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SALINITY-INDUCED CHANGES IN BRANCHIAL Na^+/K^+-ATPase ACTIVITY AND TRANSEPITHELIAL POTENTIAL DIFFERENCE IN THE BRINE SHRIMP *ARTEMIA SALINA*

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Summary

Silver staining of the adult brine shrimp, *Artemia salina*, revealed that only the metepipodites of the phyllopodia were significantly permeable to chloride and/or silver ions. The metepipodites stained in a reticulated pattern, possibly indicating areas in the cuticle over cells specialized for chloride secretion. Crude homogenates of metepipodites had very high Na^+/K^+-ATPase enzyme specific activity (ESA) which increased in proportion to the salinity of the external medium and, thus, in proportion to the need for outward salt transport in these strongly hypo-osmoregulating animals. Metepipodite ESA as a percentage of whole-body ESA increased from 7.6% in 50% sea water (SW) to 25.0% in 400% SW. Gut and maxillary gland also had high Na^+/K^+-ATPase ESAs, implicating these organs in osmoregulatory processes as well. The time courses of increases in phyllopodial and gut ESAs in brine shrimps transferred from 100% SW to 400% SW are consistent with the induction of new Na^+/K^+-ATPase; 4–7 days was required for significant increases to occur. Haemolymph ion analyses and transepithelial potential differences, measured in brine shrimp acclimated in all the SW media, indicate that chloride is actively transported out of the brine shrimp while sodium is very close to electrochemical equilibrium across the body wall. Thus, the metepipodites of the brine shrimp appear to possess cells with many functional similarities to the teleost branchial chloride cells.

Introduction

Brine shrimps, *Artemia salina*, live in some of the harshest aquatic environments known. Their ability to survive and reproduce in hypersaline waters inhabited only

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by algae, bacteria and a few species of insects has aroused much interest in the mechanisms used by these creatures to survive the ionic and osmotic stresses imposed by their environment. Conte's group has elegantly outlined the mechanisms of osmotic and ionic regulation in the nauplii of *A. salina* (reviewed in Conte, 1984), which use a special salt-secreting gland, the neck organ, to rid themselves of salts that enter by diffusion from the hypersaline medium. Further, Russler and Mangos (1978) have shown that the neck organ of *A. salina* nauplii is the major route for sodium efflux.

The mechanisms of osmotic and ionic regulation in adult *A. salina* suggested by the studies of Copeland (1967), Croghan (1958*a*,*b*,*c*,*d*,*e*), Smith (1969*a*,*b*) and Thuet *et al.* (1968) are very similar to those proposed for marine teleosts. Brine shrimps are strong hypo-osmotic regulators in all media more concentrated than approximately 30 % sea water. In these concentrated media they drink to replace water lost by osmosis to the hyperosmotic external medium and they use their gills to secrete salts which enter with the ingested medium and by diffusion (Croghan, 1958*b*,*c*,*d*). Sodium and chloride are actively transported out of the gut into the haemolymph and, presumably, water follows passively; the ions are then secreted into the medium at the gills. The metepipodites of the phyllopodia appear to be the sites of outward ion transport (Croghan, 1958*c*) and a special cell type found in these structures (the 'dark cell', Copeland, 1967), rich in mitochondria, is thought to be responsible for this ion transport. Augenfeld (1969) found that whole-body Na^+/K^+-ATPase activity in immature and adult *A. salina* increased with the salinity of the external medium. Further, based on measurements of transepithelial potential difference in brine shrimps in 100 % sea water (Smith, 1969*a*), it appears that chloride is the ion that is actively transported out of the brine shrimp by the metepipodites, while sodium is in or very close to electrochemical equilibrium across the body wall. Thus, it appears that brine shrimps, too, possess 'chloride cells' with transport characteristics similar to those in the gills of marine teleosts. A second brine shrimp, *Parartemia zietziana*, has been studied by Geddes (1975*a*,*b*,*c*) and its osmotic and ionic regulation appear to be very similar to those of *A. salina*.

The present study was undertaken to investigate further the apparent similarity between teleost and brine shrimp osmoregulatory mechanisms. Silver staining was used to show that the metepipodite cuticle has cell-sized areas of very high permeability to chloride and/or silver ions. The role of the cellular sodium pump, the Na^+/K^+-ATPase, in ion transport by the brine shrimp was investigated by assaying gills and other body parts from shrimps acclimated in media of varying salinity to determine whether enzyme specific activity increases with the salinity of the external medium, as does branchial Na^+/K^+-ATPase activity in many teleosts. Transepithelial potential differences were also measured in 50 %, 100 %, 200 % and 400 % sea water to determine whether chloride and/or sodium ions are actively transported out of the animal in these media. We report here results which confirm the functional similarities between the marine teleost gill and the metepipodites of the brine shrimp. Our data on *A. salina* metepipodites are

consistent with the transport model proposed for teleost branchial chloride cells by Zadunaisky (1984).

Materials and methods

Animals and acclimation media

Artemia salina used in this study were purchased from several commercial suppliers as San Francisco Bay Brand (Newark, California) cysts or live adults. The cysts were obtained by the vendors from both the Great Salt Lake, Utah, and the San Francisco Bay, California (Leslie Salt Co. ponds), populations of *A. salina*; live adults were from the San Francisco Bay population only.

The normal culture medium for brine shrimps was 100 % sea water (SW) which was made from a commercial synthetic sea salt mixture (Lobster Tank Salt, Dayno Manufacturing Co., Lynn, Massachusetts; 1000 mosmol kg^{-1}). Other media (50 %, 200 % and 400 % SW) were also made using this salt mixture and had final osmotic pressures of 500, 2000 and 4000 mosmol kg^{-1}, respectively. Culture medium osmotic pressure was measured at intervals of 2–3 days and adjusted by adding distilled water to replace that lost by evaporation. Cysts were hatched in 100 % SW, transferred to 5 l of clean, gently aerated medium and fed powdered tropical fish food (Staple Food, Tetra Werke, Melle, West Germany) daily. Only adult, female brine shrimps were used in this study and all animals were acclimated in the various media for at least 14 days before being used in experiments.

Silver staining

The so-called 'silver stain' was used to identify areas of the brine shrimp which have a high permeability to chloride ions and in which silver chloride precipitates when the animals are treated with a dilute solution of silver nitrate. Brine shrimps were removed from the culture medium using a wide-mouthed Pasteur pipette and rinsed for 30 s in each of three changes of deionized water to remove adherent chloride ions. The animals were then transferred to 0.5 % $AgNO_3$ for 30 s and rinsed in deionized water as before. Finally, shrimps were transferred to Kodak D-19 photographic developer (Eastman Kodak, Inc., Rochester, New York) for 30 s and rinsed in deionized water three times.

Haemolymph sampling

To avoid the difficulties of sampling haemolymph with micropipettes, a new method for rapidly sampling haemolymph from adult *A. salina* was developed for this study. Animals were removed individually from the culture medium using a wide-mouthed Pasteur pipette and gently expelled onto a dry paper towel. When all adherent culture medium had been drawn off by the paper towel, the brine shrimp was grasped firmly just behind the head from the dorsal side with jeweller's forceps, crushing the nerve cord. After a brief period of struggle the brine shrimp relaxed and straightened out; it was then held over a small plastic Petri dish filled with water-equilibrated mineral oil so as to immerse the last few millimeters of the

abdomen in the oil. Using fine scissors, the tip of the abdomen was then cut off beneath the surface of the oil as the brine shrimp was lifted up and away from the oil. This procedure caused the brine shrimp's body muscles to contract reflexly and to expel approximately 1–3 μl of haemolymph as a droplet into the oil as the body was lifted away from it. If the brine shrimp was not lifted away from the oil as the tip of the abdomen was severed, the haemolymph frequently ran up along the abdomen by capillary action and wetted the phyllopodia, making it very difficult to obtain a sample. Sampling was repeated with 5–10 individuals until 10–20 μl of haemolymph had been collected as a pooled sample for analyses.

It could be argued that haemolymph obtained by the new method would be contaminated with gut fluid, but this appears not to be the case for two reasons. First, few samples were visibly contaminated with faeces and these were not included in the pooled samples used for analyses. Second, the gut fluid of *A. salina* has been reported to be much more concentrated than the haemolymph. For example, the data reported by Croghan (1958*b*) show that gut fluid averaged 2–3 times the osmotic pressure of haemolymph in all media (e.g. shrimp in approximately 100 % SW had gut fluid averaging about 720 mosmol kg^{-1} and haemolymph averaging about 350 mosmol kg^{-1}). Haemolymph osmolality in brine shrimp acclimated in 100 % SW in the present study averaged 362 mosmol kg^{-1}, indicating that no significant contamination by the much more concentrated gut fluid had occurred.

Measurement of osmotic pressure and ion concentrations

Osmotic pressure of media and haemolymph samples was measured using a Wescor 5100 C vapour pressure osmometer. Chloride concentrations in media and haemolymph samples were measured using a coulometric titrator (Buchler–Cotlove chloridometer). Sodium concentrations in media and haemolymph samples were measured using an Orion Research Ross sodium-ion-specific electrode connected to a Beckman Φ12 pH/ISE meter; all samples were diluted and adjusted for ionic strength, and sodium concentrations were measured at pH 9.5.

Measurement of transepithelial potential difference

Transepithelial potential differences (TEPs) were measured in *A. salina* using glass microelectrodes and a plastic chamber similar to that described by Smith (1969*a*). Brine shrimps were individually removed from the culture medium using a wide-mouthed Pasteur pipette and gently expelled onto a paper towel to dry them. Pieces of human hair were tied with an overhand knot so as to form a 3–4 mm loop in the middle of each hair. One loop was then placed over the head and pulled tight just behind it; a second loop was put over the end of the abdomen and pulled tight. Thus 'lassoed', the brine shrimp was secured in the chamber by the four strands of hair protruding from it. Brine shrimps prepared in this way did not struggle and continued to make steady, vigorous swimming movements in the TEP chamber medium. Animals survived for at least 6 h in preliminary preparations; TEP measurements took less than 20 min. The swimming movements of

the brine shrimp were judged sufficient to minimize the effects of any unstirred layers which might develop during the measurement of TEP. Media were changed by siphoning approximately five times the chamber volume of new medium into the TEP chamber while the overflow was removed by suction.

TEP was measured by impaling the brine shrimp with glass microelectrodes held in a micromanipulator and viewed with a dissecting microscope. The microelectrodes were made with a Kopf model 700C electrode puller and were filled with 3 mol l^{-1} KCl; they had tip impedances of 1–5 MΩ. The microelectrode and holder were plugged into the probe end of a WPI M701 electrometer. Grounding was accomplished with a Ag/AgCl reference electrode. TEPs were displayed on a Tektronix model 5103N oscilloscope and read to the nearest millivolt from the oscilloscope screen.

Although TEPs were stable and varied little when measured at a variety of locations over the body surface, the best site for impalement without breaking the microelectrode tips was at the dorsal bases of the phyllopodia. Preliminary experiments showed that serial microelectrode impalement at the bases of successive phyllopodia did not cause the TEP to change. Impalements and TEP measurements were performed in quadruplicate for each medium to which the brine shrimp was exposed. TEPs were first measured in brine shrimps bathed in their medium of acclimation, then in the other three SW media, allowing 3–4 min for the TEP to stabilize after each change of medium.

Na^+/K^+-ATPase assay

The activity of the cellular sodium pump in various tissues of *A. salina* was measured as the enzyme specific activity (ESA) of Na^+/K^+-ATPase in crude homogenates of tissues dissected and pooled for assay. Groups of 5–10 brine shrimps were rinsed in homogenizing medium (HM; 0.25 mol l^{-1} sucrose, 6 mmol l^{-1} EDTA) and dissected into the following parts, which were pooled for homogenization and enzyme assay: heads, metepipodites, phyllopodia without metepipodites, body wall and gut. After dissection, the gut was stripped free of faeces by grasping it at one end and gently pulling it between the tips of a second pair of forceps. In some assays whole phyllopodia were used. Maxillary glands were dissected by cutting free a small patch of the body wall containing them; pairs of glands from 25 brine shrimps were pooled and homogenized for each assay. In a final assay, the ESA of the eleventh pair of metepipodites was measured; unlike metepipodites 1–10, these appendages do not stain with silver and it was of interest to determine if they had lower ESAs than the other metepipodites. The eleventh metepipodites from 40 brine shrimps acclimated in 100% SW were dissected, homogenized and assayed for ESA; metepipodites 1–10 were dissected from the same animals and served as controls.

Body parts were homogenized on ice in 0.4–1.0 ml of HM in a ground glass homogenizer and kept on ice for up to an hour until assayed. The assay media and methods were identical to those used in previous studies (Holliday, 1985). Briefly, phosphate liberated from ATP by each homogenate was measured in two reaction

media. One medium had optimum concentrations of all ions (100 mmol l^{-1} Na^+, 30 mmol l^{-1} K^+, 5 mmol l^{-1} ATP, 10 mmol l^{-1} Mg^{2+}, 20 mmol l^{-1} imidazole, pH 7.2) whereas the other lacked potassium and contained ouabain (130 mmol l^{-1} Na^+, 5 mmol l^{-1} ATP, 10 mmol l^{-1} Mg^{2+}, 20 mmol l^{-1} imidazole, 1 mmol l^{-1} ouabain, pH 7.2). After incubation at 30 °C for 15 min, the reaction was stopped and phosphate concentrations in the reaction mixtures were measured colorimetrically as the reduced phosphomolybdate complex. Enzyme specific activity was calculated as the difference in phosphate liberated by each homogenate in the two media and is expressed as μmol phosphate liberated mg^{-1} protein h^{-1}. Protein concentrations in the homogenates were measured colorimetrically using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, California) with bovine serum albumin as a standard. All chemicals used in the chemical and enzyme assays were reagent grade or better.

Statistics

Unless otherwise stated, results are expressed as mean values±S.E. Student's unpaired *t*-test was used to evaluate the significance of differences between mean values. A probability (P) value ≤ 0.05 was considered to be significant. For those points in figures without error bars, S.E. was smaller than the size of the symbol on the figure.

Results

Silver staining

The phyllopodia of silver-stained specimens of *Artemia salina* are shown in Fig. 1. The metepipodites of the phyllopodia were the only structures which took the stain, probably indicating that these areas are the major sites of chloride efflux in the animal, although other explanations (e.g. a high permeability to Ag^{2+}; see Discussion) could account for the selective staining. The metepipodites of the eleventh pair of phyllopodia did not silver stain. Interestingly, silver precipitated in a reticulated pattern in metepipodites 1–10, covering half to three-quarters of the surface area of these structures (Fig. 1A). This reticulated pattern suggests that the cuticle of the metepipodites is not uniformly permeable to chloride ions, but that certain areas of the cuticle have a higher permeability. It is important to note that brine shrimp were treated with silver nitrate for only 30 s; longer exposure to silver nitrate caused an intense, uniform staining of the metepipodites. The patchy distribution of the silver staining in the metepipodites of *A. salina* has not been previously reported and may indicate the existence of individual 'chloride cells' or rows of such cells, as the smallest patches are approximately the size of a single cell (approx. 10–20 μm in diameter; see Discussion).

Fig. 1B shows four silver-stained brine shrimps. It is clear that the metepipodites of the shrimps acclimated in 400 % SW (the two shrimps on the right) stained much more intensely than those acclimated in 50 % SW when both groups were exposed to silver nitrate for 30 s. This is consistent with the notion that the shrimps

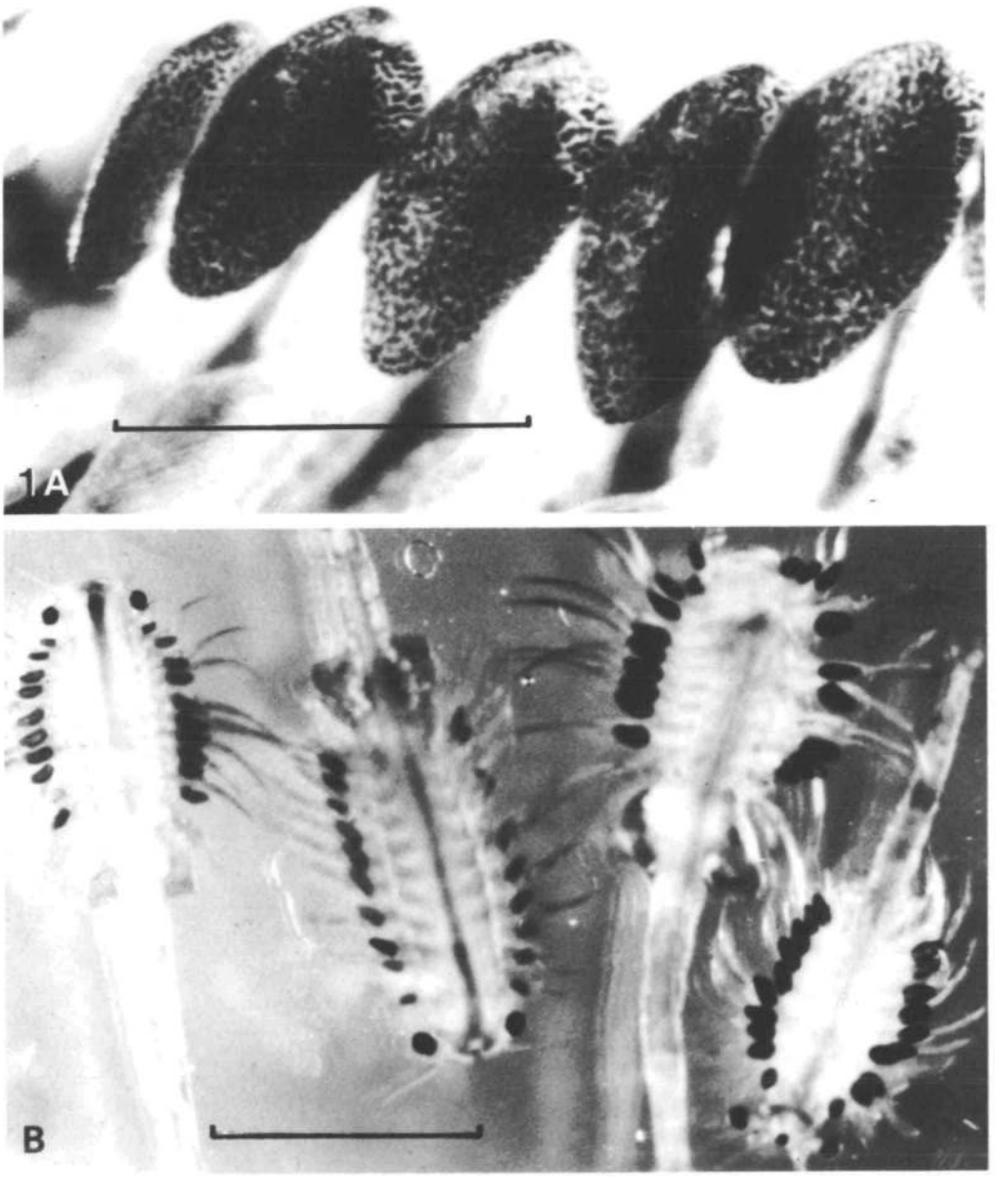

Fig. 1. Silver-stained *Artemia salina*. (A) Lateral view of phyllopodia 6–10. Note the reticulated pattern of the stain; the smallest of the dark patches is approximately 10–20 μm in diameter; (B) dorsal view of four silver-stained *A. salina*; the two animals on the left were acclimated in 50 % SW, the two on the right were acclimated in 400 % SW. Note the darker staining of the metepipodites in the two brine shrimps on the right.

Table 1. *Osmotic pressure and sodium and chloride ion concentrations in the haemolymph and external media of* Artemia salina

	External medium			Haemolymph		
% Sea water	Osmotic pressure (mosmol kg^{-1})	Sodium (mmol l^{-1})	Chloride (mmol l^{-1})	Osmotic pressure (mosmol kg^{-1})	Sodium (mmol l^{-1})	Chloride (mmol l^{-1})
50	496 (493, 499)	243 (239, 247)	273 (270, 276)	353±4(6)	135±5(5)	147 (136, 158)
100	1034 (1031, 1036)	498 (497, 499)	602 (608, 596)	362±5(6)	155±5(5)	146 (144, 148)
200	1898 (1890, 1907)	900 (898, 902)	1075 (1059, 1135)	411±3(6)	161±3(5)	156 (156, 155)
400	4012 (4001, 4024)	1946 (1940, 1952)	2216 (2223, 2210)	498±4(6)	185±5(5)	210 (203, 217)

Mean values±S.E.(N) or mean ($value_1$, $value_2$) where N is 2.

in 400 % SW were secreting chloride at a higher rate than those in 50 % SW, as would be expected.

Osmotic and ionic regulation

Table 1 shows the osmoregulatory and ionoregulatory performance of brine shrimps acclimated in four seawater media. As noted by other investigators, *A. salina* is a weak hyporegulator in 50 % SW and an increasingly strong hyporegulator in 100 %, 200 % and 400 % SW. Its haemolymph osmotic pressure and sodium and chloride concentrations increased approximately 40 % in the face of eightfold increases in the osmotic pressure and sodium and chloride concentrations in the external medium.

Transepithelial potential differences

Shown in Table 2 are the transepithelial potential differences (TEP) measured across the body wall of *A. salina* acclimated in or transferred to various seawater media. Also shown in Table 2 are the calculated transepithelial Nernstian equilibrium potentials for sodium and chloride, based on the measured concentrations of these ions in haemolymph and external media. It is clear in all cases that chloride is very far from electrochemical equilibrium across the body wall. Considering only brine shrimps acclimated and tested in the same media (boldface type in Table 2), chloride is 26–115 mV out of electrochemical equilibrium in the various media. Sodium, however, is very close to electrochemical equilibrium in all the media; TEP values are 5–7 mV less than that predicted on the basis of measured internal and external sodium ion concentrations. Thus, the TEP data strongly suggest that chloride is actively transported out of the animal, while

Table 2. *Transepithelial potential differences measured in* Artemia salina *acclimated in or acutely transferred to various seawater media and calculated Nernstian transepithelial equilibrium potentials for sodium and chloride ions in acclimated animals*

Acclimation medium	Transepithelial potential difference (mV, polarity inside) and medium in which transepithelial potential was measured				Equilibrium potential (mV, polarity inside)	
	50 % sea water	100 % sea water	200 % sea water	400 % sea water	Sodium	Chloride
50 % SW	**+10±1(4)**	+11±3(4)	+28±1(4)	+37±2(4)	+15	−16
100 % SW	+11±1(5)	**+23±1(7)**	+29±1(4)	+40±2(3)	+30	−36
200 % SW	+10±1(4)	+24±2(5)	**+37±2(5)**	+52±2(5)	+44	−50
400 % SW	+10±1(4)	+25±2(4)	+36±1(4)	**+55±1(5)**	+60	−60

* Mean values±S.E. (*N*).

For each brine shrimp, transepithelial potential difference was measured first in the medium of acclimation, then in the other three media.

Equilibrium potentials were calculated using the appropriate haemolymph and medium ion concentrations shown in Table 1.

Results for shrimps acclimated and tested in the same media are shown in bold type.

sodium is very close to being passively distributed across the body wall in all media tested.

The TEPs measured after acute transfer of brine shrimps to media other than the acclimation medium (values other than boldface type in Table 2) show interesting trends. First, when TEP was measured in media more dilute than the acclimation medium, the TEP was nearly identical to that measured in brine shrimps acclimated and measured in the same dilute medium (e.g. brine shrimps acclimated in 400 % SW and measured in 50 % SW had the same TEP as shrimps acclimated in 50 % SW and measured in 50 % SW). If it is assumed that the measured TEP is due to active ion transport, the brine shrimp seems to be capable of rapidly reducing such transport when placed in media more dilute than the acclimation medium. However, the reverse is not true. In all cases the TEP in brine shrimps measured in media more concentrated than the acclimation medium was lower than that measured in shrimps acclimated and measured in the same media (e.g. shrimps acclimated in 50 % SW and measured in 400 % SW showed a much lower TEP, 37 mV, than that of shrimps acclimated in and measured in 400 % SW, 55 mV; Table 2). This may indicate that brine shrimps are unable to 'turn on' active ion transport as rapidly as they can turn it off when the salt concentration of the external medium changes rapidly. These differences have interesting implications for the relative roles of increased activity of existing metepipodite Na^+/K^+-ATPase (enzyme activation) and synthesis of new Na^+/K^+-ATPase molecules (enzyme induction) in situations that require changes in ion transport (see Discussion).

Na^+/K^+-ATPase assay optimisation

To ensure optimal assay conditions, the Na^+/K^+-ATPases in whole phyllopodia and gut of *A. salina* reared in 100 % SW were characterized for some of their ionic and substrate requirements; these data are shown in Fig. 2. Whole phyllopodia were used rather than metepipodites because of the difficulty of dissecting enough metepipodites for the optimisation assays. Both gut and phyllopodial Na^+/K^+-ATPases showed saturation kinetics for potassium, ATP and magnesium, with saturating values of approximately 30, 5 and 3–10 mmol l^{-1}, respectively (Fig. 2A,B,C). Phosphate released from ATP was proportional to phyllopodial protein added to the reaction mixture (Fig. 2D) and the pH optimum for both phyllopodial and gut enzymes was 7.2 (Fig. 2E). Ouabain showed slightly different inhibition kinetics for the two enzymes; K_i was 9.0×10^{-6} mol l^{-1} for phyllopodia and 1.3×10^{-5} mol l^{-1} for gut. As with other crustacean Na^+/K^+-ATPases, ammonium ion could replace K^+ in support of enzyme activity; phyllopodial ESA was 92 % of control levels when K^+ was replaced by 50 mmol l^{-1} NH_4Cl in the incubation medium (data not shown in Fig. 2).

Na^+/K^+-ATPase activity in various seawater media

To assess the role of the brine shrimp Na^+/K^+-ATPase in osmoregulation, shrimps were acclimated for a minimum of 14 days in 50 %, 100 %, 200 % or 400 % SW; they were then dissected and enzyme assays were performed on various body parts (Fig. 3). Note that Fig. 3 shows data from three different groups of shrimps dissected in different ways. Na^+/K^+-ATPase enzyme specific activity (ESA) was highest in the metepipodites and this activity steadily increased in brine shrimps acclimated in increasingly concentrated media. Brine shrimp gut and maxillary gland also had relatively high ESAs and these, too, increased with the salinity of the external medium. Whole phyllopodia showed lower ESAs, probably because metepipodites constitute only a small part of the mass of the phyllopodia. Bodies, heads and phyllopodia without metepipodites had the lowest ESAs and these changed little with increasing medium salinity. Note that the significantly higher value for ESA in the heads of brine shrimps in 400 % SW is probably due to the fact that the heads contained a significant portion of gut. These data strongly implicate the metepipodites, gut and maxillary glands in osmoregulatory ion transport processes in the brine shrimp.

Fig. 4 shows a subset of data from Fig. 3. All the brine shrimps used in this assay were dissected so as to provide complete sets of heads, guts, metepipodites, phyllopodia without metepipodites and bodies. Thus, knowing the protein concentration of each homogenate and its ESA, the total body ESA and percentage of total body ESA for each dissected group of body parts could be calculated for shrimps acclimated in each of the four external media. The most pronounced change was seen in metepipodites, which increased from 7.6 % of total body ESA in 50 % SW to 25.0 % total body ESA in 400 % SW. With the exception of a low value in 50 % SW, body ESA as a percentage of total body ESA

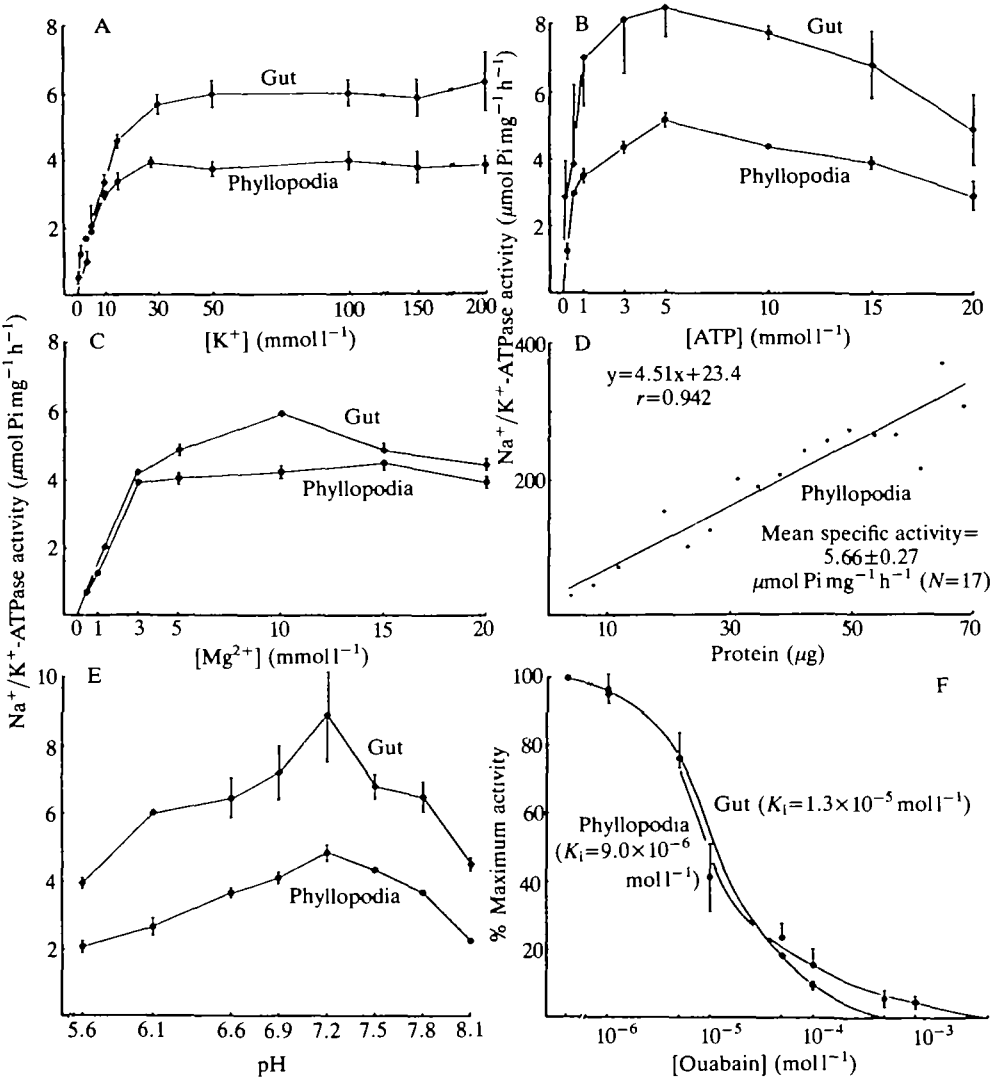

Fig. 2. Optimisation of *Artemia salina* phyllopodial and gut Na^+/K^+-ATPase assay; animals were acclimated in 100% SW. (A) The effect of K^+ concentration on activity at a constant $Na^+ + K^+$ concentration of 130 mmol l^{-1}; (B) the effect of ATP concentration on activity at a Mg^{2+} concentration of 10 mmol l^{-1}; (C) the effect of Mg^{2+} concentration on activity at an ATP concentration of 5 mmol l^{-1}; (D) the effect upon activity of phyllopodial homogenate protein added to the assay mixture; (E) the effect of pH on activity; (F) dose–response curve for ouabain inhibition in the presence of 30 mmol l^{-1} K^+. Unless otherwise indicated, the conditions were as used in the standard Na^+/K^+-ATPase assay (see Materials and methods). Mean values±S.E., N=3–4 homogenates, each made from pooled phyllopodia or guts from 5–10 brine shrimps.

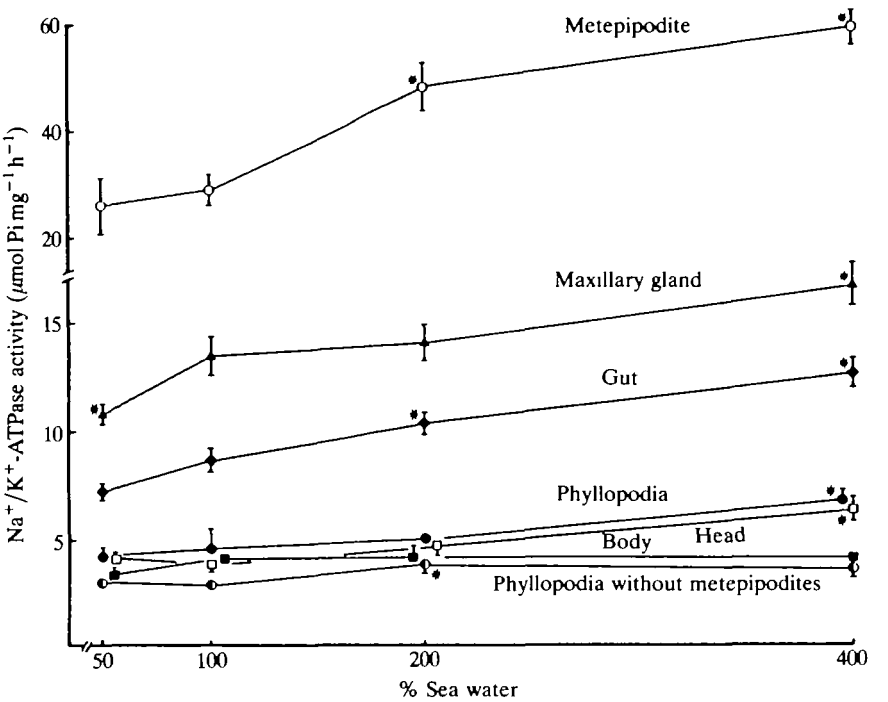

Fig. 3. Na^+/K^+-ATPase enzyme specific activity in crude homogenates of various body parts of *Artemia salina* acclimated for 14 days in seawater media. Mean values±S.E., N=4–10 homogenates, each made from pooled body parts from 5–25 brine shrimps. Asterisks indicate significant differences from 100% sea water control animals for each body part.

decreased, and heads, guts and phyllopodia without metepipodites showed no significant changes as the concentration of the external medium increased. These data strongly implicate the metepipodites in increased outward salt transport in brine shrimps acclimated in increasingly concentrated media.

The eleventh metepipodites of *A. salina* are visibly thinner than metepipodites 1–10 and they do not stain with silver. It is interesting to note that a single assay of the pooled eleventh metepipodites from 40 brine shrimps acclimated in 100% SW had an ESA of 3.2 μmol phosphate mg^{-1} h^{-1}, while a pooled sample of metepipodites 1–10 from the same animals gave a value of 14.0 μmol phosphate mg^{-1} h^{-1}. This finding reinforces the hypothesis that metepipodites of phyllopodia 1–10 are the sites of outward salt transport in the brine shrimp (see Discussion).

Time course of increased phyllopodial and gut ESA

The time courses of changes in phyllopodial and gut ESA were measured in brine shrimps raised in 100% SW and transferred to 400% SW; these data are shown in Fig. 5. Although gut ESA increased between 2 and 4 days after transfer to 400% SW, significant increases in phyllopodial and gut ESA did not occur until

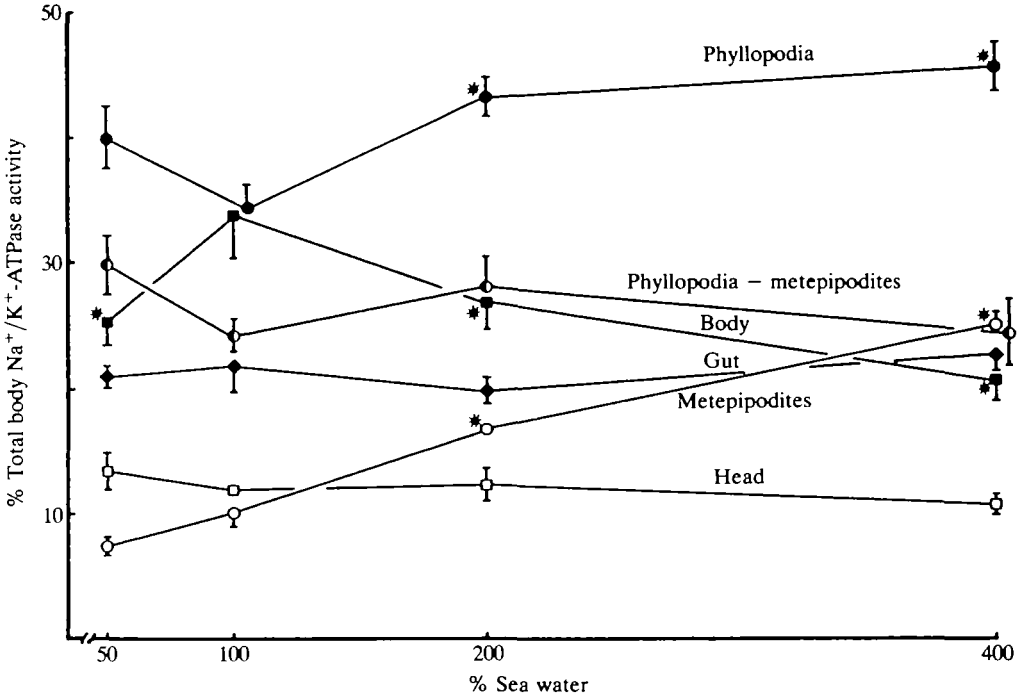

Fig. 4. Na^+/K^+-ATPase enzyme specific activity in crude homogenates of various body parts of *Artemia salina* acclimated for 14 days in seawater media. Data are recalculated as percentage of total body enzyme specific activity from a subset of the data shown in Fig. 3. Mean values±S.E., $N=4$ homogenates, each made from pooled body parts from 5–10 brine shrimps. Asterisks indicate significant differences from 100 % sea water control animals for each body part.

between 4 and 7 days after transfer. This time course would seem to implicate increased synthesis of new enzyme (enzyme induction) in the brine shrimp's response to the increased demand for outward ion transport in 400 % SW.

Discussion

The present study supports and extends the functional similarities between the gills of marine teleosts and brine shrimps in two important ways. First, as is the case in the gills of many marine teleosts (De Renzis and Bornancin, 1984), the metepipodites of *Artemia salina* are shown to have high Na^+/K^+-ATPase enzyme specific activities (ESA) that increase in proportion to the salinity of the external medium and, thus, to the need for outwardly directed ion transport in these strongly hypo-osmotic animals. This increase is dramatic, resulting in 25 % of the total body Na^+/K^+-ATPase ESA being located in the metepipodites in brine shrimps acclimated in 400 % SW (Fig. 4). Second, the range of external media in which sodium is shown to be very close to passive distribution across the body wall of *A. salina* has been extended from 100 % SW (Smith, 1969*a*) to include media

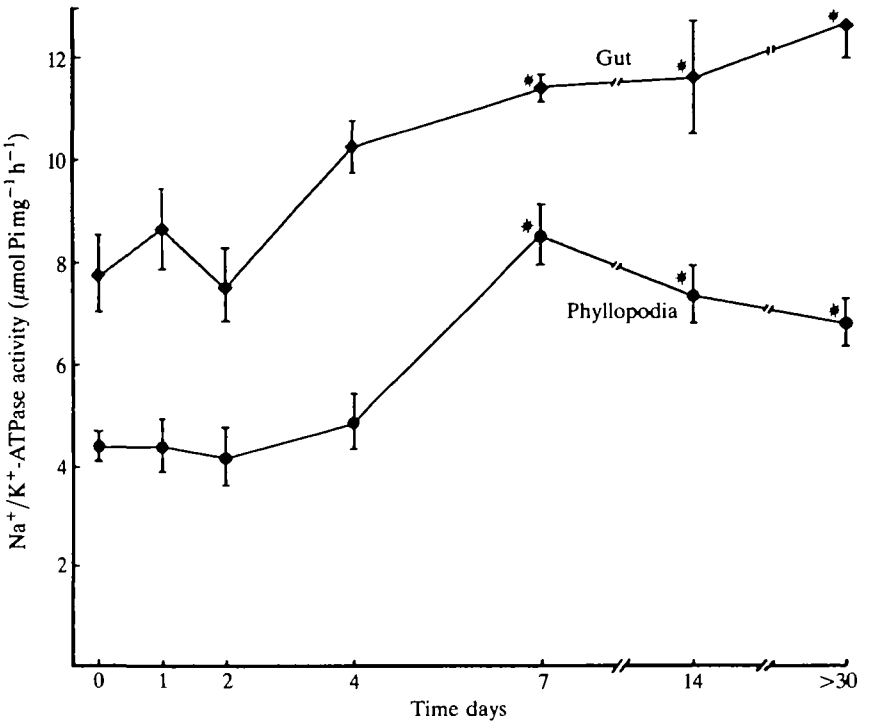

Fig. 5. Time courses of changes in phyllopodial and gut Na^+/K^+-ATPase enzyme specific activity in *Artemia salina* abruptly transferred from 100% to 400% sea water. Mean values±S.E., N=3 homogenates, each made from pooled body parts from five brine shrimps. Asterisks indicate significant differences from control values at time zero.

from 50% SW to 400% SW. Since chloride is shown to be very far from electrochemical equilibrium in all media tested, it seems likely that the brine shrimp uses a mechanism similar to that proposed by Zadunaisky (1984) for the chloride cell of the marine teleost gill. Finally, our findings lend weight to the notion that the dark cells described in *A. salina* metepipodites by Copeland (1967) and the chloride cells described in *A. salina* nauplii by Conte's group (reviewed by Conte, 1984) and by Potts and Durning (1980) in the neck organs of various branchiopods are functionally equivalent to the chloride cells of teleost gills.

Zadunaisky (1984) has proposed the mechanism illustrated in Fig. 6 to account for the function of teleost branchial and opercular chloride cells. This model explains the paradoxical finding that, although Na^+/K^+-ATPase drives ion transport out of the gill, chloride, not sodium, is the ion that is actively transported. Chloride, sodium and potassium ions are thought to enter the chloride cell *via* a basolateral cotransporter which is driven by the sodium concentration gradient across this cell membrane. Sodium is pumped back out of the cell by the basolateral Na^+/K^+-ATPase which is present at high activity in these cells (Karnaky *et al.* 1976). Chloride, which has entered the cell against its

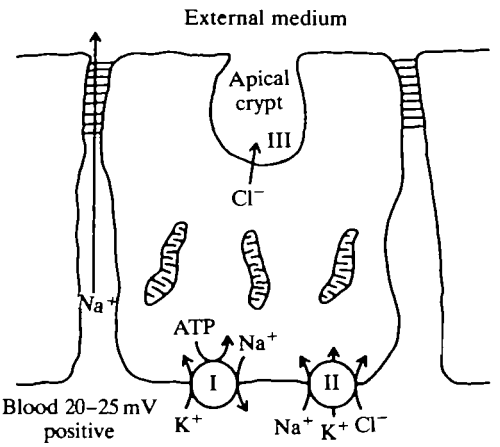

Fig. 6. A model for the teleost chloride cell (after Zadunaisky, 1984). (I) The basolateral Na^+/K^+-ATPase; (II) the basolateral $Na^+/K^+/Cl^-$ cotransporter; (III) chloride diffuses out down its electrochemical gradient at the apical crypt. Note that there is no evidence for the existence in crustacean branchial epithelial cells of the structural equivalent of the apical crypts present in teleost chloride cells.

electrochemical gradient, diffuses to the external medium down its gradient across the cell membrane of the apical crypt. The net result of the operation of the chloride cells is that the seawater teleost has a TEP of approximately 20–25 mV, inside positive (Potts, 1984). Sodium is in electrochemical equilibrium across the gill epithelium and is thought to diffuse out of the fish *via* the tight junctions between the epithelial cells. Our findings in the present study and those of Copeland (1967), Croghan (1958*c*), Smith (1969*a*,*b*) and Thuet *et al.* (1968) suggest that a very similar mechanism might be at work in the dark cells of the brine shrimp metepipodite.

The present study is the first to report the patchy distribution of silver staining in *A. salina* metepipodites. Since the smallest silver staining patches in Fig. 1A are approximately the size of typical epithelial cells (approx. 10–20 μm in diameter), it is tempting to suggest that the reticulations seen in silver-stained metepipodites correspond to chloride-permeable areas of cuticle secreted by rows of the dark cells reported by Copeland (1967) in the same species. Croghan (1958*c*) has validated the silver staining method as showing areas of high, but passive, chloride permeability in the cuticle of *A. salina* metepipodites and has suggested that these areas must be permeable if outward ion transport by these structures is to be possible. Croghan provided further evidence for this idea: he reported that developing *A. salina* add a pair of phyllopodia at each moult, but that the metepipodites of these newly formed appendages do not stain with silver until the following moult. He interpreted this to indicate that ion transport and, thus, the need for a permeable cuticle arise by epithelial differentiation at the moult after phyllopodial formation. For this reason the metepipodites of the eleventh

phyllopodia do not stain with silver; they appear at the last moult and never have a chance to develop an ion transport epithelium with a chloride-permeable cuticle. Croghan's hypothesis is supported by our finding that the eleventh metepipodites have a Na^+/K^+-ATPase ESA less than one-quarter that of metepipodites 1–10, indicating that the eleventh metepipodites do not develop the capacity to pump salts out of the haemolymph.

Although it seems likely that the silver staining technique identifies areas of high chloride permeability in *A. salina*, other mechanisms could also account for the differential staining. It is possible that the stain reveals areas of the cuticle that are generally very permeable to cations such as Ag^{2+}, which enters from the silver nitrate solution and then precipitates with chloride ion in the cuticle. If this is the case, then silver staining may identify areas of the metepipodites that are very cation-permeable and that may be the sites of passive exit of sodium ions, as in the Zadunaisky chloride cell model (Fig. 6). In this regard it is of interest that Barra *et al.* (1983), using X-ray microanalysis, have found that the precipitates which form in the branchial cuticle of silver-lactate-treated crabs, *Eriocheir sinensis*, do contain large amounts of silver and chloride. Thus, although interpretation of what the silver stain is actually showing may be problematic, it is clear that it identifies areas of relatively high ion transport activity in the cuticle.

When *A. salina* is transferred from 100 % SW to 400 % SW, the time course of changes in metepipodite Na^+/K^+-ATPase ESA is consistent with the synthesis of new enzyme in response to the increased demands for outward ion transport (i.e. several days are required; Fig. 5). Although short-term changes in the rate of ion transport probably occur, synthesis of new enzyme also appears to be necessary for increased osmoregulatory ion transport. The TEP data shown in Table 2 also lend indirect support to this idea. When transferred to media less concentrated than the acclimation medium, the TEPs decrease within 3–4 min to values very close to those in shrimps acclimated in the same dilute media. Thus, if the TEP is assumed to be due to active ion transport, such ion transport can be rapidly decreased in dilute media, suggesting rapid modulation (i.e. activation/deactivation of existing enzyme) of the Na^+/K^+-ATPase, which is assumed in the model above to drive chloride and sodium exit from the gills. However, when brine shrimps are transferred to media more concentrated than the acclimation medium, the TEP does not rapidly increase to values close to those in brine shrimps acclimated in the same concentrated media, but remains well below that of acclimated animals. This indicates that electrogenic ion transport and, presumably, the Na^+/K^+-ATPase ESA cannot be increased as rapidly as they are decreased and is consistent with the idea that Na^+/K^+-ATPase synthesis is necessary for the full response.

Morphological evidence also stresses the role of increased synthesis of Na^+/K^+-ATPase in acclimation of *A. salina* in concentrated media. When viewed edge-on in the dissection microscope, metepipodites dissected from shrimp acclimated in 400 % SW were visibly thicker than metepipodites from shrimp acclimated in 50 % SW, probably indicating hypertrophy of ion transport tissue within the metepipodites in the more concentrated medium. Copeland (1967) and Croghan (1958*c*)

have also reported thickened metepipodite epithelium in brine shrimps acclimated in concentrated media. Since brine shrimps are strong hypo-osmotic regulators in 400 % SW and, thus, tend to lose water to the external medium by osmosis, the increased thickness of the metepipodites was probably not due to osmotic swelling. Thus, it appears that the population of dark cells in the metepipodite epithelium may increase in thickness and/or multiply in response to transfer of the brine shrimp to media of high salinity. If this is so, it seems likely that these processes of hypertrophy in the metepipodite epithelium would require several days for completion.

Croghan (1959*d*), Smith (1969*b*) and Thuet *et al.* (1968) have shown that *A. salina*, like marine teleosts, drinks hypertonic medium to replace water lost to the medium by osmosis. Drinking rates were quite high (48–72 % body mass day^{-1}), but the influx of sodium and chloride *via* the gut was calculated in the last two reports above to be much smaller than influx across the body surface. Given that the concentrations of sodium and chloride in gut fluid are much lower than those in the haemolymph, Croghan (1959*d*) and Smith (1969*b*) suggested that sodium and chloride must be actively transported out of the gut fluid and that this transport might drive the reabsorption of water from the gut. The correlation between gut Na^+/K^+-ATPase ESA and medium salinity seen in the present study supports this hypothesis in that gut ESA rises with the need to increase fluid uptake from the gut. The process is presumably driven by Na^+/K^+-ATPase, but no direct measurements have been made of ion or fluid transport by the brine shrimp gut. Preliminary measurements of the TEP between the gut and haemolymph in the present study indicate that the gut is approximately 8 mV lumen-positive with respect to the haemolymph in shrimps from 50 % and 100 % SW. Thus, transport of sodium and chloride out of the gut fluid is not a strongly electrogenic process in this epithelium.

We note in closing that the brine shrimp maxillary gland has high a Na^+/K^+-ATPase ESA which increases in proportion to the salinity of the external medium, indicating that these glands may participate in osmotic or ionic regulation. Tyson (1969) has shown that the efferent duct of the maxillary gland of *A. salina* shows ultrastructural features typical of transporting epithelia. However, since the secretion of the gland has not been sampled, its role in osmotic and/or ionic balance remains unknown.

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