On the Mechanisms of Sodium Ion Transport by the Irrigated Gills of Rainbow Trout (Salmo gairdneri)

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ABSTRACT Sodium uptake by rainbow trout gills has been investigated with a small-volume system enabling rapid, successive flux measurements in different solutions. Sodium influx obeys a Michaelis-Menten type relation, with a $K_m$ of 0.46 mM, and uptake proceeds unimpaired in the absence of penetrating counterions. This suggests a coupled cation exchange. Ammonia output is about the same as the Na$^+$ influx when external [Na$^+$] is 1 mM, but at higher or lower Na$^+$ influxes, the correlation does not hold. A progressive downward shift in the pH of the irrigating medium as Na$^+$ influx increases indicates that the exchanging cation is hydrogen. In support of this, acetazolamide, which inhibits Na$^+$ uptake, also prevents the downward pH shift. The potential across the gill is about 10 mv, body fluids positive, in NaCl solutions up to 10 mM, and is little affected by changes in Na$^+$ concentration below that. Finally, evidence for locating the rate-limiting step at the outer membrane of the epithelium is presented.

INTRODUCTION

The teleost gill is a relatively simple structure (compared with e.g., a frog skin or a vertebrate kidney), and this, together with its accessibility, makes it seem a desirable organ for studying ion transport through epithelia. Much of the gill is respiratory epithelium, whose contribution to ion transport, if any, is unknown. Histological evidence indicates that the ion-transporting cells are functionally and morphologically discrete, forming a layer 2–8 cells thick around the central core of the filament (see review by Conte, 1969).

Ion transport by gills of freshwater teleosts (goldfish) was experimentally demonstrated first by Krogh (1938), although the lack of radioisotopes prevented him from estimating values for one-way fluxes. Krogh also showed that the systems for the transport of sodium and for chloride were at least partly independent of one another. When isotopes of chloride and sodium became

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generally available, values for one-way ion fluxes in freshwater were reported by several workers (Meyer, 1951; Mullins, 1950; Gordon, 1963; Garcia-Romeu and Maetz, 1964; Potts and Evans, 1967). Garcia-Romeu and Maetz (1964), working with goldfish (Carassius), confirmed Krogh's findings that sodium and chloride uptake were by independent mechanisms, and Maetz and Garcia-Romeu (1964) reported evidence for a chloride-bicarbonate exchange and a sodium-ammonium exchange to account for the independence.

Our initial purpose was to learn whether the teleost gill could be used in vivo, but under somewhat artificial conditions, as a tool for the study of basic ion transport mechanisms. Previous workers had paid close attention to minimizing stress to the animal in order to avoid the well-known "laboratory diuresis" (Maetz, 1963). Our concern was limited to the ion uptake mechanisms in the gill, consequently we accepted a high level of stress in order to achieve a small-volume system involving only the gill epithelium and mucous membranes of the mouth. In addition, preliminary evidence from the European eel (Kirschner, 1969) indicated that ion uptake is little affected in a preparation similar to ours.

Secondarily, questions regarding the validity of a Na+/NH₄⁺ exchange arose. The evidence for this exchange given by Maetz and Garcia-Romeu (1964) was impelling but indirect. They did not, for example, demonstrate a stoichiometric relation between Na⁺ uptake and NH₄⁺ output. In addition, one of their arguments for a sodium-ammonium exchange was a report by Goldstein and Forster (1961) that enzymatic mechanisms within the gill tissue could account for all the ammonia excreted by teleost gills. However, in a later paper (Goldstein, Forster, and Fanelli, 1964), it was reported that the extraction of ammonia and α-amino acids from the blood passing through the gills could account for all the gill ammonia excretion. In view of this, then, we felt it desirable to evaluate a Na⁺/NH₄⁺ exchange in our system.

METHODS AND MATERIALS
Rainbow trout (Salmo gairdneri), 150-350 g in weight, were obtained from a commercial hatchery in Soap Lake, Washington, and stored unfed at 6-9°C in continuously refiltered tap water, [Na⁺] = 0.9 mM. For measurements of sodium fluxes, individual fish were anesthetized with 0.1% tricaine methanesulfonate (TMS) and suspended dorsal side down in a holding device (Fig. 1). A 15 gauge hypodermic needle was inserted through the lower jaw into the mouth cavity and the mouth was sewn shut with a single stitch. Irrigating solution was pumped from a flask, through a stainless steel cooling coil and into the mouth cavity via the hypodermic needle. The fluid passed over the gills and dropped from the opercular opening to a collecting basin from which it was returned to the reservoir flask. The pumping rate was approximately 100 ml/min, and the total volume of fluid was 100 ml. The fish were maintained at a level of anesthesia sufficient to block most reflexes (but still allowing ven-
Circulation Collecting Basin

RESERVOIR

FIGURE 1. Gill irrigation system. Left, anterior view; the hypodermic needle is inserted through the lower jaw of the fish, and serves as the inlet for the irrigating solution. The fish is suspended dorsal side down in a cradle of rubber netting. Right, side view; the irrigating solution drops to the collecting basin and is returned by gravity to the reservoir flask.

tilation movements to continue) by adding anesthetic directly to the reservoir flask. Urethane was routinely used except in experiments in which ammonia output was measured. For these, methylpentynol (3-methyl-1-pentyn-3-ol), a nitrogen-free compound, was used. The concentration of each was about 0.1%, but in practice the exact amount was adjusted empirically to maintain the desired level of anesthesia. TMS was metabolized and/or eliminated too rapidly to be useful for maintaining anesthesia for long periods. It was possible to maintain fish under these conditions for as long as 7 hr, but few experiments lasted more than 4 hr. All flux experiments were done with the irrigating solution at 12–13°C.

Sodium influx was determined by measuring the disappearance of Na from the irrigating solution. The small volume of solution made it possible to estimate a flux in 1 hr at external concentrations below 5 mM. For higher concentrations the measurement took 2 hr. Fig. 2 shows Na uptake in a typical experiment. In such a run, the specific activity of the medium was always more than 10 times that of body fluids, so a correction for back flux of isotope was not made. The one-way sodium influx was estimated by taking a tangent to the curve of isotope uptake at the desired time and dividing by the specific activity of the medium at that time (cf. Kirschner, 1970). Net flux was measured by determining the change in total sodium concentration, and efflux was then calculated by difference.

Electrical potentials were measured with fish immersed in a 4 liter bath and narcotized with 0.004% TMS in the bath. A bridge of saturated KCl agar in 2 mm diameter polyethylene tubing was inserted through a small incision into the peritoneal cavity. A similar bridge was immersed in the bath, and both were connected through calomel electrodes to a potentiometric recorder. In order to test against possible short-circuiting, several measurements were made using the system previously described for
flux measurements, with the outside bridge held under the operculum. In this system, the water irrigating the gills did not contact the area of the incision, and the dried skin between the bridges precluded short-circuiting. The potentials measured with the two systems were comparable, but instability of the second system, due possibly to ventilation movements, made it less useful.

Sodium analyses were done on a Perkin-Elmer model 303 atomic absorption spectrophotometer. A Nuclear-Chicago autogamma scintillation counter was used for measuring $^{22}$Na. Ammonia was determined by nesslerization after microdiffusion by a modification of the method described by Seligson and Seligson (1951). A Radiometer model PHM-4b pH meter with a separate liquid-junction calomel reference electrode was used for all pH determinations.

RESULTS

Concentration Dependence of Sodium Fluxes

As part of the initial characterization of the system, sodium influx was measured as a function of external sodium concentration. The measurements were made in sodium chloride solutions, each fish being used for at least two, and usually three flux determinations for consecutive 1 hr periods at different concentrations. A series of flux measurements on each fish always included at least
one period in 1 mM NaCl. Fig. 3 illustrates the results. The curve fits the well-known Michaelis-Menten equation:

\[ J_i = \frac{J_{\max} [Na]_{out}}{K_m + [Na]_{out}} \]

where, \( J_i \) = Na\(^+\) influx, \( J_{\max} \) = maximum Na\(^+\) influx, \( K_m \) = Michaelis constant.

![Figure 3](image)

**Figure 3.** Sodium influx vs. bath [Na]. Vertical lines are standard errors of the mean. The open circles represent values from NaCl solutions, and the open squares represent sodium uptake from Cl-free solutions.

Values for the parameters were calculated from a reciprocal plot of the same data (Fig. 4). \( J_{\max} \) is 33.3 \( \mu \)Eq (100 g)-1hr-1, and \( K_m \) is 0.46 mM. These parameters were used to plot the theoretical curve represented in Fig. 3 by the dashed line. Thus sodium influx through the trout gill follows the concentration-dependent patterns shown by Brown (1962) for the frog skin, and by Shaw (1959) for the crayfish, and suggests a saturable process.

Efflux, as well as influx, increased with external concentration, as shown in Fig. 6 (Tables II and IV illustrate this, too). Since the electrochemical potential of sodium in the body fluids was practically invariant in this kind of experiment, there is no reason to expect that outward diffusion will depend on [Na]_{out}. Data of this type are usually taken to indicate that an exchange diffusion system is operative. This point will be discussed later.
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Independence of Sodium Uptake

Sodium uptake from sodium sulfate and sodium phosphate solutions was measured in a series of concentrations (0.15, 0.80, 7.5 μEq Na+/ml) to determine the effect of nonpenetrating counterions on the sodium transport system. Garcia-Romeu and Maetz (1964) showed that sulfate ions are not absorbed by goldfish gills, and flux measurements with 2PO₄ in our laboratory indicate little, if any, penetration of trout gills by this ion. Pooled values of influx measurements for three concentrations are given in Table I, and the

| Concentration | Jₑ* | Concentration | Jᵢ* | Concentration | Jᵢ* |
|---------------|-----|---------------|-----|---------------|-----|
| Cl⁻-free ‡    | 7.0±0.07 | 48.6 | 0.83±0.02 | 18.0 | 0.16±0.01 | 7.3 |
|               | n=18 | ±3.4 | n=18 | ±1.2 | n=17 | ±0.47 |
| NaCl 7.6±0.16 | 38.6 | 0.93±0.03 | 21.7 | 0.23±0.02 | 10.6 |
| n=8 | ±2.6 | n=15 | ±1.4 | n=13 | ±1.0 |
| Theoretical §  | 7.0 | 31.3 | 0.83 | 21.5 | 0.16 | 8.6 |

* μEq/(100 g)⁻¹ hr⁻¹ ± se.
‡ Pooled values from Na₂SO₄ and Na₂HPO₄/NaH₂PO₄ solutions.
§ Values taken from the concentration dependence curve in Fig. 3.
uptake rates in the two lowest concentrations are shown in Fig. 3 by the open squares.

At the two lower concentrations sodium influx is slightly below the theoretical values, but we hesitate to attach significance to this. The reduced activity coefficients of sodium in sulfate and phosphate solutions might account for the discrepancy. In the 7.0 mM range, neither the measured uptake from NaCl ($J_i = 38.6 \pm 2.6 \mu\text{Eq} (100\text{ g})^{-1}\text{hr}^{-1}$) nor that from chloride-free solutions fit the theoretical curve, but it is clear that uptake in the presence of nonpenetrating anions proceeds at least as fast as from a NaCl solution. Thus sodium influx from dilute solutions is independent of the simultaneous absorption of chloride.

![Figure 5. Transepithelial potential vs. bath [Na⁺]. Vertical lines are standard errors of the mean. Sodium concentrations milliequivalents per liter.](image)

**Transepithelial Potentials**

Under our experimental conditions bath NaCl concentrations up to 10 mM NaCl had little effect on the TEP. Fig. 5 shows that when the external solution was 0.01 mM NaCl the average TEP was 4 mV, body fluids positive. It increased only 2 mV per 10-fold increase in concentration to 1.0 mM, and showed no further change at 10 mM. This is in marked contrast to the TEP in intact frogs (Brown, 1962) and salamanders (Dietz et al., 1967) which have the same polarity but are dependent on [Na⁺] in the medium. It also conflicts with the only other reports on teleost gills, both of which show the body fluids negative to the medium (House, 1963; Maetz and Campanini, 1966).

**Flux Ratios**

Table II summarizes flux ratios and TEP's for a series of bath NaCl concentrations up to 2.4 mM. We have not corrected the fluxes for the apparent ex-
change diffusion mentioned above, because there is no assurance that this phenomenon really causes the concentration-dependent increase in efflux (see below). However, tentative calculations indicate that the efflux at [Na⁺]_{out} = 0 (presumably the true diffusive leak) is about 5 μEq (100 g)^{-1}hr^{-1}. On the assumption that exchange diffusion does occur, this value was used to correct the fluxes in Table II, and the disagreement between calculated flux ratios and those measured was little affected.

Clearly, the values at every concentration in Table II satisfy the criterion for active transport according to the Ussing flux equation (Ussing, 1949).

**TABLE II**

| [Na] μEq/mL | J_i* | J_o* | n | TEP | J_o/J_i Observed | J_o/J_i Expected |
|--------------|------|------|---|-----|-----------------|-----------------|
| 0.08±0.01    | 5.3±0.93 | 6.8±1.97 | 3 | 6.8 | 1.27            | 2290            |
| 0.24±0.03    | 9.4±0.88  | 11.8±1.31 | 7 | 7.2 | 1.26            | 776             |
| 0.97±0.06    | 19.3±1.36 | 14.3±1.53 | 14| 8.5 | 0.74            | 202             |
| 2.40±0.14    | 30.7±3.75 | 19.3±6.02 | 3 | 8.6 | 0.63            | 82              |

* μEq (100 g)^{-1}hr^{-1} ± se.
† TEP estimated from Fig. 5.
§ Flux ratio expected if diffusion only occurs.

**Ammonia Output and Sodium Influx**

The independence of sodium and chloride transport systems demands the existence of coupled ion exchanges to maintain electrical neutrality. Maetz and Garcia-Romeu (1964) concluded from work with *Carassius* that a Na⁺/NH₄⁺ exchange was the mechanism for sodium entry across the gill. In order to test for such a mechanism in trout gills, we measured sodium influx and ammonia efflux in irrigating media of three different sodium concentrations. In a small volume, open-air system such as ours, there is a possibility of losing ammonia, particularly at high pH's. In order to prevent this, fluxes were measured from sodium phosphate solutions. The pH was kept at 7.0-7.5, well below the point at which appreciable ammonia (pK_a = 9.3) loss could be expected. The three different solutions were tested in consecutive 1 hr periods, thus giving three different rates of sodium influx for each fish. Fig. 6 shows the pooled results of these experiments. Clearly, there is no correlation between sodium influx and ammonia output. In addition, ammonia output has been measured using a sodium-free solution (MgCl_2) as the irrigating medium, and ammonia output was in the normal range (30.3 ± 1.8 μEq (100 g)^{-1}hr^{-1}, 12 determinations). Normal ammonia excretion in sodium-free water has also been reported for the goldfish (de Voys, 1968). The concentrations of NH₄⁺...
in our reservoirs at the end of a 1 hr run were at a level which has been shown to inhibit ammonia excretion (Fromm and Gillette, 1968); therefore the excretion rates reported here may be slightly lower than normal.

Maetz and Garcia-Romeu (1964) were able to get marked stimulation of sodium influx in goldfish by injecting ammonium sulfate solutions. We attempted to duplicate these results with trout, and in some experiments we obtained similar results. Table III summarizes the results of three such experiments. 2 ml of 50 mM (NH$_4$)$_2$SO$_4$ were injected intraperitoneally in each fish following an initial 1 hr flux measurement in a 1 mM NaCl solution. Flux determinations were then continued for 3 consecutive hours in 1 mM NaCl. But four experiments of identical design failed to show a stimulation of influx. A few additional experiments in which ammonium solutions were continuously infused into the subintestinal vein also resulted in marked stimulation of sodium influx. Because of difficulties in keeping the fish alive, the data are not included here.

In ureotelic animals, the injection of ammonium salts causes a secondary acidosis resulting from the formation of urea and the concomitant release of equivalent hydrogen ions. Although urea formation is slight in freshwater teleosts, de Voys (1969) has reported that ammonia may exist as a carbamate in fish blood. The formation of such a compound from (NH$_4$)$_2$SO$_4$ would also release an equivalent number of hydrogen ions. In order to test for such an effect, two 1 ml injections of 100 mM (NH$_4$)$_2$SO$_4$ were given intraperitoneally 30 min apart to four fish. Blood was collected by cardiac puncture before the first and 15 min following the last injection, and pH was measured anaerobi-

![Figure 6](image-url)

**Figure 6.** Sodium and ammonia fluxes at three different rates of Na$^+$ influx. Sodium influx was changed by varying the [Na$^+$] of the irrigating solution. Note that NH$_4^+$ output is constant at all three rates of Na$^+$ uptake.
TABLE III
STIMULATION OF SODIUM INFLUX
BY AMMONIUM INJECTIONS

|   | A* | B* | C* | D* |
|---|----|----|----|----|
| J* | 23.1±1.9 | 29.4±4.9 | 29.8±1.1 | 25.7±2.2 |

* Successive 1-hr flux measurements, NH$_4^+$ injected after A.
† μEq (100 g)$^{-1}$ hr$^{-1}$ ± se.

Evidence for a Sodium/Hydrogen Exchange System

The absence of correlation between sodium influx and ammonia output meant that alternative exchanges must exist. Garcia-Romeu et al. (1969) recently described a Na$^+/H^+$ exchange through the skin of the Chilean frog, (Calyptocephalella gayi), and it appeared likely that such a coupled system might exist across the fish gill. In order to test for a correlation between hydrogen ion output and sodium uptake, flux determinations were made in 1 mM KH$_2$PO$_4$/K$_2$HPO$_4$ solutions to which Na$_2$SO$_4$ was added to give three sodium concentrations, approximately 7.0, 1.0, and 0.1 μEq/ml. Chloride-free solutions were used in order to eliminate the contribution of bicarbonate from a Cl$^-$/HCO$_3^-$ exchange (Maetz and Garcia-Romeu, 1964; Dejours, 1969). The pH of the irrigating solutions before and after each 1 hr test period was measured.

Table IV shows the pooled results of these experiments. First, in this experiment, too, there is no correlation between Na$^+$ influx and NH$_4^+$ output. Second, the pH went progressively further down as the sodium influx increased. But when unbuffered solutions were tried, pH shifts were erratic and usually upward at all rates of $J_+$. Since gills secrete mucus, as does the skin to which the irrigating medium is exposed during recirculation, the upward shift in unbuffered solutions might reflect addition of mucoprotein to the bath. This would mask the addition of hydrogen ion. In buffered solutions, the data show that H$^+$ efflux varies with Na$^+$ influx, but lacking exact stoichiometry the pH shifts can only be considered suggestive. Therefore, an independent approach was sought.

It is known that teleost gills have high concentrations of the enzyme carbonic anhydrase (Maetz, 1953), and Maetz (1956) had previously reported that acetazolamide (Diamox), a potent carbonic anhydrase inhibitor, stopped both Cl$^-$ and Na$^+$ uptake through goldfish gills. Consequently the effect of Diamox (40 mg/kg intraperitoneally) was tested on sodium uptake by trout gills. The results are shown in Table V. Sodium influx was markedly depressed. During the control period the pH shifted downward as in the preced-
TABLE IV
EFFECTS OF DIFFERENT Na+ CONCENTRATIONS ON Na+ FLUXES, NH₄⁺ OUTPUT, AND pH CHANGES

| [Jf] | [Jo] | [NH⁺⁺] | ΔpH ± SE |
|------|------|--------|----------|
| 7.0 mm Na⁺ | 33.4±6.3 | 29.8±5.7 | 15.4±2.4 | -0.31±0.08 |
|       | n=4  | n=3    | n=4      | n=4      |
| 1.0 mm Na⁺ | 17.9±1.1 | 16.0±2.9 | 17.4±0.6 | -0.07±0.04 |
|       | n=3  | n=3    | n=3      | n=3      |
| 0.1 mM Na⁺ | 6.2±1.5 | 11.6±2.8 | 12.4±1.8 | +0.07±0.08 |
|       | n=4  | n=3    | n=4      | n=4      |

* μEq (100 g)⁻¹ hr⁻¹ ± SE.

TABLE V
EFFECTS OF DIAMOX ON Na⁺ FLUXES, NH₄⁺ OUTPUT, AND pH CHANGES

| [Jf] | [Jo] | [NH⁺⁺] | ΔpH ± SE |
|------|------|--------|----------|
| 1.0 mm Na⁺ before Diamox | 18.0±1.6 | 15.7±3.6 | 15.4±1.6 | -0.15±0.03 |
|       | n=4  | n=4    | n=3      | n=3      |
| 1.0 mm after Diamox | 2.6±0.9 | 10.2±2.6 | 12.6±1.2 | +0.06±0.05 |
|       | n=4  | n=4    | n=3      | n=3      |

* μEq (100 g)⁻¹ hr⁻¹ ± SE.

ing experiment. But the data show that during inhibition of sodium uptake by Diamox the perfusion medium became slightly more alkaline. It should be noted that Diamox would also inhibit erythrocyte carbonic anhydrase, and the upward pH shift might be due, at least in part, to a decrease in CO₂ excretion. Yet the salient point is that Na⁺ uptake correlates with H⁺ excretion in this experiment as it did in the one shown in Table IV. In neither case was there any change in ammonia excretion.

DISCUSSION

A short time ago we found that the European eel (Anguilla anguilla) could withstand a long period in air under light anesthesia as long as the buccal cavity was perfused with a medium resembling the one to which the fish was adapted (Kirschner, 1969). Only a few animals were used, but it appeared that sodium and chloride fluxes were comparable to those of unrestrained fish. On being returned to aquaria they recovered quickly from the anesthetic and survived for several days at least. Our present data make it clear that the rainbow trout can be handled in the same way. When the results on eels were published the method did not appear to offer any advantage over more con-
ventational measurements on fish swimming in small chambers. But there are advantages. One is in separating the efflux into renal and gill components. The former, which may comprise more than half of the total, is not subject to the same flux-force relationships as the latter, and a flux ratio analysis, for example, would be complicated by renal loss. Similarly, studies on mechanisms (Na\(^+\)/NH\(_4\)^+ or Na\(^+\)/H\(^+\) exchanges) are unavoidably complicated by the possibility that outflow of one of the components may be partly through the kidney. A second advantage is in the use of a small volume system, enabling flux measurements to be made more rapidly and with greater accuracy than with systems employing a holding chamber.

Sodium uptake in these fish resembles that in other freshwater animals such as the crayfish (Shaw, 1959, 1960a), frog (Krogh, 1938; Brown, 1962), salamander (Alvarado and Kirschner, 1963; Dietz et al., 1967), and several other teleosts (Garcia-Romeu and Maetz, 1964; Motais, 1967). In every case influx is independent of simultaneous anion absorption, occurs through a saturable mechanism, and has a K\(_m\) near 0.5 m.

The nature of the cation exchange in fish gills is worth exploring. If the rate of ammonia output is measured in solutions to which the fish has been previously adapted, ammonia efflux appears to be of the same magnitude as sodium influx. For example, in Fig. 6, the "normal" rate of sodium influx was measured in solutions about 0.80 \(\mu\)Eq Na\(^+\)/ml, a concentration approximately equal to that of the holding tanks in which the fish were kept. The ammonia output at this concentration is not significantly different from the sodium uptake, and if this were the only solution in which sodium influx was measured, a strong case could be made for a sodium-ammonium exchange. However, if there is an obligatory coupled exchange between the two ions, stoichiometry between their fluxes should appear at all rates of sodium influx, which is demonstrably not so. Ammonia efflux lags behind sodium influx at high influx rates, exceeds it at low rates, and in fact proceeds unimpaired in sodium-free solutions. It should be pointed out, however, that NH\(_4\)^+ output is in excess of net Na\(^+\) uptake at all concentrations. If the Na\(^+\) influx includes an appreciable contribution from exchange diffusion, the total could include both Na\(^+\)/Na\(^+\) and Na\(^+\)/NH\(_4\)^+ exchanges, the latter accounting for net uptake. Such a model also demands that ammonia be excreted as both NH\(_3\) and NH\(_4\)^+, the proportion of the latter depending on the rate of sodium uptake. This would account for the constant total ammonia excreted in the face of large variations in sodium influx. It would also account for an upward pH shift when sodium uptake stops; under these conditions all the ammonia enters the external solution as the free base. But it does not explain the downward pH shift correlated with sodium influx. Exchange of Na\(^+\) for NH\(_4\)^+ in a phosphate solution around pH 7 should have little effect on its pH. On the balance, then, we believe the evidence favors a Na\(^+\)/H\(^+\) exchange.
The stimulation of sodium uptake by injections of ammonium salts, reported by Maetz and Garcia-Romeu (1964) and confirmed by us, may result, either directly or indirectly, from the resultant acidosis. Our experiments demonstrating an ammonium-induced acidosis were preliminary in nature. Until a correlation between both the onset and degree of acidosis and the stimulation of sodium influx can be demonstrated, we can make no definitive statement about the relationship. Maetz and Garcia-Romeu also report that sodium influx is inhibited by ammonium salts added to the bath. At concentrations at which they achieved inhibition, however, the NH₄⁺ to Na⁺ ratio was about 40 to 1, an excess of NH₄⁺ which makes conclusions about specific ion exchanges questionable. Shaw (1960b) argues, in the case of the crayfish, that since 1.0 mM NH₄⁺ in the bath depresses sodium uptake to as little as 20% of normal, ammonium ion may be competing for carrier sites. But he also showed that sodium uptake fell off sharply below pH 6.0, and at pH 4.0 an inhibition similar to the ammonium effect was achieved. It appears, then, that in the crayfish, H⁺ ions were 10 times as effective as NH₄⁺ in depressing sodium uptake.

Garcia-Romeu et al. (1969), in an elegantly designed series of experiments, demonstrated an exact stoichiometry between net Na⁺ uptake and H⁺ ion output in the Chilean frog, a ureotelic species. To date we have not been successful in showing a similar stoichiometry for either net Na⁺ uptake or Na⁺ influx. There are, however, two additional functions of teleost gills, gas exchange and ammonia excretion, which complicate the task. If, for example, ammonia excretion is not coupled to ion transport, then it is probably excreted as NH₃, as in the mammalian distal tubule. With a pKₐ of 9.3, it would act as a hydrogen ion trap in neutral solutions, accepting hydrogen ions not only from a Na⁺/H⁺ exchange, but also from the phosphate buffer and from carbonic acid formed by high levels of CO₂ passing outward through the gill. The problem, in showing a Na⁺/H⁺ exchange, is to account for that portion of the ammonia which has been ionized by hydrogen from the transport mechanism, and to discount that portion ionized by CO₂ excretion or hydrogen sources in the bath. At high rates of Na⁺ influx, H⁺ output is much higher than ammonia output, and the excess H⁺ should prevent the formation of (NH₄)₂HCO₃ from carbonic acid. We have evidence, from titration back to the original pH, that this is so. It is at the low rates of Na⁺ influx that the demonstration of sodium-hydrogen stoichiometry becomes difficult, for then a portion of the ammonia is available for bicarbonate formation (in effect trapping H⁺ ions from carbonic acid). It should be pointed out that the data in Table IV show fair agreement between H⁺ efflux and Na⁺ influx based on expected pH changes in a 1 mM phosphate buffer at neutral pH, and accounting for the H⁺ trapped by ammonia. Based on an average fish weight of 200 g, the first two Na⁺ influxes in Table IV represent total uptakes of 67 and 36 μEq Na⁺ in 1 hr. The corresponding H⁺ outputs, calculated as described
above, are 51 and 38 μEq. For reasons discussed above, the agreement is poor at the lowest Na⁺ influx (12 μEq Na⁺ vs. 21 μEq H⁺). The peculiar behavior of unbuffered solutions, where the shift is often upward even at high fluxes, leads us to suspect the presence of other buffering agents, possibly mucoprotein, which is copiously secreted by the teleost gill. Until this can be accounted for, unequivocal statements about sodium-hydrogen stoichiometry are unwise.

Despite the difficulties in demonstrating a sodium-hydrogen stoichiometry at all levels of sodium influx, the pH shifts occurring in phosphate solutions, the lack of correspondence between sodium and ammonium fluxes, and the depression of sodium influx following carbonic anhydrase inhibition are good evidence for a Na⁺/H⁺ exchange in the trout gill. Such a mechanism, together with the Cl⁻/HCO₃⁻ exchange described by Maetz and Garcia-Romeu (1964), links sodium chloride uptake closely with CO₂ elimination, yet makes it possible for each ion uptake system to operate independently.

The low Kₘ of sodium uptake has interesting implications with respect to the role of the Na⁺-K⁺-activated ATPase. If one considers the simplest model of a gill epithelium, a sheet one cell thick, ion movement across the tissue can be visualized in terms of two barriers, the outer cell membrane and the inner (blood border) membrane. A Na⁺-K⁺-activated ATPase has been reported in teleost gills (Epstein et al., 1967; Kamiya and Utida, 1969), and since there is no evidence for a Na⁺/K⁺ exchange between the gill and the environment in freshwater forms, the Na⁺-K⁺ ATPase must be located at the inner border, a case analogous to the frog skin model proposed by Ussing (1960). Unpublished data from our laboratory indicate that the Kₘ for the trout gill enzyme is 5–10 mM for sodium, and saturation is approached at 100 mM (with 20 mM K⁺ in the medium). But at bath sodium concentrations of 3.0 mM and below, the Kₘ for sodium transport across the gill is an order of magnitude below that of the enzyme under laboratory conditions. If the measured Kₘ of the ATPase is the same in vivo as in the test tube, the discrepancy in Kₘ's could be explained by two alternate models. The first of these is that Na⁺ movement through the outer membrane is diffusive, and the exchange with H⁺ is not closely coupled. Then, if the intracellular potential were about 60 mV negative to the medium, a range of [Na⁺] from 0.1 to 10 mM would give intracellular concentrations from about 1 to 100 mM, resulting in a Michaelis type curve for transport generated by the ATPase but reflected an order of magnitude lower to the external concentrations. There are some objections to such a model. First, it implies a variation in intracellular [Na⁺] from 1 to 100 mM, demanding an inverse variation in intracellular K⁺ or else implying serious osmotic problems for the cell. Second, an electrochemical gradient for H⁺ movement outward at neutral pH's would require an intracellular pH of 6.0 or lower. A second alternative is that the observed curve for the concentration dependence of Na⁺ influx is generated directly at the outer membrane, implying that Na⁺
movement through this boundary is not diffusive but rather involves binding at a finite number of sites. A model for Na\(^+\) uptake by the gill, incorporating the second alternative, would then include an obligatory, coupled ion exchange at the outer membrane, a source of H\(^+\) for the exchange from the action of carbonic anhydrase (which also supplies HCO\(^3\)\(^-\) for exchange with Cl\(^-\)) in the gill, and a Na\(^+\)-K\(^+\)-activated ATPase on the inner membrane to complete the transport of sodium to the body fluids.

An interesting byproduct of these experiments is the increase in Na\(^+\) efflux as [Na\(^+\)\(_{\text{out}}\)] is raised. The data suggest that exchange diffusion occurs in the trout gill. However, the question needs further examination for the following reason. Although the location of transport cells in teleost gills is still uncertain, present evidence appears to show that they are in the interlamellar regions of the filament and not in the respiratory epithelium. This means that the latter may be pure leak pathways for ions. Several studies have shown that branchial blood flows through two parallel pathways, one in the lamellae, the other in the filament core (Steen and Kruysse, 1964; Kirschner, 1969). The latter probably perfuses the transporting cells, hence mediates all of the influx and part of the efflux. The former probably contributes solely to diffusive leak through the respiratory epithelium. Randall and Wood (personal communication, D. J. Randall) have measured Na\(^+\) fluxes in trout at different levels of physical activity. They found that while influx remained steady, efflux rose as activity increased, suggesting a Na\(^+\) leak through the lamellar circulation. The lamellar microcirculation appears to be opened by norepinephrine and by high temperature, closed by acetylcholine and by low temperature. We cannot rule out the possibility that changes in environmental salinity also modify the pattern of flow. If an increase in salinity causes the respiratory capillary bed to open, ion efflux would increase as it does when norepinephrine is infused or temperature is increased (Kirschner, 1969). This would give rise to a concentration-dependent efflux as seen in Tables II and IV, but by a mechanism very different from exchange diffusion. At this time we see no simple way to eliminate one of the possibilities as long as intact animals must be used. For the present we can only state the two interpretations. If the concentration-dependent efflux is due to exchange diffusion, the \(J_i\) values in Fig. 3 are too high. A tentative correction (using 5.0 \(\mu\)Eq \((100 \text{ g})^{-1} \text{ hr}^{-1}\) as the diffusive efflux) gives a lower set of \(J_i\) and a lower \(J_{\text{max}}\) but about the same \(K_m\). In addition, the flux ratios in Table III would be too close to unity, but the corrected values differ only trivially from those shown. On the other hand, if the entire efflux is diffusive the values shown in Fig. 3 and Tables II-IV need no correction even though \(J_{\text{out}}\) represents contributions from two separate pathways. However, in either case the main conclusions seem to be unaffected by the choice of a model: sodium transport is active, saturable, and even the influx parameter, \(K_m\), is unchanged.
A second byproduct of these experiments is also worth noting. Only two measurements of a TEP in teleosts have been published, one on the blenny eel (Blennius pholis) and the other on the eel (Anguilla anguilla). In both cases when the animals were in dilute solutions the body fluids were negative to the external medium (House, 1963; Maetz and Campanini, 1966). This is in marked contrast to results obtained with frogs (Brown, 1962), and salamanders (Dietz et al., 1967). In these, the body fluids are positive when salt is transported, and some work suggests that the magnitude of the TEP depends on the rate of sodium transport (Dietz et al., 1967; Brown, 1962). We have done some preliminary work on reasons for this difference. When our animals were in solutions of 1.0 mM NaCl alone the TEP was \(-14.8 \pm 2.7\) mv \((n = 6)\), body fluids negative, in close agreement with the results on eels. But when the bath contained 0.5 mM Ca\(^{++}\) in addition to the sodium chloride, the body fluids were positive as shown in Fig. 3. Thus, the polarity of the TEP depends on the presence of Ca\(^{++}\) in the external medium, the presence of this ion producing a positive shift in the TEP. It is interesting that Chaisemartin (1966) reported that a high Ca\(^{++}\) medium gave potential shifts in a positive direction across the crayfish gill, although the sign of his potential measurement was always negative. Croghan et al. (1965) also reported negative potentials across crayfish gills, whereas Bryan (1960) measured potentials from 4.1 to 6.6 mv, body fluids positive, in the intact crayfish.

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BIBLIOGRAPHY

Alvarado, R. H., and L. B. Kirschner. 1963. Osmotic and ionic regulation in Ambystoma tigrinum. Comp. Biochem. Physiol. 10:55.

Brown, A. C. 1962. Current and potential of frog skin in vivo and in vitro. J. Cell. Comp. Physiol. 60:263.

Bryan, G. W. 1960. Sodium regulation in the crayfish Astacus fluviatilis. J. Exp. Biol. 37:83.

Chaisemartin, C. 1966. Gradients ioniques, potentiels bioélectriques et rôle de l'épithélium branchial chez Austropotamobius pallipes (Lereboullet) en état d'équilibre calcique (intemne). C.R. Hebd. Séances Acad. Sci. Paris. 160(1):305.

Contz, F. P. 1969. Salt secretion. In Fish Physiology (W. S. Hoar and D. J. Randall, editors), Academic Press, Inc., New York. 271.

Croghan, P. C., R. A. Curra, and A. P. M. Lockwood. 1965. The electrical potential difference across the epithelium of isolated gills of the crayfish Austropotamobius pallipes (Lereboullet). J. Exp. Biol. 42:463.

Dejours, P. 1969. Variations of CO\(_2\) output of a fresh-water teleost upon change of the ionic composition of the water. J. Physiol. (London). 202:113P.

De Vooys, C. G. N. 1968. Formation and excretion of ammonia in Teleostei. I. Excretion of ammonia through the gills. Arch. Int. Physiol. Biochem. 76:268.
de Voogt, C. G. N. 1969. Formation and excretion of ammonia in Teleostei. II. Occurrence and transport of ammonia in the blood. Arch. Int. Physiol. Biochem. 77:112.

Dietz, T. H., L. B. Kirchner, and D. Porter. 1967. The roles of sodium transport and anion permeability in generating transepithelial potential differences in larval salamanders. J. Exp. Biol. 46:85.

Epstein, F. H., A. I. Katz, and G. E. Pickford. 1967. Sodium- and potassium-activated adenosine triphosphatase of gills: role in adaptation of teleosts to salt water. Science (Washington). 156:1245.

Fromm, P. O., and J. R. Gillette. 1968. Effect of ambient ammonia on blood ammonia and nitrogen excretion of rainbow trout. Comp. Biochem. Physiol. 26:887.

Garcia-Romeu, F., and J. Maetz. 1964. The mechanism of sodium and chloride uptake by the gills of a fresh water fish, Carassius auratus. I. Evidence for an independent uptake of sodium and chloride ions. J. Gen. Physiol. 47:1195.

Garcia-Romeu, F., A. Salibián, and S. Pezzani-Hernández. 1969. The nature of the in vivo sodium and chloride uptake mechanisms through the epithelium of the Chilean frog, Calyptcephalella gayi (Dum. et Bibr., 1841). Exchanges of hydrogen against sodium and of bicarbonate against chloride. J. Gen. Physiol. 53:816.

Goldstein, L., and R. P. Forster. 1961. Source of ammonia excreted by the gills of the marine teleost, Myoxocephalus scorpius. Amer. J. Physiol. 200:1116.

Goldstein, L., R. P. Forster, and G. M. Fanelli. 1964. Gill blood flow and ammonia excretion in the marine teleost, Myoxocephalus scorpius. Comp. Biochem. Physiol. 12:489.

Gordon, M. 1963. Chloride exchanges in rainbow trout (Salmo gairdneri) adapted to different salinities. Biol. Bull. (Woods Hole). 124:45.

House, C. R. 1963. Osmotic regulation in the brackish water teleost, Blennius pholis. J. Exp. Biol. 40:37.

Kamiya, M., and S. Utida. 1969. Sodium-potassium activated adenosine triphosphatase activity in gills of fresh-water, marine, and euryhaline teleosts. Comp. Biochem. Physiol. 31:571.

Kirchner, L. B. 1969. Ventral aortic pressure and sodium fluxes in perfused eel gills. Amer. J. Physiol. 217:596.

Kirchner, L. B. 1970. Ionic regulation in intact animals. Amer. Zool. In press.

Krogh, A. 1938. The active absorption of ions in some freshwater animals. Z. Vergl. Physiol. 25:335.

Maetz, J. 1953. L'anhydrase carbonique dans deux téloséphales voisins. Comparaison des activités anhydrasiques chez Perca et Serranus. C.R. Hebd. Seances Acad. Sc. Paris. 147:204.

Maetz, J. 1956. Les échanges de sodium chez le poisson Carassius auratus. I. Action d'un inhibiteur de l'anhydrase carbonique. J. Physiol. (Paris). 48:1085.

Maetz, J. 1963. Physiological aspects of neurohypophysial function in fishes with some reference to the Amphibia. Symp. Zool. Soc. London. 9:107.

Maetz, J., and G. Campanini. 1966. Potentiels transepithéliaux de la branche d'angulaire in vivo en eau douce et en eau de mer. J. Physiol. (Paris). 58:248.

Maetz, J., and F. Garcia-Romeu. 1964. The mechanism of sodium and chloride uptake by the gills of a fresh-water fish, Carassius auratus. II. Evidence for NH₄⁺/Na⁺ and HCO₃⁻/Cl⁻ exchanges. J. Gen. Physiol. 47:1209.

Meyer, D. K. 1951. Na flux through the gills of goldfish. Amer. J. Physiol. 163:590.

Motais, R. 1967. Les mécanismes d'échanges ioniques branchiaux chez les teleostéens. Ann. Inst. Oceanogr. (Paris). 45:1.

Mullins, L. 1950. Osmotic regulation in fish as studied with radioisotopes. Acta Physiol. Scand. 21:303.

Potts, W., and D. Evans. 1967. Sodium and chloride balance in the killifish Fundulus heteroclitus. Biol. Bull. (Woods Hole). 133:11.

Seligson, D., and H. Seligson. 1951. A microdiffusion method for the determination of nitrogen liberated as ammonia. J. Lab. Clin. Med. 38:224.

Shaw, J. 1959. The absorption of sodium ions by the crayfish Astacus pallipes Lereboullet. I. The effect of external and internal sodium concentrations. J. Exp. Biol. 36:126.
SHAW, J. 1960 a. The absorption of sodium ions by the crayfish *Astacus pallipes* Lerebouillet. II. The effect of the external anion. *J. Exp. Biol. 37*:534.

SHAW, J. 1960 b. The absorption of sodium ions by the crayfish *Astacus pallipes* Lerebouillet. III. The effect of other cations in the external solution. *J. Exp. Biol. 37*:548.

STEEN, J., and A. KRUYSSE. 1964. The respiratory function of teleostean gills. *Comp. Biochem. Physiol. 12*:127.

USSING, H. H. 1949. The distinction by means of tracers between active transport and diffusion. *Acta Physiol. Scand. 19*:43.

USSING, H. H. 1960. The frog skin potential. *J. Gen. Physiol. 43*:135.