Na⁺-H⁺ Exchange and Na⁺ Entry across the Apical Membrane of Necturus Gallbladder

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ABSTRACT The role of Na⁺-H⁺ exchange in Na⁺ transport across the apical membrane was evaluated in Necturus gallbladder epithelium by means of intracellular Na⁺ activity (aNa⁺) and ⁸⁶Na⁺ uptake measurements. Under control conditions, complete replacement of Na⁺ in the mucosal solution with tetramethylammonium reduced aNa⁺ from 14.0 to 6.9 mM in 2 min (P < 0.001). Mucosal addition of the Na⁺-H⁺ exchange inhibitor amiloride (10⁻³ M) reduced aNa⁺ from 15.0 to 13.3 mM (P < 0.001), whereas bumetanide (10⁻⁵ and 10⁻⁴ M) had no effect. Na⁺ influx across the apical membrane was studied by treating the tissues with ouabain, bathing them in Na-free solutions, and suddenly replacing the mucosal solution with an Na-containing solution. When the mucosal solution was replaced with Na-Ringer's, aNa⁺ increased at ~11 mM/min. This increase was inhibited by 54% by amiloride (10⁻³ M, P < 0.001) and was unaffected by bumetanide (10⁻⁵ M). Amiloride-inhibitable Na⁺ fluxes across the apical membrane were also induced by the imposition of pH gradients. Na⁺ influx was also examined in tissues that had not been treated with ouabain. Under control conditions, ⁸⁶Na⁺ influx from the mucosal solution into the epithelium was linear over the first 60 s and was inhibited by 40% by amiloride (10⁻³ M, P < 0.001) and by 19% by bumetanide (10⁻⁵ M, P < 0.025). We conclude that Na⁺-H⁺ exchange is a major pathway for Na⁺ entry in Necturus gallbladder, which accounts for at least half of apical Na⁺ influx both under transporting conditions and during exposure to ouabain. Bumetanide-inhibitable Na⁺ entry mechanisms may account for only a smaller fraction of Na⁺ influx under transporting conditions, and cannot explain influx in ouabain-treated tissues. These results support the hypothesis that NaCl entry results primarily from the operation of parallel Na⁺-H⁺ and Cl⁻-HCO₃⁻ exchangers, and not from a bumetanide-inhibitable NaCl cotransporter.

INTRODUCTION
Leaky epithelia such as gallbladder, renal proximal tubule, and intestine accomplish transepithelial transport of NaCl by a neutral process. (For reviews see Diamond, 1968; Frizzell et al., 1979a; Warnock and Eveloff, 1982.) Numerous studies have demonstrated an interdependence of net transepithelial Na⁺ and...
Cl⁻ fluxes (Quay and Armstrong, 1969; Nellans et al., 1973; Frizzell et al., 1975; Cremaschi and Hénin, 1975), and an Na⁺ requirement for the maintenance of intracellular chloride activity (aCl) at a level greater than that predicted from equilibrium distribution (Duffey et al., 1978; Spring and Kimura, 1978; Reuss and Grady, 1979; Garcia-Diaz and Armstrong, 1980; Oberleithner et al., 1982). These results have been interpreted to imply the existence of direct coupling of Na⁺ and Cl⁻ fluxes through a ternary-complex NaCl cotransporter at the apical membrane (Frizzell et al., 1979). However, the possibility of neutral, coupled NaCl transport resulting from the simultaneous operation of Na⁺-H⁺ and Cl⁻-OH⁻ exchangers has been suggested (Turnberg et al., 1970; Liedtke and Hopfer, 1977) and has recently been the subject of considerable investigation (Petersen et al., 1981; Warnock and Yee, 1981a; Liedtke and Hopfer, 1982a, b). Distinguishing between these two mechanisms is difficult because many of their predictions are the same.

The conclusion that coupled NaCl entry results from a single cotransporter is based in part on the observation that the process is inhibited by furosemide or bumetanide (Eveloff et al., 1978; Ericson and Spring, 1982). Although these drugs have been demonstrated to inhibit many coupled ion transport processes, their specificity has not been established.

The case for parallel ion exchangers is also inconclusive and is based on the demonstration of both Na⁺-H⁺ and Cl⁻-OH⁻ exchange processes in apical membrane vesicles from leaky epithelia (Murcray et al., 1976; Kinsella and Aronson, 1980; Warnock and Yee, 1981a; Liedtke and Hopfer, 1982a, b). In addition, attempts to demonstrate the existence of a ternary-complex NaCl cotransporter have been unsuccessful (Liedtke and Hopfer, 1982a).

Our previous studies (Weinman and Reuss, 1982a) used extracellular and intracellular pH measurements to demonstrate the existence of Na⁺-H⁺ exchange at the apical membrane of Necturus gallbladder. This Na⁺-H⁺ exchange process occurs continuously under control conditions, but its magnitude could only be estimated crudely from the extracellular pH measurements. In the present study, we evaluate the role of Na⁺-H⁺ exchange in transapical Na⁺ transport by measurements of intracellular sodium activity (aNa) and unidirectional Na⁺ uptake in Necturus gallbladder. The results demonstrate that in ouabain-treated tissues, at least 50% of apical Na⁺ entry results from amiloride-inhibitable Na⁺-H⁺ exchange, and that under control conditions, 40% of the unidirectional Na⁺ uptake is amiloride inhibitable. Apical membrane Na⁺-H⁺ exchange is therefore a major pathway for transepithelial Na⁺ transport. Preliminary results of these studies have been reported (Weinman and Reuss, 1982b, 1983).

MATERIALS AND METHODS

Mud puppies (Necturus maculosus) were purchased from Nasco Biologicals (Ft. Atkinson, WI), kept in aquaria at ~10°C, and fed live fish. Gallbladders were removed, mounted mucosal side up, and continuously perfused on both sides in a modified Ussing chamber as previously described (Reuss and Finn, 1975, 1977). Na-Ringer's solution had the following composition (mM): 109.2 NaCl, 2.5 KCl, 1.0 CaCl₂, 1.0 Hepes. TMA-Ringer's contained 109.2 mM tetramethylammonium (TMA) Cl instead of NaCl. These solutions
were titrated with KOH and equilibrated with room air to have a final pH of 7.7. 
HCO₃⁻Ringer's was made by replacing 10 mM NaCl with 10 mM NaHCO₃ and omitting 
the Hepes. This solution was equilibrated with 1% CO₂-99% air and had a final pH of 
7.6. Amiloride was a generous gift of Merck, Sharp & Dohme, West Point, PA; bumetanide 
was a generous gift of Hoffman-La Roche, Somerville, NJ. A stock solution (10⁻⁵ M) was 
prepared in Na-Ringer's titrated to pH 9. ²²NaCl and [³H]mannitol were purchased from 
New England Nuclear, Boston, MA.

**Electrical Potential Measurements**

Transepithelial (Vₑₑ), apical membrane (Vₐₐ), and basolateral membrane (Vₑₑ) potentials 
were measured as described previously (Reuss and Finn, 1975, 1977). The serosal 
reference was an Ag-AgCl electrode connected to the solution via an Na-Ringer’s agar 
bridge. The mucosal solution potential was measured with a calomel electrode connected 
to the mucosal solution by a flowing, saturated KCl bridge. Vₑₑ was referred to the serosal 
side; Vₑₑ and Vₐₐ were referred to the respective bathing solutions. Transepithelial current 
pulses were passed via two Ag-AgCl electrodes connected to the respective solutions by 
agar bridges.

Micropipettes were pulled from 1-mm-OD inner fiberglass capillaries (Hilgenberg, 
Malsfeld, Federal Republic of Germany) on a horizontal electrode puller (Narishige, 
Japan). The pipettes were filled with either 3 M or 0.5 M KCl and had resistances of 15–
40 MΩ when filled with 3 M KCl and immersed in Na-Ringer’s. Cells were observed with 
an MS inverted microscope (Nikon, Inc., Garden City, NY) and impaled with either a 
motorized remote control micromanipulator (Stoelting, Chicago, IL) or a 3-D hydraulic 
microdrive (Narishige). Impalements with conventional microelectrodes were accepted 
when (a) the potential change upon impalement was abrupt and monotonic, (b) the cell 
potential was stable for at least 2 min, and (c) simultaneous impalement with a second, 
usually Na-selective, microelectrode revealed that both impaled cells had the same apparent 
ratio of membrane resistances (see Results).

**Intracellular Na⁺ Activity Measurements**

Intracellular Na-selective microelectrodes were constructed as described previously (Reuss 
et al., 1983). Micropipettes were pulled similarly to those used for conventional micro-
electrodes; when filled with 3 M KCl and immersed in Na-Ringer’s, they had resistances of 
10–30 MΩ. Pipettes were dried at 160°C and exposed to vapor of hexamethyldisilazane 
(Sigma Chemical Co., St. Louis, MO) for ~1 h. After cooling, the tips were filled with Na⁺ 
resin (0.2 µl). An inner pipette filled with Na-Ringer’s was then inserted into the 
back of the electrode to make contact with the resin within 100 µm of the tip. The 
electrode was backfilled with Na-Ringer’s and an Ag-AgCl wire was inserted and sealed 
in place with wax.

The Na⁺ resin used was that described by Steiner et al. (1979), and consisted of a 10% 
wt/wt solution of Na-ligand I (Fluka Chemical Co., Hauppauge, NY) in o-n-octyl oxynitro-
benzene (Alfa Products, Danvers, MA) with 0.5% Na-tetraphenylborate (Fluka Chemical 
Co.) added. Potential measurements with these electrodes were made with an FD223 
ultra-high impedance electrometer (W-P Instruments, Inc., New Haven, CT).

Slope and selectivity (Na⁺ over K⁺) for these electrodes were ~52–55 and 30–100 mV/ 
decade, respectively. It was noted that the slopes of the electrodes in pure KCl and NaCl 
solutions were frequently different. Electrodes were therefore calibrated in six solutions 
containing 120 mM KCl and various concentrations of NaCl ranging from 2 to 50 mM, 
in a method similar to that described by Armstrong and Garcia-Diaz (1980). Intracellular
Na⁺ activity was measured by impaling two cells simultaneously with a KCl-filled micro-electrode and an Na-selective micro-electrode, respectively. The potential measured by the conventional electrode (V₀), that measured by the Na-selective electrode (V₉Na), and the difference (V₉Na - V₀) were displayed on digital panel meters and a strip chart recorder (Gould, Inc., Cleveland, OH). Intracellular Na⁺ activity was determined from V₉Na - V₀ by interpolation onto the calibration curve for that particular electrode.

The use of two simultaneous impalements rather than separate single impalements at different times allowed us to have a continuous record of aNa⁺. In addition, it allowed the use of more stringent criteria to validate the impalements. These criteria have been described previously (Reuss and Weinman, 1979; Weinman and Reuss, 1982a) and involve a comparison of the voltage deflections measured by each electrode when the cell potential was changed by either transepithelial current pulses or mucosal solution ionic composition changes. An example of these criteria is presented in Fig. 1 (see Results).

**Intracellular Na⁺ Activity Measurements in Ouabain-treated Tissues**

In some experiments, gallbladders were treated with ouabain (10⁻⁶ M) on the serosal side for at least 45 min and aNa⁺ was measured during exposure to TMA-Ringer's on either the mucosal side only or on both sides. The influx of Na⁺ across the apical membrane was assessed by suddenly replacing the mucosal TMA-Ringer's with Na-Ringer's and recording the resulting changes in aNa⁺. In some tissues the Na⁺ influx rate, when the tissue was re-exposed to control Na-Ringer's, either declined or increased as a function of time. For this reason, influx rates in the presence of amiloride or bumetanide were always compared with control ones measured both before and after the exposure to the drug. In two out of nine tissues, the control influx rate changed by a factor of >4 from beginning to end of ouabain exposure. Results from these tissues were discarded.

**Unidirectional ²²Na⁺ Influx**

The flux of ²²Na⁺ from the mucosal solution into the epithelium (Jₑ) was measured in an apparatus similar to that described by others (Schultz et al., 1967; Frizzell et al., 1975). Four gallbladders were mounted on a plexiglass base, serosal side down, resting on filter paper wetted with Na-Ringer's. The mucosal surface was then isolated by placement of an upper chamber with cylindrical wells on top of the tissues. The seals were made by rubber O-rings and the exposed mucosal surfaces each had an area of 0.42 cm². The mucosal solution (200 µl) was gently stirred by bubbling with air. The bathing solution could be removed by suction and added by pipetting directly into the top of the chamber. Gallbladders mounted in this chamber were exposed on the mucosal side to Na-Ringer's for 20-30 min. After this preincubation, the mucosal solution was removed by suction and replaced by Na-Ringer's containing ²²NaCl (~15 µCi/ml) and [²³H]mannitol (~10 µCi/ml). After a timed interval (15-90 s), the tracer solution was removed by suction and the tissue was rinsed by washing for 2 s with 5 ml of isotonic sucrose solution at ~5°C. The gallbladder was then punched out, blotted gently, and transferred to a scintillation vial. Gallbladders were digested by incubation at 65°C for 1 h with 200 µl HClO₃ (60%) and 20 µl H₂O₂ (30%) (Mahin and Lofberg, 1966). After digestion and cooling, 10 ml Budget solve (Research Products International, Mount Prospect, IL) was added, and samples were counted in a Tri Carb scintillation counter (Packard Instrument Co., Inc., Downers Grove, IL) for ³H and ²²Na. ³H counts were corrected for the fraction of the ²²Na spectrum that counted in the ³H window (2.10% of the total ²²Na cpm). The ³H counting efficiency in the ²²Na window was zero. ²²Na⁺ influx was corrected for the ²²Na⁺ present in extracellular fluid contamination as estimated by the distribution of [²³H]mannitol. This was generally <5% of the total ²²Na⁺ counts. Uptake was shown to be linear for 60 s (see Results), and
uptake rates were determined from 45-s exposures. When the effects of amiloride or bumetanide on $J_{\text{Na}^+}$ were tested, the tissues were preincubated as described and exposed to drug-containing solution for 1 min prior to exposure to the drug-containing radioactive tracer solution.

**Statistics**

Results are presented as means ± SE. When specified, comparisons were made by conventional paired data analysis. Otherwise, comparisons were made by Student's $t$ test; a value of $P < 0.05$ was considered significant.

**RESULTS**

**Validation of Impalements**

Intracellular Na\(^+\) activity ($a_{\text{Na}^+}$) was determined by simultaneous impalement with two electrodes, a KCl-filled microelectrode and a liquid-membrane Na-selective microelectrode. The criteria used to validate impalements were those described previously (Reuss and Weinman, 1979; Weinman and Reuss, 1982a). The most rigorous tests consisted of demonstrating equal changes of membrane potentials of the two impaled cells both during transepithelial current pulses and during ionic substitutions of the mucosal solution. Since mucosal Na\(^+\) removal leads to an immediate change in $a_{\text{Na}^+}$, however, the depolarization produced by K\(^+\) for Na\(^+\) substitution could not be used for validation. As demonstrated in Fig. 1, mucosal Na\(^+\) replacement with TMA\(^+\) leads to a fall of $a_{\text{Na}^+}$ and a new steady state is reached in ~2 min. If at this time TMA\(^+\) is replaced with K\(^+\), very large changes in apical and basolateral membrane potentials occur that are identical in both impaled cells. This observation makes it very unlikely that the recorded values of $a_{\text{Na}^+}$ are in error because of nonspecific impalement damage of the apical membrane. All impalements in this study were validated by passage of transepithelial current pulses (Weinman and Reuss, 1982a) and only occasionally was the selectivity criterion applied.

Approximately one-third of the simultaneous conventional and Na-selective impalements that satisfied the criteria of abruptness and stability produced different basolateral membrane voltage deflections across the two impaled cells. These impalements were discarded.

**Intracellular Na\(^+\) Activity: Effects of Na\(^+\) Removal, Amiloride, and Bumetanide**

When *Necturus* gallbladder was bathed on both sides with Na-Ringer’s (1 mM Hepes), $a_{\text{Na}^+}$ was 14.1 ± 1.9 mM ($n = 52$ tissues). When tissues were bathed on both sides with 10 mM HCO\(_3\)-Ringer’s, $a_{\text{Na}^+}$ was 15.6 ± 1.2 mM ($n = 10$ tissues). In order to evaluate the mechanisms responsible for the maintenance of steady state $a_{\text{Na}^+}$, the effects of mucosal Na\(^+\) removal or mucosal addition of amiloride ($10^{-5}$ M) or bumetanide ($10^{-5}$ M) were studied. As illustrated in Fig. 2 and summarized in Table I, Na\(^+\) replacement with TMA\(^+\) reduced $a_{\text{Na}^+}$ by ~50% in 2 min. Amiloride, after 2 min, caused a 12% reduction, which was statistically significant, and bumetanide caused no measurable change. Similar results were obtained with amiloride and bumetanide in 10 mM HCO\(_3\)-Ringer’s (Table I). The initial rates of change of $a_{\text{Na}^+}$ in Na-Ringer’s, calculated over the first 20 s,
were 10.8 ± 1.6 mM/min after Na⁺ removal (n = 20) and 2.2 ± 0.4 mM/min after amiloride addition (n = 20).

Changes in \( a_{\text{Na}} \) do not provide quantitative information about the inhibition of the Na⁺ influx step by these experimental perturbations. In the case of complete Na⁺ substitution with TMA⁺, all apical Na⁺ entry has certainly stopped, but the decline of \( a_{\text{Na}} \) may be partly due to Na⁺ exit across the apical membrane. In the case of amiloride, the small magnitude of the change may in part reflect a changing basolateral Na⁺ extrusion rate or cell shrinkage, which would tend to blunt the change of \( a_{\text{Na}} \) (Spring and Ericson, 1982). In the case of bumetanide, however, the absence of any effect on \( a_{\text{Na}} \) would suggest that there has been no inhibition of Na⁺ influx. Even if cell volume decreased in parallel with a reduction of Na⁺ content, \( a_{\text{Na}} \) would be expected to decrease, since Na⁺ constitutes a small fraction of the intracellular cation pool.

Since Larson and Spring (1983) have reported that bumetanide causes a large, rapid reduction of \( a_{\text{Na}} \) at a higher concentration (10⁻⁴ M), we also tested this concentration in four experiments. No significant changes in \( a_{\text{Na}} \) were observed (\( a_{\text{Na}} \) values were 11.3 ± 1.2, 12.3 ± 1.1, and 11.7 ± 1.0 mM before, during [3 min], and after exposure to bumetanide, respectively).
Effect of Ouabain on $a_{Na}^+$

Since changes in the rate of $Na^+$ extrusion by the basolateral $Na^+-K^+$ ATPase might tend to minimize changes in $a_{Na}^+$ produced by inhibition of $Na^+$ entry, we chose to study the $Na^+$ entry step after inhibiting the basolateral pump with ouabain. After exposure to ouabain, the cells became somewhat more difficult to impale, but valid simultaneous impalements could be obtained.

The gallbladder was bathed in $Na$-Ringer's on both sides, and ouabain ($10^{-4}$ M) was added to the serosal bathing solution. After a 5-10-min lag, $a_{Na}^+$ increased roughly linearly at a rate of 0.8 mM/min (Fig. 3). This increase in $a_{Na}^+$ reduces the driving force for net $Na^+$ entry across the apical membrane and therefore makes it difficult to study this process. Hence, we reduced $a_{Na}^+$ in ouabain-treated tissues by complete replacement of $Na^+$ with $TMA^+$, either bilaterally or on the mucosal side only. When tissues were first exposed to

![Graph showing the effect of ouabain on $a_{Na}^+$.](image)

**Figure 2.** Effects of $Na$ removal, amiloride, and bumetanide on $a_{Na}^+$. Potentials were measured and transepithelial current pulses were passed as in Fig. 1. All records start with both microelectrodes in cells. At the times indicated by the bars, mucosal $Na$-Ringer's was replaced with $TMA$-Ringer's, $Na$-Ringer's plus $10^{-5}$ M amiloride, or $Na$-Ringer's plus $10^{-5}$ M bumetanide.

ouabain in Na-containing solutions and then $Na^+$ was replaced on the mucosal side only, $a_{Na}^+$ could be lowered to $\sim 20$ mM. If, however, $Na^+$ was replaced by $TMA^+$ on both sides of the epithelium, $a_{Na}^+$ could be consistently lowered to $\sim 5$ mM. The need to remove $Na^+$ from both sides in order to lower $a_{Na}^+$ could reflect either a component of $Na^+$ entry across the basolateral membrane or a serosa-to-mucosa paracellular $Na^+$ leak followed by apical membrane $Na^+$ entry.

$Na^+$ Influx in Ouabain-treated Tissues: Effects of Amiloride and Bumetanide

Tissues were treated with ouabain for at least 45 min while $a_{Na}^+$ was kept low by exposure to $TMA$-Ringer's on either the mucosal side only or on both sides of the tissue. After a steady value of $a_{Na}^+$ was measured by simultaneous impalements, the mucosal solution was suddenly changed from $TMA$-Ringer's to $Na$-Ringer's containing either no drug (control), amiloride ($10^{-5}$ M), or bumetanide ($10^{-5}$ M). The increases in $a_{Na}^+$ after 1 min were compared for these
three conditions. As illustrated in Fig. 4 and summarized in Table II, upon re-exposure of the mucosal side of the tissue to Na-Ringer's, Na⁺ enters the cells and causes a rise in aNa of ~11 mM in the first minute. This increase is inhibited by ~50% by amiloride; it is unaffected by bumetanide.

Transepithelial and cell membrane potentials before and after re-exposure to Na⁺ under these conditions are presented in Tables III and IV. It is interesting to note that there is no significant difference between control, amiloride, or bumetanide. This suggests that the mechanism of Na⁺ entry across the apical membrane is electrically silent (see Discussion).

**Effect of External pH on aNa**

The observation that ~50% of Na⁺ entry in ouabain-treated gallbladders can be inhibited by 1 mM amiloride suggests that Na⁺-H⁺ exchange accounts for a substantial fraction of entry. In principle, however, inhibition of Na⁺ entry by amiloride does not prove that it results from Na⁺-H⁺ exchange. As a result of

| Table I |

| Effect of Mucosal Solution Replacement on aNa, |
|---|
| Control solution (before) | Experimental solution | Control solution (after) | n | P |
| Heps-buffered | TMA-Ringer's | 14.0±1.2 | 6.9±0.6 | 15.8±1.2 | 21 | <0.001 |
| Amiloride (10⁻⁵ M) | 15.0±1.8 | 13.3±1.6 | 15.9±1.8 | 21 | <0.001 |
| Bumetanide (10⁻³ M) | 14.4±2.7 | 14.6±2.7 | 14.7±3.1 | 13 | NS |
| HCO₃-buffered | Amiloride (10⁻⁵ M) | 16.1±1.4 | 15.0±1.2 | 16.1±1.2 | 9 | <0.025 |
| Bumetanide (10⁻³ M) | 13.5±1.3 | 13.6±1.2 | 14.2±1.5 | 8 | NS |

Values are intracellular Na⁺ activities (in millimolar) measured in Na- or HCO₃-Ringer’s before, 2 min after solution change, and after returning to the control solution. Drugs were added to the appropriate Ringer’s solution at the indicated concentrations.

inhibiting Na⁺-H⁺ exchange, amiloride decreases intracellular pH in Necturus gallbladder (Weinman and Reuss, 1982a). Conceivably, the lower rate of Na⁺ entry in amiloride-treated tissues could result from a difference in intracellular pH after re-exposure to Na⁺. Even if the only effect of amiloride were to block Na⁺-H⁺ exchange, the intracellular acidification thus caused might inhibit another pathway for Na⁺ entry, such as NaCl cotransport. This possibility was ruled out by the experiments shown in Fig. 5 and Table V. In these experiments, the external Na⁺ concentration was adjusted to keep the Na⁺ activity ratio across the apical membrane near unity. If Na⁺-H⁺ exchange were the mechanism by which Na⁺ crosses the membrane, sizable net fluxes could then be induced by mucosal solution pH changes.

Tissues were exposed to TMA-Ringer’s with ouabain on the serosal side and TMA-Ringer’s containing 11 mM Na⁺ on the mucosal side. As shown in Fig. 5, changing the pH of the mucosal solution from 7.7 to 6.1 (HCl titration), at constant mucosal Na⁺ concentration, caused a fall in aNa, consistent with a net Na⁺ flux mediated by Na⁺-H⁺ exchange. When the experiment was repeated
with amiloride added to the pH 6.1 solution, $aNa_\text{i}$ changes were abolished. A subsequent mucosal acidification without amiloride demonstrated that the tissue retained the ability to respond to mucosal solution pH changes. The results of similar experiments in five tissues are presented in Table V. They demonstrate that pH gradients across the apical membrane induce large changes in $aNa_\text{i}$ in ouabain-treated Necturus gallbladder. These changes are abolished by amiloride.

**Unidirectional $^{22}Na^+ $ Influx**

The studies on ouabain-treated Necturus gallbladder have shown bumetanide-insensitive, amiloride-inhibitable $Na^+-H^+$ exchange to be a major mechanism of $Na^+$ flux across the apical membrane. However, it is possible that the magnitude of $Na^+-H^+$ exchange in tissues exposed to ouabain is different from that under control conditions. It was thus necessary to establish whether $Na^+-H^+$ exchange is also a sizable component of $Na^+$ entry in tissues in which the $Na^+$ pump is operative. This was accomplished by measurements of $^{22}Na^+$ influx from mucosal solution into the epithelium with methods similar to those used by others (Schultz et al., 1967; Frizzell et al., 1975).

The time course of $^{22}Na^+$ entry into the epithelium is shown in Fig. 6. During the first 60 s, influx is linear and can therefore be presumed to represent the entry process without any significant contribution of $^{22}Na^+$ backflux (Schultz et al., 1967).

The effects amiloride and bumetanide on $J_{\text{me}}$ are summarized in Table VI. $J_{\text{me}}$ was reduced by 40% by the presence of amiloride ($10^{-3}$ M) in the mucosal solution. This result is statistically significant ($P < 0.001$) and is in good quantitative agreement with the 54% reduction of $Na^+$ entry estimated from $aNa_\text{i}$ measurements in ouabain-treated tissues (Table II). Bumetanide ($10^{-5}$ M) reduced $J_{\text{me}}$ by 19%. This result is also statistically significant ($P < 0.05$) and may reflect a smaller component of $Na^+$ influx that is bumetanide inhibitable.

![Figure 3](image-url)
DISCUSSION

The importance of the results presented in this paper lies in how they relate to the mechanism of neutral NaCl entry in leaky epithelia. Na⁺ and Cl⁻ entry are linked in many systems and this observation has been interpreted to result from either a direct NaCl cotransporter (Frizzell et al., 1979a), or parallel Na⁺-H⁺, Cl⁻-OH⁻ exchangers (Turnberg et al., 1970; Liedtke and Hopfer, 1977).

The proposal of an apical membrane NaCl cotransporter is based on demonstrations of interdependence of Na⁺ and Cl⁻ fluxes and the effects of furosemide or bumetanide on cell volume changes and Na-dependent Cl⁻ fluxes or intracellular Cl⁻ activity (Quay and Armstrong, 1969; Frizzell et al., 1975, 1979b; Cremaschi and Hénin, 1975; Eveloff et al., 1978; Oberleithner et al., 1982; Ericson and Spring, 1982a). However, these arguments are inconclusive because flux interdependence can also be explained by parallel exchangers, and because the implied specificity of the loop diuretics is uncertain, particularly since

![Figure 4](image-url)

**Figure 4.** Changes in $\Delta Na$ upon sudden exposure to mucosal Na⁺ in a ouabain-treated gallbladder. These are five consecutive records obtained in a single tissue. The interval between traces is 1–4 min. The left-hand scale is $V_{Na} - V_{m}$, the right-hand one is $\Delta Na$. The tissue was perfused on both sides with TMA-Ringer's and, at the times indicated by the bars, the mucosal solution was replaced with either Na⁺-Ringer's without drugs (A, C, E), or with bumetanide (B), or amiloride (D). The deflections in the latter half of traces B, C, and D were produced by transepithelial current pulses.
TABLE II
Intracellular Na⁺ Activity Changes in Ouabain-treated Gallbladders

| Control before Drug Control after n P |
|---------------------------------------|-------------------------------------|
| Amiloride (10⁻⁵ M) 11.3±1.6 5.2±0.8 11.4±2.7 7 <0.001 |
| Bumetanide (10⁻⁵ M) 10.6±1.8 10.1±1.5 10.5±1.6 7 NS |

Values are the change in aNa⁺ (in millimolar) measured 1 min after a rapid change of the mucosal solution from TMA-Ringer’s to Na-Ringer’s. Each line represents the magnitude of this change measured in each tissue when the Na-Ringer’s contained either no drug (controls), amiloride, or bumetanide. n = number of tissues. P compares columns 1 and 2 and was determined by the paired t test.

furosemide and bumetanide inhibit purely anionic exchange processes (Brazy and Gunn, 1976; Cousin and Motais, 1976; Aronson and Seifter, 1983). Furthermore, a kinetic analysis of Cl⁻-Cl⁻ exchange at equilibrium, in intestinal luminal membrane vesicles, revealed no dependence on Na⁺, which suggests the absence of a ternary-complex NaCl cotransporter (Liedtke and Hopfer, 1982a).

Further, in two preliminary studies in renal brush-border vesicles, the existence of NaCl cotransport could not be demonstrated (Seifter et al., 1980; Warnock and Yee, 1981b).

Both Na⁺-H⁺ and Cl⁻-OH⁻ exchangers have been identified in apical membrane vesicles (Murer et al., 1976; Kinsella and Aronson, 1980; Warnock and Yee, 1981b; Liedtke and Hopfer, 1982a, b), but their existence does not prove that they are present in the apical membrane of intact cells or permit a quantitative assessment of their role in fluid transport.

Previous studies (Weinman and Reuss, 1982a) have demonstrated conclusively the existence of Na⁺-H⁺ exchange at the apical membrane of Necturus gallbladder epithelial cells under control in vitro conditions. This process was also shown to participate in the regulation of intracellular pH, but its contribution to transepithelial Na⁺ transport could not be established quantitatively. The results of this study demonstrate that Na⁺-H⁺ exchange accounts for a substantial fraction of apical Na⁺ entry, and that NaCl cotransport (estimated from the bumetanide-sensitive Na⁺ entry) is quantitatively less important. In ouabain-treated tissues, aNa⁺ increases resulting from imposed transapical membrane Na⁺ gradients are inhibited by ~50% by amiloride, and large amiloride-inhibitable aNa⁺ changes are generated by imposition of pH gradients across the apical membrane. In control tissues not treated with ouabain, 40% of unidirectional apical ²²Na uptake is inhibited by amiloride. In contrast, bumetanide had no effect on aNa⁺ changes.
The changes in transepithelial and cell membrane potentials accompanying Na⁺ entry in ouabain-treated tissues are shown in Table IV. Values are the changes in potential (in millivolts) produced by replacement of mucosal TMA-Ringer's by Na-Ringer's with or without the appropriate drug. They were measured 1 min after the solution change. n = number of tissues. Neither drug caused significant changes in $\Delta V_m$, $\Delta V_m$, or $\Delta V_n$.

Na⁺ Entry Results Predominantly from Na⁺-H⁺ Exchange

Measurements of $\Delta Na_i$ changes produced by both transapical Na⁺ and pH gradients have demonstrated that Na⁺ entry in ouabain-treated tissues results largely from Na⁺-H⁺ exchange. If the cells swell upon addition of Na⁺ to the mucosal solution, then the magnitude of the observed $\Delta Na_i$ changes would underestimate the actual Na⁺ influx. Although only 54% of the $\Delta Na_i$ change was inhibited by amiloride, the fraction of entry resulting from Na⁺-H⁺ exchange could be even greater if the kinetic constants of amiloride inhibition of Na⁺-H⁺ exchange in Necturus gallbladder are similar to those determined in renal brush-border vesicles (Kinsella and Aronson, 1981). In addition, the lack of effect of bumetanide at $10^{-5}$ M, i.e., a concentration much higher than that required to block cotransport in other systems (see Schlatter et al., 1983), indicates that

**TABLE IV**

**Changes in Transepithelial and Cell Membrane Potentials Accompanying Na⁺ Entry in Ouabain-treated Tissues**

|                | $\Delta V_m$ | $\Delta V_m$ | $\Delta V_m$ | n  |
|----------------|--------------|--------------|--------------|----|
| Control        | -35.2±2.0    | +18.3±3.1    | -17.3±2.3    | 8  |
| Amiloride (10⁻³ M) | -34.7±2.4    | +23.3±2.6    | -11.4±2.3    | 8  |
| Bumetanide (10⁻⁵ M) | -35.2±2.2    | +17.7±2.7    | -17.6±2.2    | 7  |

Values are the changes in potential (in millivolts) produced by replacement of mucosal TMA-Ringer’s by Na-Ringer’s with or without the appropriate drug. They were measured 1 min after the solution change. n = number of tissues. Neither drug caused significant changes in $\Delta V_m$, $\Delta V_m$, or $\Delta V_m$.

in ouabain-treated tissues and caused a smaller inhibition (19%) of unidirectional $^{22}$Na uptake.

**FIGURE 5.** Effect of mucosal solution pH changes on $\Delta Na_i$ in an ouabain-treated gallbladder. Simultaneous intracellular recordings with Na-selective and KCl-filled microelectrodes are shown as in Figs. 1, 2, and 4. The mucosal solution Na⁺ concentration was kept constant at 11 mM throughout. The serosal solution was TMA-Ringer’s. At the times indicated, the mucosal solution pH was changed from 7.7 to 6.1 in the presence or absence of amiloride (10⁻³ M).
TABLE V
Effect of Mucosal Solution pH on $aNa^+$ at Constant Mucosal Na+ Concentration

| Mucosal solution pH | $aNa^+$ (millimolar) | n  | P     |
|---------------------|----------------------|----|-------|
| 7.7                 | 16.3±2.7             | 8.5±1.7 | 17.7±3.5 | 5  | <0.01 |
| 6.1                 | 16.0±2.0             | 15.4±2.0 | 18.0±3.5 | 5  | NS    |

Values are intracellular Na+ activities (in millimolar) measured in tissues bathed by TMA-Ringer's on the serosal side and 11 mM Na+, 98 mM TMA+-Ringer's on the mucosal side. The first column is $aNa_+$ at mucosal solution pH 7.7. The second column is $aNa_+$ 4-5 min after mucosal solution pH change to 6.1. The third column is $aNa_+$ 4-5 min after a return to mucosal pH 7.7. In the experiments summarized in the second row, amiloride was added only to the pH 6.1 solution. n = number of tissues. P compares columns 1 and 2 and was determined by the paired t test.

under the conditions of this study, bumetanide-inhibitable ion transport processes do not play a major role in apical membrane Na+ entry.

The Na+ entry process observed in this study is also not measurably electrogenic, and therefore not electrodifusional. $aNa_+$ increased at a rate of 11 mM/min (Table II) when Na+ was suddenly added to the mucosal bathing solution. Assuming no cell volume changes, a Na+ transport mechanism carrying one positive charge per Na+ would result in a current of ~73 μA/cm² across the apical membrane. If the cells swell during Na+ entry, the hypothetical Na+ current would be even greater. Estimates of apical membrane resistance in this tissue range from 1,220 (Suzuki et al., 1982) to 4,470 Ωcm² (Frömter, 1972). The expected change in apical membrane potential resulting from this Na+ current would therefore be from 90 to 325 mV, but the observed change is only 18 mV (Table IV). Two considerations reveal that this depolarization does not

![Graph](image-url)
result from an inward Na+ current across the apical membrane. First, mucosal substitution of TMA-Ringer's with Na-Ringer's results in a large paracellular bionic potential, as reflected by a mucosal-negative $V_{m}$ change of ~35 mV. This by itself results in a depolarization of $V_{m}$ that could account for most of the observed changes (Table IV). Second, although amiloride reduced Na+ entry by ~50%, it had no significant effect on the membrane potential changes produced by the substitution of TMA-Ringer's with Na-Ringer's. We therefore conclude that the Na+ entry process illustrated in Fig. 4 is electrically silent.

**Apical Na+ Entry in the Absence of Ouabain**

Although Na+-H+ exchange is a major component of Na+ entry in ouabain-treated tissues, it is possible that the conditions of the experiment enhanced the rate of Na+-H+ exchange above that in normal transporting tissues. To test whether Na+-H+ exchange accounts for significant Na+ entry under control conditions, we measured unidirectional $^{22}$Na+ uptake from the mucosal solution into the epithelium.

As summarized in Table VI, amiloride and bumetanide reduced uptake by 40 and 19%, respectively. The effect of amiloride cannot be explained by an indirect effect on pH, because we have previously shown that intracellular acidification does not become apparent until ~2 min after exposure to the drug (see Fig. 9 of Weinman and Reuss, 1982a). We therefore conclude that in transporting gallbladders not treated with ouabain, Na+-H+ exchange still accounts for a substantial fraction of apical Na entry.

Unidirectional uptakes measured in this way are only estimates of net transepithelial Na+ uptake. The control rate of uptake, 3.3 μeq cm$^{-2}$ h$^{-1}$, is at least three times the rate of net transepithelial transport and therefore could include Na+-Na+ exchange and Na+ entry into the lateral intercellular spaces in excess of the extracellular volume marker, but the linearity of uptake (Fig. 6) argues that the latter fraction must be small. These problems make quantitative interpretation of these results difficult, but the large effect of amiloride in our study suggests that Na+-H+ exchange is a significant mechanism of Na+ entry even when the tissue has not been perturbed by exposure to Na-free solutions and/or ouabain. The smaller effect of bumetanide may indicate that there is a smaller component of Na+ influx mediated by a bumetanide-inhibitable process such as NaCl.

### Table VI

|          | $J_{m}$ (μeq cm$^{-2}$ h$^{-1}$) | n | P  |
|----------|--------------------------------|---|----|
| Na-Ringer's | 3.55±0.16                      | 15|    |
| Na-Ringer's + amiloride (10$^{-5}$ M) | 2.00±0.23                      | 9 | <0.001 |
| Na-Ringer's + bumetanide (10$^{-5}$ M) | 2.72±0.19                      | 10| <0.025 |

Values were determined from 45-s uptakes. n = number of tissues. P compares the influx rate in drug-containing solution with that in Na-Ringer's and was determined by the unpaired t test.
cotransport. However, the quantitative uncertainties of the $^{22}\text{Na}^+$ uptake experiments, the lack of any effect of bumetanide on $\text{Na}^+$ uptake as estimated from $a\text{Na}_i$ measurements, and the possibility of inhibition of other transport processes by bumetanide (vide supra), make this conclusion less certain.

*Intracellular Activity Changes in Tissues Not Treated with Ouabain*

The above results clearly implicate $\text{Na}^+-\text{H}^+$ exchange in the apical Na entry process. It therefore, at first, seems paradoxical thatamiloride addition produces only a small change in $a\text{Na}_i$ (Table I). The magnitude of the $a\text{Na}_i$ fall upon inhibition of entry is difficult to predict because it depends upon factors including changes of the rate of active basolateral $\text{Na}^+$ extrusion, the magnitude of $\text{Na}^+$ backleak through the basolateral membrane, and cell volume changes. After complete mucosal $\text{Na}^+$ removal, the initial rate of fall, 10.8 mM/min, may reflect apical membrane Na$^+$ exit as well as cessation of entry and persistent basolateral exit. For a cell height of 35 $\mu$m (Spring and Hope, 1979), ignoring cell volume changes, the calculated $a\text{Na}^+$ flux would correspond to an isotonic fluid transport rate of $\sim 22$ $\mu$l cm$^{-2}$ h$^{-1}$, i.e., a value much higher than that measured under isotonic conditions (Reuss et al., 1979; Persson and Spring, 1982).

Since pharmacological inhibition of $\text{Na}^+$ entry would not induce a backflux across the apical membrane, the rate of decline of $a\text{Na}_i$ would certainly be smaller. The initial rate of fall of $a\text{Na}_i$ after amiloride addition (2.2 mM/min) would correspond to a volume flux of $\sim 5$ $\mu$l cm$^{-2}$ h$^{-1}$, a value in good agreement with that measured recently in this tissue (Larson and Spring, 1983; Peterson and Reuss, 1983). The small magnitude of the $a\text{Na}_i$ change caused by amiloride could therefore be compatible with the blockage of a significant fraction of apical $\text{Na}^+$ entry. This result is similar to the observation of Eaton (1981) that although amiloride abolished the short-circuit current in rabbit urinary bladder, it only had a small effect on intracellular sodium activity.

*Comparison with Other Studies in Necturus Gallbladder*

The results obtained in this study do not agree with the conclusions drawn by Spring and colleagues (Ericson and Spring, 1982a, b; Larson and Spring, 1983), who measured cell volume in *Necturus* gallbladder epithelial cells. They concluded that volume regulation is the result of the operation of parallel $\text{Na}^+-\text{H}^+$ and $\text{Cl}^-\text{HCO}_3$ exchangers, and that $\text{NaCl}$ entry under control conditions is mediated by a $\text{NaCl}$ cotransporter. In addition, recent results from the same laboratory indicate large falls of both $a\text{Na}_i$ and $a\text{Cl}_i$ after addition of bumetanide to the mucosal medium (Larson and Spring, 1983).

The results reported in this paper disagree with the observation that $a\text{Na}_i$ falls after application of mucosal bumetanide. This difference cannot be explained by differences in Ringer's solution, since we obtained similar results using 10 mM $\text{HCO}_3$-Ringer's (Table I). In addition, the activity of our bumetanide was verified in isolated bullfrog cornea (reduction of short-circuit current) and in vivo rat (diuretic effect). Finally, artifactual impalements with our $\text{Na}^+$ electrodes are unlikely, given the strict adherence to our validation criteria.

An explanation of the difference between our results and those of Spring and co-workers is not apparent. One possibility is that $\text{NaCl}$ cotransport and double
exchange (Na$^+\cdot$H$^+$, Cl$^-\cdot$HCO$_3^-$) represent different transport modes of the same carriers. Perhaps as yet unidentified experimental conditions have selected one or another of these modes.

In conclusion, the results reported in this paper demonstrate that at least 50% of Na$^+$ entry across the apical membrane of Necturus gallbladder is mediated by neutral, amiloride-inhibitable Na$^+\cdot$H$^+$ exchange. A single NaCl cotransporter at the apical membrane cannot explain our observations. Whether or not Na$^+\cdot$H$^+$ exchange in parallel with Cl$^-\cdot$HCO$_3^-$ exchange explains the coupling of Na$^+$ and Cl$^-$ fluxes observed in gallbladder, and whether ternary-complex cotransport and double exchange may be different modes of operation of the same transporter, remain to be determined.

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