Effect of amiloride on sodium transport across body surfaces of freshwater animals

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Kirschner, Leonard R., Lewis Greenwald, and Theodore H. Kerstetter. Effect of amiloride on sodium transport across body surfaces of freshwater animals. Am. J. Physiol. 224(4): 832-837. 1973.—Amiloride, added to an external bathing solution at a concentration of 1 × 10^{-4} M, inhibited sodium influx in intact trout (79%), frogs (46%), and crayfish (90%). It had no effect on efflux across the body surface. Chloride fluxes, measured simultaneously, were also unaffected. Ammonia efflux was inhibited by amiloride to a lesser extent in trout (30%) and crayfish (54%). It was not excreted at measurable rates by the frog in the presence or absence of the inhibitor. Hydrogen ion excretion, calculated on the assumption that only NH_3 (not NH_4^+) was excreted, was inhibited by amiloride in all three animals. trout 56%, frogs 40%, and crayfish >90%. The data suggest that the initial step in active sodium transport across the body surface is similar in freshwater animals from at least two phyla. Under natural conditions (a dilute external medium), this step appears to require a coupled Na^+ / H^+ exchange to maintain electrical neutrality. The amiloride data also admit the possibility of NH_4^+ excretion balancing a fraction of the Na^+ influx in some animals.

ion transport; frog; trout; crayfish; ionic regulation

Krogh (13) showed that salt uptake in a number of freshwater animals comprised independent active transport systems for both Na^+ and Cl^- . He pointed out that when one ion was transported while its counterion was impermeant, electrostatic neutrality could be maintained only if the active influx was balanced by an efflux of some ion with the same charge. He suggested that Na^+ uptake might be accompanied by NH_4^+ or H^+ output, and Cl^- uptake might be accompanied by HCO_3^- output. In the intervening years, extensive research was done on isolated transport epithelia, but under the experimental conditions ordinarily employed, their characteristics were markedly different from those in situ, and Krogh's observations were not pursued. However, there has been renewed interest in ion transport in intact animals including crayfish (18), salamanders (1, 4), frogs (7, 8), and fish (6, 10, 11, 14). Krogh's observations have been fully confirmed, and recent work has raised a number of interesting questions. First, there is still some uncertainty about the identity of the ion for Na^+; both NH_4^+ (4, 14, 18) and H^+ (7, 10, 20) have been proposed in a number of animals. An unequivocal choice can be made only for the frog (Leptodactylus callimicocephalum), which excretes practically no ammonia across the skin and in which net sodium influx (J_{Na^+}) is exactly equivalent to H^+ efflux (J_{H^+}) (7). Second, there is a remarkable similarity in ion transport in completely unrelated freshwater forms. Even quantitative parameters (J_{Na^+} and K_{Na}, for example) are remarkably uniform. This suggests that the molecular mechanisms may be similar, a premise that is often assumed but infrequently tested.

The diuretic compound amiloride (N-amidino-3,5-diamino-6-chloropyrazinecarboxamide) has been shown to inhibit sodium transport in a number of vertebrate epithelia including isolated frog skin (5, 16) and toad bladder (2). It is effective in very low concentrations (10^{-8} to 10^{-5} M) when applied to the mucosal side of these preparations and appears to interfere with the uptake of Na^+ by the epithelial cells (17). This is the step which, in intact animals, requires exchange with an outwardly moving cation. Hence, it seemed that amiloride might be useful in trying to identify the ion excreted during Na^+ uptake. In addition, the question of molecular similarity could be explored by examining the behavior of different freshwater systems in its presence. The data below describe the results of such experiments on the rainbow trout (Salmo gairdneri), the crayfish (Procambarus spp.), and frog (Rana pipiens). A few observations on the salamander (Ambystoma tigrinum) were consistent with the general picture developed but will not be presented. Data on the leech (Haemopis marmorata) are mentioned in discussion but will be presented in detail in a separate publication.

A brief account of some of these results was given previously (9).

METHODS

All animals were obtained from commercial suppliers, trout (200-300 g) from a hatchery near Soap Lake, Washington, and crayfish and frogs from a supplier in Wisconsin. All were kept without feeding in dechlorinated tap water at low temperature (trout at 8 C, frogs and crayfish at 13 C).

Experimental procedures. The protocols generally followed the procedure outlined recently (12), but with specific modifications dictated by equipment design or previous work (on the frog). In the initial experiments the effect of amiloride on simultaneous fluxes of Na^+ and Cl^- was measured. Animals were placed in measured volumes of NaCl (0.9 mm) containing ^22Na^+ and ^36Cl^- . An initial sample of the medium was taken, followed by additional sam-
chloride concentration was determined by electrometric face-gills in the fish and crayfish and skin in the frogs. The entire animal was immersed in the bath, and all fluxes were those crossing the body surfaces. The temperature of the measure-ments was 25 °C.

In other experiments the animal's transport epithelium was exposed to a measured volume of external medium consisting of dilute phosphate buffer (KH$_2$PO$_4$/K$_2$HPO$_4$, 0.9 mM, pH 7.1) which contained Na$_2$SO$_4$ (0.5 mM). Phosphate and sulfate are nonpenetrating in these animals. Carrier-free $^{22}$Na$^+$ was added to the medium and an initial sample was withdrawn. Serial samples were then taken at intervals and analyzed for [Na$^+$], $^{22}$Na$^+$, NH$_4^+$, and pII. From these data values for $J_{Na^+}$, $J_{NH4^+}$, and $J_{H^+}$ were calculated. Amiloride was then added to the medium and the measurements were repeated.

Procedural differences for the three animals were as follows.

**Trout.** Animals were anesthetized with urethane or tricaine methanesulfonate, pH 7 throughout. The tricaine anesthetic was used only in experiments where pH changes were not followed. The trout were suspended in air as described in previous work (10), and the gills were irrigated with 100 ml of medium. The ratio of medium to animal (ml/g) was very small (1:2 or 1:3), and concentration changes were often large and rapid. Flux determinations were made in 1 hr; sample intervals were 20 min. The design of the box holding the fish prevented urine from entering the solution irrigating the gill. Temperature of the perfusion medium was 13 °C.

**Crayfish.** Animals were salt depleted in deionized water for several days before an experiment. The nephroprobes were blocked with dental cement just before the experiment to prevent urine from entering the medium. No anesthetic was used. The entire animal was immersed in the bath, and the ratio medium:animal was large (4:1 to 10:1), resulting in concentration changes that were slower and smaller than for the trout. However, salt depletion of the animals increased $J_{Na^+}$ and $J_{Cl^-}$ (18), which helps compensate for this. In addition, sampling intervals were extended to 1 hr and each flux determination took 2-4 hr. Temperature of the measurements was 13 °C.

**Frogs.** Animals were salt depleted prior to measurements as described earlier (8). Urine contamination was prevented by an indwelling cloacal cannula. Animals were anesthetized during cannulation and then allowed to recover. No anesthetic was used during the experiment. The frog was nearly completely submerged in the experimental medium, only the head was not covered. The medium to animal ratio was 2:1 to 3:1, and sampling intervals were extended as for the crayfish. Temperature of the measurements was 25 °C.

In all cases urine was prevented from entering the external medium, and all fluxes were those crossing the body surface—gills in the fish and crayfish and skin in the frogs.

**Analytical procedures.** Sodium concentration in samples was determined by atomic absorption spectrophotometry, and chloride concentration was determined by electrometric titration. Ammonia was distilled into dilute H$_2$SO$_4$ from the trout and crayfish samples. The acid was then nesslerized for standard ammonia determinations. Frogs excreted little NH$_3$, and measurements were made by modifying an enzymatic method (15) based on the reductive amination of $\alpha$-oxoglutarate. Disappearance of NADH was measured fluorimetrically. Prior to measuring pH, each sample was exposed to air and agitated for several minutes to facilitate loss of dissolved respiratory CO$_2$. Unless this was done, readings on some samples tended to drift upward as CO$_2$ was lost. Readings were made with a Radiometer pH 4 instrument (±0.005 pH).

An aliquot of each sample was pipetted into a counting tube and $^{22}$Na$^+$ was counted in a well-type, γ-scintillation system. $^{36}$Cl$^-$ emits no γ-radiation; hence it does not interfere even when present. In the $^{22}$Na$^+$/36Cl$^-$ experiments, a second aliquot was plated, dried, and counted with a gas-flow, thin-window geiger tube that responded to both isotopes. $^{36}$Cl$^-$ was obtained by subtracting $^{22}$Na$^+$ from the total count. A $^{22}$Na$^+$ sample was counted on both instruments to take account of the difference in counting efficiencies.

**Flux calculations.** Influxes were calculated from isotopic data using a simple two-compartment, nonsteady-state method. In a typical Na$^+$ flux experiment, $^{22}$Na$^+$ was added to the external solution, which contained about 100 μmoles of the element, and its disappearance from this compartment was followed. On moving into the animal it was rapidly diluted by $^{22}$Na$^+$ (~6,000 μmoles (100 g)$^{-1}$), and the internal specific activity was always much lower than that of the medium. For example, after four successive 1-hr periods in external media containing $^{22}$Na$^+$ at an average specific activity of 400 counts/min (μmole)$^{-1}$, the plasma specific activity for two trout was less than 4 counts/min (μmole)$^{-1}$. Thus, for these experiments, the animal could be treated as a single compartment and tracer backflux could be disregarded. Influx was obtained from the rate of change of isotopic concentration and the specific activity of the external compartment. Net ion movements were determined from concentration changes and effluxes were obtained from the conservation equation $J_{\text{net}} = J_{\text{in}} - J_{\text{out}}$. Details and limitation of this method have been described (12). $J_{\text{NH4}^+}$ was computed from changes in medium concentration. $J_{\text{H}^+}$ was calculated as follows. Changes in total acid in the medium were calculated from the pH change using the buffer concentration (0.9 mM) and $pK_a$ (7.21). We assumed that ammonia was excreted as NH$_3$ and was converted to NH$_4^+$ at the expense of H$^+$ in the external medium. Thus, H$^+$ excreted during an experiment was calculated from the sum of ($\Delta$H$^+$)$_{\text{ext}}$ + ($\Delta$NH$_3^+$)$_{\text{ext}}$. This is equivalent to assuming that H$^+$ is the ion coupled to Na$^+$ influx and that NH$_4^+$ contributes nothing. If any ammonia is excreted as NH$_3^+$, $J_{Na^+}$ will be overestimated by an amount equal to $J_{NH4^+}$. One other limitation should be noted. Both NH$_3$ and H$^+$ fluxes are calculated from concentration changes in the medium. Thus, they are net fluxes, not unidirectional effluxes. The latter would be underestimated by any backflux that occurred as media concentrations increased. This is not likely to be a problem in the frog or crayfish where external concentrations are always low. But the small volume of irrigating medium introduces some
A TROUT

FIG. 1. Effect of amiloride on Na+ and on net Na+ movement across trout gill. Figure shows data from 1 experiment on a single fish. A: Na+ disappearance from bathing medium in absence (filled circles) or presence of amiloride at 10⁻⁵ M (unfilled circles) and 10⁻⁴ M (squares). B: change in quantity of Na+ in medium with and without amiloride (symbols as in A). A decrease reflects net absorption by animal; an increase, net loss.

FIG. 2. Effect of amiloride on Cl⁻ and net Cl⁻ movement across trout gill. Figure shows data from 1 experiment on a single fish. Symbols and interpretation as in Fig. 1.

TABLE 1. Effect of amiloride on ion fluxes in the trout

<table>
<thead>
<tr>
<th>Flux</th>
<th>Control</th>
<th>Amiloride</th>
</tr>
</thead>
<tbody>
<tr>
<td>JNa⁺</td>
<td>15.7 ± 1.2</td>
<td>13.9 ± 1.4</td>
</tr>
<tr>
<td>JCl⁻</td>
<td>29.1 ± 2.1</td>
<td>6.2 ± 0.7</td>
</tr>
<tr>
<td>JNH₄⁺</td>
<td>22.4 ± 2.1</td>
<td>10.0 ± 1.5</td>
</tr>
<tr>
<td>JH⁺</td>
<td>27.1 ± 2.4</td>
<td>19.0 ± 1.6</td>
</tr>
</tbody>
</table>

Values are means ± se. Numbers in parentheses are numbers of observations.

uncertainty in experiments on the trout. If the gill is permeable to NH₄⁺, some backflux may develop during the experiments. We cannot assess the magnitude of the problem with certainty. However, the quantity of NH₄⁺ in the medium increased linearly with time through the 1-hr sampling periods, and we have therefore assumed that backflux can be neglected.

RESULTS

Figure 1 shows the effects of amiloride on Na⁺ movement in a single rainbow trout. Na⁺ influx was slightly inhibited at 10⁻⁵ M and nearly completely blocked at 10⁻⁴ M. The effect on net movement is shown in panel B where net absorption by the fish during the control period (shown by decreasing quantity in the external medium) was converted to net loss in the presence of the higher inhibitor concentration. Figure 2 shows that chloride fluxes were not changed at either concentration. Such differential action of amiloride is another illustration of the complete independence of JNa⁺ and JCl⁻, and hence of the need for a pair of exchange systems.

Values of unidirectional Na⁺ fluxes and the effluxes of NH₃ and H⁺ are presented in Table 1. Before discussing the results, the experimental design, described earlier, should be emphasized. In contrast to the experiment shown in Figs. 1 and 2, there was no chloride in the external medium, only the nonpenetrating anions phosphate and sulfate. However, there was a chloride efflux from the animal approximately equal to the loss of sodium (10, 11). This is not shown in the table and accounts for the apparent imbalance in cation fluxes. The same thing is true for data shown in Tables 2 and 3 for the crayfish and frog.

The data show that ammonia excretion, in control fish, was about equal to JNH₄⁺. If it were all excreted as NH₃, it could provide the necessary electrical coupling. On the other hand, JH⁺, calculated on the basis that all ammonia appeared as NH₃, was also nearly adequate to balance JNa⁺. The difference between JNH₄⁺ and JNH₃⁺, though small, is statistically significant, and the probable basis will be discussed below. However, the correspondence between JNH₃⁺ and either influx is sufficiently close that the control animal provides no clear choice between NH₃ and H⁺. Amiloride reduced JNH₃⁺ but had no effect on JNH₄⁺. It also depressed the mean ammonia efflux by about 30 %, but the value remained much higher than JNH₃⁺ during the period of inhibition. The calculated JH⁺ was depressed to a greater extent, and its correspondence with JNH₃⁺ was close when amiloride was present.

Figures 3 and 4 show data on simultaneous fluxes of Na⁺ and Cl⁻ in the crayfish. Because the volume of external

FIG. 3. Effect of amiloride on Na⁺ and Cl⁻ movement across crayfish body surface. Six crayfish were used in this experiment, and mean values (circles) plus 1 SEM (vertical bar) are shown. Animals were placed in amiloride-free medium containing Na⁺ and Cl⁻. At arrow, amiloride was added to medium, bringing amiloride concentration up to 10⁻⁴ M. Filled circles are for Cl⁻, and unfilled circles are for Na⁺.

FIG. 4. Effect of amiloride on Na⁺ and Cl⁻ movement across crayfish body surface. Six crayfish were used in this experiment, and mean values (circles) plus 1 SEM (vertical bar) are shown. Animals were placed in amiloride-free medium containing Na⁺ and Cl⁻. At arrow, amiloride was added to medium, bringing amiloride concentration up to 10⁻⁴ M. Filled circles are for Cl⁻, and unfilled circles are for Na⁺.

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medium was large, concentration changes were much smaller than in the trout, so we show mean values with variance estimates (SEM) for each point. In Fig. 3 it can be seen that 22Na⁺ influx was severely depressed by 10⁻⁴ M amiloride, and Fig. 4 shows that this resulted in net sodium loss from the animals. Figure 3 also shows that the same amiloride concentration had no effect on 36Cl⁻ influx, and while net uptake ceased, the animals were able to maintain a chloride steady state during inhibition of sodium transport (Fig. 4).

Table 2 shows unidirectional flux data for these animals. It is worth noting that JNa⁺ was much higher than for the trout in Table 1. This is a typical response to salt depletion; fluxes in crayfish adapted to tap water are much closer to those in the previous table. Ammonia efflux in the controls was much too small to balance sodium influx. In contrast, mean JCl⁻ was nearly as large as JNa⁺. The difference is barely significant statistically and may have the same basis as the discrepancy seen in trout. This is discussed later. Amiloride nearly abolished sodium influx with little effect on efflux. Ammonia output was inhibited, but excretion was substantial throughout the period of inhibition. In contrast, JCl⁻ was unmeasurable. In crayfish, both control and experimental groups show that the main burden of maintaining charge neutrality falls on H⁺; if NH₄⁺ makes any contribution, it is small.

Figure 5 depicts an experiment on a single animal and shows that JNa⁺ was depressed by amiloride in the frog, too. Variability was much greater among frogs than among crayfish or trout, and complete inhibition was not seen at 10⁻⁴ M. But in all measurements influx was reduced sufficiently to convert a net Na⁺ uptake to a net loss (Fig. 5B). In contrast, chloride influx was not changed by amiloride in the same animal (Fig. 6A). The net flux is lower after the drug, suggesting an increase in efflux, but this was not seen in every animal.

Table 3 shows unidirectional fluxes for our frogs. In control animals JNa⁺ was equivalent to JCl⁻; ammonia output was too small to measure, and the value shown represents the lower limit for the analytical method used.

![Image](http://ajplegacy.physiology.org/)

**TABLE 2. Effect of amiloride on ion fluxes in the crayfish**

<table>
<thead>
<tr>
<th>Flux</th>
<th>Control</th>
<th>Amiloride</th>
</tr>
</thead>
<tbody>
<tr>
<td>JNa⁺</td>
<td>20.4 ± 7.9 (9)</td>
<td>22.6 ± 2.1 (9)</td>
</tr>
<tr>
<td>JCl⁻</td>
<td>88.7 ± 9.4 (9)</td>
<td>&lt; 10 (9)</td>
</tr>
<tr>
<td>JHC⁺</td>
<td>69.1 ± 6.2 (9)</td>
<td>&lt; 4 (9)</td>
</tr>
<tr>
<td>JNH₄⁺</td>
<td>33.8 ± 4.2 (9)</td>
<td>15.5 ± 3.5 (4)</td>
</tr>
</tbody>
</table>

Values are means ±SE. Numbers in parentheses are numbers of observations.

![Image](http://ajplegacy.physiology.org/)

**FIG. 5. Effect of amiloride on 22Na⁺ and on net Na⁺ movement across intact frog skin. Figure shows data from 1 experiment on a single frog. Symbols and interpretation as in Fig. 1 (only 10⁻⁴ M amiloride was used).**

**FIG. 6. Effect of amiloride on 36Cl⁻ and on net Cl⁻ movement across intact frog skin. Symbols and interpretation as in Fig. 1 (only 10⁻⁴ M amiloride was used).**

This confirms observations on Calyptorhynchus gaim (7), merely extending them to another species of frog. Sodium influx was reduced by about 50% by amiloride, whereas efflux was unchanged. H⁺ excretion dropped exactly enough to compensate for reduced Na⁺ transport.

In trout and frogs amiloride inhibition was completely reversible; washing for a few minutes in inhibitor-free solu-
tion sufficed to restore \( J_{\text{Na}^+} \) to control levels. Reversibility was not tested directly in crayfish, but experimental animals survived for 3 months and appeared to be normal after being returned to tap water.

**DISCUSSION**

These experiments provide another demonstration that active transport of sodium and chloride are completely independent in freshwater animals. Amiloride inhibits only sodium transport and has no discernible effect on chloride transport. At \( 10^{-4} \text{ m} \), inhibition of \( J_{\text{Na}^+} \) was virtually complete in the fully aquatic fish and crayfish, but not in frogs. Perhaps inhibition in the frogs would have been greater at still higher concentrations, but this was not investigated. In none of the animals was efflux across the body surface changed; hence, permeability characteristics governing passive movement were not modified by amiloride. Comparable observations have been made on the horse leech (\textit{Haemopis marmoratis}). Amiloride reduced \( J_{\text{Na}^+} \) from 4.6 to 0.9 \( \mu \text{Eq (100 g)}^{-1} \text{hr}^{-1} \), whereas it had no effect on efflux (unpublished observations).

It has been frequently pointed out that if sodium and chloride influxes are not coupled, macroscopic electroneutrality requires that each ion be exchanged for an outward-moving ion of the same charge. Both \( \text{NH}_4^+ \) and \( \text{H}^+ \) have been suggested, since both are excreted across the body surface of aquatic animals. Evidence that appeared to favor \( \text{NH}_4^+ \) has been described recently in crayfish (18), teleost fish (14), and salamander (4). This has comprised three observations: that sodium influx and ammonia efflux are roughly equivalent in animals adapted to pond water (about 1 mM) and tested in that medium; that injected \( \text{NH}_4^+ \) stimulates sodium influx, whereas high external \( \text{NH}_4^+ \) inhibits it. However, other explanations of the \( \text{NH}_4^+ \) effects are possible (10), and the correspondence between body surface changes; hence, permeability characteristics governing passive movement were not modified by amiloride. Comparable observations have been made on the horse leech (\textit{Haemopis marmoratis}). Amiloride reduced \( J_{\text{Na}^+} \) from 4.6 to 0.9 \( \mu \text{Eq (100 g)}^{-1} \text{hr}^{-1} \), whereas it had no effect on efflux (unpublished observations).

The data in this paper also support the primacy of a Na\(^{+}/H^+\) exchange system. In crayfish \( J_{\text{Na}^+} \) was nearly equal to \( J_{\text{H}^+} \), and amiloride practically abolished both. Substantially the same effect of amiloride was seen in the trout and in the frog. It is improbable that \( \text{Na}^+ \) and \( \text{NH}_4^+ \) movements are coupled in the frog, since the animals excrete practically no ammonia across the body surface. And, although amiloride caused some depression of ammonia output in the two aquatic forms, this was too small to correspond to the change in sodium uptake. Depression of \( J_{\text{H}^+} \) was more closely correlated with inhibition of \( J_{\text{Na}^+} \) in both.

Although quantitative agreement between \( J_{\text{Na}^+} \) and \( J_{\text{Na}^+} \) was not bad, \( J_{\text{Na}^+} \) was about 20% lower than \( J_{\text{Na}^+} \) in control trout and crayfish. A probable reason for the discrepancy is passive outward diffusion of \( \text{Cl}^- \) from the animals into the \( \text{Cl}^- \)-free external solution. This will permit an influx in exchange for \( \text{HCO}_3^- \), and each of the \( \text{HCO}_3^- \) ions entering the solution will eliminate one \( \text{H}^+ \). The magnitude of this problem in the trout can be estimated from data published previously. \( J_{\text{H}^+} \) was about 15 \( \mu \text{Eq (100 g)}^{-1} \text{hr}^{-1} \) and did not vary with \( [\text{Cl}^-]_{\text{out}} \). Therefore a 200-g fish should have lost about 30 \( \mu \text{Eq} \) to the bathing medium, but titration showed 15–20 \( \mu \text{Eq} \) present. Inward \( \text{Cl}^- \) transport must have removed the rest and added an equal amount of \( \text{HCO}_3^- \). Expressed in flux units \( J_{\text{HCO}_3^-} \) was about 5–7 \( \mu \text{Eq (100 g)}^{-1} \text{hr}^{-1} \). This is exactly the deficit in \( J_{\text{H}^+} \) noted in Table 1. Comparable data for the crayfish are not available, but the same phenomenon probably accounts for part of the difference between \( J_{\text{Na}^+} \) and \( J_{\text{Na}^+} \) in this animal as well.

To this point we have discussed \( \text{NH}_4^+ \) and \( \text{H}^+ \) as if they were the sole cations available for excretion to balance sodium influx. But \( \text{K}^+ \) efflux may also play a role. In a recent paper on mosquito larvae, 33% of \( J_{\text{Na}^+} \) was shown to be balanced by the outflux of \( \text{K}^+ \) (20). Unpublished data in our laboratory have established that the trout gill is permeable to \( \text{K}^+ \), and the possibility of net outward movement cannot be dismissed. But the data presented here indicate that \( \text{H}^+ \) excretion accounts for all or most of the charge balance in our animals.

These data are also interesting from a comparative point of view. Salt uptake systems in unrelated freshwater animals show some basic similarities, which include independent active sodium and chloride transport systems and half-saturation values in the range 0.1–1.0 mM. In this paper amiloride has been shown to inhibit active sodium transport across the body surface of two classes of vertebrates and a crustacean, and it also is effective in an annelid. All of them are basically freshwater animals. In fish, frog, and crayfish, inhibition appears to involve blocking \( \text{Na}^+/\text{H}^+ \) exchange across the outer surface of the transport epithelium. These observations indicate that the first step in sodium uptake is biochemically similar in many freshwater forms.

Much of the work with amiloride and sodium transport has involved use of isolated organs (2, 5, 16, 17). Some inhibition occurred at extremely low concentrations (e.g., \( 10^{-5} \text{ m} \)), and transport was completely abolished at about \( 10^{-4} \text{ m} \). In the animals used here much higher concentrations were required, though the action was the same. As shown in Fig. 1, inhibition was complete in the trout at \( 10^{-4} \text{ m} \), but slight at \( 10^{-5} \text{ m} \). Even \( 10^{-4} \text{ m} \) did not totally inhibit in the frog. The reason for this difference is not clear. Inhibition of sodium transport across isolated frog skin appears to require \( \text{Ca}^{2+} \) (3). In the presence of 1 mM \( \text{Ca}^{2+} \), inhibition was complete at \( 10^{-4} \text{ m} \); in its absence amiloride was practically without effect. Since our external solutions contained no \( \text{Ca}^{2+} \), it appeared that this might explain the difference. However, addition of \( \text{Ca}^{2+} \) did not have the expected effect in the trout or frog; \( 10^{-6} \text{ m} \) amiloride + 1

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**References**

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10. Amiloride did not affect the rate of transport in vitro, but it did cause complete inhibition of transport in vivo. In addition, EGTA, added to the medium, did not abolish amiloride inhibition at $10^{-4}$ M in either animal (unpublished observations). Another methodological difference is that our experiments were run at [Na+]$_{out}$ = 0.9 mM, whereas most of the work in vitro used Ringer solution as an external medium. However, in one study (17) [Na+]$_{out}$ was varied and amiloride inhibition was greater, not less, when low external [Na+] bathed the outside of the isolated frog skin. At present there are no other obvious bases for explaining the discrepancy. It must be added to a list of quantitative differences between transport epithelia in vitro and in vivo, which, for the frog skin, includes the maximum rate of transport and the $K_m$ passive permeability, and the requirement for Na+/H+ exchange in vivo but not in vitro.

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