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FREE AND TOTAL CALCIUM CONCENTRATIONS IN THE BLOOD OF RAINBOW TROUT, *SALMO GAIRDNERI*, DURING 'STRESS' CONDITIONS

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SUMMARY

Measurements were made of the free and total calcium concentrations ($[Ca^{2+}]$ and Ca_t), acid-base status, haemoglobin concentration, haematocrit and plasma protein concentration in the blood of the rainbow trout, *Salmo gairdneri*. In isolated blood, $[Ca^{2+}]$ was proportional to Ca_t while $\log[Ca^{2+}]$ was inversely proportional to pH. Similar findings have been reported from human blood. During intense activity, $[Ca^{2+}]$ increased by 16 % without any significant change in Ca_t. Neither sustained exercise, hypercapnic acidosis nor hypoxia affected $[Ca^{2+}]$ or Ca_t. These data indicate that during common natural stress conditions $[Ca^{2+}]$ is maintained within a narrow range.

INTRODUCTION

The concentration of blood calcium is regarded as one of the most constant factors in mammalian biology. Calcium affects many cellular processes; for example, an increase in extracellular calcium concentration stimulates contractility in smooth muscle and heart muscle (Lüllmann, 1970; Nielsen & Gesser, 1983). Considering this, it is interesting to note that the calcium concentration in plasma may vary. Ruben & Bennett (1981) gave evidence that the total calcium, Ca_t, increases following severe exercise. Among several vertebrate species tested, the increase appeared to be more pronounced in poikilotherms with an osseous skeleton than in mammals and elasmobranchs. The effect was especially clear in trout. From a physiological point of view, however, Ca_t seems less important than the concentration of free calcium, $[Ca^{2+}]$. It should be recalled that about half of the Ca_t in human plasma is free. The remaining part is bound to protein ($\sim$40 %) or complexly bound to anions, i.e. bicarbonate ($\sim$10 %) (Siggaard-Andersen, Thode & Wandrup, 1980). The physiological importance of $[Ca^{2+}]$ is illustrated by a study of McLean & Hastings (1934) using an isolated frog heart to indicate the concentration of calcium. Under a fixed set of conditions $[Ca^{2+}]$, but not Ca_t,

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in the muscle bath could be predicted from the amount of force developed: that is, the frog heart reacts as a calcium-selective electrode. With reference to the functional importance of blood calcium and to the findings of Ruben & Bennett (1981), the purpose of the present study was to examine $[Ca^{2+}]$ and Ca_t in the rainbow trout exposed to physical exercise, hypercapnic acidosis and hypoxia. These conditions have a tendency to lower pH or otherwise affect blood parameters. Reduced pH could be of special importance, since it is suggested that this, together with an elevated lactate level, can cause a hypercalcaemia by dissolving skeletal hydroxyapatite (Ruben & Bennett, 1981). Other blood parameters such as acid-base status, haematocrit, haemoglobin concentration and plasma protein concentration were measured in order to evaluate the impact of the different treatments.

Furthermore, the inherent capacity of trout blood to modulate $[Ca^{2+}]$ was examined.

MATERIALS AND METHODS

Animals and experimental conditions

The experiments were carried out on rainbow trout, *Salmo gairdneri*, weighing between 350 and 550 g. The experimental temperature was 15 ± 1 °C. The animals were kept and fed in tanks supplied with well-aerated water for 2 months before use. To eliminate the stress of removing the fish from water for blood sampling, they were cannulated in the dorsal aorta using the technique of Soivio, Westman & Nyholm (1972) and Soivio & Nyholm (1975). During surgery, the gills were irrigated with aerated water containing $0{\cdot}1\ g\ l^{-1}$ MS222 (Sigma) anaesthetic. Each fish was allowed a 3-day recovery period in a special container restricting movements, without feeding, before any experiment was done.

Fish exposed to sustained swimming were placed in a tunnel swimming respirometer ($P_{O_2} > 145$ mmHg) and allowed an acclimation period of at least 4 h at a water speed of 10–20 cm^{-1} s before swimming for 1 h at approximately 2 body lengths s^{-1}. Fish exposed to intense activity were forced to swim to exhaustion in a 1 m^3 tank (P_{O_2} about 155 mmHg) by being prodded with a stick for about 5 min. The blood samples were collected after a recovery period of 5–10 min.

For studying the effect of hypoxia and hypercapnia as well as control conditions the fishes were kept in the special containers in which they had been placed after surgery. During hypoxia, the P_{O_2} of the water was controlled at 45 mmHg by a P_{O_2} regulator (Wood, Johansen & Weber, 1975) which opened for air equilibration when the P_{O_2} was below 45 mmHg.

The response to hypercapnia was studied in fish exposed to a P_{CO_2} of about 9 mmHg. The control fishes were maintained in well-aerated water.

Blood was sampled only once from each fish. After the first 0·2 ml had been discarded, blood was collected anaerobically with a 1·0-ml syringe which had a dead space of 30 μl and contained Ca-heparin. For studies on the modulation properties of $[Ca^{2+}]$ in isolated blood, however, the sampling was performed

percutaneously from the caudal vein of intact fish that were wrapped in a wet towel. The dead space of 3 % (v/v) was filled with Ca-heparin.

Variables measured

The measurement of free calcium ions, $[Ca^{2+}]$

The calcium electrode (F211Ca, Radiometer, Copenhagen) is highly selective when the pH is between 5 and 9 (Thode, Wandrup, Aas & Suggaard-Andersen, 1982). The electrode potential is a function of the calcium ion activity as described by the Nernst equation. In contrast with the measurement of pH, however, there is no agreed convention for the expression of individual ion activity. Therefore, the approach chosen in this study has been to give all values in concentration terms, $[Ca^{2+}]$, relative to a $CaCl_2$-NaCl standard solution. Ionic strength is the main determinator of the ion activity coefficient (calcium ion activity = activity coefficient × $[Ca^{2+}]$). Ionic strength can only be approximately evaluated by indirect methods in plasma (Siggaard-Andersen *et al.* 1980). A value of 0·160 mol l^{-1} has been chosen because it is close to the value calculated from the concentration of monovalent cations (assuming that Cl^- is the only counterion). Furthermore, this value is similar to that used in human studies, which make comparison possible. An increase or decrease in ion strength of 10 mmol l^{-1} in a solution of 0·160 mol l^{-1} will modify the $[Ca^{2+}]$ by 1·2 % (Siggaard-Andersen *et al.* 1980). Two $[Ca^{2+}]$ calibration solutions of 1·00 and 3·00 mmol l^{-1} with an ionic strength of 160 mol l^{-1} were prepared as described by Fogh-Andersen Christiansen, Komarmy & Siggaard-Andersen (1978). When the $[Ca^{2+}]$ in a sample was calculated from the potentials measured, it was assumed that the activity coefficient was similar in standards and samples since they have similar ionic strength (for calculations see, for instance, Fogh-Andersen *et al.* 1978).

The set-up was similar to that described by Fogh-Andersen *et al.* (1978) and consisted of a calcium-selective electrode mounted in series with a pH-electrode both connected to the same reference calomel electrode by a saturated KCl liquid junction. The electrodes were placed on the arm of a BMS 2 blood micro-unit (Radiometer, Copenhagen) and thermostatted to 15 ± 0·1 °C. The potentials were measured by two PHM 64 (Radiometer, Copenhagen) pH/mV-meters. A volume of 60 μl was required to fill both electrode cuvettes.

The pH electrode was calibrated using Radiometer precision buffers (6·900, 7·445; 15 °C). The electrodes were subsequently flushed with 1·00 mmol l^{-1} $[Ca^{2+}]$ standard to remove the pH-buffers which bind calcium, before a two-point calibration of the calcium electrode was done. The displayed result was read after about 2 min. After this time the potential was stable and at a maximum. This calibration gives the slope and the intercept of the linear relationship between log $[Ca^{2+}]$ and the measured potential. After each sample had been measured, the electrode was flushed with 1·00 mmol l^{-1} $[Ca^{2+}]$ standard. The effect of flushing on sample measurements was less than −0·01 mmol l^{-1}. To preserve the calcium electrode both electrodes were filled with 1·00 mmol l^{-1} $[Ca^{2+}]$ standard during stand-by.

Technical comments on measurement of [Ca^{2+}]

The potentials measured on whole blood were corrected for the suspension effect of the erythrocytes on the liquid junction using the method described by Siggaard-Andersen (1974). The effect caused an overestimation by 0·01–0·03 mmol l^{-1} before correction. The drift of the calcium electrode potential was about 0·01 mV within 1 day of measurements. The precision, given as standard deviation of 25 duplicate measurements on whole blood, was 0·017 mmol l^{-1} (~1·2 %), whereas the standard deviation of Ca_t was 0·05 mmol l^{-1} (~2·2 %). The day-to-day variation was about 0·01 mmol l^{-1} (~0·8 %) on 1-day refrigerated plasma ($N=5$), whereas it was 0·15 mmol l^{-1} (~6 %) for Ca_t on frozen samples ($N=10$, no unidirectional variation, paired t-test, $P=0·18$). Thus, the precision, at least in this study, is better for [Ca^{2+}] than for Ca_t measurement.

Other parameters

Ca_t was measured by atomic absorption (Perkin Elmer 403). Plasma was diluted 1:15 with a solution of 3·6 mmol l^{-1} La_2O_3 and 50 mmol l^{-1} HCl. The calcium standards were made from dried $CaCO_3$.

The *in vivo* P_{CO_2} and [HCO_3^-] were calculated by the Astrup method (Astrup, 1956) from the Henderson-Hasselbalch equation using an $\alpha CO_2 = 53·5$ μmol l^{-1} $mmHg^{-1}$ (at 15 °C, Siggaard-Andersen, 1974) and $pK' = 6·24$ (at 15 °C, Thomas & Hughes, 1982). The tonometers in the BMS 2 blood micro-unit (Radiometer, Copenhagen) were used for equilibrating the P_{O_2} to 375 mmHg and the P_{CO_2} to values around the expected *in vivo* value. Gas mixtures were delivered by Wösthoff pumps. Equilibrium time was 30 min. The same equilibrium procedure was used for studying the pH effect on [Ca^{2+}] in isolated blood and plasma. Plasma was obtained by centrifugation of whole blood equilibrated to a P_{CO_2} and P_{O_2} of 3 and 375 mmHg, respectively.

Haemoglobin was measured as HbO_2 using extinction coefficients of 14·4 and 15·2 $mmol^{-1}$ cm^{-1} at 542 and 577 nm (mean values from Zijlstra & Zwart, 1983). The haematocrit was measured after centrifugation for 4 min at 12 000 rev. min^{-1}. For determination of plasma protein and Ca_t the plasma obtained from haematocrit measurements was used. The protein concentration was measured with the biuret method with bovine albumin (Sigma) as a standard.

Anticoagulant

Heparin solution consisted of 1500 i.u. ml^{-1} sodium heparin (Leo), 4·5 mmol l^{-1} $CaCl_2$ and NaCl added to give an ionic strength of 0·160 mol l^{-1}. Heparin binds a significant amount of calcium but the addition of $CaCl_2$ diminishes the measurable effect on [Ca^{2+}] to less than 0·01 mmol l^{-1} at 3 % (v/v). The Ca-heparin solution was used throughout this study as an anticoagulant.

Statistics

For comparison two-tailed *t*-statistics were used and a 5 % level of significance was chosen. Linear regression was also used.

RESULTS

Influence of pH and Ca_t on $[Ca^{2+}]$ in isolated blood

Preliminary experiments showed a linear relationship between $\log[Ca^{2+}]$ and pH when pH was in the region 7·0–8·3. On this basis, slopes of $\log [Ca^{2+}]$ *versus* pH were determined by two-point equilibrium using P_{CO_2} values of 1·5 and 7·5 mmHg. At the high P_{O_2}, $\Delta\log[Ca^{2+}]/\Delta pH$ is similar in plasma and whole blood (Table 1). When exposing whole blood to the low P_{O_2}, however, this slope changed from −0·069 to −0·094. In addition $[Ca^{2+}]$ at pH = 7·8 was elevated by 0·08 mmol l^{-1}. In a separate experiment, the lowering of P_{O_2} from 375 to 15 mmHg was found to make the haematocrit (Hct) more dependent on pH in that $\Delta Hct/\Delta pH$ changed from −6 to −15 ($N=7$). Moreover, at a given pH the haematocrit was elevated at the low P_{O_2}. An enhanced dependence of the haematocrit on pH resulted in a parallel increase in the pH-dependence of the plasma volume and thus of Ca_t. This effect, together with the linear relationship between $[Ca^{2+}]$ and Ca_t (see below), will tend to lower $\Delta\log [Ca^{2+}]/\Delta pH$ and to increase $[Ca^{2+}]$ at fixed pH.

The effect of changes in Ca_t on $[Ca^{2+}]$ was studied by addition of $CaCl_2$ or of the calcium-chelating agent, ethylene glycol-bis-(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA), to plasma and whole blood. EGTA chelates calcium in a 1:1 relationship, and thereby it makes unaccessible an amount of calcium equal to the amount of EGTA added. The relationship between Ca_t and $[Ca^{2+}]$ in plasma and whole blood has a slope of 0·67 ($r=0·999$) (Fig. 1).

In vivo *experiments*

To examine the effect of intense activity, the fishes were prodded with a stick until they were exhausted (Ruben & Bennett, 1981). This treatment resulted in an

Table 1. *Effect of CO_2-acidification on $[Ca^{2+}]$*

Sample	$\Delta\log[Ca^{2+}]/\Delta pH$	$[Ca^{2+}]$(mmol l^{-1}) pH = 7·8
Plasma (375 mmHg)	−0·069 ± 0·025	1·41 ± 0·05
Whole blood (375 mmHg)	−0·069 ± 0·024	1·40 ± 0·07
Whole blood (15 mmHg)	−0·094 ± 0·031*	1·48 ± 0·06*

*Significantly different from plasma and whole blood at higher oxygen tension, paired *t*-test. Means ± 1 S.D.

Each sample consisted of blood pooled from four fishes. For each sample, whole blood and plasma were equilibrated at P_{CO_2} values of 1·5 and 7·5 mmHg giving two pH values. For whole blood this was done at P_{O_2} values of 375 and 15 mmHg, whereas plasma was exposed to the higher P_{O_2} values only. Means ± 1 S.D.

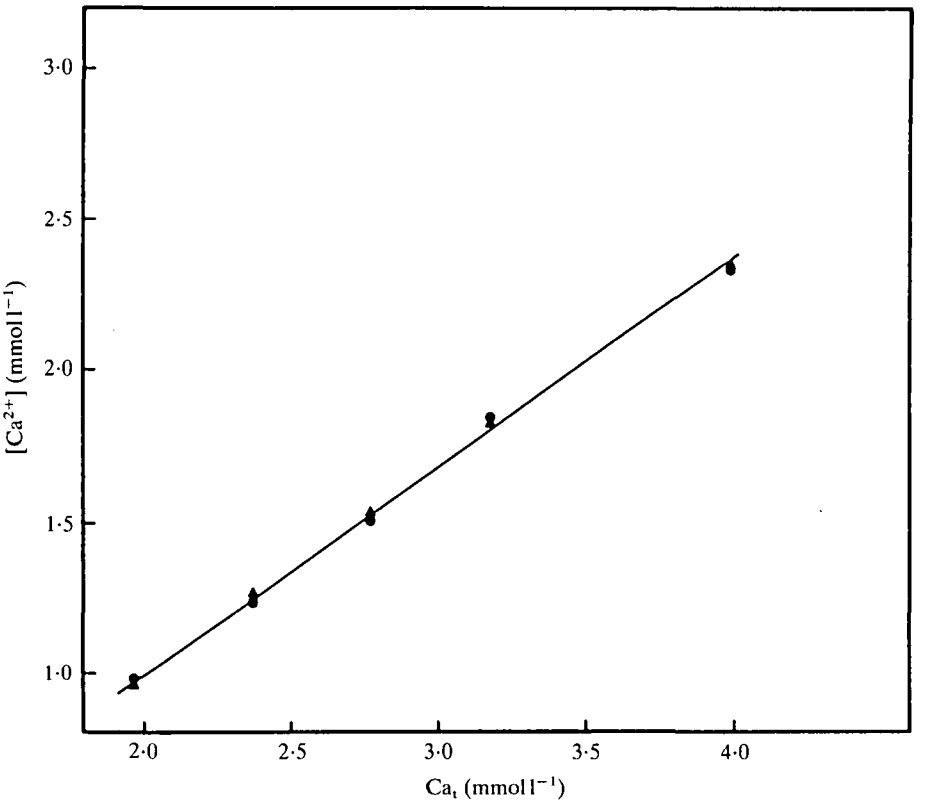

Fig. 1. The effect on $[Ca^{2+}]$ (free calcium ion concentration) of the addition of $CaCl_2$ and EGTA to whole blood (●) and plasma (▲) at pH = 7·8 (obtained by equilibrating the sample to 3 mmHg P_{CO_2} and 375 mmHg P_{O_2}). The ionic strength of the diluent was adjusted to 0·160 mol l^{-1} with NaCl. The plasma was diluted by 3 %. The plasma volume was calculated from the haematocrit. The standard deviation of duplicate measurements is within the point marks.

increase in $[Ca^{2+}]$ of 16 % as well as a drop in pH of 0·37 without any significant change in other parameters (Table 2).

To elucidate the effect of reduced blood pH, one group of fish was exposed to hypercapnia at a P_{CO_2} of 9 mmHg for 1 h. Although the blood pH fell about 0·3 and $[HCO_3^-]$ rose, no significant change in the calcium parameters occurred. The plasma protein concentration was raised by 59 % (Table 2).

Because of the elevated oxygen consumption and the reduced venous P_{O_2} associated with activity (Kiceniuk & Jones, 1977), the impact of hypoxia was examined. Because of increased P_{CO_2} caused by respiration of crowded fishes (to reduce P_{O_2}), the treatment further resulted in moderate hypercapnia. The effect of 1–1·5 h of exposure did not result in any change in $[Ca^{2+}]$ (preliminary experiments) although pH fell by 0·15. To examine the influence of prolonged hypoxia, an exposure time of 2 days was chosen. The treatment resulted in an increase in $[HCO_3^-]$ and P_{CO_2} without any change in pH, i.e. a compensated hypercapnia. Haematocrit and plasma protein concentration were elevated (Table 2).

Table 2. *$[Ca^{2+}]$, Ca_t and other blood parameters measured* in vivo *during control and 'stress' conditions*

	$[Ca^{2+}]$ ($mmol\,l^{-1}$)	Ca_t ($mmol\,l^{-1}$)	pH	P_{CO_2} (mmHg)	$[HCO_3^-]$ ($mmol\,l^{-1}$)	Hct (%)	Hb_4 ($mmol\,l^{-1}$)	Protein ($mg\,ml^{-1}$)	$\Delta\log[Ca^{2+}]/\Delta pH$
Control	1·33 ± 0·04	2·33 ± 0·16	7·87 ± 0·03	2·6 ± 0·5	6·2 ± 1·1	18·5 ± 2·4	0·83 ± 0·14	21·9 ± 6·9	−0·038 ± 0·013
Swimming	1·30 ± 0·10	2·49 ± 0·22	7·84 ± 0·11	5·0 ± 1·7*	10·5 ± 2·3*	24·7 ± 5·1*	1·15 ± 0·18*	34·0 ± 10·5	−0·064 ± 0·022
Hypercapnia	1·40 ± 0·08	2·38 ± 0·22	7·55 ± 0·08*	10·4 ± 1·6*	11·4 ± 1·5*	22·6 ± 8·9	0·92 ± 0·38	34·8 ± 5·9*	−0·062 ± 0·022
Hypoxia	1·31 ± 0·04	2·22 ± 0·18	7·84 ± 0·06	4·8 ± 0·4*	10·5 ± 1·6*	29·0 ± 7·1*	1·19 ± 0·34	33·4 ± 5·5*	−0·054 ± 0·023
Intense activity	1·54 ± 0·10*	2·60 ± 0·33	7·50 ± 0·10*	4·6 ± 2·0	5·5 ± 2·8	21·5 ± 7·5	0·79 ± 0·25	26·4 ± 12·6	−0·057 ± 0·028

*Significantly different from control at 5 %, or better.
Mean ± 1 S.D., $N = 5$ in each group. Temperature 15 °C.

The effect of sustained, but less intense, exercise was also studied. The fishes were made to swim at 2 lengths s^{-1} (about 60 cm s^{-1}) for 1 h. This speed is well below that recorded for burst activity, but about the maximum speed for sustained swimming (Webb, 1971). Table 2 shows that this activity left the calcium parameters unaffected, whereas P_{CO_2} and $[HCO_3^-]$ were increased at an unchanged pH. This indicates a compensated respiratory acidosis. An elevated P_{CO_2} is not likely to be the cause of the elevated P_{CO_2} since the water was aerated throughout the experiment. The haemoglobin and plasma protein concentration were also increased.

The results taken together revealed a negative correlation between plasma protein concentration and the pH sensitivity of $[Ca^{2+}]$ ($\Delta\log[Ca^{2+}]/\Delta pH$) ($r = -0{\cdot}57$, $P < 0{\cdot}02$). No correlation was found between plasma protein concentration and Ca_t ($r = 0{\cdot}15$, $0{\cdot}20 > P > 0{\cdot}10$).

DISCUSSION

During control conditions, both Ca_t and $[Ca^{2+}]$ were maintained within a close range. Furthermore, only intense activity to exhaustion had an effect on blood calcium, increasing $[Ca^{2+}]$ by 16 %. Ca_t remained unchanged. Neither acidosis, hypoxia nor sustained exercise influenced the calcium parameters. It should be noted, though, that the exposure times (1–1·5 h) were extended compared to the 15 min used in the examination of the effects of intense activity. In addition, an exposure time of 2 days was used during hypoxia. Exposure was prolonged in order to allow time for the extraction of skeletal calcium. However, this means that the fish, by some regulatory mechanism, might be able to regain control values of blood calcium, even though a change might have occurred in the meanwhile.

In the present study, no significant change in Ca_t appeared after intense activity. This is in contrast with the finding of Ruben & Bennett (1981) of a 72 % increase. It should be noted that they sampled the blood from the ventral aorta percutaneously in air-exposed fish. Soivio, Nikinmaa, Nyholm & Westman (1981) did not find any difference in blood Ca_t between the ventral and dorsal aorta. Furthermore, in a preliminary experiment, the percutaneous blood sampling was applied to three fishes which had been exposed to intense activity. Still, the increase in Ca_t (25 %, $2{\cdot}93 \pm 0{\cdot}06$ mmol l^{-1}) was far below the 72 % found by Ruben & Bennett (1981). A possible explanation for the difference could be that the trout in this study had a lower capacity for aerobic and anaerobic metabolism than the ones used by Ruben & Bennett (1981). The fish would therefore be exhausted at a lower $[H^+]$ in the plasma, and – according to the hypothesis of these authors – less calcium would be released from the skeleton. The post-activity pH value reported by Ruben & Bennett (1981) seems to support this explanation, but the difference in sampling technique makes a direct comparison of the pH in the two studies difficult (Nikinmaa, 1981).

In isolated blood, provided that Ca_t is unchanged, only minor modulation of

$[Ca^{2+}]$ appears possible. The effect of blood cells on $[Ca^{2+}]$ seems marginal. This conclusion is, however, complicated by the finding that a low P_{O_2} increased the change in $[Ca^{2+}]$ following a fixed change in pH. A lowering of P_{O_2} from 375 to 15 mmHg decreased $\Delta Hct/\Delta pH$ from -6 to -15. The swelling following a decrease in pH will thus be more pronounced at a lower P_{O_2} and will result in a more concentrated plasma. This, and an additional pH effect on $[Ca^{2+}]$ in the isolated plasma, will in turn result in a decreased $\Delta \log [Ca^{2+}]/\Delta pH$ at the lower P_{O_2} value. The concentrating effect of a lowering in P_{O_2} can also explain the increase of 0·08 mmol l^{-1} in $[Ca^{2+}]$ at pH = 7·8. From the information available on, for instance, heart muscle, such an increase could be expected to have only a marginal effect. The pH effect on $[Ca^{2+}]$ was too small to cause any significant increase during hypercapnia.

A possible exchange of calcium between plasma and blood cells appears not to influence plasma calcium. No difference in the linear relationship between $[Ca^{2+}]$ and Ca_t could be observed between whole blood and plasma. The observed slope of 0·67 indicates that 33 % of the change in Ca_t was buffered by plasma proteins or other calcium-binding substances. This tends to diminish the effect of an elevation of Ca_t.

Comparison with non-mammalian species is not possible, since I know of no data on the effect of pH and Ca_t on $[Ca^{2+}]$ for such species. pH appears to affect $[Ca^{2+}]$ in trout less than in human blood. In the latter, $\Delta \log[Ca^{2+}]/\Delta pH$ was $-0{\cdot}22$ (Thode *et al.* 1982), that is about 3–6 times the value of trout blood (Table 2). On the other hand, changes in Ca_t at a constant protein concentration have a greater influence on $[Ca^{2+}]$ in trout. As an example, Moore (1969) found that in human blood 47 % of a change in Ca_t occurred as a parallel change in $[Ca^{2+}]$. The corresponding figure for trout blood is 67 %. These differences in response of $[Ca^{2+}]$ to changes in either pH or Ca_t are probably a result of the lower plasma protein concentration in trout, which is less than half of that in humans. Unexpectedly, in view of the fact that the plasma proteins are expected to bind calcium, an increase of about 60 % in plasma protein concentration during hypoxia and hypercapnia affected neither Ca_t nor $[Ca^{2+}]$. This is in contrast to what has been reported for human blood (Pedersen, 1973). This might have been caused by a difference in calcium-binding properties of the proteins. Alternatively, there might be a concomitant increase in other substances occupying the calcium-binding sites, e.g. magnesium, which has a binding constant for albumin similar to that of calcium (Pedersen, 1973).

Another distinction between human and trout blood is that Ca_t is positively correlated with plasma protein concentration in humans (Pedersen, 1973). This is not observed in trout. In human as well as trout blood, $\Delta \log[Ca^{2+}]/\Delta pH$ is negatively correlated with plasma protein concentration.

As a main conclusion, the present study suggests that the blood calcium parameters in trout are kept within a narrow range during most of the conditions the trout is likely to experience in nature.

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