemodilution we observed in several low-O_2 turtles, which we attributed to possible sequestering of red cells.

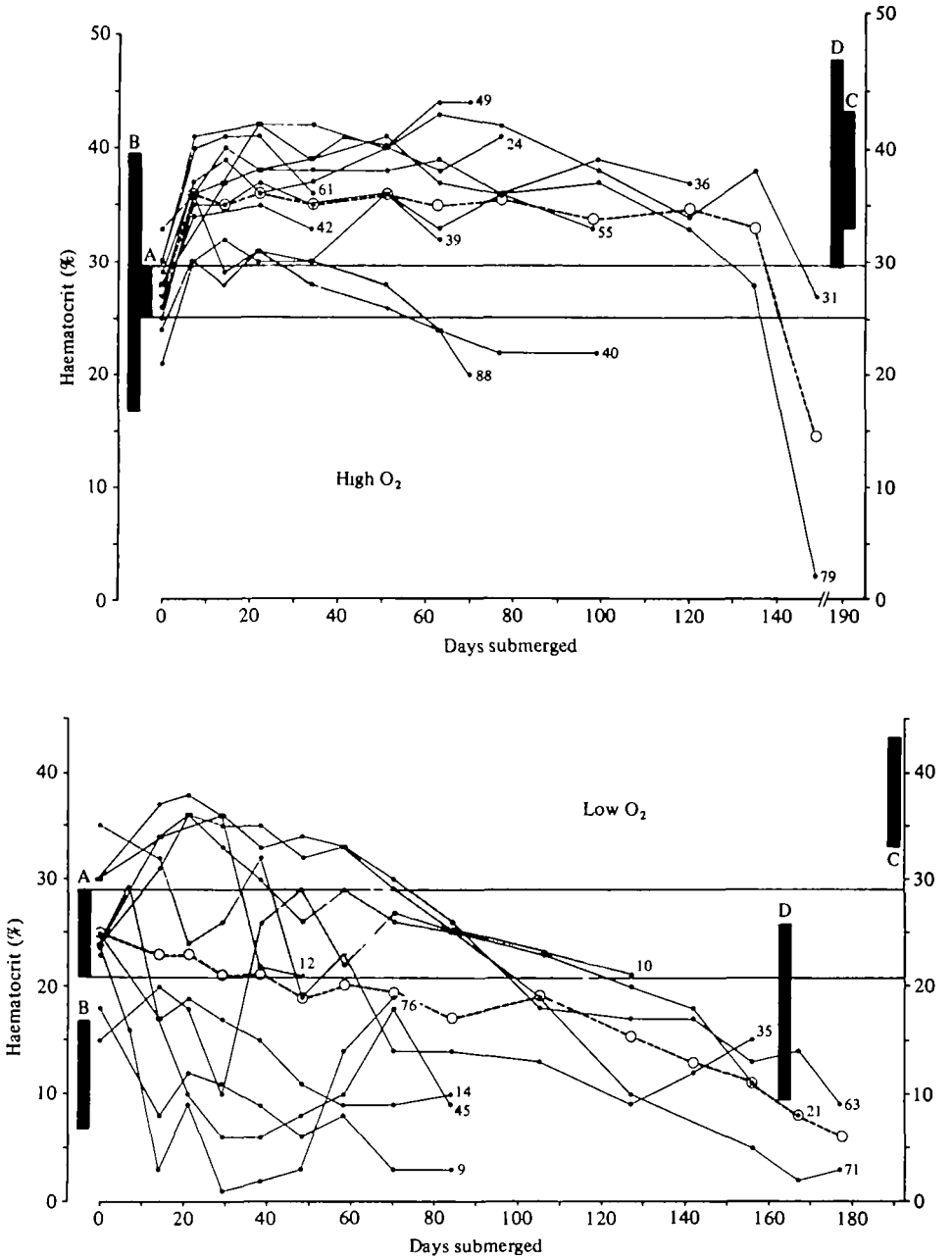

Fig. 7. Blood haematocrits of turtles at 3 °C. For explanation of graph format, see legend for Fig. 1.

Table 2. *Concentrations of lactate and calcium in the pericardial fluids of six turtles after prolonged submergence at* 3 °C

Group*	Days submerged	[Lactate] (mM)	[Calcium] (m-equiv/l)	([Ca]/[lactate]) × 100
1	77	96	40·0	41·7
3	105	138	74·8	54·2
3	106	158	83·0	52·5
4	168	169	88·6	52·4
3	142	193	92·8	48·1
4	168	216	105·8	49·0
				49·7 ± 1·8

* Experimental group number (see Materials and Methods).

Pericardial fluid, peritoneal fluid and urine. In general, the final composition of the pericardial and peritoneal fluids of the submerged turtles was similar to the plasma composition in the same animals. The normal alkaline nature of these fluids (Smith, 1929) was not observed, presumably due to the severe acidosis. There was a close correlation between plasma lactate concentration and the lactate concentrations of these fluids. For eight pairs of determinations at plasma [lactate$^-$] of 38–230 mM the relationship was:

$$[\text{lactate}^-]_{\text{PC or PN}} = -6{\cdot}15 + 0{\cdot}97[\text{lactate}^-]_{\text{Pl}};\ r^2 = 0{\cdot}96.$$

Similarly, total calcium concentration was apparently elevated in these fluids to the same extent as in plasma. Our evidence for this is indirect because we were not always able to measure calcium in pericardial (or peritoneal) fluid and plasma from the same turtles. But we did find a similarity in the ratio between total calcium and lactate in plasma and in the other fluids, and we know that lactate was equilibrated between these fluids. In Table 2, measurements of both calcium and lactate concentrations from the pericardial fluids of six turtles is presented. The ratio, ([calcium]/[lactate$^-$]) × 100, is very similar in these samples (49·7 ± 1·8) even though the range of values is large. Two peritoneal fluid samples gave a similar result. In the final plasma samples of the three anoxic turtles analysed for calcium, the same ratio, ([calcium]/[lactate$^-$]) × 100, was 48·1, 49·1 and 49·1.

Urinary bladder volume and composition was highly variable. In general, however, the high-O_2 turtles had larger fluid volumes. The bladders of seven high-O_2, catheterized turtles dissected *post mortem*, had 17·3 ± 4·4 ml of urine, while four of the six low-O_2 turtles sampled had no collectable fluid. Several of the anoxic catheterized turtles, as well as all four of the non-catheterized, low-O_2 turtles tested, had solid material in their bladders, the composition of which was not determined. Of particular significance was the presence of lactate, calcium and magnesium in the urine of all submerged turtles examined, in some cases at concentrations exceeding plasma levels. For example, the highest [lactate$^-$] observed in this study, 287 mM, was in the urine of a non-catheterized, low-O_2 turtle. This same animal had urine [Mg] of 23·3 mM, also the highest observed. In other animals, in which urine was more dilute, concentrations of these substances were less than in plasma. No pattern related to experimental treatment was apparent in these differences.

DISCUSSION

In previous studies of acid-base changes during diving, the duration of the dives were relatively short (minutes or hours) and the acute elevations of lactate were buffered extracellularly by decreases in [HCO_3^-] and, within the plasma, presumably also by decreases in protein net negative charge (Scholander, 1940; Jackson & Silverblatt, 1974). In contrast, the dives we have described in this study were of much longer duration (months) and the lactate elevations were essentially chronic. Perhaps as a consequence of the prolonged nature of the acidosis, additional response mechanisms were observed that allowed the turtles to tolerate much higher levels of lactate. Because of the more consistent and dramatic acid-base and ionic changes in the low-O_2 group in this study and because of problems with a fungal infection in the high-O_2 group (Ultsch & Jackson, 1981), we will emphasize the responses of the low-O_2 group in this discussion.

When a strong acid anion such as lactate is added to the blood, it can be balanced electrically in two ways: either by an increase in the concentration of cations or by a decrease in the concentration of anions. Effective buffering, or acid-base control, requires that an increase in [H^+] can contribute only minimally to the former mechanism. The turtles in this study apparently utilized both alternatives when lactate accumulated as a result of prolonged anoxia in the cold. Significant increases in the concentrations of the cations, potassium, calcium and magnesium were observed, while significant decreases in the concentrations of the anions, bicarbonate and chloride, occurred as well. For both theoretical (Stewart, 1978) and practical reasons, we choose in this discussion to focus on the changes occurring in strong ions. However, it should be understood that a net movement of strong cations into the plasma must be associated stoichiometrically by some combination of weak anion (such as OH^-, HCO_3^-, or CO_3^{2-}) entry and weak cation (such as H^+) exit, and similarly for the net loss from plasma of strong anions. A quantitative analysis of the responses cannot readily be made on the basis of weak ion changes because of their participation in various equilibria reactions.

The decrease in [HCO_3^-] is ordinarily the predominant extracellular response to metabolic acidosis and is a direct physical consequence of the increase in strong acid anion concentration (Stewart, 1978). Freshwater turtles have unusually high extracellular [HCO_3^-] (Smith, 1929) and are thereby able to withstand large acute increases in organic acid concentration. In short-term dives at 24 °C, most of the added lactate is buffered by HCO_3^-, with only a small fraction attributable to other mechanisms (Jackson & Silverblatt, 1974). In our study at 3 °C, on the other hand, plasma [HCO_3^-] fell from about 40 to below 5 m-equiv/l during long anoxic submergence (Ultsch & Jackson, 1981). but this accounted for less than 20% of the concurrent increase in plasma [lactate$^-$].

In contrast to HCO_3^- buffering, non-HCO_3^- buffering of turtle whole blood is not exceptional, however, as values of $\beta(\Delta[HCO_3^-]/\Delta pH)$ are 9·7–12·4 m-equiv/l unit pH (Ultsch & Jackson, 1981), which is below that of many other diving reptiles and far below that of diving mammals (Wood & Lenfant, 1976). In view of the large effect of reduced haematocrit that we observed, plasma proteins probably make only a minor contribution to this buffering.

The major ionic mechanisms we observed in response to an elevation of lactic acid involved exchanges of strong ions between the extracellular fluid and other body compartments. The changes in plasma [Na^+] and [K^+] (Figs. 1 and 2) were approximately equal but in opposite directions, and may represent a failure of the membrane Na^+/K^+ pump to maintain normal transcellular cation distribution. Plasma [K^+] increased steadily in the anoxic turtles, suggesting a leakage out of the K^+-rich intracellular compartment. The magnitude of the mean increase in plasma [K^+], from 2·4 to 9·0 m-equivl/l, was not significant in terms of overall ionic balance, but it represents a profound elevation of this ion. The decrease in plasma [Na^+] in the anoxic turtles, while a much smaller fractional change compared to K^+, was similar in absolute magnitude, and a leakage into the Na^+-poor intracellular compartment is postulated, although diffusion out of the body is also a possibility. Plasma [Na^+] was the most stable of the measured ions in the submerged turtles, and was not significantly reduced until after 3 months of apnoea in low-O_2 water. Assuming that a one-for-one counter-leakage of Na^+ and K^+ occurred between the intra- and extracellular fluids of the anoxic turtles, then the observed changes in the concentrations of these ions, while perhaps of some functional importance, were of no net consequence in terms of the acid-base state of the plasma.

The decrease in [Cl^-], on the other hand, represents a significant feature of the turtle's compensatory response to lactic acidosis, although the similarity in the response in the low and high-O_2 turtles (Fig. 3) despite the marked difference in the severity of acidosis in these two groups, makes it doubtful that this represents a controlled response. We do not know the fate of the lost Cl^-, whether into the cells or out of the body. A transcellular exchange of Cl^- for lactate would minimize the acidotic effect on the extracellular fluid, but would not be advantageous for the intracellular fluid where the lactate was produced.

In our turtles, cold exposure *per se* (group 5) had no effect on plasma [Na^+], [K^+] or [Cl^-] (cf. bars A and C in Figs. 1–3). In contrast, in previous studies of turtles able to breathe in the cold, plasma [Na^+] was reported to decrease in *Terrapene carolina* and *Chrysemys* (= *Pseudemys*) *scripta* (Hutton & Goodnight, 1957), in *Trionyx spinifer* (Dunson & Weymouth, 1965; Dunson, 1967) and in *Graptemys geographica* and *Chelydra serpentina* (Semple, Sigsworth & Stitt, 1969). There are possible explanations to account for this discrepancy with our findings. First, Hutton & Goodnight (1957) only studied one animal of each species, so a firm conclusion is difficult from their data. Second, *Trionyx spinifer* is a soft-shelled turtle with a highly permeable skin so that a greater Na^+ loss may occur in this species. Freshwater turtles can extract Na^+ but not K^+ from the surrounding water (Dunson & Weymouth, 1965; Dunson, 1967; Trobec & Stanley, 1971) and the Na^+ losses observed may have been due to inhibition of this transport process by cold. Finally, Semple *et al.* (1969) also observed a fall in both [Cl^-] and [K^+], and this general fall in ionic concentrations can be attributed largely to increases in total body water. In view of these changes, it is particularly notable that our low-O_2 turtles, despite the combined and potentially detrimental effects of anoxia, cold and net water influx (weight gain), still managed to maintain rather constant plasma [Na^+] for most of the submergence period.

The most dramatic and unexpected changes observed in this study were the in-

creases in calcium and magnesium concentrations. We assume that calcium and magnesium, probably originating in skeletal elements including the shell, entered plasma accompanied by weak anions, and that their addition to the plasma was a major mechanism acting to balance the lactate ion and minimize the fall in pH. The sum of these two elements, moreover, expressed in terms of milliequivalents, more than balance the 'cation gap' that emerged during the course of this study. The description of an ionic balance sheet is confounded, however, by the uncertainty relating to the chemistry of the calcium and magnesium response, in particular to two important aspects of this response: (1) the identity and fate of the anions accompanying the calcium and magnesium and (2) the state of calcium and magnesium in the plasma and elsewhere in the extracellular fluid, whether bound or free, and if bound, the nature of the binding compound. We measured the total concentrations of these substances in the plasma but this measurement alone cannot define the distribution between free and bound fractions. Ordinarily, the calcium and magnesium in vertebrate plasma is approximately evenly partitioned between a free, ionized fraction and a neutrally bound fraction (Marshall, 1976). Most of the latter is associated with plasma proteins, chiefly albumin. Because of the sensitivity of the heart and nervous tissue to elevated ionized calcium (Brink, 1954) and the problem of soft-tissue calcification, a predominance of this active fraction in the plasma of the anoxic turtles would seem unlikely. We observed muscular activity in these animals and we recorded blood-pressure changes to the very end of the experiment, so that the excitable tissues were certainly functional. We are faced, on the other hand, with the equally formidable problem of accounting for the charge imbalance. At this point, until experimental measurements of calcium ion activity are made, we can only briefly state alternative interpretations of our observations.

The first possibility, and the most straightforward in terms of charge balance, is that all or most of the calcium and magnesium existed as free, divalent cations. However, such extraordinarily high activities of these ions seem unlikely based on present knowledge of vertebrate excitable tissue function. A second possibility is that the calcium and magnesium were primarily in a bound state, but that the binding *per se* neutralized negative charges and thereby eliminated the cation gap. This could be accomplished by complexes with either plasma protein or with lactate. A final possibility is that the calcium and magnesium were bound to some unmeasured substance, presumably not present in the pre-dive state. Phosphate, also coming from bone and shell, could be such a substance. The final answer may well turn out to be some combination of these possibilities.

This remarkable mobilization of calcium and magnesium in the anoxic turtles may represent an extreme example of the bone demineralization that has been observed in mammals during prolonged metabolic acidosis (Lemann, Litzow & Lennon, 1966; Burnell, 1971). In mammals, however, the plasma calcium is carefully controlled and mobilized calcium is excreted, as is the acid anion. In the turtle, on the other hand, both the mobilized calcium and magnesium as well as the acid anion ($lactate^-$) are retained to a significant extent within the extracellular fluid. We do not know the source of the calcium and magnesium nor do we know the identity of the anions that accompany them, although we assume that the cations derive from bony tissues in

combination with a weak anion such as carbonate. The frog, *Rana temporaria*, as part of its compensation to experimental respiratory acidosis, mobilizes calcium carbonate from stores in its endolymphatic sacs, extensive deposits peculiar to anuran amphibians (Simkiss, 1968). It is tempting to speculate that the turtle utilizes its characteristic structure, its shell, as a source for the calcium and magnesium. The use of this structure, which accounts for about 30% of its body weight (Jackson, 1969), could represent another adaptation to anoxia that is unique among the Vertebrates. An analogous role for the exoskeleton of the crab, *Cancer productus*, in response to the acidosis produced by emersion, has recently been postulated (deFur, Wilkes & McMahon, 1980).

The presence of lactate in the percardial and peritoneal fluids at concentrations equal to those in plasma indicates that the whole extracellular fluid compartment participated in the acid buffering. In previous studies of prolonged diving at higher temperature, lactate either failed to penetrate these fluids (Penney, 1974) or was present at the end of the diving at concentrations far below plasma levels (Jackson & Silverblatt, 1974). Because these fluids are normally quite alkaline with high [HCO_3^-] and low [Cl^-] (Smith, 1929), this further indicates that the turtles at 3 °C exploited this buffer reserve, whereas at 22–24 °C they did not.

The acid load was also dealt with, but to an uncertain extent, by movement of lactate and calcium into the bladder urine. We do not know the route by which these substances entered the urine, whether from conventional kidney urine production or by direct passage from the plasma across the bladder wall. The latter possibility arises from the proven transport functions of the turtle bladder epithelium (Brodsky & Schilb, 1967). Urine production by the kidney, on the other hand, requires adequate blood flow and pressure to produce glomerular filtration. We observed, however, that cardiovascular function in the cold anoxic turtles was severely depressed. Heart rates ranged from 8 to 60 h^{-1} and mean arterial pressure averaged less than 10 mmHg (unpublished observations), and it remains to be established whether filtration can occur under these conditions. It is known, however, that frogs (*Rana clamitans*) after acclimation to 5 °C are able to produce urine by glomerular filtration, albeit at a greatly reduced rate compared to higher temperatures (Schmidt-Nielsen & Forster, 1954).

In conclusion, turtles submerged for several months in severely hypoxic water (P_{O_2} less than 5 mmHg) at 3 °C suffered a severe lactic acidosis. This acid load was buffered in the plasma by a combination of ionic changes, including the conventional reduction in HCO_3^-, but also by significant changes in the concentrations of strong ions. The notable changes in strong ions were a fall in Cl^- and an increase in calcium and magnesium levels. These effects were apparently distributed throughout the extracellular compartment. Some acid may have been excreted via the urine during the dive. Because the lactic acid originated within the cell, the major site of body fluid buffering, a problem deserving study is the nature of intracellular acid buffering and acid-base balance under these extreme conditions.

This study was supported by NSF grants PCM78-22333 (to D.C.J.) and PCM79-11609 (to G.R.U.). We thank Dr R. K. Matthews of the Brown University Department of Geology for the use of his Atomic Absorption Spectrophotometer and R. A.

Fifer for making the calcium and magnesium measurements on this machine. We also thank Dr Chung-Ja M. Cha for the amino acid analysis, Miss Patricia McLaughlin for the ammonia analysis, Dennis Lee for his technical assistance and Dr F. Ebner for the use of his osmometer.

REFERENCES

BELKIN, D. A. (1963). *Anoxia: Tolerance in Reptiles. Science, N.Y.* **139**, 492–493.

BURNELL, J. M. (1971). Changes in bone sodium and carbonate in metabolic acidosis and alkalosis in the dog. *J. clin. Invest.* **50**, 327–331.

BRODSKY, W. A. & SCHILB, T. P. (1967). Mechanism of acidification in the turtle bladder. *Fedn Proc.* **26**, 1314–1321.

BRINK, F. (1954). Role of calcium ion in neural process. *Pharmacol. Rev.* **6**, 243–298.

CHANEY, A. L. & MARBACH, E. P. (1962). Modified reagents for determination of urea and ammonia. *Clin. Chem.* **8**, 130–132.

DEFUR, P. L., WILKES, P. R. H. & MCMAHON, B. R. (1980). Non-equilibrium acid-base status in *C. productus*: role of exoskeletal carbonate buffers. *Resp. Physiol.* **42**, 247–261.

DUNSON, W. A. (1967). Sodium fluxes in freshwater turtles. *J. exp. Zool.* **165**, 171–182.

DUNSON, W. A. & WEYMOUTH, R. D. (1965). Active uptake of sodium by softshell turtles (*Trionyx spinifer*). *Science, N.Y.* **149**, 67–69.

HUTTON, K. E. & GOODNIGHT, C. J. (1957). Variations in the blood chemistry of turtles under active and hibernating conditions. *Physiol. Zoöl.* **30**, 198–207.

JACKSON, D. C. (1969). Buoyancy control in the freshwater turtle, *Pseudemys scripta elegans. Science, N.Y.* **166**, 1649–1651.

JACKSON, D. C. & SILVERBLATT, H. (1974). Respiration and acid-base status of turtles following experimental dives. *Am. J. Physiol.* **226**, 903–909.

LEMANN, J. JR., LITZOW, J. R. & LENNON, E. J. (1966). The effects of chronic acid loads in normal man: further evidence for the participation of bone mineral in the defense against chronic metabolic acidosis. *J. clin. Invest.* **45**, 1608–1614.

MARSHALL, R. W. (1976). Plasma fractions. In *Calcium, Phosphate and Magnesium Metabolism* (ed. B. E. C. Nordin), pp. 162–185. Edinburgh: Churchill Livingstone.

PENNEY, D. G. (1974). Effects of prolonged diving anoxia on the turtle, *Pseudemys scripta elegans. Comp. Biochem. Physiol.* **47** A, 933–941.

ROBIN, E. D., VESTER, J. W., MURDAUGH, H. JR. & MILLEN, J. E. (1964). Prolonged anaerobiosis in a vertebrate: anaerobic metabolism in the freshwater turtle. *J. cell. comp. Physiol.* **63**, 287–297.

SCHMIDT-NIELSEN, B. & FORSTER, R. P. (1954). The effect of dehydration and low temperature on renal function in the bullfrog. *J. cell. comp. Physiol.* **44**, 233–246.

SCHOLANDER, P. F. (1940). Experimental investigations on the respiratory function in diving mammals and birds. *Hvalrdd. Skr.* **22**, 1–131.

SEMPLE, R. E., SIGSWORTH, D. & STITT, J. T. (1969). Composition and volume of fluids in winter and summer of turtles native to Ontario, Canada. *J. Physiol., Lond.* **204**, 39*P*–40*P*.

SEMPLE, R. E., SIGSWORTH, D. & STITT, J. T. (1970). Seasonal observations on the plasma, red cell and blood volumes of two turtle species native to Ontario. *Can. J. Physiol. Pharmac.* **48**, 282–290.

SIMKISS, K. (1968). Calcium and carbonate metabolism in the frog (*Rana temporaria*) during respiratory acidosis. *Am. J. Physiol.* **214**, 627–634.

SMITH, H. W. (1929). The inorganic composition of the body fluids of the chelonia. *J. biol. Chem.* **82**, 651–661.

STEWART, P. A. (1978). Independent and dependent variables of acid-base control. *Resp. Physiol.* **33**, 9–26.

STITT, J. T., SEMPLE, R. E. & SIGSWORTH, D. W. (1971). Plasma sequestration produced by acute changes in body temperature in turtles. *Am. J. Physiol.* **221**, 1185–1188.

TROBEC, T. N. & STANLEY, J. G. (1971). Uptake of ions and water by the painted turtle, *Chrysemys picta. Copeia*, pp. 537–542.

ULTSCH, G. R. & JACKSON, D. C. (1981). Long-term submergence at 3 °C of the turtle, *Chrysemys picta bellii*, in normoxic and severely hypoxic water. I. Survival, gas exchange and acid-base status. *J. exp. Biol.* (in the Press).

WOOD, S. C. & LENFANT, C. J. M. (1976). Respiration: mechanics, control and gas exchange. In *Biology of the Reptilia* (ed. C. Gans and W. R. Dawson), Physiol. A, vol. 5, pp. 225–274. New York: Academic Press.