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THE IONIC COMPOSITION OF ION TRANSPORTING CELLS IN STONEFLY NYMPHS

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Abstract

Freshwater stonefly larvae take up sodium and chloride ions from low concentrations in water and concentrate them into haemolymph. The ions are transported by mitochondria rich cells (MRITCs) which are easily identified by their windows in the cuticle. Sodium uptake is generally inhibited at low pH but a few species of stoneflies live in acid waters. Acid tolerance is associated with presence of caviform or bulbiform MRITCs while nymphs possessing only the more common coniform cells are sensitive to acid conditions.

The intracellular concentrations of Na, K, Cl and P in coniform cells of *Perla bipunctata* and bulbiform cells of *Protonemura meyeri* have been measured by X-ray emission analysis in a scanning electron microscope (SEM). Both bulbiform and coniform cells respond to low pH by increasing the concentrations of P and of the ions Na, K and Cl, but the ionic concentrations in coniform cells soon decline at low pH and the cells fail, while bulbiform cells maintain their ionic concentrations down to a lower pH. The major transport systems on the apical and baso-lateral membranes have been identified from the effects of ions and drugs, applied to the two surfaces, on the intracellular concentrations of ions. Bulbiform and coniform cells contain similar transport systems but differ quantitatively in response to low pH.

Key Words: Plecoptera, acid precipitation, X-ray emission analysis, ion transporting epithelia, mitochondria-rich-ion-transporting cells, sodium ammonium exchange, sodium hydrogen exchange, chloride bicarbonate exchange, ouabain, ethacrynic acid.

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Introduction

August Krogh (1937, 1938) first demonstrated that freshwater animals could take up sodium and chloride ions from the medium by at least two independent active mechanisms. During the last fifty years the salient morphological features of the more active ion-transporting cells have been identified. Progress has been made in understanding the kinetics of ion uptake and the locations of the ion pumps and the ion exchangers have been identified in a few species. The most striking feature of these epithelia is their ability to transport ions across very large concentration gradients but the concentrations of intracellular ions, essential to the analysis of ion transport, have remained largely uncertain. A few measurements have been made using ion selective electrodes (Coimbra *et al.*, 1988; Zadunaisky *et al.*, 1988) but no systematic analysis has been made of ionic concentrations in ion transporting cells in freshwater animals, which maintain the largest ionic gradients, or of the effects of changes in the external medium or of drugs, on the intracellular concentrations.

The ion-transporting cells in plecopteran nymphs are readily identifiable on the gills and elsewhere (Komnick, 1977). Plecopteran nymphs are found in cold unpolluted waters and a few species are very tolerant of acid conditions. Three types of mitochondrion-rich-ion transporting cells (MRITCs) have been described in plecopteran nymphs. The bulbiform and caviform cells are confined to acid-resistant species while coniform cells are found either alone in acid-sensitive species or in combination with bulbiform or caviform cells in acid-resistant species. The thick rim of cuticle surrounding caviform cells makes it exceedingly difficult to expose the cytoplasm while still identifying the cell but both coniform and bulbiform cells can be planed obliquely at a low angle with a glass blade and analyzed by X-ray emission spectroscopy. When the window of a coniform cell or the base of the bulb of a bulbiform cell was cut obliquely it was assumed that the cytoplasm immediately below belonged to the MRITC.

The concentrations of sodium, potassium, chloride

and phosphorus in both bulbiform and coniform cells have been measured in neutral water, during exposure to low pH water, and during exposure of either the external or serosal surfaces to a variety of drugs, including ouabain, amiloride, SITS (4-acetamido-4'-isothiocyanato-stilbene 2,2'-disulphonic acid), DIDS (4-diisothiocyano-stilbene-2,2'-disulphonic acid), and ethacrynic acid and ammonium and bicarbonate ions. From the resulting changes in intracellular concentrations, the major ion pumps have been located.

Methods

Nymphs were kept at 5 °C in flowing recycled water.

Salt-free gelatine was prepared by dialysis in distilled water at 40 °C for 4 days with a trace of phenol to suppress bacterial action. The gelatine was dried *in vacuo* and dissolved in solutions containing suitable concentrations of Na, K, Cl and PO_4, so that all standards contained 22% protein comparable with that in other tissues composed of MRITCs (e.g., Bonting, 1966).

Material for analysis, together with a suitable standard, was placed on a 30° angled aluminium specimen stub, which had been coated with a thin layer of Leitz C (Neubauer) to maintain electrical contact. The stub was then pressed onto the copper block which had been cooled in liquid nitrogen to -196 °C. Just prior to use the block was moved clear of the nitrogen, but was kept in the layer or nitrogen gas just above the surface. The frozen specimens were planed by a Slee Cryomicrotome at -100 °C, to give a flat topography (Hess, 1980).

The specimens were transferred, under liquid nitrogen, to a Hexland Cryo-chamber cooled to -180 °C, attached to a JEOL JSM840A scanning electron microscope (SEM) equipped with a Hexland Cryo-stage and a Kevex energy dispersive spectroscopy (EDS) analyzer. In the cryo-chamber the material was coated by evaporating a 1 x 1.5 cm strip of aluminium foil, at 10^{-5} Torr, giving a coating of 42 $\pm$ 7.7 nm of aluminium. Analyses were carried out at a temperature of -160 °C, accelerating voltage 15 kV, specimen to detector working distance 19.5 mm, and a probe current of 0.5 nA. Areas of 1 μm were analyzed at a magnification of 10,000X (Oates, 1984). The counts were stopped when the aluminium count had reached 80,000.

Standard curves were prepared. The average regression of the elemental peak, to the aluminium peak against concentrations in mmol/kg water, was linear but standards were included in each specimen analyzed as the peak heights are sensitive to changes in take off angle.

The effects of changes at the apical surface of the cells, such as low pH, ammonium ions or drugs, were examined using whole nymphs.

Table 1. Sources of drugs and chemicals

Amiloride	Sigma Chemical Company
Diamox	Lederal Laboratories
Ethacrynic Acid	Sigma Chemical Company
Ouabain	Sigma Chemical Company
SITS	Sigma Chemical Company
DIDS	Sigma Chemical Company
Bicarbonate ions	BDH Analar as Na or Ca salts
Ammonium ions	NH_4Cl from BDH Analar
Bumetanide	Sigma Chemical Company

In order to examine the effects of changes in the haemolymph and to apply drugs to the baso-lateral membranes small pieces of cuticle containing MRITCs were mounted in Ussing chambers at 10 °C, the upper chamber containing an artificial medium containing 0.25 mmole l^{-1} Na, 0.05 mmole l^{-1} K, 0.3 mmole l^{-1} Cl, pH 7.0, the lower an artificial plecopteran saline containing 122 mmole l^{-1} Na, 11 mmole l^{-1} K, 2 mmole l^{-1} Ca, 108 mmole l^{-1} Cl, 2 mmole l^{-1} HCO_3 and sulphate and mannitol to produce an osmotic pressure of 350 mOsmole l^{-1}, pH 6.8 (Sutcliffe, 1962). For experiments with higher bicarbonate, bicarbonate was substituted for sulphate. The solution was aerated with oxygen containing 5% CO_2. Under these conditions the cells would exclude Alcian Blue for 12 hours and did not differ in ionic content from freshly prepared tissue. The drugs used and their sources are listed in Table 1. Each point on the Figs. 1 to 5 and on Tables 2 and 3 was the mean of measurements on 100 cells from 10 different animals, 10 per animal.

Results

The Effects of Low pH in the External Medium on the Intracellular Concentrations of Ions

In freshwater animals sodium ions are believed to be taken up in exchange for hydrogen ions and sodium uptake is largely inhibited at low pH (Maetz and Garcia-Romeu, 1964). Measurement of sodium uptake, using ^{22}Na, show that at low pH sodium uptake by plecopteran nymphs is instantaneously reduced although it recovers later as the animals adapt (Twitchen, 1990). Both the coniform and bulbiform cells responded to a low pH medium by an increase in the levels of phosphorus but whereas the acid resistant *Protonemura meyeri* only increased phosphorus when the pH was reduced to 3.5 (Fig. 1), *Perla bipunctata* increased phosphorus at pH 5.5 (Fig. 2), but in both species the increases were not sustained and the cells died a few days after. The level of potassium also increased (Fig. 3), as might be expected if more Na-K-ATPase were available for the baso-lateral membranes, but so, unexpectedly, did the

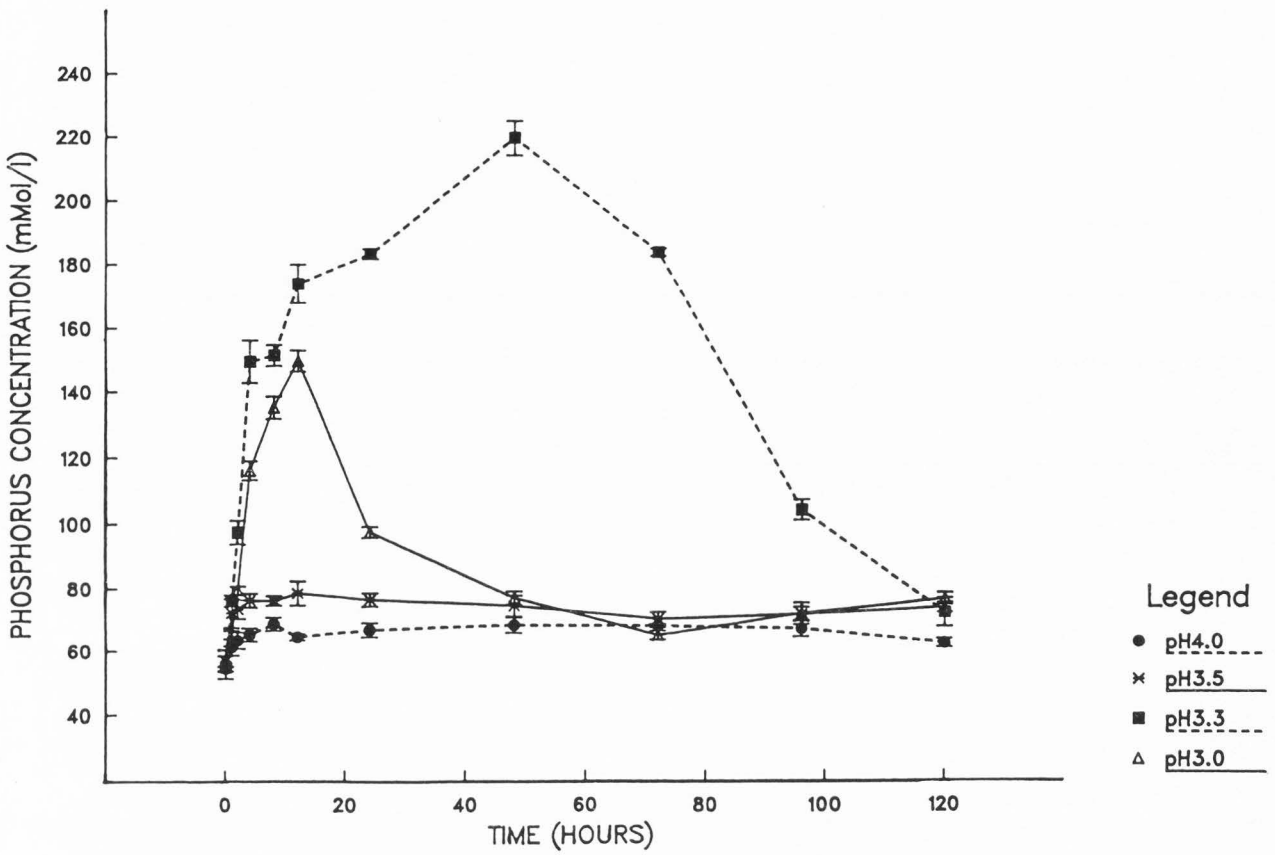

Fig. 1. The effect of pH on the intracellular concentration of phosphorus in the bulbiform MRITCs of *Protonemura meyeri*.

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concentrations of sodium and chloride (Figs. 4 and 5).

The Effect of Amiloride

Amiloride blocks the exchange of hydrogen or ammonium ions for sodium ions in many tissues (Payan, 1978, Kerstetter *et al.*, 1970). When applied to the external surface of the cells the intracellular concentration of sodium ions dropped below the level or detection as the uptake of ions was cut off, but the concentrations of phosphorus and potassium increased (Tables 2 and 3).

Bicarbonate Ions

Chloride is believed to be taken up in exchange for bicarbonate or hydroxyl ions in freshwater animals (Maetz and Garcia-Romeu, 1964). When the concentration of bicarbonate was increased on the basal membrane from 2 to 10 mmole l^{-1}, the intracellular concentrations of chloride increased but when the concentration in the external medium was increased to 5 mmole l^{-1} the intracellular concentration fell (Tables 2 and 3). Diamox (acetazolamide), which inhibits carbonic anhydrase and will therefore reduce the supply of endogenous bicarbonate ions, also reduced the intracellular concentration of chloride, whether applied basally or apically (Tables 2 and 3).

Ammonium Ions

Sodium ions can also be taken up in freshwater animals in exchange for ammonium ions produced metabolically (Maetz, 1973). When 1 mmole l^{-1} of ammonium was added to the external medium, the intracellular sodium concentration fell, perhaps because sodium uptake across the apical membrane, in exchange for ammonium ions, was reduced, while sodium continued to be removed from the cell across the basal membrane. When 0.5 mmole l^{-1} ammonium was supplied to the haemolymph side the sodium concentration increased (Tables 2 and 3).

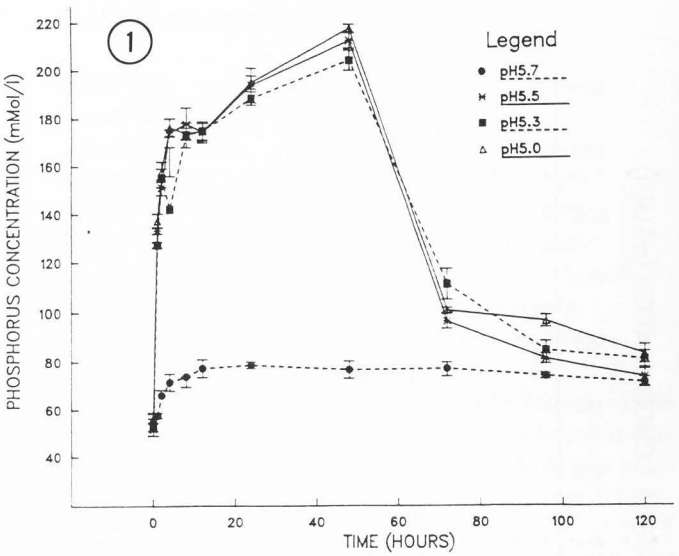
Ammonium and Amiloride

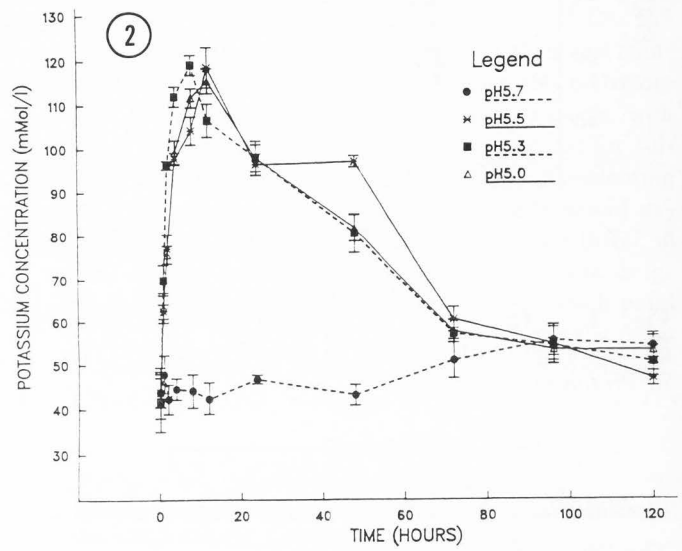
When sodium uptake was inhibited by amiloride the concentration of sodium in the cell rapidly fell below the level of detection (Tables 2 and 3), but when 0.5 mmole l^{-1} NH_4Cl was added to the saline on the basal surface, after sodium uptake had been blocked by amiloride, the intracellular sodium recovered to 23 mmole/kg water.

Table 2. Ion Concentration in Coniform MRITCs of *Perla bipunctata* in mmol/kg water ± SE

Time mins	0	20	45	90
DRUG				
Ouabain (basal)				
Na	35±6	32±4	<20*	<20*
Cl	44±5	41±3	41±4	36±4
P	55±6	47±4	26±6*	18±6*
K	55±6	26±6*	<15*	<<15*
Diamox				
Cl	43±3	28±6*	18±7*	<15*
Time hrs	0	2	4	8
Amiloride (apical)				
Na	33±4	21±6*	<20*	<20*
Cl	44±3	48±4	55±3*	66±5*
P	58±4	62±7	110±9*	141±7*
K	52±3	61±7	89±5*	83±6*
Ethacrynic acid (apical)				
Na	34±5	22±4	<20	<20
Cl	45±5	55±4	56±3	61±4
P	56±4	68±4	77±6	95±5*
K	56±4	61±7	62±6	61±4
DIDS (basal)				
Cl	44±6	68±6*	76±5*	74±4*
Bicarbonate				
Cl (apical)	45±7	34±3	27±5*	23±4*
Cl (basal)	42±2	51±2	58±2*	89±4*
Ammonium (basal)				
Na	33±5	48±6	56±5*	51±3*
K	53±3	63±4	67±2*	63±2
P	54±3	67±6	66±3	62±2
Ammonium (apical)				
Na	33±4	24±4	21±3	23±6
K	52±2	46±3	36±4*	35±2*
P	53±3	45±2*	45±6	42±3*

* P<0.05 between T=0 and starred fig.





Figs. 2 and 3. The effects of pH on the intracellular concentrations of phosphorus (**Fig. 2**) and potassium (**Fig. 3**) in the coniform MRITCs of *Perla bipunctata*.

This indicates that there are two independent sodium transport mechanisms on the apical membrane, one sensitive to amiloride, exchanging Na^+ for H^+; the other, insensitive or less sensitive to amiloride, Na^+ for NH_4^+.

Ethacrynic Acid

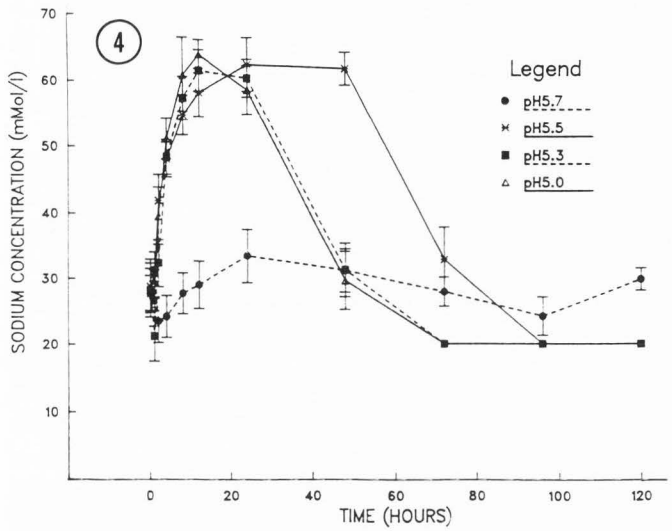
Ethacrynic acid also inhibits Na^+/H^+ exchange (Pequeux and Gilles, 1981). It proved to be effective only when applied at a concentration of 0.1 mmole l^{-1} to the apical surface. Intracellular sodium declined and intracellular potassium and phosphorus and chloride increased (Tables 2 and 3).

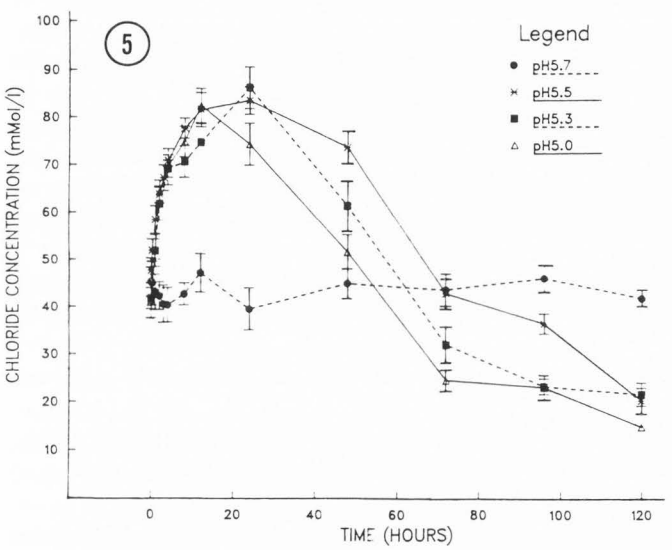
Ouabain

When 1.0 mmole l^{-1} of the Na-K-ATPase inhibitor ouabain was applied to the serosal surface the intracellular concentrations of potassium and sodium and of phosphorus all declined to low levels, but chloride remained almost unchanged (Tables 2 and 3).

SITS and DIDS

The closely related inhibitors of chloride-bicarbonate exchange, SITS and DIDS, (Siebers and Boron, 1989) were only active when applied to the serosal surfaces of bulbiform and coniform MRITCs. DIDS was the more effective inhibitor. In the presence of 1 mmol l^{-1} of DIDS intracellular chloride rose to a maximum of 79 mmol/kg water in *P. bipunctata* as chloride efflux from the cells was inhibited but potassium and sodium were not affected significantly (Tables 2 and 3).





Figs. 4 and 5. The effects of pH on the intracellular sodium (**Fig. 4**) and chloride (**Fig. 5**) concentrations in the coniform MRITCs of *Perla bipunctata*.

Table 3. Ion Concentration in Bulbiform MRITCs of *Protonemura meyeri* in mmol/kg water ± SE

Time mins	0	20	45	90
DRUG				
Ouabain (basal)				
Na	27±4	35±4	<20	<20
Cl	44±3	38±3	32±5	31±4*
P	55±3	34±3*	24±5*	25±4*
K	45±3	21±5*	<15*	<15*
Diamox				
Cl	41±2	32±3*	18±6*	<15*
Time hrs	0	2	4	8
Amiloride (apical)				
Na	28±4	<20	<20	<20
Cl	41±4	48±6	52±4*	64±3*
P	53±4	63±4	71±4*	128±8*
K	44±2	62±5*	63±5*	64±6*
Ethacrynic acid (apical)				
Na	29±4	22±5	<20	<20
Cl	41±5	53±2*	60±4*	69±4*
P	54±3	64±4	65±7	76±4*
K	43±6	55±4	55±2	58±3*
DIDS (basal)				
Cl	42±3	71±5*	76±4*	72±3*
Bicarbonate				
Cl (apical)	44±3	33±2*	28±4*	23±4*
Cl (basal)	44±4	51±5	58±3*	89±7*
Ammonium (basal)				
Na	29±4	48±4*	46±3*	49±5*
K	45±3	56±6	60±3*	61±2*
P	55±5	65±4	64±6	65±3
Ammonium (apical)				
Na	27±3	20±7	<20	<20
K	44±3	40±2	34±4*	26 3*
P	55±3	52±4	47±4	42±2*

* P<0.05 between T=0 and starred fig.

Furosemide, Bumetanide

Furosemide and bumetanide, which block Na-K-2Cl co-transport, had no detectable effect on the ionic concentration whether they were applied at either the apical or basal surfaces (Burg and Green, 1973).

Discussion

There are many similarities between the behaviour of coniform and bulbiform cells, the latter differing only quantitatively from the more pH sensitive coniform cells. After exposure to pH 5 the intracellular concentrations of Na, K, Cl and P rose initially in coniform cells but after three days the levels fell dramatically. Bulbiform cells on the other hand can tolerate pH 4 indefinitely but concentrations fell after a few days at pH 3 and the cells later died. The nymphs survived until the following moult but then died. These temporary changes would enable the nymphs of both species to tolerate short acid episodes below their normal pH ranges but would not enable them to survive prolonged exposure.

Most of the phosphorus will be present as phosphate in various compounds and the osmotic pressure exerted will be lower than the nominal concentration. The sodium, chloride and potassium ions on the other hand will

exist partly in ionic form and, although their activity coefficients will be less than unity, they must exert considerable osmotic pressures. The nominal total concentration of these ions is about 115-130 mmol/kg water in the normal cell but after 10 days at pH 3 the total had risen to 240 mmol/kg water in *P. meyeri*, compared with a total haemolymph concentration of 350 mOsmole l^{-1}. As the cells have a large surface area exposed to the haemolymph and only a small area exposed to the water, they may be presumed to be iso-osmotic with the haemolymph. However, if the cells are to remain iso-osmotic with the haemolymph, the total osmotic pressure must be regulated by some organic constituents in addition to the ions.

When the concentrations of Na, K, Cl and P all increase together at low pH, it is possible that the cells have shrunk but there are two arguments against this hypothesis. First, water movement follows concentration gradients, it does not create them; the higher concentrations should draw water into the cell. Secondly, when sodium uptake is impeded by low pH, or ethacrynic acid, P increases although the sodium concentrations fall in these circumstances. However, the possibility or some volume change cannot be ruled out.

When the stresses on MRITCs are increased by reducing the ionic concentration of the media, the density of intramembranous particles in the rectal MRITCs of the nymphs of the dragonfly, which are probably the morphological equivalent of the Na-K-ATPase, were increased (Kukulies and Komnick, 1983) and similarly the numbers of mitochondria in the epithelial cells of teleost gills were also increased (Laurent *et al.*, 1990). The increase in total P in the plecopteran larvae is likely to be indicative of an increase in cell metabolism.

Ion uptake under normal and acid conditions by freshwater fishes provides a comparable model for the plecopteran nymphs. Na^+/H^+, Na^+/NH_4^+ and Cl^- for HCO_3^- or OH^- exchange systems are well established in fish (Maetz and Garcia-Romeu, 1964; Evans, 1984) and the presence of a ouabain sensitive Na-K-ATPase on the serosal surface has also been demonstrated.

The freshwater crayfishes show a similar arrangement, with Na^+ for H^+ or NH_4^+ and Cl^- for HCO_3^- or OH^- exchanges on the apical surfaces (Ehrenfeld, 1974; Wheatly, 1989) combined with a baso-lateral Na-K-ATPase. Similar systems are also found in brackish and freshwater crabs (Burnett and Towle, 1990).

The response of the intracellular ions to drugs suggests that, in general, the ion transporting systems resemble those in freshwater fishes. For example, the decline in intracellular sodium in the presence of external amiloride is consistent with an amiloride sensitive Na^+/H^+ exchange system on the apical membrane, while the effect of Diamox is consistent with an apical HCO_3^-/Cl^- exchange system but this is evidently insensitive to DIDS. A HCO_3^-/Cl^- exchange sensitive to DIDS, is present on the serosal membranes.

The intracellular concentrations of ions in an ion-transporting epithelial cell must depend on the balance of fluxes across both the apical and serosal surfaces. When a counter ion, such as H^+, NH_4^+ or HCO_3^-, may be active at both, the effects are more complex and more difficult to interpret. The effect of serosal ammonium ions in elevating intracellular sodium may arise because the ammonium ions penetrate the cell and provide a counter ion for enhanced Na^+/NH_4^+ at the apical surface membrane via an amiloride insensitive system but they must cross the serosal, either directly or as NH_3, not via an Na^+/NH_4^+ exchange system, as that would lower the intracellular sodium. The fall in intracellular chloride, when the external concentration of bicarbonate is raised, also suggests that the apical membrane is insensitive to DIDS. The elevation of intracellular chloride when the serosal bicarbonate is raised, may arise from the enhancement or HCO_3^-/Cl^- exchange at the apical membrane. However, the bicarbonate must enter the cell, at least in part, by a DIDS sensitive exchanger on the serosal surface and there must be some other difference between the two membranes. It is likely that energy must be provided to drive chloride ions across the large electrochemical potential at the apical membrane which may therefore be more sensitive to changes in the availability of the counter ion. There are some other anomalies. At low external pH intracellular sodium increases. When sodium uptake was inhibited by amiloride the phosphorus levels rose, while intracellular sodium fell (Table 2), but following transfer to a low pH, which also inhibits sodium uptake (Twitchen, 1990), both the intracellular sodium and phosphorus increased (Figs. 2 and 4).

Sodium uptake in plecopteran nymphs follows Michaelis-Menton kinetics but following transfer to a low pH partial inhibition of sodium uptake is later compensated by an increase in f_{max}. The increase in phosphorus compounds may well represent the increase in energy supply required to restore sodium influx but it might be expected that the sodium influx would be restored when the intracellular sodium content had returned to normal. The higher levels observed (Fig. 4), suggests that low pH in the medium also interferes with sodium transport across the base of the cell as well.

Treatment with ouabain would be expected to cause a rise in intracellular sodium and a fall in intracellular potassium. Although there is an initial increase in sodium in *Protonemura meyeri*, (Table 2), all ions eventually decline in both species. This is not consistent with ion regulation in a living cell but these concentrations are found within a few microns of the apical membrane.

If the cells are dying, the apical membrane may become permeable and ions would be lost by diffusion to the external medium. This might account for both the loss of sodium and the low total concentration of ions.

The intracellular concentrations in ion transporting cells have only been measured on a few occasions, by means of ion-selective electrodes. In teleosts adapted to sea water the MRITCs excrete chloride ions to maintain a plasma concentration hypo-osmotic to sea water. Chloride is transported across the epithelium by an Na^+/Cl^- co-transporter, which utilizes the energy available from the sodium gradient across the baso-lateral membranes in order to drive chloride into the cell from the plasma (Zadunaisky *et al.*, 1988). A similar system is used by the shark rectal gland (Welsh *et al.*, 1983) but neither epithelium is closely comparable to that of the freshwater stoneflies.

A closer comparison is provided by the mantle of the freshwater lamellibranch *Anodonta cygnea* (Coimbra *et al.*, 1988). The mantle consists of two layers, one between the external medium and the haemolymph, the second separating the haemolymph from the pallial fluid, which is in contact with the shell. The haemolymph concentration in *Anodonta* is very low (57 mOsmole) and the only measurements available refer to the composition of the cells of epithelium separating the haemolymph from the pallial fluid. Here the intracellular activities were K 26.5 mmole l^{-1}, Cl 7.9 mmole l^{-1} and the chemical concentrations were K 29.4, Cl 12.9 and Na 14.0 mmole l^{-1}. When bicarbonate was removed from the haemolymph the intracellular concentration of chloride rose by 28% in 7 minutes, as in the coniform and bulbiform cells of the plecopterans when HCO_3 was added basally.

As the concentration ratios between the external medium and the haemolymph in most freshwater animals are over 1:1000 the electrical potentials required to move either cations or anions from medium to haemolymph would have to be about 200 mV, much greater than the potentials generally reported in aquatic organisms. In spite of the uncertainties with regard to the activities of the intracellular ions and the electrical potentials across the apical and baso-lateral membranes, it is possible to estimate whether or not the movements of sodium and chloride ions across the apical and basal membranes are likely to be active or passive.

Movement across the baso-lateral membrane presents few problems. Sodium will be moved out of the cell, against both an electrical and a concentration gradient, by ATP and the Na-K-ATPase system. Chloride must be moved against a concentration gradient of between 2:1 and 3:1 but will be assisted by the electrical potential created by sodium extrusion. A potential of only 30 mV would be sufficient to maintain a 3:1 concentration gradient and the potential is likely to be greater than this. Chloride efflux across the cell base is therefore likely to be passive and evidently takes place by a SITS sensitive HCO_3^--Cl^- exchanger, which may help to concentrate bicarbonate in the cell.

Transport across the much larger gradients across the apical membrane presents a greater problem. Sodium uptake will be assisted by the potential gradient, which may well exceed 60 mV, sufficient to maintain a concentration gradient of over 10:1. If the intracellular pH were lower than that of the medium a further source of energy would be available from the hydrogen ion gradient but this could not be the case in the acid waters. If ammonium were used as the counter ion, a further source of energy would be available. Stoneflies frequent pure waters where external ammonium is negligible. It is possible that the acid-resistant species turn over from hydrogen-sodium ion exchange in ammonium-sodium ion exchange at low pH.

If ammonium were accumulated to 5 or 10 μmole l^{-1} intracellularly then sodium transfer across the apical membrane might not require any local energy source if the external ammonium concentration were sufficiently low, less than 10 μmole l^{-1}. Ammonium ions might be supplied by a glutamine-glutaminase or, as they resemble potassium ions in many respects, concentrated in the cell in exchange for sodium ions. On the other hand mitochondria are present in the apical regions of the cells and might provide energy for some active pump.

Chloride must cross the apical membrane against both a high concentration gradient and against whatever potential assists the sodium ion. It could be assisted by a substantial bicarbonate gradient but a gradient of at least 1000:1 would be required to bring in chloride against a 100:1 concentration gradient and against a potential equivalent to a 10:1 concentration gradient. In acid waters the OH^--gradient between the medium and the cytoplasm might be used to bring in chloride ions as this gradient would increase as the external pH fell.

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Discussion with Reviewers

A.J. Spencer: Expression of data in terms of mmol/kg H_2O (rather than mmol/kg wet weight) requires knowledge of the dry mass fraction within your sample. You have this for your standards, but how was it derived for your specimens?

Authors: Vertebrate kidneys, avian and reptilian salt glands, and selachian rectal glands all contain around 22-25% solids, which are mainly protein. We therefore think the MRITCs are likely to be similar in composition. Aqueous standards were prepared to which salt-free protein was added. We doubt if the organic matter will be outside the range of 20-25% in MRITCs and any error is likely to be small.

G.N. Ling: There is long-standing and often-reviewed evidence that the postulated Na pump in cells like frog muscle requires a minimum energy-consumption rate far in excess of that available. This key evidence demonstrates that the Na-pump hypothesis is untenable for frog muscle and other similar cells. In addition, a new theory of selective K^+ accumulation and Na^+ exclusion as an equilibrium phenomenon, requiring no continual energy consumption was presented (GN Ling "A Physical Theory of the Living State", Blaisdell Publ Co, Waltham, MA, 1962). A subsidiary theory of active transport of ions and other solutes across frog skin, intestinal epithelium and other similar bifacial cells was briefly introduced in 1965 and in greater detail later (e.g., GN Ling, Scanning Microsc **4**, 723-736, 1990). Please comment.

Authors: These are interesting theories but within the limited scope of this paper we couldn't possibly do them justice.