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TEMPERATURE AND THE PHYSIOLOGY OF INTRACELLULAR AND EXTRACELLULAR ACID-BASE REGULATION IN THE BLUE CRAB *CALLINECTES SAPIDUS*

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SUMMARY

The ^{14}C-DMO/^{3}H-inulin method for pHi 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 pHi' (7·54 at 20 °C compared with a pHa of 7·80) was due to a highly alkaline fluid compartment (pHi = 8·23) in the carapace. At 20 °C the pHi of the heart was 7·35 and skeletal muscle pHi was 7·30, and there were small but consistent differences in the pHi of different muscle types. The change in pHa with temperature was $-0{\cdot}0151$ u °C^{-1} between 10 and 30 °C, slightly less than the slope for the neutral pH of water ($\Delta pN/\Delta T \simeq -0{\cdot}0175$ u °C^{-1}). With data corrected to constant $P_{I_{CO_2}}$, this was associated with a change in $[HCO_3^-]a$ between 10 and 20 °C ($-0{\cdot}13$ mequiv l^{-1} °C^{-1}, constant Pa_{CO_2}) and a change in Pa_{CO_2} between 20 and 30 °C ($+0{\cdot}13$ Torr °C^{-1}, constant $[HCO_3^-]a$). The disturbing effect of relatively small $P_{I_{CO_2}}$ changes on this pattern was demonstrated. $\Delta pHi/\Delta T$ slopes for all tissues except carapace were not significantly different from $pHa/\Delta T$ but generally lower than $\Delta pN/\Delta T$. The slope for the carapace was very flat and greatly influenced the 'mean whole body pHi' slope ($-0{\cdot}0062$ u °C^{-1}). In haemolymph *in vitro* at constant $P_{I_{CO_2}}$, 'passive' $\Delta[HCO_3^-]/\Delta T$ ($-0{\cdot}17$ mequiv l^{-1} °C^{-1}) was comparable to that *in vivo* between 10 and 20 °C, independent of absolute P_{CO_2}, 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 pHi associated with undershoot of Pa_{CO_2}, all of which were corrected over 24 h as $[HCO_3^-]a$ rose. During this period there was a significant net uptake

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of acidic equivalents (base output) from the environment. The relevance of 'passive' $\Delta[HCO_3^-]/\Delta T$ in an open system to the observed *in vivo* effects is discussed.

INTRODUCTION

It has been widely reported that temperature-related changes in extracellular pH (pHe) roughly parallel those of the neutral pH of water ($\Delta pN/\Delta T$ = approx. $-0{\cdot}0175\,u\,°C^{-1}$ 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 (α) of imidazole groups ('alphastat'; e.g. Reeves, 1977). Neither theory offers a complete mechanistic explanation. While imidazole is undoubtedly the most concentrated buffer *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_1' is very low ($\sim -0{\cdot}005\,u\,°C^{-1}$) relative to imidazole ($\sim -0{\cdot}018\,u\,°C^{-1}$), 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*a*,*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, $\Delta pHi/\Delta T$ varies considerably between tissues and species with numerous deviations from the $\Delta pN/\Delta T$ 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, *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*a*; McMahon *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_{ICO_2}) variation, a parameter which has not been carefully controlled in previous studies.

(ii) The 'passive' physico-chemical characteristics of haemolymph *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 *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).

(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 pHi 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 $\sim 5\ \% \ h^{-1}$. Salinity was 24–26‰, titration alkalinity was $\sim 2{\cdot}4$ 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, 1978*a*). 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 $\simeq 600$ ml min^{-1}), aeration reservoir, and heat exchanger which maintained experimental temperature within ± 1 °C and $P_{IO_2} > 120$ Torr. This closed system was necessary to monitor ^{14}C-DMO and ^{3}H-inulin excretion for calculation of 'mean whole body pHi'. 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 ($\sim 1{\cdot}1$ 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; pHi 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, pHa 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 Pa_{CO_2} and Pi_{CO_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 ^3H 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 Pi_{CO_2}) were repeated, a 2 ml terminal haemolymph sample drawn for ionic and protein analyses, and the crab killed for individual tissue pHi 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 pHi 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 ^3H-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, Pi_{CO_2}, total ammonia and titration alkalinity, the latter two allowing calculation of net acidic equivalent flux. Haemolymph samples (340 μl) for radioactivity, pHa, C_{Ta} 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 Pa_{CO_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 $P_{CO_2} = 1{\cdot}5$, 4·5 and 9·0 Torr (balance air) at 10, 20 and 30 °C; C_T 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_{CO_2} values at 20 °C were repeated at the end to check for haemolymph deterioration in two runs. Deviations from the initial values of C_T and pH were within the error of the measurements.

Analytical techniques and calculations

Haemolymph pHa, Pa_{CO_2} and water PI_{CO_2} were determined with routine electrode techniques (Radiometer). Particular care was exercised to ensure accurate P_{CO_2} electrode response at these very low P_{CO_2} levels (cf. Boutilier, Randall, Shelton & Toews, 1978); as a result, precision was approximately ±5 %. C_{Ta} was determined using a Capni-Con II (Cameron Instrument Co.), a conductometric apparatus based on the method of Maffly (1968). Values of haemolymph αCO_2 for the calculation of dissolved CO_2 (S_{CO_2}) at the appropriate temperature and salinity were taken from Truchot (1976*a*); apparent pK_1' (combining HCO_3^- and CO_3^{2-}), $[HCO_3^-]a$ and, if not measured, Pa_{CO_2}, could then be calculated from the Henderson-Hasselbalch equation. Haemolymph $[Na^+]$ and $[K^+]$ were measured with a flame photometer (Radiometer FLM3), $[Ca^{2+}]$ and $[Mg^{2+}]$ with an atomic absorption spectrophotometer (Perkin-Elmer), $[Cl^-]$ 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:

$$R = 1{\cdot}013P + 1{\cdot}97 \ (N = 18, \ r = 0{\cdot}99, \ P < 0{\cdot}001), \qquad (1)$$

where R is the refractometer reading and P the true protein concentration in $g\,100\,ml^{-1}$, 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 ^{14}C-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

(Packard 3225) and ultimately converted to d.p.m., taking combustion and counting efficiencies into account.

The trapped extracellular water (ECW) in each tissue sample was calculated as:

$$\text{ECW } (\text{g H}_2\text{O g}^{-1}) = \frac{\text{Tissue [inulin] (d.p.m. g}^{-1})}{\text{Haemolymph [inulin] (d.p.m. g}^{-1}\text{ H}_2\text{O})} \quad (2)$$

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_2O 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}H-inulin activity in haemolymph back to time 0 to obtain $[\text{inulin}]_0$. Then:

$$\text{Whole body ECW } (\text{g H}_2\text{O kg}^{-1}) = \frac{\text{Inulin injected (d.p.m.)}}{\text{Haemolymph } [\text{inulin}]_0 \text{ (d.p.m. g}^{-1}\text{ H}_2\text{O)} \times \text{body weight (kg)}}. \quad (3)$$

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

$$\text{Inulin clearance } (\text{g H}_2\text{O kg}^{-1}\text{ h}^{-1}) = \text{Whole body ECW } (\text{g H}_2\text{O kg}^{-1}) \times K(\%\text{ h}^{-1}). \quad (4)$$

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

$$\text{Whole body ECW } (\text{g H}_2\text{O kg}^{-1}) = \frac{^3\text{H-inulin injected (d.p.m.)} - \Sigma\text{excreted (d.p.m.)} - \Sigma\text{sampled (d.p.m.)}}{\text{Haemolymph [inulin] (d.p.m. g}^{-1}\text{ H}_2\text{O)} \times \text{body weight (kg)}}, \quad (5)$$

where Σexcreted represents measured losses to the water and Σsampled 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:

$$\text{pHi} = \text{pK}_{\text{DMO}} + \log\left\{\frac{[\text{DMO}]\text{i}}{[\text{DMO}]\text{e}}\,(1 + 10^{(\text{pHa}-\text{pK}_{\text{DMO}})}) - 1\right\}, \quad (6)$$

assuming pHa to be representative of extracellular pHe and where [DMO]i and [DMO]e were the ^{14}C-DMO activities (d.p.m.$g^{-1}H_2O$) in intracellular and extracellular fluids respectively, and pK_{DMO} at the appropriate temperature was taken from Heisler *et al.* (1976). For pHi in individual tissues, [DMO]i was calculated as:

$$[\text{DMO}]\text{i (d.p.m.g}^{-1}\text{H}_2\text{O}) = \quad (7)$$

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

and for 'mean whole body pHi' as:

$$[\text{DMO}]\text{i (d.p.m.g}^{-1}\text{H}_2\text{O}) = \quad (8)$$

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

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 NH_3 and NH_4^+ 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 ^{14}C-DMO and ^{3}H-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

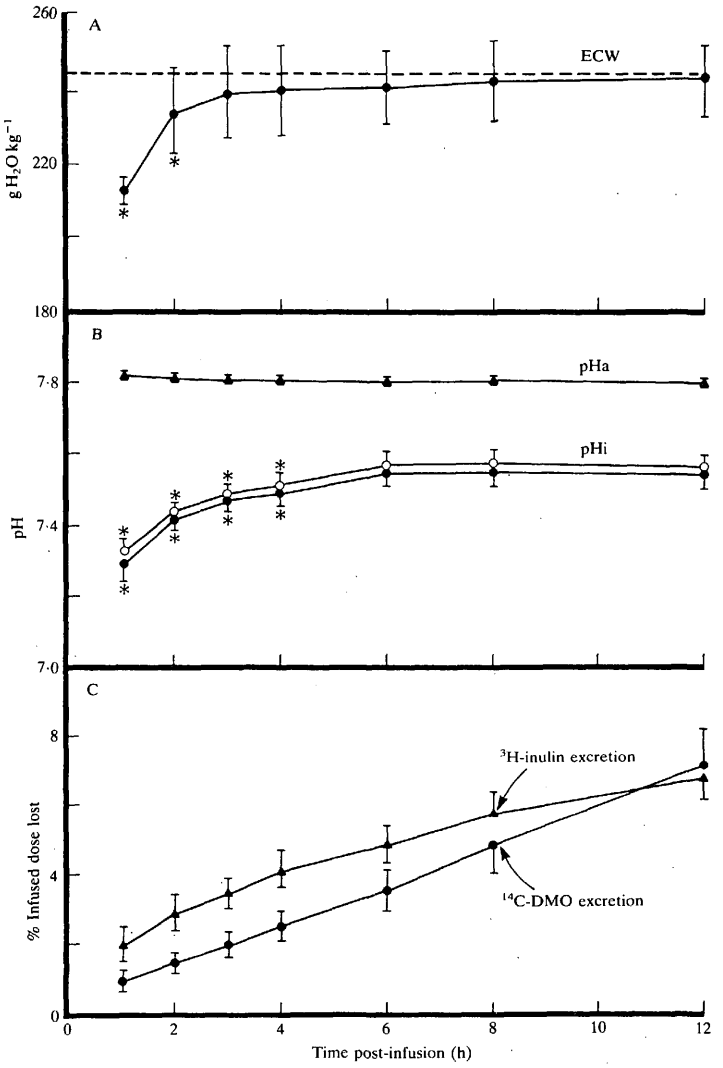
A
ECW
260
220
180
g H2O kg-1
B
pHa
pHi
7·8
7·4
7·0
pH
C
3H-inulin excretion
14C-DMO excretion
8
4
0
% Infused dose lost
0
2
4
6
8
10
12
Time post-infusion (h)

Fig. 1

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 pHi' 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 pHi, as shown by comparison of the pHi 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 C_{Ta} and calculated Pa_{CO_2} (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 pHi' would have been overestimated by 0·23 u and ECW by 18 %. Loss of ^{14}C-DMO by catabolism was evidently insignificant, since no ^{14}C-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 Pi_{CO_2} with temperature in the recirculating

Fig. 1. The behaviour of ^{3}H-inulin and ^{14}C-DMO in *Callinectes sapidus* at 20 °C. Means ± 1 S.E.M. ($N = 11$). (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 (△) and 'mean whole body intracellular pH' with time after injection. Values of pHi calculated using the 'instantaneous' estimate of ECW at that time (○) 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 ^{3}H-inulin and ^{14}C-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 ($\bar{X} \pm 1$ S.E.M.)*

	10 °C ($N = 8$)	20 °C ($N = 11$)	30 °C ($N = 14$)
$K(^3$H-inulin) (% h^{-1})	0·844 ± 0·104	0·813 ± 0·070	1·604 ± 0·317
^{3}H-inulin clearance (g H_2O kg^{-1} h^{-1})	1·94 ± 0·23	2·02 ± 0·25	5·02 ± 1.01*†
Measured ^{3}H-inulin excretion at 12 h (% infused dose)	9·61 ± 1·86	6·79 ± 0·65	15·54 ± 3·02*†
Measured ^{14}C-DMO excretion at 12 h (% infused dose)	10·63 ± 2·26	7·10 ± 1·16	16·32 ± 2·44†

* 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 $P_{I_{CO_2}}$ 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 (Pa_{CO_2}-$P_{I_{CO_2}}$) were identical at comparable temperatures in the two systems, so Pa_{CO_2} and C_{Ta} 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_{CO_2} levels characteristic of water breathers, relatively small temperature-related changes in $P_{I_{CO_2}}$ 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 $P_{I_{CO_2}} = 0.81$ Torr have been slightly adjusted to the same $P_{I_{CO_2}}$ as the 10 and 20 °C data (0·60 Torr), assuming an unchanged Pa_{CO_2}-$P_{I_{CO_2}}$ gradient and unchanged pHa. (Thus at 30 °C, Pa_{CO_2} was adjusted from 2·75 to 2·54 Torr, and $[HCO_3^-]a$ from 4·10 to 3·79 mequiv l^{-1}.) The overall $\Delta pHa/\Delta T$ was -0.0151 u $°C^{-1}$. Between 10 and 20 °C, haemolymph $[HCO_3^-]a$ fell significantly ($\Delta[HCO_3^-]a/\Delta T = -0.13$ mequiv l^{-1} $°C^{-1}$) but did not change at 30 °C (Fig. 3B), whereas Pa_{CO_2} was identical at 10 and 20 °C, but doubled at 30 °C ($\Delta Pa_{CO_2}/\Delta T = +0.13$ Torr $°C^{-1}$; Fig. 3C). The factorial analysis of Fig. 3A illustrates that between 10 and 20 °C, $\Delta pHa/\Delta T$ could be almost entirely attributed to a $[HCO_3^-]a$ change, and between 20 and 30 °C to a Pa_{CO_2} change, and thus to an increase in the P_{CO_2} gradient across the gills. *Callinectes* haemolymph *in vivo*

Fig. 2. The extracellular (arterial) acid-base status of *Callinectes sapidus* acclimated to 10 ($N = 8$), 20 ($N = 7$–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_{CO_2} (circles) and inspired P_{CO_2} (triangles).

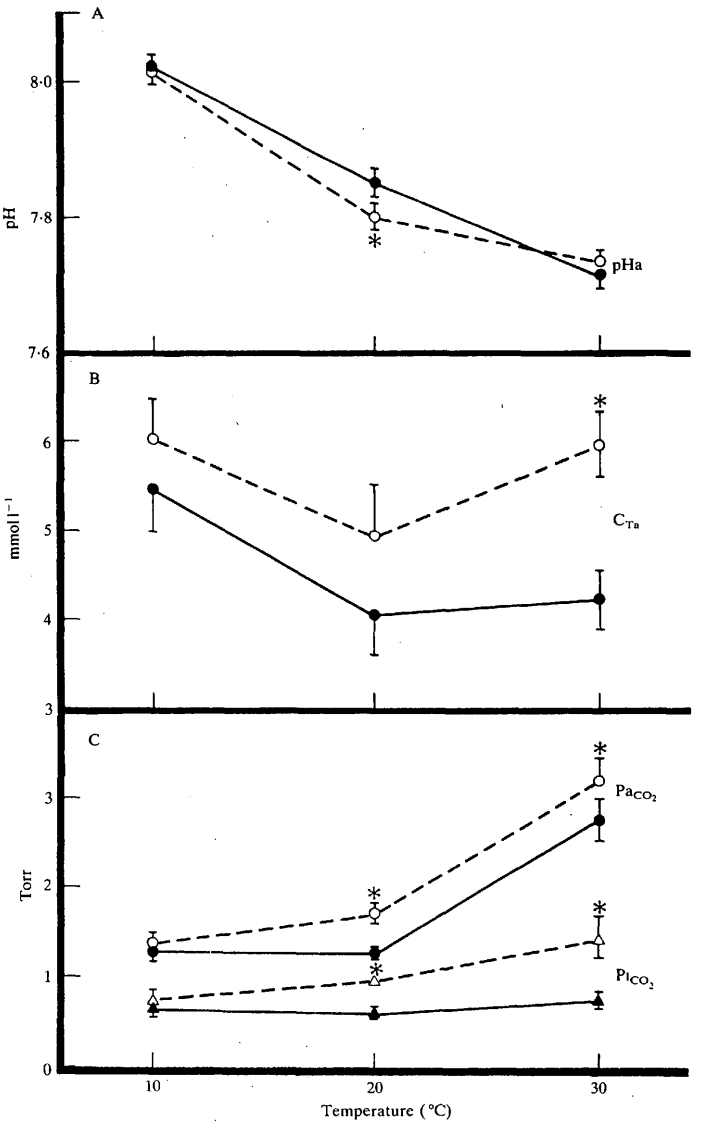
A
8·0
7·8
7·6
pH
pHa
B
6
5
4
3
mmol l⁻¹
C_{Ta}
C
3
2
1
0
Torr
Pa_{CO_2}
PI_{CO_2}
10
20
30
Temperature (°C)

Fig.2

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 ($\bar{X} \pm 1$ S.E.M.)*

	10 °C ($N = 8$)	20 °C ($N = 11$)	30 °C ($N = 14$)
Cheliped muscle	13·00 ± 0·98	14·14 ± 0·90	16·31 ± 1·10*
Walking leg muscle	12·52 ± 1·12	11·69 ± 0·74	13·76 ± 0·60
Dark backfin muscle	13·36 ± 0·99	12·93 ± 0·62	16.02 ± 0·88*†
Light backfin muscle	11·09 ± 0·62	11·79 ± 0·37	12·83 ± 0·56
Heart	20·22 ± 1·40	24·75 ± 1·63	25·11 ± 1·20*
Carapace	12·53 ± 1·93	7·86 ± 0·49*	12·36 ± 0·92†
Haemolymph protein (g 100 ml^{-1})	6·31 ± 0·49	5·35 ± 0·61	5·76 ± 0·28

*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_{CO_2} 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 CO_2/HCO_3^- pK_1', 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 H_2O 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 H_2O 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 mequiv l^{-1} between 10 and 20 °C with no further change at 30 °C (Table 3). Concentrations of Ca^{2+} and Mg^{2+} fell by a greater relative proportion between 10 and 20 °C, and then rose again at 30 °C (significant only for Ca^{2+}). 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 ($N = 8$), 20 ($N = 7$–11) and 30 °C ($N = 14$) in terms of the components of the Henderson-Hasselbalch equation. The data represent measurements from crabs in the flow-through system at constant $P_{CO_2} = 0·60$ 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_{CO_2}. (D) Apparent pK_1' of the HCO_3^-/CO_2 system, determined experimentally. (E) Dissolved carbon dioxide concentration.

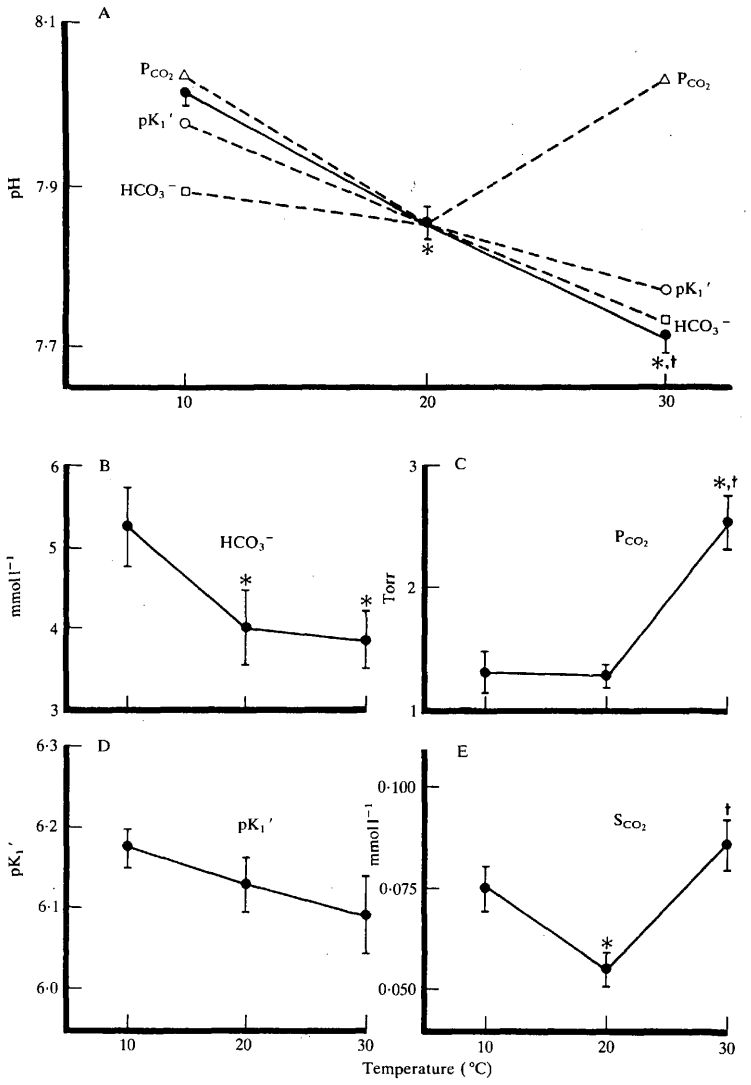
A
8·1
7·9
7·7
pH
P_{CO_2}
pK_1'
HCO_3^-
*
*,†
10
20
30
B
6
5
4
3
mmol l^{-1}
HCO_3^-
C
3
2
1
Torr
P_{CO_2}
D
6·3
6·2
6·1
6·0
pK_1'
E
0·100
0·075
0·050
mmol l^{-1}
S_{CO_2}
t
Temperature (°C)

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 ($\bar{X} \pm 1$ S.E.M.)*

	10 °C ($N = 8$)	20→10 °C ($N = 10$)	20 °C ($N = 6$)	30 °C ($N = 14$)
Na^+	424 ± 4	401 ± 5*†	391 ± 4*	393 ± 6*
Cl^-	444 ± 4	434 ± 8†	411 ± 3*	416 ± 10*
Ca^{2+}	22·8 ± 0·6	22·6 ± 0·6†	17·2 ± 1·7*	21·4 ± 0·2*†
Mg^{2+}	24·7 ± 0·8	26·8 ± 0·7†	21·1 ± 2·3	24·3 ± 0·9
K^+	7·5 ± 0·2	7·3 ± 0·3†	8·5 ± 0·3*	8·9 ± 0·2*

*Significantly different ($P < 0·05$) from 10 °C value.
†Significantly different ($P < 0·05$) from 20 °C value.

1978), here defined as $[Na^+ + Ca^{2+} + Mg^{2+} + K^+ - Cl^-]$ in mequiv l^{-1}, was ~30 mequiv l^{-1} (or about seven-fold $[HCO_3^-]$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 ($\bar{X} \pm 1$ S.E.M.)*

	N	m	b	r	P
Haemolymph	33	−0·0151 ± 0·0012	8·162 ± 0·009	0·92	< 0·001
Heart	32	−0·0163 ± 0·0022	7·629 ± 0·019	0·78	< 0·001
Skeletal muscles	33	−0·0132 ± 0·0015†	7·562 ± 0·009	0·89	< 0·001
cheliped	33	−0·0141 ± 0·0012†	7·608 ± 0·010	0·90	< 0·001
walking leg	28	−0·0125 ± 0·0016†	7·527 ± 0·013	0·84	< 0·001
light backfin	33	−0·0124 ± 0·0012†	7·489 ± 0·009	0·88	< 0·001
dark backfin	28	−0·0121 ± 0·0014†	7·553 ± 0·012	0·87	< 0·001
Carapace	39	−0·0083 ± 0·0032*†	8·388 ± 0·024	0·39	< 0·05
Mean whole body	28	−0·0062 ± 0·0024*†	7·645 ± 0·019	0·45	< 0·05

*Significantly different ($P < 0·05$) from $\Delta pN/\Delta T$.
†Significantly different ($P < 0·05$) from haemolymph $\Delta pHa/\Delta T$.

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

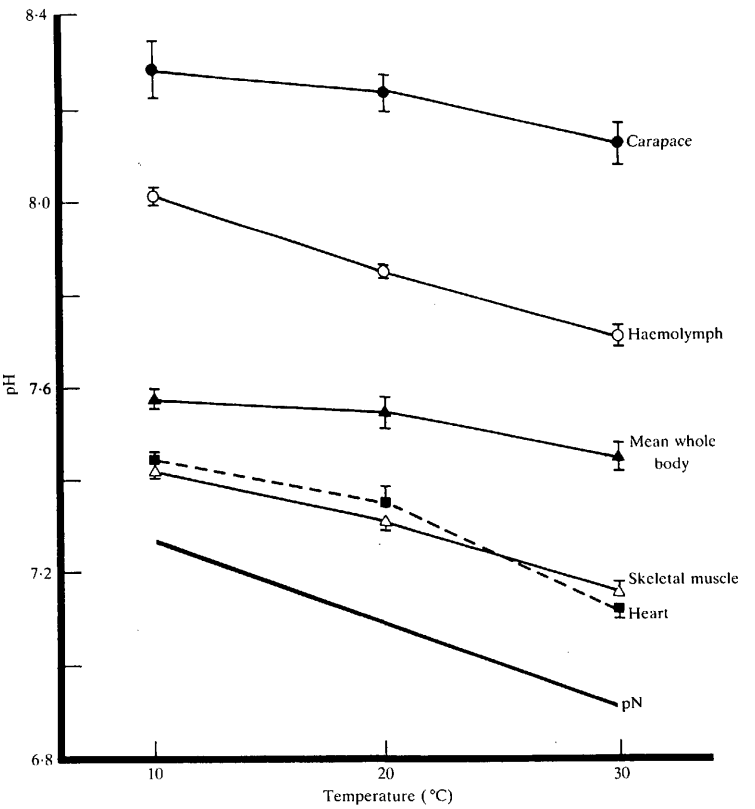

Fig. 4. Intracellular pH in various tissues and 'mean whole body pHi' of *Callinectes sapidus* acclimated to 10 ($N = 8$), 20 ($N = 7$–11 except carapace where $N = 17$) and 30 °C ($N = 14$). Means ± 1 S.E.M. Skeletal muscle values are averages for dark and light backfin, cheliped and walking leg muscles. The relationships for haemolymph (pHa; flow-through system measurements) and the pH of neutrality (pN) are included for comparison.

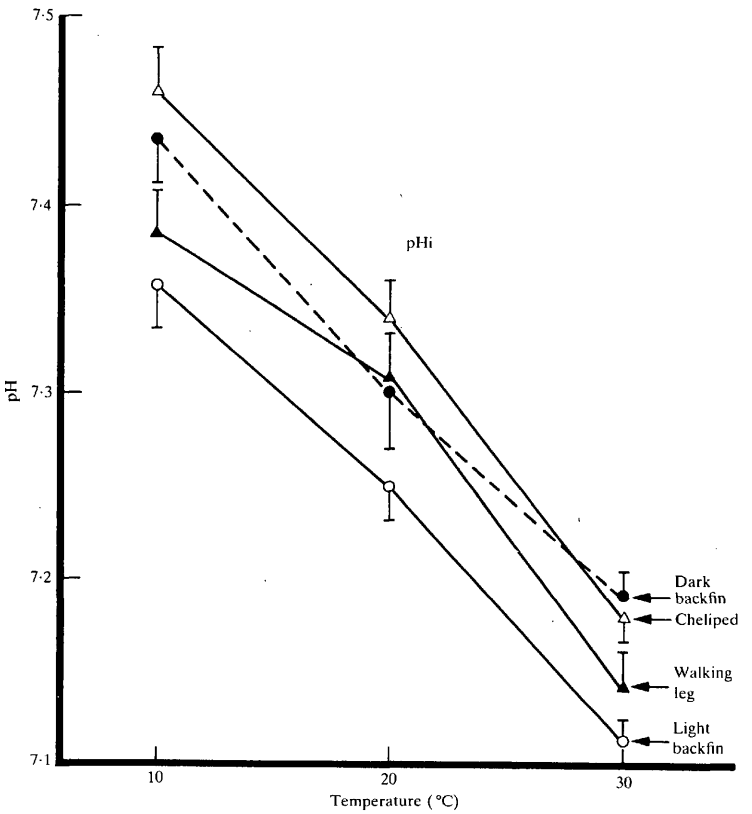

Fig. 5. Intracellular pH in four skeletal muscle tissues of *Callinectes sapidus* acclimated to 10 ($N = 7$–8), 20 ($N = 6$–11) and 30 °C ($N = 14$). Means ± 1 S.E.M.

(Roos & Boron, 1981), these inter-muscle pHi differences may reflect relative mitochondrial volume.

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 ($-0{\cdot}0222 \pm 0{\cdot}0051$ *vs* $-0{\cdot}0093 \pm 0{\cdot}0050$ u °C^{-1}) and walking leg ($-0{\cdot}0167 \pm 0{\cdot}0033$ *vs* $-0{\cdot}0076 \pm 0{\cdot}0036$ 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

($\Delta pHa/\Delta T = -0{\cdot}0151$ u °C^{-1}). However $\Delta pHi/\Delta T$ values for all skeletal muscle tissues were significantly less than for constant relative alkalinity (i.e. $\Delta pN/\Delta T \simeq -0{\cdot}0175$ u °C^{-1}). The slope for carapace was extremely flat, and this was clearly reflected in the slope for 'mean whole body pHi' (Fig. 4; Table 4); both were significantly less than either $\Delta pHa/\Delta T$ or $\Delta pN/\Delta T$.

Temperature responses of haemolymph in vitro

When haemolymph was equilibrated at constant P_{CO_2} *in vitro* (i.e. open system conditions), $[HCO_3^-]$ fell as temperature rose (Fig. 6B). Although the absolute $[HCO_3^-]$ increased with greater P_{CO_2}, $\Delta[HCO_3^-]/\Delta T$ was independent of P_{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 H^+ ions from the haemocyanin, resulting in the removal of HCO_3^- from the system as gaseous CO_2. As such it is considered a 'passive', physico-chemical effect – i.e. not involving 'active' regulation of P_{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 vivo* (Table 2), this 'passive' $\Delta[HCO_3^-]/\Delta T$ would have been about $-0{\cdot}17$ mequiv l^{-1} °C^{-1} (Fig. 7A), slightly greater than the *in vivo* slope of $-0{\cdot}13$ mequiv l^{-1} °C^{-1} at constant Pa_{CO_2} between 10 and 20 °C (Fig. 3B,C). The change in haemolymph pH with temperature *in vitro* associated with this constant $\Delta[HCO_3^-]/\Delta T$ was proportionately greater at lower P_{CO_2} values (Fig. 6A), as could also be predicted from the Henderson-Hasselbalch equation. However, even at the lowest P_{CO_2} tested (1·5 Torr), $\Delta pH/\Delta T$ ($-0{\cdot}0071$ u °C^{-1}; Fig. 6A) was less than half that observed *in vivo* ($-0{\cdot}0151$ u °C^{-1}; Fig. 3A) between 10 and 20 °C where Pa_{CO_2} was similarly constant. This difference was explained partly by the lower Pa_{CO_2} *in vivo* (1·2 Torr), since small changes in P_{CO_2} have large effects on pH in this range, and partly by the greater variability *in vitro*.

The non-bicarbonate buffer value ($\beta = -\Delta[HCO_3^-]/\Delta pH$) was calculated from the linear change in $[HCO_3^-]$ with pH at the three equilibration levels of P_{CO_2}. At all three temperatures, β 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 [$0{\cdot}18 \pm 0{\cdot}03$ (5) mmol l^{-1}]. The value of β clearly increased with temperature, the regression slope rising significantly from $1{\cdot}819 \pm 0{\cdot}121$ at 10 °C to $2{\cdot}637 \pm 0{\cdot}201$ slykes 100 ml^{-1} g^{-1} protein at 30 °C. The mean values of β *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 vivo* was entirely a $[HCO_3^-]$a rather than a Pa_{CO_2} adjustment (Fig. 3); the 'passive' generation of basic equivalents by haemolymph at constant P_{CO_2} *in vitro* was large enough to explain this $\Delta[HCO_3^-]a/\Delta T$, 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 Pa_{CO_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 PI_{CO_2} in the recirculating system, the more important component was a 50% decrease in the

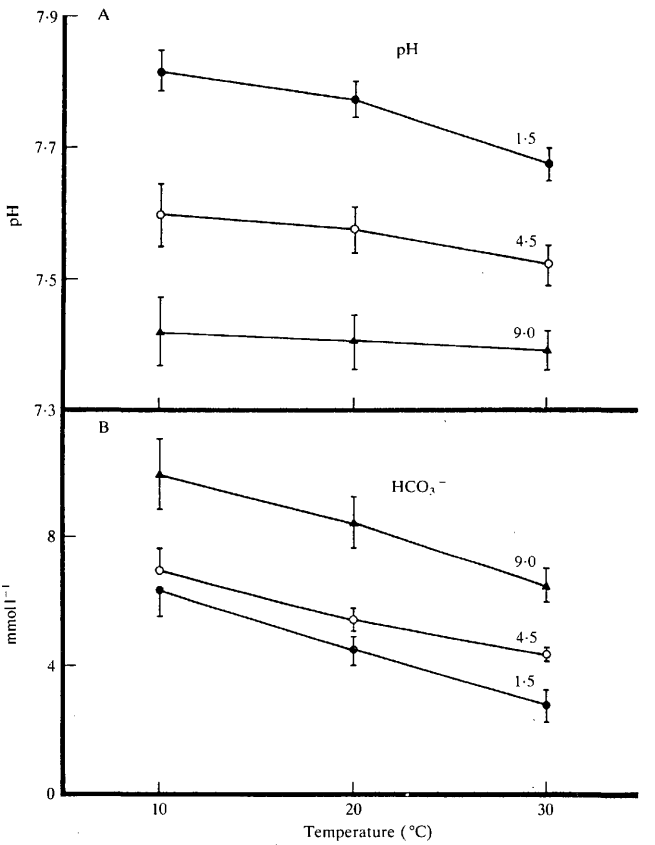

Fig. 6. Changes in (A) pH and (B) bicarbonate concentrations in haemolymph samples of *Callinectes sapidus* equilibrated *in vitro* at 10, 20 and 30°C in an open system to P_{CO_2} = 1·5 (N = 6), 4·5 (N = 4) and 9·0 Torr (N = 6), balance air.

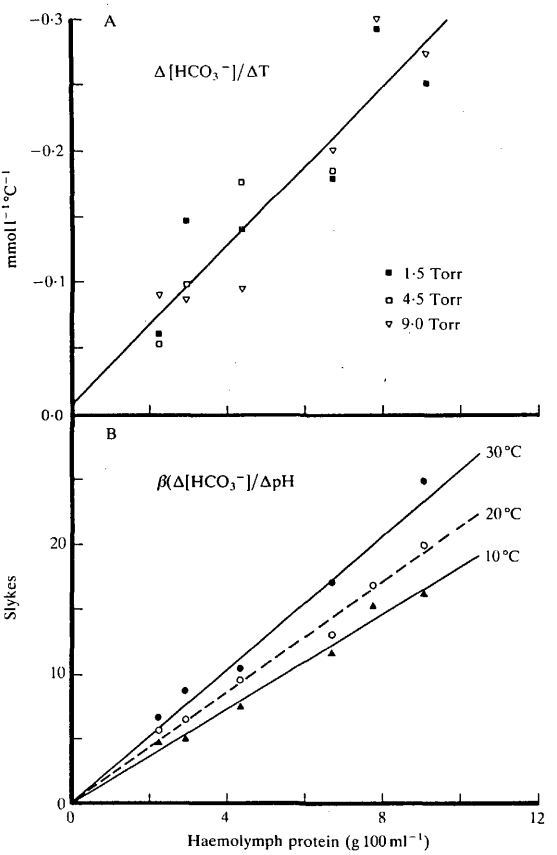

Fig. 7. Relationships between physico-chemical characteristics and total protein concentration in haemolymph samples of *Callinectes sapidus* equilibrated *in vitro*. (A) Passive change in bicarbonate concentration per degree Centigrade in an open system at P_{CO_2} = 1·5 (■), 4·5 (□) and 9·0 (▽) Torr, balance air. The fitted regression line is: y = −0·03039x−0·0095 (r = −0·92, P < 0·001, N = 16). (B) Non-bicarbonate buffer value (β) at 10 (▲), 20 (○) and 30 °C (●). The fitted regression lines are: 10 °C, y = 1·819x+0·066 (r = 0·99, P < 0·01, N = 6); 20 °C, y = 2·086x+0·385 (r = 0·99, P < 0·01, N = 6); 30 °C, y = 2·637x+0·228 (r = 0·99, P < 0·01, N = 5).

Pa_{CO_2}-PI_{CO_2} gradient across the gills (Fig. 8C). Initially, C_{T_a} 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 PI_{CO_2}, which contributed to the decrease in Pa_{CO_2}, and

thereby masked an effective $[HCO_3^-]a$ adjustment. Had the change in $P_{I_{CO_2}}$-Pa_{CO_2} gradient alone occurred (i.e. absence of $P_{I_{CO_2}}$ change), together with the observed pHa change, $[HCO_3^-]a$ would have risen with a slope of $-0{\cdot}15$ mequiv $l^{-1}°C^{-1}$ between 20 and 10°C over the first hour. The net effect of this combined Pa_{CO_2} and $[HCO_3^-]a$ adjustment was a large initial overshoot in pHa, which rose with a slope of $-0{\cdot}0218$ u $°C^{-1}$ during the first hour (Fig. 8A). Thereafter pHa gradually fell, accompanied by a slow rise in both C_{Ta} and Pa_{CO_2}, the latter reflecting an increase in the $P_{I_{CO_2}}$-Pa_{CO_2} gradient. By 24 h, this P_{CO_2} gradient had returned to the 20°C value, C_{Ta} had risen significantly, and pHa had reached a level which gave a 20 to 10°C slope of $-0{\cdot}0104$ u $°C^{-1}$. Adjusting the data to constant $P_{I_{CO_2}}$ as before, the effective $\Delta[HCO_3^-]a/\Delta T$ value between 20 and 10°C after 24 h was $-0{\cdot}18$ mequiv $l^{-1}°C^{-1}$. 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 $4{\cdot}79 \pm 0{\cdot}50$ (10) g $100\,ml^{-1}$ 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 $-0{\cdot}0164$ u $°C^{-1}$, but this decreased to $-0{\cdot}0077$ u $°C^{-1}$ by 24 h, close to the value observed in temperature-acclimated crabs (Table 4). Assuming P_{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 $P_{I_{CO_2}}$ against a background of $[HCO_3^-]i$ accumulation. At constant $P_{I_{CO_2}}$, the effective $\Delta[HCO_3^-]i/\Delta T$ between 20 and 10°C after 24 h was $-0{\cdot}12$ mequiv $l^{-1}°C^{-1}$.

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 $+200\,\mu$equiv $kg^{-1}\,h^{-1}$. The net uptake of acidic equivalents over the 24 h period following the temperature change amounted to $2240\,\mu$equiv kg^{-1} relative to the control rate at 20°C.

DISCUSSION

Methodology for pHi measurements

The ^{14}C-DMO/^{3}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; Hōbe, 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

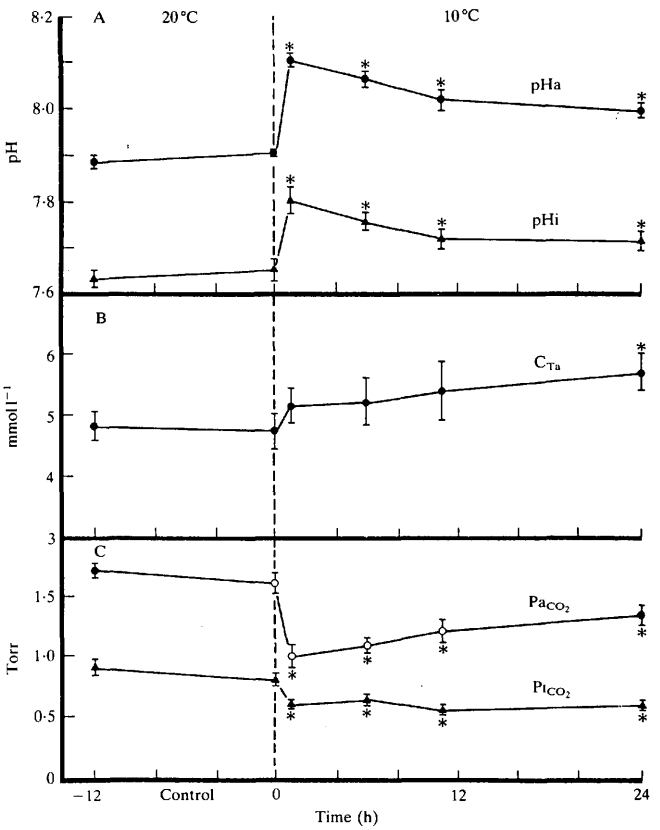

Fig. 8. The influence of an acute change in temperature from 20 down to 10 °C on extracellular (arterial) and intracellular acid-base status in *Callinectes sapidus* acclimated to 20 °C ($N = 10$). Means ± 1 S.E.M. *Indicates value significantly different ($P < 0{\cdot}05$) from mean of two control 20 °C values. (A) Extracellular and 'mean whole body' intracellular pH. (B) Extracellular total carbon dioxide content. (C) Extracellular P_{CO_2} (●, measured values; ○, calculated values) and inspired P_{CO_2} (measured values).

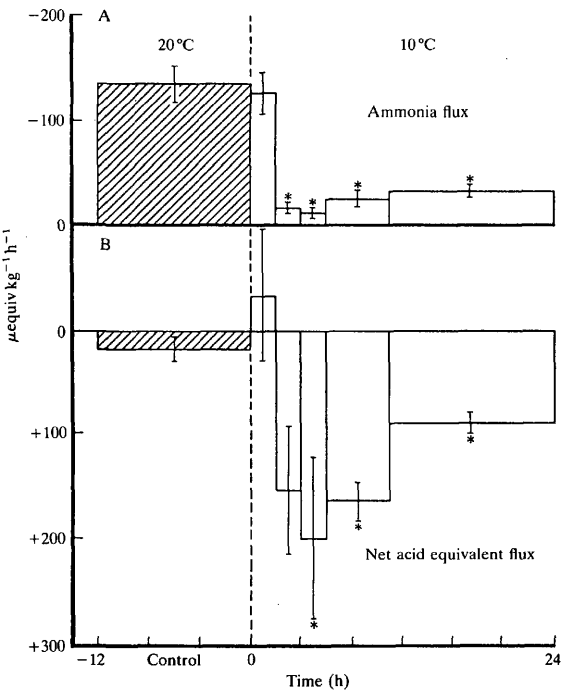

Fig. 9. The influence of an acute change in temperature from 20 down to 10 °C on (A) ammonia and (B) net acidic equivalent flux to the environment in *Callinectes sapidus* acclimated to 20 °C ($N = 10$). Means ± 1 S.E.M. *Indicates value significantly different ($P < 0.05$) from 20 °C control value.

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 pHi' and ECW. The measured DMO excretion rates in *Callinectes* were 1·2- to 2·8-fold greater than in trout (Hōbe *et al.* 1984). The route of inulin loss is exclusively *via* the antennal gland (Cameron & Batterton, 1978*b*; 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 pHi in isolated crustacean muscles (Boron & Roos, 1976; Rodeau, 1982). Mean pHi 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

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 pHi 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 $CaCO_3$ 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' $[HCO_3^-]$ 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 CO_2 (mainly CO_3^{2-}) store in the whole carapace was $2{\cdot}09\times10^6$ μequiv kg^{-1} body weight ($1{\cdot}04\times10^6$ μmol l^{-1}; Cameron & Wood, 1985) or over 1000 times the HCO_3^- 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 $\Delta pHa/\Delta T$ for acclimated *Callinectes* was $-0{\cdot}0151$ u $°C^{-1}$, close to that for constant relative alkalinity ($\simeq -0{\cdot}0175$ u $°C^{-1}$).

This may be compared with previous values for *Callinectes* of −0·0120 (Cameron & Batterton, 1978*a*) 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 u °C^{-1} for *Cancer* (McMahon *et al.* 1978). Haemolymph [HCO_3^-] fell with rising temperature in all studies but with a slope varying widely from −0·085 mequiv l^{-1} °C^{-1} (Truchot, 1973) to −1·29 mequiv l^{-1} °C^{-1} (Howell *et al.* 1973). P_{CO_2} either rose a lot (Truchot, 1973, 1978), a little (Cameron & Batterton, 1978*a*), remained the same (McMahon *et al.* 1978) or fell (Howell *et al.* 1973). In none of these previous studies was P_{ICO_2} closely controlled or measured. In our experience, despite vigorous aeration, P_{ICO_2} tends to increase with temperature in recirculating systems (cf. Fig. 2). Because of the very low P_{CO_2} levels and gradients across the gills in crabs, minor changes in P_{ICO_2} 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_{ICO_2} from 0·60 to 1·20 Torr would have decreased pHa from 7·85 to 7·68; after metabolic compensation, [HCO_3^-]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_{ICO_2} was stable (Fig. 3). The comparison of recirculating and flow-through system data in Fig. 2 emphasizes the possible errors involved.

At constant P_{ICO_2}, the haemolymph acid-base adjustment in acclimated *Callinectes* was almost entirely the result of a [HCO_3^-]a change between 10 and 20 °C and a Pa_{CO_2} 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. HCO_3^- exchange) to an aerial mode of regulation (i.e. ventilatory P_{CO_2} control) at higher temperature, an explanation which cannot apply to *Callinectes*. Rather, it appears that between 10 and 20 °C, the [HCO_3^-]a change could be explained by the 'passive' effect of temperature on H^+ dissociation from haemocyanin in an open system (see below), and between 20 and 30 °C, the Pa_{CO_2} 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_2) is unaffected by temperature in *Carcinus* (Truchot, 1978) and *Callinectes* (Cameron & Batterton, 1978*a*). However, as Truchot (1978) points out, Pa_{CO_2} may depend on (expired) $P\bar{E}_{CO_2}$, which in turn varies inversely with αCO_2, the latter decreasing with temperature. Thus the fall in $P\bar{E}_{O_2}$ at high temperature observed by Cameron & Batterton (1978*a*) in *Callinectes* was probably associated with a rise in $P\bar{E}_{CO_2}$.

Intracellular parameters versus *acclimation temperature*

In *Callinectes*, as in most other poikilotherms which have been examined (see Introduction), pHi in individual tissues fell with increasing acclimation temperature (Figs 4, 5; Table 4). For most tissues, $\Delta pHi/\Delta T$ was less than $\Delta pN/\Delta T$ but not significantly different from $\Delta pHa/\Delta T$, 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 pHi

independently of pHe, presumably by transmembrane acidic equivalent flux. Without a detailed knowledge of the extent of such fluxes, metabolic adjustments, $P_{I_{CO_2}}$ 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 pHi on 'mean whole body pHi' contributed greatly to the low ΔpHi/ΔT of the latter.

The HCO_3^- pools within acclimated crabs at 10, 20 and 30°C (Table 5) were estimated by assuming that pHi was internally uniform and that P_{CO_2} was equilibrated across the extracellular/intracellular interface, and by using intracellular pK_1' values taken from pH *vs* pK_1' regressions fitted to the *in vitro* haemolymph data. The analysis indicated that over half the 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 $[HCO_3^-]$ 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 $[HCO_3^-]$a with rising temperature from 10 to 20°C in acclimated crabs *in vivo* need not reflect active mechanisms of 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 HCO_3^- and the resulting CO_2 would leave in an open, constant P_{CO_2} system. In accordance with this interpretation, $\Delta[HCO_3^-]/\Delta T$ in the open system was independent of P_{CO_2} (Figs 6B, 7A) but directly related to haemolymph protein concentration (Fig. 7A). This 'passive' $\Delta[HCO_3^-]/\Delta T$ of $-0{\cdot}17$ mequiv l^{-1} °C^{-1} at normal *in vivo* protein levels was sufficient to explain the observed *in vivo* change of $-0{\cdot}13$ mequiv l^{-1} °C^{-1} at constant Pa_{CO_2} between 10 and

Table 5. *An estimate of the extracellular, intracellular and total HCO_3^- pools in* Callinectes sapidus *at three different acclimation temperatures*

	10 °C	20 °C	30 °C
Extracellular			
pH	8·017	7·852	7·712
$[HCO_3^-]$ (μequiv ml^{-1})	5·26	3·99	3·79
Volume (ml kg^{-1})	251	266	347
HCO_3^- pool (μequiv kg^{-1})	1320	1061	1315
Intracellular			
pH	7·571	7·541	7·449
$[HCO_3^-]$ (μequiv ml^{-1})	2·19	1·65	2·22
Volume (ml kg^{-1})	446	425	368
HCO_3^- pool (μequiv kg^{-1})	977	701	817
Total			
HCO_3^- pool (μequiv kg^{-1})	2297	1762	2132

20°C in acclimated animals (Fig. 3B). The Henderson-Hasselbalch equation illustrates that at constant 'passive' $\Delta[HCO_3^-]a/\Delta T$, the accompanying change in pHa will be greater, the lower the absolute value of Pa_{CO_2}, because of relative changes in the $[HCO_3^-]a/S_{CO_2}$ ratio. In practice in *Callinectes*, it can be calculated that 'passive' $\Delta HCO_3^-/\Delta T = 0{\cdot}17$ mequiv $l^{-1}°C^{-1}$ between 10 and 20°C would produce $\Delta pHa/\Delta T \simeq \Delta pN/\Delta T$ at $Pa_{CO_2} = 1{\cdot}0$ Torr, which was close to the observed situation between 10 and 20°C (Fig. 3). At higher Pa_{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 Pa_{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 (1978*a*) showing an *in vitro* open system slope of $-0{\cdot}12$ mequiv $l^{-1}°C^{-1}$ in *Callinectes* haemolymph, close to their *in vivo* slope of $-0{\cdot}16$ mequiv $l^{-1}°C^{-1}$, and the report of Randall & Cameron (1973) that $\Delta pH/\Delta T$ in trout blood *in vitro* was $-0{\cdot}019$ u $°C^{-1}$ at 0–1 Torr, but $-0{\cdot}005$ and $-0{\cdot}004$ u $°C^{-1}$ at 11 or 24 Torr.

Like $\Delta[HCO_3^-]/\Delta T$ in the open system, haemolymph β 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 (1976*b*) also demonstrated a linear relationship between total protein and β in *Carcinus* haemolymph. However, in the present study, β, 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 β per unit haemoglobin rose by 48% between 21·5 and 37°C, a proportionally similar change to that of haemolymph *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 β should be re-evaluated (e.g. Heisler & Neumann, 1980; Cameron, 1984*b*).

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 μequiv 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 $\Delta[HCO_3^-]i/\Delta T$ slope of $-0{\cdot}12$ mequiv $l^{-1}°C^{-1}$ 24 h after the acute change, the actual elevation in

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 $-0{\cdot}17$ mequiv $l^{-1}\,°C^{-1}$. This may not be unreasonable, as passive extracellular $\Delta[HCO_3^-]/\Delta T$ seemed to vary in parallel with β (Fig. 7), and intracellular β is typically three- to four-fold greater than extracellular β in poikilotherms (e.g. Heisler & Neumann, 1980; Cameron & Kormanik, 1982). However, other explanations are equally possible (e.g. metabolic adjustments, shifts in buffer composition, Pi_{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 & Kormanik (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.

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