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### Research Article

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# Induction of Hypertrophy in Cultured Proximal Tubule Cells by Extracellular NH<sub>4</sub>Cl

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## Abstract

Ammonia production increases in several models of renal hypertrophy in vivo. The present study was designed to determine whether ammonia can directly modulate the growth of renal cells in the absence of a change in extracellular acidity. In serum-free media NH<sub>4</sub>Cl (0–20 mM) caused JTC cells and a primary culture of rabbit proximal tubule cells to hypertrophy (increase in cell protein content) in a dose-dependent fashion without a change in DNA synthesis. Studies in JTC cells revealed that the cell protein content increased as a result of both an increase in protein synthesis and a decrease in protein degradation. Total cell RNA content and ribosome number increased after NH<sub>4</sub>Cl exposure and the cell content of the lysosomal enzymes cathepsin B and L decreased. Inhibition of the Na<sup>+</sup>/H<sup>+</sup> antiporter with amiloride did not prevent the hypertrophic response induced by NH<sub>4</sub>Cl. The results indicate that ammonia is an important modulator of renal cell growth and that hypertrophy can occur in the absence of functioning Na<sup>+</sup>/H<sup>+</sup> antiport activity.

## Introduction

Renal hypertrophy occurs in such diverse clinical states as (a) protein loading, (b) NH<sub>4</sub>Cl-induced metabolic acidosis, (c) hypokalemia, (d) diabetes mellitus (type 1), and (e) reduction of renal mass (1). However, the signaling events that cause the kidney to hypertrophy in these disorders are presently unknown. An adaptation of the renal tubule that characterizes several models of renal hypertrophy is an increase in the activity of the brush border Na<sup>+</sup>/H<sup>+</sup> exchanger (2–8). However, there are no studies that have addressed the question of whether renal hypertrophy can occur in the absence of functioning Na<sup>+</sup>/H<sup>+</sup> antiport activity.

It has not been previously recognized that in addition to an increase in Na<sup>+</sup>/H<sup>+</sup> antiport activity many models of renal hypertrophy, i.e., protein loading, NH<sub>4</sub>Cl loading, hypokalemia, diabetes mellitus (type 1), and a reduction in renal mass have in common an increase in ammonia production and/or excretion per nephron (2, 9–17). In addition, calcium restriction and deoxycorticosterone acetate administration cause renal hypertrophy and are associated with an increase in renal ammonia production and/or excretion (18–22). The finding that there is increased ammonia production and/or excretion

in models of renal hypertrophy is intriguing given recent evidence in nonrenal cells that suggests that ammonia may be an important modulator of cell growth. Specifically, extracellular NH<sub>4</sub>Cl has been recently shown to cause an increase in cell protein content in cultured astrocytes (23) and human gingival fibroblasts (24). In addition, several studies have documented that extracellular NH<sub>4</sub>Cl decreases the rate of protein degradation in cultured rat hepatocytes (25), rat fibroblasts (26), and mouse macrophages (27). Other studies have shown that (a) the ammonium ion modulates ribosomal RNA synthesis during *Xenopus laevis* embryogenesis (28), (b) ammonia is secreted during slime mold development and affects cAMP content (29), (c) ammonia affects spore cell differentiation and modulates stalk cell formation (30), and (d) ammonia exposure results in the stimulation of protein and DNA synthesis in sea urchin eggs (31). The results of these studies suggest that ammonia, either alone or in concert with other growth factors, may play a pivotal role as a growth modulator in renal cells.

Lotspeich (9, 32) and Halliburton (33) demonstrated that rats administered NH<sub>4</sub>Cl intravenously developed acidemia and renal hypertrophy. Systemic acidemia resulted from the hepatic conversion of ammonium to urea, a process that consumes bicarbonate and prevents blood ammonia levels from increasing above normal. Animals administered ammonium citrate failed to develop acidemia because of conversion of the citrate ion to bicarbonate, and in addition the animals did not develop renal hypertrophy. On the basis of these results it was argued that systemic acidemia induced the hypertrophic response. Studies by Haas and Simpson (34) have demonstrated that chronic metabolic acidosis induced by NH<sub>4</sub>Cl administration in the rat increases the rate of renal cortical protein synthesis. Van Thiel et al. (35) demonstrated that rats fed ethanol chronically developed renal hypertrophy, and suggested that renal growth may have resulted from the metabolic acidosis that the animals developed as a result of ethanol ingestion. Unlike in vivo acidemia, in vitro acidemia has never been demonstrated to induce cell hypertrophy (increased cell protein content in the absence of an increase in DNA synthesis) but instead results in either a decreased rate of cell growth in nonrenal cells (36) or an increased rate of DNA synthesis in renal cortical cells (37). A possible explanation of these discrepant results is that NH<sub>4</sub>Cl and ethanol administration in vivo caused acidemia, which subsequently resulted in an increase in renal ammonia production, and that the increased renal tissue ammonia concentration rather than the acidemia per se induced the hypertrophic response. Given the normal acid-base status of ammonium citrate-treated animals, renal ammonia-genes would not have been expected to increase above normal.

The present study was designed to test the hypothesis that ammonia in the absence of extracellular acidemia can modulate renal cell growth. In addition, experiments were performed to determine whether normally functioning Na<sup>+</sup>/H<sup>+</sup> antiport activity is required for hypertrophy to occur. The JTC

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cell line derived from monkey kidney proximal tubule, and cultured rabbit primary proximal tubule cells were used as model systems.

## Methods

### Culture of JTC cells

JTC cells were obtained from Dr. Paul A. Insel, Department of Pharmacology, University of California, San Diego. Cells (passage 12–25) were plated at a density of  $\sim 5 \times 10^5$  cells/76-cm<sup>2</sup> flask and maintained in a complete growth medium composed of DME, 10% newborn calf serum, 50 IU/ml penicillin, and 50  $\mu$ g/ml streptomycin. Cultures were maintained in an incubator at 37°C in 95% air–5% CO<sub>2</sub>, pH 7.4. The cells were harvested with trypsin and then seeded onto either sterile glass coverslips (for studies requiring the monitoring of intracellular pH [pH<sub>i</sub>])<sup>1</sup> or 35-mm petri dishes. In all studies, unless indicated, after the cells became confluent the monolayers were bathed for 96 h in serum-free DME (medium A) to achieve quiescence before altering the medium bathing the cells. To prevent cellular H<sup>+</sup> efflux from lowering the external pH, after the cells achieved quiescence the medium bathing the cells was changed twice daily.

### Culture of rabbit tubule cells

Primary cultures of rabbit proximal tubule epithelial cells were prepared as described previously (38, 39). Cells were grown to confluence in DME/Ham's F12 medium (1:1) supplemented with transferrin (5  $\mu$ g/ml), insulin (5  $\mu$ g/ml), and hydrocortisone (50 nM). Insulin and hydrocortisone were removed from the medium for 48 h before the study to achieve quiescence. After the cells achieved quiescence the medium bathing the cells was changed twice daily to prevent the external pH from changing.

### Measurement of cell protein content

**JTC cells.** Cell protein content was measured according to Sedmark and Grosberg (40) using BSA as a standard. Cell number was measured on the same petri dishes that were used for the cell protein assay. After the 96-h serum-free period (medium A) the cells were bathed in either serum-free DME (medium A), serum-free DME + 40 mM mannitol (medium B), or serum-free DME + 20 mM NH<sub>4</sub>Cl (medium C) before study. Although the effective osmolality of the medium containing 40 mM mannitol exceeds the effective osmolality of the medium containing 20 mM NH<sub>4</sub>Cl, 40 mM mannitol was used as an upper limit to rule out an effect of medium osmolality changes on cell protein content. To determine the time course of change in cell protein, the cell protein content was measured after 24, 48, 72, and 96 h exposure to these solutions. In a separate study the dose dependence of NH<sub>4</sub>Cl addition (0–20 mM) on cell protein content was determined after 96 h exposure to NH<sub>4</sub>Cl. In another series of experiments the effect of acute NH<sub>4</sub>Cl exposure (10 s) on cell protein content was measured 96 h later. Control JTC cell protein content was found to vary from  $\sim 1.6$  to  $2.2 \times 10^{-4}$   $\mu$ g/cell; therefore, control cell protein measurements were always made with each experimental protocol.

**Rabbit primary proximal tubule cells.** After 48 h exposure to medium containing only transferrin, the cells were bathed in various concentrations of NH<sub>4</sub>Cl (0–20 mM) in medium containing only transferrin for 96 h, at which time the cell protein content was measured.

### Measurement of protein synthetic rate

After the 96-h serum-free period (medium A) the rate of protein synthesis was measured in cells bathed in either medium A, B, or C. Measurements were made after 24, 48, 72, and 96 h exposure. On the

day of the study the DME medium bathing the cells was replaced by labeling medium identical to media A, B, and C except that leucine-free media (LFM) were used and supplemented with [<sup>3</sup>H]leucine (1  $\mu$ Ci/ml, sp act 45 Ci/mmol). The monolayers were exposed to these media for either 4 h or in separate experiments for 16 h. After the labeling period the medium was replaced with LFM (otherwise identical to media A, B, and C) and supplemented with 2 mM leucine (chase medium) for 3 h. Then the monolayers were washed three times with the same media. The cells were harvested (trypsinized) and a cell count was performed on a 10- $\mu$ l aliquot, followed by a TCA precipitation of the cell suspension, and radioactivity was then measured in the TCA-insoluble phase of the suspension. In the time-course studies the protein synthetic rate was expressed as counts/minute per cell after 16 h uptake. In a second series of experiments, to determine the dose-dependent effect of NH<sub>4</sub>Cl exposure on protein synthesis, JTC cells were exposed for 72 h to medium A plus varying concentrations of NH<sub>4</sub>Cl (0–20 mM). In these studies the cells were exposed to [<sup>3</sup>H]leucine for either 4 h, or in separate experiments for 16 h.

### Measurement of protein degradation rate

After the 96-h serum-free period (medium A) the rate of protein degradation was measured in cells bathed in either medium A, B, or C. Measurements of the protein degradation rate were made at 24, 48, 72, and 96 h exposure. To measure the rate of protein degradation the cells were exposed for 18 h to media identical to medium A, B, or C except that LFM was used and supplemented with [<sup>3</sup>H]leucine (1  $\mu$ Ci/ml, sp act 45 Ci/mmol). After the labeling period the media were replaced by LFM otherwise identical to media A, B, and C and supplemented with 2 mM leucine (chase media). After a 3-h incubation the monolayers were washed three times with the same media, then 1 ml of fresh chase medium was added to each dish for 3 h. The medium was removed and 1 mg of bovine albumin per dish was added. The protein in the media was precipitated with 10% TCA and the suspension was centrifuged. The counts in the supernatant TCA-soluble (amino acid) and -insoluble (protein) fractions were measured. The cells were also exposed to 10% TCA, the suspension was centrifuged, and the counts were measured in the TCA-soluble (amino acid) and -insoluble (protein) fractions. Protein degradation rates were expressed as the ratio of the counts in the medium in the TCA-soluble (medium amino acid) fraction to the counts in the cells in the TCA-insoluble (cell protein) fraction that were released over 3 h. Separate studies revealed that the counts in the medium from the TCA-insoluble (medium protein) fraction were constant and  $< 0.2\%$  of the cell TCA-insoluble (cell protein) fraction. In separate experiments JTC cells were exposed to varying concentrations of NH<sub>4</sub>Cl (0–20 mM) for 72 h to determine the dose-dependent effect of NH<sub>4</sub>Cl exposure on protein degradation.

### Measurement of cell size, blastogenic index, and RNA content using flow cytometry

JTC cells were bathed in medium A for 96 h and then exposed to medium A, B, or C for various periods of time. The cell monolayers were then trypsinized, washed twice with PBS, resuspended in 70% ethanol, and stored at 4°C. For each sample, equal aliquots were washed twice with PBS. One aliquot was resuspended in 0.1 ml PBS with 1.0 mg/ml RNase A. The other was resuspended in PBS. Both aliquots were incubated at 37°C for 30 min. Each sample was then stained with propidium iodide (1.0 ml of a 50  $\mu$ g/ml stock in hypotonic citrate; 41). Samples were allowed to equilibrate with the dye for 24 h at 4°C before analysis on an EPICS V cell sorter (Coulter Electronics Inc., Hialeah, FL). The following histograms were generated for each sample: (a) forward angle light scatter to estimate cell size (42), and (b) linear fluorescences gated from the forward angle light scatter to perform a cell cycle analysis, assess the blastogenic index (43), and measure the RNA content per cell (44). A minimum of 10,000 cells were analyzed from each sample. Cell cycle analysis was performed by plotting the number of cells in the sample versus the quantity of DNA per cell (as measured by propidium iodide fluorescence). The blastogenic

index, which is defined as the percentage of cells in the population that are in the S + G<sub>2</sub> + M phase of the cell cycle, was calculated using standard programs on the MDADS computer (Coulter Electronics Inc.). An estimate of the total cellular RNA content was determined by the difference in the G<sub>1</sub> peak of the samples treated with and without RNase.

#### Measurement of DNA synthetic rate

[<sup>3</sup>H]Thymidine (2 μCi/ml, sp act 35 Ci/mmol; ICN Radiochemicals, Irvine, CA) was added directly to the medium and cells were incubated for 2 h at 37°C. [<sup>3</sup>H] Counts incorporated into TCA-precipitable material were measured in JTC cells exposed initially to medium A for 96 h and then to medium A + various concentrations of NH<sub>4</sub>Cl (0–20 mM) for the next 96 h.

#### Measurement of lysosomal enzyme cathepsins L and B

JTC cells were bathed in medium A for 96 h and then exposed to various concentrations of NH<sub>4</sub>Cl (0–20 mM). At 72 h exposure measurements of the cell content of the cathepsin L and B lysosomal enzymes were made (45). Standard curves were generated using 7-amino-4-methylcoumarin (NMc; Enzyme Systems Products, Livermore, CA) in various concentrations from 0.5 to 4 μM. NMc standards were made by diluting aliquots of a 100 μM of NMc dissolved in DMSO with the assay buffer. The assay buffer contained 11.0 mM Na<sub>2</sub>HPO<sub>4</sub>, 55.2 mM KH<sub>2</sub>PO<sub>4</sub>, and 4.0 mM EDTA. 10-mM stock solutions of the two lysosomal enzyme substrates used in the lysosomal assay, Z-Phe-Arg-NMc (for cathepsin L) and Z-Arg-NMc (for cathepsin B), were made by dissolving each compound in DMSO to a final concentration of 10 mM. On the day of the study the stock solutions of the lysosomal enzyme substrates were diluted to 1 mM with 0.1% Brij 35. The stop solution used in the assay contained 100 mM sodium monochloroacetate in a sodium acetate buffer consisting of 30 mM sodium acetate and 70 mM acetic acid, pH 4.3. To assay the cell lysosomal enzyme content the cell monolayers were trypsinized and the suspension centrifuged. The pellet was resuspended in PBS. A cell count was performed on a 10-μl aliquot. The suspension was recentrifuged and the supernatant was removed. The pellet was resuspended in the assay buffer with 0.2% Triton X and the cell suspension was frozen for 15 min and then thawed in a 37°C water bath. Equal volumes of the cell homogenate and substrate were mixed and incubated at 37°C in a water bath (for 20 min). Then the mixture was taken out and placed on ice and the reaction was stopped by adding ice-cold stop solution. The fluorescence of samples was measured in a fluorometer (LS-5; Perkin-Elmer Corp., Norwalk, CT) at an excitation wavelength of 365 nm and an emission wavelength of 450 nm using 3-nm slits. The enzyme activity was expressed as picomoles of NMc released per minute per cell.

#### Measurement of pH<sub>i</sub>

JTC cells were grown to confluency on glass coverslips. pH<sub>i</sub> was measured with 2',7'-bis-(carboxyethyl)-5,6 carboxyfluorescein (BCECF) as previously described using an LS-5 fluorometer (46). Calibration of intracellular BCECF fluorescent excitation ratios was performed after each experiment using high-K<sup>+</sup> nigericin solutions set to various external pH values (46).

#### Measurement of Na<sup>+</sup>/H<sup>+</sup> antiport activity

The cells were acid-loaded by exposure to and subsequent removal of NH<sub>4</sub>Cl (20 mM) in the absence of sodium in HEPES-buffered solutions (47). Cell buffer capacity (β) was measured after removal of NH<sub>4</sub>Cl in the absence of sodium (47). The amiloride-inhibitable, Na<sup>+</sup>-dependent rate of H<sup>+</sup> efflux was calculated according to the formula H<sup>+</sup> efflux = β × dpH<sub>i</sub>/dt where dpH<sub>i</sub>/dt is the rate of amiloride-inhibitable, Na<sup>+</sup>-dependent pH<sub>i</sub> recovery measured in the initial 45 s.

#### Solutions

Steady state of pH<sub>i</sub> was measured in cells bathed in a solution containing 115 mM NaCl, 2.5 mM K<sub>2</sub>HPO<sub>4</sub>, 25 mM NaHCO<sub>3</sub>, 1 mM CaCl<sub>2</sub>,

1 mM MgCl<sub>2</sub>, and 5 mM glucose. The solution was gassed with 95% O<sub>2</sub>/5% CO<sub>2</sub>, pH 7.4 (solution A). In separate experiments pH<sub>i</sub> was measured in cells bathed in a solution otherwise identical to solution A containing 2 or 20 mM NH<sub>4</sub>Cl. To measure the rate of Na<sup>+</sup>/H<sup>+</sup> antiport activity the cells were loaded with BCECF in a Na<sup>+</sup>-free solution containing 20 mM NH<sub>4</sub>Cl for 5 min: 120 mM TMACl, 2.5 mM K<sub>2</sub>HPO<sub>4</sub>, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 5 mM glucose, and 5 mM HEPES and gassed with 100% O<sub>2</sub>, pH 7.4 (solution B). Then NH<sub>4</sub>Cl was removed by bathing the cells in a solution containing 140 mM TMACl, 2.5 mM K<sub>2</sub>HPO<sub>4</sub>, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 5 mM glucose, and 5 mM HEPES and gassed with 100% O<sub>2</sub>, pH 7.4 (solution C). The Na<sup>+</sup>-dependent recovery rate was measured after the readdition of a solution identical to solution C except that TMACl (140 mM) was replaced with NaCl (140 mM) (solution D).

#### Materials

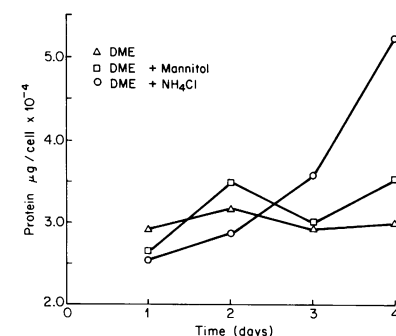
BCECF-AM was from Molecular Probes Inc., Junction City, OR; [<sup>3</sup>H]leucine and [<sup>3</sup>H]thymidine were from ICN Radiochemicals; propidium iodide and RNase were from Calbiochem-Behring Corp., San Diego, CA; DME and FCS were from Gibco Laboratories, Grand Island, NY; leucine, TCA, DNase 1, amiloride HCl, DMSO, and Triton X-100 were from Sigma Chemical Co., St. Louis, MO; mannitol, NH<sub>4</sub>Cl, and EDTA were from Fisher Scientific Co., Pittsburgh, PA; NMc, Z-Phe-Arg-NMc, and Z-Arg-NMc were from Enzyme Systems Products; and sodium monochloroacetate and Brij 35 were from Eastman Kodak Co., Rochester, NY.

#### Statistics

The results are expressed as mean ± SEM. The unpaired *t* test was used as required to compare two group means. When a protocol involved more than three group means Dunnett's *t* test (48) was used to compare each experimental group with the same control group. *n* refers to the number of cell monolayers used to derive a mean value for a given protocol. Monolayers were assigned randomly to the experimental groups.

#### Results

**Effect of NH<sub>4</sub>Cl on cell protein content.** The protein content of the cells bathed in medium A, B, or C for 24–72 h was not significantly different. However, at 96 h the protein content of the cells exposed to 20 mM NH<sub>4</sub>Cl (medium C) was significantly increased (5.24 ± 0.47 × 10<sup>-4</sup> μg/cell, *n* = 4, *P* < 0.01 vs. DME; *P* < 0.02 vs. DME + 40 mM mannitol; Fig. 1), whereas the cell protein content had remained unchanged in cells bathed in media A and B. Since renal cortical cells are not exposed to 20 mM ammonium in vivo, JTC cells were exposed to either 0 or 2 mM NH<sub>4</sub>Cl to determine whether hypertrophy



**Figure 1.** Effect of NH<sub>4</sub>Cl on cell protein content in JTC cells (time course). JTC cells were exposed to serum-free DME (medium A) for 96 h and then to either serum-free DME (medium A), serum-free DME + 40 mM mannitol (medium B), or serum-free DME + 20 mM NH<sub>4</sub>Cl (medium C). Cell protein content

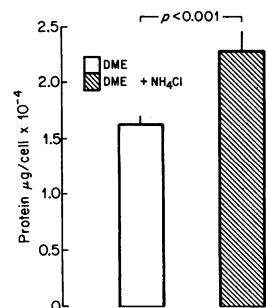
was measured after 24, 46, 72, and 96 h. Each point represents the mean of four determinations.

could be induced by this more physiologically relevant concentration of  $\text{NH}_4\text{Cl}$ . As shown in Fig. 2, 96 h exposure to DME + 2 mM  $\text{NH}_4\text{Cl}$  caused the cell protein content to increase from  $1.63 \pm 0.09 \times 10^{-4} \mu\text{g}/\text{cell}$  ( $n = 4$ ) to  $2.28 \pm 0.18 \times 10^{-4} \mu\text{g}/\text{cell}$  ( $n = 5$ ),  $P < 0.001$ . To determine the dose dependence of this effect cells were exposed to various concentrations of  $\text{NH}_4\text{Cl}$  (0–20 mM) in the presence (Fig. 3 a) and absence (Fig. 3 b) of serum, and the cell protein content was measured after 96 h exposure. As depicted in Fig. 3 a,  $\text{NH}_4\text{Cl}$  exposure caused a dose-dependent increase in cell protein content without plateauing at 20 mM. As shown in Fig. 3 b, the results were qualitatively similar in the serum-free studies. To rule out the possibility that  $\text{NH}_4\text{Cl}$  was only capable of inducing hypertrophy in a continuous renal cell line such as JTC cells, further experiments on a primary culture of rabbit proximal tubule cells were performed. As shown in Fig. 4, after 96 h exposure  $\text{NH}_4\text{Cl}$  (0–20 mM) induced a dose-dependent increase in cell protein in a primary culture of rabbit proximal tubule cells. Protein content increased in a dose-dependent fashion from  $5.80 \pm 0.53 \times 10^{-4} \mu\text{g}/\text{cell}$  ( $n = 4$ ) to  $12.9 \pm 0.73 \times 10^{-4} \mu\text{g}/\text{cell}$  ( $n = 4$ ),  $P < 0.01$ . As in JTC cells, the effect did not plateau at 20 mM  $\text{NH}_4\text{Cl}$ .

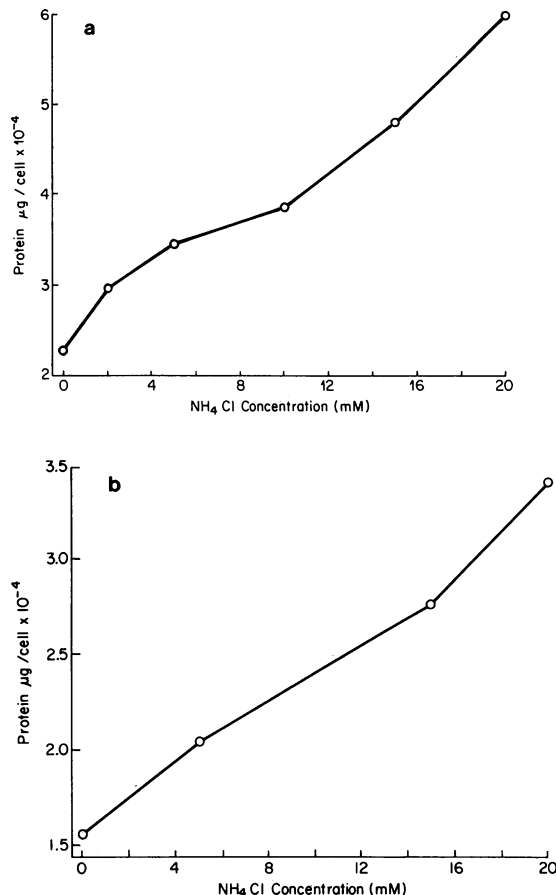
In a previous study of proximal tubule cells an inverse relationship was documented between cell number and cell protein content, when cell number decreased to  $< \sim 50\%$  of the control value (49). As summarized in Table I, only in 15 and 20 mM  $\text{NH}_4\text{Cl}$  was cell number significantly decreased in JTC cells, suggesting that at these higher doses a decrease in cell density could have contributed in part to the observed increase in cell protein content.

**Effect of acute  $\text{NH}_4\text{Cl}$  exposure on cell protein content.** It is well documented that  $\text{NH}_4\text{Cl}$  exposure results in an initial rapid rise in  $\text{pH}_i$ , which peaks in  $< 5$  s and is due to the rapid cellular influx of  $\text{NH}_3$  (47, 50). The rise in  $\text{pH}_i$  is followed immediately by a return of  $\text{pH}_i$  to baseline due to the influx of  $\text{NH}_4^+$  and stimulation of  $\text{Cl}^-/\text{base}$  exchange (46). It has previously been shown that a rise in  $\text{pH}_i$  resulting from a stimulation of  $\text{Na}^+/\text{H}^+$  antiport activity is an important signaling event that results in growth of sea urchin eggs (51).

To determine whether the acute rise in  $\text{pH}_i$  induced by exposure to 20 mM  $\text{NH}_4\text{Cl}$  was sufficient to cause cell hypertrophy, JTC cells were acutely exposed to serum-free DME + 20 mM  $\text{NH}_4\text{Cl}$  for 10 s and thereafter serum-free DME only. In this protocol  $\text{pH}_i$  increased  $\sim 0.5$  pH units after  $\text{NH}_4\text{Cl}$  exposure and returned to baseline after the removal of  $\text{NH}_4\text{Cl}$  (data not shown).  $\text{pH}_i$  did not decrease below the baseline value because the short exposure did not permit sufficient  $\text{NH}_4^+$  to enter the cells. 96 h after the acute exposure to 20 mM

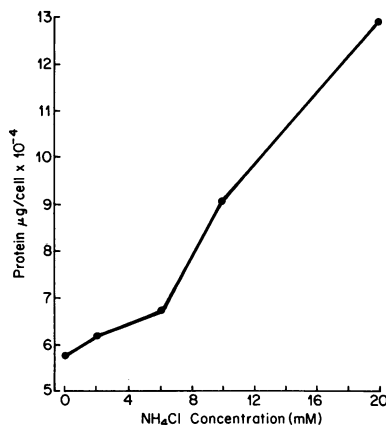


**Figure 2.** Effect of 2 mM  $\text{NH}_4\text{Cl}$  on JTC cell protein content (96 h exposure). JTC cells were exposed to serum-free DME (medium A) for 96 h and then to either serum-free DME (medium A) or serum-free DME + 2 mM  $\text{NH}_4\text{Cl}$  for the following 96 h. 2 mM  $\text{NH}_4\text{Cl}$  induced a significant increase in cell protein content.



**Figure 3.** Effect of  $\text{NH}_4\text{Cl}$  on cell protein content in JTC cells (dose response). JTC cells were exposed to DME + various concentrations (0–20 mM) of  $\text{NH}_4\text{Cl}$  for 96 h in the presence (a) and absence (b) of serum. Each point represents the mean of at least three determinations.

$\text{NH}_4\text{Cl}$ , protein content was  $2.27 \pm 0.10 \times 10^{-4} \mu\text{g}/\text{cell}$  ( $n = 5$ ), which was not significantly different from control cells,  $2.22 \pm 0.11 \times 10^{-4} \mu\text{g}/\text{cell}$  ( $n = 5$ ). These results suggest that the acute rise in  $\text{pH}_i$  resulting from  $\text{NH}_4\text{Cl}$  exposure is not sufficient to induce hypertrophy in JTC cells.



**Figure 4.** Effect of  $\text{NH}_4\text{Cl}$  on cell protein content of rabbit proximal cells in primary culture (96 h exposure). The cells were exposed to serum-free DME/Ham's F12 medium (1:1) in the absence of insulin and hydrocortisone for 48 h and thereafter to the same medium + various concentrations of  $\text{NH}_4\text{Cl}$  (0–20 mM) for the next 96 h. Each point represents the mean of four determinations.

Table I. Effect of NH<sub>4</sub>Cl on Cell Number (96 h Exposure)

NH <sub>4</sub> Cl	Cell number
mM	×10 <sup>4</sup> /35-mm dish*
0	65.0±4.4
2	62.2±4.7
5	54.3±4.8
10	59.9±4.4
15	50.7±2.3 <sup>‡</sup>
20	46.4±4.8 <sup>‡</sup>

\* The results represent the mean of three to five studies. For each study, three to six culture dishes were used to calculate the value for each experimental group.

<sup>‡</sup> P < 0.05 vs. 0 mM NH<sub>4</sub>Cl.

**Measurement of DNA synthetic rate, blastogenic index, and cell cycle analysis.** Changes in cell protein content induced by NH<sub>4</sub>Cl exposure may have resulted from a stimulation of cell proliferation (hyperplastic response). Therefore, thymidine incorporation was measured as an index of DNA synthesis to determine whether NH<sub>4</sub>Cl induced a hyperplastic vs. a hypertrophic response. As Fig. 5 depicts, thymidine incorporation/cell was unaffected by exposure of the cells for 96 h to various concentrations of NH<sub>4</sub>Cl (0–20 mM), which indicates that NH<sub>4</sub>Cl induces an in vitro model of cell hypertrophy rather than hyperplasia. To confirm the interpretation that NH<sub>4</sub>Cl exposure does not induce hyperplasia in JTC cells, a cell cycle analysis was performed using the flow cytometer (Fig. 6) and the blastogenic index was calculated after 24, 48, and 72 h exposure to either medium A, B, or C (Table II). As depicted in Table II, after 24 h in serum-free media the blastogenic index was ~ 15% in all experimental groups and subsequently decreased to < 10% after 72 h in serum-free media. The results indicate that the percentage of cells in the population that were in G<sub>0</sub> was not affected by NH<sub>4</sub>Cl exposure. These results complement the [<sup>3</sup>H]thymidine incorporation data and indicate that NH<sub>4</sub>Cl exposure does not induce hyperplasia in JTC cells.

**Effect of NH<sub>4</sub>Cl on cell size.** Further experiments were performed to determine whether in addition to increasing the protein content of JTC cells, NH<sub>4</sub>Cl increases the cell size.

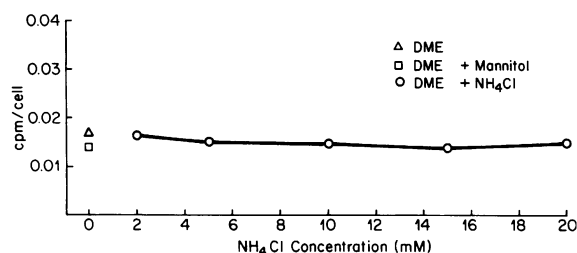


Figure 5. Effect of NH<sub>4</sub>Cl on DNA synthesis in JTC cells as assessed by 2-h [<sup>3</sup>H]thymidine incorporation (96 h exposure). JTC cells were bathed in serum-free DME (medium A) for 96 h and the rate of DNA synthesis was measured after the cells were exposed for the next 96 h to serum-free DME + various concentrations of NH<sub>4</sub>Cl (0–20 mM). Each point represents the mean of at least four determinations. The results indicate that NH<sub>4</sub>Cl does not induce cell hyperplasia.

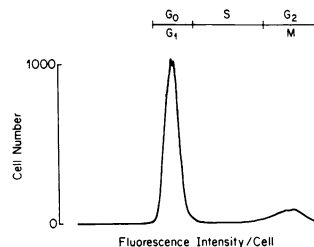


Figure 6. Cell cycle analysis of JTC cells bathed in serum-free DME medium (medium A) for 96 h. Note that most of the cells are in G<sub>0</sub>.

Using the forward angle light scattering technique, the cell size was assessed qualitatively in a flow cytometer in cells exposed to medium A, B, or C. As depicted in Fig. 7, the size distribution of cells bathed in 20 mM NH<sub>4</sub>Cl (medium C) was increased.

**Effect of NH<sub>4</sub>Cl on protein synthetic rate.** Increase in cell protein content after exposure of JTC cells to NH<sub>4</sub>Cl could have been due to an increase in the protein synthetic rate and/or a decrease in the protein degradation rate. To clarify this issue the protein synthetic rate was assessed by measuring [<sup>3</sup>H]leucine incorporation in 16 h. Table III shows that after 24 h exposure to NH<sub>4</sub>Cl the rate of protein synthesis had increased significantly. The increase in protein synthesis remained elevated up to 96 h exposure. Cells were then exposed to various concentrations of NH<sub>4</sub>Cl (0–20 mM) for 72 h. As summarized in Table IV, NH<sub>4</sub>Cl caused a dose-dependent increase in protein synthesis (4 and 16 h [<sup>3</sup>H]leucine incorporation). These results indicate that the increase in JTC cell protein content induced by NH<sub>4</sub>Cl results in part from a stimulation of the protein synthetic rate.

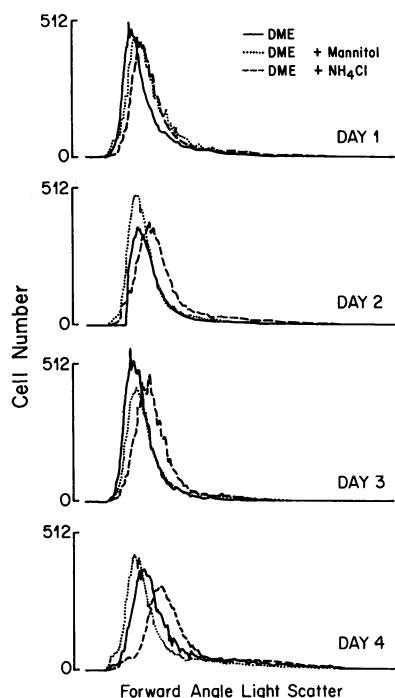
**Effect of NH<sub>4</sub>Cl on cytoplasmic RNA content.** To determine whether an increase in the protein synthetic rate was associated with an increase in cytoplasmic RNA, total cytoplasmic RNA content was measured using a flow cytometric assay after 72 h exposure to medium A, B, or C. As depicted in Fig. 8, NH<sub>4</sub>Cl exposure significantly increased the RNA content of the cells. Associated with an increase in cell RNA content was an increase in ribosome number as depicted in Fig. 9, a and b).

**Effect of NH<sub>4</sub>Cl on the protein degradation rate.** NH<sub>4</sub>Cl has been previously been shown to decrease the rate of protein degradation in cultured hepatocytes (25), rat fibroblasts (26), and mouse macrophages (27), presumably due to its effect on lysosomal pH (26; see Discussion). Therefore, further studies were performed to determine whether the protein degradation rate was decreased in JTC cells exposed to NH<sub>4</sub>Cl. The results are expressed as percentage of total cell protein that was de-

Table II. Blastogenic Index

	Time (h) after serum removal		
	24 h	48 h	72 h
DME	15.0%	5.8%	8.8%
DME + mannitol (40 mM)	17.4%	7.7%	7.7%
DME + NH <sub>4</sub> Cl (20 mM)	18.7%	4.7%	6.9%

Blastogenic index is defined as the percentage of cells in the population that are in the S + G<sub>2</sub> + M phase of the cell cycle. Each result was calculated by measuring a total of 10,000 cells.



**Figure 7.** Cell size analysis (time course). Population cell size was analyzed qualitatively using the flow cytometer forward angle light scattering technique (see Methods). Cells were exposed to serum-free DME (medium A) for 96 h and then to either serum-free DME (medium A), serum-free DME + 40 mM mannitol (medium B), or serum-free DME + 20 mM  $\text{NH}_4\text{Cl}$  (medium C) for 24, 48, 72, or 96 h. The mean population cell size increased in the  $\text{NH}_4\text{Cl}$ -treated group at 48, 72, and 96 h when compared with cells exposed to medium A or B ( $P < 0.01$ ).

graded in 3 h because of the well-documented linear relationship between cell protein content and the protein degradation rate. As Table V shows, by 24 h exposure the percent of cell protein degraded in the  $\text{NH}_4\text{Cl}$ -treated cells was significantly decreased. In a separate series of experiments the dose dependence of this effect was determined after 72 h exposure to 0–20 mM  $\text{NH}_4\text{Cl}$ . As summarized in Table VI, by 24 h exposure  $\text{NH}_4\text{Cl}$  decreased the protein degradation rate in JTC cells. The inability to detect a significant increase in cell protein content until 96 h exposure (Fig. 1) despite a significant change in cell protein turnover before this time is probably due to the greater sensitivity of the protein turnover studies.

**Effect of  $\text{NH}_4\text{Cl}$  on lysosomal enzymes content (cathepsins B and L).** It has previously been suggested that  $\text{NH}_4\text{Cl}$  decreases the protein degradation rate in hepatocytes by inhibiting lysosomal enzyme function (25). This presumably occurs as a result of a rise in lysosomal pH induced by the passive entry of  $\text{NH}_3$ . An elevation in lysosomal pH inhibits the lysosomal reuptake of mannose-6-phosphate-coupled enzymes and increases the cellular efflux of these enzymes (52). JTC cells were exposed to  $\text{NH}_4\text{Cl}$  (0–20 mM) for 72 h after which measurements of lysosomal enzyme activity were made. As

shown in Table VII, the cathepsin B activity decreased from  $0.51 \pm 0.001$  ( $n = 4$ ) to  $0.28 \pm 0.001$  pmol/min per cell ( $n = 5$ ),  $P < 0.01$ , in a dose-dependent fashion. Similarly, cathepsin L activity decreased from  $0.43 \pm 0.001$  ( $n = 4$ ) to  $0.17 \pm 0.001$  pmol/min per cell ( $n = 5$ ),  $P < 0.01$ , as the  $\text{NH}_4\text{Cl}$  concentration increased from 0 to 20 mM. In addition, as depicted in Fig. 9, a and b  $\text{NH}_4\text{Cl}$  exposure resulted in lysosomal swelling and the accumulation of electron-dense material in the lysosomes.

**Effect of  $\text{NH}_4\text{Cl}$  on  $\text{pH}_i$ .** In the present study cell hypertrophy was induced by  $\text{NH}_4\text{Cl}$  exposure in the absence of extracellular acidemia. To determine whether  $\text{pH}_i$  was altered in cells chronically exposed to  $\text{NH}_4\text{Cl}$ ,  $\text{pH}_i$  was measured with BCECF. Since a hypertrophic response was detected in cells exposed to the more physiologic concentration of 2 mM  $\text{NH}_4\text{Cl}$ ,  $\text{pH}_i$  was measured initially at this concentration. JTC cells were bathed for 96 h in medium A to achieve quiescence and then bathed in either medium A or serum-free DME + 2 mM  $\text{NH}_4\text{Cl}$  for up to 96 h. As depicted in Fig. 10 a,  $\text{pH}_i$  was unaffected by 2 mM  $\text{NH}_4\text{Cl}$  exposure, indicating that cell hypertrophy can occur in the absence of intracellular or extracellular acidemia. In cells exposed to 20 mM  $\text{NH}_4\text{Cl}$  for 96 h (Fig. 10 b)  $\text{pH}_i$  was slightly decreased at 24 and 48 h exposure but was not significantly different from control at the remaining time points.

**Effect of  $\text{NH}_4\text{Cl}$  on the rate of  $\text{Na}^+/\text{H}^+$  antiport activity.** JTC cells were bathed in medium A for 96 h. Then the rate of  $\text{Na}^+/\text{H}^+$  antiport activity was measured in cells exposed to either medium A or serum-free DME + 2 mM  $\text{NH}_4\text{Cl}$  for 24 and 72 h. As depicted in Fig. 11, the rate of  $\text{Na}^+/\text{H}^+$  activity was not significantly affected by exposure to 2 mM  $\text{NH}_4\text{Cl}$ . After exposure of JTC cells to 2 mM  $\text{NH}_4\text{Cl}$ , the lack of detectable increase in  $\text{Na}^+/\text{H}^+$  antiport activity may have been due to the insensitivity of the methodology. Therefore, JTC cells were exposed to 20 mM  $\text{NH}_4\text{Cl}$  (medium C) and the rate of  $\text{Na}^+/\text{H}^+$  antiport activity was measured after 24 and 72 h exposure. The results shown in Fig. 11 demonstrate that unlike 2 mM  $\text{NH}_4\text{Cl}$ , after 72 h exposure to 20 mM  $\text{NH}_4\text{Cl}$  the rate of  $\text{H}^+$  efflux via the  $\text{Na}^+/\text{H}^+$  antiporter increased significantly from 7.93 to 0.49 mM/min ( $n = 5$ ) to  $12.4 \pm 2.20$  mM/min ( $n = 3$ ),  $P < 0.05$ .

**Effect of  $\text{NH}_4\text{Cl}$  on cell protein in amiloride-treated cells.** To determine whether stimulation of the plasma membrane  $\text{Na}^+/\text{H}^+$  antiporter was required for cell hypertrophy to occur, JTC cells were exposed to either serum-free DME (medium A), serum-free DME + 20 mM  $\text{NH}_4\text{Cl}$  (medium C), serum-free DME + 1 mM amiloride, or DME + 20 mM  $\text{NH}_4\text{Cl}$  plus 1 mM amiloride for 96 h. The basal cell protein content in cells

**Table III.** Protein Synthetic Rate (Counts per Minute/Cell; 16-h Uptake)

	n	Exposure time			
		24 h	48 h	72 h	96 h
DME	4	$0.25 \pm 0.02$	$0.21 \pm 0.02$	$0.29 \pm 0.02$	$0.26 \pm 0.02^*$
DME + mannitol (40 mM)	4	$0.27 \pm 0.01^\ddagger$	$0.20 \pm 0.01$	$0.28 \pm 0.02$	—
DME + $\text{NH}_4\text{Cl}$ (20 mM)	4	$0.35 \pm 0.01^{\S  }$	$0.33 \pm 0.02^{  \#}$	$0.41 \pm 0.02^{\#  **}$	$0.37 \pm 0.02^{\#§}$

\*  $n = 8$ ;  $^\ddagger n = 3$ ;  $^\S P < 0.01$  DME +  $\text{NH}_4\text{Cl}$  (20 mM) vs. DME;  $^{||} P < 0.01$  DME +  $\text{NH}_4\text{Cl}$  (20 mM) vs. DME + mannitol (40 mM);  $^\# P < 0.05$  DME +  $\text{NH}_4\text{Cl}$  (20 mM) vs. DME;  $^{**} P < 0.02$  DME +  $\text{NH}_4\text{Cl}$  (20 mM) vs. DME.

Table IV. Increase in Protein Synthetic Rate after 72 h Exposure to NH<sub>4</sub>Cl

NH <sub>4</sub> Cl mM	Uptake of [ <sup>3</sup> H]leucine	
	cpm/cell; 4-h uptake	cpm/cell; 16-h uptake
0	0.071±0.001 (n = 4)	0.21±0.01 (n = 4)
2	0.068±0.002 (n = 5)	0.23±0.01 (n = 5)
5	0.079±0.002 (n = 5)	0.30±0.01 (n = 2)
10	0.088±0.002 (n = 5)	0.31±0.02 (n = 4)
20	0.094±0.002 (n = 5)	0.36±0.03 (n = 4)

bathed in serum-free DME was  $1.56 \pm 0.11 \times 10^{-4}$   $\mu\text{g}/\text{cell}$  ( $n = 6$ ). In the cells exposed to 20 mM NH<sub>4</sub>Cl cell protein content increased to  $3.42 \pm 0.18 \times 10^{-4}$   $\mu\text{g}/\text{cell}$  ( $n = 6$ ) (Fig. 12; 119±16% increase,  $P < 0.001$ ). Protein content of cells exposed to serum-free DME + 1 mM amiloride was  $1.99 \pm 0.15 \times 10^{-4}$   $\mu\text{g}/\text{cell}$  ( $n = 4$ ), which was not significantly different from the protein content of the cells exposed to serum-free DME only. In cells bathed in 20 mM NH<sub>4</sub>Cl + 1 mM amiloride the protein content increased significantly to  $4.12 \pm 0.25 \times 10^{-4}$   $\mu\text{g}/\text{cell}$  ( $n = 5$ ) (Fig. 12; 107±21% increase,  $P < 0.001$ ). These studies indicate that (a) amiloride treatment does not alter basal cell protein content, and (b) amiloride exposure does not inhibit the hypertrophic response induced by NH<sub>4</sub>Cl. Further studies were performed to determine whether the Na<sup>+</sup>/H<sup>+</sup> antiporter in cells exposed to 1 mM amiloride for 96 h remained inactivated since it seemed possible that the antiporter may have upregulated in cells chronically exposed to amiloride, thereby potentially overcoming its inhibitory effect. As depicted in Fig. 13, cells chronically exposed to amiloride (96 h) still failed to demonstrate antiport activity in the presence of amiloride (1 mM) after acute intracellular acidification using the NH<sub>4</sub>Cl prepulse technique (47). These results suggest that normal functioning Na<sup>+</sup>/H<sup>+</sup> antiport activity is not a necessary requirement for the hypertrophic response induced by exogenous NH<sub>4</sub>Cl.

## Discussion

Lotspeich and Halliburton first demonstrated that NH<sub>4</sub>Cl loading in the rat in vivo resulted in renal hypertrophy (9, 32,

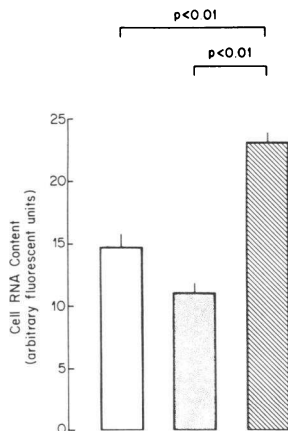


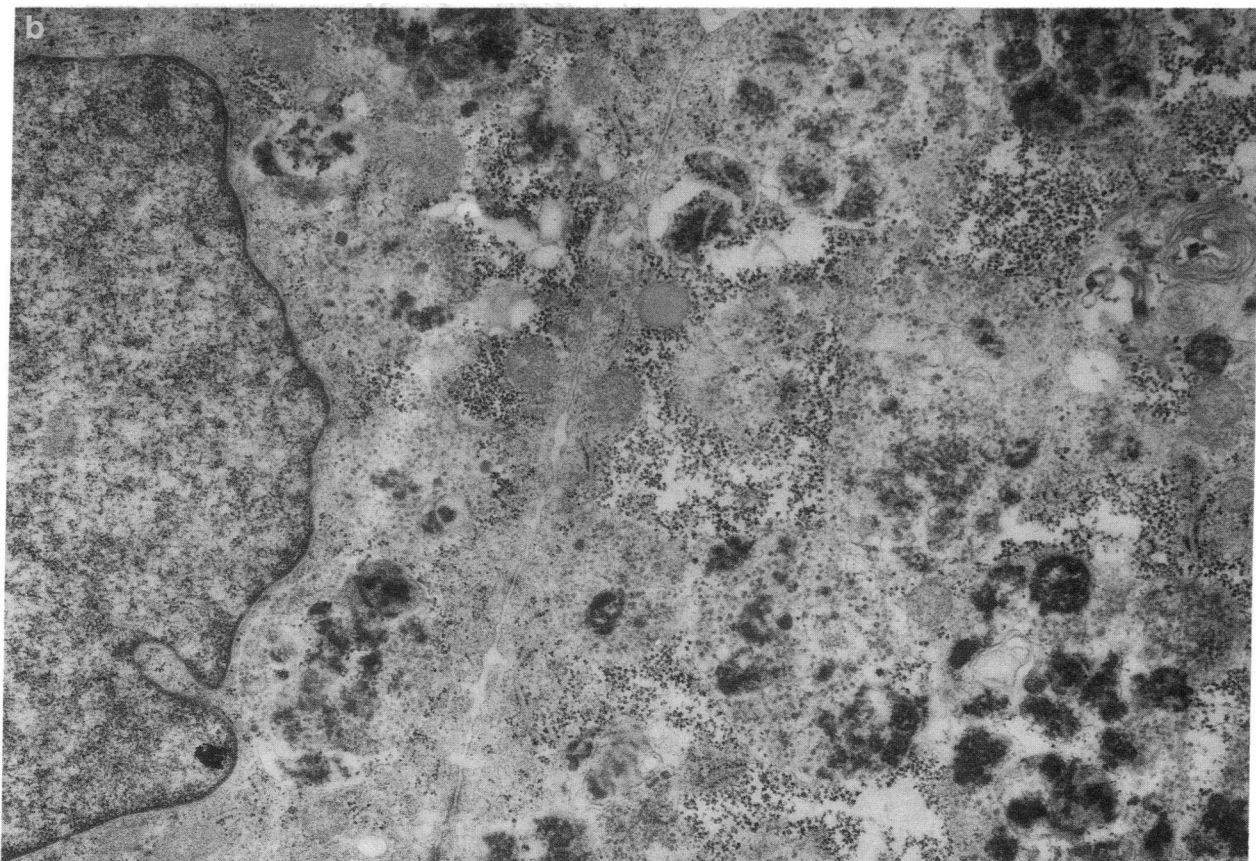
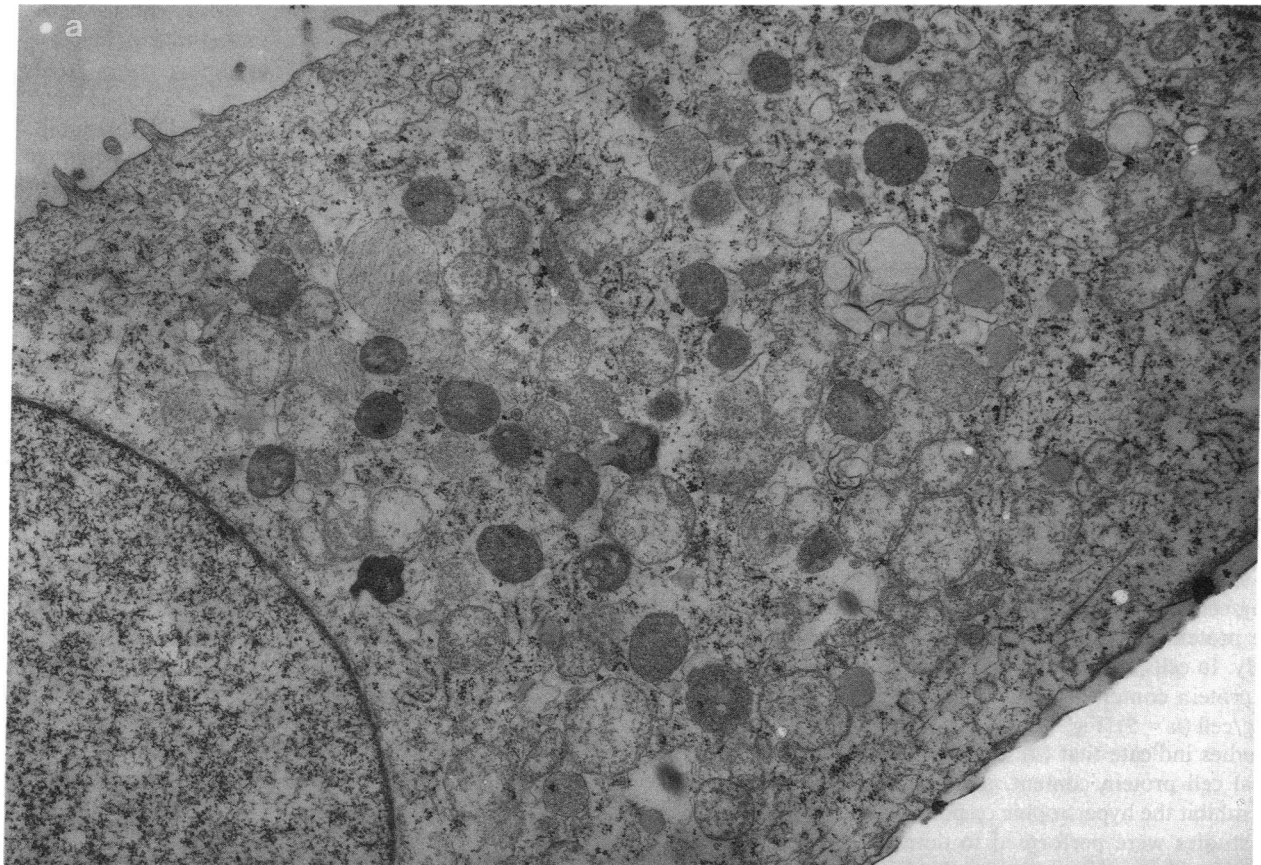
Figure 8. Effect of NH<sub>4</sub>Cl on JTC cell cytoplasmic RNA content (72 h exposure). JTC cells were exposed to serum-free DME (medium A) for 96 h and then to either serum-free DME (medium A), serum-free DME + 40 mM mannitol (medium B), or serum-free DME + 20 mM NH<sub>4</sub>Cl (medium C) for the next 72 h. Cytoplasmic RNA content was significantly increased at 72 h exposure to NH<sub>4</sub>Cl. Open bars, DME; stippled bars, DME + mannitol; hatched bars, DME + NH<sub>4</sub>Cl.

33). The increase in renal mass was associated with an increase in renal RNA content in the absence of an increase in DNA content, whereas ammonium citrate ingestion increased renal weight minimally and failed to alter RNA and DNA content. These results were interpreted as having demonstrated that acidosis per se caused the renal hypertrophy. In a recent study of cultured renal proximal tubule cells where the effect of changes in medium pH on cell growth were assessed in vitro, lowering the medium pH from 7.4 to 6.8 resulted in a hyperplastic response (increase in DNA synthesis as assessed by [<sup>3</sup>H]thymidine incorporation) (37). However, as mentioned, in vivo acidosis is associated with renal cell hypertrophy in the absence of an increase in DNA content. There are no reports of changes in medium pH resulting in hypertrophy of cells of renal or extrarenal origin. The results of the present study suggest the possibility that metabolic acidosis in vivo induces renal hypertrophy not as a result of the acidemia per se, but via an increase in renal ammoniogenesis. It is of interest that respiratory acidosis, which fails to stimulate renal ammoniogenesis to the same extent as metabolic acidosis (53), is associated with only minimal renal hypertrophy (54).

In the present study 2 mM NH<sub>4</sub>Cl was found to induce renal cell hypertrophy. Although the intracellular and peritubular capillary ammonia concentration has not yet been measured in vivo, the concentration of ammonia in the lumen of the rat proximal tubule in vivo has been measured in several micropuncture studies and varies from ~ 0.4 to 2.8 mM (16, 55–58). The ammonia concentration in the proximal tubule lumen increases to ~ 1.3 to 3.3 mM in rats with metabolic acidosis (55–57), ~ 5.6 mM in rats with reduced renal mass (16), and 4.1 mM in rats with potassium depletion (58). In the unstirred layer adjacent to the apical membrane the ammonia concentration may be greater. Therefore, the dose of 2 mM NH<sub>4</sub>Cl, which was found to induce hypertrophy in the present study, is within the physiologic range. In the inner medulla the concentration of ammonia is greater. The concentration of ammonia in rat papillary vasa recta blood is ~ 2 mM and increases to 6 mM in metabolic acidosis (59). In the tubule lumen at the bend of Henle's loop and the tip of the collecting duct in the rat the ammonia concentration is 2.5 and 34 mM, respectively (59). During metabolic acidosis these values increase to 9.4 and 74 mM, respectively (59). It would be of interest to determine whether cells derived from tubules in the inner medulla that are exposed to higher concentrations of ammonia in vivo manifest less hypertrophy when exposed to NH<sub>4</sub>Cl (0–20 mM) than cells derived from cortical tubules (i.e., dose response is shifted to the right).

An increase in cell protein content can result from an increase in protein synthesis and/or a decrease in protein degradation. Most efforts to determine the cause of increased cell protein content in models of renal hypertrophy have focused on factors that modulate protein synthesis. In vivo renal hypertrophy resulting from either a reduction in renal mass or metabolic acidosis is associated with an increase in protein synthesis (34, 60). The rate of protein degradation has not been measured in these models or any other in vivo or in vitro model of renal hypertrophy. It is of interest that insulin, which has been reported to decrease the rate of protein degradation (61), causes rabbit proximal tubule cells to hypertrophy in vitro (62). The results of the present study provide the first evidence that in addition to an alteration in protein synthesis, a decrease in protein degradation may be an important mecha-





**Figure 9.** Electron micrograph of a JTC cell exposed for 96 h to serum-free DME (medium A) and then to either serum-free DME (medium A) (a) or serum-free DME + 10 mM  $\text{NH}_4\text{Cl}$  (b) for the following 96 h.  $\text{NH}_4\text{Cl}$  exposure results in a marked increase in ribosome number and lysosomal swelling. The lysosomes accumulate electron-dense material which is presently unidentified.  $\times 10,000$ .

Table V. Protein Degradation Rate (Percent/Cell per 3 h)

	n	Exposure time			
		24 h	48 h	72 h	96 h
DME	4	3.0±0.1	2.6±0.1	4.0±0.1	3.4±0.35*
DME + mannitol (40 mM)	4	3.2±0.3	3.0±0.1	4.1±0.1	—
DME + NH <sub>4</sub> Cl (20 mM)	4	2.1±0.1 <sup>‡§</sup>	1.5±0.04 <sup>‡§</sup>	2.1±0.1 <sup>‡§</sup>	1.5±0.16 <sup>‡  </sup>

\* n = 6; ‡ P < 0.01 DME + NH<sub>4</sub>Cl (20 mM) vs. DME; § P < 0.01 DME + NH<sub>4</sub>Cl (20 mM) vs. DME + mannitol (40 mM); || n = 9.

nism by which total cell protein content can increase in renal cells. The importance of modulating the rate of protein degradation has been well documented in models of liver growth such as liver regeneration, replacement of hemopoietic tissue by liver parenchymal tissue postnatally, and recovery from protein deprivation (63). In all these examples of liver growth a decrease in the rate of protein degradation is the most important cause of the increase in cell protein content. Several studies have documented the ability of weak bases, e.g., ammonia, methylalanine, and chloroquine, in nonrenal cells to elevate intralysosomal pH, which prevents mannose-6-phosphate-coupled enzymes from being targeted to the lysosomes (52). It is probably via this mechanism that NH<sub>4</sub>Cl inhibits protein degradation in hepatocytes (25), rat fibroblasts (26), and mouse macrophages (27). These results complement the present finding that NH<sub>4</sub>Cl decreased the protein degradation rate and the cell content of the lysosomal enzymes cathepsin L and B in JTC cells in a dose-dependent fashion. Whether the inhibition of lysosomal proteolysis can fully account for the decrease in protein degradation induced by NH<sub>4</sub>Cl requires further study. Since the concentration of ammonia increases axially from the cortex to the inner renal medulla, it might be expected that the lysosomal enzyme content of the nephron segments in the inner medulla would be markedly decreased. In addition, as has been demonstrated in cultured rat fibroblasts (26) and the present study, NH<sub>4</sub>Cl exposure causes lysosomal swelling, which has been attributed to the osmotic entry of NH<sub>4</sub>Cl (a weak base) into the acidic lysosomal compartment. Whether the lysosomes in tubule segments in the inner medulla are more swollen in vivo than more proximal nephron segments is unknown. In addition to lysosomes, the Golgi apparatus maintains a low internal pH which is important for the trafficking of proteins to their proper location in polarized cells. NH<sub>4</sub>Cl exposure by raising the internal pH in the Golgi apparatus inhibits these processes (64). It would be of

interest to measure the lysosomal permeability to NH<sub>3</sub>/NH<sub>4</sub><sup>+</sup>, lysosomal enzyme content, lysosomal pH, and Golgi pH in tubules originating from the inner medulla to determine whether these nephron segments have somehow adapted to cope with the high concentration of ammonia to which they are exposed in vivo.

In addition to decreasing the protein degradation rate, exogenous NH<sub>4</sub>Cl exposure caused JTC cell protein content to increase as a result of increased protein synthesis. Ammonia has been reported to increase the rate of protein synthesis in sea urchin eggs (31) and differentiated astrocytes (23). Although the mechanism for NH<sub>4</sub>Cl-induced increase in protein synthesis was not investigated in the present study, recent experiments in sea urchin eggs indicate that ammonia increases the rate of protein synthesis via a pH-independent effect (31). Other amines such as tricaine and benzocaine which do not raise pH<sub>i</sub> were found to stimulate protein synthesis. In the present study the increase in protein synthesis was associated with an increase in total cell RNA content. An increase in protein synthesis and total RNA have been described in the in vivo models of hypertrophy associated with a reduction in renal mass and metabolic acidosis (9, 32–34, 60). Protein degradation rates have not been assessed in these models of renal hypertrophy. It would be of interest to measure protein synthetic and degradation rates in various in vivo human models of renal hypertrophy in an attempt to classify these disorders into mechanisms of increase in protein content.

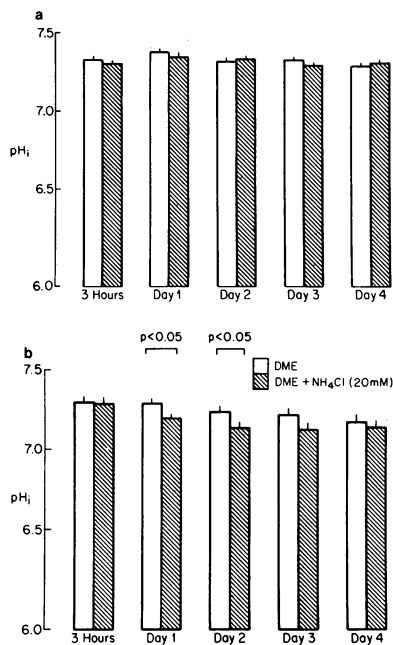
In the present study ammonia was added exogenously to proximal tubule cells. The question of whether an increase in endogenous ammonia production can also induce hypertrophy was not addressed. Endogenous ammonia production from glutamine in the proximal tubule, unlike exogenous ammonia addition, is associated with an increase in ATP production, bicarbonate production, possibly gluconeogenesis, and potentially an increase in Na<sup>+</sup>/NH<sub>4</sub><sup>+</sup> exchange (65, 66). In vivo,

Table VI. Decrease in Protein Degradation Rate after 72 h Exposure to NH<sub>4</sub>Cl

NH <sub>4</sub> Cl	n	Percent/cell per 3 h
mM		
0	5	2.9±0.1
2	3	2.6±0.2
5	5	2.4±0.1
10	5	2.3±0.1
20	5	2.1±0.1

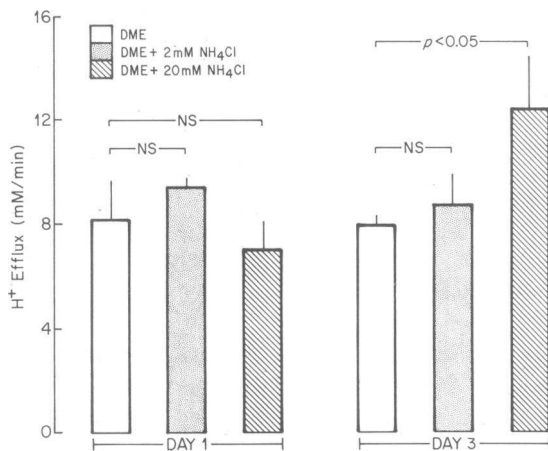
Table VII. Effect of NH<sub>4</sub>Cl on Lysosomal Enzyme Activity (72 h Exposure)

NH <sub>4</sub> Cl	n	Cathepsin B activity	Cathepsin L activity
mM		pmol/min per cell	pmol/min per cell
0	4	0.51±0.001	0.43±0.001
2	5	0.46±0.001	0.39±0.001
5	4	0.37±0.001	0.36±0.001
10	5	0.31±0.001	0.22±0.001
20	5	0.28±0.001	0.17±0.001

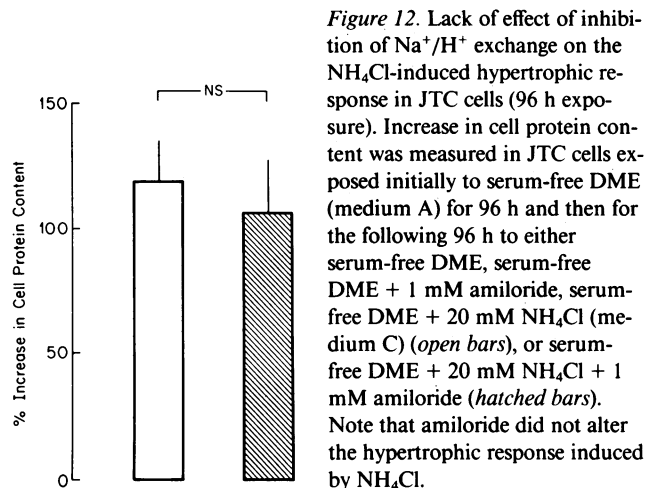


**Figure 10. a.** Lack of effect of chronic  $\text{NH}_4\text{Cl}$  exposure (2 mM) on  $\text{pH}_i$ . JTC cells were bathed in serum-free DME (medium A) for 96 h. The cells were then bathed in either medium A (open bars) or serum-free DME + 2 mM  $\text{NH}_4\text{Cl}$  (hatched bars) for up to 96 h.  $\text{pH}_i$  was measured on JTC cell monolayers with BCECF. After the initial transient increase in  $\text{pH}_i$  induced by  $\text{NH}_4\text{Cl}$  exposure (not shown),  $\text{pH}_i$  was not significantly different from control during chronic  $\text{NH}_4\text{Cl}$  exposure. **b.** In cells exposed to serum-free DME +  $\text{NH}_4\text{Cl}$  (medium C)

for 96 h,  $\text{pH}_i$  was decreased slightly at 24 and 48 h exposure but was not significantly different from control at the remaining time points. The data represent the mean of at least three measurements. Each measurement was obtained from a separate cell monolayer.



**Figure 11.** Effect of  $\text{NH}_4\text{Cl}$  exposure on rate of  $\text{Na}^+/\text{H}^+$  exchange. JTC cells were bathed in serum-free DME (medium A) for 96 h. The rate of  $\text{H}^+$  efflux via the  $\text{Na}^+/\text{H}^+$  exchanger was measured in JTC cells exposed for an additional 24 h and 72 h exposure to either serum-free DME, serum-free DME + 2 mM  $\text{NH}_4\text{Cl}$ , or serum-free DME + 20 mM  $\text{NH}_4\text{Cl}$ . To acid load the cells, on the day of study the monolayers were exposed to 20 mM  $\text{NH}_4\text{Cl}$  in the absence of  $\text{Na}^+$  (solution B) for 5 min. After the removal of  $\text{NH}_4\text{Cl}$  (solution C),  $\text{pH}_i$  decreased due to the rapid efflux of  $\text{NH}_3$ . The cells were then exposed to 140 mM NaCl (solution D). The amiloride-inhibitible,  $\text{Na}^+$ -dependent rate of  $\text{pH}_i$  recovery was measured ( $dp\text{H}_i/dt$ ) in the initial 45 s. Cell buffer capacity ( $\beta$ ) was measured and  $\text{H}^+$  efflux was calculated (see Methods). 2 mM  $\text{NH}_4\text{Cl}$  did not alter the rate of  $\text{Na}^+/\text{H}^+$  activity after 24 or 72 h exposure. 20 mM  $\text{NH}_4\text{Cl}$  significantly increased the rate of  $\text{Na}^+/\text{H}^+$  antiport activity after 72 h exposure. The results represent the mean of at least three measurements. Each measurement was obtained from a separate cell monolayer.

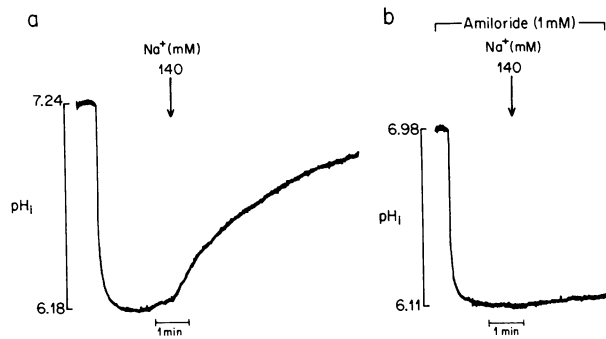


**Figure 12.** Lack of effect of inhibition of  $\text{Na}^+/\text{H}^+$  exchange on the  $\text{NH}_4\text{Cl}$ -induced hypertrophic response in JTC cells (96 h exposure). Increase in cell protein content was measured in JTC cells exposed initially to serum-free DME (medium A) for 96 h and then for the following 96 h to either serum-free DME, serum-free DME + 1 mM amiloride, serum-free DME + 20 mM  $\text{NH}_4\text{Cl}$  (medium C) (open bars), or serum-free DME + 20 mM  $\text{NH}_4\text{Cl}$  + 1 mM amiloride (hatched bars). Note that amiloride did not alter the hypertrophic response induced by  $\text{NH}_4\text{Cl}$ .

since the increase in the interstitial ammonia concentration in models of renal hypertrophy occurs primarily as a result of an increase in endogenous proximal tubule ammonia production, other segments of the nephron that also hypertrophy would be exposed to a higher concentration of ammonia exogenously. Further studies are required to assess the role of these factors in modifying the hypertrophic response.

Although the results of the present study indicate that  $\text{NH}_4\text{Cl}$  can induce hypertrophy in vitro in the presence or absence of other serum growth factors, the results of studies by Griendling et al. suggest that  $\text{NH}_4\text{Cl}$  may interact with growth factors to modulate the phosphoinositol pathway (67). In particular, these authors have reported that ammonia enhances the stimulation by angiotensin II of diacylglycerol formation in vascular smooth muscle cells. Recent studies of rats with renal hypertrophy secondary to a reduction of renal mass and type 1 diabetes mellitus have documented an increase in brush border protein kinase C activity (68, 69). Further studies are required to determine whether exposure to ammonia ± angiotensin II can increase protein kinase C activity in renal brush border vesicles.

Chronic hypokalemia has long been known to both increase renal ammonia production (13, 14) and induce renal hypertrophy (70). It was previously thought that hypokalemia causes intracellular acidosis which then stimulates ammonia production. That intracellular acidosis has been postulated to induce renal hypertrophy is in keeping with Lotspiech's hypothesis that extracellular acidosis causes renal hypertrophy. However, recent studies of proximal tubules derived from chronically  $\text{K}^+$ -depleted rats suggest that  $\text{pH}_i$  is not different from controls and yet these tubules produce more ammonia and glucose than control tubules (71). The mechanism by which a decrease in extracellular potassium modifies renal cell growth in vitro has been addressed. The results of these experiments indicate that an autocrine growth factor is released by BSC-1 cells when the extracellular  $\text{K}^+$  concentration is reduced (72). Unlike the hypertrophic effect of ammonia on JTC cells and rabbit proximal tubule cells, the autocrine factor produced by BSC-1 cells appears to be mitogenic, making it less likely that ammonia plays an important role as a growth factor in this in vitro model of renal cell growth. In addition, the in vivo rat  $\text{K}^+$  depletion model differs from the remnant kidney model in that in the former model: (a) morphologic changes mainly



**Figure 13.** Effect of chronic amiloride exposure (96 h) on JTC cell  $\text{Na}^+/\text{H}^+$  antiporter activity.  $\text{Na}^+/\text{H}^+$  antiporter activity was measured in cells exposed initially to serum-free DME (medium A) for 96 h and then to serum-free DME + 20 mM  $\text{NH}_4\text{Cl}$  (medium C) for the following 96 h in the absence (a) or presence (b) of 1 mM amiloride.  $\text{pH}_i$  was acutely acidified using the  $\text{NH}_4\text{Cl}$  prepulse technique. After continuous exposure to 20 mM  $\text{NH}_4\text{Cl}$  + 1 mM amiloride for 96 h the  $\text{Na}^+/\text{H}^+$  antiporter remained inactivated.

occur in the inner medulla, and (b) hyperplastic changes are present (1). Although factors other than an increase in ammonia production are probably involved in the  $\text{K}^+$  depletion model in vivo, of interest are the recent studies that have suggested that in the rat remnant kidney model and in the rat model of hypokalemic nephropathy, ammonia may cause interstitial nephritis by activation of the alternate complement pathway (70, 73). The authors found that administration of bicarbonate which decreased ammoniogenesis resulted in an amelioration of the histologic findings. In the chronic hypokalemic model of renal hypertrophy bicarbonate administration decreased the extent of renal hypertrophy despite the same degree of hypokalemia and more severe alkalemia (70). This latter finding complements the results of the present study in that bicarbonate administration according to the hypothesis presented in this report may have decreased the degree of renal hypertrophy via an inhibition of renal ammoniogenesis. It would be of interest to determine whether bicarbonate administration (or other maneuvers that would suppress ammoniogenesis) decreases the degree of renal hypertrophy in the remnant kidney model and other in vivo models of renal hypertrophy.

Several clinical models of renal hypertrophy (reduction in renal mass, type 1 diabetes mellitus, protein loading) are associated with an early increase in the single nephron glomerular filtration rate (SNGFR). The mechanism by which an increase in tubule flow rate could initiate renal hypertrophy is unknown. It has been suggested that an increase in SNGFR could induce renal hypertrophy by stimulating  $\text{Na}^+$  transport, perhaps via the  $\text{Na}^+/\text{H}^+$  antiporter (1, 2). The results of the present study, in addition to the recent finding that L-triiodothyronine increases  $\text{Na}^+/\text{H}^+$  exchange activity in the absence of cell hypertrophy (74), suggest that elevated  $\text{Na}^+/\text{H}^+$  exchange activity demonstrated in several clinical models of hypertrophy may be a consequence rather than a cause of the hypertrophic response, and other mechanism(s) by which an increase in SNGFR could induce renal hypertrophy ought to be considered.

It has previously been demonstrated that an increase in SNGFR secondary to a reduction in renal mass is associated

with an increase in the entry of ammonia into the proximal tubule lumen (16). Whole kidney ammonia production/glomerular filtration rate increases after a reduction in renal mass (15). In addition, the proximal tubule luminal ammonia concentration increases (16). In the isolated perfused mouse proximal tubule an increase in the luminal flow rate increases ammonia production (75). Given the rapidity by which ammonia production can be modulated by changes in proximal tubule flow, it seems possible that alterations in ammonia production could be the necessary link between a rise in SNGFR and subsequent renal hypertrophy.

Protein loading in the remnant kidney model results in a further increase in SNGFR,  $\text{Na}^+/\text{H}^+$  antiporter activity, ammonia production/excretion, and increased renal hypertrophy (1, 2, 10). Protein loading could stimulate renal ammonia production by (a) increasing the flux of ammoniogenic precursors to the kidney, (b) lowering systemic pH, or (c) increasing the proximal tubule luminal flow rate as a result of an increase in SNGFR. It remains to be determined whether, as in the hypokalemic model of renal hypertrophy, inhibition of ammonia production by bicarbonate administration can decrease the degree of renal hypertrophy in protein-loaded animals.

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