J. Membrane Biol. 88, 55–66 (1985)

The Journal of
Membrane Biology

Evaluation of Ion Gradient-Dependent H^+ Transport Systems in Isolated Enterocytes from the Chick

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Summary. The experiments reported here evaluate the capability of isolated intestinal epithelial cells to accomplish net H^+ transport in response to imposed ion gradients. In most cases, the membrane potential was kept constant by means of a K^+ plus valinomycin "voltage clamp" in order to prevent electrical coupling of ion fluxes. Net H^+ flux across the cellular membrane was examined at pH 6.0 (the physiological lumenal pH) and at pH 7.4 using methylamine distribution or recordings of changes in media pH. Results from both techniques suggest that the cells have an Na^+/H^+ exchange system in the plasma membrane that is capable of rapid and sustained changes in intracellular pH in response to an imposed Na^+ gradient. The kinetics of the Na^+/H^+ exchange reaction at pH 6.0 [K_t for Na^+ = 57 mM, V_{max} = 42 mmol H^+/liter 3OMG (3-O-methylglucose) space/min] are dramatically different from those at pH 7.4 (K_t for Na^+ = 15 mM, V_{max} = 1.7 mmol H^+/liter 3OMG space/min). Experiments involving imposed K^+ gradients suggest that these cells have negligible K^+/H^+ exchange capability. They exhibit limited but measurable H^+ conductance. Anion exchange for base equivalents was not detected in experiments performed in media nominally free of bicarbonate.

Key Words epithelial ion transport · Na^+/H^+ exchange · Cl^-/OH^- exchange · intracellular pH · H^+ flux measurements · methylamine distribution as a cellular pH sensor

Introduction

Transmural movement of Na^+ and Cl^- across renal and intestinal epithelia exhibit mutually dependent fluxes. Two mechanisms of coupling have been proposed. One envisions coupling as the result of a brush-border biological cotransport system for NaCl which requires the simultaneous presence of both ions for activity (Quay & Armstrong, 1969; Nellans et al., 1973). The other suggests coupling is the result of concomitant function of two separate antiport systems, one for Na^+/H^+ and another for Cl^-/OH^- (or Cl^-/HCO_3^-). The two systems are regarded as coupled via transmembrane changes in pH (Turnburg et al., 1970).

Numerous studies have provided evidence for the existence of an Na^+/H^+ exchange reaction in the brush-border membrane of many epithelial cells. In brush-border membrane vesicles isolated from intestine or renal tubules, such electroneutral exchange accounts for a significant fraction of Na^+ flux across the membrane (Murer et al., 1976; Kinsella & Aronson, 1980, 1981; Knickelbein et al., 1983) and is capable of significantly altering intravesicular pH when Na^+ gradients are imposed (Kinsella & Aronson, 1980; Burnham et al., 1982; Warnock et al., 1982; Sabolic & Burckhardt, 1983; Cassano et al., 1984). Studies with gallbladder epithelium also suggest the presence of Na/H exchange events in the apical membrane of that tissue (Weinman & Reuss, 1984).

Similar attempts to document the presence of anion exchange capability have provided less consistent results, however. Anion tracer studies with brush-border vesicles have shown that pH gradients can cause overshoots of Cl^- accumulation in rabbit renal cortex membranes (Warnock & Yee, 1981). In rat ileal membranes, imposed pH gradients have also been shown to drive transient Cl^- gradients (Leidtke & Hopfer, 1982; Shiuan & Weinstein, 1984) or to increase Br^- flux (Murer et al., 1980). On the other hand, similar experiments with brush-border membrane vesicles in other laboratories have demonstrated limited or no capacity for imposed anion gradients to alter intravesicular pH in rat and rabbit renal cortex membranes (Kinsella & Aronson, 1980; Sabolic & Burckhardt, 1983) or to diminish the net H^+ efflux caused by the Na^+/H^+ exchanger in rat jejunal and renal cortex membranes (Cassano et al., 1984). Exchange reactions involving OH^- could not be shown to catalyze a significant fraction of Cl^- tracer flux in rabbit renal cortex membranes, however (Seifter et al., 1984). Results from *Necturus* gallbladder vary depending on the state of the cells (Ericson & Spring, 1982*a*,*b*). Many of the differences in results may be explained

by tissue and species differences, but it is not clear whether some ambiguity regarding anion exchange capability relates to artifacts induced during vesicle preparation or to problems related to inadequate control of membrane potential ($\Delta\psi$) during the flux measurement with consequent electrogenic Cl^- and H^+ fluxes.

The experiments reported here examine the H^+ transport systems present in the plasma membrane of epithelial cells isolated from the small intestine of the chick. The isolated cells allow an evaluation of the types of transport systems present in the entire plasma membrane, since all faces of the membrane are exposed to the suspension medium. While the cells cannot be used to localize transport events to the apical or basolateral membrane, they do allow evaluation of the existence and relative magnitude of specific types of transport systems. The large intracellular volume (relative to membrane vesicle volumes) allows certain kinetic experiments to be accomplished more readily than with vesicles. Additionally, the use of intact cells circumvents some of the questions regarding possible alteration of inherent membrane properties which accompany the process of cell lysis, divalent cation precipitation and resealing of membrane vesicles. Cells depleted of ATP also offer the opportunity for experiments in which relatively stable gradients of ions and/or $\Delta\psi$ may be experimentally imposed (relative to the experimental interval) and consequent advantages relating to systematic evaluation of the effect of the individual driving forces ($\Delta\mu$ and $\Delta\psi$) operative on a given system.

Abbreviations

HEPES —N-2-hydroxyethyl piperazine-N-2-ethanesulfonic acid
MES —2[N-morpholino]ethanesulfonic acid
$H_o^+(H_i^+)$ —extra(intra) cellular proton concentration
$pH_o(pH_i)$ —extra(intra) cellular pH
$Na_o^+(Na_i^+)$—extra(intra) cellular sodium
FCCP —carbonylcyanide *p*-trifluoromethoxylphenylhydrazone
3OMG —3-O-methylglucose
TMA^+ —tetramethylammonium
gluc —gluconate
$\Delta\psi$ —membrane potential difference

Materials and Methods

Cell Isolation and ATP Depletion

Cell isolation was as described previously using hyaluronidase to enzymatically release enterocytes from the small intestine of 3- to 6-week-old (White Rock or White Leghorn) chicks (Kimmich, 1970). Cells were released from the intestine by gentle agitation of the tissue and were washed twice by centrifugation. The cell isolation media were 280 to 300 mOsm in every experiment except when the osmolarity was varied systematically in order to evaluate the effect on cell volume. All osmolarities were measured with a Fiske freezing-point depression osmometer. Components of the cell isolation media depended on the particular experiment and are given in the legends to the appropriate figures. In addition to the component shown in the legends, all media contained 1 mM $MgSO_4$, 1 mM $CaSO_4$ and 1 mg/ml bovine serum albumin (Sigma—fraction 5). All solutions were prepared fresh each day and titrated to the required pH at 37°C with TMAOH or H_2SO_4 immediately prior to use.

Cells were depleted of cellular ATP as described previously (Carter-Su & Kimmich, 1979) to facilitate equilibration of the cells with the isolation medium. Cell suspensions were incubated with 30 μM rotenone and 200 μM ouabain for 10 to 15 min at 37°C and aliquots were placed on ice. If the cells were to be kept in a medium without buffer, the pH of the cell supernatant was continuously monitored prior to being placed on ice, and kept at the desired pH by titration with TMAOH or H_2SO_4. Media and ATP-depleted cells were prewarmed for 5 min at 37°C prior to mixing to initiate the indicated isotopic fluxes.

Isotopic Tracers

Changes in intracellular pH were determined by monitoring changes in the amount of ^{14}C-methylamine (New England Nuclear) accumulated in the cellular compartment. The fidelity of this H^+ gradient sensor has been evaluated previously in the chick cell preparation.[1] Fluxes were initiated by diluting cells into the appropriate medium containing 10 μM methylamine in a final volume of 1.0 ml. Aliquots of the incubation suspension (100 μl) were taken at the indicated intervals and were placed in 4 ml of ice-cold medium to stop subsequent flux. Cells were pelleted by a 15-sec centrifugation at 8000 × *g*, and then washed by centrifugation with an additional 2 ml of ice-cold medium prior to solubilization with Liquiscint® (National Diagnostics) and scintillation counting (Beckman LS-230). Specific activity (cpm/nanomole) for each experiment was determined by counting an aliquot of the medium. The amount of methylamine in the cellular compartment is expressed relative to the amount taken up by cells incubated with no imposed ion gradients or as a concentration based on measurements of cell volume.

Cell volume was determined by measuring the equilibrium distribution space for 100 μM of the metabolically inert sugar ^{14}C-3-O-methylglucose (3OMG, Amersham) in the presence of 200 μM phlorizin (K and K) in order to inhibit concentrative uptake via the Na^+-dependent transport system. This sugar is transported by a serosal facilitated diffusion system and takes 5 to 7 min to equilibrate at 37°C (*data not shown*). Measurements of 3OMG space were performed in a final volume of 0.5 ml and at least two samples were taken following a 10-min incubation interval. The cell pellet was washed and counted by procedures similar to those described for methylamine uptake. Cells retained 99% of accumulated ^{14}C-3OMG during the 2-min wash procedure (fractional rate of loss of 0.005 min^{-1}). The cell pellets collected during the experiments typically represented 1 to 3 μl of 3OMG space (2.62 ± 0.12 μl 3OMG space/mg biuret protein, mean ± SE, n = 20 experiments) (Gornall et al., 1949).

pH-Meter Recordings

In many experiments, the pH of the cell suspension was monitored continuously. This was performed using a Ross semi-micro

[1] M.H. Montrose and G.A. Kimmich. 1985. Quantitative use of weak bases for estimation of cellular H^+ gradients. *Am. J. Physiol. (in press)*.

combination pH electrode (Orion Research) connected to a Fisher Accumet pH meter (model 720) and a Heath recorder (model SR-205).

Recordings were taken while the electrode was immersed in 1.5 to 2.1 ml of medium in a thermostated polypropylene vessel. Temperature control was provided by a Lauda K2/R circulator while the medium was mixed by a small Teflon® stir bar. Fluxes at 37°C were initiated by the addition of prewarmed cells to the titration vessel containing prewarmed medium titrated to the desired pH with H_2SO_4 or TMAOH.

With 2.1 ml of solution stirring in the titration vessel, the $t_{1/2}$ of the electrode response is approximately 0.7 sec for jumps of 0.1 to 0.4 pH units. Initial rates of H^+ efflux from cells, calculated from tangents to the pH-meter tracings, were always less than half of the maximal "rate" observed when an aliquot of acid was added to simulate a pH excursion of similar magnitude. Experiments with fewer cells or higher extracellular buffering capacity gave identical calculated flux values.

In order to estimate extracellular buffering capacity, a cell-free supernatant was collected following an experiment and titrated at 37°C with 10 mM HNO_3. Medium buffering capacity was always evaluated at the pH of the experiment (pH 6.0 or 7.4) using excursions of less than 0.2 pH units.

All chemicals for which the supplier is not specifically mentioned were purchased from Sigma Chemical Co. (St. Louis). Digitonin was added from a stock solution of 0.5% in water to a final concentration of 0.002% (200 μM). FCCP was added from a stock of 1 mM in 50% ethanol to a final concentration of 10 μM. Both of these stock solutions were titrated to either pH 7.4 or 6.0 when they were prepared to avoid artifactual changes in pH-meter recordings due to their addition. Monensin was added from an ethanolic stock solution of 50 μM to a final concentration of 0.5 μM. Nigericin was added from an ethanolic stock solution of 0.1 mg/ml to a final concentration of 1 μg/ml. Valinomycin was added from an ethanolic solution of 3 mg/ml to a final concentration of 10 μg/ml. TMAgluconate and $TMANO_3$ were made by mixing equimolar concentrations of the appropriate acid and base. Results are presented as mean values ± SE of the mean.

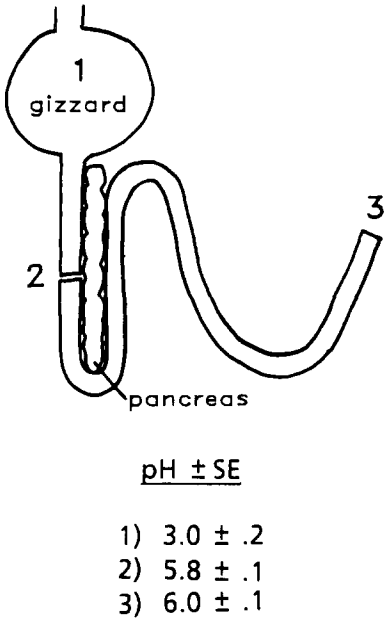

Fig. 1. The pH of chick gastrointestinal tract at different loci. The length of small intestine from points two to three is used for isolation of enterocytes. Under these conditions, blood pH is 7.3 and the colon pH is 7.2. The mean (± SE) pH of 14 chicks is presented for the three indicated regions of tissue

Results

Prior to evaluating the general classes of ion gradient-dependent H^+ or OH^- transport systems in isolated chick enterocytes, the pH of the lumenal contents of chick gut was determined. Experiments evaluating net H^+ (OH^-) movement could then be performed at pH values similar to those which occur physiologically. Figure 1 is a schematic representation of the proximal portion of the chick small intestine. Typically, cells are isolated from the region lying between points 2 and 3 on the diagram. It is a segment which includes a distal portion of the duodenum, but is mostly composed of the section of small intestine comparable to the jejunum/ileum of mammals (these segments are not separately defined in the avian intestine; Sturkie, 1965). The extremely low pH of the gizzard (3.0) is largely neutralized by the pancreatic secretions such that the lumenal pH remains near 6.0 throughout the segment lying between points 2 and 3. These values of pH are similar to those reported in other avian species and in older living chickens (Sturkie, 1965).

The capability of the cell membrane to allow net movement of acid or base equivalents in response to imposed ion gradients was evaluated at pH 6.0 and pH 7.4 using cells that had been depleted of ATP by prior treatment with rotenone. This treatment has been shown to reduce ATP levels by >95% which allows extensive manipulation of ion gradient conditions across the cell membrane (Carter-Su & Kimmich, 1979, 1980; Kimmich et al., 1985*a*). The $\Delta\psi$ was "clamped" near zero by incubation of the cell population with 40 mM K^+ and 10 μg/ml valinomycin during the ATP depletion interval as well as throughout the experimental observation interval. By preventing changes in $\Delta\psi$, ion fluxes which might otherwise occur due to electrophoretic movement (electrical coupling) can be eliminated.

We have shown previously that ^{14}C-methylamine distribution across the plasma membrane of these isolated cells is a reliable method for defining the magnitude of changes in intracellular pH.[1] Figure 2 shows the redistribution of ^{14}C-methylamine which occurs in response to various imposed ion gradients using ATP-depleted cells that have been incubated in media at either pH 6.0 (Fig. 2*A*) or 7.4 (Fig. 2*B*). As expected, the incubation interval required for steady-state distribution of methylamine depends on the extracellular pH employed. This relates to pH-dependent changes in the concentration

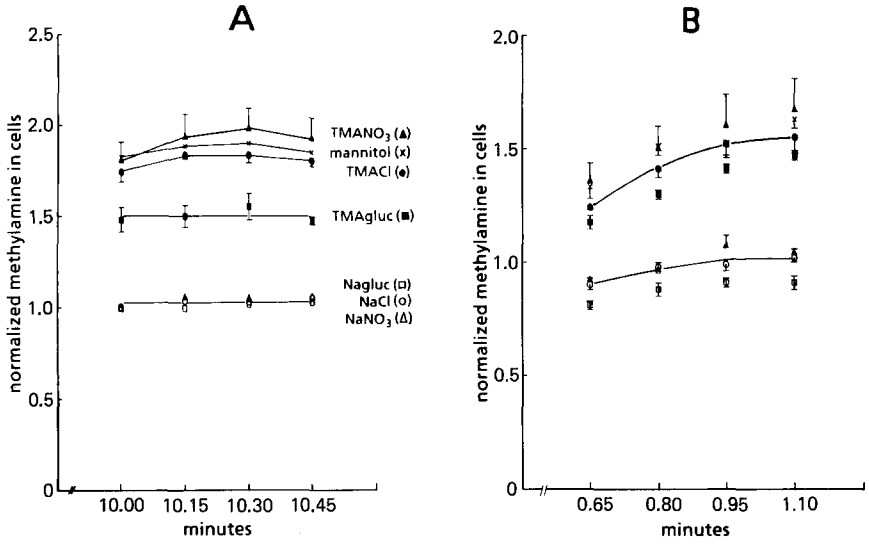

Fig. 2. Effect of experimentally imposed ion gradients on ^{14}C-methylamine distribution across the membrane of ATP-depleted intestinal cells. The cells were isolated and ATP-depleted in medium containing 90 mM NaCl, 40 mM KNO_3, and valinomycin. At time zero, 81 mM of the indicated salt (or 148 mM mannitol) was substituted for 81 mM NaCl. Only standard errors larger than the symbol are presented. (*A*) The isolation medium also contained 25 mM MES-TMA, and all media were titrated to pH 6.0. Cellular methylamine concentrations were based on a 3OMG space measurement for each different ion gradient condition. The methylamine level of cells maintained in NaCl medium was 10.3 ± 0.1 μmol/liter 3OMG space (n = four experiments). (*B*) The isolation medium also contained 5 mM HEPES-TMA and 30 mM mannitol. When methylamine uptake was initiated, cells were exposed to media with 20 mM HEPES-TMA replacing mannitol. All media were titrated to pH 7.4. The methylamine level of cells maintained in NaCl medium was 15.5 ± 0.6 μmol/liter 3OMG (n = three experiments)

of the highly permeant unionized form of methylamine. At pH's below 9.5 this concentration increases as an exponential function of pH so that at pH 7.4 the equilibration time (0.4 min) is more than 10 times faster than at pH 6.0 (5 to 7 min). Because the experiments are performed under conditions of constant extracellular pH, the results demonstrate that specific ion gradients are capable of producing rapid changes in intracellular pH which can persist for several minutes. For instance, when cells preequilibrated in NaCl are diluted into a medium in which the Na^+ was replaced by TMA in order to create an outward Na^+ gradient ($[Na^+]_i > [Na^+]_o$) there is nearly a doubling of cellular methylamine uptake at the steady state (i.e. a rise in cellular $[H^+]$). If both Na^+ and Cl^- are replaced (by mannitol or $TMANO_3$) the same change is observed in pH_i suggesting that the imposition of a Cl^- gradient does not compromise the shift in cellular H^+ induced by an imposed Na^+ gradient. This, in turn, indicates the absence of Cl^-/OH^- or other Cl^--dependent exchange systems which can modify cellular pH under these conditions. Alternatively, if only a Cl^- gradient is created ($[Cl^-]_i > [Cl^-]_o$) by dilution into $NaNO_3$ or Na gluconate there is no change in the distribution of methylamine from that observed by keeping the cells in NaCl. This again implies that Cl^--driven transport systems which can alter cellular pH have no detectable function for this experimental situation. The same general results are observed when the extracellular pH is 7.4 (Fig. 2*B*) or at pH 6.0 (Fig. 2*A*) although the magnitude of the cellular pH change was somewhat lower at the higher pH. In particular, the results suggest that at both pH values, an Na^+/H^+ exchange reaction is present which produces changes in intracellular pH in response to an Na^+ gradient. The data suggest negligible Cl^-/OH^- exchange capability at either pH in the sense that an imposed Cl^- gradient does not modify either methylamine distribution or the response to a Na^+ gradient. The attenuated response for cells exposed to TMAgluconate at pH 6.0 is believed to be due to increased buffering capacity of the cell cytosol due to the slow permeation of gluconate.

Approximately 5 to 7 min is required for methylamine to equilibrate at pH 6.0. Over this time interval, when ion gradients have been imposed, the cells may shrink or swell which by itself might alter the observed cellular uptake of methylamine. Therefore, in the experiments presented in Fig. 2*A*, uptake of ^{14}C-3OMG was used to measure cell volume for each different set of ion gradient conditions. When phlorizin is present, this sugar rapidly equilibrates via a facilitated diffusion transport system localized in the serosal boundary of the cell

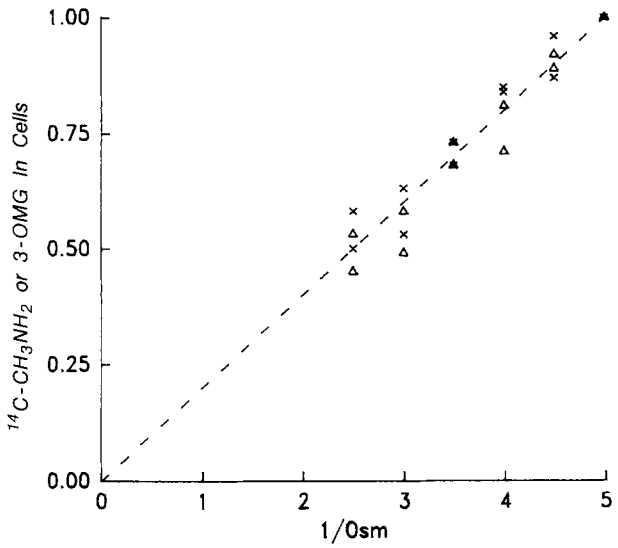

Fig. 3. Effect of medium osmolarity on cellular uptake of ^{14}C-methylamine or ^{14}C-3OMG. The cells were isolated and ATP-depleted at pH 6.0 in 200 mOsm medium containing 25 mM MES-TMA and 80 mM $TMANO_3$. Cells were exposed to higher osmolarities by dilution into media with up to 200 mM cellobiose added and either ^{14}C-methylamine (×) or ^{14}C-3OMG + 200 μM phlorizin (Δ). The data points are the averages of triplicate samples taken after 10 min of incubation from two experiments. The average level of methylamine in these experiments was 15.3 ± 0.3 μmol/liter 3OMG space (n = 12 determinations)

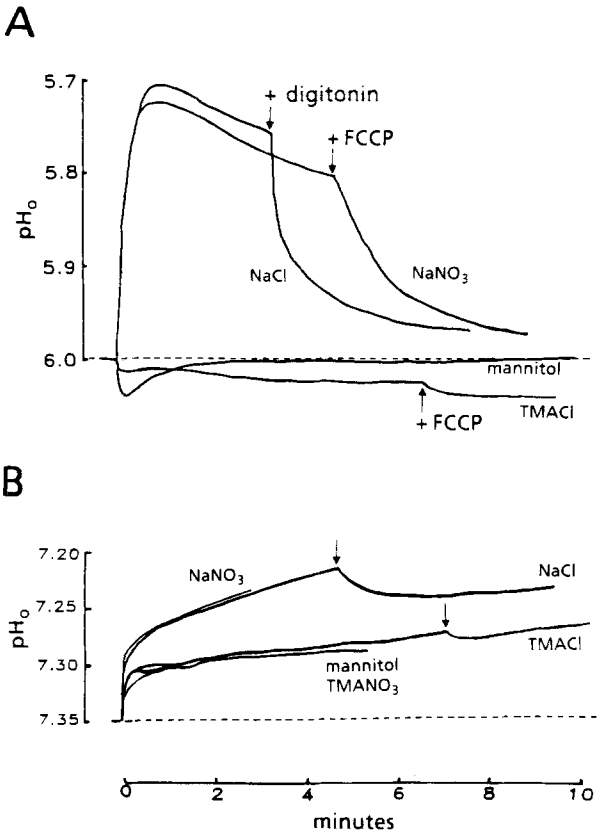

Fig. 4. Effect of experimentally imposed ion gradients on extracellular pH. The cells were isolated and ATP-depleted in a medium containing 215 mM mannitol, 40 mM KNO_3 and valinomycin. At time zero, cells were diluted into a medium in which 81 mM of the indicated salt replaced 154 mM mannitol. Addition of digitonin or FCCP was made at the times indicated by the arrow. Only FCCP was added in (*B*). (*A*) Cells were isolated and ATP-depleted at pH 6.0. A 0.1 pH unit excursion of the supernatant was equivalent to 0.1 μmol of added HNO_3. (*B*) All media also contained 3 mM HEPES-TMA, and cells were isolated and ATP-depleted at pH 7.4. A 0.1 pH unit excursion was equivalent to 0.5 μmol of added HNO_3

(Kimmich, 1981). All data have been normalized to correct for the small changes in cell volume which occurred due to the imposed ion gradients. In order to show that this procedure correctly compensates for changes in cell volume, cells were isolated at pH 6.0 in low osmolarity medium (200 mOsm) and were then equilibrated with 3OMG or methylamine in higher osmolarity media (cellobiose added) for 10 min. The levels of both tracers observed in the cellular pellet following this incubation are shown in Fig. 3. The simplest interpretation of the data in Fig. 3 is that cellobiose is completely impermeant over the 10-min incubation interval and that the cellular accumulation of both 10 μM methylamine and 100 μM 3OMG is within an osmotically active space with negligible binding of either probe. The data suggests that artifacts due to changes in cell volume do not contribute to the lack of observable anion exchange capability in the methylamine experiments.

In order to corroborate and extend the results of the methylamine experiments, changes in medium pH were monitored directly with the use of a pH electrode. By continuously monitoring changes in pH of lightly buffered medium, it is possible to detect and quantitate net fluxes of protons across the plasma membrane. Figure 4 shows examples of typical results obtained by this method in the presence of imposed (inwardly directed) ion gradients and in the presence of 40 mM K^+ plus valinomycin to clamp the membrane potential (Kimmich & Randles, 1984). Large pH_o changes can be induced by imposing gradients of Na^+ salts (NO_3^- or Cl^-). These pH changes are reversed by addition of 200 μM (0.002%) digitonin or 10 μM FCCP. In contrast, TMACl gradients are ineffective in altering medium pH compared to cells maintained in mannitol. The cell-induced drift in pH at the more alkaline pH (Fig. 4*B*) is probably due to ongoing metabolic substrate oxidation in the cells. The rate of drift is temperature dependent, but is not sensitive to the presence of 20 mM 2-deoxyglucose (*data not shown*). In Fig. 4*B*, the initial rapid change in pH upon intro-

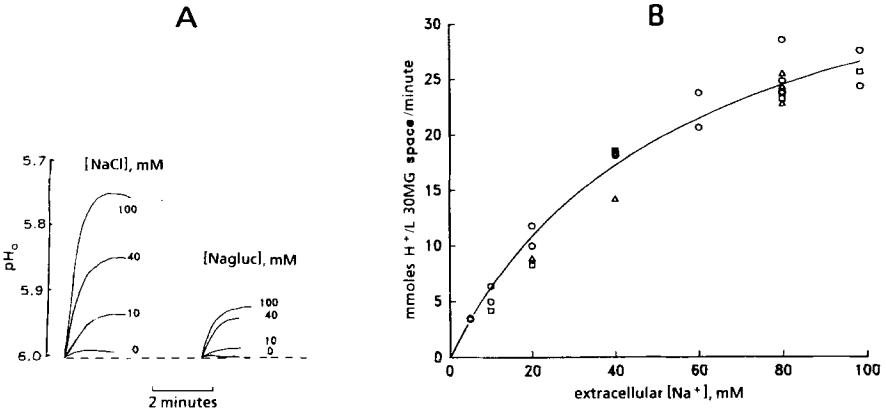

Fig. 5. Relationship between extracellular Na^+ concentration and the initial rate of H^+ efflux for ATP-depleted intestinal cells. The experiments were performed as described in Fig. 4(*A*), in which TMA^+ was substituted for Na^+ and the inwardly directed anion gradient was kept constant at 82 mM. (*A*) Effect of medium buffering capacity on estimation of initial rates of Na^+/H^+ exchange at pH 6.0. Results are shown with Cl^- and gluconate$^-$ in the presence of the indicated Na^+ gradient. (*B*) Compilation of eight experiments relating initial H^+ efflux to the extracellular Na^+ concentration. The anion component of the imposed gradient was either Cl^- (○), gluconate (□), or NO_3^- (△). Sodium replaced TMA^+ at the indicated concentrations. The line is a nonlinear least-squares fit of the data to the Michaelis-Menten equation. The best-fit parameters are V_{max} = 41.6 mmol H^+/liter 3OMG space/min, and K_t = 36.6 mM Na^+

duction of cells illustrates the difficulty in maintaining cells at a constant pH near 7.4 with the lightly buffered media used for this experimental protocol. Note that at either pH the presence of a Cl^- gradient does not modify the excursion induced by a Na^+ gradient.

Even with the increased time resolution of the pH-meter recordings, the evidence suggests that the cells have negligible Cl^-/OH^- exchange capability at pH 6.0 and 7.4. In the absence of anion exchange for base equivalents, it is predicted that the initial rate of H^+ efflux in response to an imposed Na^+ gradient should be the same for a variety of counteranions. In order to test this prediction, the Na^+ activation kinetics of net H^+ efflux were evaluated at pH 6.0. The initial rate of H^+ efflux was calculated from recordings of changes in medium pH (as shown in Fig. 5*A*) and from values from the buffering capacity of the medium. The data in Fig. 5*A* demonstrate the effects of the buffering capacity of gluconate salts on the rate and magnitude of pH excursions with varying Na^+ gradients. However, as shown in Fig. 5*B*, no significant differences were noted in terms of initial rates of Na^+-induced H^+ efflux in media with NO_3^-, gluconate$^-$ or Cl^- gradients as the primary anion. This was true even at Na^+ concentrations as low as 10 mM.

At pH 7.4, the unidirectional influx of $^{22}Na^+$ remains linear for an interval of approximately 40 sec when various Na^+ gradients are experimentally imposed (Kimmich & Randles, 1984). This implies that the $\Delta\mu_{Na}$ remains relatively consistent over this interval. Fortunately, cellular uptake of ^{14}C-methylamine reaches a transient steady state within 20 to 40 sec under the same experimental conditions. In addition, steady-state methylamine levels, at constant pH_o, should be directly proportional to pH_i. Taken together, these facts imply that relative methylamine levels at pH 7.4 will reflect relative flux values catalyzed by the Na^+/H^+ exchange system. Therefore we have used short-term methylamine distribution to evaluate relative levels of activity of the Na^+/H^+ exchanger at different extracellular Na^+ concentrations ($[Na^+]_i$ = 0). An advantage of using methylamine for this purpose is that fewer cells may be used than in pH-meter experiments, so that the medium pH may be better controlled. An example of this type of experiment is shown in Fig. 6*A*, where the methylamine level is shown to change in response to changes in the imposed Na^+ gradient. Three such experiments are averaged in Fig. 6*B*, where the change in methylamine levels is expressed as a function of extracellular Na^+ concentration.

It is possible to convert the relative units in Fig. 6*B* to actual H^+ flux by using an estimate for initial rates of H^+ efflux derived from the data. In order to correct for the pH drift at pH 7.4, several measurements were taken between 0.25 and 1.0 min after the addition of cells. Subtraction of this Na^+-independent change in proton production from the total H^+ change at each time point provided a value for Na^+-dependent flux. These data indicate a rate of 1.4 ± 0.4 mmol H^+/liter 3OMG space/min in the presence of 81 mM extracellular Na^+ and 0 mM intracellular Na^+ (three experiments). Using the

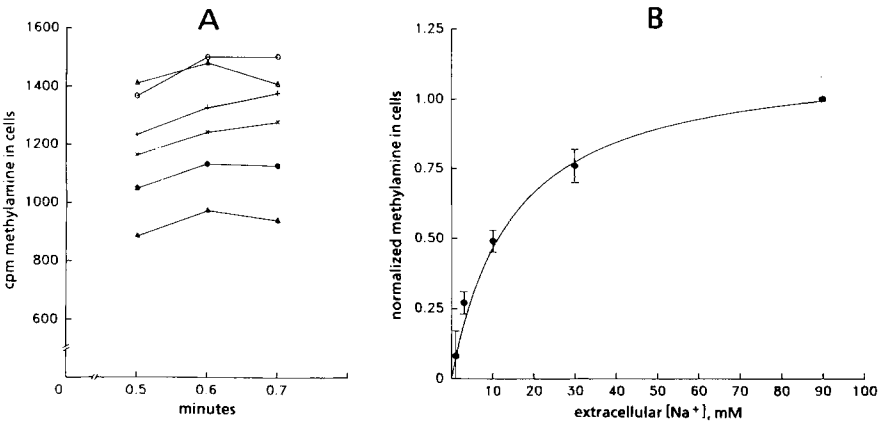

Fig. 6. ^{14}C-Methylamine distribution (transient steady state) at different extracellular Na^+ concentrations ($[Na^+]_i$ = 0) at pH 7.4. The experiments were performed as described in Fig. 2(*B*) where TMACl was substituted for NaCl. (*A*) Cells were exposed to 0 (○), 1 (△), 3 (+), 10 (×), 30 (●), or 90 (▲) mM Na^+. (*B*) The three time points shown in (*A*) were averaged for each estimate of relative methylamine level, and the data were expressed relative to the difference in methylamine accumulation between 0 and 90 mM Na^+ conditions. Three experiments are compiled. The line is a nonlinear fit of the data to the Michaelis-Menten equation. The best-fit parameters are V_{max} = 1.16 normalized units, and K_t = 14.9 mM Na^+

equation of best fit to the data of Fig. 6*B*, this implies that the V_{max} is approximately 1.7 mmol H^+/liter 3OMG space/min.

Monensin and certain other ionophores can be used to confer proton/cation exchange capacity of many membrane systems. Figure 7*A* shows that isolated chick intestinal cells also respond to monensin. If amiloride is present to largely inhibit the natural Na^+/H^+ exchange system, an imposed inward gradient of $NaNO_3$ produces only a modest change in extracellular pH (lower trace). When monensin is added, a considerably larger pH_o excursion is observed and addition of digitonin causes an abrupt relaxation of the established H^+ gradient and a return of the pH tracing to the original baseline. In the absence of amiloride, an imposed gradient of either NaCl or $NaNO_3$ causes a large change in pH_o (upper two traces) which is not enhanced by subsequent addition of monensin. This implies that the intrinsic Na^+/H^+ antiport system has established a thermodynamic equilibrium between the transmembrane Na^+ and H^+ gradients. This in turn implies that the native exchanger dominates the fluxes of Na^+ or H^+ by all other routes under these conditions.

In order to demonstrate that monensin does not catalyze appreciable K^+/H^+ exchange under similar conditions, inwardly directed K^+ gradients were imposed on the cells and the effects of monensin and nigericin (a known K^+/H^+ exchanger) were compared. As shown in Fig. 7*B*, addition of monensin causes negligible changes in medium pH whereas the addition of nigericin causes a net H^+ efflux which is dissipated by adding FCCP. In four experiments similar to those shown in Fig. 2*A* (pH 6.0), we have also shown that monensin cannot disturb the change in distribution of methylamine induced by an imposed Na^+ gradient (control = 0.62 ± 0.02; + monensin = 0.66 ± 0.02). Again this suggests an Na^+/H^+ exchange process in the cell membrane which is near thermodynamic equilibrium. In contrast, in three experiments at pH 7.4 (protocol identical to Fig. 2B), the relative methylamine distribution was significantly altered by addition of monensin (control = 1.53 ± 0.06; + monensin = 1.92 ± 0.08). This reflects the sharply lower capacity (V_{max}) the natural Na^+/H^+ exchanger exhibits at pH 7.4.

A final set of experiments evaluated the possibility that the plasma membrane might have significant H^+ conductance and/or K^+/H^+ exchange capability. For this purpose cells were isolated and ATP-depleted in medium containing 155 mM KCl and then pH electrode recordings were taken in either the absence or presence of an imposed K^+ gradient (extracellular TMA^+ replacing K^+ at time zero). As shown in Fig. 8*A*, changes in medium pH due to the addition of nigericin are dependent on the presence of an imposed K^+ gradient. These results suggest that K^+ and protons are near equilibrium across the membrane following the preincubation with K^+, and that the cells have negligible native K^+/H^+ exchange capability. The small change in pH due to the addition of FCCP suggests that the cells maintain a small H^+ gradient across the membrane (possibly due to a Donnan potential). As shown in Fig. 8*B*, when H^+ conductance is established (FCCP added) there is a redistribution of protons

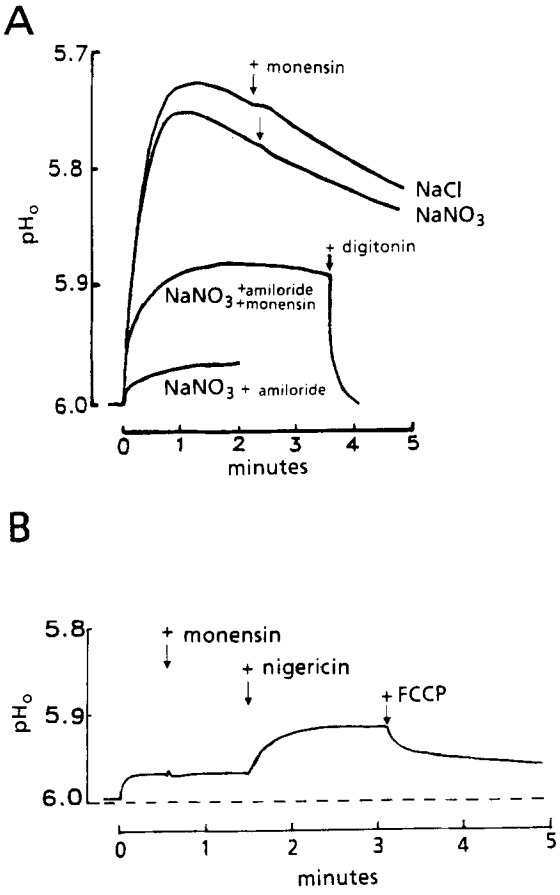

Fig. 7. (*A*) Effects of amiloride and monensin on H^+ fluxes induced by imposed Na^+ gradients. The experiments were performed as described in Fig. 4(*A*). Amiloride was added to the indicated cell suspensions 5 min prior to dilution creating the ion gradients. Cells were exposed to ionophores at the times indicated by the arrows. (*B*) Effect of monensin and nigericin on H^+ efflux in the presence of an imposed K^+ gradient. Cells were isolated and ATP-depleted at pH 6.0 in medium containing 300 mM mannitol. At time zero, they were diluted into a medium with 133 mM KCl replacing 257 mM mannitol and the medium pH was monitored. Ionophores were added at the indicated times

across the membrane if a K^+ gradient is present. Also shown in Fig. 8*B*, the addition of valinomycin produces a significant electrophoretic driving force for H^+ flux under these conditions. In Fig. 8*C*, the use of valinomycin alone (in the presence of a K^+ gradient) produces only a small net flux of protons (four experiments) which can be markedly enhanced by subsequent addition of FCCP. This suggests that the native membrane contains only limited H^+ conductance which can respond to the valinomycin-induced change in membrane potential. As expected, if the cells have only slight native H^+ conductance, increasing the H^+ conductance of valinomycin-treated cells with FCCP causes a dramatic change in response to the electrical driving force.

Discussion

Chick enterocytes that have been depleted of cellular ATP maintain expression of a variety of transport processes demonstrable in normally energized cells or in isolated membrane vesicles. ATP-depleted cells have been used previously to examine the electrogenicity and stoichiometry of the Na^+/glucose cotransporter (Carter-Su & Kimmich, 1979; Kimmich, 1983; Kimmich & Randles, 1984; Kimmich et al., 1985*b*). They have also been used to examine Na^+-dependent acidic amino-acid transport (Wingrove & Kimmich, 1983) and ion conductance pathways (Kimmich et al., 1985*a*). An advantage of using cells depleted of ATP is that they offer the possibility for careful experimental control of the thermodynamic driving forces which energize transport (membrane potential and ion-gradient conditions) in a manner similar to approaches used with vesicle preparations. The cellular compartment is much larger than vesicular space, however, which allows for much longer intervals of linearity in many undirectional flux measurements. This in turn allows greater reliability of quantitative measurements relating flux dependence to $\Delta\psi$ or $\Delta\mu_{Na^+}$ parameters. In addition the cell preparation represents an experimental system in which the cell membrane has not undergone lysis and resealing with the attendant questions regarding possible modification of transport properties. The latter point has become increasingly important since it has been shown recently that relative rates of vesicular Na^+ transport routes (Sabolic & Burckhardt, 1984) and relative ion permeabilities (Wright, 1984) are dependent in part on the membrane isolation procedure. Intact cells also represent the smallest tissue element capable of establishing a steady-state $\Delta\tilde{\mu}_{Na^+}$ for allowing evaluation of transport events under physiological investigation conditions.

The lumenal contents of chick small intestine have a pH near 6.0. Despite the acidic contents of the lumen, the intracellular pH of a normally energized cell is probably maintained near pH 7.0 to facilitate metabolic activity in these glycolytically active cells (Kimmich, 1970; Roos & Boron, 1981). This suggests that the brush-border membrane of the chick intestinal cell may be responsible for maintaining a significant H^+ gradient *in vivo*. If the intracellular Na^+ activity for these cells is similar to that determined with ion-selective microelectrodes for intact tissue, an Na^+/H^+ exchanger in the chick brush-border membrane at partial ionic equilibrium

($Na_o^+/Na_{a_i}^+ = H_o^+/H_i^+$) would maintain intracellular pH near 7.0 (Armstrong et al., 1979; Garcia-Diaz & Armstrong, 1980; Fisher & Spring, 1984; Weinman & Reuss, 1984). If a Cl^-/OH^- (or Cl^-/HCO_3^-) exchange reaction was operative in the brush-border membrane at the same rate as Na^+/H^+ exchange, then intracellular pH would be drawn to values near pH 6.0. On this basis, the suggestion for dual-exchanger capability of equal capacity as a mechanism for the coupling of Na^+ and Cl^- fluxes across the brush-border membrane seems unlikely. This prediction is supported by the present work in which Cl^-/OH^- exchange activity was not detected.

On the other hand, we were able to demonstrate Na^+/H^+ antiport readily by several criteria. In order to rule out electrophoretic fluxes as the basis of the supposed coupling it was necessary to use a K^+-valinomycin "voltage-clamp" approach. The efficacy of the clamping procedure is indicated by the fact that substituting Cl^- for the less permeant gluconate anion (Figs. 2 and 3) did not alter the magnitude of the Na^+-dependent H^+ gradients established. Dissipation of the H^+ gradient would have been expected due either to an ineffective clamp or to significant Cl^-/OH^- antiport. The data thus rules out either possibility. Also, we have recently described measurements for the relative permeabilities of a number of ions in the isolated anion intestinal cell preparation (Kimmich et al., 1985*a,b*). By employing the Goldman-Hodgkin-Katz equation (Goldman, 1949) in conjunction with these relative permeabilities it is possible to estimate that the largest change in membrane potential in these experiments ($TMANO_3$ replacing mannitol) is approximately 6 mV. A change in potential of this small magnitude would induce a change in electrophoretic flux of no more than 11% for fluxes acting in accordance with the Goldman flux equation.

Because the quality of the voltage-clamping procedure precludes electrophoretic coupling, the results from experiments using methylamine distribution or the pH electrode suggest the presence of a directly coupled Na^+/H^+ exchange reaction in the plasma membrane of the isolated cells. The lack of further response to monensin at pH 6.0 suggests that the Na^+/H^+ exchange accounts for the major proportion of total Na^+ and H^+ flux in the ATP-depleted cells. These results support data reported previously from brush-border membrane vesicle preparations of intestinal and renal tissue (Murer et al., 1976; Kinsella & Aronson, 1980; Warnock et al., 1982; Knickelbein et al., 1983).

The unidirectional H^+ efflux due to Na^+/H^+ exchange is insensitive to inwardly directed anion gradients. This implies that the chick intestinal Na^+/H^+ exchange is not a partial reaction of directly coupled Na^+, H^+ and Cl^- transport of the type reported for invertebrate neurons and muscle fibers (Thomas, 1977; Russell et al., 1983). Instead, the chick intestinal exchanger is similar in characteristics to the rabbit renal exchanger (Kinsella & Aronson, 1980). In addition, the Na^+ activation kinetics for the chick intestinal exchanger can be well fit by the Michaelis-Menten equation. This is consistent with results derived from the use of fluorescent sensors of H^+ gradients (Warnock et al., 1982; Ives et al., 1983) and intracellular pH (Grinstein et al., 1984) to study the Na^+/H^+ exchanger of mammalian cells.

The Na^+ activation kinetics of net H^+ flux by the chick Na^+/H^+ exchanger change dramatically with different experimental conditions. The results

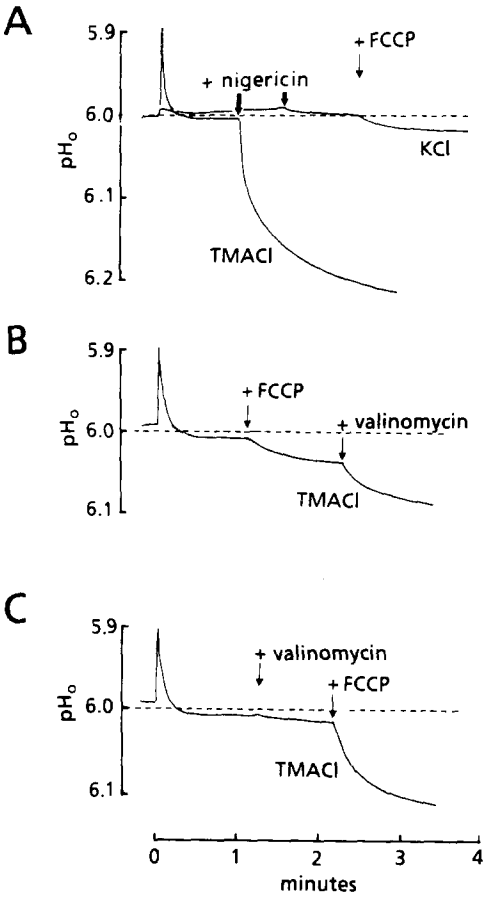

Fig. 8. Effects of nigericin, FCCP and valinomycin on H^+ fluxes in cells with an imposed K^+ gradient. Cells were isolated and ATP-depleted at pH 6.0 in medium containing 155 mM KCl. At time zero, cells were exposed to medium of identical composition (indicated by KCl in the figure), or with 133 mM TMACl replacing 133 mM KCl (indicated by TMACl). Nigericin, FCCP or valinomycin were added at the indicated times. The brief spike of acidification is always observed when an outwardly directed K^+ gradient is introduced, but its origin is unknown

suggest that the K_t for Na_o^+ changes from 57 to 15 mM as the medium pH is changed from 6.0 to 7.4. This change in K_t is similar to that observed by Kinsella and Aronson (1981) in rabbit renal brush-border vesicles as the extravesicular pH is changed from pH 6.6 to 7.5.

Nord et al. (1984) have suggested that changes in H^+-gradient conditions (rather than pH *per se*) are responsible for the observed differences in K_t in rabbit renal vesicles. However, the observed change in K_t in the chick intestinal cells is probably not due to different H^+-gradient conditions because two lines of evidence suggest that the intracellular pH of ATP-depleted cells is approximately the same as extracellular pH. Addition of FCCP to ATP-depleted cells does not cause a marked shift in either methylamine distribution (*data not shown*) or medium pH, which would be expected if the cells maintained a significant H^+ gradient. Second, the equilibrium levels of 10 μM methylamine observed in ATP-depleted cells at the two pH values are close to expected values for $pH_i = pH_o$ (10.3 ± 0.1 μmol/liter 3OMG space, pH 6.0; 15.5 ± 0.6 μmol/liter 3OMG space, pH 7.4).

Other laboratories have reported that when H^+ is equilibrated across the rabbit brush-border vesicle membrane, the V_{max} of transport decreases approximately 10-fold between pH 6.0 and 7.5 (Kinsella & Aronson, 1981; Aronson et al., 1983), which is consistent with our observations. Our data is not consistent with that of Nord et al. (1984), however, who observed no effect on V_{max} in rabbit membrane vesicles due to changes in extracellular pH. It is not known whether the alteration in V_{max} of the chick Na^+/H^+ exchanger is due to an internal H^+ modifier site as proposed by Aronson et al. (1982), or has some other mechanistic basis.

Detailed examination of the effect of anion gradients on the H^+ overshoot caused by Na^+/H^+ exchange at pH 6.0 suggest that anion gradients neither contribute to the net H^+ efflux rate nor disturb the equilibrium established by the Na^+/H^+ exchange reaction. The small differences observed in net H^+ flux when NO_3^- or Cl^- are present may be attributed to minor changes in $\Delta\psi$ that are not controlled by the K^+ plus valinomycin "voltage clamp." It is possible to demonstrate limited but measurable H^+ conductance in the plasma membrane of the cells (Fig. 8) which is consistent with the latter possibility.

Because the experiments at pH 7.4 were conducted at physiological $[OH^-]$ but low $[HCO_3^-]$, the data suggest that any physiologically important anion exchange for base equivalents does not utilize OH^- as a substrate. The absence of added CO_2 in the present experiments does not allow us to rigorously exclude the possibility of Cl^-/HCO_3^- exchange capability in the cells. It *is* possible to exclude an exchange system with high affinity for HCO_3^- since atmospheric CO_2 will produce ~1 mM HCO_3^- in all of the solutions used for these experiments. Since the experiments were performed using whole intestinal epithelial cells, they additionally suggest a lack of Cl^-/OH^- exchange capability in both the brush-border and basolateral membranes. A similar conclusion relating just to brush-border anion exchange was reached by Cassano et al. (1984) from experiments using brush-border vesicles from jejunal and renal cortex epithelia. Our data for intact cells suggest that the divalent cation precipitation techniques used to isolate membrane vesicles are not responsible for inactivation of Cl^-/OH^- exchange capability.

The experiments involving monensin demonstrate that the Na^+/H^+ exchanger is responsible for a very large fraction of the total Na^+ and H^+ flux of the cells at pH 6.0. The overshoot of H^+ flux caused by the Na^+/H^+ exchange reaction approaches a partial ionic equilibrium which is uncompromised in any significant way by leak fluxes of protons or OH^- by other routes. Addition of monensin to ATP-depleted cells at pH 7.4 caused a significant increase in the efficiency of conversion of an imposed Na^+ gradient to a H^+ gradient, however, suggesting that the Na^+/H^+ exchange is much further from partial ionic equilibrium at the more alkaline pH.

The data from experiments in which K^+ gradients are imposed suggest that the cells do not catalyze K^+/H^+ exchange at pH 6.0. In the absence of nigericin, the cells are not capable of using the potential energy represented by an imposed K^+ gradient to produce a measurable net H^+ flux. The H^+ flux observed after addition of nigericin demonstrates that the K^+ gradient is not rapidly dissipated. The magnitude of the pH change under these conditions illustrates the magnitude of the net H^+ flux that would be expected if significant K^+/H^+ exchange were occurring.

Other experiments using K^+ gradients suggest that the cells have only a slight H^+ conductance. Manipulation of the membrane potential with valinomycin produced a slow net H^+ flux consistent with a conductance pathway for H^+ (or OH^-). Addition of FCCP to increase membrane H^+ conductance caused a large net H^+ flux in response to a valinomycin-induced $\Delta\psi$. These data are different from those reported for vesicle preparations, where the addition of H^+ ionophores produced a relatively small change in the net H^+ flux under similar conditions (Sabolic & Burckhardt, 1983; Cassano et al., 1984). It is possible that the membrane vesicle prep-

aration procedures enhance membrane H^+ permeability, as suggested by Sabolic and Burckhardt (1983) and by Cassano et al. (1984).

In summary, the ATP-depleted cells demonstrate Na^+/H^+ exchange and H^+ conductance, but show no evidence of K^+/H^+ exchange or Cl^-/OH^- exchange capability. The Na^+/H^+ exchange reaction in the chick cells has many of the features previously demonstrated in vesicle preparations (activation at acidic pH, sensitivity to pH of V_{max} and K_t for Na^+). The results also demonstrate that the Na^+/H^+ exchange reaction is capable of rapid alterations in cellular pH despite cellular buffering capacity. The lack of Cl^-/OH^- exchange supports conclusions derived from numerous vesicle experiments with epithelial and related tissue and suggests that vesicle preparation techniques or microenvironments within the vesicle are not responsible for the lack of observable Cl^-/OH^- exchange with those experimental models.

This work was supported by a grant from the National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases, AM15365.

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Received 8 April 1985; revised 17 July 1985