Stimulation of Intestinal Na^+/H^+ Exchange by Cell Volume Changes during Fasting and Refeeding in Rats (43907)

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Abstract. **Intestinal cell water was studied in rats that have been fasted then refed, two conditions that are known to decrease and increase, respectively, proliferation of the intestinal epithelium. Cell water decreased (15%) during fasting and returned to normal with refeeding. The amiloride-sensitive sodium uptake, which estimates uptake through the Na^+/H^+ exchange was higher in the ileum than the jejunum, but the jejunal uptake, unlike the ileal, was significantly increased in fasted/refed rats than in normally fed or fasted ones. Osmotic shrinkage of intestinal cells followed by restitution of their cell volume stimulated the Na^+/H^+ exchange in all of the three groups of animals, but the increase was most prominent in the fasted and the fasted/refed groups. Also, shrinkage of cultured jejunal crypt cells (IEC-6) by a hypertonic solution increased intracellular alkalinization that was inhibited by amiloride. The results provide evidence for a relationship between the change in intestinal cell size, such as that which occurs during fasting/refeeding, and the activation of the Na^+/H^+ antiport system. This may represent one of the signals that initiates intestinal proliferation in the fasting/refeeding state.** [P.S.E.B.M. 1995, Vol 209]

The small intestinal mucosal cell mass is normally in a state of balance between cell replication and cell loss. Several factors play a role in replication, migration, and shedding of the intestinal epithelial cells (1). The response of the rat intestinal mucosa to fasting and refeeding has been a valuable experimental model that has contributed greatly to the understanding of intestinal growth (1–5). Fasting, which reduces significantly the proliferative capacity of the intestinal epithelium (2, 4), leads to a reduction of the mucosal cell mass which reverses rapidly with refeeding (3–5). The process of restoration of the mucosal mass is influenced by a number of factors but an important known stimulus for regeneration is the physical presence of nutrients in the gut lumen. The mechanism by which luminal nutrients stimulate proliferation is, however, unclear. In the fasting rat, intraluminal perfusion of glucose, amino acids, or even sodium chloride alone, causes equal stimulation of intestinal growth (3). This suggests that the mechanism of stimulation of growth is independent of the nutritive effect of the solutes perfused and local physical factors in the intestinal lumen contribute, at least in part, to initiating the proliferative response. This study was intended to test the hypothesis that fasting and refeeding alter intestinal cell size and cause activation of the Na^+/H^+ antiport, one of the signals which is known to initiate proliferation. Fasting is reported to decrease the size of the intestinal mucosa (2, 6, 7) and in many cell systems other than the intestine a change in cell size produces activation of the Na^+/H^+ exchange (8–11).

Materials and Methods

Animals. Male Sprague-Dawley rats from the Charles River Breeding Labs (Wilmington, MA), weighing 200–250 g were used. They were housed in our animal facility and fed rodent chow *ad libitum* for at least a week before being used. Three groups of rats were tested, a normally fed group, a group fasted for 48 hr then tested, and a group that was similarly fasted then refed for 48 hr. Water was allowed during the fasting periods. In the *in vivo* experiments described below, the rats were anesthetized with intraperitoneal injections of Na-pentobarbital (50 mg/Kg). In other *in*

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vitro studies, the rats were sacrifice with an injection of a larger dose of the barbiturate.

Measurements of Intestinal Cell Volume. The method of Esposito *et al.* (12) was used to determine the cell water content of the intestinal epithelial cells *in vivo*. The jugular vein of an anesthetized rat was cannulated and a priming dose of ^{14}C-polyethyleneglycol (1.5 μCi/100 g rat body weight, Dupont NEN Research Products) was injected. This was followed by intrajugular infusion of 0.5 ml of isotonic saline solution containing 1.5 μCi/100 g of rat body weight. The infusion was perfused slowly during a 30-min period. The labeled extracellular marker had a MW of 4000 and a specific activity of 0.7 mCi/g. At the end of the perfusion a blood sample was taken from the other jugular vein and the rat was killed and segments of its intestines were resected and cut open along their mesenteric borders. The luminal contents of some of the segments were flushed with isotonic saline and the contents of other segments were cleared by gentle blotting with cotton swabs and filter paper (Whatman No1) using a dissecting microscope for adequate magnification. The mucosa was scraped off with a glass slide, weighed, then extracted in 0.1 *N* HNO_3. The tissues residue after extraction was dried in an oven at 90°C. Cell water was determined from the radioactivity in the total tissue extracts, the plasma concentration of the isotope and from the wet and dry weights of the tissues as described previously (13).

Na^+ Uptake Measurements. Experiments were designed to measure the Na^+ uptake that is mediated via the Na^+/H^+ antiport. They were done in *in vivo* conditions since they were intended to test the changes of the antiport as they relate to the fasting/refeeding states of the animals and to the changes in the intestinal cell size during fasting and refeeding. We used a method that we described previously (14) in measuring the influx of amino acids across the mucosal border of the *in vivo* rat intestine. The method was adapted to measurement of the initial Na^+ uptake rate across the mucosal border of the intestine. Briefly, two adjacent segments of intestine of anesthetized rat were cannulated at both ends then flushed with a choline-Ringer solution containing 134 m*M* choline chloride, 20 m*M* NaCl, 3 m*M* K_2HPO_4, and 3.6 m*M* Tris-HCL (pH 7.4). The segments were perfused by the choline-Ringer solutions containing 0.1 m*M* ouabain and 1 m*M* furosemide, and in some cases 2 m*M* amiloride (Sigma Chemical Co., St. Louis, MO). Perfusion was continued for a period of 20 min at a rate of 0.5 ml/min using a perfusion pump (Model 975; Harvard Apparatus, Mills, MA). At the end of the preincubation period, uptake across each of the segments was measured as described in details previously (14). Briefly, the segment was perfused at a rapid rate of 5 ml/min (normally for 30 sec) with a test solution containing the choline Ringer to which is added 0.1 m*M* ouabain, 1 m*M* furosemide (Sigma) and tracer quantities of ^{14}C-PEG and $Na^{22}Cl$ (Dupont NEN Research Products). The second segment was similarly tested except that an additional 2 m*M* of amiloride (Sigma) was placed in the preincubation and test media. In preliminary experiments N-methyl-isobutyl amiloride was used and it produced similar results as amiloride. Sodium uptake was calculated from the Na^{22} content in tissue extracts after making a correction for the ^{14}C-PEG space (14). The amiloride sensitive sodium uptake was estimated from the difference between the total uptake measured in one of the intestinal segments and the uptake in a sister segment perfused with amiloride-containing solutions.

Validation of the Method of Measuring Sodium Uptake *in Vivo*. Three sets of experiments were performed to validate the Na^{22} uptake method. First, the appearance of Na^{22} in the portal and systemic blood was measured after instilling the sodium isotope in the gut lumen. As shown in Figure 1, Na^{22} was not detectable in the portal or systemic blood samples obtained at 17 and 30 sec but began to appear in the portal blood at 45 sec and increased significantly beyond 60 sec. The amount of tracer reaching the tissue during the first 30 sec represented therefore the entry of sodium into the intestinal cell (i.e., influx). The second set of experiments measured the time course of the uptake of $Na^{22}Cl$ (20 m*M*) into the intestinal cell. The cumulative uptake of labeled sodium at 10, 20, 30, 40, and 60 sec was measured and as shown in (Fig. 2) uptake as a function of time was linear at least during the initial 40 sec. Similar linear uptakes/time curves (data not shown) were observed during the initial 40 sec of perfusion of 30 and 60 m*M* concentrations of NaCl. A concentration curve of the uptake of Na^{22} versus the concentration of sodium chloride in the test solution represented the third group of experiments performed

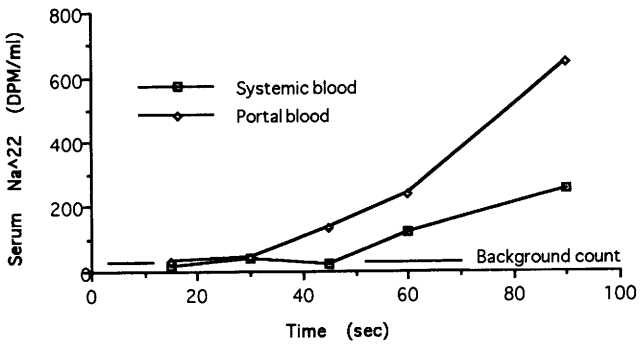

Figure 1. The time course of appearance of $Na^{22}Cl$ in the systemic and portal blood of a rat after perfusing the isotope into its jejunal lumen. The perfusate consisted of a choline-Ringer's solution containing 134 m*M* choline chloride, 20 m*M* NaCl, 3 m*M* K_2HPO_4, 3.6 m*M* Tris-HCL, 0.1 m*M* ouabain, and 1 m*M* furosemide (pH 7.4). Each point is the average of three determinations. The line represents the mean $Na^{22}Cl$ background count.

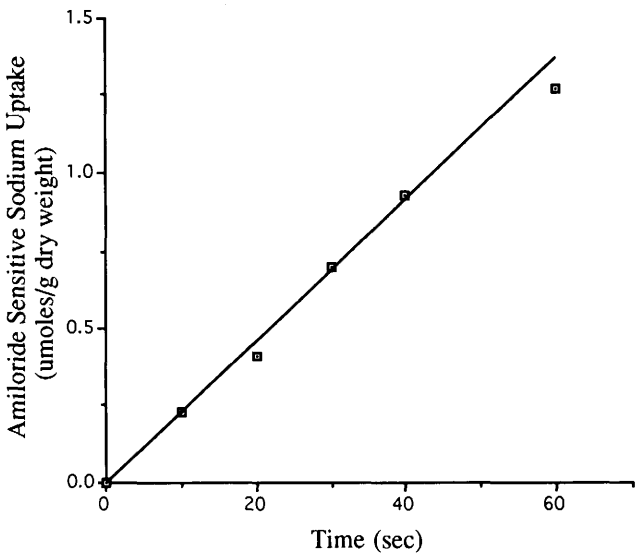

Figure 2. The time course of the uptake of $Na^{22}Cl$ by rat jejunal segments. Each point is the mean of four determinations, each representing the difference between the uptake measured during perfusion with choline-Ringer's and the uptake measured in the presence of the same solution but containing amiloride (2 m*M*). The choline-Ringer's solution contained 134 m*M* choline chloride, 20 m*M* NaCl, 3 m*M* K_2HPO_4, 3.6 m*M* Tris-HCL, 0.1 m*M* ouabain, and 1 m*M* furosemide (pH 7.4).

(Fig. 3). There is a tendency to saturation at low NaCl concentrations, while linearity in the uptake rate occurs with increasing sodium concentrations. The linearity may be related to an increased entry of Na^+ into the intestine through diffusional processes since the extracellular-to-intracellular Na^+ concentration gradient increases with the increase in NaCl concentration of the test media. The V_{max} and K_m of this transport process are 111 μmoles/hr/g dry weight and 16 m*M*, respectively. These finding justified, therefore, the choice for using a low concentration of Na^+ (20 m*M*)

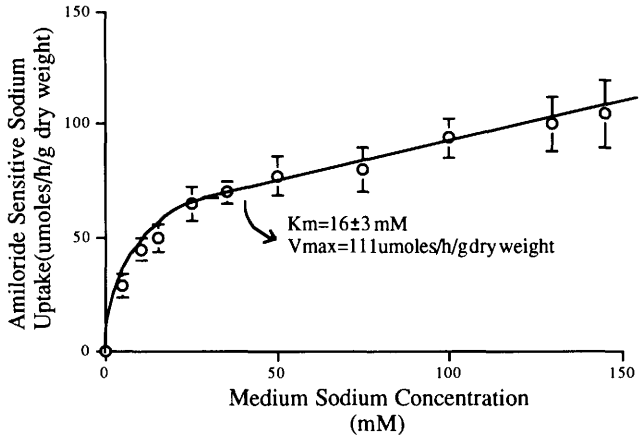

Figure 3. $Na^{22}Cl$ uptake as a function of luminal NaCl concentration. Sodium concentration was varied by substituting NaCl for choline chloride in the choline Ringer's solution. A 30-sec time period was used in all the of the uptake studies shown. The data at each of the sodium concentrations used is the mean ± SE of at least four determinations.

in all of the subsequent measurements of the Na^{22} uptake. Moreover, at this low sodium concentration, the passive entry of Na^+ into the intestinal cell was limited during the brief uptake period. All of the above studies provided, therefore, validity for the uptake measurement technique that was used in this investigation.

Measurement of Intracellular pH in the Cultured Intestinal Crypt Cell Line. Because of difficulties in the isolation of viable and homogeneous populations of rat intestinal cells that would be suitable for testing the changes in intestinal cell volume and for measuring their intracellular pH, we used a cultured rat jejunal crypt cell (IEC-6) line. This cell line was obtained from the American Type Culture Collection (Rockville, MD). The cells were cultured in 75-cm polystyrene flasks containing Dulbecco's Modified Eagle's Medium (DMEM, supplemented with glutamine 4 m*M*, insulin 10 units/ml, dialyzed fetal bovine serum 10%, penicillin 50 U/ml, streptomycin 50 ug/ml, and amphotericin B2.5 ug/ml [All from GIBCO, Grand Island, NY]). The cells were harvested by trypsinization and resuspended at 1×10^7 cells/ml in (in mM) 124 NaCl, 5 KCl, 0.5 $CaCl_2$, 5.5 glucose, and 20 HEPES (pH 7.3). Changes in intracellular pH were measured using the fluorescent dye 2',7'-bis-(2-carboxyethyl)-5,6-carboxy fluorescein (BCECF) (15). IEC-6 cells were incubated with 4.3 m*M* of the tetraacetoxymethyl ester of BCECF (BCECF-AM; Molecular Probes, Inc., Eugene, OR) for 30 min at 37°C. The cells were then washed twice and resuspended at a final concentration of 2×10^6/ml. Fluorescent measurements were made in an SLM 8000 spectrofluorimeter (SLM Instruments, Urbana, IL). Fluorescence was calibrated over the pH range 6.4 to 7.8 using nigericin (15).

Results

The sodium uptake studies are summarized in Table I. Total and amiloride inhibited uptakes of sodium are not shown, but their difference, the amiloride sensitive uptake, is presented. In Condition A, E, and I, where the intestinal segments were preincubated and tested in choline-Ringer, no significant difference in sodium uptake was noted between the normally fed and the fasted rats, but uptake was minimally, but statistically, increased in the fasted/refed animals, a finding that agrees with the results of Kikuchi *et al.* (16). Sodium uptake was also higher in the ileum than the jejunum in all three groups but, unlike the jejunum, it was not increased in the fasted/refed animals. In Condition B, F, and J, where the intestinal segments were preincubated with a hypertonic choline-Ringer and tested with an isotonic Ringer solution (i.e., shrinkage then restoration of cell volume), there was a significant increase in uptake in the three rat groups studied. The increase was most remarkable in the fasted/refed ani-

Table I. Amiloride-Sensitive Sodium Uptake in Intestinal Segments of Rats That Have Been Normally Fed, Fasted for 48 Hr, or Fasted for 48 Hr then Refed for 48 Hr

	Preincubation solution	Test solution	Sodium uptake (μmoles/hr/g dry weight)	
			Jejunal	Ileal
Normally fed rats	(A) C-R	C-R	55 ± 6	114 ± 12
	(B) C-R + M	C-R	94 ± 9[a]	132 ± 11
	(C) C-R	C-R + M	52 ± 5	
	(D) C-R + M	C-R + M	66 ± 6	
Fasted rats	(E) C-R	C-R	64 ± 8	137 ± 24
	(F) C-R + M	C-R	154 ± 11[b]	150 ± 25
	(G) C-R	C-R + M	72 ± 9	
	(H) C-R + M	C-R + M	76 ± 10	
Fasted/refed rats	(I) C-R	C-R	85 ± 11[a]	145 ± 26
	(J) C-R + M	C-R	188 ± 13[c]	162 ± 29
	(K) C-R	C-R + M	97 ± 10	
	(L) C-R + M	C-R + M	93 ± 9	

Note. The constituents of the preincubation and test solutions were either choline-Ringer (C-R), which contained 134 m*M* choline chloride, 20 m*M* NaCl, 3 m*M* K_2HPO_4, 3.6 m*M* Tris-HCL, 0.1 m*M* ouabain, and 1 m*M* furosemide, or C-R containing an additional 150 m*M* of mannitol (C-R + M). Values are means ± SE of six determinations. Significance was measured using Student's *t* test.
[a] $P < 0.05$ compared with jejunal uptake in Condition A.
[b] $P < 0.01$ compared with jejunal uptake in Condition (E).
[c] $P < 0.01$ compared with jejunal uptake in Condition I.

mals and to a lesser but significant degree in the fasted group. Such stimulation, again, did not occur in the ileum. No significant difference in sodium uptake was noted in Group C, G, and K, where the segments were preincubated in an isotonic choline-Ringer and tested with a hypertonic medium, or in Group D, H, and L where preincubation and testing were done using a hypertonic medium. These studies suggest that shrinking and restitution of the cell volume of the jejunal cells causes activation of the amiloride-sensitive Na^+-uptake which was more apparent in the fasted than the normally fed animals and most evident in the fasted/refed group.

In interpreting the results in Table I that are described above, the effect of fasting and refeeding on the intestinal cell size was evaluated in the studies in Table II. As shown, fasting decreases the wet/dry weight ratio and the cell water content of the rat jejunal mucosa. A 15%–16% decrease occurred and was only evident in tissues that have not been rinsed with the wash solution prior to testing. These findings suggest that exposure of the shrunken cells to the wash solution rapidly restores their normal cell size during washing, a feature that could explain why shrinkage of intestinal cells during fasting has not been frequently reported. Refeeding of the fasted rats restores the cell water and wet/dry weight ratio of the cells to normal (Table II).

Reduction in the intestinal cell size during fasting makes it necessary to maintain the state of the reduced cell size during the sodium uptake measurements. For this purpose the studies shown in Figure 4 were done. Cell size was measured in the presence of Krebs phosphate buffer containing an additional concentration of 50, 100, 150, and 200 m*M* of mannitol. The addition of 150 m*M* mannitol to the isotonic Ringer's solution produces a 16% reduction in cell volume, a similar reduction as that observed in the fasting animal (Table II).

Table II. Effect of Fasting and Refeeding on Rat Jejunal Wet-to-Dry Weight Ratio and Intestinal Cell Water

	Normally fed, Rinsed	Fasting		Refed	
		Rinsed	Not rinsed	Rinsed	Not rinsed
Wet/dry weight ratio	4.0 ± 0.2	4.1 ± 0.2 (+3%)	3.4 ± 0.1[a] (−15%)	4.2 ± 0.2 (+5%)	4.0 ± 0.2 (0%)
Cell water (μl/mg dry weight)	4.1 ± 0.13	4.0 ± 0.12 (−2%)	3.4 ± 0.08[a] (−16%)	4.2 ± 0.14 (+1%)	4.0 ± 0.16 (−2%)

Note. Wet/dry weight ratio and cell water were measured in isolated sheets of mucosal strips. Strips were either rinsed with isotonic saline solution or cleaned with cotton swabs and filter paper to avoid changes in tissue or cell water that occur with washing and rinsing the tissues prior to experimentation. Values are means ± SE of 48 observations in eight rats. Percentages in parentheses are comparisons with the controls.
[a] $P < 0.01$ comparing the rinsed and not rinsed tissues (Student's *t* test).

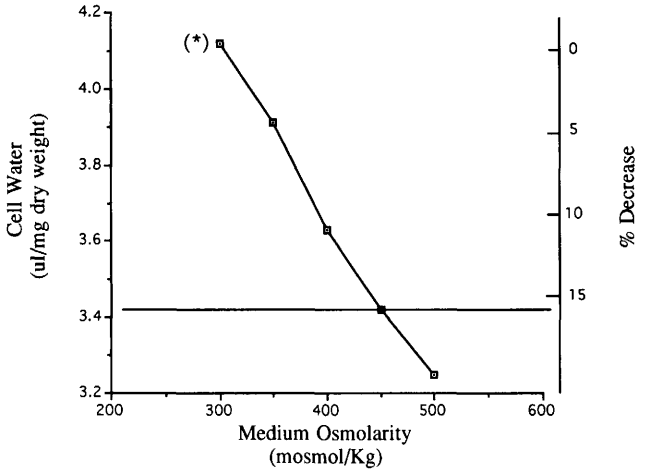

Figure 4. Cell volume change of jejunal mucosa bathed in media containing varying concentrations of mannitol. Mucosal strips were incubated for 30 min *in vitro* in a Krebs phosphate buffer (pH 7.4, 37°C) that was oxygenated by 100% oxygen and containing varying concentrations of mannitol. *The control value without mannitol. The medium osmolarities at 350, 400, 450, and 500 mosmol/kg were adjusted by the addition of mannitol in increments of about 50 m*M*, as adjusted by osmometry. Each point is the average of 20 determinations on tissues of four animals.

Sorbitol and sodium chloride were also tested and they, like mannitol, produced shrinkage of the intestinal cells (Table III). A Na-free, choline substituted medium caused a small but insignificant reduction in cell volume. Based on these findings, the uptake experiments in the fasting rat were performed under conditions that maintained the shrunken state of the intestinal epithelium during the wash and test periods (i.e., preincubation with a choline-Ringer's solution containing 150 m*M* mannitol). Positive and negative controls for these experiments were performed in both the normally fed and the fasted/refed animals.

Table III. Effect of Varying Hypertonic Solutions on Cell Water Content of Rat Jejunal Mucosal Strips

Solutions	Cell water (μl/mg dry weight)	*n*	% decrease
KPB	4.23 ± 0.07	24	—
Choline KPB	4.01 ± 0.06	24	5
KPB + 150 m*M* Mannitol	3.64 ± 0.09[a]	21	14
KPB + 150 m*M* Sorbitol	3.76 ± 0.10[a]	24	11
KPB + 126 m*M* NaCl	3.11 ± 0.07[a]	16	26

Note. Intestinal mucosal strips were incubated in the listed solutions for 10 min. The Krebs phosphate buffer (KPB) composition was: 140 m*M* Na^+, 8 m*M* K^+, 2.6 m*M* Ca^{2+}, 1 m*M* Mg^{2+}, 140 m*M* Cl^-, 1 m*M* SO_4^{2+}, 8 m*M* $H_2PO_4^-$ + HPO_4^{2-}, and 5 m*M* glutamine (pH 7.4). Choline KPB is a Na^+-free choline substituted buffer solution. Values are means ± SE. *n* = number of determinations of tissues from four to five animals.
[a] $P < 0.01$ compared with control strips incubated in KPB (Student's *t* test).

To validate the above findings further, specifically, that a cell volume change can influence the Na^+/H^+ antiport in intestinal cells we tested the effect of a hypertonic NaCl solution on intracellular acidification of IEC-6 cells. The results of these experiments are presented in Figure 5 which shows intracellular alkalinization increasing by the addition of 126 m*M* NaCl to the incubation medium of these cells. The increase was abolished by amiloride, suggesting that it is caused by the extrusion of H^+ through the antiport. The effect on intracellular alkalinization was not reproducible in the complete absence of NaCl from the incubation solution (data not shown).

Discussion

The results presented provide evidence for the presence of a relationship between changes in the intestinal cell size that occur during fasting/refeeding and activation of the Na^+/H^+ antiport system. We have no evidence, at present, that activation of the antiport system may enhance proliferation of intestinal cells. This relationship, however, is well established in cells other than the intestine (9, 15, 17–20). Furthermore, Ulrich-Baker *et al.* (21) have observed that treatment of rats with amiloride blocked the activation of ornithine decarboxylase (ODC) activity and DNA synthesis in rat jejunum, and Na^+/H^+ exchange activity was also found to be significantly greater in rats that are fasted then refed than in the fasted controls (16). These findings support our observations and suggest that activation of the Na^+/H^+ antiport increases ODC activity and DNA synthesis in the rat intestine.

The method of measurement of the initial uptake of sodium into the intestinal mucosa *in vivo* was evaluated in this study and optimized in various ways. Sodium enters the mucosal membrane of the intestine by at least four mechanisms: the Na^+/H^+ antiport system, the Na^+-nonelectrolyte coupled system, neutral

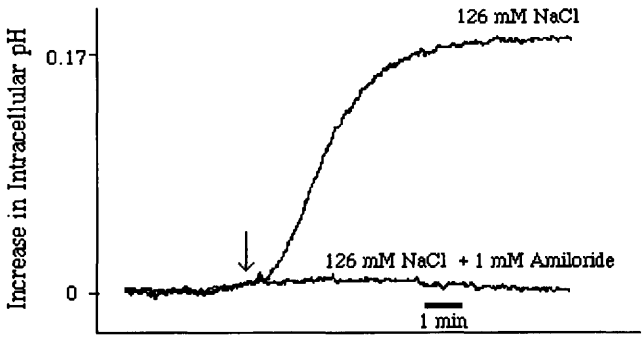

Figure 5. Increase in the intracellular pH of the cultured intestinal crypt cells (IEC-6 cells) during exposure to a hypertonic solution. The cells were suspended in 124 m*M* NaCl, 5 m*M* KCl, 0.5 m*M* $CaCl_2$, 5.5 m*M* glucose, and 20 m*M* HEPES. At the arrow shown, NaCl (126 m*M*) was added to shrink the cells. In some experiments, 1 m*M* amiloride was added 2 min before the addition of the NaCl. The graphs shown in the figure are representative of three separate but similar experiments.

NaCl transport, and diffusion into the mucosal cells possibly through a sodium channel. To minimize sodium uptake through the Na^+-nonelectrolyte system, glucose and/or amino acids were omitted from the test solutions. Furosemide was used to block, at least to some extent, the entry of sodium through the NaCl coupled system (22). Diffusion and/or flux through a sodium channel were minimized by using a relatively low luminal concentration of sodium to reduce the inwardly directed gradient of sodium between the lumen and the intestinal cell. Ouabain was also used to minimize the extrusion of Na^{22} from the transporting cells during the uptake period.

The results presented bring out several important observations. First is that the amiloride-sensitive Na uptake and therefore the Na^+/H^+ antiport system are normally more prominent in the ileum than the jejunum, but the jejunal uptake is more responsive to stimulation by fasting/refeeding and/or changes in the osmolarity of the luminal contents of the intestine. The second observation is that the osmotically induced acute changes in cell volume (Conditions B, F, and J of Table I) cause stimulation of the amiloride sensitive sodium uptake in the normally fed, fasted, and fasted/refed rats. This increase was most prominent in the fasted/refed and in the fasted rat groups and least prominent in the normally fed animals. This suggests that priming of the antiport may occur during fasting/refeeding making the intestine more responsive to stimulation. The third observation is that fasting causes a reduction in cell volume which returns to normal with refeeding (Table II). These findings suggest that fasting and refeeding stimulate the jejunal Na^+/H^+ antiport by causing changes in cell volume similar to those that are osmotically induced. The proposed mechanism of stimulation of the antiport by an osmotically driven effect cannot rule out, however, the possibility that other controlling factors (1) such as neurohumoral regulation, intraluminal polyamines, and an increase in mitogenic genes like c-*fos* and c-*jun* (23) may be directly involved in causing the changes in intestinal replication in response to fasting and refeeding. It is unclear at present whether similar cell volume changes occur in other physiologic or pathophysiologic conditions such as in the short bowel syndrome or in the shunted bowel of patients with intestinal bypass conditions. Further studies are needed to test the importance of cell volume changes in intestinal growth and to establish the relationship between the antiport and cell proliferation.

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