

# Independence of Apical Membrane Na<sup>+</sup> and Cl<sup>-</sup> Entry in *Necturus* Gallbladder Epithelium

LUIS REUSS

From the Department of Physiology and Biophysics, Washington University School of Medicine,  
St. Louis, Missouri 63110

**ABSTRACT** Transepithelial fluid transport ( $J_v$ ) and intracellular Na<sup>+</sup> and Cl<sup>-</sup> activities ( $a_{Na_i}$ ,  $a_{Cl_i}$ ) were measured in isolated *Necturus* gallbladders to establish the contribution of different proposed apical membrane entry mechanisms to transepithelial salt transport. In 10 mM HCO<sub>3</sub>-Ringer's,  $J_v$  was  $13.5 \pm 1.1 \mu\text{l}\cdot\text{cm}^{-2}\cdot\text{h}^{-1}$ , and was significantly reduced by a low bicarbonate medium and by addition of amiloride ( $10^{-3}$  M) or SITS ( $0.5 \times 10^{-3}$  M) to the mucosal bathing solution. Bumetanide ( $10^{-5}$  M) was ineffective. Bilateral Na<sup>+</sup> removal abolished  $J_v$ . The hypothesis of NaCl cotransport was rejected on the basis of the following results, all obtained during mucosal bathing solution changes: (a) during Na<sup>+</sup> removal,  $a_{Na_i}$  fell 4.3 times faster than  $a_{Cl_i}$ ; (b) during Cl<sup>-</sup> removal,  $a_{Cl_i}$  fell 7.5 times faster than  $a_{Na_i}$ ; (c) amiloride ( $10^{-3}$  M) reduced  $a_{Na_i}$  at a rate of  $2.4 \pm 0.3$  mM/min, whereas  $a_{Cl_i}$  was not changed; (d) bumetanide ( $10^{-5}$  M) had no significant effects on  $J_v$  or  $a_{Cl_i}$ . The hypothesis of Na-K-Cl cotransport was rejected for the same reasons; in addition, K<sup>+</sup> removal from the mucosal bathing solution (with concomitant Ba<sup>2+</sup> addition) did not alter  $a_{Na_i}$  or  $a_{Cl_i}$ . The average rate of NaCl entry under normal transporting conditions, estimated from  $J_v$ , assuming that the transported fluid is an isosmotic NaCl solution, was  $22.5 \text{ nmol}\cdot\text{cm}^{-2}\cdot\text{min}^{-1}$ . Upon sudden cessation of NaCl entry, assuming no cell volume changes,  $a_{Na_i}$  and  $a_{Cl_i}$  should fall at an average rate of 4.8 mM/min. To compare this rate with the rates of Na<sup>+</sup> and Cl<sup>-</sup> entry by ion exchange, the Na<sup>+</sup> or Cl<sup>-</sup> concentration in the mucosal bathing solution was reduced rapidly to levels such that electroneutral cation or anion exchange, respectively, should cease. The rate of Na<sup>+</sup> or Cl<sup>-</sup> entry before this maneuver was estimated from the initial rate of fall of the respective intracellular ionic activity upon the mucosal solution substitution.  $a_{Na_i}$  and  $a_{Cl_i}$  decreased at initial rates of  $3.7 \pm 0.4$  and  $5.9 \pm 0.8$  mM/min, respectively. The rate of fall of  $a_{Na_i}$  upon reduction of external [Na] was not affected by amiloride ( $10^{-3}$  M), and the rate of fall of  $a_{Cl_i}$  upon reduction of external [Cl] was unchanged by SITS ( $0.5 \times 10^{-3}$  M), which indicates that net cation or anion exchange was, in fact, abolished by the changes in Na<sup>+</sup> and Cl<sup>-</sup> gradients, respectively. I conclude that double exchange (Na<sup>+</sup>/H<sup>+</sup> and Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup>) is the predominant or sole mechanism of apical membrane NaCl entry in this epithelium.

## INTRODUCTION

The precise mechanism of NaCl entry across the apical cell membrane of epithelia that transport salt by an electroneutral process has been a subject of considerable controversy (Frizzell et al., 1979; Warnock and Eveloff, 1982; Spring and Ericson, 1982; Turnberg et al., 1970; Liedtke and Hopfer, 1977; Petersen et al., 1981; Weinman and Reuss, 1984; Reuss and Costantin, 1984). In *Necturus* gallbladder epithelium, electroneutral NaCl entry has been attributed to a ternary-complex carrier-mediated process (Ericson and Spring, 1982; Spring and Ericson, 1982*a*) and to the simultaneous, but independent, operation of  $\text{Na}^+/\text{H}^+$  and  $\text{Cl}^-/\text{HCO}_3^-$  exchangers (Baerentsen et al., 1983; Machen and Zeuthen, 1983; Weinman and Reuss, 1982, 1984; Reuss and Costantin, 1984). The results reported in the latter three studies have demonstrated that these exchangers operate in the apical membrane of *Necturus* gallbladder epithelial cells under control conditions. In particular, it was shown that in tissues pretreated with ouabain and incubated in a Na-free medium, addition of  $\text{Na}^+$  to the mucosal solution resulted in a rapid rise of intracellular  $\text{Na}^+$  activity, which was amiloride sensitive and bumetanide insensitive. In addition, in tissues not treated with ouabain,  $^{22}\text{Na}^+$  influx (mucosal solution into epithelium) was inhibited ~40% by amiloride and ~20% by bumetanide (Weinman and Reuss, 1984). In other experiments,  $\text{Cl}^-$  removal from the mucosal solution caused intracellular alkalization, which was partly inhibited by the disulfonic stilbene SITS. Furthermore, intracellular acidification or alkalization (obtained by exposure to permeant acid or base, respectively) resulted in large, rapid changes in intracellular  $\text{Cl}^-$  activity, in directions consistent with  $\text{Cl}^-/\text{HCO}_3^-$  exchange (Reuss and Costantin, 1984).

Hence, the results obtained from Na and Cl removal experiments and from the use of inhibitors (amiloride, disulfonic stilbenes) suggested that double exchange may be the principal, if not the only, mechanism of NaCl entry. However, it is conceivable that the ouabain treatment employed in some of the experiments referred to above resulted, directly or indirectly, in alterations of the modes or rates of ion transport at the apical membrane. The issue is complicated further by the possibility of apical membrane  $\text{Na}^+$  and/or  $\text{Cl}^-$  entry pathways other than those listed above, such as Na-K-Cl cotransport, as demonstrated for fish intestine (Musch et al., 1982) and suggested for *Necturus* gallbladder (Davis and Finn, 1983). In addition, the magnitude of electrodiffusional  $\text{Na}^+$  entry is unclear (Van Os and Slegers, 1975; Reuss and Finn, 1975*b*; Graf and Giebisch, 1979).

The purposes of the present experiments were: (a) to determine the degree of coupling between  $\text{Na}^+$  and  $\text{Cl}^-$  fluxes at the apical membrane; (b) to assess further the fractions of  $\text{Na}^+$  entry and  $\text{Cl}^-$  entry that can be attributed to  $\text{Na}^+/\text{H}^+$  and  $\text{Cl}^-/\text{HCO}_3^-$  exchange, respectively; (c) to ascertain the contribution of possible parallel pathways (NaCl cotransport, Na-K-Cl cotransport, and amiloride-sensitive conductive  $\text{Na}^+$  entry) to NaCl entry.

The main experimental technique employed to address these issues was the measurement of intracellular ionic activities ( $\text{Na}^+$  and  $\text{Cl}^-$ ) under conditions designed to inhibit, stop, or reverse the net flux of one or the other ion across

the apical membrane. Transport rates were estimated from the initial changes of intracellular activities and compared with the rates of fluid transport ( $J_v$ ), measured under similar conditions by a new technique with good time resolution.

The results indicate that the estimated rates of Na<sup>+</sup> and Cl<sup>-</sup> entry by double exchange account for the normal rates of isosmotic fluid transport; there is an amiloride-insensitive electrodiffusional Na<sup>+</sup> pathway, but its contribution to transepithelial transport is very small; the contributions of NaCl and Na-K-Cl cotransport to salt entry, if any, are unmeasurably small. A preliminary account of these results has been presented (Reuss, 1984).

#### MATERIALS AND METHODS

Mud puppies (*Necturus maculosus*) were purchased from Nasco Biologicals (Ft. Atkinson, WI) and kept as previously described (Weinman and Reuss, 1984). Gallbladders excised from animals anesthetized with Tricaine methanesulfonate were mounted in a modified Ussing chamber as previously described (Reuss and Finn, 1975a, 1977). The control bathing solution contained (in mM): 90 NaCl, 10 NaHCO<sub>3</sub>, 2.5 KCl, 1.8 CaCl<sub>2</sub>, 1.0 MgCl<sub>2</sub>, and 0.5 NaH<sub>2</sub>PO<sub>4</sub>. It was gassed with a 1% CO<sub>2</sub>/99% air mixture and had a pH of 7.6. Ionic substitutions of Na<sup>+</sup> with tetramethylammonium (TMA<sup>+</sup>) and Cl<sup>-</sup> with cyclamate were isomolar. Amiloride was a generous gift from Merck, Sharp & Dohme, West Point, PA; bumetanide was a generous gift from Hoffmann-LaRoche, Inc., Nutley, NJ; SITS (4-acetamido-4'-isothiocyano-stilbene-2,2'-disulfonic acid) was purchased from Pierce Chemical Co., Rockford, IL.

#### *Measurements of Electrical Potentials*

Transepithelial ( $V_m$ ) and cell membrane potentials (apical:  $V_{mc}$ ; basolateral:  $V_{cs}$ ) were measured in general as described previously (Reuss and Finn, 1975a). The serosal reference electrode was an Ag-AgCl pellet separated from the solution by a short Ringer's-agar bridge. The mucosal electrode was a calomel half-cell connected to the bathing solution by a flowing, saturated KCl bridge (Reuss and Costantin, 1984). To prevent K<sup>+</sup> concentration changes in the mucosal bathing medium, the rate of mucosal superfusion was kept high.  $V_m$  was referred to the serosal side;  $V_{mc}$  and  $V_{cs}$  were referred to the respective bathing solutions. Transepithelial current pulses were passed by means of two Ag-AgCl electrodes to measure transepithelial resistance and apparent ratio of cell membrane resistances, and to validate the impalements (Weinman and Reuss, 1982).

Intracellular microelectrodes were prepared from inner-fiber borosilicate glass, as described before. Membrane potential microelectrodes were filled with 3 or 0.5 M KCl. Microscopic observation of the tissue and impalements were performed as previously described (Weinman and Reuss, 1982, 1984; Reuss and Costantin, 1984).

#### *Measurements of Intracellular Na and Cl Activities*

Simultaneous impalements with conventional and ion-sensitive microelectrodes were employed. Na<sup>+</sup>- and Cl<sup>-</sup>-sensitive microelectrodes were constructed and calibrated as previously described (Reuss et al., 1983; Weinman and Reuss, 1984; Reuss and Costantin, 1984).

After the two impalements were validated (Weinman and Reuss, 1982), the mucosal bathing solution was rapidly changed, usually for 3 min, to one in which Na<sup>+</sup> or Cl<sup>-</sup> was partly or completely replaced, or to one containing amiloride, bumetanide, or SITS. At the end of this period, the tissue was exposed again to the control Ringer's solution. Since only the mucosal bathing solution was changed and the mucosal solution electrode was a

flowing, saturated KCl bridge, no liquid junction potential corrections were made. The activity coefficients of the solutions and intracellular ionic activities were calculated as described before (Reuss et al., 1983).

#### *Measurements of Fluid Transport Rate*

For measurements of fluid transport rate ( $J_v$ ,  $\mu\text{l}\cdot\text{cm}^{-2}\cdot\text{h}^{-1}$ ), the tissues were mounted, mucosal surface up, in the same chamber used for the intracellular microelectrode experiments. A thin layer ( $\sim 200\ \mu\text{m}$ ) of solution was placed on top of the tissue and covered with a thick layer of paraffin oil pre-equilibrated with water. A conventional microelectrode was advanced, at a  $30^\circ$  angle, from the oil into the mucosal solution by means of a hydraulic microdrive (MO-103; Narishige, Tokyo, Japan) or a piezoelectric micromanipulator (Inchworm, Burleigh Instruments, Inc., Fishers, NY). In either case, the position at which the microelectrode made contact with the solution could be electrically determined and read with a  $2\text{-}\mu\text{m}$  resolution. By withdrawal and advance of the microelectrode at  $\sim 1\text{-min}$  intervals, the position of the oil-Ringer's interface was determined. The change in height of the fluid layer ( $\Delta h$ ) as a function of time was determined from the change in the longitudinal advancement ( $\Delta a$ ) required to make contact with the Ringer's, from  $\Delta h = 0.5\ \Delta a$ , where 0.5 is the sine of the angle of the microelectrode axis with respect to the horizontal ( $30^\circ$ ). The fluid transport rate was determined from the slope of the linear regression of  $\Delta h$  as a function of time. Measurements were carried out for periods of at least 8 min, usually at 1-min intervals.

Inasmuch as the oil was not pre-equilibrated with  $\text{CO}_2$ , luminal solution alkalization would be expected from a net  $\text{CO}_2$  flux from the mucosal fluid layer into the oil. In three experiments, the pH of the solution was measured with a liquid membrane pH microelectrode during superfusion, after partial removal of the mucosal fluid and oil addition, and again during Ringer's superfusion. It was found that the pH rose in  $<1$  min by an average of 0.16 unit, but returned to a value within  $\pm 0.02$  unit of the control one in 3 min. At this time, the paraffin oil is probably equilibrated with the 1%  $\text{CO}_2$  supplied by the rapid superfusion of the serosal side. All measurements of  $J_v$  in  $\text{HCO}_3^-/\text{CO}_2$ -buffered Ringer's were started at least 3 min after covering the Ringer's with oil.

#### *Statistics*

Results are presented as means  $\pm$  SEM. Unless stated otherwise, statistical comparisons were made by conventional  $t$  tests on paired data. A value of  $P < 0.05$  was considered significant.

### RESULTS

#### *Fluid Absorption*

The rationale for developing the  $J_v$  measuring technique described in Materials and Methods was, first, to be able to make quantitative comparisons between the fluid transport rate and rates of change in intracellular activities under comparable conditions, and second, to improve the time resolution of the measurements. Gravimetric, horizontal capillary, and volume-marker techniques previously used in this tissue (Van Os and Slegers, 1975; Reuss et al., 1979; Bello-Reuss et al., 1981; Zeuthen, 1982; Larson and Spring, 1983; Petersen and Reuss, 1983) have a poor time resolution.

Typical measurements of  $J_v$  in two experimental conditions are shown in Fig. 1. In all experiments, as illustrated in the figure, linearity was excellent. Fig. 2

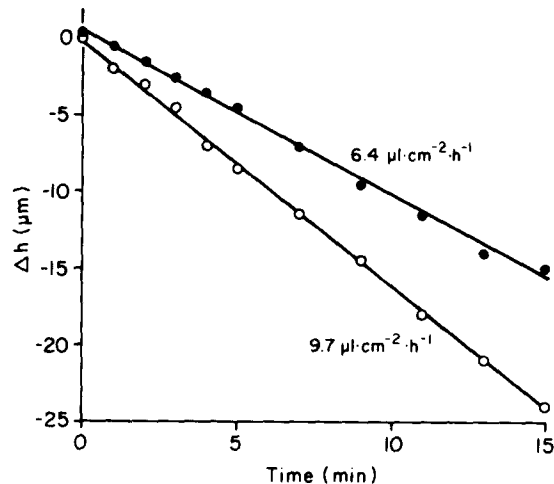


FIGURE 1. Determination of  $J_v$  in a single tissue, incubated in  $\text{HCO}_3$ -Ringer's on both sides (open symbols) and in HEPES-Ringer's (filled symbols). Data plotted are the changes in height of the oil-Ringer's interface (ordinate) as a function of time (abscissa). In both experiments, measurements were started 3 min after depositing the oil on the Ringer's layer.  $J_v$  values shown were calculated from the slopes of the regression lines obtained by a least-squares fit.

summarizes results obtained under control conditions ( $\text{HCO}_3$ -Ringer's) and in a variety of experimental situations. Bilateral isomolar replacement of  $\text{Na}^+$  with  $\text{TMA}^+$  abolished fluid transport. This result rules out a number of potential artifacts of the measuring technique.  $J_v$  in  $\text{HCO}_3$ -Ringer's, measured in a total of 12 tissues, was  $13.5 \pm 1.1 \mu\text{l}\cdot\text{cm}^{-2}\cdot\text{h}^{-1}$ . In paired experiments, it decreased significantly and reversibly in low bicarbonate bathing media ( $\Delta = 36\%$ ) and also when amiloride ( $10^{-3}$  M) or SITS ( $0.5 \times 10^{-3}$  M) was added to the mucosal solution ( $\Delta = 52$  and  $59\%$ , respectively). Bumetanide addition to the mucosal solution ( $10^{-5}$  M) did not alter  $J_v$  significantly.

Assuming that the absorbate is a 100 mM NaCl solution, the average rate of  $\text{Na}^+$  and  $\text{Cl}^-$  entry at the apical membrane in  $\text{HCO}_3$ -Ringer's is  $22.5 \text{ nmol}\cdot\text{cm}^{-2}\cdot\text{min}^{-1}$ . If  $\text{Na}^+$  or  $\text{Cl}^-$  entry ceased suddenly, assuming a cell height of  $35 \mu\text{m}$  (Spring and Hope, 1979) and neglecting changes in cell volume, the intracellular  $\text{Na}^+$  or  $\text{Cl}^-$  concentration should fall at an initial rate of 6.4 mM/min, which is equivalent to an initial rate of fall of intracellular activity of 4.8 mM/min.

#### *Rate of Na<sup>+</sup> Entry by Na<sup>+</sup>/H<sup>+</sup> Exchange*

Since  $\text{Na}^+/\text{H}^+$  exchange is an electroneutral process (Weinman and Reuss, 1982, 1984), the net  $\text{Na}^+$  flux across this pathway depends on the thermodynamic  $\text{Na}^+$  and  $\text{H}^+$  activities in the cell and the mucosal bathing solution, and is independent of the membrane potential. The four activities are known (Weinman and Reuss, 1982, 1984; Reuss and Costantin, 1984). Hence, it is possible to change experimentally one of them to a value calculated to result in cessation of  $\text{Na}^+$  entry via  $\text{Na}^+/\text{H}^+$  exchange. This was done by decreasing the  $\text{Na}^+$  concentration in the

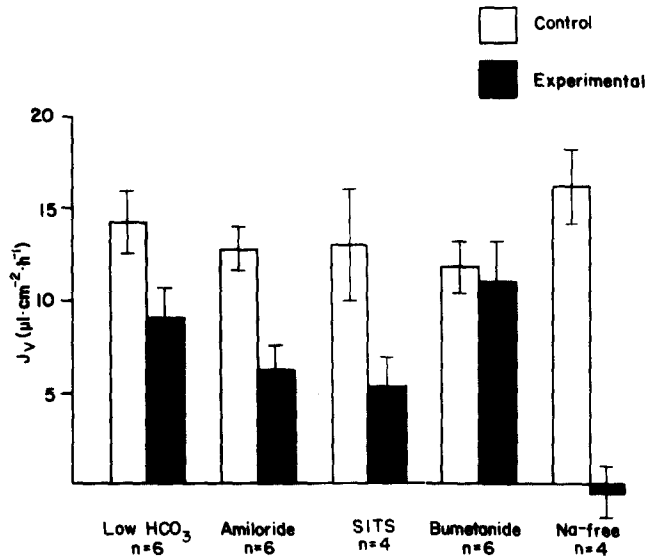


FIGURE 2. Effects of HEPES-Ringer's ( $[\text{HCO}_3^-] < 1 \text{ mM}$ ), mucosal addition of amiloride ( $10^{-3} \text{ M}$ ), SITS ( $0.5 \times 10^{-3} \text{ M}$ ), or bumetanide ( $10^{-5} \text{ M}$ ), and bilateral  $\text{Na}^+$  removal ( $\text{TMA}^+$  substitution) on  $J_v$ . Each pair of columns shows means  $\pm$  SEM of  $J_v$  values obtained in the same tissues under control conditions ( $\text{HCO}_3^-$ -Ringer's, no drug addition, light bars) and during exposure to the experimental solution indicated (dark bars). The effects of low  $\text{HCO}_3^-$ -Ringer's, amiloride, SITS, and  $\text{Na}^+$  removal were statistically significant ( $P < 0.05$  or better).

mucosal solution. If  $\text{Na}^+/\text{H}^+$  exchange is the only mechanism of  $\text{Na}^+$  entry, then the initial rate of fall of the intracellular  $\text{Na}^+$  content should be equal to the rate of extrusion across the basolateral membrane present immediately before the ionic substitution. From the extracellular and intracellular pH values in  $\text{HCO}_3^-$ -Ringer's (Reuss and Costantin, 1984) and the mean value of  $a\text{Na}_i$  ( $\sim 10 \text{ mM}$ , Table I), it can be shown that at a mucosal solution  $\text{Na}$  concentration of  $10 \text{ mM}$ , the driving force across the  $\text{Na}^+/\text{H}^+$  exchanger is approximately zero. However, since the extracellular  $\text{Cl}^-$  activity under these conditions is much greater than the intracellular one ( $72$  vs.  $20 \text{ mM}$ ), the driving force for  $\text{NaCl}$  cotransport still favors entry. In addition, net electrodiffusional  $\text{Na}^+$  entry would also occur because of the large, cell-negative, apical membrane voltage.

The effect of a reduction of mucosal  $[\text{Na}^+]$  from  $100$  to  $10 \text{ mM}$  is illustrated in Fig. 3. Concomitantly with the cell membrane potential changes produced by this maneuver, which are mainly caused by a  $\text{Na}^+/\text{TMA}^+$  paracellular biionic potential (Reuss and Finn, 1975b), a rapid change  $a\text{Na}_i$  is observed, which is reversed when the tissue is re-exposed to the control Ringer's solution. Table I (A) summarizes results from eight such experiments. The  $a\text{Na}_i$  fell initially at a rate of  $3.7 \pm 0.4 \text{ mM/min}$ . This measurement may underestimate the rate of  $\text{Na}^+$  entry, first, because if cell volume decreases, the change in  $a\text{Na}_i$  is minimized, and second, because if the  $\text{Na}^+$  pump is below saturation under control conditions (Graf and Giebisch, 1979), small falls in  $a\text{Na}_i$  can result in significant and rapid

TABLE I  
Effect of Reducing Mucosal Solution [Na] on  $a\text{Na}_i$

	$V_{ms}$	$V_{cs}$	$a\text{Na}_i$	$\Delta a\text{Na}_i/\Delta t$
	mV	mV	mM	mM/min
(A) Control	$-0.3 \pm 0.2$	$-69.0 \pm 1.6$	$11.0 \pm 0.3$	$-3.7 \pm 0.4$
10 mM Na	$27.0 \pm 2.8^*$	$-67.9 \pm 2.4$	$7.2 \pm 0.4^*$	$5.3 \pm 1.5$
Control	$-0.5 \pm 0.2$	$-69.9 \pm 2.7$	$10.5 \pm 0.5$	
(B) Control	$-0.2 \pm 0.3$	$-70.8 \pm 2.8$	$9.3 \pm 0.9$	$-3.8 \pm 0.8$
10 mM Na + amiloride	$23.0 \pm 3.9^*$	$-70.6 \pm 3.0$	$6.1 \pm 0.6^*$	$6.3 \pm 3.4$
Control	$-0.5 \pm 0.3$	$-71.4 \pm 2.6$	$8.6 \pm 0.6$	

Data shown are means  $\pm$  SEM obtained under control conditions, 3 min after replacing control Ringer's with a 10 mM Na medium (TMA substitution) and 3 min after returning to control Ringer's.  $V_{ms}$ , transepithelial potential;  $V_{cs}$ , basolateral membrane potential;  $a\text{Na}_i$ , intracellular  $\text{Na}^+$  activity. In B, 1 mM amiloride was added to the 10 mM Na solution. A:  $n = 8$ ; B:  $n = 5$ . The last column depicts the initial rates of change of  $a\text{Na}_i$  during the two solution substitutions (Ringer's to 10 mM Na and 10 mM Na to Ringer's). \* Significantly different from control before or after ( $P < 0.005$ ).

falls in pump rate, therefore tending also to minimize the fall in  $a\text{Na}_i$ . To reduce the error generated by these two mechanisms, the slope of the  $V_{\text{Na}} - V_{cs}$  trace (see Fig. 3) was measured over as short a segment as possible, but because of the response time of the  $\text{Na}^+$ -sensitive electrode and noise in the voltage records, its duration could not be less than  $\sim 20$  s. It is interesting to note, however, that reducing mucosal [Na] to 10 mM did not cause a measurable reduction in cell volume, measured optically (C. W. Davis and A. L. Finn, personal communication). Also shown in Table I is the rate of increase of  $a\text{Na}_i$  upon restoration of the control mucosal bathing solution. As expected, this rate exceeds that observed when external [Na] is decreased. The reason is that upon exposure to the control [Na] after a period of exposure to 10 mM [Na], the net driving force favoring  $\text{Na}^+$  entry through the  $\text{Na}^+/\text{H}^+$  exchanger is increased by both the reduction of  $a\text{Na}_i$  (Table I) and the acidification of the cell (Weinman and Reuss, 1982). In addition, the fall of  $\text{pH}_i$  may stimulate  $\text{Na}^+/\text{H}^+$  exchange kinetically (Aronson et al., 1982). Finally, Table I (B) summarizes data which prove that reducing mucosal [Na] to 10 mM abolishes the net  $\text{Na}^+$  flux across the luminal membrane. If the net flux is in fact zero under these conditions, inhibition of the transporter at the time of the reduction in external [Na] should not alter the initial rate of fall of  $a\text{Na}_i$ . As shown, addition of amiloride (1 mM) did not alter the initial rate of fall of  $a\text{Na}_i$  produced by the reduction in mucosal [Na]. That amiloride inhibits  $\text{Na}^+$  entry at the concentration employed in these experiments has been shown before in tissues incubated in HEPES-Ringer's (Weinman and Reuss, 1984). In addition, it inhibits fluid absorption (Fig. 2) and causes a rapid, significant fall of  $a\text{Na}_i$  in  $\text{HCO}_3^-$ -Ringer's (100 mM Na), as shown below. In conclusion, inhibition of  $\text{Na}^+/\text{H}^+$  exchange by thermodynamic means causes a fall of  $a\text{Na}_i$  equivalent to 75% of the estimated control Na transport rate; since this value is certainly an underestimate, it is clear that the  $\text{Na}^+/\text{H}^+$  exchanger accounts for most or all of  $\text{Na}^+$  transport across the apical membrane under physiologic conditions.

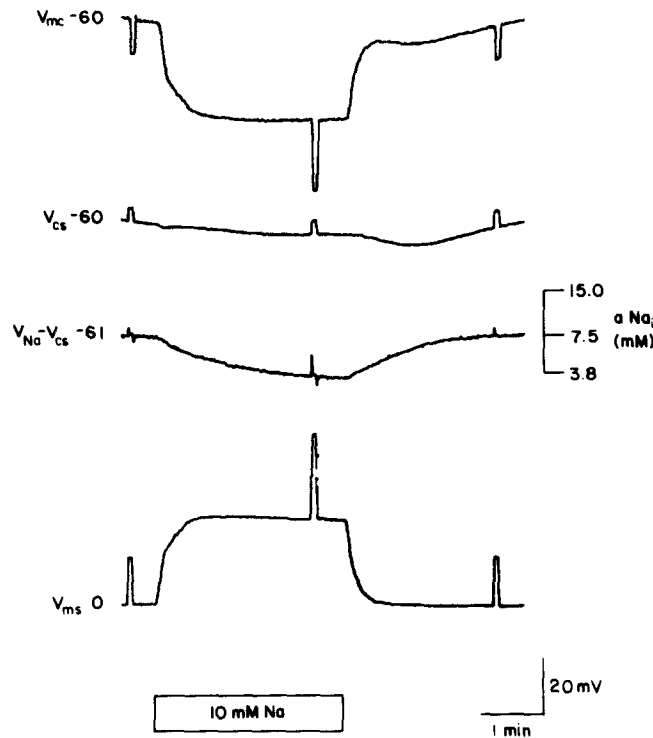


FIGURE 3. Effects of reducing mucosal  $\text{Na}^+$  concentration to 10 mM on membrane potentials and intracellular  $\text{Na}^+$  activity ( $a\text{Na}_i$ ). The record was obtained during simultaneous impalements with a conventional and an  $\text{Na}$ -sensitive microelectrode. In this and similar figures, the traces represent:  $V_{mc}$ , apical membrane potential;  $V_{cs}$ , basolateral membrane potential;  $V_{Na} - V_{cs}$ , difference between intracellular voltages measured by the  $\text{Na}$ -sensitive and the conventional intracellular microelectrode;  $V_{ms}$ , transepithelial potential difference. Initial values are indicated on the left. Cell membrane voltages were referred to the adjacent solution; transepithelial potential difference was referred to the serosal solution. The voltage deflections are the result of transepithelial pulses, at 2-min intervals, to compare ratio of cell membrane resistance of the two impaled cells, for validation purposes (see Weinman and Reuss, 1982). Note that the steady state deflections in the differential trace are negligible. Reducing mucosal  $[\text{Na}]$  for the period indicated by the bar caused large changes in  $V_{mc}$  and  $V_{ms}$ , a slight hyperpolarization of  $V_{cs}$ , and a rapid fall of  $a\text{Na}_i$ . All of these changes were reversible upon restoration of the control mucosal  $\text{Na}^+$  concentration. The cell membrane voltage transients after re-exposure to control Ringer's were observed in three of eight experiments.

#### *Effect of Amiloride on $\text{Na}^+$ Entry*

We have shown previously (Weinman and Reuss, 1984) that amiloride (1 mM) reduces  $\text{Na}^+$  entry in tissues incubated in HEPES-Ringer's (<1 mM external  $\text{HCO}_3^-$ ). We also showed that amiloride causes a reduction of  $a\text{Na}_i$  in tissues incubated in a 10 mM  $\text{HCO}_3^-/1\%$   $\text{CO}_2$  medium (Weinman and Reuss, 1984), but the composition of that solution was slightly different from the one employed in



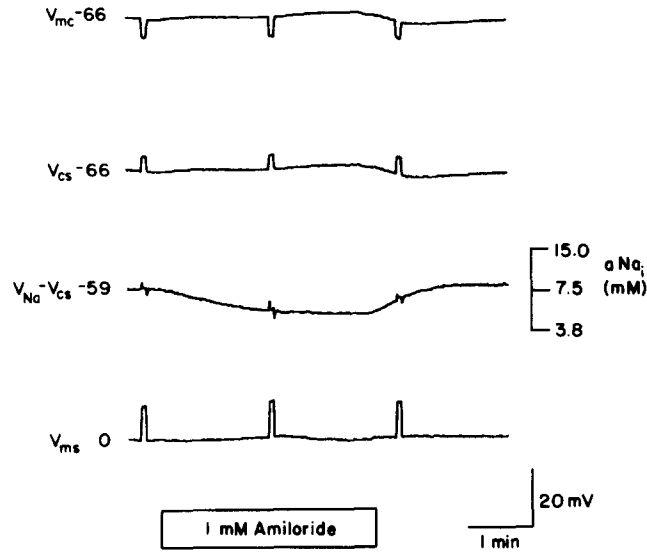


FIGURE 4. Effect of amiloride ( $10^{-3}$  M, added to the mucosal solution during the period indicated by the bar) on membrane potentials and  $aNa_i$ . Symbols and protocol are the same as in Fig. 3.

the present studies. Therefore, in the present experiments, the effect of amiloride of  $aNa_i$  was determined in tissues bathed in a 10 mM  $HCO_3^-$ -Ringer's solution of composition identical to that employed for the  $J_v$  measurements. Fig. 4 shows a typical record of the effect of amiloride on electrical parameters and  $aNa_i$ . Changes in cell membrane potentials during this short period consist of a slow depolarization (see Weinman and Reuss, 1982). The fall of  $aNa_i$  is rapid and reversible. As summarized in Table II, the initial rate of fall of  $aNa_i$  was  $2.4 \pm$

TABLE II  
Effects of Amiloride ( $10^{-3}$  M) on  $aNa_i$  and  $aCl_i$

		$V_{ms}$	$V_{cs}$	$aNa_i$	$\Delta aNa_i/\Delta t$
		mV	mV	mM	mM/min
(A)	Control	$-0.3 \pm 0.1$	$-69.5 \pm 2.2$	$10.4 \pm 0.7$	$-2.4 \pm 0.3$
	Amiloride	$-0.5 \pm 0.2$	$-69.8 \pm 2.3$	$7.7 \pm 0.7^*$	$3.3 \pm 0.7$
	Control	$-0.5 \pm 0.1$	$-68.4 \pm 2.5$	$10.1 \pm 0.7$	
		$V_{ms}$	$V_{cs}$	$aCl_i$	
		mV	mV	mM	
(B)	Control	$-0.6 \pm 0.2$	$-67.6 \pm 1.2$	$21.0 \pm 1.5$	
	Amiloride	$-0.3 \pm 0.2$	$-66.6 \pm 2.7$	$20.8 \pm 1.7$	
	Control	$-0.5 \pm 0.2$	$-68.7 \pm 1.2$	$20.7 \pm 1.7$	

Symbols and protocol are the same as in Table I. Amiloride ( $10^{-3}$  M) was added to the control  $HCO_3^-$ -Ringer's (middle line in A and B). Na measurements,  $n = 6$ ; Cl measurements,  $n = 7$ .

\* Significantly different from control before or after ( $P < 0.001$ ). Slopes of  $aCl_i$  changes in amiloride were unmeasurably small. See Figs. 4 and 7.

0.3 mM/min, i.e., ~65% of the rate of fall observed when  $\text{Na}^+/\text{H}^+$  exchange was stopped (Table I). At this concentration, amiloride produced a 52% inhibition of  $J_v$  (Fig. 2).

That amiloride-sensitive  $\text{Na}^+$  entry occurs at least predominantly by  $\text{Na}^+/\text{H}^+$  exchange is supported by the demonstration that amiloride reduces or reverses luminal acidification (Weinman and Reuss, 1982; Reuss and Costantin, 1984) and produces intracellular acidification (Weinman and Reuss, 1982). However, these data do not rule out the possibility that amiloride also inhibits other modes of  $\text{Na}^+$  entry, e.g., electrodiffusion or  $\text{NaCl}$  cotransport.

If amiloride blocked electroneutral  $\text{NaCl}$  cotransport, then  $a\text{Cl}_i$  should fall immediately after exposure to the drug at a rate similar to the rate of fall of  $a\text{Na}_i$ . This was tested in experiments in which  $a\text{Cl}_i$  was measured before, during, and after exposure to 1 mM amiloride. As illustrated in Fig. 5 and summarized in Table II (B), no significant effect was observed in 3 min. These results rule out the possibility that amiloride inhibits  $\text{NaCl}$  cotransport and are consistent with the original interpretation of the mechanism of action of the drug, i.e., inhibition of apical membrane  $\text{Na}^+/\text{H}^+$  exchange.

#### Rate of $\text{Cl}^-$ Entry by $\text{Cl}^-/\text{HCO}_3^-$ Exchange

$\text{Cl}^-/\text{HCO}_3^-$  exchange is also an electrically silent process (Reuss and Constantin, 1984). Therefore, the rate of  $\text{Cl}^-$  entry under control conditions can be estimated by measuring the initial change of intracellular  $\text{Cl}^-$  activity upon decreasing  $[\text{Cl}]$  in the mucosal solution to a value at which the net force across the anion exchanger is zero. The same assumptions concerning exit rate, steady state  $\text{Cl}^-$  content, and cell volume are applied in this case. Hence, the measurement again provides a minimum estimate of the control rate of  $\text{Cl}^-$  entry. A typical experiment in which mucosal  $[\text{Cl}]$  was reduced from 98 to 29 mM is shown in Fig. 6.

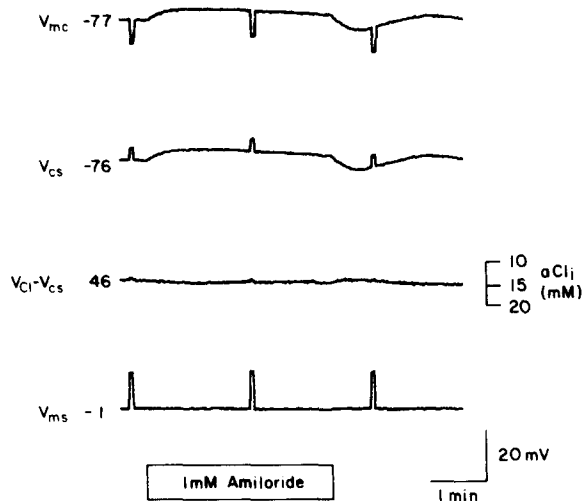


FIGURE 5. Effects of amiloride ( $10^{-5}$  M, added to the mucosal solution) on membrane potentials and  $a\text{Cl}_i$ . Symbols and protocol are the same as in Figs. 3 and 4. Note the negligible effect of the diuretic on  $a\text{Cl}_i$ . Compare with Fig. 4.

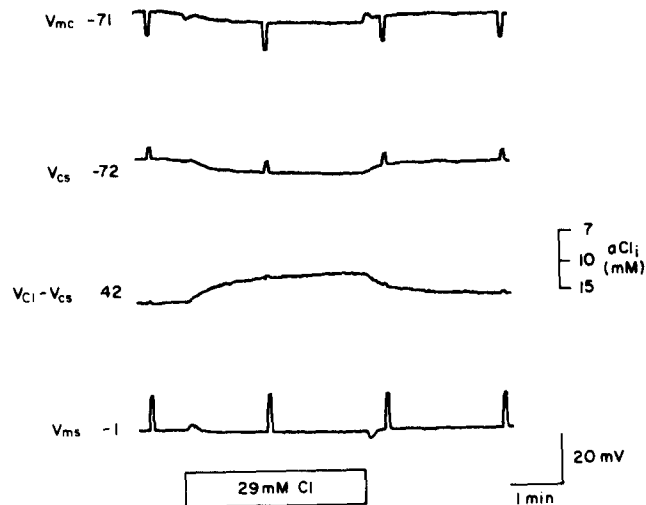


FIGURE 6. Effect of reducing mucosal solution  $\text{Cl}^-$  concentration to 29 mM (for the period indicated by the lower bar) on membrane potentials and  $a\text{Cl}_i$ . Symbols and protocol are the same as in Figs. 3–5.

As in the case of  $\text{Na}^+$ , a rapid fall of  $a\text{Cl}_i$  was observed. Concomitant with this change of  $a\text{Cl}_i$ , the cell membranes hyperpolarized. Possible mechanisms for this hyperpolarization are: (a) intracellular alkalinization, because of decreased cell-to-lumen  $\text{HCO}_3^-$  transport in exchange for  $\text{Cl}^-$ , which increases cell membrane  $\text{K}^+$  permeability (Petersen and Reuss, 1983; Garcia-Diaz et al., 1983; Reuss and Costantin, 1984), and (b) an increase in intracellular  $\text{Ca}^{2+}$  activity, for instance from cell shrinkage, which would cause increases in  $P_{\text{K}}$  at both cell membranes. A Ca-activated  $\text{K}^+$  conductance has been shown to exist at both cell membranes in this epithelium (Bello-Reuss et al., 1981). As summarized in Table III,  $a\text{Cl}_i$  fell at an initial rate of  $5.9 \pm 0.8$  mM/min and rose, after re-exposure to the control mucosal  $\text{Cl}^-$  concentration, at an initial rate of  $13.6 \pm 3.3$  mM/min. These differences can be explained by the fall of  $a\text{Cl}_i$  and the rise of  $a\text{HCO}_3^-$  during the period of exposure to low  $\text{Cl}^-$  (Reuss and Costantin, 1984), which

TABLE III  
Effect of Reducing Mucosal Solution  $[\text{Cl}^-]$  on  $a\text{Cl}_i$

		$V_{\text{ms}}$	$V_{\text{cs}}$	$a\text{Cl}_i$	$\Delta a\text{Cl}_i/\Delta t$
		mV	mV	mM	mM/min
(A)	Control	$-0.4 \pm 0.1$	$-72.6 \pm 3.2$	$14.0 \pm 0.5$	$-5.9 \pm 0.8$
	29 mM Cl	$-1.1 \pm 0.4^*$	$-78.2 \pm 3.3^*$	$10.3 \pm 0.4^*$	$13.6 \pm 3.3$
	Control	$-0.3 \pm 0.3$	$-74.0 \pm 3.8$	$13.8 \pm 0.1$	
(B)	Control	$0.5 \pm 0.2$	$-74.8 \pm 3.8$	$14.1 \pm 0.4$	$-5.8 \pm 0.7$
	29 mM Cl + SITS	$-0.9 \pm 0.7$	$-81.0 \pm 4.8^*$	$10.0 \pm 1.9^*$	$14.6 \pm 2.8$
	Control	$0.4 \pm 0.2$	$-76.3 \pm 4.3$	$13.6 \pm 1.2$	

Symbols and protocol are the same as in Table I.  $\text{Cl}^-$  was substituted with cyclamate. In B, 0.5 mM SITS was added to the low-Cl solution. A:  $n = 5$ ; B:  $n = 4$ .

\* Significantly different from control ( $P < 0.025$ ).

both result in a larger driving force for  $\text{Cl}^-$  entry at this time, as compared with control conditions. The measured rate indicates that all of  $\text{Cl}^-$  entry can be accounted for by  $\text{Cl}^-/\text{HCO}_3^-$  exchange. Validation of this technique, i.e., a demonstration that the net  $\text{Cl}^-$  flux across the apical membrane was abolished, was obtained by measuring the rate of fall of  $a\text{Cl}_i$  produced by reducing mucosal  $[\text{Cl}]$  to 29 mM with concomitant addition of 0.5 mM SITS. The presence of SITS did not alter the rate of fall of  $a\text{Cl}_i$  produced by the reduction in mucosal solution  $[\text{Cl}]$  (Table III, B). That SITS inhibits apical membrane  $\text{Cl}^-/\text{HCO}_3^-$  exchange has been shown by measurements of extracellular and intracellular pH (Reuss and Costantin, 1984) and by the reduction of  $J_v$  (Fig. 2). In addition, when mucosal  $[\text{Cl}]$  was reduced to nominally zero, the rate of fall of  $a\text{Cl}_i$  was significantly lower in the presence of SITS than in its absence (Fig. 7). In seven paired experiments, upon  $\text{Cl}^-$  removal,  $a\text{Cl}_i$  fell initially at rates of  $10.3 \pm 1.3$  and  $6.2 \pm 0.9$  mM/min in the absence and presence of 0.5 mM SITS, respectively ( $P < 0.005$ ).

#### *Are $\text{Na}^+$ and $\text{Cl}^-$ Entry Obligatorily Coupled?*

The possibility of multiple modes of operation of the apical membrane  $\text{Na}^+$  and  $\text{Cl}^-$  transporters (ternary complex, linked double exchange, independent double exchange) has been proposed by Cremaschi et al. (1983) on the basis of tracer

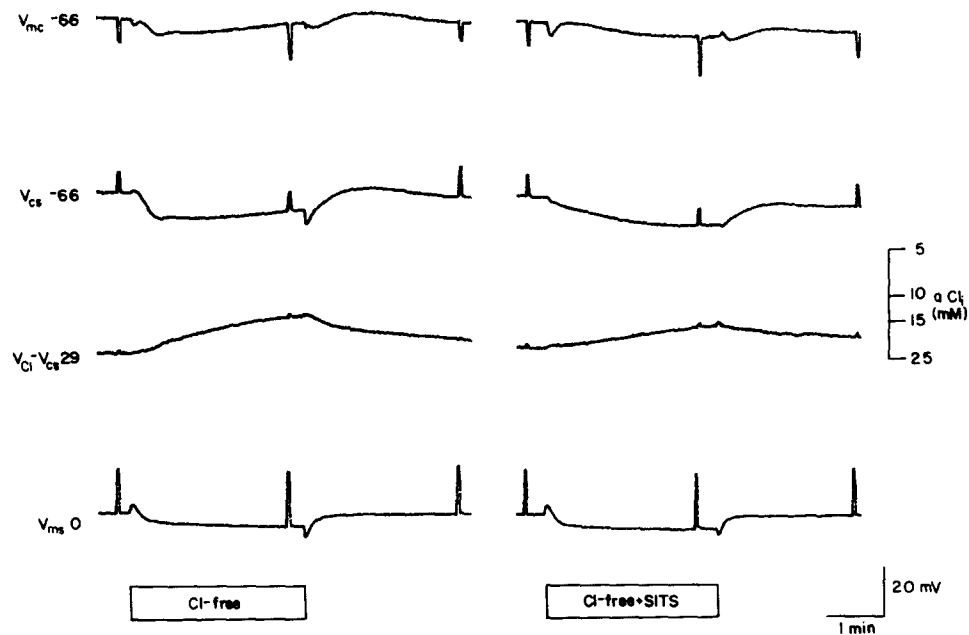


FIGURE 7. Effects of  $\text{Cl}^-$  removal from the mucosal solution on membrane potentials and  $a\text{Cl}_i$  in the absence (left) and in the presence of SITS (right). Both experiments were performed with the microelectrodes in the same cells. The two panels are separated by a 10-min interval. SITS was added to the mucosal solution only, at a concentration of  $0.5 \times 10^{-3}$  M, at the time of reduction of mucosal  $[\text{Cl}]$ . Note the reduction in the rate of fall of  $a\text{Cl}_i$  in the presence of SITS.

flux kinetic studies in rabbit gallbladder. Hence, the possibility that the two exchangers present in our system are directly linked, i.e., that they can only operate simultaneously, needed further examination.

We have already shown (Reuss and Costantin, 1984) that prolonged Cl<sup>-</sup> removal from the bathing media does not reduce the luminal acidification driven by an apical membrane Na<sup>+</sup> gradient oriented inward. Further support against interdependence of the transporters was obtained by studying the effects of complete removal of Na<sup>+</sup> or Cl<sup>-</sup> from the mucosal solution on *aNa<sub>i</sub>* and *aCl<sub>i</sub>*. In the example shown in Fig. 8, it is clear that when Na<sup>+</sup> is removed from the mucosal solution, *aNa<sub>i</sub>* falls much faster than when Cl<sup>-</sup> is removed. In the six experiments summarized in Table IV (A), *aNa<sub>i</sub>* fell 4.3 times faster than *aCl<sub>i</sub>*. Similarly, upon Cl<sup>-</sup> removal, the rate of fall of *aCl<sub>i</sub>* was 7.5 times greater than that of *aNa<sub>i</sub>* (Table IV, B). Baerentsen et al. (1983) also found large differences between the initial rates of fall of *aNa<sub>i</sub>* and *aCl<sub>i</sub>* upon Na<sup>+</sup> or Cl<sup>-</sup> removal from the mucosal side. These results, taken together with the different effects of amiloride on *aNa<sub>i</sub>* and *aCl<sub>i</sub>*, rule out the possibility that NaCl cotransport is a significant apical entry pathway in this epithelium.

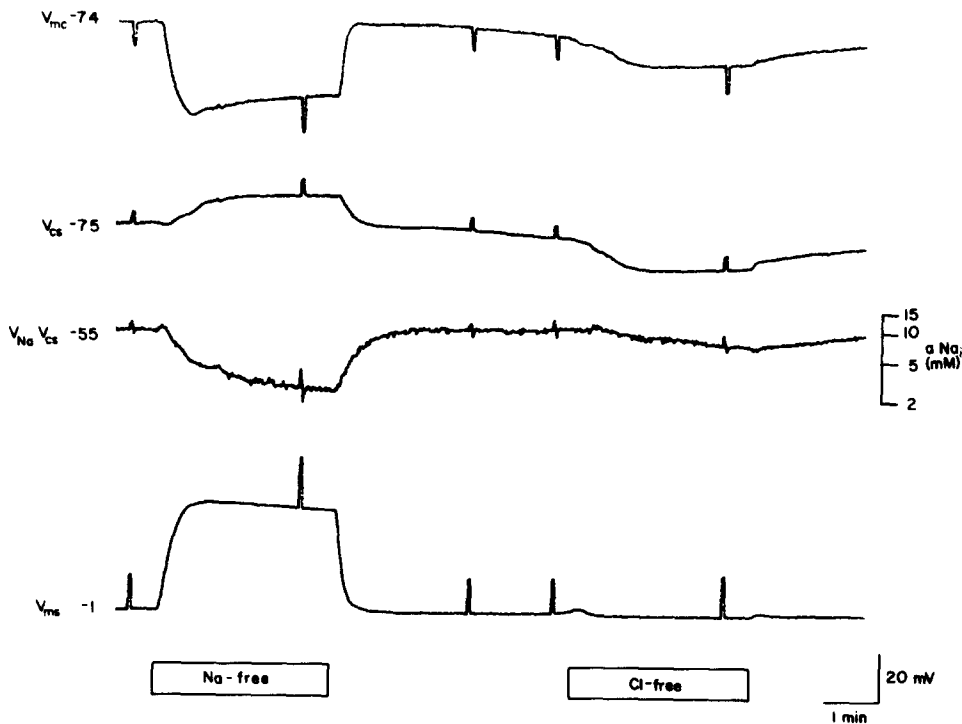


FIGURE 8. Effects of mucosal Na<sup>+</sup> removal (left) and mucosal Cl<sup>-</sup> removal (right) on membrane potentials and *aNa<sub>i</sub>*. Symbols and protocols are the same as in the preceding figures. Note the large difference in the rates of fall of *aNa<sub>i</sub>* in response to these perturbations.

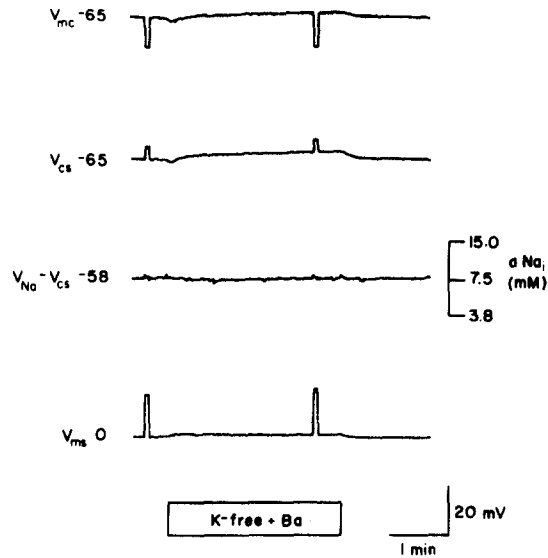


FIGURE 9. Lack of effect of mucosal K<sup>+</sup> removal on  $aNa_i$ . During the period indicated by the bar, KCl was removed from and BaCl<sub>2</sub> (5 mM) was added to the mucosal solution. Note the slight cell membrane depolarization and the lack of change of  $aNa_i$ .

*Is Apical Membrane Na<sup>+</sup> and/or Cl<sup>-</sup> Transport K<sup>+</sup> Dependent?*

Na-K-Cl cotransport seems to be the predominant mechanism of NaCl entry in some segments of the loop of Henle (Greger and Schlatter, 1981) and in flounder intestinal epithelium (Musch et al., 1982). In *Necturus* gallbladder, elevating luminal [K] in the presence of Na has been shown to increase cell volume, an observation that, among other possibilities, could indicate the presence of such a cotransport mechanism at the luminal membrane (Davis and Finn, 1983). For these reasons, the effect of complete K<sup>+</sup> removal from the mucosal solution on

TABLE IV  
*Effects of Na or Cl Removal from the Mucosal Solution on  $aNa_i$  or  $aCl_i$*

	$V_{ms}$	$V_{cs}$	$aNa_i$	$\Delta aNa_i/\Delta t$	$aCl_i$	$\Delta aCl_i/\Delta t$
	mV	mV	mM	mM/min	mM	mM/min
(A) Control	-0.2±0.1	-70.8±1.3	10.7±0.8	-6.5±1.7	19.5±1.5	-1.5±0.4
Na-free	36.5±2.2*	-68.7±1.8	5.1±0.8*		18.5±1.6	
Control	-0.4±0.2	-71.7±1.3	10.6±0.9		20.0±1.8	
(B) Control	-0.1±0.3	-76.1±1.1	9.6±0.9	-1.6±0.4	18.8±2.7	-12.0±1.9
Cl-free	-1.4±0.3*	-85.6±2.1*	7.2±1.3*		7.8±1.3*	
Control	-0.4±0.2	-77.4±0.8	9.0±0.9		17.7±1.9	

Symbols and protocol are the same as in Table I. A:  $n = 12$  (membrane potentials),  $n = 6$  ( $aNa_i$ ),  $n = 6$  ( $aCl_i$ ); B:  $n = 10$  (membrane potentials),  $n = 5$  ( $aNa_i$ ),  $n = 5$  ( $aCl_i$ ).  $\Delta a_i/\Delta t$  = initial rate of fall of intracellular activity upon Na<sup>+</sup> or Cl<sup>-</sup> removal.

\* Significantly different from controls before and after ( $P < 0.025$ ).

TABLE V  
Effects of K<sup>+</sup> Removal from the Mucosal Solution on aNa<sub>i</sub> and aCl<sub>i</sub>

		V <sub>ms</sub>	V <sub>cs</sub>	aNa <sub>i</sub>
		mV	mV	mM
(A)	Control	0.0±0.3	-68.8±2.6	9.7±1.5
	K-free + Ba	0.8±0.4*	-66.0±2.6*	9.6±1.3
	Control	0.1±0.3	-69.8±2.1	10.3±1.4
		V <sub>ms</sub>	V <sub>cs</sub>	aCl <sub>i</sub>
		mV	mV	mV
(B)	Control	-0.6±0.2	-70.0±1.0	19.3±1.0
	K-free + Ba	0.5±0.2*	-69.4±1.5	19.3±1.3
	Control	-0.4±0.2	-72.3±0.8	18.5±1.4

Symbols and protocol are the same as in Table I. A and B: n = 5. Ba<sup>2+</sup> was added (middle line in A and B) at a 5-mM concentration.

\* Significantly different from control (P < 0.01).

intracellular Na<sup>+</sup> and Cl<sup>-</sup> activities was investigated. To reduce K<sup>+</sup> efflux across the luminal membrane, Ba<sup>2+</sup>, which reduces P<sub>K</sub> at this membrane (Reuss et al., 1981), was added at a concentration of 5 mM.

As shown in Fig. 9, aNa<sub>i</sub> remained unchanged. Data from experiments in which either aNa<sub>i</sub> or aCl<sub>i</sub> was measured under these conditions are summarized in Table V. K<sup>+</sup> removal had no significant effect on the intracellular activity of either ion.

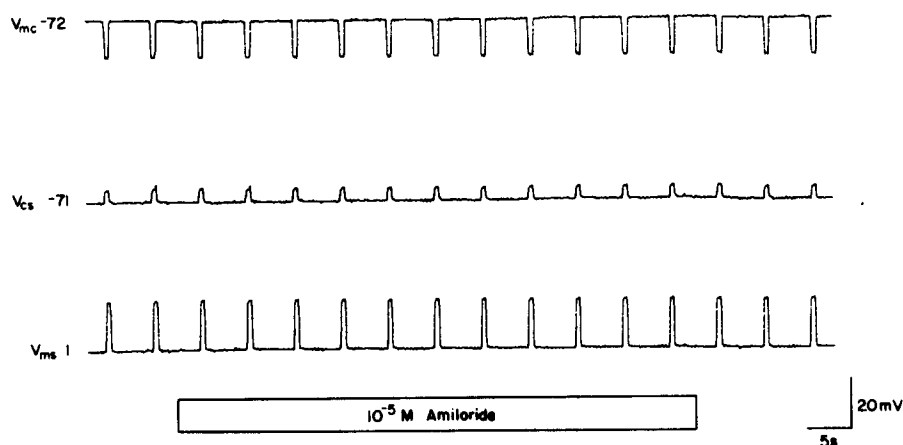


FIGURE 10. Lack of effect of amiloride (10<sup>-5</sup> M, added to the mucosal solution during the period indicated by the bar) on membrane potentials and cell membrane resistance ratio. Pulses were applied at 5-s intervals (0.5 s duration). After correction for series resistances, the apparent ratio of cell membrane resistances can be calculated from the deflections in the V<sub>mc</sub> and V<sub>cs</sub> traces.

*Is There an Amiloride-sensitive Apical Membrane Na<sup>+</sup> Conductance?*

A relatively small apical membrane Na<sup>+</sup> conductance has been demonstrated in this epithelium by intracellular microelectrode techniques (Van Os and Slegers, 1975; Reuss and Finn, 1975*b*; Graf and Giebisch, 1979). To establish whether amiloride inhibits this pathway, cell membrane potentials and apparent cell membrane resistance ratios were measured before, during, and after exposure of the mucosal surface of the tissue to a concentration of the drug (10<sup>-5</sup> M) that essentially abolishes Na<sup>+</sup> entry and hyperpolarizes the apical membrane in epithelia such as frog skin (Helman and Fisher, 1977; Nagel, 1980). As shown in Fig. 10, there was no effect on membrane potentials or on the resistance ratio. In six such experiments,  $V_{mc}$  values were  $-70.0 \pm 1.7$  and  $-70.6 \pm 1.8$  and apparent  $R_a/R_b$  values were  $2.86 \pm 0.25$  and  $2.93 \pm 0.28$  before and during amiloride, respectively. Both experimental values were not statistically different from the control ones. Hence, the apical Na<sup>+</sup>-conductive pathway is not measurably sensitive to amiloride. Similar negative results with 10<sup>-4</sup> M amiloride have been reported by Van Os and Slegers (1975).

#### DISCUSSION

##### *Fluid Transport Measurements*

The electrical method described offers clear advantages as compared with more conventional ones used in epithelial organs. In particular, the same in vitro mounting procedure can be used for both  $J_v$  determinations and microelectrode experiments. In addition, the time resolution of this technique is excellent. The main disadvantages are that the mucosal fluid volume is small and static and that unpredictable changes in the height of the fluid column are frequently recorded shortly after placing the oil on top of the physiologic salt solution. These transients may be caused at least in part by oil flow toward the tissue at the edge of the preparation, which in the experiments described here was sealed by a Sylgard disk.

The values of  $J_v$  in 10 mM HCO<sub>3</sub>-Ringer's ranged from 6 to 19  $\mu\text{l}\cdot\text{cm}^{-2}\cdot\text{h}^{-1}$  (average 13.5  $\mu\text{l}\cdot\text{cm}^{-2}\cdot\text{h}^{-1}$ ). Inasmuch as the degree of stretch of the preparations cannot be precisely controlled, this variability can result in part from differences in the true transporting area. The direct paired comparison shown in Fig. 2 demonstrates that in *Necturus* gallbladder, as in mammalian gallbladder, HCO<sub>3</sub> stimulates fluid transport. Amiloride, added to a final concentration of 10<sup>-3</sup> M to the lumen, inhibited fluid absorption significantly and reversibly, in agreement with the results of Frederiksen (1983) in rabbit gallbladder and of Heintze et al. (1972) in guinea pig gallbladder. SITS (0.5 × 10<sup>-3</sup> M) also inhibited fluid absorption, by ~59%. Parr and Martin (1984) have recently reported a 36% inhibition of fluid absorption by SITS (10<sup>-3</sup> M) in rabbit gallbladder. Bumetanide (10<sup>-5</sup> M, luminal addition) was ineffective. These data are in direct contradiction with some of the results and conclusions of Spring and associates (Spring and Ericson, 1982; Ericson and Spring, 1982*a, b*; Larson and Spring, 1983). These authors claim that fluid transport in this tissue is the result of a ternary-complex NaCl entry process at the apical membrane, which is amiloride insensitive and bumetanide sensitive ( $K_i \sim 10^{-9}$  M). It could be argued that the effect of amiloride



in the present experiments is not at the apical entry step, but at an intracellular site, e.g., because of inhibition of the Na,K pump (Soltoff and Mandel, 1983). This clearly cannot be the case, however, because fluid transport inhibition was concomitant with a decrease, not an increase, of  $a\text{Na}_i$ .

#### *Tests for NaCl and Na-K-Cl<sub>2</sub> Cotransport at the Apical Membrane*

The possibility of apical membrane NaCl entry by cotransport was subjected to stringent tests in these experiments. Since it is clear that NaCl entry is an electroneutral process (Reuss and Grady, 1979; Garcia-Diaz and Armstrong, 1980), a ternary-complex (Na-Cl-carrier) entry mechanism would require stoichiometric (1:1) transport on Na<sup>+</sup> and Cl<sup>-</sup>. Therefore, removal of either Na<sup>+</sup> or Cl<sup>-</sup> from the luminal solution should cause initial decreases of intracellular activities of both ions at similar rates (not necessarily at identical rates, because of the possibilities of [a] rapidly established differences in Na<sup>+</sup> and Cl<sup>-</sup> basolateral extrusion rates, and [b] parallel backleak pathways at the apical membrane). In contrast with this expectation, when Na was removed,  $a\text{Na}_i$  fell at a rate 4.3 times faster than the initial fall of  $a\text{Cl}_i$ ; conversely, when Cl was removed,  $a\text{Cl}_i$  fell 7.5 times faster than  $a\text{Na}_i$  (Table IV; see also Baerentsen et al., 1983). These results suggest independent entry processes, but could still be consistent with the possibility of NaCl cotransport (Ericson and Spring, 1982a) if a parallel pathway is activated (or inserted into the apical membrane) at the time of or shortly after the mucosal solution substitution. A possible mechanism consistent with this view would be the following sequence of events: Na<sup>+</sup> removal reverses the net NaCl flux through the cotransporter, causing a decrease in cell volume, which in turn results in activation of the Na<sup>+</sup>/H<sup>+</sup> and Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchangers; because of the orientation of the chemical gradients ( $\text{Na}_i > \text{Na}_o$ ,  $\text{Cl}_o > \text{Cl}_i$ ), such activation would increase both Na<sup>+</sup> exit and Cl<sup>-</sup> entry across the apical membrane, thereby accounting for the large differences between the rates of fall of  $a\text{Na}_i$  and  $a\text{Cl}_i$ . A similar mechanism could operate for the case of Cl<sup>-</sup> removal from the mucosal bathing medium.

Several arguments can be raised against the above explanation. First, if NaCl cotransport is bumetanide sensitive, as claimed by others (Larson and Spring, 1983), addition of this drug to the mucosal solution should inhibit NaCl entry and thus produce three measurable effects: fall of  $a\text{Na}_i$ , fall of  $a\text{Cl}_i$ , and inhibition of  $J_v$ . In our hands, however, bumetanide had no effects on these parameters (Fig. 2, Results; see also Weinman and Reuss, 1984). As stated above, these observations do not agree with those of Larson and Spring (1983), who found rapid reductions of intracellular Na<sup>+</sup> and Cl<sup>-</sup> activities and cessation of fluid transport upon exposure to bumetanide. Second, in the absence of primary changes in ion gradients, amiloride inhibits fluid absorption and causes a rapid decrease in  $a\text{Na}_i$ , while not affecting  $a\text{Cl}_i$ . This result cannot be reconciled with the hypothesis of amiloride-sensitive NaCl cotransport, because if amiloride blocks it (and if this is the mechanism of fall of  $a\text{Na}_i$  and  $J_v$ ),  $a\text{Cl}_i$  should fall. Third, when the mucosal solution [Na] is reduced to 10 mM, addition of amiloride should enhance the rate of fall of  $a\text{Na}_i$ , if entry is by NaCl cotransport and is amiloride sensitive. However, no change in this rate was observed, an

observation consistent with  $\text{Na}^+$  entry by  $\text{Na}^+/\text{H}^+$  exchange, since at the mucosal solution  $[\text{Na}]$  employed, the net driving force on the transporter is close to zero.

In addition, Ericson and Spring (1982a) observed that ouabain-induced cell swelling was abolished when mucosal  $[\text{Na}]$  or  $[\text{Cl}]$  was reduced to 10 mM. This observation is hard to reconcile with a simple NaCl cotransport model, because when the concentration of only one of the two ions is thus reduced, the combined chemical gradients still favor entry.

In sum, the data presented do not support the hypothesis of a ternary-complex mechanism of NaCl entry. Electrically silent cotransport of Na, K, and Cl (e.g., Na-K- $\text{Cl}_2$  transport) can also be ruled out by the same experimental arguments advanced against the possibility of NaCl cotransport. In addition, neither  $a\text{Na}_i$  nor  $a\text{Cl}_i$  was affected upon mucosal solution  $\text{K}^+$  removal.

#### *Test for Amiloride-sensitive $\text{Na}^+$ Conductance*

The possibility that amiloride blocks apical membrane electrodiffusional  $\text{Na}^+$  entry was also ruled out by the demonstration of a lack of effect on cell membrane potentials and apparent cell membrane resistance ratios. The possibility of an amiloride-sensitive electrodiffusional  $\text{Na}^+$  entry pathway in rabbit and guinea pig gallbladder epithelia has been proposed by Cremaschi and Meyer (1982) on the basis of cell membrane hyperpolarization and an increase of the ratio of cell membrane resistances (apical/basolateral) upon addition of a low concentration of the drug ( $\sim 10^{-5}$  M) to the mucosal solution. It should be noted, however, that the electrical effects were only measurable after a delay of 2 min. This time course is very different from the rapid action of amiloride on membrane potentials in epithelia such as frog skin (Helman and Fisher, 1977; Nagel, 1980). In addition, according to the  $J_v$  measurements of Frederiksen (1983), at this concentration amiloride would not inhibit fluid transport. Finally, if a reduction of apical membrane  $P_{\text{Na}}$  took place in *Necturus* gallbladder after a 2-min delay, it is obvious that such effect could not be responsible for the immediate fall in  $a\text{Na}_i$  (Fig. 4) or in unidirectional tracer Na uptake (Weinman and Reuss, 1984). Delayed effects of amiloride are difficult to interpret, because the drug has been shown to permeate biological and artificial membranes (Dubinsky and Frizzell, 1983) and to inhibit, among other enzymes, the Na,K-activated ATPase (Soltoff and Mandel, 1983). In sum, previous results indicate that there is a small but measurable apical membrane  $P_{\text{Na}}$  in *Necturus* gallbladder epithelium, which makes a small contribution to  $\text{Na}^+$  entry. The present results show that this pathway is insensitive to amiloride at concentrations of the drug that block  $\text{Na}^+$  channels in frog skin (Lindemann and Van Driessche, 1977; Helman and Fisher, 1977; Nagel, 1980). At a higher concentration ( $10^{-3}$  M), amiloride depolarizes the cells (Fig. 4). This effect is probably secondary to a reduction of  $P_{\text{K}}$  caused by the intracellular acidification produced by the drug (Weinman and Reuss, 1982).

#### *Rates of Apical Membrane $\text{Na}^+$ and $\text{Cl}^-$ Transport by Double Exchange*

The essential conclusion of the preceding discussion is that NaCl cotransport and/or Na-K- $\text{Cl}_2$  cotransport cannot account for the normal rates of entry of NaCl from mucosal solution to cells in the transporting *Necturus* gallbladder

epithelium. Na<sup>+</sup> electrodiffusion seems also to be a minor component of Na<sup>+</sup> entry. Inasmuch as we have shown that Na<sup>+</sup>/H<sup>+</sup> and Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchangers exist at this membrane (Weinman and Reuss, 1982, 1984; Reuss and Costantin, 1984), it is natural to hypothesize that these exchangers are the pathways for NaCl entry. Further support for this hypothesis was obtained by demonstrating that the rates of net ion transport across the apical membrane attributable to the exchangers are in agreement with the rates of transepithelial salt transport.

Machen and Zeuthen (1983) attempted to obtain a quantitative estimate of the rates of exchanger-mediated entry of Na<sup>+</sup> and Cl<sup>-</sup> by measuring rates of change of pH<sub>i</sub> in response to removal of Na<sup>+</sup> or Cl<sup>-</sup> from the lumen. Their results are difficult to interpret quantitatively because no measurements of intracellular buffering power were given, and because their experimental procedure (complete luminal Na<sup>+</sup> or Cl<sup>-</sup> removal) not only stops entry, but reverses the Na<sup>+</sup> and Cl<sup>-</sup> fluxes. Nevertheless, their observations are qualitatively consistent with ours. The correct procedure is to reduce [Na] or [Cl] in the mucosal solution to values such that the net driving force acting on the respective countertransporter becomes zero, as done in the present studies. Under these conditions, the initial rates of change of intracellular activities will provide an indication of the entry rates under control conditions, i.e., immediately before the ionic substitution. Later on, as pH<sub>i</sub> and *a*Na<sub>i</sub> or *a*Cl<sub>i</sub> change, the driving forces will change as well and hence the changes in intracellular activities will not reflect the control transport rates. The results of these experiments were entirely consistent with the hypothesis presented, i.e., that double exchange is the main or sole mechanism of NaCl entry. Upon reducing luminal [Na] to stop Na<sup>+</sup>/H<sup>+</sup> exchange, *a*Na<sub>i</sub> fell at an average initial rate of 3.7 mM/min, which is equivalent to ~75% of the control rate of Na<sup>+</sup> entry, estimated from *J<sub>v</sub>* measurements. These results are in excellent agreement with previous estimates obtained with different methods (Weinman and Reuss, 1984). In the Cl<sup>-</sup> experiments, the initial rate of fall was on the average 5.9 mM/min, i.e., ~20% higher than that predicted from the control *J<sub>v</sub>* values if Cl<sup>-</sup> entry suddenly ceased. Because of the variability of *J<sub>v</sub>* values from tissue to tissue and the inherent limitations of the technique, the significance of the observed difference between Na<sup>+</sup> and Cl<sup>-</sup> transport rates is uncertain. If real, a possible explanation could be a rapid reduction in basolateral Na<sup>+</sup> pumping or an increase in basolateral Na<sup>+</sup> entry secondary to the fall in *a*Na<sub>i</sub>. The latter possibility, i.e., a significant basolateral membrane Na<sup>+</sup> backleak, is supported by the observation that *a*Na<sub>i</sub> stays at relatively high values in ouabain-treated tissues after nominally complete Na<sup>+</sup> removal from the mucosal side (Weinman and Reuss, 1984).

#### *Mechanism of Linkage of Na<sup>+</sup> and Cl<sup>-</sup> Entry*

The results described show that NaCl entry occurs by double exchange, but do not explain why Na<sup>+</sup> and Cl<sup>-</sup> entry proceed at the same rates under normal transporting conditions, or why the net flux of one of the ions is abolished by removal of the other (Spring and Hope, 1979). Several possibilities for this synchronization are apparent. For instance, the coupling could be in principle entirely thermodynamic, i.e., if Na<sup>+</sup> is decreased in the lumen, pH<sub>i</sub> falls, HCO<sub>3</sub><sup>-</sup><sub>i</sub>

falls, and the rate of  $\text{Cl}^-$  entry, which below saturation depends directly on  $\text{HCO}_3^-$ , is decreased as well. Conversely, if luminal  $\text{Cl}^-$  is reduced, the cell alkalizes and  $\text{Na}^+$  entry is decreased because of the lower cytosolic  $\text{H}^+$  activity. Needless to say, these mechanisms operate in the right direction, but they do not explain quantitatively the fact that net entry across the parallel pathway eventually stops when either  $\text{Na}^+$  or  $\text{Cl}^-$  is removed. Under these conditions, the average changes in  $\text{pH}_i$  were from 7.59 to 7.37 ( $\text{Na}^+$  removal; Weinman and Reuss, 1982) and from 7.53 to 7.89 ( $\text{Cl}^-$  removal; Reuss and Costantin, 1984). To stop  $\text{Na}^+$  transport,  $\text{pH}_i$  should rise to a value given by  $\text{pH}_o + \log \text{Na}_o/\text{Na}_i$ , i.e.,  $\sim 8.5$ ; to stop  $\text{Cl}^-$  transport,  $\text{pH}_i$  should fall to a value given by  $\text{pH}_o - \log \text{Cl}_o/\text{Cl}_i$ , i.e.,  $\sim 7.0$ . These values are slight underestimates of the required changes, because the control intracellular activities were employed for the calculation, and, as seen in Table IV,  $a\text{Na}_i$  and  $a\text{Cl}_i$  fell upon mucosal removal of the counterion. Hence, since the required  $\text{pH}_i$  changes are much greater than the observed ones, it is safe to conclude that a  $\text{pH}_i$ -dependent, purely thermodynamic mechanism of coupling of  $\text{Na}^+$  and  $\text{Cl}^-$  fluxes cannot explain completely the absolute dependency of steady state transport of one ion on the presence of the other. Attractive possibilities, which will require further experimental work, include a  $\text{pH}_i$  dependence of the kinetic parameters of the apical exchangers, such as that reported for the  $\text{Na}^+/\text{H}^+$  exchanger of proximal renal tubule apical membrane vesicles (Aronson et al., 1982), or of the basolateral extrusion mechanisms. In this regard, the pump-mediated  $\text{Na}^+$  flux in rabbit urinary bladder appears to be highly dependent on  $\text{pH}_i$  (Eaton et al., 1984).

In sum, apical membrane  $\text{Na}^+/\text{H}^+$  and  $\text{Cl}^-/\text{HCO}_3^-$  exchange account quantitatively for transepithelial salt transport in *Necturus* gallbladder. Under appropriate experimental manipulations, the  $\text{Na}^+$  and  $\text{Cl}^-$  fluxes across the apical membrane can be dissociated, which indicates that these transporters are not obligatorily linked. The long-term equivalence of transepithelial  $\text{Na}^+$  and  $\text{Cl}^-$  fluxes is not the result of purely thermodynamic coupling mediated by  $\text{pH}_i$ , but requires kinetic effects at the apical membrane, the basolateral membrane, or both.

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