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SODIUM EXTRUSION BY A FISH ACCLIMATED TO SEA WATER: PHYSIOLOGICAL AND BIOCHEMICAL DESCRIPTION OF A Na-FOR-K EXCHANGE SYSTEM

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INTRODUCTION

Homer Smith (1930) first proposed that teleost fish that are acclimated to sea water extrude unwanted sodium ions via some extra-renal system since he found that the fish kidney was unable to form a urine that contained a greater sodium concentration than the blood. It has now been generally accepted that the site of this extra-renal sodium extrusion is the gill tissue (Maetz, 1971). In recent years the advent of the use of radio-isotopes has allowed workers to examine the magnitude of this sodium extrusion and its kinetic properties (Motais, Garcia Romeu & Maetz, 1966; Evans, 1967, 1969; Potts & Evans, 1967; Potts *et al.* 1967; Maetz, 1969, 1971; Maetz & Evans, 1972).

It has been shown that in a wide array of cell types unidirectional sodium movement is often coupled with potassium movement in the opposite direction (Stein, 1967), and Maetz (1969) has shown that such a system may also be involved in sodium extrusion by bony fish. This hypothesis was prompted by three lines of physiological evidence from experimentation on the European flounder, *Platichthys flesus* (Maetz, 1969): (1) the isotopically measured influx of potassium was equivalent to the calculated net extrusion of sodium ions; (2) the absence of external potassium ions decreased the extrusion of sodium ions so that the blood sodium concentration increased; (3) the addition of potassium ions to the external medium stimulated the efflux of sodium ions from the fish.

These physiological data corroborated biochemical data (Epstein, Katz & Pickford, 1967; Kamiya & Utida, 1968; Jampol & Epstein, 1970; Motais, 1970) which showed that bony fish acclimated to sea water have relatively high concentrations of the enzyme Na-K-activated ATPase in gill tissue. The correlation between this enzyme and Na-for-K exchange systems is well documented (Skou, 1965).

It is still unclear whether the conclusions drawn from the investigation of the European flounder can be extended to other species of fish. Potts & Fleming (1971) found that when the plains killifish, *Fundulus kansae*, was acclimated to 150% sea water, the efflux of radioactive sodium was unaffected by removal of potassium ions from the external medium.

In an effort to extend the physiological study of sodium extrusion mechanisms to

another species of teleost fish, and to correlate its kinetic characteristics with th biochemical kinetic characteristics of the Na-K-activated ATPase extracted from gill tissue, we have investigated these parameters in the euryhaline eleotrid fish *Dormitator maculatus* (the fat sleeper).

MATERIALS AND METHODS

(1) *Capture and laboratory care of experimental animals*

Mature individuals (5–15 g) of the fat sleeper, *Dormitator maculatus*, were caught by hand-trawl in freshwater canals west of Vero Beach, Palm Beach County, Florida. They were maintained in brackish water aquaria in the laboratory under approximately normal day-length, at 24 ± 1 °C and were fed Tetramin (Tetrawerke Co.) daily. One week before a given experiment individuals were transferred to either sea water (500 mM-Na/l) or fresh water (~ 1 mM-Na/l).

(2) *Physiological studies*

To determine the gross efflux of sodium ions from individual fish, they were injected, under MS-222 anaesthesia, with approximately 2 μc ^{22}Na carried in 10 μl of isotonic saline solution. Individual fish were then transferred to 300 ml of sea water contained in a small flask with the appropriate aeration. At intervals thereafter 25 ml samples of the external baths were removed and the radioactivity was assayed in a Packard 'Armac' scintillation detector attached to a Packard Tri-Carb Model 2001 scintillation spectrometer. At the end of the counting period the sample was returned to the experimental flask. At the end of the experiment (usually 1–4 h post-injection) a final 25 ml sample was assayed as well as the fish. The rate constant (K) of efflux was then calculated from the following formula:

$$K = 1/T \ln Q_0/Q_t,$$

where K is expressed as a fraction of the exchangeable sodium space per hour, T is the length of the experimental period in hours, Q_0 is the radioactivity in the fish at the start of the experimental period and Q_t is the radioactivity in the fish at the end of the experiment. While Q_t is measured directly, Q_0 is the sum of the final radioactivity in the fish and the total radioactivity in the bath at the end of the experiment. The exchangeable sodium space was approximated by the total sodium content of fish acclimated to sea water since it can be shown that this value is of the same order as the isotopically measured sodium space (Potts *et al.* 1967; Maetz *et al.* 1967). Total body sodium was determined by homogenization of previously washed, dried and weighed fish in a known volume of distilled water with a Virtis Model 23 homogenizer. The homogenate was made up to 500 ml with distilled water. An aliquot was centrifuged and the sodium concentration of a known volume was determined with an Instrumentation Laboratories Model 143 flame photometer with an internal lithium standard. The total body sodium was expressed in μM-Na/g fish.

The gross efflux of sodium was then taken as the product of the rate constant and the total body sodium and was expressed as μM-Na g^{-1} h^{-1}.

The rate of oral ingestion of the medium was determined by placing individual fish into seawater baths (usually 1 l) which were labelled with ^{125}I-Glofil (Abbott Laboratories). Glofil has been shown to function as a biologically inert marker of water

movement in the same way as inulin (Elwood, Sigman & Treger, 1967). At the end of 1 h the fish was removed and placed in 1 l of non-radioactive sea water. After another 1 h period the fish was removed, blotted dry with paper towelling and weighed, and its radioactivity was monitored in the 'Armac' scintillation detector. The rate of oral ingestion of the medium was then calculated by comparing the radioactivity in the fish with the radioactivity per ml of the loading bath. The drinking rate was expressed in μl g^{-1} h^{-1} and the oral ingestion of sodium was calculated by multiplying the drinking rate by the sodium concentration of the experimental bath and expressed as μM-Na g^{-1} h^{-1}.

To determine the effect on sodium efflux either of removal of potassium ions from the external bath or addition of ouabain to the bath, the experimental fish was treated as above except that after 1 h in sea water it was carefully transferred to another flask containing either potassium-free sea water (Pantin, 1962) or sea water containing 10^{-4} M ouabain.

To determine the effect on sodium extrusion of the addition of potassium ions a flow system similar to that described by Motais *et al.* (1966) and Evans (1969) was used. Tygon tubing connected in series a peristaltic pump, a small glass chamber containing the fish and a small glass jar placed inside the 'Armac' scintillation detector; 400 ml of solution was circulated through this system at a rate sufficient to renew the entire volume every 30 sec. The radioactivity appearing in the external bath containing the ^{22}Na-injected fish was recorded via a Packard Model 380 recording ratemeter attached to the Packard scintillation detector and spectrometer described previously. The rate of appearance of radioactivity in the external bath was recorded as a slope for a period of from 3 to 5 min. At the end of that period the pump was stopped, the glass chamber containing the fish removed from the flow system, and the system flushed with tap water, emptied, and then filled with 400 ml of the next solution in a new glass chamber. The fish was transferred to a new bath which was then attached to the flow system. The pump was started again and a new slope of appearance of radioactivity recorded. The entire transfer procedure could be completed within 60–90 sec. In experiments the fish transferred from sea water to tap water or low salinities was rinsed in tap water for a few seconds to remove external ionic contamination before transferral. Using this system one can investigate the rapid effects on radioactive sodium efflux of transfers from sea water to tap water and then to tap water containing various concentrations of KCl.

(3) *Biochemical studies. To extract Na-K-activated ATPase*

Individual fish were immobilized by section of the spinal cord. The gills were removed, held in ice-cold homogenization medium and *in toto* homogenized in 2·0 ml of medium containing 0·25 M sucrose, 0·30 M histidine and 5 mM disodium EDTA at pH 6·8. Homogenization was carried out in a Ten-Broeck tissue grinder immersed in an ice bath and attached to a Thomas homogenizer drive. The homogenate was either assayed immediately or fractionated by centrifugation.

Gill tissue homogenate (25 μl containing 60–250 μg protein) was added to 3·7 ml of incubation medium of 10 mM imadazole buffer, pH 7·8, containing either 100 mM-NaCl and 20 mM-KCl or 120 mM-NaCl. The reaction was initiated by the addition of 0·1 ml of a 10 mM disodium ATP and 10 mM $MgCl_2$ solution. Incubation was at

Table 1. *Rate constant of sodium efflux, total body sodium, and oral ingestion rate of seawater-acclimated* Dormitator maculatus

Rate constant of sodium efflux (fraction of body sodium/h)	Total body sodium (μM/g fish)	Oral ingestion rate (μl/g fish)
0·92 ± 10(8)*	52 ± 9·5(11)	2·1 ± 1·2(8)

* Mean ± S.D. (no. of fish).

37 °C for 15 min. The reaction was terminated by the addition of 1·0 ml of 20% TCA. The concentration of inorganic phosphate (P_i) was then determined by the Fiske & Subbarow procedure (Fiske & Subbarow, 1925). The Na-K-activated portion of total ATPase activity is defined as the difference between the P_i liberated in the presence and absence of potassium. The assays of each control or experiment were performed in triplicate. Under these conditions ATP hydrolysis was a linear function of time and enzyme concentration. Hydrolysis of the ATP in the potassium-free medium is referred to as residual ATPase. Protein contents were determined by the Lowry procedure as modified by Leggett & Bailey (1967).

To determine the effect of differing concentrations of K^+ on the activity of ATPase the standard assay was performed while varying the concentration of KCl in the imadazole incubation medium from 0 to 25 mM.

RESULTS

(1) *Gross sodium movements and oral ingestion of sodium*

Table 1 presents the results of the determinations of the efflux of sodium, the total body sodium content, and the rate of oral ingestion of the medium. The gross efflux of sodium (rate constant of efflux times the total body sodium) is 48 μM-Na g^{-1} h^{-1}, twice that described for the European flounder, *Platichthys flesus* (Motais, 1967; Maetz, 1969, 1971), indicating an even greater ion permeability than that already described for other seawater-acclimated teleosts (Motais & Garcia Romeu, 1972). The rate of oral ingestion of the medium is relatively low and accounts for a sodium influx of only 1·0 μM-Na g^{-1} h^{-1}, approximately 2% of the gross sodium influx if one assumes that the experimental animal is in sodium balance so that the sodium influx equals the sodium efflux during the course of the experiment.

(2) *Effect of external potassium ions on sodium efflux*

Table 2 presents the results of experiments comparing the efflux of radioactive sodium from fish placed into full-strength sea water and then into potassium-free sea water for an additional 1 h. It is obvious that the lack of external potassium ions is correlated with a marked reduction of efflux of sodium from the fish.

(3) *Effect of* 10^{-4} M *ouabain on sodium efflux*

Table 2 also presents the results of experiments testing the effect of the addition of 10^{-4} M ouabain to the external sea-water bath. The relative flux is calculated from the

Table 2. *Effect of transfer to potassium-free sea water or sea water containing ouabain on the rate of sodium efflux from* Dormitator maculatus

	Relative sodium efflux (% sea-water control efflux)	
Sea water	Potassium-free sea water	Sea water + 10^{-4} ouabain
100	78 ± 7(13)	86 ± 8(8)

Table 3. *Residual magnesium-activated and sodium-potassium-activated adenosine triphosphatase specific activities in whole gill homogenates of freshwater-adapted and seawater-adapted* Dormitator maculatus

Acclimation medium	Residual (μM P_i mg protein^{-1} h^{-1})	Na-K-activated (μM P_i mg protein^{-1} h^{-1})
Fresh water	4·71 ± 1·74(6)	1·63 ± 0·33(6)
Sea water	4·50 ± 0·84(7)	4·59 ± 0·82*(6)

* $P \ll 0{\cdot}001$ when compared with freshwater ATPase levels.

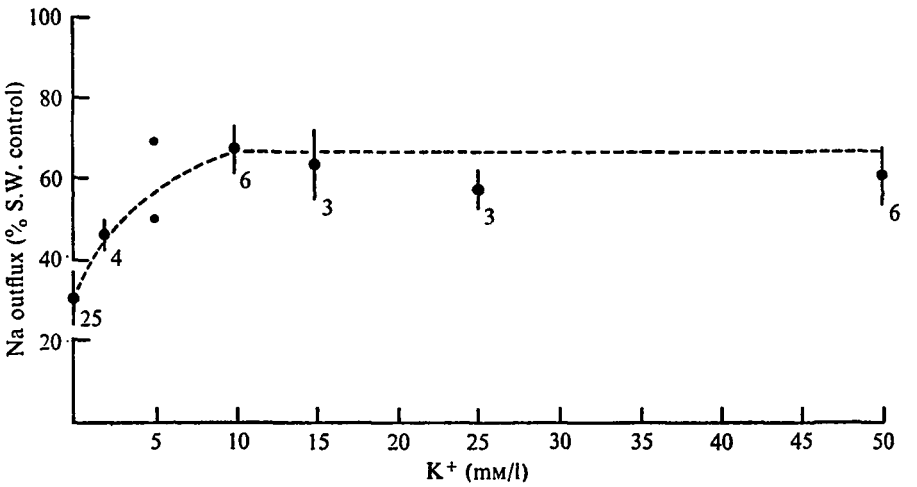

Fig. 1. The effect of external potassium ions on the relative sodium efflux from *Dormitator maculatus*.

rate constant of efflux for 2 h after transfer of the fish to the ouabain-seawater bath. It is evident that the presence of ouabain in the external bath significantly reduces sodium efflux.

(4) *Effect of the addition of potassium ions on the efflux of sodium ions*

Using the flow system described in Materials and Methods, the radiosodium efflux from experimental fish was followed during rapid transfers from sea-water baths to freshwater baths and then to various KCl solutions. Fig. 1 presents the results of such experiments. It is obvious that rapid transfer to freshwater solutions (taken as 0 mM-KCl) is followed by a rapid decline in sodium efflux to only 30% of the seawater level, and subsequent transfer into various KCl solutions results in a stimulation of sodium

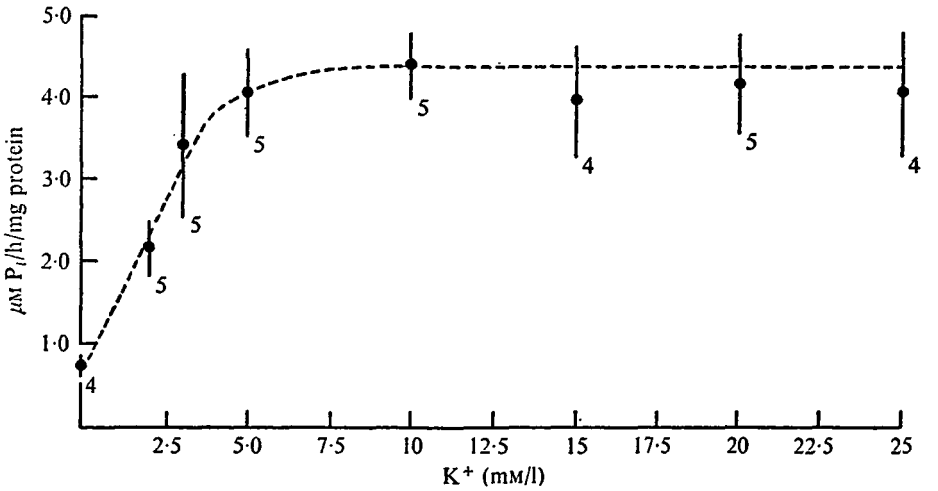

Fig. 2. The effect of external potassium ions on the relative specific activity of the Na-K-activated ATPase extracted from the gill tissue of *Dormitator maculatus*.

efflux that is dependent on the KCl concentration up to 5–10 mM-KCl and then levels off with no further increase in sodium efflux up to external KCl concentrations of 50 mM/l.

(5) *Biochemical assay for Na-K-activated ATPase*

Table 3 presents the results of ATPase activity determinations and shows that while the residual (Mg^{2+} dependent) ATPase levels are the same in gill tissue from freshwater-acclimated and seawater-acclimated fish, there is a marked and statistically significant increase in the levels of Na-K activated ATPase when the animals are acclimated to seawater.

(6) *Potassium stimulation of Na-K-activated ATPase activity*

The results of these determinations are presented in Fig. 2 and show that the activity of the enzyme is K-dependent up to a concentration of 5–10 mM-K/l, after which a plateau of activity is reached.

DISCUSSION

The physiological experiments indicate quite strongly that, as in *Platichthys* (Maetz, 1969), at least some of the gross efflux of sodium ions from the seawater-acclimated fat sleeper is controlled by external potassium ions. Removal of external potassium ions or the addition of the known Na-K exchange inhibitor ouabain (Skou, 1965) decreases the sodium efflux by approximately 20%. The simplest explanation for these findings is that 20% of 48 μM-Na g^{-1} h^{-1} or approximately 10 μM-Na g^{-1} h^{-1} is being extruded from the fat sleeper in exchange for external potassium ions and that the exchange system is coupled with the enzyme Na-K-activated ATPase. It is interesting to note that this rate of K-dependent sodium extrusion is 10 times that found for *Platichthys* (Maetz, 1969) and to compare this K-dependent sodium extrusion rate with the rate of sodium uptake via oral ingestion. Maetz (1969) proposed that the drinking influx should be equivalent to the net

extrusion rate of sodium or uptake of potassium and, indeed, found that these three parameters were approximately equal in *Platichthys*. If one compares the oral influx of sodium with the K-dependent sodium extrusion rate of 10 μM-Na g^{-1} h^{-1} it is clear that, at least in *Dormitator*, the net extrusion rate of sodium (linked to potassium) is 10 times the drinking influx of sodium ions. Therefore, in the fat sleeper, the net diffusional influx of sodium must play a major role in the sodium load of this species while in *Platichthys* it is of minor importance.

A correlate of the model that sodium extrusion is linked to potassium uptake is that addition of the latter ion to distilled water solutions should promote the efflux of sodium ions from radioactively labelled fish which have been rapidly transferred from sea water to fresh water to KCl solutions. The data in Fig. 1 support this hypothesis. Immediately after the labelled fish is placed into a freshwater solution the efflux of sodium ions falls to approximately 30% of the sea water level. This phenomenon has been called the 'sodium-free effect' and was first described in marine fish by Motais *et al.* (1966) and has since between extended to other species of marine teleost (Evans, 1967, 1969; Potts, Foster & Stather, 1970). This 'sodium-free effect' was initially proposed to be due to an obligatory exchange diffusion of sodium-for-sodium across the fish gill – a phenomenon which had no role in the sodium balance of the fish but which would terminate immediately after removal of sodium ions from the external bath. Recent work on the crustacean *Artemia* (Smith, 1969) has indicated that the 'sodium-free effect' could also be ascribed to electrical potential changes across the gill tissue caused by removal of external sodium ions. It is still not clear what is the underlying mechanism of this phenomenon in marine fish (Motais & Garcia Romeu, 1972), but it is clear that it, plus any other external-ion-linked sodium efflux accounts for 70% of the sodium efflux from the fat sleeper. The 30% of the gross efflux (or 15 μM-Na g^{-1} h^{-1}) that remains immediately after the fish is transferred to fresh water must be the combination of diffusional, urinary and anal sodium loss. The possibility of an unlinked, electrogenic, active efflux of sodium accounting for some of this 15 μM-Na g^{-1} h^{-1} cannot be ruled out at the present time. The rapid addition of potassium ions to the external bath of a fish immediately after it had been transferred to fresh water should then 'turn on' any linked Na-K transport that is possible across the fish gill. It is obvious from examining Fig. 1 that the addition of external potassium ions does drive sodium ions out of the fish at a rate that is dependent on the concentration of potassium ions supplied. It is interesting to note that the addition of 10 mM-K/l (equal to the concentration of K in normal sea water) to the outside bath increases the relative sodium efflux to approximately 65% of the sea-water efflux. Thus the addition of 10 mM-K/l to the external bath stimulates sodium extrusion by approximately 35% (65% minus 30%) of the control sea-water flux. This means that under the conditions of no external sodium ions, Na-K-linked flux amounts to 17 μM-Na g^{-1} h^{-1} – a value much above that calculated from the potassium-free experiments (10 μM-Na g^{-1} h^{-1}). The discrepancy between the two determinations of the magnitude of the sodium extrusion rate linked to potassium uptake is probably due to interference with the Na-K-transporting system by the external sodium ions present in the potassium-free experiments. If external sodium ions can compete for the same transporter as the external potassium ions, then removal of potassium ions while the sodium ions are still present will be followed by a decline in sodium efflux by a value

somewhat less than would be expected if sodium ions were removed at the same time. A similar situation was described by Maetz (1969) for *Platichthys*.

Fig. 1 shows that the external potassium-stimulated sodium efflux is dependent on amount of external potassium at low potassium concentrations but that it reaches a plateau above a potassium concentration of 5–10 mM/l. The resulting hyperbolic curve can be defined by the Michaelis–Menten equation which indicates a saturable carrier system. The K_m of this system is approximately 2 mM-K/l – a value nearly identical with that described for the *Platichthys* Na/K exchange system by Maetz (1969).

Biochemical studies

Table 3 indicates that, as in other euryhaline teleosts that have been examined (Epstein *et al.* 1967; Kamiya & Utida, 1968; Motais, 1970), *Dormitator* gill tissue contains significantly more Na-K-activated ATPase when the fish is acclimated to sea water than when it is acclimated to fresh water. It is not clear what function the fresh water ATPase performs. It may be concerned with the supposed volume-regulatory system of the gill cells in general, or it may be concerned with the active influx of sodium ions, a freshwater adaptation that may also be functioning when the animal is acclimated to sea water (Motais, 1970; Evans, 1973). Other workers have generally assumed that the seawater Na-K-activated ATPase is concerned with the sodium-extrusion system since parallel changes have been described in the enzymatic activity and branchial ion exchanges after hypophysectomy (Epstein *et al.* 1967), cortisol treatment (Epstein, Cynamon & McKay, 1971) or actinomycin treatment (Motais, 1970). However, other data question this hypothesized linkage between the physiological extrusion and the gill enzyme. Motais & Isaia (quoted in Motais & Garcia Romeu, 1972) have found that addition of ouabain to the external medium had no effect on the sodium extrusion of *Platichthys* or the sea perch, *Serranus*. In addition, while the rapid sodium fluxes common to the European eel, *Anguilla anguilla*, in the sea-water state appear soon after this species is transferred from fresh water to sea water (48 h), the enzymic activity of the gill tissue of the Japanese eel, *Anguilla japonica*, reaches sea-water levels one week after transfer from fresh water to sea water (Motais & Garcia Romeu, 1972). It therefore appears, at least in some species, that the functional linkage between sodium extrusion and Na-K-activated ATPase may not be as strong as was formerly proposed (Maetz, 1969).

On the other hand, our evidence supports the conclusion that in *Dormitator* the gill enzyme is indeed associated with the net extrusion of sodium ions. If one examines the hyperbolic curve of K^+ activation of the enzyme in Fig. 2, the calculated K_m again approaches 2 mM-K/l – a value nearly identical with the K_m of potassium activation of sodium extrusion. This similarity between the physiological K_m and biochemical K_m has also been described for the human erythrocyte (Post, Merrit & Kinsolving, 1960) and certainly is a strong indication that the physiological and biochemical phenomena are linked (Skou, 1965). In addition, 10^{-4} M ouabain inhibits the extrusion of sodium ions by *Dormitator* (see above) as well as reducing the activity of the enzyme by 80% after 1 h of incubation (Mallery & Evans, unpublished results).

Thus, it appears that, at least in *Dormitator*, the linkage between the physiological extrusion of sodium ions and the gill Na-K activated ATPase may be firmly established.

Bonting, Simon & Hawkins (1961) and Bonting & Caravaggio (1963) have shown in a variety of tissues a direct correlation between the activity of Na-K-activated ATPase the rate of sodium or potassium transport. This relationship does not seem to hold for gill tissue from various species of teleost fish. While the flounder (*Platichthys*) is excreting only approximately 1 μm-Na g^{-1} h^{-1} and maintains an enzyme level of up to 78 μM P_i mg protein^{-1} h^{-1} (Langford & Maetz, personal communication) the fat sleeper (*Dormitator*) excretes at least 10 times this amount of sodium per hour and has a gill enzyme level of only 4·5 μM P_i mg protein^{-1} h^{-1}. While differences in biochemical technique will account for some of this discrepancy, it is clear that no direct correlation between rates of sodium transport and enzymatic activity exists. The reasons for this discrepancy are unknown at the present time.

SUMMARY

1. The effect of external potassium ions on the extrusion of sodium ions by the seawater-acclimated fat sleeper, *Dormitator maculatus*, was investigated.

2. Removal of external potassium ions reduced the efflux of sodium from the fish by 22% while addition of 10^{-4} M ouabain reduced the efflux of sodium ions by 14%.

3. Addition of potassium ions to distilled-water baths into which fish were rapidly transferred stimulated sodium extrusion in a manner which could be described by the Michaelis–Menten equation. The K_m of this potassium-stimulated sodium efflux was approximately 2 mM-K/l.

4. The calculated rate of sodium extrusion was 10 times the oral ingestion of sodium ions.

5. Biochemical assays of the levels of the enzyme Na-K-activated ATPase extracted from gill tissue determined that seawater-acclimated fish had 3 times the enzymic activity that fish acclimated to freshwater had.

6. *In vitro* potassium stimulation of the extracted Na-K-activated ATPase showed Michaelis–Menten kinetics with a K_m of approximately 2 mM-K/l.

7. It is concluded that the extrusion of sodium ions by *Dormitator maculatus* acclimated to sea water is coupled with potassium uptake and is mediated by the enzyme Na-K-activated ATPase.

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