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ADAPTATIONS TO A TERRESTRIAL EXISTENCE BY THE ROBBER CRAB *BIRGUS LATRO*

VII. THE BRANCHIAL CHAMBER AND ITS ROLE IN URINE REPROCESSING

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Summary

The branchial chambers of the terrestrial anomuran *Birgus latro* L. were examined as potential sites for urine reprocessing. The antennal glands opened at the bases of the second antennae within the anterior extension of the branchial chambers. The adjacent mouth parts and scaphognathites and the cuticle lining the ventral folds of the branchiostegite bear hydrophilic hairs. The hairs retained urine in the branchial chamber and conducted it to either the gills or the mouth. Irrigation of the branchial chambers with artificial urine demonstrated the strong net uptakes of Na^+ and Cl^- to be similar at approximately 4.5 μmol h^{-1} g^{-1} from full-strength urine, diluting the fluid to a threshold below 20 mmol l^{-1}.

Na^+/K^+-ATPase and Ca^{2+}-ATPase activities of gill homogenates indicated V_{max} values (65–127 and 11 nmol min^{-1} mg^{-1} protein, respectively) similar to those of marine species but low K_m values (sodium K_m=0.2–2.4 mmol l^{-1}, potassium K_m=0.03–0.2 mmol l^{-1}, calcium K_m=4–9 μmol l^{-1}), which are more characteristic of freshwater species. The gills are considered to be a site of ion reclamation from the urine and this is achieved by specific morphological, biochemical and behavioural adaptations.

Introduction

The urine of crabs is normally near-iso-osmotic with their haemolymph (see Mantel and Farmer, 1983; Greenaway, 1988, for reviews) and salts lost by this route are replaced, in aquatic species, by absorption of ions from the water. Terrestrial crabs lack this source of ions; their drinking water is generally dilute and ion intake in the food represents the only major ion source available. Major loss of salt in the urine, therefore, is not sustainable and recovery of these ions is essential to maintain balance. In terrestrial brachyurans, the released urine is not,

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in fact, lost. Instead, it is passed into the branchial chambers where reabsorption of ions occurs and an appropriately diluted excretory fluid is released (Wolcott and Wolcott, 1982, 1984, 1985; Greenaway and Nakamura, 1991) so that ionic balance may be maintained even on a low-salt diet (Wolcott and Wolcott, 1988). The site of ion absorption in the branchial chambers of terrestrial brachyurans is probably the gills, which have an ultrastructure characteristic of ion-transporting tissue (Copeland, 1968; Storch and Welsch, 1975; Greenaway and Farrelly, 1990) and possess high levels of ion-transporting enzymes (Towle, 1981). The walls of the branchial chambers are highly modified for gas exchange and are definitely not suited to an ion-transporting function (Taylor and Greenaway, 1979; Farrelly and Greenaway, 1987; Greenaway and Farrelly, 1990; C. A. Farrelly, unpublished data).

Iso-osmotic urine is also produced by the antennal organs of the anomuran *Birgus latro*, and again this fluid is reprocessed post-renally in the branchial chambers so that the excretory fluid finally released is quite dilute (Greenaway and Morris, 1989; Greenaway *et al.* 1990). When only fresh water is available for drinking, *Birgus* maintains a low water turnover which, together with the low concentration of the final excretory fluid, minimises salt loss by this route (Greenaway *et al.* 1990).

From the above evidence it is quite clear that the branchial chambers are the site from which ion recovery from the urine occurs, both in terrestrial brachyurans and in anomurans. In this study further clarification of urine reprocessing function is sought in the anomuran *Birgus latro*. First, the anatomy of the branchial chambers and the location of the antennal gland opening are examined in order to establish how urine might be directed to the branchial chambers and the capacity of these structures to receive and hold urine. Second, the ability of the branchial chamber system to absorb salts is studied experimentally by perfusing the chambers with artificial urine of known composition and following changes in composition of the perfusate. Third, the ion-transporting potential of the walls of the branchial chambers and the gills was investigated by measuring the activities and characteristics of the ion-transporting enzymes present.

Materials and methods

Animal collection and maintenance

Specimens of *Birgus latro* (250–500 g) were collected under permit from the Australian Territory of Christmas Island (location 10°28′S, 105°38′E). The crabs were individually packed and air-freighted to the University of New South Wales, Sydney, within 3 days of collection. They were maintained individually, in plastic fish boxes, in a humidified constant-temperature room at 25 °C and supplied with fresh fruit, vegetables, dry dog biscuits and fresh water. Food, but not water, was withdrawn 24 h prior to experimental observations.

Location of the urinary aperture and branchial morphology

The morphology of the second antennae, the surrounding mouthparts and the

branchial chambers was examined in animals that had been chilled until sluggish and also in preserved specimens.

Measurement of net uptake of Cl^- and Na^+ from the branchial chamber

Cannulae were inserted through the anterior branchiostegite of *Birgus latro* (350–500 g), near the junction between the anterior and posterior gills, at least 24 h prior to any experiment. The crab rested on a stainless-steel mesh above a collecting funnel in a humidified glass chamber thermostatted to 25 °C. Saline with a composition similar to that of urine [=artificial urine (AU), composition in mmol l^{-1}: NaCl 300, KCl 8, $MgCl_2$ 15, $CaCl_2$ 15], or a dilution thereof, was delivered to each branchial chamber at a rate of 4.5 ml min^{-1} using a peristaltic pump with twin heads. Fluid overflowed from the branchial chambers, collected in the funnel under the animal and was recirculated. Filters in the lines prevented occlusion of the cannulae with debris and samples could be removed *via* a Luer tap fitting in the return line. The chamber was covered to minimise disturbance and the crab was allowed to settle for approximately 30 min before commencing circulation and for a further 20 min after introducing the known volume (35–40 ml) of saline to the system.

In initial uptake experiments ^{51}Cr-labelled EDTA was employed as an extracorporeal fluid volume marker. Three consecutive spikes of radioisotope were added. The first was included in the AU introduced initially and its dilution permitted determination of any volume of fluid already resident in the branchial chambers (commonly 0–1 ml). A second spike introduced at time zero (0 h) enabled accurate determination of fluid in the chamber at the start of the experiments, whilst the third spike enabled circulating volume to be checked at 1 h. After the system had been drained, residual isotope was washed out and permitted the volume of fluid retained in the branchial chambers, filters and tubing (=residual volume R) to be estimated. Samples (1 ml) were taken at 0 and 1 h for the determination of Na^+ and Cl^- concentration and at 15-min intervals (150 μl) for determination of radioactivity. Suitable corrections were made for the volumes of the samples removed.

The isotope studies showed that the circulating volume decreased markedly within the first 30 min, occasionally by more than 50 %, and remained essentially constant thereafter. In subsequent experiments the ^{51}Cr-labelled EDTA was omitted and the mean volume during the uptake period was estimated as follows. Saline was introduced, allowed to circulate for 15–20 min and the system was then drained. A measured volume (35–40 ml, V1) of fresh saline was introduced and allowed to circulate for 15–20 min. Samples of known volume (about 1 ml) were withdrawn at 0 h and 0.5 h (S1 and S2) for the determination of [Na^+] and [Cl^-]. The system was then drained into a measuring cylinder and weighed (V2). Ion uptake could then be calculated knowing the volume of fluid [$V=R+(V1-S1+V2+S2)/2$ where R is the residual volume after draining (14.0 ml kg^{-1})]. This method permitted consecutive estimates of rates of ion

uptake on one animal and, by manipulating the concentration of the AU, measurements were obtained over a range of concentration.

The concentrations of Na^+ were measured using a Varian 175 atomic absorption spectrophotometer (Greenaway, 1989). Chloride concentrations were measured with a CMT 10 chloride titrator (Radiometer, Copenhagen) and ^{51}Cr using a Bicron well scintillation detector and Ortec counting equipment.

ATPase activity

Tissue acquisition

The tissues investigated for ATPase activity were (i) the branchiostegal lining divided into dorsal (lung) and ventral portions and (ii) the gills separated into the posterior five gills and the anterior nine gills. Crabs were cooled at 4°C until completely torpid and killed by rapid removal of the heart. Tissues were then dissected and immediately washed in homogenisation buffer. The pooled tissues from both sides were weighed and sliced finely in glass vessels containing 10 times the tissue volume of cold buffer. This material was then homogenised using 50 hand turns in ground-glass homogenisers (Wheaton) and held at 4°C until assayed (within 12 h) for one or more specific ATPase activities. The homogenisation buffer was a 25 mmol l^{-1} Tris/acetate buffer containing, in mmol l^{-1}: sorbitol, 250; EDTA, 6; phenylmethylsulphonyl fluoride (PMSF), 0.2; dithiothreitol, 0.1, and aprotinin at 100 units ml^{-1} (Sigma, 1991). All reagents were of analytical grade or better. All glassware was washed in 1% EDTA and rinsed copiously in 'reverse osmosis' water polished with a MilliQ water system.

Assay

ATPase activity was determined in (i) a buffer of the following composition, in mmol l^{-1}: $MgCl_2$, 6; NaCl, 100; KCl, 10; Tris, 25; and adjusted with acetic acid to pH 7.4 and (ii) the same buffer without KCl but containing 5 mmol l^{-1} ouabain, which specifically inhibited Na^+/K^+-ATPase. The difference in activities of the two assays could then be attributed to Na^+/K^+-stimulated ATPase activity. The reaction was initiated by adding 55 μl of ATP (35 mmol l^{-1} Na_2–ATP) to 550 μl of appropriate buffer and 25 μl of homogenate. The ATP solutions used in this and all subsequent assays were prepared from vanadium-free salts. All reactants were pre-equilibrated to the assay temperature of 25°C. After 40 min the reaction was stopped by adding 150 μl of trichloroacetic acid (TCA) (0.6 mol l^{-1}). The resulting precipitate was centrifuged and the supernatant assayed for inorganic phosphate, as a measure of ATPase activity. Inorganic phosphate (P_i) was determined in all cases using a test kit (Sigma 661–11 and 661–8) based on the Fiske and Subbarow method. The test was routinely calibrated using six P_i standards in the concentration range 0–4.0 mmol l^{-1}. Absorbances were determined using a spectrophotometer (LKB Ultraspec II, model 4050 UV/Vis).

The activity of Ca^{2+}-stimulated ATPase was determined using a similar two-buffer system. The composition of the first buffer, in mmol l^{-1}, was NaCl, 100;

ouabain, 0.1; NaN_3, 5; Tris, 20; $MgCl_2$, 6; $CaCl_2$, 0.7; EGTA, 0.5; and oligomycin 5 mg l^{-1} (a mitochondrial Ca^{2+}-ATPase inhibitor). The buffer was adjusted to pH 7.4 with acetic acid. The 'blank' buffer had the same composition but without the $CaCl_2$. The efficacy of the oligomycin treatment was tested in fractions derived by density gradient centrifugation (see Towle, 1981; Henry, 1988, for centrifugation). The oligomycin was found to have no effect on activity of fractions containing membrane Na^+/K^+-ATPase activity but completely inhibited those fractions associated with mitochondria (cytochrome *c* oxidase marker, see Wharton and Tzagoloff, 1967). The reaction was initiated at 25°C by adding Na_2–ATP (35 mmol l^{-1}), as described above for the Na^+/K^+-ATPase assay.

The activity of HCO_3^--dependent ATPase was determined in salines of the following composition, in mmol l^{-1}: $NaHCO_3$, 10; NaCl, 10; NaN_3, 5; $MgCl_2$, 0.5; Tris, 30; ouabain, 1; oligomycin at 5 mg l^{-1} adjusted to pH 7.8 with acetic acid. The inhibitory control buffer had a similar composition except that the NaCl was replaced by KSCN. In this assay, at 25°C, 50 μl of homogenate and 850 μl of buffer were preincubated for 15 min and the reaction was initiated with 100 μl of Na_2–ATP (5 mmol l^{-1}). The reaction was allowed to proceed for 30 min and stopped with 150 μl of TCA (10 % *v*/*w*). The resulting precipitate was centrifuged and the supernatant used to estimate liberated P_i, as described above.

The dependence of each of the ATPase activities investigated on the concentration of the stimulating ion was tested by carrying out the assays using salines based on those above but in the presence of various concentrations of the appropriate salt. For the Na^+/K^+-ATPase the concentration of K^+ was varied, at constant $[Na^+]$, using nine concentrations in the range 1–50 mmol l^{-1}. In a separate series, the concentration of Na^+ was also varied, at constant $[K^+]$, using 10 concentrations within the range 0–400 mmol l^{-1}. For the $[Na^+]$-dependent assays the reaction was initiated using 35 mmol l^{-1} Ca^{2+}–ATP. The inhibitory buffer contained none of the specific ion being investigated. Calcium dependency was investigated by preparing a range of Ca^{2+}–EGTA buffers (see Ghijsen *et al.* 1980) to provide concentrations in the range 0–400 μmol l^{-1}. The $[HCO_3^-]$ dependency of ATPase activity was determined using HCO_3^- solutions in tightly stoppered flasks and reaction tubes within the range 0.1–100 mmol l^{-1}.

Since the tissue homogenates contained significant amounts of Na^+, K^+ and Ca^{2+}, the concentrations of these elements were determined for each homogenate and included in subsequent calculations. The homogenates were assumed to be equilibrated with atmospheric P_{CO_2}. The data resulting from these determinations were then analyzed for maximal rate, if sufficient activity was present, according to the Lineweaver–Burke or Eadie–Hofstee plots to permit calculation of V_{max} and K_m.

Results

Morphological investigations

The nephropores (urinary apertures) were situated at the bases of the filiform

(second) antennae (coxa of protopodite in Fig. 1). They were covered by the anterior margin of the branchiostegite and opened posteriorly into the anterior branchial chamber (Fig. 1). The urinary apertures were fringed with hydrophilic hairs and lay in close juxtaposition to the exopodites and endites of the maxillae and maxillipeds and to the mobile scaphognathites. These parts also bear hydrophilic hairs, as does the anterior branchial chamber. In chilled crabs, fluid was released from the nephropores (Greenaway *et al.* 1990; H. H. Taylor, P. Greenaway and S. Morris, in preparation) and immediately wicked into these hairs. Fluid collected in glass micropipettes applied to the nephropores or to any of the neighbouring hair-covered structures had identical osmolality and ionic

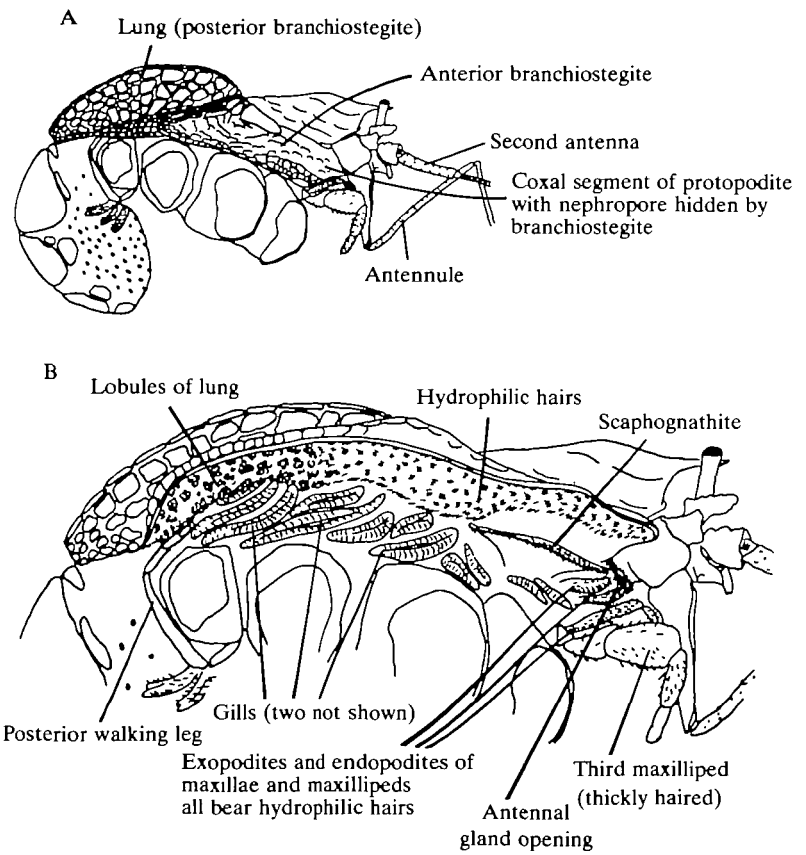

Fig. 1. (A) Morphology of the branchiostegite and anterior appendages of *Birgus latro*, lateral view with legs omitted. (B) Lateral view of the branchial chamber of *Birgus latro* with the branchiostegite on the right-hand side reflected upwards to expose the inner surface of the branchiostegite. Note the distinction between the anterior hair-lined portion of the branchiostegite and the posterior respiratory portion. The nephropore (antennal gland opening) faces posteriorly and opens directly into the branchial chamber. (Drawing made from a 500 g animal, approximately 20 cm in length excluding appendages, as shown.)

composition (H. H. Taylor, P. Greenaway and S. Morris, unpublished data). These properties indicate that urine could be conducted either posteriorly into the branchial chamber, and thus to the gills, or forward to the mouth. Most of the gills depended into the anterior ventral fold of the branchiostegite (Fig. 1) which was also hair-lined and quite moist. There is an abrupt transition between the anterior–ventral and the posterior–dorsal expansions of the branchiostegite, which forms the lung. The structure of the lung was as previously described (Harms, 1932; Storch and Welsch, 1984) with a hairless lining evaginated to form lobate respiratory trees that greatly amplify the surface area of the branchiostegal membrane.

Ion-dependent ATPase activities

Na^+/K^+-ATPase activity was detected in all four homogenates tested. There was no significant difference between sodium-dependent and potassium-dependent maximal velocities (V_{max}) and the two data sets are combined in Table 1. The affinities (K_m) for Na^+ and K^+, however, differed significantly ($P<0.05$) between tissues (Table 1). Statistical analysis (Students *t*-test, $P<0.05$) of the protein-specific Na^+/K^+-ATPase activity demonstrated a number of differences. First, specific activity in the gills was higher than in the branchiostegal tissues and, second, greater total activity was present in the branchiostegite, a considerably larger tissue mass (Table 1).

Sodium-dependent activity of the four tissue preparations increased with increasing Na^+ concentration up to approximately $50\,mmol\,l^{-1}$, but further increases in Na^+ concentration were sharply inhibitory (one-way analysis of variance, ANOVA, and Tukey's HSD test) (Fig. 2). Na^+/K^+-ATPase activity was maximally stimulated by relatively low (cf. Na^+) concentrations of potassium and V_{max} was obtained at $[K^+]<10\,mmol\,l^{-1}$; concentrations in excess of $50\,mmol\,l^{-1}$

Table 1. *Maximal Na^+/K^+-ATPase activity (V_{max}) in gill and branchiostegal tissue*

			Branchiostegite	
	Anterior gills (N=11)	Posterior gills (N=12)	Dorsal (N=12)	Ventral (N=9)
V_{max} (nmol min^{-1} mg^{-1} protein)	127±27.0[a]	65±8.5[b]	47±8.0[b]	44±6.4[b]
V_{max} (nmol min^{-1} mg^{-1} fresh mass)	23.6±5.2[c]	14.2±2.8[c,d]	6.3±0.9[e]	8.2±1.1[d,e]
Total organ activity (μmol min^{-1})	10.4±2.5[f]	9.9±1.8[f]	6.5±1.0[f]	29.6±3.8[g]
Sodium K_m (mmol l^{-1})	0.21±0.08[h]	2.37±0.99[i]	0.89±0.81[j]	1.81±1.2[k]
Potassium K_m (mmol l^{-1})	0.03±0.02[h]	0.21±0.19[i]	0.05±0.05[j]	0.11±0.16[k]

Different superscripts (a–g) indicate significant differences between means within a row ($P<0.05$) and superscripts (h–k) indicate significant differences between means within a column ($P<0.05$).

Values are mean±S.E.M.

Specific activities are presented in terms of protein content and tissue fresh mass.

Total activities have been calculated for the gills and branchiostegites.

Affinity values (K_m) for Na^+ and K^+ are also provided.

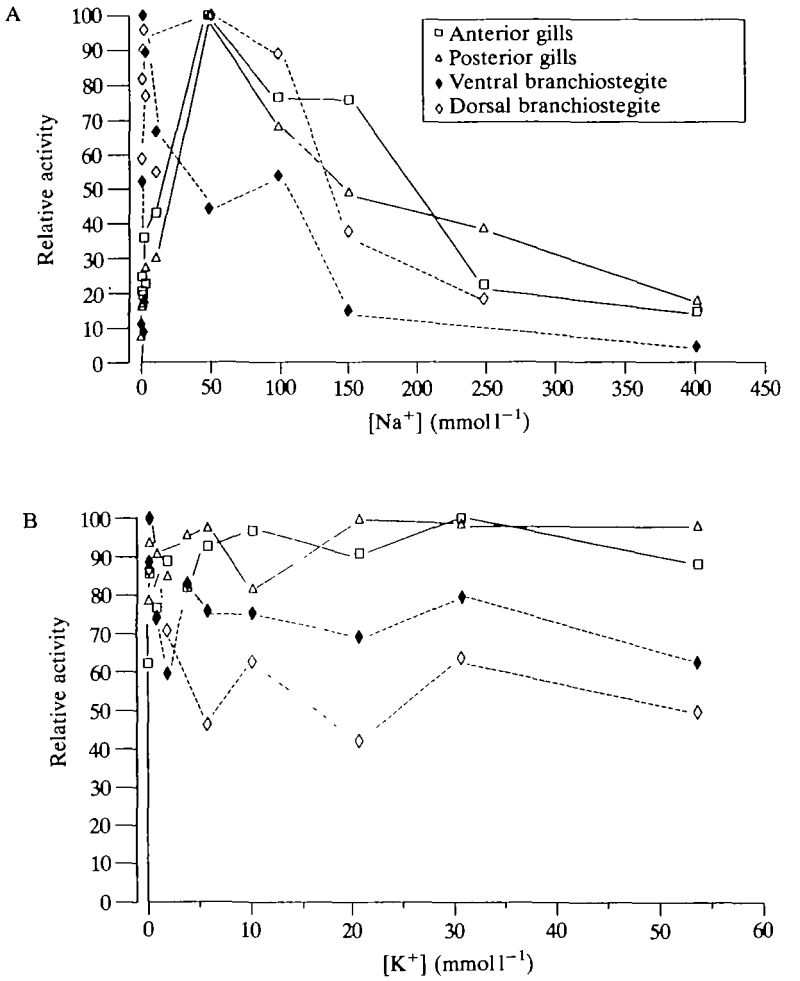

Fig. 2. Relative ATPase activities of homogenates of gill and branchiostegal tissues from *Birgus latro*. (A) Relative activity as a function of $[Na^+]$ at constant $[K^+]$ (10 mmol l^{-1}). (B) Relative activity as a function of $[K^+]$ at constant concentrations of Na^+ (100 mmol l^{-1}). Details of assay conditions are provided in the text. Error bars have been omitted for clarity (for absolute values and errors see Table 1) (N=4–6 for each of the eight curves shown).

did not increase the rate further. Indeed, there is an indication that higher levels of K^+ inhibited branchiostegal, although not gill, ATPase, but this was not confirmed statistically (two-way ANOVA). Statistical differences were found between the affinities for K^+ and Na^+, ranging from approximately 0.2 to 2.4 mmol l^{-1} for Na^+, with the greatest affinity occurring in the anterior gills. For K^+, the values ranged from approximately 0.03 to 0.2 mmol l^{-1}, an order of magnitude lower.

The activity of calcium-dependent ATPase in each of the two gill homogenates was very similar and Lineweaver–Burke analysis indicated a V_{max} of 10–11 nmol min^{-1} mg^{-1} protein. Much lower maximal activities of Ca^{2+}-ATPase were

measured in the branchiostegal tissues, 2.4±0.9 (S.D., N=6) and 1.4±0.7 nmol $min^{-1} mg^{-1}$ FW (where FW is fresh mass) in the ventral and dorsal tissue, respectively (cf. 4.6±0.8 nmol $min^{-1} mg^{-1}$ FW in the gills). The affinity for Ca^{2+} was high, with K_m values between 4 and 9 μmol l^{-1}. A HCO_3^--dependent ATPase activity was measured in the lung tissue homogenates (observed maximal rate 22.4 and 7.4 nmol $min^{-1} mg^{-1}$ protein for ventral and dorsal tissue, respectively), but not in gill homogenates.

Uptake of extracorporeal Na^+ and Cl^- within the branchial chambers

On commencement of branchial irrigation the crabs ceased activity and adopted a characteristic stance for the duration of the irrigation period.

Net uptake of Na^+ and Cl^- were measured from four different concentrations of artificial urine (AU) spanning the range of concentration normally found in the branchial chambers (Table 2). The mean net uptake (J_{net}) of both ions decreased in non-linear fashion with decreasing concentration and at the lowest concentrations a small net loss of Na^+ and Cl^- to the AU occurred (Table 2, Fig. 3). The data did not conform to Michaelis–Menten kinetics, but values for maximum uptake and ion affinity obtained by graphical estimation indicated maximum net uptakes (J_{max}) of approximately 4.5 and 4.1 μmol $h^{-1} g^{-1}$ for Na^+ and Cl^-, respectively. Using these values, the corresponding half-maximal concentrations (K_m) were 58 and 72 mmol l^{-1}. It must be emphasised that these are 'apparent' affinities for 'net' uptake.

Below a particular concentration of the AU no net uptake of Na^+ and Cl^- by the animal was possible and net loss occurred. The AU concentration at which no net exchange occurred was estimated to be 15.1 mmol l^{-1} for Na^+ and 19.6 mmol l^{-1} for Cl^- (Fig. 3). Clearly, the uptake kinetics of both Na^+ and Cl^-

Table 2. *The uptake of Na^+ and Cl^- from artificial urine introduced into the branchial chambers by a recirculating irrigation system*

	1	2	3	4	
$[Cl^-]$ (mmol l^{-1})	310±10.2 (6)	104.5±6.7 (3)	37.8±4.5 (7)	15.9±0.95 (5)	(total N=21)
Cl^- uptake (μmol $g^{-1} h^{-1}$)	4.3±1.9	2.3±1.4	0.9±0.37	−0.1±0.04	
$[Na^+]$ (mmol l^{-1})	254.5±10.3 (4)	78.9±5.1 (3)	27.3±0.8 (4)	12.4±0.4 (4)	(total N=15)
Na^+ uptake (μmol $g^{-1} h^{-1}$)	4.7±1.5	2.7±0.7	0.55±0.01	−0.01±0.11	

The four columns (1–4) provide the mean concentration for each of the four artificial urines at the start of the measurement period and the mean uptake rate determined (N for each concentration is shown in parentheses).
Values are mean±S.E.M.

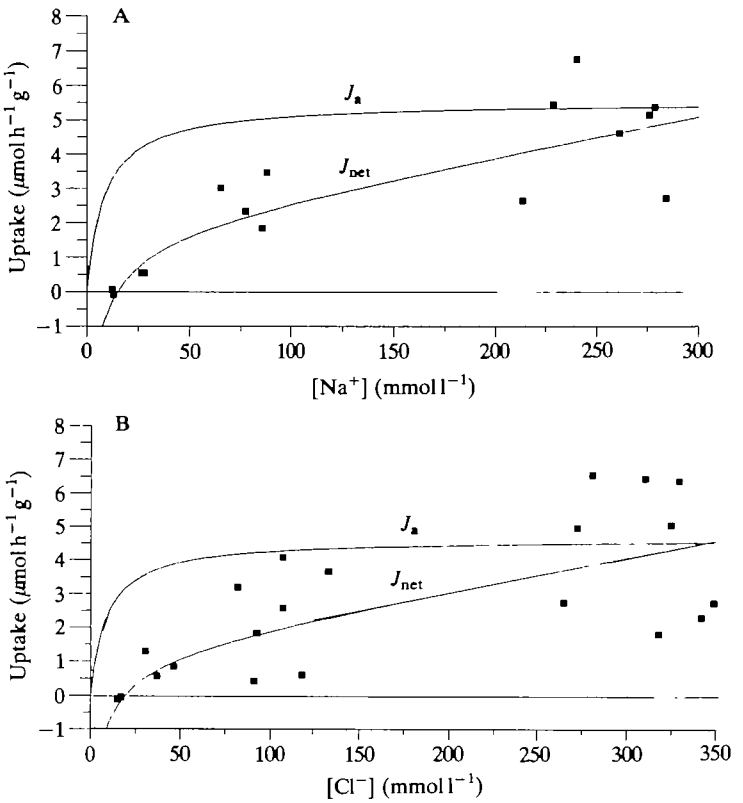

Fig. 3. The uptake of Na^+ and Cl^- from artificial urine solutions by *Birgus latro* as a function of their concentrations. (A) Sodium uptake (total $N=15$). (B) Chloride uptake (total $N=21$). J_{net}, net uptake of either Na^+ or Cl^-; J_a, the active component of uptake. For details of the construction of the curves see text.

were influenced by an active transport component and a passive diffusive component, as described by the leaky pump model. To determine the characteristics of the active component it was assumed that at Na^+ or Cl^- concentrations greater than 80 mmol l^{-1} the active transport component (i.e. ATPase) would be working at maximal velocity. The mean slope of $\delta J_{net}/\delta[Na^+]$ above 80 mmol l^{-1} would then be due to diffusion alone (J_d). J_d was taken as zero when the AU concentration equalled the blood concentration; accordingly, using these slope and intercept values, J_d could then be determined for any concentration. The active component (J_{net}) was derived according to Shaw (1959) using the relationship:

$$J_{net} = \frac{J_{max}C}{K_m+C} + J_d \,, \qquad (1)$$

where C is $[Na^+]$ or $[Cl^-]$ in the AU and J_{max} (maximal rate) is $J_{net}-J_d$ at Na^+ or Cl^- concentrations greater than 300 mmol l^{-1} (assumed maximal rate). The above

equation can be solved for K_m when $J_{net}=0$ (i.e. the concentration at which net uptake was zero, see above).

Using the derived K_m value, active uptake (J_a) can be calculated:

$$J_a = \frac{J_{max}C}{K_m + C}. \quad (2)$$

The plots corresponding to the derived dependencies of J_{net} and J_a are shown in Fig. 3. The two data sets, linearized according to equation 1 and subjected to regression analysis, provided r values of 0.85 and 0.71 for Na^+ and Cl^-, respectively. The resulting plot for J_a exhibited the hyperbolic curve typical of Michaelis–Menten kinetics (Fig. 3). The K_m values determined for J_a were 8.4 and 9.1 mmol l^{-1} for Na^+ and Cl^-, respectively. The corresponding maximal uptake rates (J_{max}) were 5.51 and 4.64 μmol $h^{-1} g^{-1}$. Covariance analysis revealed no difference between Na^+ and Cl^- for either the concentration dependency of uptake or the rate of net uptake. When J_{net} is 0, diffusive losses from the animal were calculated to be 3.54 and 3.17 μmol $h^{-1} g^{-1}$ for Na^+ and Cl^-, respectively.

Discussion

Morphological and functional correlates

The morphology of the branchial chamber of *Birgus latro* was found to be consistent with earlier descriptions (Harms, 1932; Semper, 1878). All reports agree that it is the anterior gills that are most firmly and completely held in the thickly haired, ventral fold of the branchiostegite and that this fold routinely appears quite moist.

In *Birgus latro* the position of the nephropore ensures that urine is voided directly into the branchial chamber. The hydrophilic hair lining can conduct fluid released from the nephropores into the ventral fold of the branchial chamber or, in association with hairs on the mouthparts, towards the mouth. The position of the anterior gills, in the ventral fold of the branchial chamber, allows urine to be held in close contact with the lamellae for extended periods. The morphology of the posterior–dorsal section of the branchial chamber is not, however, indicative of a major role in ionic regulation and it is likely that the epithelium functions principally in gas exchange (Semper, 1878; Harms, 1932; Storch and Welsch, 1984).

Na^+ and Cl^- uptake – the putative Na^+/K^+ pump

The high Na^+/K^+-ATPase activity of the gill homogenates suggests the involvement of the branchial epithelium in the active transport of Na^+ and K^+. The maximum activity recorded in the present study of 127±27 nmol $min^{-1} mg^{-1}$ was similar to, but greater than, the specific activity of 86–107 nmol $min^{-1} mg^{-1}$ reported by Towle (1981). Specific activities of Na^+/K^+-ATPase from marine species vary between 80 and 500 nmol $min^{-1} mg^{-1}$ (e.g. D'Orazio and Holliday, 1985; Holliday, 1985; Towle and Mangum, 1985) and the values for *Birgus latro* fall

into this range. The gills of *Birgus latro* do not, therefore, possess a K^+/Na^+ pump of unusual specific activity.

In *Uca minax*, which is essentially an aquatic osmoregulator, the branchiostegal membrane exhibits only approximately 12% of the specific Na^+/K^+-ATPase activity shown by the gills (Wanson *et al.* 1984), suggesting a minimal contribution to ion uptake. The measured specific activity of branchiostegal Na^+/K^+-ATPase of *Birgus latro*, however, was approximately 47% that of the gills (considerably higher than the 15% reported for the species previously, Towle, 1981). The significant Na^+/K^+-ATPase activity found in the branchial chamber lining of *Birgus* suggests that the chamber has an important role in Na^+ uptake. This point is emphasised if total organ activities rather than specific activities are considered (Table 1).

Raising $[Na^+]$ above 50 mmol l^{-1} progressively inhibited the activity of Na^+/K^+-ATPase, as has been reported for marine, freshwater and amphibious species (Siebers *et al.* 1985; Wanson *et al.* 1984; Harris and Bayliss, 1988; Quinn and Lane, 1966). This optimum of 50 mmol l^{-1} is close to the $[Na^+]$ expected in crustacean cells (Gilles and Pequeux, 1983) and it is this concentration that will determine active Na^+ uptake.

The Na^+/K^+-ATPase in the basolateral membrane of gill epithelia (Siebers *et al.* 1982; Towle and Kays, 1986) may substitute NH_4^+ for K^+ (Towle and Hølleland, 1987) and Towle (1981) suggested that the Na^+/K^+-ATPase in *Birgus* gills may function in the excretion of NH_4^+. More recent data, however, reveal that *Birgus* is uricotelic and excretes negligible amounts of ammonia in excretory fluid (Greenaway and Morris, 1989).

The affinity for K^+ of the Na^+/K^+-ATPase from gill homogenates of *Birgus* (Table 1) was similar to the single value of 0.064 mmol l^{-1} reported earlier (Towle, 1981). The K_m values for Na^+/K^+-ATPase from anterior gills were lower than values found for some marine species, e.g. *Uca pugnax* (Holliday, 1985) and *Uca minax* (Wanson *et al.* 1984), but within the range reported for some freshwater species (Harris and Bayliss, 1988). The net uptake experiments, however, indicate that *Birgus* would be unable to achieve a net gain of $NaCl^-$ from fresh water owing to its high permeability.

Na^+/K^+-ATPase and ion-transport function are generally concentrated in the posterior gills of both aquatic and terrestrial brachyurans (e.g. Copeland, 1968; Spencer *et al.* 1979; Holliday, 1985), but in *Birgus* the anterior gills exhibited the greater specific activity. This is presumably because the anterior gills are those more likely to be bathed in urine in *Birgus*.

Two very different fluids may enter the branchial chamber of *Birgus*: fresh water, which may be 'spooned' into the chamber during drinking, and urine from the antennal organs (Greenaway *et al.* 1990). For *Birgus* drinking fresh water, the Na^+ and Cl^- concentrations in the urine are similar ($[Na^+]$=371 mmol l^{-1} and $[Cl^-]$ 373 mmol l^{-1}) and slightly higher than in the haemolymph (Greenaway *et al.* 1990; H. H. Taylor, P. Greenaway and S. Morris, unpublished data). The excretory fluid (P) that eventually leaves the animal often has a markedly different

composition from that of the urine, and *Birgus* drinking fresh water release P that is extremely hypo-osmotic (Greenaway and Morris, 1989; Greenaway *et al.* 1990). Thus, the gills of *Birgus* are bathed initially with a solution of high osmolality (800 mosmol kg^{-1}) but which rapidly falls to less than 200 mosmol kg^{-1}. Some *Birgus*, indeed, produced P containing less than 10 mmol l^{-1} NaCl, a testament to the efficacy of the active uptake mechanism. Urine entering the branchial chamber would cause a net diffusive flux into the animal (calculated for Na^+ as 1.19 and Cl^- as 0.82 μmol h^{-1} g^{-1}) and J_{net} would initially be 6.60 and 5.37 μmol h^{-1} g^{-1}, respectively (Fig. 3), but this flux would rapidly become negative as net uptake lowered the concentration of the fluid in the branchial chambers. When the crabs are supplied with saline drinking water, ion reclamation ceases rapidly (Greenaway *et al.* 1990) and modulation of ATPase activity in response to changing blood concentration seems likely. In some aquatic brachyurans, modulation of Na^+/K^+-ATPase is mediated by a blood-borne factor (Savage and Robinson, 1983; Mantel, 1985; Sommer and Mantel, 1988; Trausch *et al.* 1989).

Uptake of Na^+ and Cl^- in the branchial chamber

The affinity of the Na^+ uptake system (K_m 8.4 mmol l^{-1}) resembled that of crustaceans from the sea or brackish water, rather than from fresh water (e.g. Sutcliffe, 1975; Greenaway, 1981, 1989; Mantel and Farmer, 1983). Similarly, the minimum equilibrium concentration for Na^+ (approx. 15 mmol l^{-1}) is very much higher than that found in freshwater crustaceans (Table 3 in Greenaway, 1989), and these two factors indicate that the ion-transport system in the gills of *Birgus* has not been greatly modified from that characteristic of marine species.

HCO_3^--stimulated ATPase and its importance to Cl^- uptake

The absence of HCO_3^--ATPase in the gills of *Birgus* from Christmas Island contrasts with an activity of approximately 70 nmol min^{-1} mg^{-1} protein reported by Towle (1981) for gills of *Birgus* collected from the Palau Islands. Interestingly, the branchial chamber lining in both studies showed similar activities of HCO_3^--ATPase.

In view of these differing results, the assay used here was verified using the brachyuran *Leptograpsus variegatus*, collected locally in Sydney. Homogenates from gills of these crabs were treated as described for *Birgus* but then subjected to sucrose gradient density centrifugation [10 %–40 % (*w*/*v*) sucrose gradient; EDTA, 6 mmol l^{-1}; Tris, 25 mmol l^{-1}, pH 7.0; 100 000 *g*, Beckman SW28 rotor for 30 min at 4 °C]. Of the 10 fractions collected, the lower five showed cytochrome *c* oxidase activity and contained 90 % of the HCO_3^--ATPase activity expressed with respect to protein. The remaining 10 % of activity co-sedimented with Na^+/K^+-ATPase activity and the membrane fraction. The crude homogenate had a mean activity of 7.61 nmol min^{-1} mg^{-1}.

As this assay functioned as expected, it must be concluded that no significant HCO_3^--ATPase activity existed in the gill homogenate from *Birgus*. The gills of *Birgus latro* contain carbonic anhydrase, however, which Morris and Greenaway

(1990) suggested might function in the production of bicarbonate to facilitate HCO_3^-/Cl^- exchange. The data suggest that active Cl^-/HCO_3^- pumping would be restricted to the ventral branchiostegal membrane. In *Leptograpsus* gills, most of the HCO_3^-/Cl^--ATPase activity was associated with the mitochondrial fraction and was unavailable for membrane-transport processes. Similar data for *Birgus* (Towle, 1981) indicate that approximately 60% of the HCO_3^--ATPase activity was associated with mitochondria (see also DePew and Towle, 1979).

The present study provided no evidence for active Cl^- uptake by Cl^-/HCO_3^--ATPase. The data for Cl^- uptake from the branchial chambers of *Birgus*, however, clearly showed saturation kinetics in addition to a diffusive component, and uptake of Cl^- was similar to that of Na^+. As with Na^+, the characteristics of Cl^- transport are reminiscent of a marine decapod rather than a freshwater species. Studies of isolated perfused crab gills indicated that Cl^- uptake was not dependent on the Na^+/K^+-ATPase (Onken and Graszynski, 1989; Péqueux and Gilles, 1988), but rather that Cl^- uptake occurred across the apical membrane *via* Cl^-/HCO_3^- antiport. Péqueux and Gilles (1988) suggest the presence of a Na^+/H^+ antiport. Onken and Graszynski (1989) have proposed that Cl^- uptake (and, by implication, Na^+ uptake) by crab gills is dependent on carbonic anhydrase activity in the gill to provide the H^+ and HCO_3^- from CO_2 hydration (see also Henry and Cameron, 1983) and this would seem to be the case for *Birgus*.

Calcium-dependent ATPase activity

The final excretory fluid released by *Birgus* normally has a much lower calcium concentration than either the urine or the haemolymph and a mechanism for the absorption of Ca^{2+} must be present in the branchial chambers (Greenaway and Morris, 1989). Measurements in this study demonstrated the presence of calcium-dependent ATPase activity in both the anterior and posterior gills with rather low levels in branchiostegal tissue. The specific activity of the Ca^{2+}-ATPase was lower than that of the Na^+/K^+-ATPase, but still exceeded 10 nmol min^{-1} mg^{-1}, while the affinity for Ca^{2+} of the Ca^{2+}-ATPase was very high (K_m<9 μmol l^{-1}), especially in the anterior gills.

There are few published data for Ca^{2+}-ATPase in crustaceans with which values for *Birgus* can be compared. Previous K_m values near 1 mmol l^{-1} (Cameron, 1989) probably represent generalized phosphatase activity and not specific Ca^{2+}-ATPase activity. The K_m value for calcium of 4–9 μmol l^{-1} obtained for *Birgus* is consistent with a basolateral location of a Ca^{2+}-extruding pump, and a high-affinity Ca^{2+}-ATPase (K_m=6–34 nmol l^{-1}) has also been found in the gills of the supratidal brachyuran *Leptograpsus variegatus* (M. A. Morris and P. Greenaway, unpublished data). Using microsomal preparations in addition to homogenates these workers showed that this Ca^{2+}-ATPase activity occurred in the same membrane preparations as the Na^+/K^+-ATPase activity, confirming the membrane location of the Ca^{2+}-ATPase. The Ca^{2+}-ATPase activity of approximately 10 nmol min^{-1} mg^{-1} in *Birgus* was essentially the same as the 6.2 nmol min^{-1} mg^{-1} measured for *Leptograpsus*.

Available information suggests that the Ca^{2+}-ATPase in *Birgus* is concerned with basolateral extrusion of Ca^{2+} from the cell into the haemolymph rather than apical uptake of calcium from branchial chamber fluid. There is a very large concentration gradient for calcium from the branchial chamber fluid into the cell, which could facilitate passive entry across the apical membrane, or some other mechanism may be involved.

In summary, the branchial chambers are morphologically suited for the retention of urine released from the antennal organs and the animal possesses behavioural mechanisms that restrict activity (and hence spillages) when urine is present in the branchial chambers. Ion-transport mechanisms, necessary for reclamation of salts from the urine, are present in the ventral branchiostegite and gills and these structures are probably responsible for the high rates of ion absorption measured.

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