J. Membrane Biol. 87, 67–75 (1985)

The Journal of
Membrane Biology
© Springer-Verlag 1985

# Effects of Internal and External pH on Amiloride-Blockable $Na^+$ Transport across Toad Urinary Bladder Vesicles

Haim Garty, Ethan D. Civan*, and Mortimer M. Civan**
Department of Membrane Research, The Weizmann Institute of Science, Rehovot 76100, Israel

**Summary.** We have examined the effect of internal and external pH on $Na^+$ transport across toad bladder membrane vesicles. Vesicles prepared and assayed with a recently modified procedure (Garty & Asher, 1985) exhibit large, rheogenic, amiloride-sensitive fluxes. Of the total $^{22}$Na uptake measured 0.5–2.0 min after introducing tracer, 80 ± 4% (mean ± SE, $n = 9$) is blocked by the diuretic with a $K_I$ of $2 \times 10^{-8}$ M. Thus, this amiloride-sensitive flux is mediated by the apical sodium-selective channels. Varying the internal (cytosolic) pH over the physiologic range 7.0–8.0 had no effect on sodium transport; this result suggests that variation of intracellular pH *in vivo* has no direct apical effect on modulating sodium uptake. On the other hand, $^{22}$Na was directly and monotonically dependent on external pH. External acidification also reduced the amiloride-sensitive efflux across the walls of the vesicles. This inhibition of $^{22}$Na efflux was noted at external $Na^+$ concentrations of both 0.2 μM and 53 mM.

These results are different from those reported with whole toad bladder. A number of possible bases for these differences are considered and discussed. We suggest that the natriferic response induced by mucosal acidification of whole toad urinary bladder appears to operate indirectly through one or more factors, presumably cytosolic, present in whole cells and absent from the vesicles.

**Key Words** sodium channels · pH dependence · mucosal acidification · intracellular pH · apical $Na^+$ entry · $Na^+$ permeability

## Introduction

Transcellular sodium transport proceeds across tight epithelia in two steps (Koefoed-Johnsen & Ussing, 1958), apical entry through ion-selective, amiloride-inhibitable channels and basolateral extrusion through the Na,K-exchange pump. Several lines of evidence suggest that over time frames of seconds to minutes, this transport is primarily regulated at the apical plasma membrane. A great number of factors have been considered to play possible roles in this regulation, among them the ionic compositions of the intracellular and extracellular fluids (Civan, 1983). One particularly striking phenomenon has been the marked stimulation of mucosal-to-serosal sodium movement across the urinary bladder of the toad produced by acidification of the mucosal medium (Leaf, Keller & Dempsey, 1964). Whether this stimulation reflects an extracellular or an intracellular effect, whether the mode of action is direct or indirect, and the possible physiologic significance of the phenomenon have been unclear.

In the present work, we have been concerned primarily with the question whether mucosal acidification increases sodium transport by a direct membrane effect, and secondarily with the sidedness of the effect, mucosal or intracellular. It is now feasible to monitor the intracellular pH of tight epithelia noninvasively with pH-selective microelectrodes (M. Duffey, E. Kelepouris, K. Peterson-Yantorno & M.M. Civan, *unpublished observations*) and with $^{31}$P (Bond et al., 1981; Nunnally et al., 1983; Lin, Shporer & Civan, 1984) and $^{19}$F NMR spectroscopy (Civan et al., 1985). However, it must be emphasized that study of whole cell preparations cannot rigorously establish whether changes in pH or other single isolated parameters exert direct effects on the sodium channel or whether such effects reflect complex interactions with other intracellular processes.

We have approached the problem by measuring amiloride-blockable $^{22}Na^+$ fluxes in apical membrane vesicles rather than in whole epithelium. In isolated membrane preparations, the channels are uncoupled from cytoplasmic factors and only direct pH effects are expected to influence their $Na^+$ permeability. In order to assess apical $Na^+$ conductance, we have utilized a recently described procedure permitting measurement of

* *Present address:* College of Arts and Sciences, University of Pennsylvania, Philadelphia, PA 19104.

** *Permanent address:* Departments of Physiology and Medicine, University of Pennsylvania School of Medicine, Richards Building/G4, Philadelphia, PA 19104.

amiloride-blockable $^{22}Na$ uptake in anatomically heterogeneous populations of toad bladder vesicles (Garty, Rudy & Karlish, 1983; Garty, 1984; Garty & Asher, 1985). With this approach, $^{22}Na$ uptake primarily by the apical vesicles can be measured by taking advantage of the selective permeability characteristics of the apical membranes. Specifically, sodium uptake is measured in the presence of a negative inside membrane potential imposed preferentially across the apical vesicles by a chemical gradient either of NaCl or of KCl in the presence of valinomycin. In contrast, little $^{22}Na$ is taken up by the basolateral vesicles since their transmural electrical gradients are dissipated by the relatively large $Cl^-$ conductances of the basolateral membranes. The $^{22}Na$ uptake by these vesicles has been demonstrated to be ion-selective, rheogenic, and under favorable conditions largely inhibitable by low concentrations of amiloride ($K_I = 2 \times 10^{-8}$ M) (Garty & Asher, 1985). The amiloride-blockable flux in vesicles can also be inhibited by $Ca^{2+}$ ions. From the sidedness of the $Ca^{2+}$ and amiloride effects, it has been concluded that the membranes of these apical vesicles are "right-side out" with respect to their cellular orientation (Garty, 1985). In addition to uptake, it is possible to monitor amiloride-sensitive tracer efflux from these vesicles induced by membrane depolarization.

Using these procedures, we have succeeded in measuring the effects of separately varying intra- ($pH_i$) and extravesicular pH ($pH_o$) on radioactive sodium uptake. We have found that alterations in cytosolic and external pH exert direct membrane effects which are different both from each other and from the observations reported for whole toad urinary bladder.

## Materials and Methods

### Preparation of Vesicles

Specimens of the toad *Bufo marinus* of mixed sex and Mexican origin (Lemberger, Oshkosh, WI) were doubly pithed and deblooded by transventricular perfusion with approximately 500 ml of a Ringer's solution consisting of (in mM): NaCl, 110.0; $CaCl_2$, 1.0; $MgCl_2$, 0.5; $KH_2PO_4$ and $K_2HPO_4$, 3.5; at a pH of 7.5. The urinary bladders were excised and rinsed several times in an ice-cold homogenizing medium containing (in mM): KCl, 90; sucrose, 45; $MgCl_2$, 5; EGTA [ethyleneglycol-bis($\beta$-aminoethyl ether)-N,N,N',N'-tetraacetic acid], 1; and Tris-HCl, 5 or 20; at a pH of 7.8.

The epithelium was scraped off the underlying connective tissue with a glass slide, and the cells were dispersed in the homogenizing medium by rapidly drawing them in and ejecting them out from a Pasteur pipette. The cell suspension was washed twice in the homogenizing medium at 0°C and then incubated for 30–45 min at 25°C, after which the cells were broken by applying 6 sec of shear forces with a polytron homogenizer (Ystral GmbH, Göttingen, FRG). Intact cells and nuclei were separated into a pellet by centrifuging the homogenates at 1,000 × *g* for 5 min, following which the supernatant solutions were centrifuged for a further hour at 0°C at 27,000 × *g*. The microsomal pellets were resuspended in minimal volumes of the homogenizing solution for experimental study within 24 hr.

### Assay of $^{22}Na$ Transport

Influx and efflux measurements of $^{22}Na$ transfer were conducted at 25°C as previously described (Garty, 1984; Garty & Asher, 1985). The microsomal preparation, containing 50–100 $\mu$g protein, was first eluted through short Dowex (50 WX8) columns (50–100 mesh, Tris form) with 0.7–1.4 ml 175 mM sucrose. This initial step established a chemical gradient for potassium across the vesicular walls by exchanging external cation for Tris and diluting the external medium with isotonic sucrose solution. The pH of the eluent was adjusted with small volumes of Tris buffer, and valinomycin was added to a final concentration of 3 $\mu$M. Aliquots were thereupon immediately distributed among vials containing approximately 4 $\mu$Ci ml$^{-1}$ $^{22}Na$ (0.2–0.4 $\mu$M $Na^+$), 20 mM buffer (Tris or MES), and either 1.5 $\mu$M amiloride or the pure sucrose. Unless otherwise specified, the total and amiloride-insensitive $^{22}Na$ uptakes were measured by taking one (at 1 min) or more (at 0.5 and 2 min) timed aliquots (150 $\mu$l, 4–25 $\mu$g protein) from the radioactive suspensions, transferring them to Dowex columns precoated with bovine serum albumin, and eluting the vesicles with 1.5 ml ice-cold sucrose solution into counting vials. The initial rate of sodium uptake was calculated as pmol $^{22}Na$ · mg protein$^{-1}$ · min$^{-1}$. In efflux measurements, the vesicles were first allowed to accumulate $^{22}Na$ for 10–15 min. After removing one aliquot, the residual vesicle suspension was diluted 1 : 2 with a medium containing 110 mM of either NaCl or KCl and either amiloride or diluent and sampled again 1–2 min later. The protein content of the sample was determined (Bradford, 1976) with a separate aliquot of the eluted vesicles. The $^{22}Na$ was assayed with a $\beta$-counter.

### pH Clamping

In experiments assessing the effects of $pH_o$ on $^{22}Na$ transport, the vesicles were prepared to contain 20 mM Tris-HCl at a pH of 7.7–7.8, unless otherwise specified. Elution of the microsomes through the Dowex columns acidified the external medium by exchanging Tris for $K^+$. Therefore, the pH of the eluent was then readjusted to 7.8 by adding a minimal volume of Tris base. At zero time, the external pH was clamped to the desired value with the 20 mM buffer (Tris or MES) present in the radioactive solution. In this way, the pH gradient was established at the same time that the radioactive tracer was introduced.

In experiments that assessed the effects of $pH_i$, the vesicles were prepared to contain 5 mM Tris-HCl, divided into several aliquots and incubated for at least 2 hr at different pH values. The external pH was readjusted to 7.8–8.0 after eluting the vesicles through the Dowex column, i.e., approximately 30 sec before initiating the experiment.

### Fluorescence Measurements

Measurements of intracellular pH were conducted with the pH indicator pyranine (8-hydroxy-1,3,6-pyrenetrisulfonate) (Kano &

Fendler, 1978; Clement & Gould, 1981). The sample was excited at 465 nm and the fluorescence measured at 510 nm with a luminescence spectrometer (LS-5, Perkin Elmer Corp., Norwalk, CT).

### Chemicals

Valinomycin, EGTA, FCCP (carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone) and bovine serum albumin (fraction V) were obtained from the Sigma Chemical Company (St. Louis, MO), Dowex beads from Fluka AG (Buchs, Switzerland), pyranine from Kodak (Rochester, NY), and $^{22}$NaCl (carrier-free, 1.66 mCi · ml$^{-1}$) from Amersham Radiochemicals (Buckinghamshire, UK). The amiloride was a gift from Merck, Sharp and Dohme (GmbH, Munich, FRG).

## Results

### Time Course of Proton Equilibration

One aim of the present study was to determine the effects on $^{22}$Na transport induced by separately clamping the pH of the intra- and extravesicular phases. Such measurements are meaningful only if the rate of proton equilibration across the walls of the vesicles is no faster than the rate of experimental sampling. The feasibility of the study with the current vesicles was indicated by the data of Fig. 1. The fluorescent dye pyranine was entrapped at a concentration of 1 mM within vesicles at the time of their formation. The molecule's fluorescence is dependent upon pH. When the external pH was reduced, the measured fluorescence underwent an initial abrupt fall, presumably reflecting the effect of acidification on dye adsorbed onto the external vesicular surface. Subsequently, the fluorescence underwent a second slower phase of decline. We interpret this slower rate of change of fluorescence as reflecting the rate of proton transfer and pH change of the intravesicular fluid. This interpretation is supported by the observation that adding FCCP, a proton ionophore, abruptly accelerated the rate of change of fluorescence. The combined presence of the proton ionophore (FCCP) and the potassium ionophore (valinomycin) abolished the second slow phase of pH equilibration but not the initial rapid response to subsequent alkalinization and acidification of the extravesicular medium.

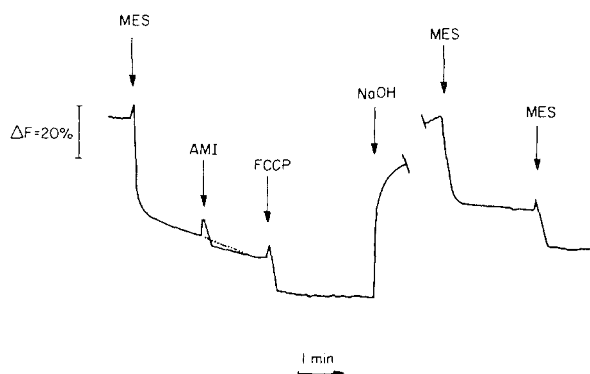


**Fig. 1.** Effect of external pH on internal pH of vesicles prepared from toad urinary bladder. The preparation consisted of 130 μl of vesicles containing 1 mM pyranine in the homogenization solution, 750 μl of 6% sucrose solution, and 3 μl of a 1 mM valinomycin solution at an external and internal pH of 7.9. Following external acidification by adding 20 μl of 0.5 M MES solution (first arrow labeled *MES*), the extravesicular pH fell to 6.5 and the fluorescence sharply fell. Thereafter, a slower phase was noted in which the fluorescence continued to decline. This rate of fall of fluorescence was slightly reduced by the addition of 50 μl of amiloride solution (2 mM, *AMI*). However, following the subsequent addition of 3 μl of 1 mM FCCP, the fluorescence promptly declined to a minimal stable value. At the arrow labeled *NaOH*, 0.5 M NaOH was added in two steps; the first was the addition of 10 μl, and the second (*not shown*) was the addition of 3 μl solution. In the presence of valinomycin and FCCP, changes in external pH induced rapid changes in fluorescence without the appearance of the second slow phase noted earlier in the trace; at the second and third arrows labeled *MES*, two volumes of 20 μl each of 0.5 M MES were added sequentially

### Effect of $pH_o$

$^{22}$Na uptake was measured in two parallel aliquots of vesicles containing either no amiloride or 1.5 μM amiloride. The uptake through the amiloride-sensitive channels was calculated as the difference in $^{22}$Na content between the two aliquots. In our early experiments, the magnitude of the amiloride-sensitive uptake was low and variable with respect to the background amiloride-insensitive level. In the later series of 12 experiments, the modified procedure of Garty and Asher (1985) was used to prepare the vesicular suspensions. When care was exercised to: (i) reduce the extracellular free calcium concentration to well under $10^{-8}$ M before scraping the epithelial cells from the mucosal surface, and (ii) preincubate the scraped cells at 25°C for 30–45 min, the assays were far more reproducible, and uptake through the high-affinity channels accounted for 80 ± 4% (mean ± SE, $n = 9$) of the total measured $^{22}$Na uptake. The data of Figs. 1, 3, 4*C* and 5, and those obtained while changing $pH_i$ in one preparation, were collected during the early phase of the study. All of the other results presented were obtained during the course of the later series of experiments.

Figure 2*A* and Table 1 present the effect of external pH on $^{22}$Na uptake through the amiloride-sensitive channels, obtained from measurements of aliquots of vesicles at six different values of $pH_o$,

**Table 1.** Dependence of initial $^{22}Na$ uptake on external and internal pH

| Variable | pH | $^{22}Na$ content (cpm/sample) | | | |
|---|---|---|---|---|---|
| | | Without amiloride | | With amiloride | |
| | | Intravesicular | Total suspension | Intravesicular | Total suspension |
| $pH_o$ | 8.25 | 4447 | 76,004 | 834 | 74,166 |
| | 7.9 | 3005 | 87,739 | 770 | 80,210 |
| | 7.45 | 2043 | 89,217 | 467 | 89,345 |
| | 7.1 | 1059 | 82,899 | 331 | 95,930 |
| | 6.6 | 765 | 92,489 | 214 | 101,579 |
| | 6.1 | 401 | 98,621 | 158 | 106,240 |
| $pH_i$ | 9.0 | 2418 | 82,523 | 418 | 94,457 |
| | 8.5 | 2809 | 98,854 | 427 | 94,386 |
| | 8.0 | 8538 | 96,543 | 596 | 89,970 |
| | 7.0 | 8306 | 90,111 | 546 | 102,981 |
| | 6.5 | 6237 | 98,462 | 499 | 92,553 |
| | 6.0 | 2269 | 96,544 | 352 | 97,685 |

Unreduced results from which the data points of Fig. 2 were calculated. Counting time was 1 min for all samples. The volumes were 150 and 15 $\mu$l for the samples of vesicles and total suspensions, respectively.

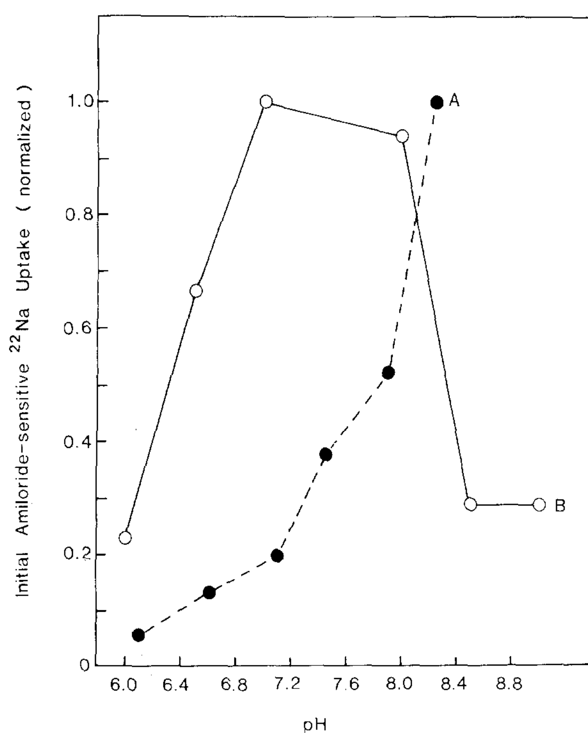


**Fig. 2.** Dependence of initial amiloride-sensitive $^{22}Na$ uptake on external and internal pH. (*A*) Dependence on external pH (closed circles): The internal pH was fixed at 7.8, and the external pH was varied from 6.1 to 8.25 in six separate aliquots of vesicular suspensions. The data were obtained 1 min after exposure to $^{22}Na$ and have been normalized to the uptake measured at $pH_o$ = 8.25, 11.0 pmol · $min^{-1}$ · mg $protein^{-1}$. (*B*) Dependence on internal pH (open circles): The external pH was clamped at 7.8 and the internal pH was varied from 6.0 to 9.0 in six separate aliquots of vesicles. The results were obtained 0.5 min after adding $^{22}Na$ and have been normalized to the value of absolute uptake measured at $pH_i$ = 7.0, 19.1 pmol · $min^{-1}$ · mg $protein^{-1}$

while clamping $pH_i$ at 7.8. Qualitatively similar results were obtained in five other experiments conducted at two to four different values of external pH. Over the pH range studied, the initial $^{22}Na$ uptake was strongly and monotonically dependent on $pH_o$; the highest uptake was noted at the highest external pH applied. In principle, the decreased uptake observed at acidic pH could reflect either a decrease in the $Na^+$ channel conductance or a depolarization of the electrical membrane potential driving the tracer uptake (e.g., by opening nonspecific conductive pathways). The second possibility can be excluded by comparing efflux measurements conducted after external acidification with those carried out after depolarizing the vesicle membranes (Fig. 3). Increasing the pH of the external fluid bathing preloaded vesicles from 6.7 to 7.9 triggered an increased rate of $^{22}Na$ uptake (Fig. 3*A*). If, however, the microsomes were preincubated at a $pH_o$ of 7.9 and then acidified, net tracer accumulation ceased but did not reverse sign (Fig. 3*B*). This behavior is in striking contrast to the large amiloride-sensitive ejection of intravesicular $^{22}Na$ induced by depolarizing the driving membrane potential with high external $Na^+$ (Fig. 3*C*). These differences clearly indicate that the dependence of flux on $pH_o$ shown in Fig. 2 reflects changes in $Na^+$ permeability and not in membrane potential.

## Effect of Internal pH

Changing the intravesicular pH had different effects on the $^{22}Na$ uptake through the amiloride-sensitive channels from those induced by varying $pH_o$. In

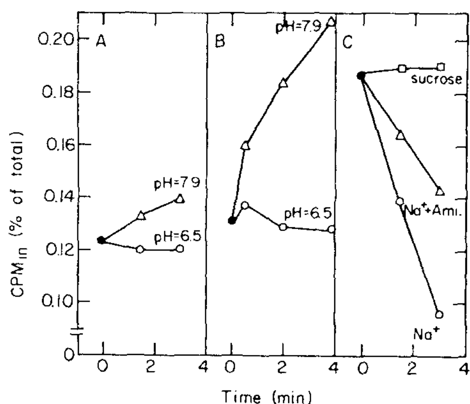


**Fig. 3.** Dependence of $^{22}Na$ efflux on ionic composition of external medium. Sodium was first accumulated by the vesicles during a period of 15 min in the presence of valinomycin and a chemical gradient for $K^+$. (*A*,*B*) The preincubation was conducted at a pH of either 7.7 (*A*) or 6.7 (*B*) before changing the external pH either to 7.9 (triangles) or to 6.5 (circles). (*C*) The preincubation was conducted at 7.9, following which the suspensions were administered with either 5 mM NaCl [with (triangles) or without (circles) 175 μM amiloride] or an equal volume of 6% sucrose (squares)

these experiments, the intravesicular pH was changed by preincubating the vesicles for at least 2 hr in buffers at the desired pH before clamping the external pH to 7.8–8.0 and initiating the assay. The results of one experiment conducted at six different pH values are presented in Fig. 2 and Table 1. Similar results were obtained with three other preparations studied at three to five values of internal pH. In these additional experiments, measurements were also conducted with the $pH_i$ fixed at 7.1, 7.2, 7.7 and 7.9. Over the pH range from approximately 6.8 to 8.0, a peak uptake of $^{22}Na$ was observed, measured 0.5 min after exposing the suspension to radioactive sodium. At pH values above or below this range, the intravesicular $Na^+$ content was lower at the same time point. However, the mechanisms responsible for the reduced $^{22}Na$ content at the lower and higher ends of the pH spectrum were different. At very low values of internal pH, the amiloride-sensitive sodium uptake was appreciably greater after 2.0 min incubation than after 0.5 min, as was generally noted in all other experiments of the present study. The ratio of the amiloride-sensitive $^{22}Na$ uptake at 2.0 min to that at 0.5 min varied from 1.75 to 1.97 at four values of pH, over the pH range 6.0 to 8.0, with a mean ratio (± SE) of 1.85 ± 0.04. In contrast, at internal pH values of 8.5 and 9.0, the corresponding ratios were considerably reduced, to 1.23 and 1.30, respectively. The more rapid attainment of a stable reduced plateau of amiloride-sensitive $^{22}Na$ uptake is consistent with the concept that intravesicular alkalinization above a pH of 8 induced nonspecific shunting across the walls of the vesicles. The fact that this phenomenon

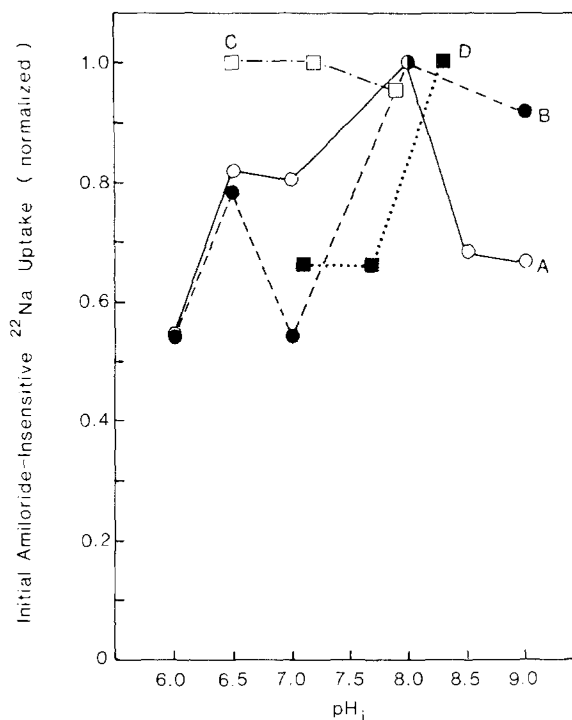


**Fig. 4.** Initial amiloride-insensitive uptake of $^{22}Na$ as a function of internal pH. The symbols *A–D* identify data from four different preparations. In each case, the vesicles were preincubated for at least 2 hr in a suitable buffer solution in order to fix $pH_i$ at the desired value. The experiment was then initiated when the external pH was clamped to 7.8–8.0. The sodium uptake was assayed 0.5 min after exposing the vesicles to $^{22}Na$ in experiments *A*,*B* and *D*, and after 1.5 min in experiment *C*. The results have been normalized to the maximal rates of measured uptake (in pmol · $min^{-1}$ · mg $protein^{-1}$): (*A*) 0.72, (*B*) 1.58, (*C*) 1.25 and (*D*) 1.21

was not observed in response to extravesicular alkalinization is a further indication that protons equilibrate relatively slowly across the vesicular walls; i.e., measurements of $^{22}Na$ uptake taken approximately a minute after changing external pH are indeed meaningful.

The precise nature of the shunting produced by large alkalinizations of the intravesicular fluid cannot be specified from the current data. However, three observations suggest that raising $pH_i$ above 8.0 reduces the ionic selectivity of the amiloride-sensitive channel, permitting shunting through this channel. First, unlike the amiloride-sensitive $^{22}Na$ uptake, the amiloride-insensitive uptake does not display relative saturation after short times at high internal pH. For the experiment of Fig. 2 (curve *B*), the ratio of the amiloride-insensitive uptake at 2.0 min to that at 0.5 min at an internal pH of 8.5 and of 9.0 were 2.51 and 2.42, respectively, insignificantly different from the values (2.44–2.64, mean ± SE = 2.56 ± 0.04) measured at the four values of $pH_i$ over the range 6.0 to 8.0. Second, the amiloride-insensitive sodium uptake does not appear to be dependent upon internal pH (Fig. 4). Both of these observa-

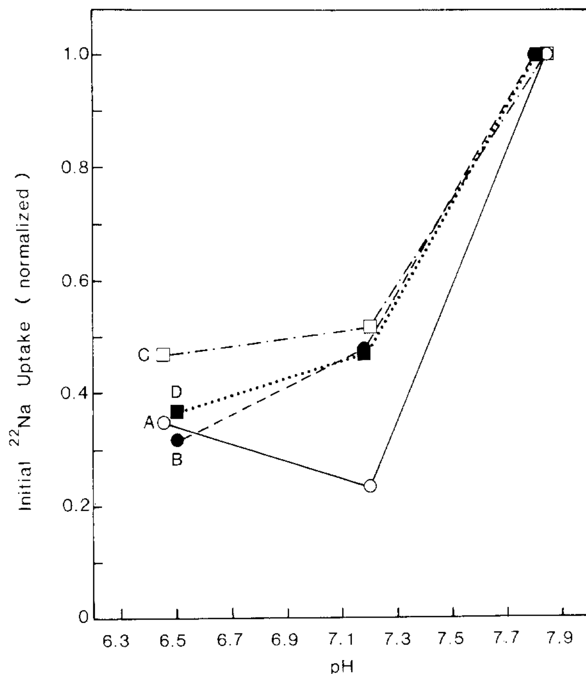


**Fig. 5.** Initial amiloride-sensitive (*A*,*B*) and amiloride-insensitive (*C*,*D*) uptakes in the presence or absence of transmembrane gradients of pH. In this early experiment (conducted before the preparative modifications discussed in the test were initiated), $^{22}Na$ uptake through the amiloride-sensitive and amiloride-insensitive pathways were similar in magnitude. This permitted a meaningful comparison of the pH-dependence of the two uptakes in the same preparation. The data of *A* and *C* were carried out by initially incubating the vesicles at a pH of 7.2, and then rapidly changing the external pH to one of three values, thus establishing a pH gradient across the walls of the vesicles. The results identified as *B* and *D* were collected 5.5 hr after incubating the aliquots at the new values of external pH; in this case, $pH_i$ and $pH_o$ should have been equal when tracer was added. Each sample for radioactive assay was taken 1.5 min after exposing the vesicles to $^{22}Na$. The data have been normalized to the maximal measured rates of uptake, which were (in pmol · $min^{-1}$ · mg $protein^{-1}$): (*A*) 1.20, (*B*) 0.60, (*C*) 1.12 and (*D*) 1.07

tions suggest that the amiloride-insensitive uptake of $^{22}Na$ takes place in a minor population of vesicles and that changes in the magnitude of this background uptake cannot explain the development of significant transvesicular shunting in the vesicle population containing the amiloride-sensitive sodium channels. The third observation is that simultaneous changes in both $pH_i$ and $pH_o$ produce changes in $^{22}Na$ uptake similar to those induced by changing $pH_o$ alone (Fig. 5). If internal alkalinization had resulted in the development of new shunt pathways across the walls of the vesicles, this depolarization would be expected to persist, irrespective of the effects of external alkalinization on the amiloride-sensitive pathways. It seems more plausible that, in the presence of external alkalinization, the selectivity of the physiologic $Na^+$ channels is retained.

## Dependence on External $Na^+$ and Competition with External $H^+$

The dependence of $^{22}Na$ transfer across the walls of the vesicles both upon internal and external pH is different from that reported for whole toad bladder (Leaf, Keller & Dempsey, 1964; Park & Fanestil, 1983). One possible basis for these differences could have been the low concentration of external $Na^+$ used in most of the current studies. As pointed out by Li and Lindemann (1981), the effect of mucosal acidification could be to reduce the degree of inhibition of apical $Na^+$ entry produced by higher concentrations of mucosal $Na^+$ (of the order of 20–30 mM; Fuchs, Larsen & Lindemann, 1977).

The most direct approach to examine this possibility would be to measure $^{22}Na$ uptake in the presence of large mucosal concentrations of $Na^+$. This tack is not practicable because of the high intrinsic $Na^+$ permeability of the vesicles through the amiloride-sensitive channels. Addition of less than 1 mM of either $Na^+$ or $K^+$ to the valinomycin-treated vesicles inhibits $^{22}Na$ uptake substantially; in both cases, 50% inhibition is reached at approximately 50 $\mu$M external cation. This inhibition appears to reflect depolarization of the membrane, which can be observed at such low external cation concentrations because of the very much higher permeability of the membranes of interest to $Na^+$ and to $K^+$ (with valinomycin present) than to $Cl^-$ and Tris. Because of this technical limitation, $^{22}Na$ influx measurements could not be meaningfully conducted at external $Na^+$ concentrations above 50 $\mu$M. However, at this concentration, $^{22}Na$ uptake was examined at external pH values of 8.3 and 7.2 and compared with the results obtained in the same experiment in the absence of added $Na^+$. Despite the partial absolute inhibition of amiloride-sensitive uptake at the higher external sodium concentration, mucosal acidification produced the same fractional inhibition (ca. 35%) in both cases.

An alternative approach to examine the pH dependence of the $Na^+$ permeability at high external sodium concentrations was to measure the $^{22}Na$ efflux from vesicles suspended in solutions containing different values of pH. As documented by Fig. 3*C*, efflux measurements are entirely practicable under conditions of partial membrane depolarization. Table 2 presents the amiloride-sensitive sodium efflux measured in the presence of 53 mM external $Na^+$ or $K^+$ at an external pH of either 8.3 or 6.1. Irrespective of the major external cation, acidification pro-

**Table 2.** $^{22}Na$ efflux from preloaded vesicles as a function of external cation and external pH

| Cation | pH | Rates of efflux (pmol · $min^{-1}$ · mg $protein^{-1}$) | |
|---|---|---|---|
| | | Amiloride-sensitive | Amiloride-insensitive |
| $Na^+$ | 8.3 | 2.2 | 0.9 |
| | 6.1 | 1.4 | 1.4 |
| $K^+$ | 8.3 | 2.8 | 1.2 |
| | 6.1 | 1.5 | 1.3 |

After preloading the vesicles for 10 min with $^{22}Na$ at external and internal pH values of 7.8, $pH_o$ was changed to either 8.3 or 6.1, and 53 mM $Na^+$ or $K^+$ was added to the external medium. Measurements of $^{22}Na$ content were obtained just before and 2 min after the experimental perturbations.

duced a large decrease in amiloride-sensitive efflux, qualitatively similar to that noted in the absence of added external cation. Therefore, the observed pH dependence in the present work is not simply a reflection of the specific experimental conditions under which most of the current assays were conducted.

## Discussion

Leaf and coworkers (1964) were the first to document that acidification of the mucosal fluid selectively increases the short-circuit current and transepithelial mucosal-to-serosal flux of $^{22}Na$ across the urinary bladder of the toad. This stimulation of short-circuit current has been confirmed both in toad bladder (Park & Fanestil, 1983) and in skins from some (Li & Lindemann, 1981), but not all (Ussing, 1949; Linderholm, 1952; Schoffeniels, 1955; Snell & McIntyre, 1960; Funder, Ussing & Wieth, 1967; Cuthbert, 1976; Mandel, 1978) species and subspecies of frogs. The rapid onset of the effect (within seconds) has suggested that the site of action is indeed at the apical permeability barrier (Li & Lindemann, 1981). Studies of the distribution of DMO (5,5-dimethyl-2,4-oxazolidine-$^{14}C$) between whole urinary bladders and the extracellular bath had suggested that mucosal acidification is unaccompanied by any shift in intracellular pH (Leaf, Keller & Dempsey, 1964); however, the significance of these early analyses is limited because of the large contributions of the subepithelial cellular components to the total values measured with whole tissues (Macknight, Civan, & Leaf, 1975). The mechanism of action has also been uncertain. Park and Fanestil (1983) have suggested that the phenomenon reflects the protonation of two sites on the sodium channel, while Li and Lindemann (1981) have suggested that the effect arises from a decrease in the self-inhibition of mucosal $Na^+$ effected at a single regulatory site. Thus, the precise site (intra- or extracellular) and mode of action (direct or indirect) of mucosal acidification have been unclear.

The present manuscript presents measurements of the amiloride-blockable $Na^+$ fluxes across the walls of vesicles obtained from toad urinary bladder, conducted at different values of internal and external pH. Over the physiologic range of cytosolic pH in toad bladder and frog skin (Bond et al., 1981; Nunnally et al., 1983; Lin et al., 1984; Civan et al., 1984) of 7.0–8.0, the channel conductance is independent of internal (cytosolic) pH; only above and below this physiologic range were effects of changing $pH_i$ noted. Thus, it seems that *in vivo* variations of the intracellular pH do not play a direct role in regulating apical sodium permeability. In contrast to the trapezoidal dependence of $Na^+$ flux on $pH_i$ (Fig. 2), the rate of $^{22}Na$ uptake was directly and monotonically dependent upon external pH over the entire range of values examined (Fig. 2). This behavior is altogether different from the pH dependence of $Na^+$ transport across the intact toad bladder described above. This difference in behavior does not arise from a membrane depolarization elicited by external acidification, reducing the driving force for $^{22}Na$ uptake. This possibility has been excluded by measuring $^{22}Na$ efflux as a function of external pH. Acidification of the external phase stabilized the intravesicular $^{22}Na$ content (Fig. 3*A* and *B*). In contrast, membrane depolarization produced by adding external $Na^+$ (Fig. 3*C*) or external $K^+$ induces a rapid amiloride-blockable tracer efflux.

In principle, at least four classes of mechanisms could be responsible for the stimulation of transepithelial $Na^+$ transport induced by mucosal acidification of whole urinary bladders. Not all of these mechanisms would be operative in the vesicle preparation studied, leading to differences in the results obtained with the two preparations.

First, mucosal acidification could cause direct titration of fixed charges near or within the $Na^+$ channels, as suggested by Leaf and coworkers (1964) and by Park and Fanestil (1983). This mechanism should be operative both in the vesicles and in the whole cell preparation. However, the pH dependences observed in the two cases are very different. We conclude that, unless the channels in the intact cells have been modified in preparing the isolated vesicles, direct protonation of the $Na^+$ channel cannot account for the natriferic effect of mucosal acidification.

A second possible mechanism of action would be a direct competition between protons and $Na^+$ for sites within the sodium-selective channels of the

apical membrane. This concept receives some support from the observation of Palmer (1982) that the amiloride-sensitive channels of whole toad bladder display an order of magnitude greater affinity for $H^+$ than for $Na^+$. Again, this mechanism should be operative both in vesicles and in whole cells. However, were this effect actually operative in the present preparation, we would have expected that increasing the extracellular $Na^+$ concentration 250-fold from 0.2 to 50 $\mu$M should have abolished the influx inhibition effected by increasing the external $H^+$ concentration 13-fold [from 5 nM (pH 8.3) to 63 nM (pH 7.2)], contrary to observation. The untenability of this interpretation is even more strikingly evident from the measurements of $^{22}$Na efflux. In this case, raising the external $Na^+$ concentration five orders of magnitude (from 0.2 $\mu$M to 53 mM) did not prevent external acidification from blocking sodium movement through the amiloride-sensitive pathways.

A third possible basis for the stimulation of $Na^+$ transport across whole tissues could be the protonation of gating sites on either side of the membrane. The best-documented such site is that responsible for sodium self-inhibition, located at the external surface of the apical membrane (Fuchs et al., 1977). Li and Lindemann (1981) have actually suggested that mucosal acidification stimulates sodium transport by reducing sodium self-inhibition. This concept was supported by their observation that mucosal acidification increases the affinity of the apical sodium channels to mucosal $Na^+$. However, direct measurements of the dependence of acid stimulation on mucosal sodium concentration have not yet been reported for whole tissues. This specific mechanism was certainly not operative in the current vesicle preparation. As noted above, external acidification reduced the rate of $^{22}$Na efflux both in the presence of 53 mM external $Na^+$ and in the absence of added external $Na^+$. However, it should be appreciated that this entire class of mechanisms of action cannot be rigorously excluded as being possibly operative *in vivo*. Although the amiloride-sensitive, rheogenic $Na^+$ channels of the vesicles prepared are functionally similar to those of whole tissues, the preparative procedures may conceivably have produced disruption or dissociation of gating sites normally present.

Subject to the forestated caveat, the data suggest that mucosal acidification exerts its natriferic effect across whole epithelia by stimulating an additional chemical event, such as phosphorylation of a regulatory site (Jard & Bastide, 1970; Kirchberger, Schwartz & Walter, 1972; Schwartz et al., 1974). Such a process would not have been observed with the current suspensions of vesicles. *In vivo*, the triggering of this putative event could proceed by titration of an external membrane site or by the rapid influx of protons altering the local pH of an intracellular site.

This work was supported in part by research grants from the American Cystic Fibrosis Foundation and the Israeli Academy of Sciences and Humanities to H.G. and from the National Institutes of Health (AM 20632) to M.M.C.

## References

Bond, M., Shporer, M., Peterson, K., Civan, M.M. 1981. $^{31}$P nuclear magnetic resonance analysis of toad urinary bladder. *Mol. Physiol.* **1:**243–263

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:**248–254

Civan, M.M. 1983. Epithelial Ions and Transport: Application of Biophysical Techniques. Wiley-Interscience, New York

Civan, M.M., Lin, L.-E., Peterson-Yantorno, K., Taylor, J., Deutsch, C. 1984. Intracellular pH of perfused single frog skin: Combined $^{19}$F and $^{31}$P NMR analysis. *Am. J. Physiol.* **247:***C*506–*C*510

Clement, N.R., Gould, J.M. 1981. Pyranine (8-hydroxy-1,3,6-pyrenetrisulfonate) as a probe of internal aqueous hydrogen ion concentration in phospholipid vesicles. *Biochemistry* **20:**1534–1538

Cuthbert, A.W. 1976. Importance of guanidinium groups for blocking sodium channels in epithelia. *Mol. Pharmacol.* **12:**945–957

Fuchs, W., Larsen, E.H., Lindemann, B. 1977. Current-voltage curve of sodium channels and concentration dependence of sodium permeability in frog skin. *J. Physiol. (London)* **267:**137–166

Funder, J., Ussing, H.H., Wieth, J.O. 1967. The effects of $CO_2$ and hydrogen ions on active Na transport in the isolated frog skin. *Acta Physiol. Scand.* **71:**65–76

Garty, H. 1984. Amiloride blockable Na fluxes in toad bladder membrane vesicles. *J. Membrane Biol.* **82:**269–279

Garty, H ., Asher, C. 1985. Ca dependent, temperature sensitive, regulation of Na channels in tight epithelia. A study using membrane vesicles. *J. Biol. Chem. (in press)*

Garty, H., Rudy, B., Karlish, S.J.D. 1983. A simple and sensitive procedure for measuring isotope fluxes through ion-specific channels in heterogeneous populations of membrane vesicles. *J. Biol. Chem.* **258:**13094–13099

Jard, S., Bastide, F. 1970. A cyclic AMP-dependent protein kinase from frog bladder epithelial cells. *Biochem. Biophys. Res. Commun.* **39:**559–566

Kano, K., Fendler, J.H. 1978. Pyranine as a sensitive pH probe for liposome interiors and surfaces. pH gradients across phospholipid vesicles. *Biochim. Biophys. Acta* **509:**289–299

Kirchberger, M.A., Schwartz, I.L., Walter, R. 1972. Cyclic 3',5'-AMP-dependent protein kinase activity in toad bladder epithelium. *Proc. Soc. Expt. Biol. Med.* **140:**657–660

Koefoed-Johnsen, V., Ussing, H.H. 1958. The nature of the frog skin potential. *Acta. Physiol. Scand.* **42:**298–308

Leaf, A., Keller, A., Dempsey, E.F. 1964. Stimulation of sodium transport in toad bladder by acidification of mucosal membrane. *Am. J. Physiol.* **207**:547–552

Li, J.H.-Y., Lindemann, B. 1981. Dependence of apical Na-transport in frog skin. *Adv. Physiol. Sci.* **3**:151–155

Lin, L.-E., Shporer, M., Civan, M.M. 1985. $^{31}P$ NMR analysis of perfused single frog skins. *Am. J. Physiol.* **248**:*C*177–*C*180

Linderholm, H. 1952. Active transport of ions through frog skin with special reference to the action of certain diuretics. *Acta Physiol. Scand.* **27 (Suppl. 97)**:1–144

Macknight, A.D.C., Civan, M.M., Leaf, A. 1975. The sodium transport pool in toad urinary bladder epithelial cells. *J. Membrane Biol.* **20**:365–386

Mandel, L.J. 1978. Effects of pH, Ca, ADH, and theophylline on kinetics of Na entry in frog skin. *Am. J. Physiol.* **235**:*C*35–*C*48

Nunnally, R.L., Stoddard, J.S., Helman, S.I., Kokko, J.P. 1983. Response of $^{31}P$-nuclear magnetic resonance spectra of frog skin to variations in $P_{CO_2}$ and hypoxia. *Am. J. Physiol.* **245**:*F*792–*F*800

Palmer, L.G. 1982. Ion selectivity of the apical membrane Na channel in the toad urinary bladder. *J. Membrane Biol.* **67**:91–98

Park, C.S., Fanestil, D.D. 1983. Functional groups of the Na channel: Role of carboxyl and histidyl groups. *Am. J. Physiol.* **245**:*F*716–*F*725

Schoffeniels, E. 1955. Influence du pH sur le transport actif de sodium a travers la peau de grenouille. *Arch. Int. Physiol. Biochim.* **63**:513–530

Schwartz, I.L., Shlatz, L.J., Kinne-Saffran, E., Kinne, R. 1974. Target cell polarity and membrane phosphorylation in relation of the mechanism of action of antidiuretic hormone. *Proc. Natl. Acad. Sci. USA* **71**:2595–2599

Snell, F.M., McIntyre, O.R. 1960. The effects of carbon dioxide and hydrogen ion on sodium transport in isolated frog skin. *Biochim. Biophys. Acta* **41**:89–98

Ussing, H.H. 1949. The active ion transport through the isolated frog skin in the light of tracer studies. *Acta. Physiol. Scand.* **17**:1–37

Received 29 January 1985; revised 5 June 1985