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Seawater Teleosts: Evidence for a Sodium-Potassium Exchange in the Branchial Sodium-Excreting Pump

Abstract. *The net sodium extrusion rate by the gill of the seawater-adapted euryhaline flounder is identical to the potassium influx. The excretion of sodium is blocked in K^+-free seawater solutions. The instantaneous sodium outflux readjustment pattern of flounders transferred from seawater to solutions of various sodium chloride or potassium chloride concentrations is consistent with the hypothesis of a linkage between Na^+ outflux and K^+ influx through a common exchange carrier. External Na^+ and K^+ compete for this common carrier. It is suggested that the exchange diffusion mechanism (linkage of sodium influx and outflux) and the high internal sodium turnover rate which characterizes all seawater teleosts are the results of this competitive process. The sodium-potassium dependent adenosine triphosphatase system occurring in the gill of the seawater teleosts may play a central role in this sodium-potassium exchange pump.*

Homer Smith (*1*) showed that marine teleosts maintain their water balance by drinking, absorbing water and electrolytes in the gut, and eliminating the monovalent ions by the gills. More recently, dynamic studies of salt balance, made by using radioactive tracers (*2*), demonstrated that sodium and chloride turnovers involve amounts of salt more than ten times greater than can be accounted for by gut absorption and gill excretion. My observations concerning the mechanism of the branchial sodium pump have been obtained on the seawater-adapted flounder *Platichthys flesus*.

The most recent evaluations of the drinking rate of flounder (*3*) give a value of 192 ± 35 μl hr^{-1} per 100 g of body weight (mean and S.E. for $N = 11$) at 17°C, the temperature at which all the present flux measurements have been made. Assuming that all the Na^+ swallowed is absorbed, the Na^+ concentration of the seawater in our tanks being 520 mmole/liter, the gut absorption and gill excretion rate of Na^+ amount to 100 ± 18 μmole hr^{-1} per 100 g. All the subsequent flux values will be given in these units. The gill sodium outflux (f_{out}) as deduced from the turnover rate (*4*) amounts to 2600 μmole and direct measurement of this outflux (*5*) gave a closely similar value: 2623 ± 90 μmole ($N = 12$). The net flux of sodium across the gill represents therefore only about 3.5 percent of the unidirectional fluxes, and the ratio f_{in}/f_{out} is nearly 1.

By rapid transfer experiments permitting the comparison of the sodium unidirectional fluxes in media of various sodium concentrations, Motais, Garcia Romeu, and Maetz (*4*) demonstrated that about 85 percent of the sodium outflux as well as the total sodium influx are dependent on the external sodium concentration in a manner suggesting Michaelis-Menten saturation kinetics. An exchange-diffusion carrier with a low affinity for sodium (K_m about 400 mmole) allowing for the coupling of sodium influx and most of the sodium outflux has been suggested.

According to this hypothesis, adaptation of the fish to any change of external salinity would be accompanied by a variation in the quantity of available carrier in the gill epithelium. Indirect evidence suggests a close correlation between the exchange-diffusion process and the sodium-excreting pump (*6*). This pump is a potassium-sodium exchange pump. External sodium, the concentration of which is about 50 times that of potassium in seawater, competes for this exchange carrier. The exchange-diffusion effect arises as a result.

The K^+ influx, measured with the help of the tracer ^{42}K added to the outside medium in a closed circuit (*7*), was found to be 120 ± 23.5 μmole ($N = 4$), a value identical to the net sodium excretion rate given above.

Rapid transfer experiments were performed on flounders alternately placed in K^+-seawater and K^+-free seawater for comparison of the sodium outfluxes in these media (*8*). A small but consistent reduction of the sodium outflux was observed in the absence of external potassium (-2.95 percent ± 0.44; $N = 6$; $P < .01$). This reduction amounts to 80 ± 12 μmole, a value similar to the net extrusion rate of sodium. If the animals are kept for 24 hours in renewed K^+-free seawater, an increase of the internal sodium is observed, the plasma sodium level augmenting by 17.5 ± 2.42 mmole/liter ($N = 8$; $P < .001$). The sodium space also increases: 46.1 ± 3.63 ml/100 g compared with 34.2 ± 2.55 ($N = 8$; $P < .05$), the value previously reported for the seawater-adapted flounder (*4*). The rate of increase of the internal sodium is about 110 μmole hr^{-1} per 100 g, a value that would be expected if the sodium-excreting mechanism failed. After return to K-seawater for 48 to 72 hours, the internal sodium concentration declines by 27.8 ± 6.32 mmole/liter, the decrease being highly significant ($P < .01$; $N = 8$). This decline can be explained by the sodium pump being "turned on" again upon addition of external potassium. The sodium turnover rate remains high in K-free seawater, for the sodium outflux of the fish kept in this medium was 2125 ± 354 μmole ($N = 7$) which is only a slightly smaller value than that reported for control fish (see above), the difference not being significant. Furthermore a net potassium loss of 36 ± 5.5 μmole ($N = 7$), is observed in Na-free seawater. It is evident that suppression of external potassium does not abolish the sodium-sodium exchange mechanism, but this is not the vital mechanism for the osmoregulation of the fish.

The existence of a K-Na exchange mechanism is further demonstrated by rapid transfer experiments of the fish permitting the comparison of the sodium outfluxes in seawater, in deion-

ized water, or in various NaCl or KCl solutions at concentrations ranging from 5 to 50 mmole/liter. Figure 1 illustrates two typical experiments and Fig. 2 summarizes the results of 27 experiments of this kind. In deionized water, an "instantaneous" reduction bringing down the outflux to 14.4 ± 1.20 percent ($N = 27$) of the control value in seawater is observed, confirming our previous observations (*4*). As a second "delayed" reduction would occur if the fish were kept in waters of lower salinities (*4*), all the testings of the fish in the various media have to be made within 20 to 30 minutes. If this condition is met, the outflux of the fish during the third or fourth experimental period upon return to seawater remains within 90 to 100 percent of the control value. As seen in Fig. 2, the extent of the outflux reduction varies almost linearly with the external concentrations after transfer to NaCl solutions in the range 5 to 50 mmole.

Transfer to media at concentrations higher than 50 m*M* had previously shown (*4*) that the outflux varies according to a hyperbolic function of the Michaelis-Menten type as discussed above. Figures 1 and 2 show that potassium chloride solutions in the lower range of concentrations are even more effective than the sodium chloride solutions in "driving the sodium out" of the fish. The reduction of the control outflux is much less and the difference is highly significant ($P < .001$) for the 5 and 10 m*M* solutions and less significant ($P < .05$) for the 25 m*M* concentration. For the 50 m*M* solutions, however, Na^+ and K^+ are equally efficient in driving sodium out of the fish. If the assumption is made that in the K^+ as in the Na^+ solutions the sodium outflux over and above that observed in deionized water is equal to the K^+ or

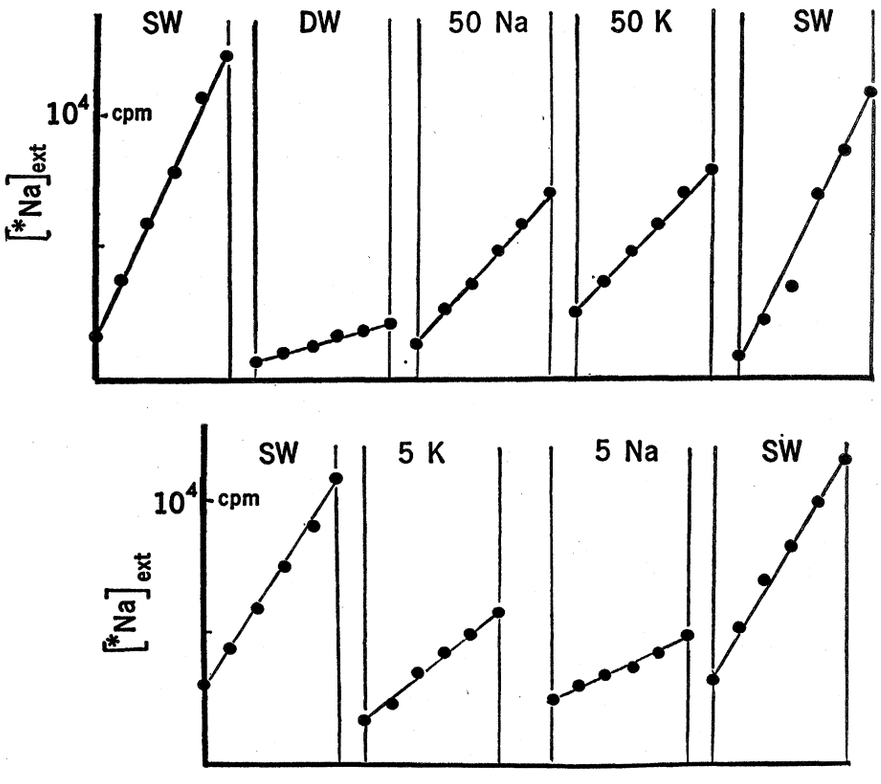

Fig. 1. Comparison of ^{24}Na appearance rates of seawater-adapted flounders transferred to various external media, showing external radioactivity in counts per minute as a function of time. (Top) After transfer from seawater (*SW*) to deionized water (*DW*) the residual outflux is 15 percent of the control level. Transfer from *DW* to 50-mmole solutions of NaCl (*50* Na) or KCl (*50* K) is followed by an identical increase of the outflux reaching about 60 percent of the control level. Return to *SW* shows instantaneous reversibility of the outflux readjustment. Duration of the successive periods is 9 minutes; they are separated by rinsing periods of 1½ to 3 minutes. (Bottom) After transfer to a 5-mmole KCl solution the sodium outflux is 50 percent of the control level, while in a 5-mmole NaCl solution the sodium outflux reaches only 20 percent of the control level.

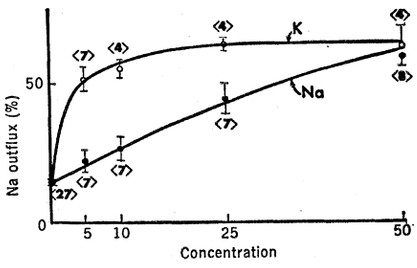

Fig. 2. Sodium outfluxes (in percentage of the control level in seawater) as a function of the external potassium or sodium concentration of the transfer medium in millimoles per liter.

Na^+ influx, it follows that the curves depicted in Fig. 2 correspond to the K^+ influx or Na^+ influx as a function of their respective external concentrations. In comparing the curves, it can be seen that the K^+ influx is almost maximal at the lowest concentration tested (5 m*M*). Apparently the affinity of the carrier for K^+ is very high ($K_m \cong 2$ to 3 mmole), in contrast to the low affinity observed for sodium ($K_m \cong 400$ mmole).

There is, furthermore, compelling evidence for a competition between Na^+ and K^+ for this carrier. The K^+-dependent sodium outflux observed in the 10 m*M* KCl solution represents about 40 percent of the total outflux observed during the control period in seawater and corresponds to a 1075 μmole flux, wihch presumably is exchanged against an equal K^+ influx. The K^+ influx in seawater (containing 10 mmole of potassium) is, however, only about 120 μmole. At equal external K^+ concentrations, the K^+ influx would be eight to nine times higher in the absence of external sodium. Such a discrepancy suggests a competition between both ionic species for a common carrier. Such a phenomenon has been demonstrated in yeast cells (*9*). Further evidence also points to a similar situation for the gill of the seawater-adapted flounder.

Comparative outflux measurements were also made on flounders transferred from seawater to solutions containing either NaCl or KCl alone or a mixture of both ions at varying molar ratios. For example, when 5 m*M* solutions are compared, it appears that in the K solution the observed sodium outflux is reduced to 51.9 ± 2.91 percent of the control level against 22.1 ± 4.07 percent in the Na solution. In the equimolar mixture of $Na^+ + K^+$, however, the observed level is 36.5 ±

2.50 percent ($N = 6$). As the difference is highly significant ($P < .01$), this demonstrates that a mixture of both ions is less effective than K^+ alone in driving sodium out of the fish. This result and similar observations made for mixtures containing 5 mmole of K^+ together with 10, 25, or 50 mmole of Na^+ are indirect evidence that the K^+ influx is reduced in the presence of Na^+ ions.

In conclusion, our observations concerning the branchial sodium pump in seawater teleosts suggest that the operating mechanism is a Na-K exchange pump similar to that described for many cells (*10*). In the red cells and muscle cells an exchange diffusion mechanism also operates in parallel to the Na-K exchange pump, and part of the exchange diffusion process may be explained in terms of competition between Na^+ and K^+ for a common carrier (*11*).

One problem raised by the present investigation concerns the fate of the K^+ ions entering the gill in exchange for Na^+. Comparison of the K^+ influx and outflux in vivo for flounders in seawater shows that both fluxes are identical. Measurement of the K^+ outflux (*12*) gave a value of 145 ± 46 μmole ($N = 4$), not significantly different from that given above for the influx. The gill is obviously the major route for K^+ influx and outflux, for the renal loss of K^+ is negligible (*13*) and the drinking rate accounts for only about a 2 μmole uptake.

The main question raised by the present observations concerns the role of the Na-K activated adenosine triphosphatase activity which has been found in the teleostean gill by several authors. In *Anguilla japonica* (*14*) and *Fundulus heteroclitus* (*15*), but apparently not in *Anguilla anguilla* (*16*), the enzyme activity is higher in the seawater-adapted animals. Recent observations on many species, including the European (*17*) and American eels (*18*), confirm, however, the difference between seawater- and freshwater-adapted animals. In freshwater teleosts the branchial salt pumping mechanism exhibits high specificity for Na^+ against K^+ ions (*19*). The augmentation of the adenosine triphosphatase activity observed during adaptation to high external salinity probably coincides with the differentiation of the salt-excreting Na-K exchange and the Na-Na exchange mechanism. Furthermore, parallel variations of the enzyme activity and gill ionic turnover in seawater either after hypophysectomy (*15*) or after actinomycin D treatment (*17*) are also suggestive of an essential role of this enzyme system in sodium extrusion by the gill.

J. MAETZ

Département de Biologie,
Commissariat à l'Energie Atomique,
Station Zoologique,
06, Villefranche-sur-Mer, France

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7. To obtain the influx (in μeq hour^{-1}) of the fish the disappearance rate of external ^{42}K (in count min^{-1} hour^{-1}) was divided by the mean specific radioactivity (count min^{-1} μeq^{-1}) of potassium. Techniques and calculations are similar to those given by J. Maetz [*J. Physiol. (Paris)* **48**, 1085 (1956)] for measuring Na influx in freshwater fish.
8. The technique is outlined in R. Motais, F. Garcia Romeu, and J. Maetz (*4*) and R. Motais (*2*); Fig. 1 also illustrates an application of this procedure for different experimental conditions. Composition of artificial seawater: NaCl, 500 mmole; $CaCl_2$, 10 mmole; $MgSO_4$, 30 mmole; $MgCl_2$, 20 mmole; with or without KCl, 10 mmole.
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19. A. Krogh, *Osmotic Regulation in Aquatic Animals* (Cambridge Univ. Press, New York, 1939); J. Maetz and F. Garcia Romeu, *J. Gen. Physiol.* **47**, 1209 (1964): Table VI shows no consistent effect of the sudden increase of external K^+ concentration to one that is 50 times higher than that of Na^+ on the Na net flux of the goldfish. Recent unpublished experiments also confirm the absence of effect of K^+ on the Na^+ influx of both goldfish and eels in fresh water.
20. I thank Professor L. B. Kirschner who inspired this investigation by discussing possible models for the role of Na-K activated adenosine triphosphatase in fish gills during his sabbatical leave in my laboratory. Professor R. Motais has kindly placed unpublished data at my disposal. The technical assistance of R. Tanguy and S. Lenkauer is gratefully acknowledged.

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Trypsin and Papain Covalently Coupled to Porous Glass: Preparation and Characterization

Abstract. *Trypsin and papain have been covalently linked to porous glass particles. The resulting insolubilized enzymes show increased thermal stability and can be employed for extended periods of time without loss of activity.*

Insolubilized enzymes have been prepared by various methods, including polymerization onto organic polymer lattices and attachment to polymers of amino acids (*1*), coupling to cellulose (*2*) and polystyrene derivatives (*3*), and immobilization in starch (*4*) and acrylamide gels (*5*). These insoluble enzyme derivatives are either trapped in or coupled to organic carriers. Organic carriers are subject to microbial attack, and they will swell or contract depending on *p*H and other solvent conditions. Enzyme stability will be affected accordingly.

The insolubilized enzyme derivatives described here were prepared with the use of porous glass, an inorganic carrier. With the aid of an intermediate coupling agent, I have covalently bonded enzymes to many inorganic materials.

Inorganic carriers in general, and glass in particular, are not subject to microbial attack; they do not change configuration over an extensive *p*H range or under various solvent conditions; and they have a higher modulus of elasticity than organic polymers do. The enzyme-glass derivatives show increased thermal stability over enzymes insolubilized on organic carriers, and