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SHORT COMMUNICATION

INTRACELLULAR BUFFERING BY DIPEPTIDES AT HIGH AND LOW TEMPERATURE IN THE BLUE CRAB *CALLINECTES SAPIDUS*

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Intracellular buffering is provided mainly by bicarbonate, phosphates and proteins (see Burton, 1978). A part may also be played by free histidine and small peptides containing histidine. Although the free histidine content of most animal tissues is low, a variety have been found to possess substantial amounts of histidine-containing dipeptides, most commonly *N*-β-alanyl-L-histidine, either without substitutions (carnosine) or methylated at the 1-position (anserine) or at the 3-position (ophidine or balenine). At 25 °C the pK of carnosine is 6·83 whereas that of anserine is 7·04, so the combination of appropriate pK and high concentration leads to significant intracellular buffer action.

The distribution of these dipeptides has been studied in many vertebrates, but few invertebrates. Where high concentrations occur, one form usually predominates. Thus, terrestrial mammals and amphibians have mostly carnosine in skeletal muscle, fish and birds primarily anserine (Lukton & Olcott, 1958; Davey, 1960; Crush, 1970; Abe, 1983). Ophidine (or balenine) seems common, oddly enough, to reptiles and cetaceans. The only published data for invertebrates appear to be those of Lukton & Olcott (1958), showing small amounts of carnosine ($<1\ \mu mol\ g^{-1}$) in muscles from four molluscs and two crustaceans, and anserine only from the oyster.

Concentrations of these compounds can be high: Davey (1960) reported concentrations of 40–50 $\mu mol\ g^{-1}$ in several birds and cetaceans; Lukton & Olcott (1958) found only slightly lower concentrations in a variety of fish; and Crush (1970) and Abe *et al.* (1985) reported high concentrations in some fish and other animals. Davey (1960) calculated that 40 % or more of the buffering in muscle could be attributed to these dipeptides, and Abe *et al.* (1985) found a range from 7 % in trout to over 60 % in marlin. Several workers have remarked upon the inverse correlation between dipeptide content and aerobic metabolism: the dipeptides seem more important in muscles which are used mostly for burst

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activity and which rely upon glycolytic metabolism with its attendant lactic acid production (Davey, 1960; Abe *et al.* 1985).

The objective of the present study was to measure the concentrations and buffer contributions of amino acids and histidine-containing dipeptides in crab muscle tissue at both high and low acclimation temperatures. The aerobic capability of blue crab muscle is modest, and the concentration of lactic acid rises rapidly after exercise (Booth *et al.* 1982), characteristics of vertebrate tissues that have significant dipeptide buffering. Blue crabs are very active at high temperatures, but nearly torpid at low temperatures. Crabs also utilize intracellular free amino acids to balance the osmotic pressure of intra- and extracellular fluids (Bishop, 1977). Finally, since the bulk of the intracellular anion complement consists of protein, intracellular ions were measured as an index of changes in net protein charge at different temperatures.

Intermoult blue crabs (*Callinectes sapidus* Rathbun) of both sexes were collected from local waters and transferred to laboratory holding tanks provided with aeration and biological filtration systems. The animals were fed *ad libitum* with cut shrimp and fish, and temperature was changed by approximately 2°C per day until temperatures of 10 ± 1 and 30 ± 1°C were reached for two separate groups. Acclimation was continued for at least 3 weeks in each group.

To correct for trapped extracellular fluid in the tissue samples, 3–5 μCi of ^{14}C-labelled mannitol was injected into each animal 8–12 h prior to euthanasia. Two blood samples were drawn from the infrabranchial sinuses, a 0·5 ml sample for acid–base analysis (pH and total CO_2), and a 3–5 ml sample for analysis of radioactivity, ion and amino acid concentrations.

After withdrawing the blood samples, the crabs were quickly killed, then samples of skeletal muscle were dissected from the ventrolateral region of the abdomen and blotted free of excess blood. When the muscle from the last leg (the swimmeret muscle, or 'backfin') was used, care was taken to dissect out only the lighter-coloured muscle. Three tissue samples from each crab were placed in 4 vols of a chilled 70 % solution of methanol in water, and three in 4 vols of 0·1 mol l^{-1} HNO_3. The samples in methanol were homogenized on ice with a Potter–Elvehejm Teflon/glass homogenizer (15 strokes, 350 revs min^{-1}); the samples in 0·1 mol l^{-1} HNO_3 were homogenized by hand in a glass/glass homogenizer. The homogenates were centrifuged at low speed (1500 *g*) for 15 min at 4°C. An additional 2 ml of iced 70 % methanol was added to the pellet with mixing, the sample recentrifuged, and the washings added to the original supernatant. The extracts were then freeze-dried (Speed-Vac), redissolved in 2 ml of 40 mmol l^{-1} Li_2CO_3 (pH 9·5) and stored frozen. The nitric acid supernatants were stored frozen for later analysis.

For amino acid analysis, the extracts were dansylated by a modified version of the procedure described by Wiedmeier *et al.* (1982). A 0·5 ml sample was reacted overnight at room temperature with 0·5 ml of dansyl chloride (1·5 mg ml^{-1} CH_3CN), then extracted twice with 0·5 ml of *n*-heptane to remove unreacted reagent and hydrolysis products. The dansylated samples were then filtered

through a 0·2 μm filter and injected onto a reverse-phase HPLC column (Altex ODS, 5 μm C18). Mobile phase A was 10 mmol l^{-1} sodium acetate, pH 4·18 plus 5 % tetrahydrofuran (THF), and phase B was 90 % acetonitrile and 5 % THF. The gradient was: 10 to 40 % B in 30 min; 40 % B for 15 min; 40 to 100 % B in 3 min; 100 % B for 12 min; and a re-equilibration with 10 % B for 8 min between samples. Dansyl-amino acid derivatives were detected as relative fluorescence with excitation at 350 nm and detection at 530 nm. Amino acid and dipeptide standards (Sigma) were used to verify retention times and to quantify peak areas. All reagents were HPLC grade and the water used had a resistivity greater than 18 MΩ · cm (Milli-Q).

Portions of the blood samples were added to 0·5 ml of water and 5 ml of scintillation cocktail so that the concentration of the extracellular marker could be measured. A 2 ml portion of the blood sample was added to an equal volume of 0·1 mol l^{-1} HNO_3, centrifuged to remove precipitated proteins, then diluted as appropriate for the various ion analyses. Chloride was measured by amperometric titration (Buchler–Cotlove), sodium and potassium by flame photometry, and calcium and magnesium by atomic absorption. Portions of the acidified tissue extracts were treated similarly, except that the portion for scintillation counting was first neutralized with NaOH. By comparing the radioactivity of blood and tissue extracts, a correction factor for trapped extracellular fluid in the tissue samples was calculated. Using this correction factor and the blood ion concentrations, corrections to the tissue ion concentrations could be made to correct for trapped extracellular ions.

The mean pH values at 10 and 30 °C were 8·100 ± 0·027 and 7·877 ± 0·035. Total CO_2 decreased from 5·20 ± 0·22 mmol l^{-1} at 10 °C to 4·59 ± 0·41 mmol l^{-1} at 30 °C, and the calculated P_{CO_2} rose from 123 to 251 Pa (0·92 to 1·88 mmHg; $N = 10$ for all).

Six amino acids (Gly, Pro, Tau, Arg, Ala and Gln, Table 1) accounted for approximately 93 % of the total free amino acid (FAA) pool in muscle and 80 % of FAA in blood from both temperature acclimation groups. Histidine and lysine each constituted less than 1 % of the FAA pool in both blood and tissue. Wheatly (1985) reported roughly similar proportions of the major amino acids in whole tissue samples (i.e. intra- and extracellular solutes combined) from intermoult blue crabs. Carnosine accounted for slightly more than 10 % of the FAA in blood, but was only a minor component of the tissue pool (Table 1). The concentration of carnosine increased by almost 60 % from 10 to 30 °C ($P < 0·05$). Anserine was not detectable in any sample.

From titrations of pure carnosine at appropriate ionic strength, a buffer value of $-0·48\,(\text{mmol}\,l^{-1})^{-1}$ was derived, and this was used to calculate the buffer contribution. Alcoholic extract buffering (dB/dpH) for the muscle samples was −15·46 ± 0·70 at 10 °C and increased to −18·65 ± 0·60 at 30 °C ($P < 0·05$, $N = 10$), of which carnosine accounted for 10 % and 13 %, respectively. Assuming that the total intracellular buffering lies in the range 25–50 mequiv l^{-1} pH unit^{-1}, buffering by the amine fraction represents approximately 30–50 % of the total. From

Table 1. *Amino acid concentrations in blood (N = 3) and muscle tissue (N = 10) of crabs acclimated to 10 and 30°C*

	Blood		Muscle	
Amino acid	10°C	30°C	10°C	30°C
Gly	0·89	1·13	96·98 ± 12·9	87·85 ± 15·99
Pro	0·48	0·48	27·16 ± 12·96	28·19 ± 9·24
Tau	0·38	0·47	17·49 ± 5·37	16·69 ± 4·19
Ala	0·55	0·31	17·10 ± 2·58	14·26 ± 1·75
Arg	0·16	0·43	12·67 ± 2·15	25·85 ± 4·74
Gln	0·20	0·43	11·44 ± 5·02	12·94 ± 3·14
Carn	0·37	0·51	3·33 ± 1·03	5·22 ± 0·68
Glu	0·05	0·10	3·15 ± 2·18	2·57 ± 2·11
Hpr	0·0	0·10	1·16 ± 0·50	0·6 ± 1·20
Asn	0·0	0·0	0·86 ± 0·59	0·70 ± 0·33
Val	0·03	0·04	0·81 ± 0·36	0·93 ± 0·22
His	0·0	0·0	0·76 ± 0·06	0·30 ± 0·34
Asp	0·0	0·09	0·65 ± 0·63	1·27 ± 0·45
Lys	0·09	0·02	0·54 ± 0·08	0·63 ± 0·15
Thr	0·0	0·01	0·53 ± 0·20	1·07 ± 0·53
Leu, Ile	0·02	0·02	0·50 ± 0·17	0·59 ± 0·13
Tyr	0·05	0·08	0·43 ± 0·20	0·45 ± 0·16
Ser	0·03	0·11	0·38 ± 0·17	0·48 ± 0·32
Phe	0·02	0·0	0·30 ± 0·14	0·26 ± 0·08
Met	0·0	0·0	0·28 ± 0·13	0·66 ± 0·32
Orn	0·0	0·0	0·07 ± 0·02	0·06 ± 0·01
Trp	0·01	0·0	0·03 ± 0·02	0·14 ± 0·15
Total	3·32	4·29	196·92 ± 19·90	201·71 ± 8·19

Carn, carnosine; Hpr, hydroxyproline.
Concentrations are in mmol kg^{-1} wet mass, means for blood and ±1 S.E. for tissue samples.

examination of the free amino acid composition and the buffer contribution of carnosine, most of this buffering must come from small- to intermediate-sized peptides/proteins soluble in cold 70 % methanol.

Concentrations of the principal inorganic ions in both blood and intracellular fluid of skeletal muscle showed few changes upon acclimation from 10 to 30°C (Table 2). There were small but statistically significant increases in blood $[Na^+]$ and $[K^+]$, but no other significant differences between blood and tissue ions at the two temperatures. The anion gap, defined as the sum of measured cations minus chloride, was slightly higher in blood and muscle at 30°C, but the differences were not significant.

Based upon studies of vertebrate muscles, increased intracellular buffering appears to be characteristic of muscles engaged in 'burst' activity, i.e. short-term anaerobic activity which results in appreciable lactic acid production (Abe *et al.* 1985; Castellini & Somero, 1981). Crabs generally have fairly low aerobic capability; fatigue is quickly reached, and lactic acid appearance in the blood is

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