SUMMARY

The 14C-DMO/3H-inulin method for pHj was critically assessed in intact Callinectes and found to be reliable provided adequate equilibration time and significant radiolabel excretion were taken into account. An unusually high 'mean whole body pHj' (7.54 at 20°C compared with a pHa of 7.80) was due to a highly alkaline fluid compartment (pHj = 8.23) in the carapace. At 20°C the pHj of the heart was 7.35 and skeletal muscle pHj was 7.30, and there were small but consistent differences in the pHj of different muscle types. The change in pHa with temperature was —0.0151 p.p.m.—1 between 10 and 30°C, slightly less than the slope for the neutral pH of water (ΔpN/ΔT = —0.0175 p.p.m.—1). With data corrected to constant Pico2, this was associated with a change in [HCO3—]a between 10 and 20°C (—0.13 mequiv l—1°C—1, constant Pico2) and a change in Pico2 between 20 and 30°C (+0.13 Torr°C—1, constant [HCO3—]a). The disturbing effect of relatively small Pico2 changes on this pattern was demonstrated. ΔpHj/ΔT slopes for all tissues except carapace were not significantly different from pHa/ΔT but generally lower than ΔpN/ΔT. The slope for the carapace was very flat and greatly influenced the 'mean whole body pHj' slope (—0.0062 p.p.m.—1). In haemolymph in vitro at constant Pico2, 'passive' Δ[HCO3—]/ΔT (—0.17 mequiv l—1°C—1) was comparable to that in vivo between 10 and 20°C, independent of absolute Pico2, and directly related to total protein concentration. Haemolymph non-bicarbonate buffer value (β) was similarly related to protein, but increased with temperature. Crabs subjected to an acute 20→10°C shift showed initial overshoots of pHa and pHj associated with undershoot of Pico2, all of which were corrected over 24 h as [HCO3—]a rose. During this period there was a significant net uptake...
of acidic equivalents (base output) from the environment. The relevance of 'passive' $\Delta [\text{HCO}_3^-]/\Delta T$ in an open system to the observed \textit{in vivo} effects is discussed.

\textbf{INTRODUCTION}

It has been widely reported that temperature-related changes in extracellular pH (pHe) roughly parallel those of the neutral pH of water ($\Delta \text{pH}/\Delta T = \text{approx. } -0.0175 \text{ u}^\circ \text{C}^{-1}$ between 10 and 30°C). The phenomenon has been variously explained as the maintenance of 'constant relative alkalinity' (e.g. Howell, Baumgardner, Bondi & Rahn, 1970) or of constant fractional dissociation ($\alpha$) of imidazole groups ('alphastat'; e.g. Reeves, 1977). Neither theory offers a complete mechanistic explanation. While imidazole is undoubtedly the most concentrated buffer \textit{in vivo}, regulation of the CO$_2$/HCO$_3^-$ system represents the principal physiological control of acid-base status. The temperature dependence of its effective $pK_a$ is very low ($\sim -0.005 \text{ u}^\circ \text{C}^{-1}$) relative to imidazole ($\sim -0.018 \text{ u}^\circ \text{C}^{-1}$), necessitating changes in P$_{CO_2}$, [HCO$_3^-$], or both. In general, air-breathing ectotherms operate a constant CO$_2$ content system such that P$_{CO_2}$ is varied by active regulation of ventilation at more or less stable [HCO$_3^-$] (cf. Reeves, 1977). The very low O$_2$ vs CO$_2$ capacitance of water obviously restricts ventilatory control of P$_{CO_2}$ in aquatic ectotherms; a range of different patterns of P$_{CO_2}$ and [HCO$_3^-$] variation with temperature has been reported (cf. Heisler, 1980; Cameron, 1984\textit{a},\textit{b}). Studies on intracellular acid-base status are relatively few, and no clear temperature pattern has yet emerged. While intracellular pH (pHi) generally falls as temperature rises, $\Delta \text{pHi}/\Delta T$ varies considerably between tissues and species with numerous deviations from the $\Delta \text{pH}/\Delta T$ slope (Malan, Wilson & Reeves, 1976; Heisler, Weitz & Weitz, 1976; Heisler, Neumann & Holeton, 1980; Heisler, 1980; Cameron & Kormanik, 1982; Walsh & Moon, 1982).

The present study examined the influence of temperature on both pHe and pHi in the blue crab, \textit{Callinectes sapidus}. Temperature effects on pHe have been previously studied in aquatic crabs, but conflicting results have emerged (Truchot, 1973, 1978; Howell, Rahn, Goodfellow & Herreid, 1973; Cameron & Batterton, 1978\textit{a}; McMahon \textit{et al.} 1978). There are no previous reports on temperature effects on pHi in any aquatic invertebrate. The present investigation focused on the following points.

(i) The relative importance of extracellular P$_{CO_2}$ and [HCO$_3^-$] changes, with particular attention to possible artifacts induced by inspired P$_{CO_2}$ (P$_{CO_2}$) variation, a parameter which has not been carefully controlled in previous studies.

(ii) The 'passive' physico-chemical characteristics of haemolymph \textit{in vitro} in an open system. The previous studies have described closed system, constant CO$_2$ content characteristics, but these may not be particularly relevant to the \textit{in vivo} situation which more closely resembles an open system in a water breather.

(iii) Critical assessment of the $^{14}$C-DMO/$^3$H-inulin technique for pHi determination (Waddell & Butler, 1959) by examination of marker equilibration, distribution, excretion and possible metabolism. Its only previous use in intact crabs produced peculiarly high values for 'mean whole body pHi' (Cameron, 1981).
Temperature vs ICF and ECF pH in blue crab

(iv) The $\Delta$pHi/$\Delta$T relationships in different tissues, and their influence on the response of the whole body.

(v) The pattern of pHe and pH adjustment following acute temperature change, and the importance of acidic equivalent exchange with the environment in the overall response.

MATERIALS AND METHODS

Experimental animals

Adult intermoult blue crabs, Callinectes sapidus Rathbun (140–450 g), freshly caught in sea water near Port Aransas, Texas, were held, with daily feeding, in running sea water at seasonal temperature (16–24°C). Five to ten days prior to experimentation, the crabs (groups of 6–10 in 600-l tanks) were acclimated to the experimental temperatures of 10, 20 or 30°C, without food. The acclimation sea water was filtered through a charcoal/sand bed and replaced at ~5% h$^{-1}$. Salinity was 24–26%, titration alkalinity was ~2.4 mequiv l$^{-1}$, $P_{O_2}$ > 140 Torr, and $P_{CO_2}$  < 1 Torr. On the day before an experiment, the crabs were fitted with neoprene septa over the pericardial space for arterial haemolymph sampling (Cameron & Batterton, 1978a). They were then allowed to recover overnight in the experimental chambers which were flushed with sea water from the acclimation tanks at ~600 ml min$^{-1}$. These chambers were shielded Plexiglas boxes only slightly larger than the crab itself. A removable port allowed access to the pericardial septum for haemolymph sampling without disturbance. One hour before an experiment, the chamber was connected into a closed recirculating system comprising a pump (output = 600 ml min$^{-1}$), aeration reservoir, and heat exchanger which maintained experimental temperature within ± 1°C and $P_{O_2}$ > 120 Torr. This closed system was necessary to monitor $^{14}$C-DMO and $^3$H-inulin excretion for calculation of 'mean whole body pH'. In the 20°C studies only, the air leaving the aeration reservoir was bubbled through a 50 cm, 500 ml column of 1 mol l$^{-1}$ KOH to trap any $^{14}$C-labelled CO$_2$ which may have resulted from metabolism of $^{14}$C-DMO.

Experimental protocols

Constant temperature studies

Extracellular (i.e. arterial haemolymph) and intracellular acid-base status were examined in crabs acclimated to 10°C ($N = 8$), 20°C ($N = 29$) and 30°C ($N = 14$). At time 0, the volume of the recirculating system was adjusted to a known level (~1.1 litre) and the crab injected with 20 μCi $^3$H-inulin (Amersham, ECF marker) and 4 μCi $^{14}$C-DMO (5,5-dimethyl-2,4-oxazolidinedione; ethyl acetate-free; New England Nuclear; pH marker) in 150 μl of 400 mmol l$^{-1}$ NaCl via the pericardial septum. At 20°C, the temperature at which the behaviour of the markers was examined in detail, simultaneous water, KOH and haemolymph samples (340 μl) were withdrawn at 1, 2, 3, 4, 6, 8 and 12 h. The water and KOH samples (after neutralization with boric acid) were assayed for $^3$H and $^{14}$C radioactivity and the haemolymph samples for radioactivity, pH and total carbon dioxide content ($C_{Ta}$).
At 12 h, an additional 200 μl haemolymph sample and a water sample from the inflow of the experimental chamber were analysed for $P_{\text{ACO}_2}$ and $P_{\text{ICO}_2}$ respectively. To ensure that water ammonia remained below 200 μmol l$^{-1}$, the system was flushed (2 min) immediately after the 4 h and 8 h samples; additional water samples were then taken to keep track of total $^3$H and $^{14}$C losses by the animal. To assess possible differences in acid-base status caused by the closed recirculating system, the experimental chambers were returned to an open flow-through system pumped directly from the acclimation tanks for the period from 12–24 h. At 24 h, all haemolymph measurements (and water $P_{\text{ICO}_2}$) were repeated, a 2 ml terminal haemolymph sample drawn for ionic and protein analyses, and the crab killed for individual tissue pH measurements. On the basis of the 20 °C results, the KOH trap and the 1, 2, 3 and 4 h samples were not employed in the 10 and 30 °C studies; the protocols were otherwise identical.

In order to minimize pH disturbance due to struggling, crabs were killed as rapidly as possible by removal of the legs and dorsal carapace, and excision of the heart. Tissue samples (100–500 mg) were dissected out, thoroughly blotted, and then dried to a constant weight at 80 °C in tared paper cups used for subsequent sample oxidation. The following tissues (number of samples per animal) were analysed: cheliped muscle (6–8); walking leg muscle (3–4); heart (1); carapace (from the pericardial and upper branchial chamber regions, scraped clean of soft tissue; 4–6); and dark (2–4) and light (2–4) ‘backfin’ muscle. The ‘backfin’ constitutes the swimming muscle of the 5th periopod, the colour difference between light and dark regions resulting from greater mitochondrial density in the latter (Tse, Govind & Atwood, 1983). The dissection was carried out in a tared dish lined with absorbent paper; subsequent drying to a constant weight yielded the total body water content.

**Acute temperature change study**

Extracellular and whole body intracellular acid-base status and acidic equivalent flux to the environment were followed in 20 °C-acclimated crabs ($N = 10$) subjected to a rapid shift to 10 °C. The closed recirculating systems were operated at higher volume (~3.0 l) and flushes carried out at 12 h intervals to minimize possible disturbing effects on the measured flux rates. For the same reason, 12 h were allowed to elapse between the injection of the $^3$H-inulin/$^{14}$C-DMO stock (as above) and the start of the 12 h control flux period at 20 °C. Thereafter, water temperature was lowered to 10 °C over 45 min, the start of which represented time 0. Experimental fluxes were then measured over the following 0–2, 2–4, 4–6, 6–11 and 11–24 h intervals. At each time, water samples were assayed for radioactivity, $P_{\text{ICO}_2}$, total ammonia and titration alkalinity, the latter two allowing calculation of net acidic equivalent flux. Haemolymph samples (340 μl) for radioactivity, pH, $C_{T_k}$ and protein analyses were drawn at the start and end of the control period, and at 1, 5, 11 and 24 h of the experimental period. Additional 200 μl aliquots were analysed for $P_{\text{ACO}_2}$ at the first control and 24 h experimental sample times. Finally, 2 ml terminal haemolymph samples were drawn for ionic analyses, and the crabs then killed and dried to a constant weight for total body water content.
In vitro haemolymph studies

The physico-chemical characteristics of haemolymph in an open system *in vitro* were examined using the blood of six crabs acclimated to 20°C. These crabs were selected to span the full range of haemolymph protein concentration observed in the *in vivo* experiments. Approximately 15 ml of venous blood was drawn from the arthrodial membranes of each animal and allowed to clot in a glass tube. After clot disruption and centrifugation at 12,000 g for 10 min, the haemolymph was decanted into a spinning tonometer supplied with humidified gas mixtures from Wösthoff pumps. The haemolymph was serially equilibrated to P<sub>CO₂</sub> = 1.5, 4.5 and 9.0 Torr (balance air) at 10, 20 and 30°C; C<sub>T</sub> and pH were measured in duplicate after 40–60 min. The order of different temperatures was varied without any obvious effect on the results. As total tonometry time was 10–12 h, two initial P<sub>CO₂</sub> values at 20°C were repeated at the end to check for haemolymph deterioration in two runs. Deviations from the initial values of C<sub>T</sub> and pH were within the error of the measurements.

Analytical techniques and calculations

Haemolymph pHi, P<sub>CO₂</sub>, and water P<sub>CO₂</sub> were determined with routine electrode techniques (Radiometer). Particular care was exercised to ensure accurate P<sub>CO₂</sub> electrode response at these very low P<sub>CO₂</sub> levels (cf. Boutilier, Randall, Shelton & Toews, 1978); as a result, precision was approximately ±5%. C<sub>1a</sub> was determined using a Capni-Con II (Cameron Instrument Co.), a conductometric apparatus based on the method of Maffly (1968). Values of haemolymph αCO₂ for the calculation of dissolved CO₂ (S<sub>CO₂</sub>) at the appropriate temperature and salinity were taken from Truchot (1976a); apparent pK<sub>HCO₃⁻</sub> (combining HCO₃⁻ and CO₃<sup>2⁻</sup>), [HCO₃⁻]<sub>a</sub> and, if not measured, P<sub>CO₂</sub>, could then be calculated from the Henderson-Hasselbalch equation. Haemolymph [Na<sup>+</sup>], [K<sup>+</sup>], [Ca<sup>2+</sup>], and [Mg<sup>2+</sup>] were measured with a flame photometer (Radiometer FLM3), [Ca<sup>2+</sup>] and [Mg<sup>2+</sup>] with an atomic absorption spectrophotometer (Perkin-Elmer), [Cl<sup>⁻</sup>] with a chloridometer (Buchler-Cotlove) and inorganic phosphate by phosphomolybdate reduction (Sigma, 1981). A linear calibration curve was constructed relating the total protein content of *Callinectes* haemolymph measured by a micro-Lowry method (Sigma, 1982) to the reading of the same samples on a Goldberg refractometer (American Optical TS meter), which is calibrated for human plasma. The resulting regression relationship:

\[
R = 1.013P + 1.97 \quad (N = 18, \ r = 0.99, \ P < 0.001),
\]

where R is the refractometer reading and P the true protein concentration in g 100 ml<sup>⁻¹</sup>, was routinely used to estimate P by refractometry.

Dried tissue, haemolymph and injection stock samples were combusted with a sample oxidizer (Packard 306), allowing separate assay of ³H and ¹⁴C-radioactivity. Water samples were counted directly in fluor. Dual label quench correction was performed using the external standard ratio and internal standardization (when required). All radioactivities were measured by a liquid scintillation counter.
The trapped extracellular water (ECW) in each tissue sample was calculated as:

\[
\text{ECW (g H}_2\text{O g}^{-1}) = \frac{\text{Tissue [inulin] (d.p.m. g}^{-1})}{\text{Haemolymph [inulin] (d.p.m. g}^{-1} \text{H}_2\text{O})}
\]

and tissue ICW as the difference between total tissue water and ECW. Since the haemolymph protein concentration did not vary significantly with temperature (Table 2), a single value for haemolymph water content (92.26 g H\text{O 100 g}^{-1}; determined by drying aliquots from 10 animals) was used in all calculations. In any event, variations in this parameter within the normal range are a negligible source of error relative to other factors (e.g. sampling, counting errors).

The whole body ECW was determined in two different ways. A ‘mean’ estimate was calculated in the usual manner by extrapolating the linear portion of a plot of ln \(^{3}\text{H}-\text{inulin} \) activity in haemolymph back to time 0 to obtain [inulin]\(_{0}\). Then:

\[
\text{Whole body ECW (g H}_2\text{O kg}^{-1}) = \frac{\text{Inulin injected (d.p.m.)}}{\text{Haemolymph [inulin]}_{0} \text{ (d.p.m. g}^{-1} \text{H}_2\text{O}) \times \text{body weight (kg)}}
\]

Inulin clearance, an estimate of the rate of primary urine formation (Cameron & Batterton, 1978b) could then be calculated from the slope of this line (K):

\[
\text{Inulin clearance (g H}_2\text{O kg}^{-1} \text{ h}^{-1}) = \frac{\text{Whole body ECW (g H}_2\text{O kg}^{-1})}{K(\% \text{ h}^{-1})}.
\]

An ‘instantaneous’ estimate of whole body ECW at each time was calculated as:

\[
\text{Whole body ECW (g H}_2\text{O kg}^{-1}) = \frac{^{3}\text{H}-\text{inulin injected (d.p.m.)} - \Sigma \text{excreted (d.p.m.)} - \Sigma \text{sampled (d.p.m.)}}{\text{Haemolymph [inulin] (d.p.m. g}^{-1} \text{H}_2\text{O}) \times \text{body weight (kg)}}
\]

where \( \Sigma \text{excreted} \) represents measured losses to the water and \( \Sigma \text{sampled} \) refers to measured losses via blood sampling. The whole body ICW was estimated as the difference between total body water and ECW.

Intracellular pH was calculated according to the equation:

\[
\text{pHi} = pK_{\text{DMO}} + \log \left\{ \frac{[\text{DMO}]}{[\text{DMO}]} \left( 1 + 10^{p[H^+] - pK_{\text{ion}}} \right) \right\}.
\]
Temperature vs ICF and ECF pH in blue crab

assuming pHa to be representative of extracellular pH and where [DMO]i and [DMO]e were the 14C-DMO activities (d.p.m. g⁻¹ H₂O) in intracellular and extracellular fluids respectively, and pKDMO at the appropriate temperature was taken from Heisler et al. (1976). For pHi in individual tissues, [DMO]i was calculated as:

$$\text{[DMO]}_i \text{(d.p.m. g}^{-1}\text{H}_2\text{O)} =$$

$$\frac{\text{Tissue [DMO]} \text{(d.p.m. g}^{-1}\text{H}_2\text{O}) - \text{ECW (g} \text{H}_2\text{O g}^{-1}\text{)} \times [\text{DMO}]\text{e (d.p.m. g}^{-1}\text{H}_2\text{O)}}{\text{ICW (g} \text{H}_2\text{O g}^{-1}\text{)}}$$

and for ‘mean whole body pHi’ as:

$$\text{[DMO]}_i \text{(d.p.m. g}^{-1}\text{H}_2\text{O)} =$$

$$\frac{\text{DMO(i) (d.p.m.)} - \text{Σ(e) (d.p.m.)} - \text{Σ(s) (d.p.m.)} - \text{[DMO]}\text{e (d.p.m. g}^{-1}\text{H}_2\text{O}) \times \text{WB ECW (g} \text{H}_2\text{O})}{\text{Whole body ICW (g} \text{H}_2\text{O)}}$$

where (i) = injected, (e) = excreted, (s) = sampled and WB = whole body.

In view of the large number of separate measurements involved in the calculations, the precision of individual tissue pHi determinations is estimated as about ±0·02 units, and of ‘whole body pHi’ values, about ±0·04 units.

The net flux of acidic equivalents to the environment was calculated as the sum of the change in titratable acidity of the water and the ammonia flux, signs considered (cf. Maetz, 1973). This method does not distinguish between ammonia movement in the NH₃ and NH₄⁺ forms, nor between the net excretion of acidic equivalents and the net uptake of basic equivalents, or vice versa (cf. McDonald & Wood, 1981). Fortunately this does not matter in terms of the net acid-base budget of the animal. Water titration alkalinity (for titratable acidity flux) was determined by titration to pH = 4·00 with 0·02 mol l⁻¹ HCl as described by McDonald & Wood (1981); ammonia was measured by the phenol hypochlorite method of Solorzano (1969).

Data have been expressed as means ±1 s.e.m. (N) where N equals the number of animals sampled, and Student’s two-tailed paired and unpaired t-tests were used for comparisons within and between groups respectively. Lines were fitted by standard least squares regression, simple Pearson’s correlation coefficients and standard errors for slopes and intercepts were calculated, and differences in slope were assessed by analysis of covariance. A 5% significance level was employed throughout.

RESULTS

Behaviour of 14C-DMO and 3H-inulin in Callinectes

At 20°C, the initial mixing phases for both compounds lasted 3–4 h; thereafter, plots of log haemolymph radioactivity against time were linear. For inulin this equilibration period was illustrated by the initial rise in the ‘instantaneous’ estimate
Fig. 1

A

\[ \text{\(\delta\)H}_{2}\text{O}\text{kg}^{-1} \]

B

pHa

pHi

C

\% Infused dose lost

\(\text{\(^{3}\text{H}\)-inulin excretion} \)

\(\text{\(^{14}\text{C}\)-DMO excretion} \)

Time post-infusion (h)
Temperature vs ICF and ECF pH in blue crab

of whole body ECW (Fig. 1A), which from 3 h onwards was not significantly different from the 'mean' estimate based on the log extrapolation technique using 6–24 h data. The calculated 'mean whole body pH' rose more gradually to a plateau at 6–12 h (Fig. 1B), reflecting a slightly longer equilibration phase for DMO. Incomplete inulin distribution was of minor importance relative to incomplete DMO distribution in underestimating pH, as shown by comparison of the pH values calculated using the two different ECW estimates (Fig. 1B). Notably, the pHa-pHi gradient was only ~0.25 u (Fig. 1B), about half that seen in most other animals, but very similar to the previous report of Cameron (1981) for two air-breathing crabs. Haemolymph pHa remained stable throughout (Fig. 1B), but both CTa and calculated PaCO₂ (not shown) were slightly elevated at 1–4 h relative to later sample times, presumably in response to the injection and/or sampling. For these reasons, haemolymph samples were taken only from 6 h onwards in subsequent studies at 10 and 30°C. At 10°C, only the 8 h and 12 h data could actually be used since DMO equilibration at this colder temperature was not complete at 6 h.

There was significant excretion of both DMO and inulin in the 12 h study period (Fig. 1C). Efflux rates varied widely amongst animals but were generally correlated for DMO and inulin and more or less linear over time. The mean losses were comparable at 10 and 20°C but significantly higher at 30°C (Table 1). For inulin, measured losses to the water were very similar to those predictable from K, the rate constant of disappearance from the haemolymph (Table 1), confirming that inulin was lost from the ECW by excretion and not by penetration of the ICW. Inulin clearance rates were 2.5-fold higher at 30 than at 10 or 20°C, indicating a much greater rate of primary urine formation (Table 1). Radiolabel removal via haemolymph sampling was an additional, though much smaller, route of DMO and inulin loss, amounting to 10–30% of the excretion loss rates. Failure to account for these two sources of loss would have introduced significant error. For example, at 30°C, 'mean whole body pH' would have been overestimated by 0.23 u and ECW by 18%. Loss of ¹⁴C-DMO by catabolism was evidently insignificant, since no ¹⁴C-radioactivity appeared in the KOH traps.

Extracellular parameters versus acclimation temperature

The haemolymph pHa fell with increasing temperature in Callinectes in the usual manner (Fig. 2A). The overall acid-base vs temperature pattern, however, differed significantly between crabs sampled in the closed recirculating systems and the same crabs sampled 12 h later in the open flow-through systems. These effects were attributable to a significant rise in PtCO₂ with temperature in the recirculating

---

**Fig. 1.** The behaviour of ³H-inulin and ¹⁴C-DMO in Callinectes sapidus at 20°C. (A) Changes in the 'instantaneous' estimate of whole body extracellular fluid volume (see equation 5) with time after injection. The dotted line indicates the 'mean' estimate of ECW calculated by log extrapolation of the 6–24 h data (see equation 3) (see text for details). *Indicates 'instantaneous' values significantly different (P<0.05) from the 'mean' value. (B) Changes in extracellular (arterial) pH (ΔpH) and 'mean whole body intracellular pH' with time after injection. Values of pH, calculated using the 'instantaneous' estimate of ECW at that time (C) and the 'mean' estimate calculated by log extrapolation of the 6–24 h data (○) are shown. *Indicates value significantly different (P<0.05) from the plateau at 6–12 h. (C) The cumulative excretion of ³H-inulin and ¹⁴C-DMO into the external sea water with time after injection.
Table 1. Various indices of radiolabel excretion in Callinectes sapidus at three different acclimation temperatures ($X \pm 1$ S.E.M.)

<table>
<thead>
<tr>
<th>Temperature</th>
<th>10°C (N = 8)</th>
<th>20°C (N = 11)</th>
<th>30°C (N = 14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K^\text{H}(\text{inulin})$ (% h$^{-1}$)</td>
<td>0.844 ± 0.104</td>
<td>0.813 ± 0.070</td>
<td>1.604 ± 0.317</td>
</tr>
<tr>
<td>$^3$H-inulin clearance (g H$_2$O kg$^{-1}$ h$^{-1}$)</td>
<td>1.94 ± 0.23</td>
<td>2.02 ± 0.25</td>
<td>5.02 ± 1.01†</td>
</tr>
<tr>
<td>Measured $^3$H-inulin excretion at 12 h (% infused dose)</td>
<td>9.61 ± 1.86</td>
<td>6.79 ± 0.65</td>
<td>15.54 ± 3.02†</td>
</tr>
<tr>
<td>Measured $^{14}$C-DMO excretion at 12 h (% infused dose)</td>
<td>10.63 ± 2.26</td>
<td>7.10 ± 1.16</td>
<td>16.32 ± 2.44†</td>
</tr>
</tbody>
</table>

*Significantly different ($P < 0.05$) from 10°C value. †Significantly different ($P < 0.05$) from 20°C value.

systems, from 0.74 Torr at 10°C to 1.45 Torr at 30°C, whereas $P_{\text{CO}_2}$ was much more stable in the flow-through systems (0.60 Torr at 10 and 20°C, 0.81 Torr at 30°C) (Fig. 2C). Haemolymph to water gradients ($P_{\text{aCO}_2}$-$P_{\text{wCO}_2}$) were identical at comparable temperatures in the two systems, so $P_{\text{aCO}_2}$ and $C_{\text{a}}$ were consistently higher under recirculating conditions (Fig. 2B,C). Relative to the flow-through situation, these crabs appeared to be in a state of slight respiratory acidosis, compensated at 30°C but not at 20°C (Fig. 2A). Thus, at the low $P_{\text{CO}_2}$ levels characteristic of water breathers, relatively small temperature-related changes in $P_{\text{CO}_2}$ can have marked effects on acid-base status which could easily be confused with direct temperature effects.

The haemolymph acid-base data for the flow-through situation are shown as the standard components of the Henderson-Hasselbalch equation in Fig. 3. The 30°C data at $P_{\text{aCO}_2} = 0.81$ Torr have been slightly adjusted to the same $P_{\text{aCO}_2}$ as the 10 and 20°C data (0.60 Torr), assuming an unchanged $P_{\text{aCO}_2}$-$P_{\text{wCO}_2}$ gradient and unchanged $p_{\text{Ha}}$. (Thus at 30°C, $P_{\text{aCO}_2}$ was adjusted from 2.75 to 2.54 Torr, and $[\text{HCO}_3^-]_{\text{a}}$ from 4.10 to 3.79 mequiv l$^{-1}$.) The overall $\Delta p_{\text{Ha}}/\Delta T$ was $-0.015$ l°C$^{-1}$. Between 10 and 20°C, haemolymph $[\text{HCO}_3^-]$ fell significantly ($\Delta[\text{HCO}_3^-]/\Delta T = -0.13$ mequiv l$^{-1}$°C$^{-1}$) but did not change at 30°C (Fig. 3B), whereas $P_{\text{aCO}_2}$ was identical at 10 and 20°C, but doubled at 30°C ($\Delta P_{\text{aCO}_2}/\Delta T = +0.13$ Torr°C$^{-1}$; Fig. 3C). The factorial analysis of Fig. 3A illustrates that between 10 and 20°C, $\Delta p_{\text{Ha}}/\Delta T$ could be almost entirely attributed to a $[\text{HCO}_3^-]_{\text{a}}$ change, and between 20 and 30°C to a $P_{\text{CO}_2}$ change, and thus to an increase in the $P_{\text{CO}_2}$ gradient across the gills. *Callinectes* haemolymph in vivo

---

Fig. 2. The extracellular (arterial) acid-base status of *Callinectes sapidus* acclimated to 10°C (N = 8), 20°C (N = 7–11) and 30°C (N = 14). Means ± 1 s.e.m. Data taken in the recirculating system (open symbols, dotted lines) and from the same crabs 12 h later in the flow-through system (closed symbols, solid lines) are shown separately. *Indicates values significantly different ($P < 0.05$) between the two measurement conditions at the same temperature. (A) pH. (B) Total carbon dioxide content. (C) Extracellular $P_{\text{CO}_2}$ (circles) and inspired $P_{\text{CO}_2}$ (triangles).
Temperature vs ICF and ECF pH in blue crab

Fig. 2

A

Temperature (°C)

pHa

B

Temperature (°C)

C

Temperature (°C)

PaCO₂

Pco₂

Na⁺

Fig. 3

8-0

7-8

7-6

6

5

4

3

2

1

0

10

20

30
Table 2. Trapped extracellular water, expressed as a percentage of total tissue water content, in different tissues, and haemolymph protein concentration of Callinectes sapidus at three different acclimation temperatures (X ± 1 S.E.M.)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>10°C</th>
<th>20°C</th>
<th>30°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cheliped muscle</td>
<td>13.30 ± 0.98</td>
<td>14.14 ± 0.90</td>
<td>16.31 ± 1.10*</td>
</tr>
<tr>
<td>Walking leg muscle</td>
<td>12.52 ± 1.12</td>
<td>11.69 ± 0.74</td>
<td>13.76 ± 0.60</td>
</tr>
<tr>
<td>Dark backfin muscle</td>
<td>13.36 ± 0.99</td>
<td>12.93 ± 0.62</td>
<td>13.02 ± 0.88†</td>
</tr>
<tr>
<td>Light backfin muscle</td>
<td>11.09 ± 0.62</td>
<td>11.79 ± 0.37</td>
<td>12.83 ± 0.56</td>
</tr>
<tr>
<td>Heart</td>
<td>20.22 ± 1.40</td>
<td>24.72 ± 1.63</td>
<td>23.31 ± 1.20*</td>
</tr>
<tr>
<td>Carapace</td>
<td>12.53 ± 1.93</td>
<td>7.86 ± 0.49*</td>
<td>12.36 ± 0.92†</td>
</tr>
<tr>
<td>Haemolymph protein (g 100 ml⁻¹)</td>
<td>6.31 ± 0.49</td>
<td>5.35 ± 0.61</td>
<td>5.76 ± 0.28</td>
</tr>
</tbody>
</table>

* Significantly different (P<0.05) from 10°C value.
† Significantly different (P<0.05) from 20°C value.

appeared to operate as an open, constant P_C0₂ system between 10 and 20°C, and as a closed, constant C_T system between 20 and 30°C in temperature-acclimated animals. As expected, the apparent CO₂/HCO₃⁻ pKᵢ', determined experimentally, changed with a relatively low slope (−0.004 u°C⁻¹; Fig. 3D) and therefore made only a small contribution to the pHₐ vs temperature relationship (Fig. 3A).

Whole body ECW was similar at 10°C [232.1 ± 12.1 (8) g H₂O kg⁻¹] and 20°C [245.3 ± 12.1 (11)] amounting to 35% of total body water, but increased significantly by one-third at 30°C [320.1 ± 16.9 (14)]. This increase occurred entirely at the expense of the ICW, because total body water remained the same at the three temperatures (670–688 ml H₂O kg⁻¹). There was also a significant increase in trapped ECW at 30°C, in cheliped and dark backfin muscles, heart and carapace (Table 2). Total haemolymph protein concentration (mainly haemocyanin) did not vary significantly with temperature (Table 2), suggesting that synthesis of new haemocyanin may have accompanied the rise in ECW at 30°C. Haemolymph [Na⁺] and [Cl⁻] both fell by ~30 meq l⁻¹ between 10 and 20°C with no further change at 30°C (Table 3). Concentrations of Ca²⁺ and Mg²⁺ fell by a greater relative proportion between 10 and 20°C, and then rose again at 30°C (significant only for Ca²⁺). K⁺, present only at very low levels, was significantly greater at 20 and 30 than at 10°C. Interestingly, similar electrolyte levels to those in the 10°C-acclimated crabs were seen in animals only 24 h after acute transfer from 20 to 10°C in the temperature change experiment (Table 3). The strong ion difference (Stewart, Fig. 3. An analysis of the factors contributing to differences in extracellular (arterial) pH in Callinectes sapidus acclimated to 10 (X = 8), 20 (X = 7–11) and 30°C (X = 14) in terms of the components of the Henderson-Hasselbalch equation. The data represent measurements from crabs in the flow-through system at constant Pi<sub>H₂</sub>CO₃ = 50 Torr (see text for details). Means ± 1 S.E.M. * Indicates values significantly different (P<0.05) from 10°C value; † from 20°C value. (A) pH. The pH values at 10 and 30°C are also shown as predicted by the Henderson-Hasselbalch equation using the mean values for all components measured at that temperature, except for the component noted, where the 20°C value is used. (B) Bicarbonate concentration. (C) P<sub>H₂</sub>CO₃. (D) Apparent pK<sub>ᵢ</sub>' of the HCO₃⁻/CO₂ system, determined experimentally. (E) Dissolved carbon dioxide concentration.
Temperature vs ICF and ECF pH in blue crab

Fig. 3
Table 3. Inorganic electrolyte levels (mequiv l⁻¹) in extracellular fluid of Callinectes sapidus at three different acclimation temperatures and 24 h after an acute change from 20 to 10°C (X ± 1 S.E.M.)

<table>
<thead>
<tr>
<th></th>
<th>10°C  (N = 8)</th>
<th>20°C → 10°C (N = 10)</th>
<th>20°C  (N = 6)</th>
<th>30°C  (N = 14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺</td>
<td>424 ± 4</td>
<td>401 ± 5†</td>
<td>391 ± 4*</td>
<td>393 ± 6*</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>444 ± 4</td>
<td>434 ± 8†</td>
<td>411 ± 3*</td>
<td>416 ± 10*</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>22.8 ± 0.6</td>
<td>22.6 ± 0.6†</td>
<td>17.2 ± 1.7*</td>
<td>21.4 ± 0.2†</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>24.7 ± 0.8</td>
<td>26.8 ± 0.7†</td>
<td>21.1 ± 2.3</td>
<td>24.3 ± 0.9</td>
</tr>
<tr>
<td>K⁺</td>
<td>7.9 ± 0.2</td>
<td>7.3 ± 0.3†</td>
<td>8.5 ± 0.3*</td>
<td>8.9 ± 0.2‡</td>
</tr>
</tbody>
</table>

*Significantly different (P<0.05) from 10°C value.
†Significantly different (P<0.05) from 20°C value.

1978), here defined as [Na⁺+Ca²⁺+Mg²⁺+K⁺−Cl⁻] in mequiv l⁻¹, was ~30 mequiv l⁻¹ (or about seven-fold [HCO₃⁻]a; Fig. 3A) and did not vary significantly with temperature. These electrolyte patterns seen during short-term laboratory acclimation were generally very similar to those occurring in Callinectes during long-term seasonal temperature acclimation in the wild (Lynch, Webb & Van Engel, 1973; Colvocoresses, Lynch & Webb, 1974).

Intracellular parameters versus acclimation temperature

The remarkably high ‘mean whole body pHi’, and resultant small pHa-pHi gradient, was explained (see Discussion) by the discovery of a large, highly alkaline fluid compartment in the carapace (Fig. 4). Whether or not this is a true intracellular compartment is uncertain, but it was not penetrated by inulin, and with a pHi 0.3–0.4 u above pHa, served as an important sink for DMO. In one immediately pre-moult crab at 30°C, pHi in the soft underlying new carapace was 7.55 (relative to 8.33 in the old hard carapace) pointing to a role for mineralization in the origin of this high pHi (or vice versa; Cameron & Wood, 1985).

Table 4. Regression parameters in the format pH = mT (°C) + b for haemolymph and various intracellular compartments in temperature-acclimated Callinectes sapidus over the range 10–30°C (X ± 1 S.E.M.)

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>m</th>
<th>b</th>
<th>r</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemolymph</td>
<td>33</td>
<td>-0.0151 ± 0.0012</td>
<td>8.162 ± 0.009</td>
<td>0.92</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Heart</td>
<td>32</td>
<td>-0.0163 ± 0.0022</td>
<td>7.629 ± 0.019</td>
<td>0.78</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Skeletal muscles</td>
<td>33</td>
<td>-0.0132 ± 0.0015†</td>
<td>7.562 ± 0.009</td>
<td>0.89</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>cheliped</td>
<td>33</td>
<td>-0.0141 ± 0.0012†</td>
<td>7.608 ± 0.010</td>
<td>0.90</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>walking leg</td>
<td>28</td>
<td>-0.0125 ± 0.0016†</td>
<td>7.527 ± 0.013</td>
<td>0.84</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>light backfin</td>
<td>33</td>
<td>-0.0124 ± 0.0012†</td>
<td>7.489 ± 0.009</td>
<td>0.88</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>dark backfin</td>
<td>28</td>
<td>-0.0121 ± 0.0014†</td>
<td>7.553 ± 0.012</td>
<td>0.87</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Carapace</td>
<td>39</td>
<td>-0.0083 ± 0.0032‡</td>
<td>8.388 ± 0.024</td>
<td>0.39</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Mean whole body</td>
<td>28</td>
<td>-0.0062 ± 0.0024‡‡</td>
<td>7.645 ± 0.019</td>
<td>0.43</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

*Significantly different (P<0.05) from ΔpHa/ΔT.
†Significantly different (P<0.05) from haemolymph ΔpHa/ΔT.
Heart and skeletal muscle tissues had a more usual pHa-pHi gradient ≈ 0.5–0.6 u (Fig. 4). Nevertheless, there was significant heterogeneity within skeletal muscle. At all temperatures, pHi was consistently lower in light than in dark backfin or cheliped, while walking leg was intermediate (Fig. 5). As the colour difference between light and dark backfin results from greater mitochondrial density in the latter (Tse et al. 1983), and mitochondrial pH is generally greater than cytosol pH
Intracellular pH fell significantly with increasing temperature in all compartments (Figs 4, 5; Table 4). There was a general tendency for a greater slope between 20 and 30°C than between 10 and 20°C, but this was significant only for heart (−0.0222 ± 0.0051 vs −0.0093 ± 0.0050 u °C⁻¹) and walking leg (−0.0167 ± 0.0033 vs −0.0076 ± 0.0036 u °C⁻¹). Over the whole range of 10–30°C, the slopes for all tissues except carapace were not significantly different from those for haemolymph (Roos & Boron, 1981), these inter-muscle pH differences may reflect relative mitochondrial volume.
Temperature vs ICF and ECF pH in blue crab

(\(\Delta \text{pHi}/\Delta T = -0.0151 \text{ u}^\circ \text{C}^{-1}\)). However \(\Delta \text{pHi}/\Delta T\) values for all skeletal muscle tissues were significantly less than for constant relative alkalinity (i.e. \(\Delta \text{pN}/\Delta T = -0.0175 \text{ u}^\circ \text{C}^{-1}\)). The slope for carapace was extremely flat, and this was clearly reflected in the slope for ‘mean whole body pH’ (Fig. 4; Table 4); both were significantly less than either \(\Delta \text{pHa}/\Delta T\) or \(\Delta \text{pN}/\Delta T\).

Temperature responses of haemolymph in vitro

When haemolymph was equilibrated at constant \(\text{P}_{\text{CO}_2}\) in vitro (i.e. open system conditions), \([\text{HCO}_3^-]\) fell as temperature rose (Fig. 6B). Although the absolute \([\text{HCO}_3^-]\) increased with greater \(\text{P}_{\text{CO}_2}\), \(\Delta [\text{HCO}_3^-]/\Delta T\) was independent of \(\text{P}_{\text{CO}_2}\) but a direct function of haemolymph protein concentration (Fig. 7A). This is interpreted as a direct consequence of rising temperature increasing the dissociation of \(\text{H}^+\) ions from the haemocyanin, resulting in the removal of \(\text{HCO}_3^-\) from the system as gaseous \(\text{CO}_2\). As such it is considered a ‘passive’, physico-chemical effect – i.e. not involving ‘active’ regulation of \(\text{P}_{\text{CO}_2}\) and/or acidic equivalent exchange by the animal. The wide range of haemolymph protein levels, selected to span the normal range of occurrence, explained most of the variability in the averaged data of Fig. 6. At the mean protein concentration observed in vitro (Table 2), this ‘passive’ \(\Delta [\text{HCO}_3^-]/\Delta T\) would have been about \(-0.17\) mequiv \(1^\circ \text{C}^{-1}\) (Fig. 7A), slightly greater than the in vivo slope of \(-0.13\) mequiv \(1^\circ \text{C}^{-1}\) at constant \(\text{P}_{\text{CO}_2}\) between 10 and 20°C (Fig. 3B,C). The change in haemolymph pH with temperature in vitro associated with this constant \(\Delta [\text{HCO}_3^-]/\Delta T\) was proportionately greater at lower \(\text{P}_{\text{CO}_2}\) values (Fig. 6A), as could also be predicted from the Henderson-Hasselbalch equation. However, even at the lowest \(\text{P}_{\text{CO}_2}\) tested (1.5 Torr), \(\Delta \text{pH}/\Delta T\) \((-0.0071 \text{ u}^\circ \text{C}^{-1}\); Fig. 6A) was less than half that observed in vivo \((-0.0151 \text{ u}^\circ \text{C}^{-1}\); Fig. 3A) between 10 and 20°C where \(\text{P}_{\text{CO}_2}\) was similarly constant. This difference was explained partly by the lower \(\text{P}_{\text{CO}_2}\) in vivo (1.2 Torr), since small changes in \(\text{P}_{\text{CO}_2}\) have large effects on pH in this range, and partly by the greater variability in vitro.

The non-bicarbonate buffer value (\(\beta = -\Delta [\text{HCO}_3^-]/\Delta \text{pH}\)) was calculated from the linear change in \([\text{HCO}_3^-]\) with pH at the three equilibration levels of \(\text{P}_{\text{CO}_2}\). At all three temperatures, \(\beta\) was a linear function of total haemolymph protein concentration (Fig. 7B), with intercepts not significantly different from zero. This is not surprising, as the only other likely non-bicarbonate buffer, inorganic phosphate, was present in negligible quantities \([0.18 \pm 0.03 (5) \text{ mmol}^1^\text{m}^{-3}\)]. The value of \(\beta\) clearly increased with temperature, the regression slope rising significantly from 1.819 \pm 0.121 at 10°C to 2.637 \pm 0.201 slykes 100 ml\(^{-1}\) g\(^{-1}\) protein at 30°C. The mean values of \(\beta\) in vivo based on these relationships and the haemolymph protein levels of Table 2 were \(~11.5\) slykes at 10 and 20°C and \(~15.4\) slykes at 30°C.

Responses to an acute change in temperature

In acclimated animals, the acid-base change between 20 and 10°C in vitro was entirely a \([\text{HCO}_3^-]\)a rather than a \(\text{P}_{\text{CO}_2}\) adjustment (Fig. 3); the ‘passive’ generation of basic equivalents by haemolymph at constant \(\text{P}_{\text{CO}_2}\) in vitro was large enough to explain this \(\Delta [\text{HCO}_3^-]\)a/\(\Delta T\), and the ECW did not change. The response to a step change from
20 down to 10°C was therefore followed in the whole animal to identify the time course of adjustment and to assess the magnitude of any acidic equivalent exchange with the environment which might occur.

An immediate drop in $P_{\text{aCO}_2}$ was the most prominent extracellular effect of the rapid decrease from 20 to 10°C (Fig. 8C), very different from the situation seen in fully acclimated animals (Figs 2, 3). While this was partially due to a fall in $P_{\text{tCO}_2}$ in the recirculating system, the more important component was a 50% decrease in the

![Graph](image-url)

Fig. 6. Changes in (A) pH and (B) bicarbonate concentrations in hemolymph samples of *Callinectes sapidus* equilibrated in vitro at 10, 20 and 30°C in an open system to $P_{\text{CO}_2} = 1.5$ (N = 6), 4.5 (N = 4) and 9.0 Torr (N = 6), balance air.
Temperature vs ICF and ECF pH in blue crab

Fig. 7. Relationships between physico-chemical characteristics and total protein concentration in haemolymph samples of Callinectes sapidus equilibrated in vitro. (A) Passive change in bicarbonate concentration per degree Centigrade in an open system at $P_{CO_2} = 1.5$ ( ), $4.5$ ( ) and $9.0$ ( ) Torr, balance air. The fitted regression line is: $y = -0.03039x - 0.00095 (r = -0.92, P < 0.001, N = 16)$. (B) Non-bicarbonate buffer value ($\beta$) at $10$ ( ), $20$ ( ) and $30^\circ$C ( ). The fitted regression lines are: $10^\circ$C, $y = 1.819x + 0.066 (r = 0.99, P < 0.01, N = 6)$; $20^\circ$C, $y = 2.086x + 0.385 (r = 0.99, P < 0.01, N = 6)$; $30^\circ$C, $y = 2.637x + 0.228 (r = 0.99, P < 0.01, N = 6)$.

$P_{CO_2} - P_{CO_2}$ gradient across the gills (Fig. 8C). Initially, $C_{Ta}$ rose only slightly and non-significantly (Fig. 8B), so the intact animal in some ways initially behaved in a manner similar to a classic in vitro closed system. However the situation was complicated by the fall in $P_{1CO_2}$, which contributed to the decrease in $P_{CO_2}$, and
thereby masked an effective \( [\text{HCO}_3^-]_a \) adjustment. Had the change in \( P_{\text{CO}_2}-P_{\text{acO}_2} \) gradient alone occurred (i.e. absence of \( P_{\text{CO}_2} \) change), together with the observed pHa change, \( [\text{HCO}_3^-]_a \) would have risen with a slope of \(-0.15\text{ mequiv l}^{-1}\text{°C}^{-1}\) between 20 and 10°C over the first hour. The net effect of this combined \( P_{\text{acO}_2} \) and \( [\text{HCO}_3^-]_a \) adjustment was a large initial overshoot in pHa, which rose with a slope of \(-0.0218\text{ u°C}^{-1}\) during the first hour (Fig. 8A). Thereafter pHa gradually fell, accompanied by a slow rise in both \( C_{\text{tr}} \) and \( P_{\text{acO}_2} \), the latter reflecting an increase in the \( P_{\text{CO}_2}-P_{\text{acO}_2} \) gradient. By 24 h, this \( P_{\text{CO}_2} \) gradient had returned to the 20°C value, \( C_{\text{tr}} \) had risen significantly, and pHa had reached a level which gave a 20 to 10°C slope of \(-0.0104\text{ u°C}^{-1}\). Adjusting the data to constant \( P_{\text{CO}_2} \) as before, the effective \( [\text{HCO}_3^-]_a/\Delta T \) value between 20 and 10°C after 24 h was \(-0.18\text{ mequiv l}^{-1}\text{°C}^{-1}\). Thus after 24 h, the extracellular in vivo situation was very similar to that predicted by the immediate ‘passive’ response of haemolymph in an open system in vitro to this same temperature change. Throughout the experiment, there was no significant change in haemolymph protein concentration, which averaged \( 4.79 \pm 0.50 \text{ (10) g} 100\text{ ml}^{-1} \) in this group.

‘Mean whole body pHi’ followed a very similar pattern to pHe, with an initial overshoot followed by later decline (Fig. 8A), though the absolute changes were smaller. Over the first hour, \( \Delta pHi/\Delta T \) was \(-0.0164\text{ u°C}^{-1}\), but this decreased to \(-0.0077\text{ u°C}^{-1}\) by 24 h, close to the value observed in temperature-acclimated crabs (Table 4). Assuming \( P_{\text{CO}_2} \) equilibration across the extracellular/intracellular interface, this pattern can be explained by the same factors as in the ECW – i.e. an initial, and later corrected, undershoot of \( P_{\text{CO}_2} \) against a background of \( [\text{HCO}_3^-]_i \) accumulation. At constant \( P_{\text{CO}_2} \), the effective \( [\text{HCO}_3^-]_i/\Delta T \) between 20 and 10°C after 24 h was \(-0.12\text{ mequiv l}^{-1}\text{°C}^{-1}\).

At 20°C, the titratable flux was just balanced by the ammonia flux, so the net acidic equivalent flux between the crab and the external sea water was not significantly different from zero (Fig. 9B). Two hours after the temperature shift from 20 to 10°C, this situation changed dramatically. A significant net uptake of acidic equivalents (or excretion of basic equivalents) from the environment began (Fig. 9B), accompanied by a 90% decrease in ammonia output (Fig. 9A). By 24 h, the ammonia output had recovered only slightly. The acidic equivalent flux, while still significantly elevated, had dropped to about half the peak level of \(+200\mu\text{equiv kg}^{-1}\text{h}^{-1}\). The net uptake of acidic equivalents over the 24 h period following the temperature change amounted to \( 2240\mu\text{equiv kg}^{-1} \) relative to the control rate at 20°C.

**DISCUSSION**

**Methodology for pHi measurements**

The \(^{14}\text{C}-\text{DMO}/\text{H-inulin} \) technique worked satisfactorily in *Callinectes*, providing certain precautions were observed. The first was adequate marker equilibration time, 4–6 h at 20°C (Fig. 1) and up to 8 h at 10°C, comparable or slightly longer than those for teleost fish (Cameron & Kormanik, 1982; Walsh & Moon, 1982; Höbe, Wood & Wheatly, 1984) and crayfish (Gaillard & Malan, 1983). Most of this delay probably reflected convective mixing in the ECW, since, at least
in fish tissues, DMO equilibration time across the extracellular/intracellular boundary is rapid (<15 min; Walsh & Moon, 1983; C. L. Milligan & C. M. Wood, unpublished data). An additional problem in crustaceans may be slow marker penetration of the carapace, the pHi of which so markedly influenced 'mean whole body pHi' (see below). Nevertheless, the plateaux reached in the whole body pHi and ECW curves by 12 h (Fig. 1A,B) clearly indicated that marker distribution in

Fig. 8. The influence of an acute change in temperature from 20 down to 10°C on extracellular (arterial) and intracellular acid-base status in Callinectes sapidus acclimated to 20°C (N=10). Means ± 1 S.E.M. *Indicates value significantly different (P<0.05) from mean of two control 20°C values. (A) Extracellular and 'mean whole body' intracellular pH. (B) Extracellular total carbon dioxide content. (C) Extracellular PCO₂ (•, measured values; ○, calculated values) and inspired PCO₂ (measured values).
the carapace was at equilibrium by the time (24 h) this tissue was sampled. A second important precaution was correcting for the significant excretion of $^{14}$C-DMO and $^{3}$H-inulin which occurred before equilibration; the neglect of this could result in considerable overestimation of 'mean whole body pH' and ECW. The measured DMO excretion rates in Callinectes were 1.2- to 2.8-fold greater than in trout (Höbe et al. 1984). The route of inulin loss is exclusively via the antennal gland (Cameron & Batterton, 1978b; Wheatly, 1984) and the close correlation of DMO and inulin loss rates in individual crabs suggests that this is also true for DMO.

Compared with microelectrodes, DMO yields slightly lower but consistent values of pH in isolated crustacean muscles (Boron & Roos, 1976; Rodeau, 1982). Mean pH values in several soft tissues of intact Callinectes (heart, cheliped muscle; interpolated to 13°C; Fig. 4; Table 4) in the present study were very similar to those
Temperature vs ICF and ECF pH in blue crab

obtained by Gaillard & Malan (1983) in the crayfish Astacus at 13°C using the same methodology. The pHa-pHi gradient of 0.5–0.6 u for skeletal muscle in Callinectes was in the normal range, but that for ‘mean whole body’ was only about half as large as normally found in vertebrates (Roos & Boron, 1981). Cameron (1981) reported an identical situation in two air-breathing crabs and suspected either a large heterogeneity of the overall ICW or a systematic error. The current results show that the former is the correct explanation, because there is a large, highly alkaline (pHi = 8.1–8.3) fluid compartment in the carapace markedly influencing the whole body pH value (Fig. 4; Table 4).

Carapace pHi

Waddell & Bates (1969) have noted that a subcompartment with a high pHi may bias the estimate of ‘mean whole body pHi’ with the DMO method because the distribution of DMO is a logarithmic, rather than a linear, function of pH. A rough estimate of the extent of such bias may be made for the 20°C data using the total weight and water content of Callinectes carapace reported by Cameron & Wood (1985), the trapped ECW corrections of Table 2, the whole body ICW, and assuming that the mean pHi of the whole carapace was equal to that of the carapace areas sampled and that the mean pHi of all soft tissues was equal to that of skeletal muscle (Fig. 4). Given these conditions, the true ‘mean whole body pHi’ should have been 7.51, whereas the estimate based on equation (8) was 7.54, indicating that the upward bias, while present, was not particularly serious.

It is uncertain whether the carapace pHi values were representative of true intracellular fluid or simply of a bounded fluid space not penetrated by inulin. True cellular pHi values in this range (8.1–8.3; Fig. 4; Table 4) are unusual but not unprecedented (Roos & Boron, 1981). The cuticular epidermis certainly contains some cells (Dennel, 1960), but it is unlikely that all the carapace water (~90%, Table 2; equal to about 91 ml kg⁻¹ body weight, Cameron & Wood, 1985) lying outside the trapped ECW is contained within cells, as assumed in the pHi calculation. Whatever the exact nature of this pool, it is probably in dynamic equilibrium with the massive CaCO₃ stores which occur in the carapace (Cameron & Wood, 1985), and either or both may be an important source of mobilizable base for extracellular buffering. Calculated ‘intracellular’ [HCO₃⁻] in the carapace fluid space was ~12 mequiv l⁻¹ at 20°C in comparison to ~4 mequiv l⁻¹ in the haemolymph and ~1 mequiv l⁻¹ in skeletal muscle. The measured CO₂ (mainly CO₃²⁻) store in the whole carapace was 2.09×10⁶ μequiv kg⁻¹ body weight (1.04×10⁸ μmol l⁻¹; Cameron & Wood, 1985) or over 1000 times the HCO₃⁻ content of the rest of the body. There is now considerable evidence that mobilization of carapace buffer base can occur during acidosis in crabs (Defur, Wilkes & McMahon, 1980; Henry, Kormanik, Smatresk & Cameron, 1981; Wood & Randall, 1981); this whole area clearly deserves further investigation.

Extracellular parameters versus acclimation temperature

In the present study the value of ΔpHa/ΔT for acclimated Callinectes was -0.0151 u°C⁻¹, close to that for constant relative alkalinity (= -0.0175 u°C⁻¹).
This may be compared with previous values for *Callinectes* of −0.0120 (Cameron & Batterton, 1978a) and −0.0260 (Howell *et al.* 1973), for *Carcinus* of −0.0142 (Truchot, 1978), −0.0162 (Truchot, 1973) and −0.0190 (Howell *et al.* 1973), and −0.0180 umol°C−1 for *Cancer* (McMahon *et al.* 1978). Haemolymph [HCO₃⁻] fell with rising temperature in all studies but with a slope varying widely from −0.085 mequiv°C−1 (Truchot, 1973) to −1.29 mequiv°C−1 (Howell *et al.* 1973). P<sub>CO₂</sub> either rose a lot (Truchot, 1973, 1978), a little (Cameron & Batterton, 1978a), remained the same (McMahon *et al.* 1978) or fell (Howell *et al.* 1973). In none of these previous studies was P<sub>CO₂</sub> closely controlled or measured. In our experience, despite vigorous aeration, P<sub>CO₂</sub> tends to increase with temperature in recirculating systems (cf. Fig. 2). Because of the very low P<sub>CO₂</sub> levels and gradients across the gills in crabs, minor changes in P<sub>CO₂</sub> can have very large effects on pHa, which may or may not be compensated. For example, using the 20°C data of Fig. 3, an increase in P<sub>CO₂</sub> from 0-60 to 1-20 Torr would have decreased pHa from 7-85 to 7-68; after metabolic compensation, [HCO₃⁻]ₐ would have risen from 3-99 to 5-92 mequiv l⁻¹. These effects are larger than measured differences at temperatures 10°C apart when P<sub>CO₂</sub> was stable (Fig. 3). The comparison of recirculating and flow-through system data in Fig. 2 emphasizes the possible errors involved.

At constant P<sub>CO₂</sub>, the haemolymph acid-base adjustment in acclimated *Callinectes* was almost entirely the result of a [HCO₃⁻]ₐ change between 10 and 20°C and a P<sub>CO₂</sub> change between 20 and 30°C. This pattern has previously only been observed in two amphibious crabs (*Cardisoma* and *Coenobita*; McMahon & Burggren, 1981), where it was attributed to a switch from an aquatic (i.e. HCO₃⁻ exchange) to an aerial mode of regulation (i.e. ventilatory P<sub>CO₂</sub> control) at higher temperature, an explanation which cannot apply to *Callinectes*. Rather, it appears that between 10 and 20°C, the [HCO₃⁻]ₐ change could be explained by the ‘passive’ effect of temperature on H<sup>+</sup> dissociation from haemocyanin in an open system (see below), and between 20 and 30°C, the P<sub>CO₂</sub> change could be explained by classic closed system behaviour of the haemolymph (Reeves, 1977). It is not clear why the latter should occur in a water breather. Relative ventilation (as expressed by the ventilatory convection requirement for O<sub>2</sub>) is unaffected by temperature in *Carcinus* (Truchot, 1978) and *Callinectes* (Cameron & Batterton, 1978a). However, as Truchot (1978) points out, P<sub>CO₂</sub> may depend on (expired) P<sub>ECO₂</sub>, which in turn varies inversely with α<sub>CO₂</sub>, the latter decreasing with temperature. Thus the fall in P<sub>ECO₂</sub> at high temperature observed by Cameron & Batterton (1978a) in *Callinectes* was probably associated with a rise in P<sub>ECO₂</sub>.

**Intracellular parameters versus acclimation temperature**

In *Callinectes*, as in most other poikilotherms which have been examined (see Introduction), pH<sub>i</sub> in individual tissues fell with increasing acclimation temperature (Figs 4, 5; Table 4). For most tissues, ΔpH<sub>i</sub>/ΔT was less than ΔpN/ΔT but not significantly different from ΔpHa/ΔT, and there was some evidence of changing slope over the full 10–30°C range. A general fall in pH with temperature is to be expected in virtually any buffer system. In intact crayfish, Gaillard & Malan (1983) have demonstrated considerable capacity within individual tissues to regulate pH<sub>i</sub>.
Temperature vs ICF and ECF pH in blue crab

independently of pHe, presumably by transmembrane acidic equivalent flux. Without a detailed knowledge of the extent of such fluxes, metabolic adjustments, \( P_{\text{CO}_2} \) changes and the relative proportions of protein (imidazole), phosphate and bicarbonate buffers in the cytosol, no definite conclusions can be drawn. However, in the carapace, where buffering by the bicarbonate system undoubtedly predominated, the very flat temperature slope was probably a direct consequence of the low temperature sensitivity of this buffer system. The marked influence of carapace pH on 'mean whole body pH' contributed greatly to the low \( \Delta \text{pHi}/\Delta T \) of the latter.

The \( \text{HCO}_3^- \) pools within acclimated crabs at 10, 20 and 30°C (Table 5) were estimated by assuming that pH was internally uniform and that \( P_{\text{CO}_2} \) was equilibrated across the extracellular/intracellular interface, and by using intracellular \( \text{pK}' \) values taken from pH vs \( \text{pK}' \) regressions fitted to the in vitro haemolymph data. The analysis indicated that over half the \( \text{HCO}_3^- \) pool lay in the extracellular compartment at each temperature, and that the total pool varied by less than 30% at the three temperatures. The rise in the total pool between 20 and 30°C did not indicate that the pattern of \( [\text{HCO}_3^-] \) regulation in the intracellular compartment was different from that of the extracellular compartment, but rather that the volume of the ECW relative to the ICW had increased.

**Temperature responses of haemolymph in vitro**

The fall in \( [\text{HCO}_3^-] \) with rising temperature from 10 to 20°C in acclimated crabs *in vivo* need not reflect active mechanisms of \( \text{HCO}_3^- \) regulation. These changes could result simply from the 'passive' effect of temperature on \( H^+ \) dissociation from haemocyanin, presumably from the histidine residues. These released \( H^+ \) ions would combine with \( \text{HCO}_3^- \) and the resulting \( \text{CO}_2 \) would leave in an open, constant \( P_{\text{CO}_2} \) system. In accordance with this interpretation, \( \Delta [\text{HCO}_3^-]/\Delta T \) in the open system was independent of \( P_{\text{CO}_2} \) (Figs 6B, 7A) but directly related to haemolymph protein concentration (Fig. 7A). This 'passive' \( \Delta [\text{HCO}_3^-]/\Delta T \) of \(-0.17 \text{ mequiv} \cdot \text{kg}^{-1} \cdot \text{°C}^{-1} \) at normal *in vivo* protein levels was sufficient to explain the observed *in vitro* change of \(-0.13 \text{ mequiv} \cdot \text{kg}^{-1} \cdot \text{°C}^{-1} \) at constant \( P_{\text{CO}_2} \) between 10 and 30°C.

**Table 5. An estimate of the extracellular, intracellular and total \( \text{HCO}_3^- \) pools in Callinectes sapidus at three different acclimation temperatures**

<table>
<thead>
<tr>
<th></th>
<th>10°C</th>
<th>20°C</th>
<th>30°C</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Extracellular</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>8.017</td>
<td>7.852</td>
<td>7.712</td>
</tr>
<tr>
<td>( [\text{HCO}_3^-] ) (mequiv ml(^{-1}))</td>
<td>5.26</td>
<td>3.99</td>
<td>3.79</td>
</tr>
<tr>
<td>Volume (ml kg(^{-1}))</td>
<td>251</td>
<td>266</td>
<td>347</td>
</tr>
<tr>
<td>( \text{HCO}_3^- ) pool (mequiv kg(^{-1}))</td>
<td>1320</td>
<td>1061</td>
<td>1315</td>
</tr>
<tr>
<td><strong>Intracellular</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>7.571</td>
<td>7.541</td>
<td>7.449</td>
</tr>
<tr>
<td>( [\text{HCO}_3^-] ) (mequiv ml(^{-1}))</td>
<td>2.19</td>
<td>1.65</td>
<td>2.22</td>
</tr>
<tr>
<td>Volume (ml kg(^{-1}))</td>
<td>446</td>
<td>425</td>
<td>368</td>
</tr>
<tr>
<td>( \text{HCO}_3^- ) pool (mequiv kg(^{-1}))</td>
<td>977</td>
<td>701</td>
<td>817</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \text{HCO}_3^- ) pool (mequiv kg(^{-1}))</td>
<td>2297</td>
<td>1762</td>
<td>2132</td>
</tr>
</tbody>
</table>
20°C in acclimated animals (Fig. 3B). The Henderson-Hasselbalch equation illustrates that at constant 'passive' \( \Delta[A(HCO_3^-)]/\Delta T \), the accompanying change in pHa will be greater, the lower the absolute value of \( P_{CO_2} \), because of relative changes in the \( [HCO_3^-]/S_{CO_2} \) ratio. In practice in Callinectes, it can be calculated that 'passive' \( \Delta[HCO_3^-]/\Delta T = 0.17 \text{ mequiv deg}^{-1} \) between 10 and 20°C would produce \( \Delta \text{pH}_{\text{a}}/\Delta T \approx \Delta \text{pH}_{\text{a}}/\Delta T \) at \( P_{CO_2} = 1-0 \text{ Torr} \), which was close to the observed situation between 10 and 20°C (Fig. 3). At higher \( P_{CO_2} \), as at 30°C (Fig. 3C), the influence of this 'passive' mechanism on pHa would be greatly reduced (e.g. Fig. 6A) and thereby overwhelmed by 'active' mechanisms such as the \( P_{CO_2} \) adjustment itself.

A similar passive mechanism has recently been identified in tuna blood (S. F. Perry, unpublished data) but appears to have been overlooked in previous studies on water breathers, since open systems have been little studied. Two exceptions are the data of Cameron & Batterton (1978a) showing an \textit{in vitro} open system slope of \(-0.12 \text{ mequiv deg}^{-1} \) in Callinectes haemolymph, close to their \textit{in vivo} slope of \(-0.16 \text{ mequiv deg}^{-1} \), and the report of Randall & Cameron (1973) that \( \Delta \text{pH}/\Delta T \) in trout blood \textit{in vitro} was \(-0.019 \text{ u deg}^{-1} \) at 0-1 Torr, but \(-0.005 \) and \(-0.004 \text{ u deg}^{-1} \) at 11 or 24 Torr.

Like \( \Delta[HCO_3^-]/\Delta T \) in the open system, haemolymph \( \beta \) was also a linear function of protein concentration (Fig. 7B), which seems reasonable as the same buffer sites could be involved in both phenomena. Truchot (1976b) also demonstrated a linear relationship between total protein and \( \beta \) in Carcinus haemolymph. However, in the present study, \( \beta \), at constant protein concentration, clearly increased with temperature, an effect which could result from slight conformational changes in the haemocyanin molecule opening up more buffer sites. In rats, Saborowski, Lang & Albers (1973) found that the extracellular \( \beta \) per unit haemoglobin rose by 48% between 21.5 and 37°C, a proportionally similar change to that of haemolymph \textit{in vitro} between 10 and 30°C (Fig. 7B). A similar effect has recently been documented in purified human haemoglobin solutions and attributed to the increased participation of carbamino groups on valine residues in buffering at higher temperatures (Castaing, Bursaux & Poyart, 1982). Whether this is a feature of proteins in general remains to be seen. If so, analyses of temperature vs acid-base status which assume constant values of \( \beta \) should be re-evaluated (e.g. Heisler & Neumann, 1980; Cameron, 1984b).

**Responses to an acute change in temperature**

In the 24 h period following an acute shift from 20°C down to 10°C, crabs took up 2240 \( \mu\text{equiv kg}^{-1} \) net acidic equivalents from the environment (Fig. 9B). Thus the animal effectively excreted base (i.e. acidic equivalent uptake) at a time when it was accumulating \( HCO_3^- \) internally. However, as outlined above, the 'passive' change in haemolymph [\( HCO_3^- \)] with temperature at constant \( P_{CO_2} \) would account for all the observed \( HCO_3^- \) accumulation in the ECW. One possible explanation is that the 'passive' readjustment of the intracellular buffer pools produced more \( HCO_3^- \) than was needed for pHi adjustment, and this excess was effectively moved into the external environment by 'active' mechanisms. With an effective \( \Delta[HCO_3^-]/\Delta T \) slope of \(-0.12 \text{ mequiv deg}^{-1} \) 24 h after the acute change, the actual elevation in
Temperature vs ICF and ECF pH in blue crab

the intracellular $HCO_3^-$ pool was only $+530 \mu$equiv kg$^{-1}$. In fully acclimated crabs, the intracellular elevation was even smaller ($+276 \mu$equiv kg$^{-1}$; Table 5). If this is the correct explanation, then 'passive' intracellular $\Delta[HCO_3^-]/\Delta T$ would have to be approximately three- to four-fold greater than the observed extracellular value of $-0.17$ mequiv L$^{-1}$°C$^{-1}$. This may not be unreasonable, as passive extracellular $\Delta[HCO_3^-]/\Delta T$ seemed to vary in parallel with $\beta$ (Fig. 7), and intracellular $\beta$ is typically three- to four-fold greater than extracellular $\beta$ in poikilotherms (e.g. Heisler & Neumann, 1980; Cameron & Kornmanik, 1982). However, other explanations are equally possible (e.g. metabolic adjustments, shifts in buffer composition, $P_{CO_2}$ variation); again more detailed intracellular studies are obviously needed to settle the question.

The only comparable study on crabs is that of Truchot (1978) who found no change in net acidic equivalent flux after *Carcinus* was subjected to a 20 to 10°C shift. However, Truchot did not take ammonia flux into account. If this were reduced by 90% in *Carcinus* as in *Callinectes* (Fig. 9A), his results would have been very similar to ours. On the other hand Heisler (1978) and Cameron & Kornmanik (1982) measured smaller net acidic equivalent fluxes of opposite sign (ammonia considered) in dogfish and catfish respectively subjected to similar temperature decreases. The reasons for these differences are unknown.

The pattern of acid-base regulation initially seen after the acute temperature shift was very different from that seen in acclimated animals. This difference was characterized particularly by an initial undershoot of the $P_{CO_2}$ gradient across the gills and associated overshoot of pHa and pHi, both of which were corrected over the following 24 h (Fig. 8). Whether this resulted from acute temperature effects on metabolism, ventilation or both cannot be determined at present. Nevertheless the observation emphasizes both the complexity of the adjustments made by the animal and the importance of acclimation time in defining the pattern of these adjustments.

We thank Anna M. García for excellent technical assistance, Drs S. Gaillard and A. Malan for helpful communication and access to their manuscript prior to publication, and Drs S. F. Ferry and C. E. Booth for constructive comments. The work was supported by NSF Grant PCM80-20982 to JNC and an NSERC operating grant to CMW. CMW's visit to Port Aransas was supported by an NSERC International Collaborative Research Fellowship.

REFERENCES


Temperature vs ICF and ECF pH in blue crab


