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THE EFFECT OF EXTERNAL SODIUM AND CALCIUM CONCENTRATIONS ON SODIUM FLUXES BY SALT-DEPLETED AND NON-DEPLETED MINNOWS, *PHOXINUS PHOXINUS* (L.)

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SUMMARY

The relationship between sodium influx and external sodium concentration in *Phoxinus* is complex and unusual. In non-depleted fish the relationship is approximately that given by the Michaelis–Menten equation of enzyme kinetics. However, the K_m value (a measure of the affinity of the sodium uptake mechanism for sodium) is very high (3 mmol l^{-1}), indicating a low affinity of the uptake mechanism for sodium.

On sodium depletion, the relationship between sodium influx and external sodium concentration changes to produce a curve which has a stepped appearance, and is unusual in that the maximum influx is not increased above that in non-depleted fish. The overall K_m alters very little; however, the K_m for the lower part of the curve is very low (0·05 mmol l^{-1}).

A model is proposed to explain these results in the form of two sodium uptake mechanisms working in parallel across the gill. The second carrier is only active when the fish is sodium-depleted and kept in low external sodium concentrations.

Neither the external sodium concentration nor the external calcium concentration has any direct effect on sodium efflux. However, fish depleted in 1 mmol l^{-1} calcium have a lower sodium efflux than fish depleted in distilled water.

Calcium appears to reduce the permeability of the gill to ions such as sodium. Since calcium has no effect on sodium influx, changes in gill permeability do not involve the sodium influx mechanism.

INTRODUCTION

The relationship between sodium influx and external sodium concentration in many freshwater animals studied to date is approximately that given by the Michaelis–Menten equation (Potts & Parry, 1963):

$$I = \frac{KC_{ext}}{K_m + C_{ext.}}, \quad (1)$$

where I is influx, K is maximum influx, K_m is the external sodium concentration for half maximum influx and $C_{ext.}$ is the external sodium concentration. When K_m is low

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the affinity of the uptake mechanism for sodium is high and *vice versa*. This type of relationship has been reported for a variety of aquatic animals (Shaw, 1959; Stobbart, 1960; Sutcliffe, 1967; Tarbit, 1967; Wright, 1975). The implications of this type of relationship are as follows: (i) sodium combines, according to the Law of Mass Action, with a limited amount of substance (carrier molecule) situated in the transporting membrane; (ii) the rate of transport of sodium is proportional to the amount of sodium–carrier molecule complex so formed.

Most freshwater animals studied so far respond to sodium depletion by increasing K in equation 1, i.e. more carrier molecule is apparently synthesized, but its affinity for sodium (K_m) is not altered.

During investigation of the effects of some heavy metals on ionic regulation in the minnow *Phoxinus phoxinus* the characteristics of its sodium balance mechanisms were studied in relation to external sodium and calcium concentrations. The unusual characteristics of these mechanisms form the subject of this paper.

The terms influx and efflux refer in what follows to unidirectional movements of ions measured with radioactive tracers, and the terms uptake and loss refer to net movements of ions.

MATERIALS AND METHODS

Minnows were collected by net from the River Wansbeck, near Scot's Gap in Northumberland. The fish were kept in large (1·5 m×0·3 m×0·45 m) glass holding tanks at 5–20°C. These had gravel bottoms and contained rooted and floating aquatic plants; they were filled with Newcastle-upon-Tyne tap water and were aerated *via* air stones. The tanks were lit by 'natural light' strip lights using a 12 h: 12 h light: dark regime. The fish were fed daily on re-wetted freeze-dried tubifex and kept at low densities as disease spread rapidly in crowded conditions. Individual fish were kept for short periods and frequent collecting was necessary. Fish were kept in the aquarium for at least 1 week to adapt before being used in experiments.

The minnows were depleted of ions by treating them, in 2-l polystyrene boxes, with three changes of distilled water. Each change lasted about 36 h. During depletion the fish were not fed and were maintained at a density of about 1 g of fish to 75 ml of water. Non-depleted fish were fed and kept for about 5 days in a small aquarium filled with tap water.

Influx measurements

After the period of adaptation the fish were netted, rinsed in distilled water and placed in a sodium chloride solution containing ^{22}Na, at a specific activity of about 30×10^6 counts min^{-1} $mmol^{-1}$ (approximately 100 μCi $mmol\,l^{-1}$) for 10 min. Experiments on sodium influx with time showed that adsorbtion of ^{22}Na had no effect on the values for sodium influx over a 10-min period. These experiments also showed that disturbing the fish had no effect on sodium influx since there was no change in influx over a 30-min period, a period long enough for sodium regulation to return to normal after disturbance in *Carassius auratus* (Tarbit, 1967).

On removal from the radioactive solution the fish was rinsed quickly in three changes of distilled water, which took less than 30 s. The excess water was dried from the fish's surface and opercular cavity with filter paper and the fish was killed by severing the spinal column just behind the head.

The fish were weighed (average mass approximately 0·2 g), dried to constant weight at 60 °C and then ashed at 450 °C for 12 h in platinum or zirconium crucibles. The ash was dissolved in 0·5 ml of dilute Analar hydrochloric acid and transferred by teat pipette to a 1 ml steel planchette. The crucible and pipette were rinsed onto the planchette and the liquid was evaporated off. 100 μl of 0·9 mol l^{-1} dextrose in 10 % Teepol were used on the planchette as a spreader. The activity on the planchette was counted with a low-background end-window Geiger counter used in conjunction with a Betaplan 50 automatic sample changer, a Panax scaler and Kienzle printer.

The experiments were designed so that: (1) the ratio of external to internal sodium was large (between 5 and 100), (2) the animals were in the radioactive solution for only a short time (10 min), (3) changes in internal and external sodium concentrations were small, less than 0·5 %.

These conditions ensured that (a) influx was not affected by changes in external sodium concentration and (b) back movement of tracer was negligible, so that the accumulation of radioactivity in the animal effectively measured influx only.

Efflux measurements

Larger fish (0·8 g) were used in these experiments than in the influx experiments. However, no significant differences were observed between large and small fish. Rates of influx and efflux per gram were similar. The sodium concentrations established following transfer to distilled water were also similar.

Efflux measurements were originally performed by loading the fish for 48 h in tap water labelled with ^{22}Na (the specific activity of the whole fish increased very slowly after this time), rinsing them and then leaving them in 400 ml of unlabelled tap water for 8 h. However, this caused a large diuresis over the first 30 min. To avoid this, fish were loaded, depleted (for experiments on depleted fish only), then individual fish were placed in 400 ml of distilled water (depleted fish) or unlabelled tap water (non-depleted fish) for 2–3 h. The water was then siphoned off to a level just deep enough to allow the fish to swim (about 75 ml). The experimental solution was then slowly added to increase the volume to about 500 ml. The process was repeated twice; the volume was finally adjusted to 400 ml and the fish left for about 5 h. Results are therefore from undisturbed fish and the large volume ensured minimum change in the external solution and little back flux, hence efflux only was measured. A sample of the external solution was then evaporated at 100 °C and its radioactivity counted with a Beckman liquid scintillation counter.

Not all the internal sodium is readily exchangeable with external sodium. The specific activity of that fraction of internal sodium which is readily exchangeable was determined in the following way. Fish were loaded and then depleted (depleted fish only), then placed for 5 h in distilled water in polypropylene beakers. The sodium concentration and radioactivity of the solution were measured and the specific

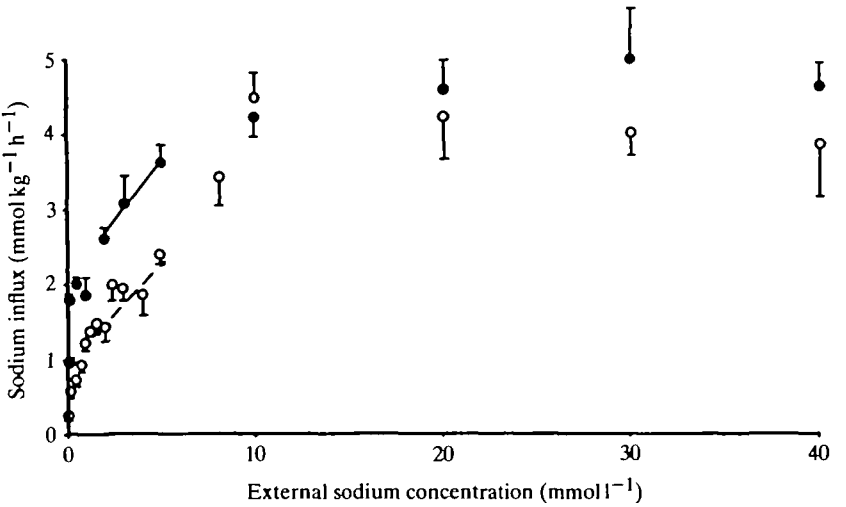

Fig. 1. The relationship between sodium influx and external sodium concentration for depleted fish (●) and non-depleted fish (○). Each point is the mean of data from at least six fish and the bars each represent one standard error. The lines are the lines of linear regression for the results between 2·0 and 5·0 mmol l^{-1} sodium for depleted fish and 1·5 and 5·0 mmol l^{-1} sodium for non-depleted fish.

activity of the exchanging sodium was calculated. Specific activities found in this way were used to calculate sodium efflux from the fish. Influx and efflux were expressed as mmol kg^{-1} wet mass h^{-1}.

RESULTS

Fig. 1 shows the effect of increasing external sodium concentration on sodium influx in non-depleted fish; the graph shows a saturable uptake resembling the Michaelis–Menten curve of enzyme kinetics. However, saturation was not reached until the external sodium concentration was above 10 mmol l^{-1} – at least 20 times higher than that of the fish's natural environment. The K_m value was about 3 mmol l^{-1}, indicating a relatively poor affinity of the sodium uptake mechanism for sodium in comparison with other freshwater animals (Wright, 1975). Fig. 2 shows the results obtained at low external sodium concentrations. These results fit well with those in Fig. 1.

There was no significant difference in the maximum influx (K) in depleted and non-depleted fish with increasing external sodium concentrations, at 10, 20, 30 and 40 mmol l^{-1} ($P > 0{\cdot}05$, t-test) (Fig. 1). Linear regression analysis was carried out on data from depleted and non-depleted fish between 1·5 and 5 mmol l^{-1}. This portion of the curve approximates to a straight line and shows that there is no significant difference ($P > 0{\cdot}06$) in the rise in influx between these concentrations in depleted and non-depleted fish, although the fluxes are at different levels.

Sodium influx in depleted fish at low external sodium concentrations was very different from that in non-depleted fish (Fig. 2). Linear regression analysis on data

between 0·125 and 1·0 mmol l^{-1}, where the curve approximates to a straight line, showed that while the data for non-depleted fish gave a significant slope above the horizontal ($P < 0{\cdot}001$) the data for depleted fish did not (Fig. 2), with the regression line for these data sloping down slightly.

At lower sodium concentrations the levels of influx in depleted fish were much higher than in non-depleted fish. However, the maximum sodium influx was unaltered by salt depletion. This resulted in a very small change in the overall K_m of the two curves. However, the K_m for the curve for depleted fish below 1 mmol l^{-1} sodium is as low as 0·05 mmol l^{-1}. The overall results for depleted fish, therefore, have a stepped appearance as shown in Fig. 3. Possible explanations for this are discussed later.

It is apparent from these results that sodium depletion causes a large increase in the affinity of the sodium uptake mechanism in the range of sodium concentrations found in the fish's natural environment.

Efflux

At low external sodium concentrations sodium efflux was unaltered by changes in external concentration (Fig. 4). At high external sodium concentrations efflux increased rapidly as the external sodium concentration increased (Fig. 4). This is probably not a direct effect of sodium on the carrier mechanism. In high external sodium concentrations (and indeed in low ones in the case of depleted fish) the fish will gain a considerable amount of sodium since influx levels will be high and the

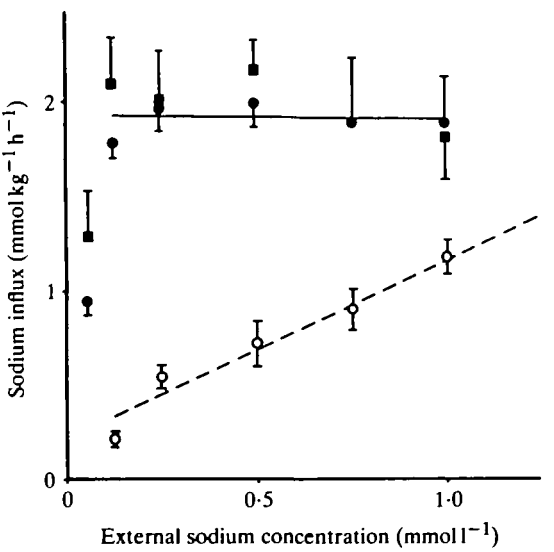

Fig. 2. The relationship between sodium influx and external sodium concentration for depleted fish (●), fish depleted in 1 mmol l^{-1} calcium chloride (■) and non-depleted fish (○). Each point is the mean of data from at least six fish and the bars each represent one standard error. The lines are the lines of linear regression for the results between 0·125 and 1·25 mmol l^{-1} sodium.

procedure takes several hours. For example, a 1-g fish in 10 mmol l^{-1} NaCl would have a net influx of over 3 mmol kg^{-1} h^{-1} resulting in an increase in internal sodium of 0·012 mmol after 5 h, a 27 % increase in the total sodium content of the fish. It

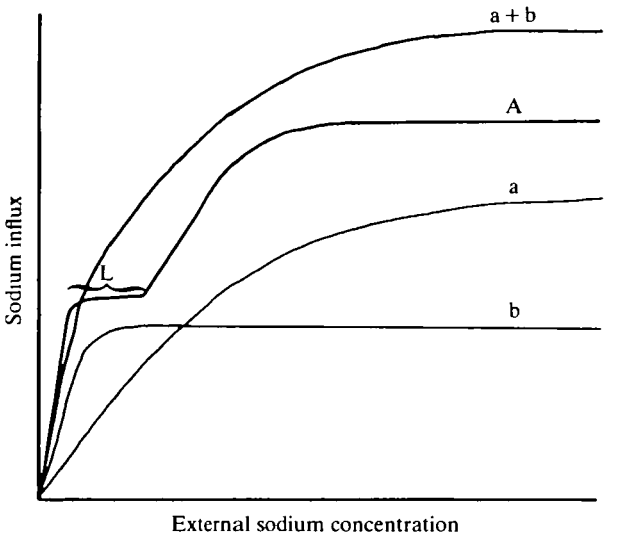

Fig. 3. A is a representation of the changes in sodium influx in depleted fish over the full range of external sodium concentrations used. Line a + b shows the result of adding two Michaelis–Menten curves a and b. For an explanation of L, see the Discussion.

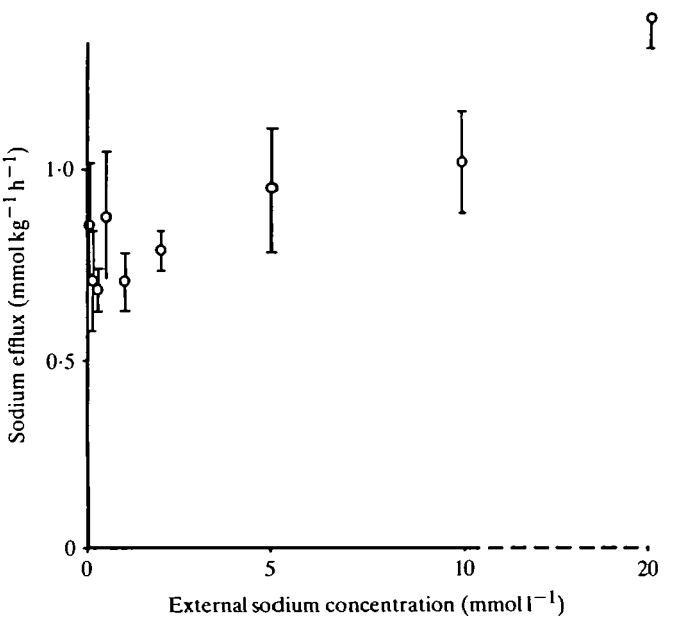

Fig. 4. The relationship between sodium efflux from non-depleted fish and external sodium concentration. Each point is the mean of data from six fish and the bars represent one standard error.

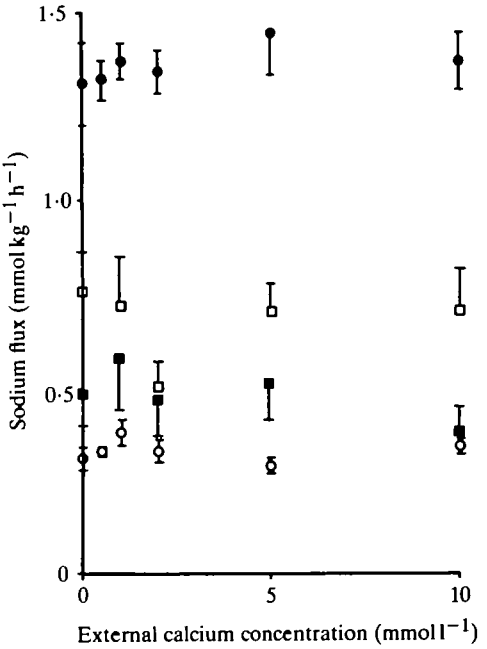

Fig. 5. The relationship between sodium influx in 0·1 mmol l^{-1} sodium and external calcium concentration for depleted fish (●) and non-depleted fish (○), and the relationship between sodium efflux and external calcium concentration for depleted fish (■) and non-depleted fish (□). Each point is the mean of data from six fish and the bars represent one standard error.

seems likely that the observed increase in efflux is due to an increase in internal sodium levels, although a link between influx and efflux cannot be ruled out. This is supported by the fact that efflux from depleted fish, which will have lower internal sodium levels, into distilled water ($0{\cdot}45 \pm 0{\cdot}04$ mmol kg^{-1} h^{-1}, ±S.E.M., $N = 18$) was lower than that from non-depleted fish ($0{\cdot}70 \pm 0{\cdot}05$ mmol kg^{-1} h^{-1}, $N = 60$).

Sodium efflux could be reduced still further to $0{\cdot}29 \pm 0{\cdot}04$ mmol kg^{-1} h^{-1}, $N = 12$ by depleting the fish in 1 mmol l^{-1} calcium chloride. However calcium had no effect on sodium efflux when non-depleted or depleted (in distilled water) fish were placed in calcium chloride solutions of increasing concentration (Fig. 5).

DISCUSSION

Most freshwater animals respond to sodium depletion by increasing the maximum sodium influx (K), while the affinity of the uptake mechanism (K_m) for sodium remains unchanged. However, ammocoete *Lampetra planeri* show a change in K_m for sodium (Morris & Bull, 1970), and *Carassius auratus* show a change in K_m for chloride (De Renzis & Maetz, 1973). In this respect these animals are similar to *Phoxinus*, for which more extensive data (Figs 1, 2) are available. Since changes in K_m are now known in a cyclostome and two species of teleost, it seems possible that such changes may be more widespread in fish than had been thought.

The relationship between sodium influx and external sodium concentration in *Phoxinus* is complex (Fig. 3). Several possible models for the observed pattern of sodium influx in depleted and non-depleted *Phoxinus* may be suggested. Any model must account for: (i) the high influx in depleted fish at low external sodium concentrations; (ii) the fact that fluxes in depleted and non-depleted fish are very similar above about 2 mmol l^{-1}; (iii) the approximately Michaelis–Menten nature of the relationship between influx and external concentrations; (iv) the apparently prompt change in the K_m of the sodium uptake mechanism of depleted fish resulting from changes in external sodium concentration.

The possible models fall into two categories: (1) a single carrier molecule which is modified with respect to its affinity (K_m) for sodium, or is modified with respect to both K_m and the number of sodium attachment sites; (2) a second sodium carrier, with a much higher affinity for sodium than that of the normal carrier, which is activated in response to low internal sodium levels.

The second model seems more probable. It is difficult to imagine how the affinity of a single carrier could be instantaneously increased by changes in internal sodium level and instantaneously reduced by changes in external sodium level.

The assumption that there are two sodium carriers working in parallel across the gill of the depleted fish does not explain the stepped appearance of the curve of Fig. 3, since the result of adding two Michaelis–Menten curves is simply to produce another Michaelis–Menten-type curve.

To arrive at a stepped curve it is necessary to postulate a rapid inhibition of the second (high-affinity) carrier at external concentrations greater than 0·1 mmol l^{-1}, the level portion of the curve (L in Fig. 3) resulting from a balance between the decreasing effect of the high-affinity carrier and the increasing effect of the low-affinity carrier as external sodium concentration increases.

The results from the experiments on sodium efflux show that external sodium concentration has no effect on sodium efflux. The results do, however, suggest that internal sodium concentration has a strong influence on sodium efflux.

The investigation of the effect of calcium on sodium efflux produced apparently contradictory results since depletion in 1 mmol l^{-1} calcium chloride reduced sodium efflux to a lower level than that in fish depleted in distilled water, while calcium added to the experimental solutions had no effect on sodium efflux from depleted and non-depleted fish. Similar results were obtained by Cuthbert & Maetz (1972) for *Carassius auratus*. Their explanation, which follows, seems also to be applicable to *Phoxinus*. Calcium in the gill epithelium reduces the permeability of the gill to ions such as sodium. When the calcium is removed, by depletion in distilled water, the permeability of the gill increases and more sodium is lost. When the depleted fish are placed in a solution containing calcium, the calcium is only slowly replaced in the gill epithelium, and so sodium efflux remains at its elevated level.

Since calcium has no effect on sodium influx it would appear that in *Phoxinus* changes of gill permeability do not involve the sodium influx mechanisms.

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