J. exp. Biol. (1982), 99, 425–445
With 5 figures
Printed in Great Britain

RESPONSES TO HYPERSALINE EXPOSURE IN THE EURYHALINE CRAYFISH *PACIFASTACUS LENIUSCULUS*

I. THE INTERACTION BETWEEN IONIC AND ACID–BASE REGULATION

BY MICHÈLE G. WHEATLY AND B. R. McMAHON

Department of Biology, University of Calgary, 2500 University Drive NW, Calgary, Alberta, Canada T2N 1N4

(*Received* 30 *September* 1981 – *Accepted* 10 *February* 1982)

SUMMARY

Haemolymph iono- and osmoregulation and acid–base balance were recorded after 48 h exposure at 15 °C to a range of increasing ambient salinities (0, 25, 50 and 75% sea water) in the euryhaline crayfish *Pacifastacus leniusculus* (Dana). Except for K^+, concentrations of all measured inorganic ions and osmolality were significantly elevated in 50 and 75% SW. When compared with ambient changes there was evidence of a transition from hyper- to hypoionic regulation above 44% SW. Ca^{2+} was regulated for a constant blood–medium difference. A progressive reduction in total CO_2 was recorded; pH was maintained except in 75% SW where a haemolymph acidosis developed. To permit calculation of CO_2 tension (P_{CO_2}), carbon dioxide solubility coefficient (α_{CO_2}) and the apparent first dissociation constant of carbonic acid (pK'_1) were experimentally determined *in vitro*. α_{CO_2} decreased progressively with acclimation salinity but was unaffected by circulating protein. pK'_1 decreased as a function both of physiological pH and increasing haemolymph ionic strength. P_{CO_2}, calculated using these empirical constants, progressively decreased with high-salinity acclimation. The resulting 'hypocapnic alkalosis' was partially offset by a metabolic acidosis, whose correlation with extracellular anisosmotic and intracellular isosmotic regulation is discussed.

INTRODUCTION

Variation in ambient salinity exerts profound effects on iono- and osmoregulation, acid–base balance, respiratory gas exchange and intermediary metabolism in decapod crustaceans. Previous investigations have focused largely on the effects of external dilution in marine species. A. C. Taylor (1977) and E. W. Taylor, Butler & Al-Wassia (1977) both examined the ventilatory and cardiovascular responses of *Carcinus maenas* to dilute environments, and the effects of hyposaline exposure on O_2 transport for this species have been assessed by Truchot (1973) and for *Callinectes sapidus* by Weiland & Mangum (1975). Changes in O_2 uptake were related to amino acid metabolism by Gilles (1973) and the overall changes in intermediary metabolism during salinity stress

have been extensively reviewed by Huggins & Munday (1968) and Schoffeniels & Gilles (1970).

The present study is predominantly concerned with the interaction between ionic and acid–base regulation. Hydrogen ion concentration is determined by three independent quantities, the difference between strong acid anions and base cations (S.I.D.), the CO_2 partial pressure (P_{CO_2}) and the total weak acid present (Stewart, 1978). The S.I.D. may be adjusted by both branchial and urinary ion exchange mechanisms. P_{CO_2} may be directly affected by alterations in ventilatory/circulatory flows and additionally by changes in the chemical equilibria arising indirectly from ionic disturbance. Finally, total haemolymph protein levels may fluctuate with external salinity affecting haemolymph buffering capacity. An increase in haemolymph pH during exposure to hyposaline environments was reported in *Carcinus* (Truchot, 1973, 1981) and in *Callinectes* (Weiland & Mangum, 1975). This alkalosis was due to excess base related to modifications in ion exchange rather than respiration, since P_{CO_2} was maintained.

In the present study, the contrasting problem of hypersaline exposure in a euryhaline freshwater species was investigated. Successful invasion of fresh water demands active uptake of ions, production where possible of a hyposmotic urine, and reduction in boundary permeability in order to maintain a hyperosmotic condition of the blood (Kirschner, 1979). As the external concentration is increased, equilibration with haemolymph is attained, whereafter the nature of the response may alter to ensure hyporegulation of body fluids. By comparison, the problem of hyposaline exposure is essentially unidirectional.

The species selected for study was the western North American crayfish *Pacifastacus leniusculus*, which extends into brackish water up to 13‰ along regions of the Pacific coast in Oregon and Washington (Miller, 1960). The ability of this species to regulate over a wide range of salinities was recently demonstrated by Kerley & Pritchard (1967) and contrasts with the lower salinity tolerance exhibited by most freshwater species (cf. Hermann, 1931; Lienemann, 1938; Bryan, 1960).

Changes in haemolymph acid–base status resulting from exposure to a range of increasing ambient salinities were examined and correlated with ion- and osmoregulatory capacity. In the course of the investigations, the constants pK_1' and α_{CO_2} were derived experimentally under controlled *in vitro* conditions and used for accurate calculation of P_{CO_2} via the Henderson–Hasselbalch equation. Values have been published for the brachyuran crab *C. maenas* (Truchot, 1976) but problems inherent in their application to other species have recently been presented (Wilkes, deFur & McMahon, 1980; deFur, Wilkes & McMahon, 1980).

MATERIALS AND METHODS

1. *Experimental animals and maintenance*

Experiments were performed on adult *Pacifastacus leniusculus leniusculus* (Dana, 1852), identified from taxonomic criteria of Miller (1960), of either sex and wet weight 57·3–106·8 g, which were obtained from commercial sources (Monterey Bay Hydroculture, California) and judged to be in intermoult stage C (Drach & Tchernigovtzeff, 1967). Prior to experimentation they were housed for at least two weeks in holding

tanks 120 × 62 × 10 cm deep continuously provided with flowing well-aerated, dechlorinated Calgary tap water (*ca.* 750 ml min^{-1}) maintained at the acclimation temperature of 15 ± 1 °C, and fed twice weekly on chopped smelt.

2. *Salinity regime*

Crayfish were exposed to experimental salinities equivalent to 25, 50 and 75% sea water, which were obtained by diluting artificial sea water (Instant Ocean – s.g. 1·020–1.023 at 24 °C) with tap water (see Table 1). As a change in salinity will alter the acid–base status of the medium and thereby the extracellular balance (Dejours, Armand & Gendner, 1978; Truchot, Toulmond & Dejours, 1980), ambient pH and titratable alkalinity were also continually monitored, the latter by HCl titration following the procedure of Strickland & Parsons, 1965 (see Truchot *et al.* 1980).

3. *Experimental protocol*

Crayfish were housed individually in chambers of dimensions 21 × 14 × 7 cm deep (volume 2 l) supplied at a continuous rate of 40 ml min^{-1} with recirculated well-aerated water (O_2 tension (P_{O_2}) ≃ 136 torr–elevation 1048 m), maintained at the experimental temperature. The salinity was raised rapidly and without animal disturbance by switching to an alternative circulation.

Haemolymph was anaerobically sampled from submerged crayfish in fresh water or acclimated for 48 h to each of the experimental salinities, analysed immediately for osmolality, pH and total CO_2, and subsamples frozen rapidly for ion analysis. Individual animals were thus sampled once only in view of the reported detrimental effect on acid–base balance of repetitive sampling (Truchot, 1975; McMahon, Butler & Taylor, 1978).

Pre-(venous, *v*) and post-(arterial, *a*) branchial samples were removed respectively from the ventral sinus via the arthrodial membrane at the base of the right 4th walking leg and from the pericardial sinus through a small hole (< 1 mm) drilled through the carapace above the pericardium and lateral to the heart, which had been prepared and sealed with a latex diaphragm one week prior to sampling (see McDonald, 1977).

4. *Analytical procedures*

(*a*) In vivo

Osmolality and inorganic ions. Osmolality was determined on a 7 μl subsample using a vapour pressure osmometer (Wescor-5100 B). Inorganic ions were routinely analysed on frozen subsamples, preliminary comparison with test measurements on fresh haemolymph having indicated that changes had not resulted from freezing. Cations were analysed by atomic absorption spectrophotometry (Jarrell-Ash 850) using single-element hollow cathode lamps (Corning). Mg^{2+} and Ca^{2+} were diluted 1 in 500 with 0·1% $LaCl_3.6H_2O$, and K^+ and Na^+ were diluted 1 in 500 and 4000 respectively in 0·1% CsCl, to suppress chemical interference occurring in an air/acetylene flame and calibrated against similarly diluted certified stock solution (Fisher). Cl^- concentration was determined on a 20 μl subsample using a digital chloridometer by coulometric titration with silver ions (Searle Buchler 4-2500).

Acid–base status. pH was determined on a 50 μl subsample using a liquid junction capillary electrode (Radiometer, G299A) connected to an acid–base analyser (Radio-

meter, PHM71) and calibrated against precision buffers. Total CO_2 (Σ_{CO_2}) was measured on a 40 μl subsample either using the Cameron technique (1971) or a Corning 960 CO_2 analyser. Results using both methods on the same sample agreed to $\pm$ 2%. CO_2 tension (P_{CO_2}) was calculated using values for solubility coefficient of CO_2 (α_{CO_2}) and pH-dependent first apparent-dissociation constant of carbonic acid (pK_1') experimentally determined *in vitro*.

(*b*) In vitro *determination of* α_{CO_2} *and* pK_1'

Measured changes in haemolymph ionic strength will affect CO_2 solubility and equilibrium constants as outlined in Albers (1970). The use of correct values in the calculation of P_{CO_2} is imperative, since an error of 0·01 unit in pK_1' for example can result in an error of 2·3% (Severinghaus, Stupfel & Bradley, 1956). The values derived for the shore crab by Truchot (1976) cannot be employed for freshwater decapods whose haemolymph ionic concentrations and ion ratios are markedly different, since they lack correction for pH dependence (see Albers & Pleschka, 1967). Furthermore, Truchot's salinity-dependent nomograms, which were calculated for varying external [Na^+] by assuming a constant ratio between values in sea water and serum, did not take into consideration variations in haemolymph buffering capacity which the present investigation demonstrates to be an important response to salinity acclimation. Whilst a new operational approach has proved useful for calculation of P_{CO_2} in freshwater crustaceans (Wilkes *et al.* 1980; Morgan & McMahon, 1982) the method is limited to a prescribed set of experimental conditions, and in the present study where four experimental media are involved it was considered necessary to derive these constants experimentally.

α_{CO_2}. α_{CO_2} was determined in haemolymph from crayfish in fresh water or acclimated for 48 h in each experimental salinity by the method outlined in Truchot (1976). Briefly, solubility was determined in a salt-free solution in which precipitated proteins are redissoluble below the isoelectric point during the necessary acidification of the blood (Redfield, Mason & Ingalls, 1932). This indirect method was based on the assumption that solubility in whole haemolymph can be considered as that of a solvent modified by the presence of protein.

Serum was dialysed for 36 h against four changes of distilled water and two changes of $1{\cdot}2 \times 10^{-3}$N-HCl at 4 °C. Wheatly & McMahon (1982*b*) demonstrated that the deterioration of haemocyanin over this time period was minimal. Protein concentration was assayed according to the dye-binding method of Bradford (1976) using Coomassie Brilliant Blue G-250, and Σ_{CO_2} was determined at 15 °C after 40 min equilibration with 40% CO_2 (P_{CO_2} *ca.* 300 torr) in gently shaken round-bottomed tonometers. α_{CO_2} was similarly determined in acidified distilled water and saline solutions corresponding to the ionic levels found in the haemolymph after equilibration to the experimental salinities, with the intention of applying to these a protein-concentration-dependent correction.

pK_1'. The first and second apparent carbonic acid dissociation constants are defined as:

$$pK_1' = pH - \log \frac{[HCO_3^-]}{\alpha_{CO_2} . P_{CO_2}} \quad \text{and} \quad pK_2' = pH - \log \frac{[CO_3^{2-}]}{[HCO_3^-]}$$

pK_1' was determined gasometrically. Haemolymph from individual animals accli-

mated in each of the experimental salinities was declotted and sequentially equilibrated to P_{CO_2} 1·32, 2·64, 5·28, 7·92 and 10·56 torr for FW, or 1·32, 3·96, 7·92 and 13·20 torr for 25, 50 and 75% SW (all 21% O_2) for 30 min with the addition of 1 μg ml^{-1} of carbonic anhydrase. A 100 μl aliquot was analysed for pH and Σ_{CO_2} at each P_{CO_2} level. CO_2 buffering capacity was estimated in each acclimation salinity by calculating the mean buffer value ($\Delta[HCO_3^- + CO_3^{2-}]/\Delta pH$) and plotting a line of this gradient at the mean native $[HCO_3^- + CO_3^{2-}]$ and at physiological pH.

Since in crustaceans $[CO_3^{2-}]$ can, depending on pH, form an appreciable part of measured Σ_{CO_2} and yet cannot be determined independently of $[HCO_3^-]$, pK_2' was estimated from Lyman (1956), for a sea water of corresponding ionic strength, corrected for pH dependence (−0·102 per pH unit according to Albers & Pleschka, 1967) and used to assess the contribution of CO_3^{2-} to Σ_{CO_2} using the second dissociation equation. pK_1' was then calculated at a range of pH values, by substitution of corrected $[HCO_3^-]$, measured pH, equilibration P_{CO_2} and experimentally determined α_{CO_2} into the first dissociation equation.

P_{CO_2} values calculated using these constants and others existing in the literature were compared.

Values throughout are expressed as mean ± 1 S.E.M. and significance at the 5% level as calculated by Student's *t*-test (unpaired) is denoted.

RESULTS

No mortality resulted from exposure of *P. leniusculus* to any of the salinities for the duration of the present experiment.

Osmolality and inorganic ions

Osmolality and concentrations of inorganic ions in postbranchial haemolymph are given for each experimental salinity in Table 1. Afferent and efferent values were not significantly different.

Except for K^+, which remained constant throughout, all major inorganic ions were significantly elevated in 50% SW and osmolality likewise in 75% SW. These increases however were modest in comparison with ambient changes. Fig. 1 indicates the difference between blood and medium ion levels and osmolality. In fresh water, *Pacifastacus* maintains the ECF hyperosmotic and hyperionic with respect to ambient. With the exception of Ca^{2+}, a similar trend was observed in all ions and osmolality during hypersaline exposure. Essentially, the magnitude of the blood–medium difference became progressively reduced and increasingly negative, implying a change to hypoionic regulation. Regression analysis determined that the transition point occurred at 427 mOsm kg^{-1} external osmolality (approximately 44% SW). Isoionicity with respect to Cl^- was attained at this salinity (corresponding to an external $[Cl^-]$ of 204·5 m-equiv l^{-1}). The medium did not become isoionic with respect to either Na^+ or K^+ until 51% SW (external concentrations of 254·6 and 4·6 m-equiv l^{-1} respectively). $[Mg^{2+}]$ in the haemolymph in fresh water is maintained only marginally hyperionic, and equilibration with the external milieu occurred at 5·08 m-equiv l^{-1} external concentration and thereafter was maintained increasingly hypoionic.

Ca^{2+} regulation departed from this overall pattern. Levels in local tap water and

Table 1. *Measured concentrations of inorganic cations and anions and osmolality in post-branchial haemolymph of* Pacifastacus *in fresh water and exposed for* 48 *h in dilutions of sea water*

(The values given in parentheses are the major ionic composition, titration alkalinity and pH of Calgary tap water and dilutions of artificial sea water used for acclimation experiments. Ammonium ion concentration represents the accumulation over 48 h recirculation. Values are expressed as mean ±S.E.M. with *n* numbers listed at the head of each column, and asterisks denote significance from FW values.)

	FW $n = 11$ ($n = 8$)	25% SW $n = 11$ ($n = 8$)	50% SW $n = 7$ ($n = 8$)	75% SW $n = 6$ ($n = 8$)
$[Na^+]$ (m-equiv l^{-1})	199·31 ±15·98 (1·33 ±0·04)	207·48 ±17·77 (129·70 ±8·40)	256·67* ±6·72 (250·40 ±6·40)	289·83* ±4·80 (381·60 ±10·20)
$[K^+]$ (m-equiv l^{-1})	4·51 ±1·15 (0·01 ±0·00)	4·68 ±0·91 (2·19 ±0·09)	±4·14 (±4·03 ±0·85 (±0·41)	4·88 ±0·83 (7·13 ±0·20)
$[Mg^{2+}]$ (m-equiv l^{-1})	5·46 ±0·36 (3·12 ±0·04)	5·64 ±0·38 (21·80 ±1·80)	7·44* ±0·86 (40·80 ±6·80)	9·98* ±0·28 (73·40 ±4·20)
$[Ca^{2+}]$ (m-equiv l^{-1})	15·52 ±2·26 (6·44 ±1·64)	15·56 ±2·38 (6·82 ±0·12)	20·52* ±1·36 (14·72 ±1·84)	28·08* ±3·24 (22·20 ±2·86)
$[Cl^-]$ (m-equiv l^{-1})	169·69 ±6·72 (1·46 ±0·07)	174·40 ±7·77 (142·70 ±2·10)	210·63* ±8·89 (275·00 ±8·30)	267·72* ±17·10 (419·10 10·20)
Osmolality (mOsm kg^{-1})	366·3 ±21·3 (low)	410·3 ±7·9 (259·8 ±1·3)	430·3 ±22·4 (498·8 ±7·1)	470·1* ±14·1 (717·8 ±8·8)
$[NH_4^+]$ (μ-equiv l^{-1})	(0·25 ±0·06)	(4·50 ±0·12)	(low)	(1·15 ±0·07)
Titration alkalinity (m-equiv l^{-1})	(0·91 ±0·08)	(0·63 ±0·04)	(1·20 ±0·14)	(1·93 ±0·27)
pHw	(7·39 ±0·40)	(7·78 ±0·09)	(7·94 ±0·12)	(8·00 ±0·05)

25% SW were essentially similar and in both cases haemolymph concentrations were 6–8 m-equiv higher. This differential was maintained during hypersaline exposure.

Acid–base status – pH and Σ_{CO_2}

No significant change in pH followed exposure to either 25 or 50% SW (Table 2), although a slight (non-significant) alkalosis was observed in mean pH in 25% SW. A significant acidosis, greater in pre- (0·147) than postbranchial haemolymph (0·122 pH units) resulted from 48 h exposure to 75% SW. A progressive reduction in Σ_{CO_2} accompanied these changes and became significant around 50–75% SW.

In vitro *determinations*

α_{CO_2}. Protein concentration, which was 63·59 ± 6·17 (15) mg ml^{-1} in fresh water decreased significantly to 37·30 ± 5·44 (5), 44·62 ± 2·17 (16) and 46·75 ± 2·71 (15) mg ml^{-1} respectively in 25, 50 and 75% SW. Circulating levels however did not influence CO_2 solubility since the α_{CO_2} values measured in salt-free acidified haemolymph protein solutions in no case differed from the value of 0·0576 mmol l^{-1} $torr^{-1}$ measured in acidified distilled water. Solubility in acidified salines equivalent in ionic strength to haemolymph after acclimation to the experimental salinities (from Table 1)

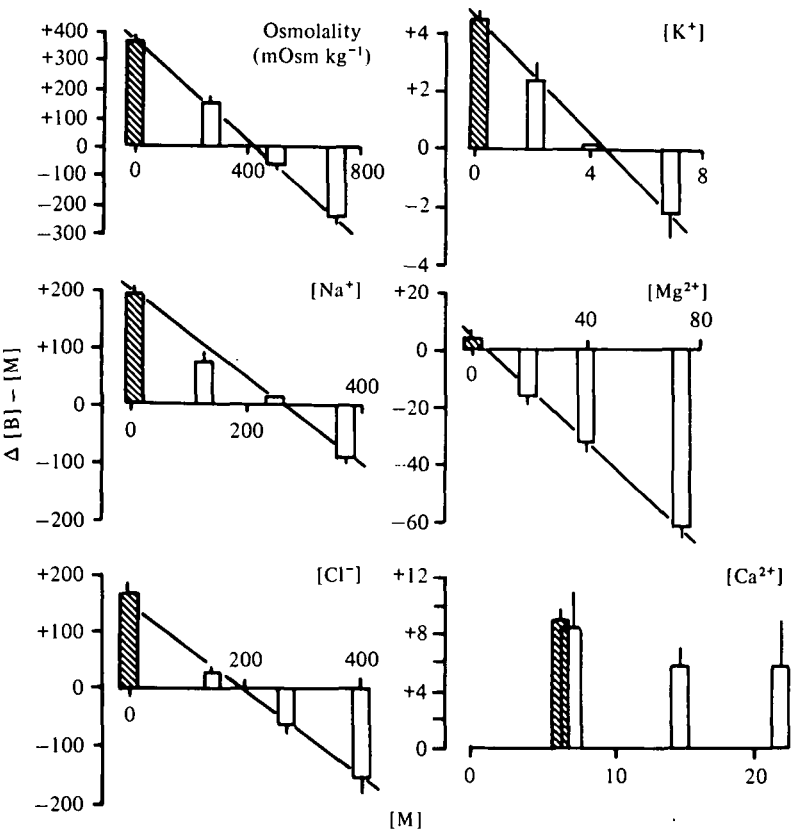

Fig. 1. Variation of blood–medium differences (Δ[B] – [M]) in osmolality and major inorganic ion concentrations in postbranchial haemolymph (ordinate) against levels in the external milieu ([M]-abscissa) for *Pacifastacus* in fresh water (cross-hatched) and after 48 h acclimation in 25, 50 and 75 % SW. Values are expressed as mean ±S.E.M. (*n* values given in Table 1) and the units of all axes m-equiv l^{-1} unless otherwise stated. Regression lines indicated have the following equations:

Osmolality	$\Delta[B]-[M] = 366{\cdot}8-0{\cdot}859\,[M]$	$r = 0{\cdot}999$	for $n = 4$
Na^+	$\Delta[B]-[M] = 189{\cdot}9-0{\cdot}746\,[M]$	$r = 0{\cdot}996$	
Cl^-	$\Delta[B]-[M] = 155{\cdot}6-0{\cdot}761\,[M]$	$r = 0{\cdot}994$	
K^+	$\Delta[B]-[M] = 4{\cdot}4-0{\cdot}966\,[M]$	$r = 0{\cdot}994$	
Mg^{2+}	$\Delta[B]-[M] = 4{\cdot}7-0{\cdot}931\,[M]$	$r = 0{\cdot}999$	

Table 2. *pH and total CO_2 content (Σ_{CO_2}) of post-(a) and prebranchial (v) haemolymph of* Pacifastacus *acclimated to FW and 25, 50 and 75 % SW*

(Values expressed as mean ±S.E.M. with number of observations in parentheses. Asterisks denote significance from settled FW values.)

	FW	25 % SW	50 % SW	75 % SW
pH_a	7·954 ±0·024 (9)	8·000 ±0·033 (8)	7·964 ±0·037 (7)	7·832* ±0·015 (6)
pH_v	7·941 ±0·025 (9)	7·953 ±0·024 (8)	7·911 ±0·041 (7)	7·794* ±0·022 (6)
Σ_{a,CO_2} (mmol l^{-1})	8·77 ±1·21 (11)	5·92 ±1·31 (5)	4·92* ±0·95 (10)	2·93* ±1·47 (9)
Σ_{v,CO_2} (mmol l^{-1})	8·92 ±0·34 (11)	6·12 ±1·04 (5)	5·26* ±1·49 (10)	3·00* ±1·87 (9)

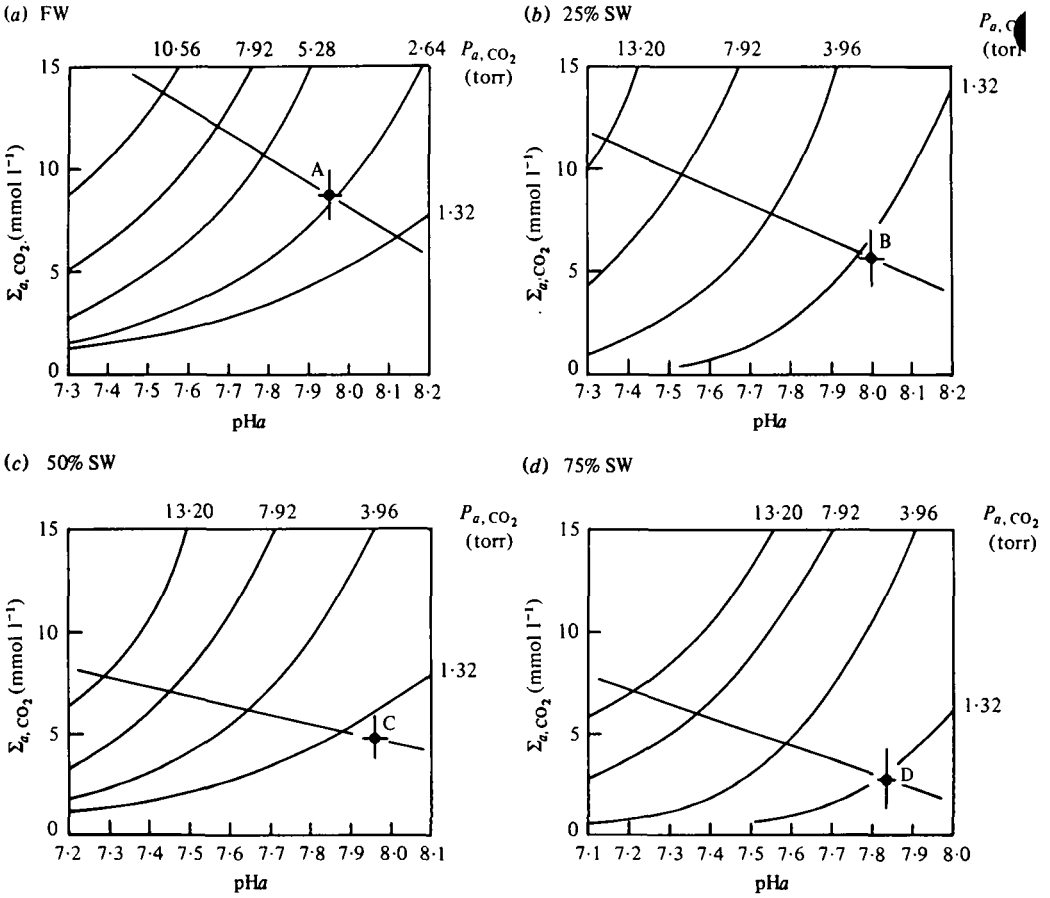

Fig. 2. CO_2 buffer curves constructed from haemolymph measurements on a number of individual *Pacifastacus* in fresh water (*a*) or acclimated for 48 h in 25, 50 or 75 % SW (*b*, *c*, *d* respectively). Mean buffer values in FW, 25, 50, 75 % SW were $-11{\cdot}59 \pm 1{\cdot}96$ (n = 11), $-8{\cdot}55 \pm 1{\cdot}92$ (n = 5), $-4{\cdot}73 \pm 0{\cdot}78$ (n = 10) and $-7{\cdot}02 \pm 1{\cdot}53$ (n = 9) mmol l^{-1} pH unit^{-1}. Lines of these slopes were constructed to pass through mean values of physiological pH (pH$_a$) and total CO_2 (Σ_{a,CO_2}) – A–D (taken from Table 2). The resulting CO_2 buffer lines have the following equations:

FW	Σ_{a,CO_2}	$= 100{\cdot}99 - 11{\cdot}59\ pH_a$
25 % SW		$= 74{\cdot}14 - 8{\cdot}55$
50 % SW		$= 42{\cdot}48 - 4{\cdot}73$
75 % SW		$= 57{\cdot}75 - 7{\cdot}02$

The curved CO_2 isopleths were fitted by linearizing log Σ_{CO_2} against log pH plots according to the method of Wilkes *et al.* (1980).

was therefore assumed to be representative of that in whole serum. Mean values of $0{\cdot}0563 \pm 0{\cdot}0002$ (5), $0{\cdot}0561 \pm 0{\cdot}0002$ (5), $0{\cdot}0554 \pm 0{\cdot}0003$ (5) and $0{\cdot}0544 \pm 0{\cdot}0005$ (5) mmol l^{-1} torr^{-1} were measured in FW, 25, 50 and 75 % SW respectively, indicating a progressive decrease in CO_2 solubility with acclimation salinity.

pK'_1. CO_2 buffer curves combined from a number of individual animals in each acclimation salinity (Fig. 2) demonstrated that, accompanying the progressive reduction in circulating [$HCO_3^- + CO_3^{2-}$] during hypersaline exposure, there was also a

Table 3. *Estimations of* pK_2' *from Lyman* (1956) (*corrected for variation in pH and ionic strength as outlined in methods section*) *determined at pH levels from Fig.* 2 *of the points of intersection of* CO_2 *isopleths and buffer lines. These data were then employed in the calculation of* pK_1' (*see Fig.* 3)

	pH	Estim. pK_2'
FW	8·124	9·70
	7·969	9·72
	7·788	9·74
	7·668	9·76
25 % SW	7·971	9·70
	7·756	9·73
	7·524	9·76
	7·343	9·78
50 % SW	7·884	9·62
	7·638	9·64
	7·453	9·67
	7·275	9·69
75 % SW	7·817	9·47
	7·574	9·49
	7·350	9·52
	7·194	9·54

reduction in the CO_2 buffer value (expressed as $\Delta[HCO_3^- + CO_3^{2-}]/\Delta pH$) which declined from −11·6 in FW to −8·56, −4·73 and −7·02 in 25, 50 and 75 % SW respectively. Equations describing each of the curved CO_2 isopleths were obtained by linearizing the plot of log Σ_{CO_2} *vs.* log pH for all the animals in each acclimation salinity using the operational procedure devised by Wilkes *et al.* (1980). The points of intersection of these with the buffer line were employed in the calculation of pK_1' over the given pH range. Table 3 indicates the values for pK_2' which were used in the estimation of the ratio $[CO_3^{2-}]/[HCO_3^-]$. Having deducted molecular CO_2 (i.e. $\alpha_{CO_2} \times P_{CO_2}$-using experimentally determined α_{CO_2} and equilibration P_{CO_2}) from measured Σ_{CO_2}, $[HCO_3^-]$, and thereby pK_1', could be estimated from the determined ratio.

Variation with pH. The regression analyses for pH dependence of pK_1' in fresh water, 25 and 50 % SW (see legend to Fig. 3) show reasonable closeness of fit, and there is good correlation between haemolymph salinity ($[Cl^-] \times 1{\cdot}807$) and both Y intercept and gradient as follows.

$$\text{Y intercept} = -0{\cdot}449 + 0{\cdot}711\ (\text{salinity}); \quad r = 0{\cdot}995, n = 3$$

$$\text{Gradient} = -0{\cdot}908 + 0{\cdot}096\ (\text{salinity}); \quad r = 0{\cdot}999, n = 3$$

From these data the pH dependence of pK_1' in 75 % acclimation salinity was predicted as

$$pK_1' = 11{\cdot}633 - 0{\cdot}727\ (\text{pH})$$

since that determined experimentally exhibited a poor correlation coefficient ($pK_1' = 6{\cdot}138 + 0{\cdot}015$ (pH); $r = 0{\cdot}228$).

Operational pK_1' decreased as a function of pH (see Fig. 3), and the pH dependence increased with ionic strength of the haemolymph. This contention is supported by comparison of the FW and 25 % SW lines, where gradients were similar (−0·138 and

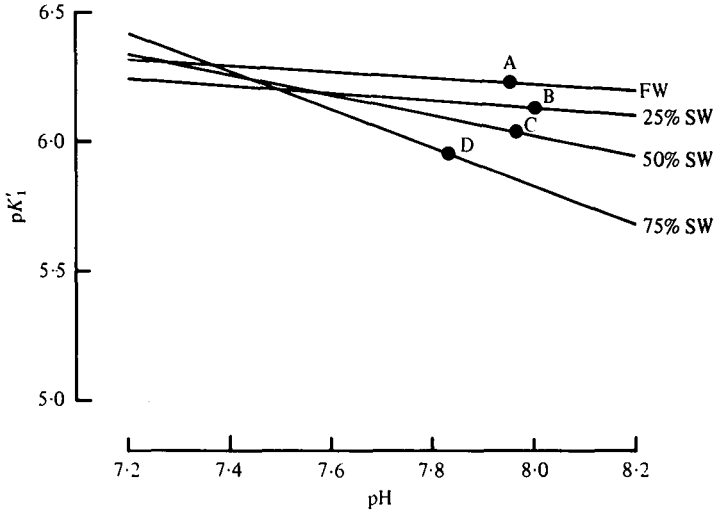

Fig. 3. Operational pK_1' of carbonic acid as a function of pH in haemolymph from *Pacifastacus* acclimated in FW, 25, 50 or 75 % SW. Points A–D indicate physiological pH at each acclimation salinity. Regression equations:

FW	pK_1' =	7·319 − 0·138 (pH) (n = 4)	r = 0·951
25 % SW	=	7·336 − 0·152	0·628
50 % SW	=	9·087 − 0·382	0·960
75 % SW	=	11·633 − 0·727	*Consult text

−0·152 per pH unit respectively) yet ion concentrations were unchanged (Table 1). The difference in elevation of these two lines indicates that other changes in blood composition may affect the absolute values of pK_1'. At physiological values of pH, operational pK_1' became progressively reduced with increasing acclimation salinity.

Variation with ionic strength. According to Debye-Hückel (1923) and Lewis & Randall (1923), the behaviour of strong electrolytes (in this case the haemolymph) can be described by the mathematical formula.

$$\mu = \tfrac{1}{2}\Sigma C v^2$$

where μ is the ionic strength of the haemolymph and C and v are the molal concentration and valency of each ionic species. This equation was used to calculate the ionic strength of *Pacifastacus* haemolymph in each experimental salinity, using concentrations of inorganic ions given in Table 1 and a value for $[SO_4^{2-}]$ of 4·98 m-equiv l^{-1}, determined in fresh water for *Procambarus* by Morgan & McMahon (1982), assuming that it would increase in similar proportions to $[Cl^-]$ in hypersaline media. μ was calculated as 0·2306, 0·2366, 0·2892 and 0·3500 in FW and 25, 50 and 75 % SW respectively. The reduction in pK_1', interpolated at physiological pH from Fig. 3, with μ is shown in Fig. 4.

Calculation of P_{CO_2}. Experimentally determined values for pK_1', corrected for pH and ionic strength, were then used in conjunction with estimated pK_2' and measured values of pH, Σ_{CO_2} and α_{CO_2} to solve the Henderson–Hasselbalch equations for $[HCO_3^-]$, $[CO_3^{2-}]$ and ultimately P_{CO_2} (Table 4). Calculated values are also given using pK_1' values for sea waters of comparable ionic strength taken from Lyman (1956)

Table 4. *Values of* $[HCO_3^-]$, $[CO_3^{2-}]$ *and* P_{CO_2} *calculated at physiological pH and* Σ_{CO_2} *in postbranchial haemolymph using* pK_1' *experimentally derived in the present investigation for* Pacifastacus *acclimated in fresh water and* 25, 50 *and* 75% *sea water*

(For comparison, the same values have been computed using pK_1' values taken from: (1) Lyman (1956) for sea water solutions of comparable salinity. (2) Truchot (1976) for *Carcinus* haemolymph at an acclimation salinity corresponding to ionic concentrations found in *Pacifastacus* haemolymph assuming the relationship described between $[Na^+]$ and external concentration for *Carcinus* given in Truchot (1973).)

	pH_a	Σ_{a,CO_2} (mmol l^{-1})	α_{CO_2} (mmol l^{-1} $torr^{-1}$)	Estimated pK_2'	$[CO_3^{2-}]_a/[HCO_3^-]_a$		pK_1'	$[HCO_3^-]_a$ calculated (m-equiv l^{-1})	$[CO_3^{2-}]_a$ calculated (m-equiv l^{-1})	P_{a,CO_2} calculated (torr)
Fresh water	7·954	8·77	0·0563	9·72	0·0171		6·223	8·468	0·1448	2·794
						1	6·242	8·461	0·1447	2·917
						2	6·133	8·496	0·1450	2·279
25% SW	8·000	5·92	0·0561	9·70	0·0200		6·119	5·729	0·1150	1·343
						1	6·239	5·707	0·1140	1·764
						2	6·126	5·729	0·1150	1·365
50% SW	7·964	4·92	0·0554	9·61	0·0226		6·044	4·756	0·1075	1·032
						1	6·213	4·729	0·1070	1·514
						2	6·173	4·746	0·1070	1·210
75% SW	7·832	2·93	0·0544	9·47	0·0230		5·937	2·829	0·0651	0·662
						1	6·173	2·803	0·0650	1·130
						2	6·104	2·812	0·0650	0·967

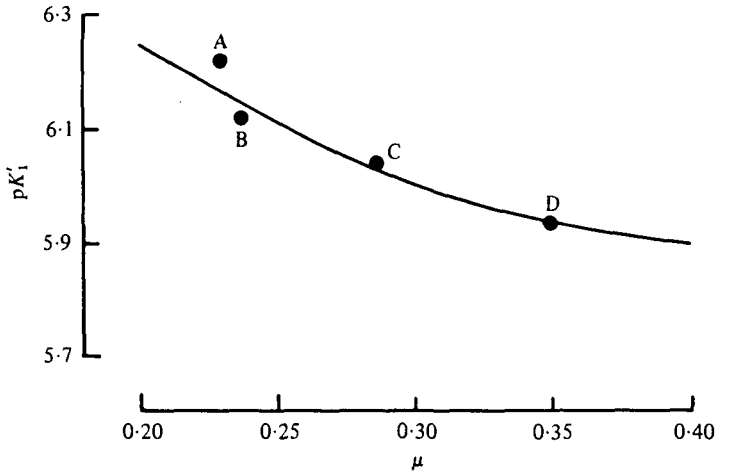

Fig. 4. pK_1' as a function of ionic strength (μ) of the haemolymph in *Pacifastacus* acclimated to FW(A) and 25, 50 or 75 % SW (B, C, D). The curve fitted has the regression equation

$$pK_1' = 7{\cdot}239 - 2{\cdot}211\sqrt{\mu}\ (r = 0{\cdot}951, n = 4)$$

and also from Truchot (1976) at a salinity corresponding to ion levels found in *Pacifastacus*, assuming the relationship between $[Na^+]$ and external concentration for *Carcinus* in Truchot (1973). This analysis attempted to highlight sources of potential inconsistencies in the calculation of CO_2 tension. From *in vivo* measurements of pH and Σ_{CO_2} it is evident that during hypersaline exposure P_{CO_2} progressively decreased; $[HCO_3^-]$ and $[CO_3^{2-}]$ were also reduced.

DISCUSSION

In the discussion which follows *in vitro* determinations have been critically appraised independently of *in vivo* measurements which resulted from hypersaline exposure.

In vitro

α_{CO_2}. The mean value of α_{CO_2} measured in acidified distilled water (0·0576 mmol l^{-1} $torr^{-1}$) agrees with values reported by Truchot (1976) and Bartels & Wrbitzky (1960) at 15 °C. All, for some reason, yield lower values than 0·0593 mmol l^{-1} $torr^{-1}$ measured gravimetrically by Murray & Riley (1971), although Van Slyke *et al.* (1928) postulate that the depression of α_{CO_2} caused by acidification was accountable for only 0·01 % of the reduction.

This investigation confirmed that circulating protein levels do not affect CO_2 solubility, which is effectively a function of ionic strength of the haemolymph. The α_{CO_2} values determined agree well with predictions from Bohr's original data derived from Buch (1951) and corrected in accordance with Lyman (1956) for solutions of NaCl, and also with the more recent data of Murray & Riley (1971) for solutions of comparable salinity.

Agreement with Truchot's data (1976) for haemodilution in *Carcinus* is not so good.

When exposed to dilute media, *Carcinus* exhibits some reduction in [Na^+], but never to the level recorded in *Pacifastacus* in fresh water. For this reason, in order to use the salinity nomograms constructed by Truchot, considerable extrapolation must be employed. The values of 0·0543, 0·0542, 0·0539 and 0·0537 mmol l^{-1} $torr^{-1}$, which correspond to FW and 25, 50 and 75% SW respectively, are 4% lower than the measured values.

pK'_1. One potential source of error in the determination of pK'_1 was the estimation of pK'_2 which was unavoidable in the absence of a method for separately determining [HCO_3^-] and [CO_3^{2-}]. In particular, in view of the data obtained for pK'_1 (i.e. Fig. 3), the pH dependence which was taken from work on mammalian blood (Albers & Pleschka, 1967) may have been underestimated, especially at increased ionic strengths. None the less, the ratio of [CO_3^{2-}]/[HCO_3^-] is never greater than 3%, and the resulting errors may be insignificant.

In all experimental salinities pK'_1 decreased with haemolymph pH. The pH dependence in FW and 25% SW was similar to that observed in mammalian blood, but appeared to increase with the ionic strength of the haemolymph, although there is little in the literature with which to compare this finding. In addition, factors other than ionic strength may well affect the absolute levels of pK'_1 calculated.

In *Carcinus*, Truchot (1976) described the variation of pK'_1 with μ as

$$pK'_1 = 6{\cdot}419 - 0{\cdot}5\sqrt{\mu},$$

the same equation for NaCl + $NaHCO_3$ solutions of differing ionic strength as that of Hastings & Sendroy (1925), where 6·419 is the true carbonic acid first dissociation constant extrapolated to $\mu = 0$ (Harned & Davis, 1943). The discrepancy observed in comparison with the present investigation may be explained by procedural differences. Truchot's determinations were made in serum dialysed against saline solutions of varying ionic strength and may mask the effects on pK'_1 of changes in other haemolymph constituents. Secondly, Hastings & Sendroy's equation is only valid for μ values below 0·2, above which complications arise due to changing dielectric properties of the solvent. Lastly, the estimation of μ in the present investigation may not have taken into account certain unidentified polyvalent ions. Moreover, ionic interactions in biological fluids may well be more complex than in aqueous ionic solutions.

Empirical values for pK'_1 are consistently lower than those predicted by Lyman (1956) for sea water solutions of comparable salinity. The determination of pK'_1 from Truchot's nomograms meets with similar difficulties in interpretation as were experienced for α_{CO_2}, and yields values generally higher than those experimentally determined, though exhibiting less variation with acclimation salinity. The extent of the error inherent in their use in the determination of CO_2 tension is demonstrated in Table 4. Values for pK'_1 from Lyman (1956) vary from 6·242 (FW) down to 6·173 (75% SW) and result in calculated P_{CO_2}s which are 4·4, 31·4, 46·7 and 70·6% higher than those calculated in the present investigation in fresh 75% SW, the discrepancy becoming exacerbated at low P_{CO_2}. Truchot's (1976) values for pK'_1 show still less variation as a function of ionic strength. The pK'_1 derived in FW results in an 18·4% underestimation of P_{CO_2}, those in 50 and 75% SW in 17·3 and 46·1% overestimations respectively, while the 25% SW value provides an accurate estimation.

Direct measurement of P_{CO_2} is difficult owing to the low levels of P_{CO_2} characteristic of waterbreathers (Rahn, 1966), and is complicated by rapid clotting in this species. A technique for direct measurement of P_{CO_2} has recently been described (deFur *et al.* 1980) and is to be used to confirm calculated values on haemolymph from high salinity acclimated animals which exhibits reduced tendency for agglutination (Troup *et al.* 1961).

In vivo. Although chronic tolerance of hypersaline exposure has not been tested in the present study, our animals survived in 75% SW for 48 h. Kerley & Pritchard (1967) have reported an LD 50 of 8 days in 90% SW. In contrast, although *Potamobius astacus* survived 6 weeks exposure to 50% SW, it rapidly succumbed at higher levels (Hermann, 1931), and Kentall & Schwarz (1964) report LD 50 values of 72–96 h for *Orconectes virilis* and *Cambarus bartonii* in 50% SW. The greater salinity tolerance of *P. leniusculus* is associated with an ability for hypo/hyperosmotic regulation, which is found in some other crustaceans (e.g. *Pachygrapsus crassipes* – Prosse, Green & Chow, 1955), but is not unexpected since this species extends into coastal river outlets (Smith, 1959), although rarely in salinities above $\frac{1}{3}$ sea water (Miller, 1960).

The measured levels of haemolymph osmolality and major inorganic ions show good agreement with values for other freshwater crayfish (see Taylor & Wheatly, 1982; Morgan & McMahon, 1982; Wilkes & McMahon, 1982). The suspected mechanisms of hyper-regulation should perhaps be clarified before considering how they may be adapted for regulation in iso- or hyperosmotic media. The crayfish shows two characteristics diagnostic of complete adaptation to fresh water. First, a reduction in ECF concentration minimizes the gradient for H_2O and NaCl diffusion (although water permeability is high) and the production of a dilute urine (implicating renal filtrate reabsorption) reduces salt loss. NaCl is actively transported across the branchial epithelium by ion exchange mechanisms, hydration of metabolic CO_2 producing the counterions (H^+ and HCO_3^-) at a rate commensurate with normal ion transfer (Kirschner, 1979).

The increases in osmolality and inorganic ion levels during hypersaline exposure were significant but modest when compared to ambient changes, indicating a switch to hyporegulation. Hyperosmotic regulators generally become isosmotic as the external concentration is raised (see Lienemann, 1938; Bryan, 1960). Facing now the reverse problem of desiccation and salt loading, these animals must drink the medium and actively extrude NaCl. Kerley & Pritchard (1967) found that *Pacifastacus* does not extensively drink the medium even in hypersaline conditions. Cells structurally analogous to chloride cells of fish gills have been observed in crustaceans by Copeland (1967) and may account for Cl^- extrusion. Na^+ extrusion is more controversial. Na^+/K^+ ATPase may be important, as may reversal of the Na^+/NH_4^+ exchange, which operates in low salinity (Henry & Mangum, 1980). Generally, renal salt extrusion is less important, and the best these animals can do is to produce an isosmotic urine (Potts & Parry, 1964). Kerley and Pritchard's study, which examined intracellular osmotic regulation during hypersalinity, concluded that osmotic regulation in *Pacifastacus* does not involve shifts in water between body fluid compartments. The relative contribution of these mechanisms, however, awaits a critical analysis of renal and branchial function in saline-stressed animals, perhaps incorporating radioisotope flux studies.

More pertinent to the present investigation is the integration of ionic/osmotic and acid–base regulation. It has been established relatively recently that the principal mechanism by which pH and bicarbonate are maintained in waterbreathing animals is not the ventilatory control of P_{CO_2} but adjustment of the strong ion difference (Cameron, 1976, 1978, 1980), which can be adjusted by changes in the relative rates of Cl^-/HCO_3^- and Na^+/H^+ exchange at the gills (and kidney). Whilst rates of exchange were not measured in the present investigation, certain conclusions can be drawn from the data.

The changes in acid–base balance resulting from hypersaline exposure were complex, and varied with the degree of salinity imposed. pH was maintained in 25 and 50% SW, an acidosis developed in 75% SW. The response in 75% SW directly contrasts with a haemolymph alkalosis reported by Truchot (1973), Taylor (1977) and Weiland & Mangum (1975) for marine crabs undergoing hyposmotic stress. The accompanying increase in circulating bicarbonate in these studies contrasts with the progressive reduction observed in the present study along with a pioneer investigation on eel migration from fresh to salt water (Fontaine & Boucher-Firly, 1933*a*, *b*), where a similar reduction occurred. The magnitude of the $[HCO_3^- + CO_3^{2-}]$ change observed in *Carcinus* on transfer from 36 to 12‰ is comparable with that found in the present study, although the pH increase was more extreme (0·4 pH unit – Truchot, 1973). Weiland & Mangum's data on *Callinectes* (1975) are more conservative and more closely parallel the findings in *Pacifastacus*.

In contrast to the present study, where a progressive reduction in P_{CO_2} was calculated during hypersaline exposure, Truchot (1981) noted that after long-term exposure to external dilution (4 days) *Carcinus* haemolymph P_{CO_2} was unchanged, from which he concluded that the observed pH variation was largely independent of ventilation and resulted from a base excess due to altered ionoregulation. Closer analysis of his data with time reveal that there is some contribution of CO_2 to the overall change in acid–base status. In order to discuss his findings in the context of our own, a 'Davenport' analysis of the relative contributions of CO_2 and acids/bases to the overall pH change in postbranchial haemolymph is illustrated in Fig. 5. Because of the effect of ionic strength on α_{CO_2}, pK_1' and pK_2', and thereby the location of P_{CO_2} isopleths, the classical analyses performed by Woodbury (1965), Davenport (1974) and more recently Wood, McMahon & McDonald (1977) cannot be employed. On Fig. 5, which indicates the mean acid–base status after 48 h acclimation in 25, 50 and 75% SW (pts. B, C, D respectively) in comparison with FW(A), CO_2 isopleths for 1–7 torr have been plotted from the constants experimentally determined above. It is encouraging to observe that these agree well with isopleths constructed from the original data using the operational approach of Wilkes *et al.* (1980). An additional complication was the progressive reduction in buffering capacity indicated by a decrease in the slope of the CO_2 buffer line. This may be partially ascribed to the lowered circulating protein levels and other non-bicarbonate buffers, such as NH_4^+, whose involvement will be implicated below (see Wheatly & McMahon, 1982*a*).

Respiratory disturbances are typically characterized by translation along a buffer line in the direction of increasing or decreasing P_{CO_2}, whereas metabolic disturbances are represented by translation along a P_{CO_2} isopleth (i.e. increasing or decreasing $[HCO_3^-]$ at constant P_{CO_2} – see Wood *et al.* 1977). More frequently, as seen in the

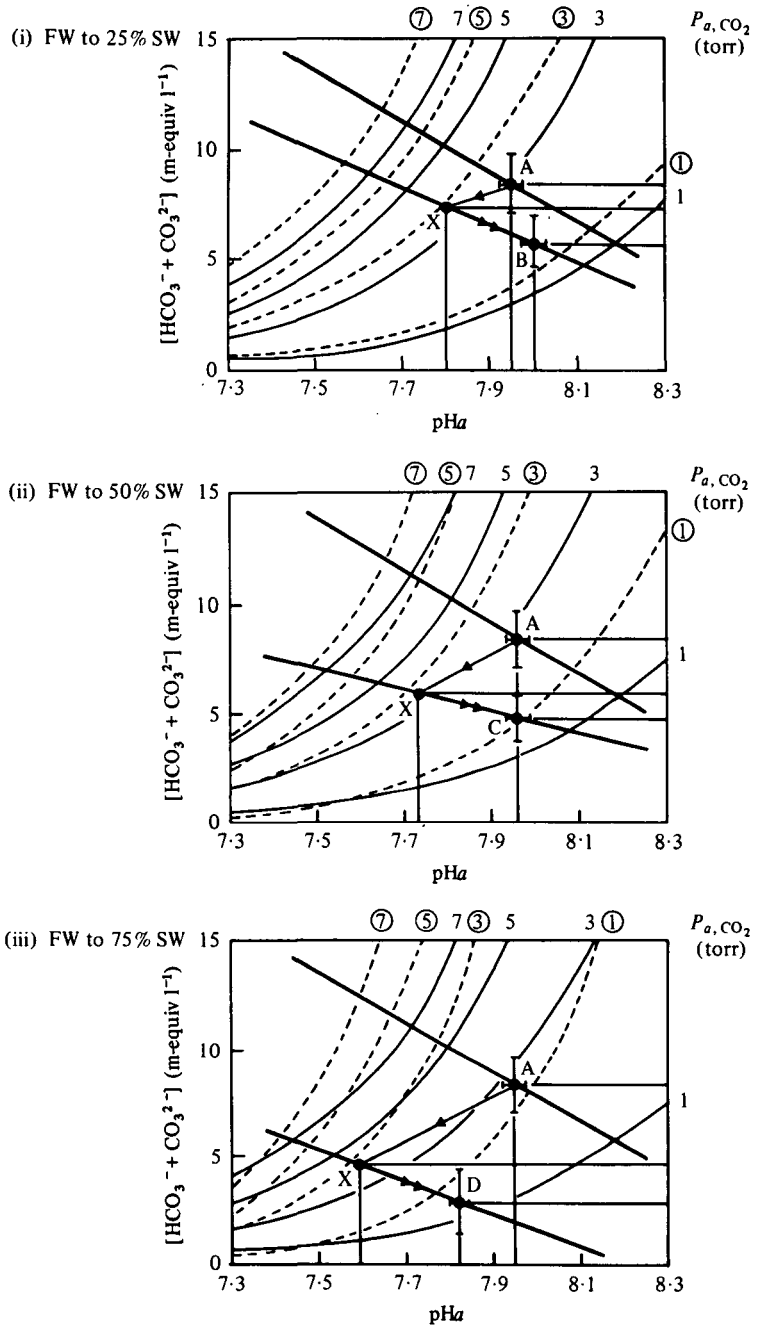

Fig. 5. Analyses of the relative contribution of the metabolic acidosis (AX) and hypocapnic alkalosis (XB, C, D) to the overall change in acid–base status in postbranchial haemolymph of *Pacifastacus* following acclimation for 48 h in (i) 25 % SW (transformation AB) (ii) 50 % SW (AC) and (iii) 75 % SW (AD) in comparison with settled fresh water values. Variation around mean values is indicated for pH_a and $[HCO_3^- + CO_3^{2-}]_a$. Horizontal displacements AX and XB, C, D indicate changes in pH of metabolic and respiratory origin respectively and vertical displacements AX and XB, C, D give a similar indication for circulating $[HCO_3^- + CO_3^{2-}]$. The diagonal lines are the combined CO_2 buffer lines in each acclimation salinity. The CO_2 isopleths have been fitted using pK_1' and α_{CO_2} values experimentally determined in the present investigation. Solid lines apply to FW points, broken lines (P_{CO_2} levels encircled) correspond in each case to the experimental salinity.

present example, the overall pH change represents a combination of both. On transfer from FW to each acclimation salinity, an acidosis of metabolic origin is seen when comparing points of equal P_{CO_2} on the two CO_2 buffer lines (i.e. A–X), in addition to a 'respiratory alkalosis' which from now on, and for reasons outlined below, will be referred to as a 'variably compensated hypocapnic alkalosis' (i.e. X–B, C, D).

These metabolic and respiratory origins of the overall alteration in acid–base balance can be quantitatively assessed. In 25% SW the magnitude of the hypocapnic alkalosis (0·20 pH unit), which accounts for $\frac{2}{3}$ of the overall reduction in bicarbonate, exceeds the metabolic acidosis (0·14 pH unit) and causes a slight haemolymph alkalosis. On transfer to 50% SW, (which incidentally is near to the point of equilibration of the haemolymph with the external medium) the two exhibit similar magnitudes of 0·23 pH unit, resulting in complete compensation and a maintenance of pH. The bicarbonate lost in excess to CO_2 buffering (1·3 m-equiv l^{-1}) is 2·5 m-equiv l^{-1}. Lastly, the acidosis observed on acclimation to 75% SW constitutes a metabolic acidosis of 0·34 pH unit counteracted by a hypocapnic alkalosis of 0·22 pH unit: CO_2 buffering in this case accounts for 1·8 m-equiv l^{-1} of the overall reduction (5·6 m-equiv l^{-1}). In summary therefore, whilst the magnitude of the hypocapnic alkalosis remains relatively constant, that of the metabolic acidosis increases with hypersaline exposure, accounting for proportionally more of the observed reduction in circulating bicarbonate.

The fact that these observed reductions in haemolymph P_{CO_2} are not due to changes in ventilation, and therefore not of 'respiratory' origin in the accepted sense, is substantiated by recent ventilation studies in our laboratory (see Wheatly & McMahon, 1982*a*), which demonstrate that significant hyperventilation only occurs in 75% SW. In both 25 and 50% SW, gill ventilation volume and rate are markedly reduced in comparison with FW. We postulate that this part of the acid–base disturbance depends essentially on changes in the acid–base status of the water (i.e. on changes in titration alkalinity, CO_2 capacitance coefficient, and ultimately on ionic movements through the gill epithelium), which explains the preferred use of the terminology 'hypocapnic alkalosis'.

An inevitable corollary of hypersaline exposure as seen in Table 1 is an increase in the water titration alkalinity (TA), which will determine the shape and position of the CO_2 dissociation curve so that the water capacitance coefficient for CO_2 (i.e. βw_{CO_2} – Piiper, Dejours, Haab & Rahn, 1971) increases. Blood P_{CO_2} will depend not only on production and excretion rates of CO_2 but also on β_{CO_2}. Assuming that the rate of CO_2 output by the gill does not alter as a result of environmental salinity (Taylor (1977) found this to be true for O_2), then inspired and expired CO_2 tensions will be lower (since $\beta w_{CO_2} = \Delta C w_{CO_2}/\Delta P w_{CO_2}$), as will haemolymph tensions (see Dejours *et al.* 1978; Dejours & Armand, 1980; Truchot *et al.* 1980).

A closer look at Truchot's data for *Carcinus* (1981) reveals that prior to complete adjustment of extracellular osmotic concentration an uncompensated hypercapnic acidosis was measured, as one would predict from changes in the acid–base status of the water arising from external dilution. In this case, the change in haemolymph P_{CO_2} was only transient and returned to normal when base output by the gill increased sufficiently to increase once again the TA of the water passing over the gill.

A careful examination of acid–base and other ionic fluxes across the gill will elucidate whether the acid–base changes occurring during hypersaline exposure in *Pacifastacus* are linked to extracellular anisosmotic regulation or to metabolic adjustments involved in intracellular isosmotic regulation. In both *Callinectes* (Weiland & Mangum, 1975) and *Carcinus* (Truchot, 1981), the pattern of pH change with time closely resembled that of blood osmolality and chloride, in the former case actually extending as far as seasonal differences, in that winter animals have more concentrated blood of a lower pH. Since the haemolymph bicarbonate concentration was found to increase under hypo-osmotic exposure in *Carcinus*, the involvement of branchial ion exchanges as the source of the acid–base disequilibria would implicate an apparent acid excretion into the water. Measurement of acid–base exchanges between the crab and the external medium indicating net base excretion, however, strongly support a tissular origin of the metabolic disturbance associated with environmental salinity (Truchot, 1981).

It is well known that alteration of the pool of non-essential free amino acids in crustaceans participates in intracellular isosmotic regulation during salinity acclimation (Gilles, 1979). Mangum *et al.* (1976) suggested that the elevation of blood pH in dilute media resulted from protonation of NH_3 produced in the tissues from enhanced amino acid catabolism by deamination (Jeuniaux & Florkin, 1961) and diffusion into the haemolymph, there accounting perhaps for the measured base efflux.

In a separate investigation, a reduction in circulating [NH_4^+] during hypersaline exposure in *Pacifastacus* was coupled with an elevation in free amino acids (see Wheatly & McMahon, 1982*a*) and may contribute to the observed reduction in S.I.D., since it can be substituted as a counterion for Na^+ exchange (Towle, 1974). The only problem in the present investigation is the maintained hypocapnic alkalosis. This would preclude the notion of significant acid output by the gill during hypersaline exposure, which can be predicted from the findings of Truchot (1981) and should return the P_{CO_2} to resting levels. Obviously this aspect of the physiological response to hypersalinity merits further investigation.

The authors gratefully acknowledge financial support provided by Postdoctoral Research Fellowships awarded by the University of Calgary and Alberta Heritage Foundation for Medical Research to M.G.W. and NSERC grant A 5762 to B.R.M.

REFERENCES

ALBERS, C. (1970). Acid–base balance. In *Fish Physiology*, vol. IV (ed. W. S. Hoar and D. J. Randall), pp. 172–208. New York: Academic Press.

ALBERS, C. & PLESCHKA, K. (1967). Effect of temperature on CO_2 transport in elasmobranch blood. *Respir. Physiol.* **2**, 261–273.

BARTELS, H. & WRBITZKY, R. (1960). Bestimmung des CO_2-Absorptions koeffizienten zwischen 15 und 38 °C in Wasser und Plasma. *Pflügers Arch. ges. Physiol.* **271**, 162–168.

BRADFORD, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254.

BRYAN, G. W. (1960). Sodium regulation in the crayfish *Astacus fluviatilis*. III. *J. exp. Biol.* **37**, 113–128.

BUCH, K. (1951). Das Kohlensäuregleichgewichtssystem im Meereswasser. *Havsforsk Inst. Skr., Helsingf.* **151**.

CAMERON, J. N. (1971). Rapid method for determination of total carbon dioxide in small blood samples. *J. appl. Physiol.* **31**, 632–634.

CAMERON, J. N. (1976). Branchial ion uptake in Arctic grayling: resting values and effects of acid–base disturbance. *J. exp. Biol.* **64**, 711–725.

CAMERON, J. N. (1978). Regulation of blood pH in teleost fish. *Respir. Physiol.* **33**, 129–144.
CAMERON, J. N. (1980). Body fluid pools, kidney function and acid–base regulation in the freshwater catfish *Ictalurus punctatus*. *J. exp. Biol.* **86**, 171–185.
COPELAND, D. E. (1967). A study of the salt secreting cells in the brine shrimp (*Artemia salina*). *Protoplasma* **63**, 363–384.
DANA, J. D. (1852). *United States Exploring Expedition During the Years 1838–1842 under the Command of Charles Wilkes, U.S.N. Crustacea*, vol. 13. pp. 685. Philadelphia: C. Sherman.
DAVENPORT, H. W. (1974). *The ABC of Acid-Base Chemistry*, 6th ed. Chicago: The University of Chicago Press.
DEBYE, P. & HÜCKEL, E. (1923). *Physik. Z.* **24**, 185, as quoted by Hastings & Sendroy (1925).
DEJOURS, P. & ARMAND, J. (1980). Hemolymph acid–base balance as a function of the oxygenation and acid–base balance of the ambient water in the crayfish *Astacus leptodactylus*. *Respir. Physiol.* **41**, 1–11.
DEJOURS, P., ARMAND, J. & GENDNER, J. P. (1978). Importance de la régulation de l'équilibre acide–base de l'eau ambiante pour l'étude des échanges respiratoires et ioniques des animaux aquatiques. *C. r. hebd. Séanc. Acad. Sci. Paris.* **287**, 1397–1399.
DRACH, P. & TCHERNIGOVTZEFF, C. (1967). Sur la méthode de détermination des stades d'intermue et son application générale aux crustacés. *Vie et Milieu* **18** A, 595–607.
FONTAINE, M. & BOUCHER-FIRLY, S. (1933*a*). Sur la réserve alcaline du sang des Poissons. *C. r. hebd. Séanc. Acad. Sci. Paris.* **196**, 1439–1440.
FONTAINE, M. & BOUCHER-FIRLY, S. (1933*b*). Influence des variations de salinité sur la réserve alcaline du sang des Poissons. *C. r. Séanc. Soc. Biol.* **113**, 306–308.
DEFUR, P. L., WILKES, P. R. H. & MCMAHON, B. R. (1980). Non-equilibrium acid–base status in *C. productus*: role of exoskeletal carbonate buffers. *Respir. Physiol.* **42**, 247–261.
GILLES, R. (1973). Oxygen consumption as related to the amino-acid metabolism during osmoregulation in the blue crab *Callinectes sapidus*. *Neth. J. Sea. Res.* **7**, 280–289.
GILLES, R. (1979). Intracellular organic osmotic effectors. In *Mechanisms of Osmoregulation in Animals* (ed. R. Gilles), pp. 111–154. Chichester, New York, Brisbane, Toronto: John Wiley.
HARNED, H. S. & DAVIS, R. (1943). The ionization constant of carbonic acid in water and the solubility of carbon dioxide in aqueous salt solution from 0–50 °C. *J. Am. Chem. Soc.* **65**, 2030–2037.
HASTINGS, A. B. & SENDROY, J., Jr. (1925). The effect of variation in ionic strength on the apparent first and second dissociation constants of carbonic acid. *J. biol. Chem.* **65**, 445–455.
HENRY, R. P. & MANGUM, C. P. (1980). Salt and water balance in the oligohaline clam *Rangia cuneata*. I. Anisosmotic extracellular regulation. *J. exp. Zool.* **211**, 1–10.
HERMANN, F. (1931). Über den Wasserhaushalt des Flusskrebses (*Potamobius astacus* Leach). *Z. vergl. Physiol.* **14**, 479–524.
HUGGINS, A. K. & MUNDAY, K. A. (1968). Crustacean metabolism. In *Advances in Comparative Physiology and Biochemistry*, **3** (ed. O. Lowenstein), pp. 271–378. New York and London: Academic Press.
JEUNIAUX, C. & FLORKIN, M. (1961). Modification de l'excrétion azotée du crabe chinois au cours de l'adaptation osmotique. *Archs. int. Physiol. Biochim.* **69**, 385–386.
KENTALL, A. & SCHWARZ, F. J. (1964). Salinity tolerances of two Maryland crayfishes. *Ohio J. Sci.* **64**, 403–409.
KERLEY, D. E. & PRITCHARD, A. W. (1967). Osmotic regulation in the crayfish *Pacifastacus leniusculus*, stepwise acclimated to dilutions of seawater. *Comp. Biochem. Physiol.* **20**, 101–113.
KIRSCHNER, L. B. (1979). Control mechanisms in crustaceans and fishes. In *Mechanisms of Osmoregulation in Animals* (ed. R. Gilles), pp. 157–222. Chichester, New York, Brisbane, Toronto: John Wiley.
LEWIS, G. N. & RANDALL, M. (1923). *Thermodynamics and the Free Energy of Chemical Substances*, 2nd ed. (rev. by K. S. Pitzer and L. Brewer), New York: McGraw-Hill (1961).
LIENEMANN, L. J. (1938). The green gland as a mechanism for osmotic regulation in the crayfish *Cambarus clarkii* Girard. *J. cell. comp. Physiol.* **11**, 149–161.
LYMAN, J. (1956). Buffer mechanisms of seawater. Ph.D. Thesis University of California, Los Angeles, pp. 196.
MANGUM, C. P., SILVERTHORN, S., HARRIS, J. L., TOWLE, D. W. & KRALL, A. R. (1976). The relationship between blood pH, ammonia excretion and adaptation to low salinity in the blue crab, *Callinectes sapidus*. *J. exp. Zool.* **195**, 129–136.
MCDONALD, D. G. (1977). Respiratory physiology of the crab *Cancer magister*. Ph.D. Thesis, University of Calgary, pp. 87.
MCMAHON, B. R., BUTLER, P. J. & TAYLOR, E. W. (1978). Acid–base changes during recovery from disturbance and during long term hypoxic exposure in the lobster *Homarus vulgaris*. *J. exp. Zool.* **205**, 361–370.
MILLER, G. C. (1960). The taxonomy and certain biological aspects of the crayfish of Oregon and Washington. Master's thesis, Oregon State University, Corvallis, Oregon, pp. 144–147.
MORGAN, D. O. & MCMAHON, B. R. (1982). Acid tolerance and effects of sublethal acid exposure on

iono-regulation and acid–base status in two crayfish *Procambarus clarki* and *Orconectes rusticus*. *J. exp. Biol.* (In the Press.)

MURRAY, C. N. & RILEY, J. P. (1971). The solubility of gases in distilled water and sea water. IV. Carbon dioxide. *Deep Sea Res.* **18**, 533–541.

PIIPER, J., DEJOURS, P., HAAB, P. & RAHN, H. (1971). Concepts and basic quantities in gas exchange physiology. *Respir. Physiol.* **13**, 292–304.

POTTS, W. T. W. & PARRY, G. (1964). *Osmotic and Ionic Regulation in Animals*, pp. 278–329. Oxford, London, New York, Paris: Pergamon Press.

PROSSER, C. L., GREEN, J. W. & CHOW, T. S. (1955). Ionic and osmotic concentrations in blood and urine of *Pachygrapsus crassipes* acclimated to different salinities. *Biol. Bull. Mar. biol. Lab., Woods Hole*, **109**, 99–107.

RAHN, H. (1966). Aquatic gas exchange theory. *Respir. Physiol.* **1**, 1–12.

REDFIELD, A. C., MASON, E. D. & INGALLS, E. N. (1932). The influence of hydrochloric acid on the combination of oxygen with the haemocyanin of *Limulus polyphemus*. *J. cell. comp. Physiol.* **1**, 93–112.

SCHOFFENIELS, E. & GILLES, R. (1970). Osmoregulation in aquatic arthropods. In *Chemical Zoology: Arthropoda*, vol. 5A (eds. M. Florkin and B. T. Scheer), pp. 255. New York: Academic Press.

SEVERINGHAUS, J. W., STUPFEL, M. & BRADLEY, A. F. (1956). Variations of serum carbonic acid pk_1' with pH and temperature. *J. appl. Physiol.* **9**, 197–200.

SMITH, R. I. (1959). Physiological and ecological problems of brackish waters. *Marine Biology, Proceedings 20th Annual Biological Colloquium, Oregon State College, April* 1959, p. 60–69. Oregon State College Office of Publications, Corvallis, Oregon.

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

STRICKLAND, J. D. H. & PARSONS, T. R. (1965). A manual of sea water analysis. Fisheries Research Board of Canada, Bulletin no. 125, 2nd ed., Ottawa.

TAYLOR, A. C. (1977). The respiratory responses of *Carcinus maenas* (L.) to changes in environmental salinity. *J. exp. mar. Biol. Ecol.* **29**, 197–210.

TAYLOR, E. W., BUTLER, P. J. & AL-WASSIA, A. (1977). The effect of a decrease in salinity on respiration, osmoregulation and activity in the shore crab *Carcinus maenas* (L.) at different acclimation temperatures. *J. comp. Physiol.* **119**, 155–170.

TAYLOR, E. W. & WHEATLY, M. G. (1982). The effects of dehydration during aerial exposure on distribution of water and ions in the freshwater crayfish *Austropotamobius pallipes* (Lereboullet). *J. comp. Physiol.* (Submitted.)

TOWLE, D. W. (1974). Equivalence of gill Na^+ and K^+ ATPase from blue crabs acclimated to high and low salinity. *Amer. Zool.* **14**, 1259.

TROUP, S. B., REED, C. F., MARINETTI, G. V. & SWISHER, S. N. (1961). The platelet lipids: their identification, quantification and behaviour in clotting systems *in vitro*. In *Blood Platelets* (Henry Ford Hospital Intern. Symp.) (ed. S. A. Johnson, R. W. Monto, J. N. Robuck and R. C. Horn), pp. 265–275. Boston: Little, Brown.

TRUCHOT, J.-P. (1973). Fixation et transport de l'oxygène par le sang de *Carcinus maenas*: variations en rapport avec diverses conditions de température et de salinité. *Neth. J. Sea. Res.* **7**, 482–495.

TRUCHOT, J.-P. (1975). Blood acid–base changes during experimental emersion and reimmersion of the intertidal crab *Carcinus maenas* (L.). *Respir. Physiol.* **23**, 351–360.

TRUCHOT, J.-P. (1976). Carbon dioxide combining properties of the blood of the shore crab *Carcinus maenas* (L.): carbon dioxide solubility coefficient and carbonic acid dissociation constants. *J. exp. Biol.* **64**, 45–57.

TRUCHOT, J.-P. (1981). The effect of water salinity and acid–base state on the blood acid–base balance in the euryhaline crab *Carcinus maenas* (L.). *Comp. Biochem. Physiol.* **68** A, 555–561.

TRUCHOT, J.-P., TOULMOND, A. & DEJOURS, P. (1980). Blood acid–base balance as a function of water oxygenation: a study at two different ambient CO_2 levels in the dogfish, *Scyliorhinus canicula*. *Respir. Physiol.* **41**, 13–28.

VAN SLYKE, D. D., SENDROY, J., JR, HASTINGS, A. B. & NEILL, J. M. (1928). The solubility of carbon dioxide at 38 °C in water, salt solution, serum and blood cells. *J. biol. Chem.* **78**, 765–799.

WEILAND, A. L. & MANGUM, C. P. (1975). The influence of environmental salinity on haemocyanin function in the blue crab, *Callinectes sapidus*. *J. exp. Zool.* **193**, 265–274.

WHEATLY, M. G. & MCMAHON, B. R. (1982*a*). Respiration and ionoregulation in the euryhaline crayfish *Pacifastacus leniusculus* on exposure to high salinity: an overview. In *Freshwater Crayfish*, vol. v (ed. C. R. Goldman). Davis, California. (In the Press.)

WHEATLY, M. G. & MCMAHON, B. R. (1982*b*). Responses to hypersaline exposure in the euryhaline crayfish *Pacifastacus leniusculus*. II. Modulation of haemocyanin O_2 binding *in vitro* and *in vivo*. *J. exp. Biol.* **99**, 447–467.

WILKES, P. R. H., DEFUR, P. L. & MCMAHON, B. R. (1980). A new operational approach to P_{CO_2} determination in crustacean hemolymph. *Respir. Physiol.* **42**, 17–28.

WILKES, P. R. H., & MCMAHON, B. R. (1982). Effects of maintained hypoxic exposure in the crayfish *Orconectes rusticus*. II. Modulation of haemocyanin O_2 affinity. *J. exp. Biol.* (In the Press.)

WOOD, C. M., MCMAHON, B. R. & MCDONALD, D. G. (1977). An analysis of changes in blood pH following exhausting activity in the starry flounder *Platichthys stellatus*. *J. exp. Biol.* **69**, 173–185.

WOODBURY, J. W. (1965). Regulation of pH. In *Physiology and Biophysics* (ed. T. C. Ruch and H. D. Patton). Philadelphia: W. B. Saunders.