# Regulation of Ammonia Production by Mouse Proximal Tubules Perfused In Vitro
## Effect of Luminal Perfusion

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## Abstract

To investigate factors regulating ammonia ($NH_3$) production by isolated defined proximal tubule segments, we examined the rates of total $NH_3$ ($NH_3 + NH_4^+$) production by individual proximal tubule segments perfused in vitro under a variety of perfusion conditions. Segments consisting of late convoluted and early straight portions of superficial proximal tubules were incubated at 37°C in Krebs-Ringer bicarbonate (KRB) buffer containing 0.5 mM L-glutamine and 1.0 mM sodium acetate, pH 7.4. The rate of total ammonia production was calculated from the rate of accumulation of total $NH_3$ in the bath. The total ammonia production rate by unperfused proximal segments was 6.0±0.2 (±SE) pmol/mm per minute, which was significantly lower than segments perfused at a flow rate of 22.7±3.4 nl/min with KRB buffer (21.5±1.4 pmol/mm per minute; $P < 0.001$) or with KRB buffer containing 0.5 mM L-glutamine (31.9±2.5; $P < 0.001$). The rate of $NH_3$ production was higher in segments perfused with glutamine than in segments perfused without glutamine ($P < 0.01$).

The perfusion-associated stimulation of $NH_3$ production was characterized further. Analysis of collected luminal fluid samples revealed that the luminal fluid total $NH_3$ leaving the distal end of the perfused proximal segment accounted for 91% of the increment in $NH_3$ production observed with perfusion. Increasing the perfusion flow rate from 3.7±0.1 to 22.7±3.4 nl/min by raising the perfusion pressure resulted in an increased rate of total $NH_3$ production in the presence or absence of perfusate glutamine (mean rise in rate of total $NH_3$ production was 14.9±3.7 pmol/mm per minute in segments perfused with glutamine and 7.8±0.9 in those perfused without glutamine). In addition, increasing the perfusion flow rate at a constant perfusion pressure increased the rate of luminal output of $NH_3$. Total $NH_3$ production was not affected by reducing perfusate sodium concentration to 25 mM and adding 1.0 mM amiloride to the perfusate, a condition that was shown to inhibit proximal tubule fluid reabsorption. These observations demonstrate that the rate of total $NH_3$ production by the mouse proximal tubule is accelerated by perfusion of the lumen of the segment, by the presence of glutamine in the perfusate, and by increased perfusion flow rates. The increased rate of $NH_3$ production with perfusion seems not to depend upon normal rates of sodium reabsorption. The mechanism underlying the stimulation of $NH_3$ production by luminal flow is unknown and requires further study.

## Introduction

Ammonia production and its excretion by the kidney promote the maintenance of acid-base homeostasis (1, 2; reviews). Results of micropuncture studies have suggested that the proximal tubule is a major site for ammonia production and addition to the tubular fluid (3–6). Microanalysis of enzymatic activity along the nephron has shown that glutaminase, glutamate dehydrogenase, and α-ketoglutarate dehydrogenase, important enzymes in glutamine catabolism to ammonia and other metabolites (2, 7), reside in the proximal tubule (8, 9). Direct measurements of ammonia production rates made in unperfused isolated rat nephron segments have demonstrated that proximal tubules produced ammonia from glutamine at a substantial rate, and that the early segments of the proximal tubule, namely, the S1 and S2 segments, were the only portions of the rat nephron observed to increase ammonia production in response to metabolic acidosis (10).

Biochemical studies on unperfused isolated nephron segments have provided insight into the localization of a variety of biochemical processes, including ammonia production (9–11). Nevertheless, this approach may be criticized because the transporting activity of such unperfused dissected tubules may differ greatly from tubules that are perfused. Indeed, the lumen of an unperfused tubule incubated in vitro becomes very narrow or collapses with time so that transport across the tubular epithelium may be subnormal. As a result, the activity of biochemical processes that may be influenced by transport activity will differ in unperfused and perfused nephron segments.

The standard free-flow micropuncture technique has provided important information about the handling of ammonia by accessible portions of the nephron in situ (3–6). The main advantage of this technique is that functioning nephrons may be examined in a natural environment with preservation of spatial relationships with other nephron segments, interstitium, and vasculature. The limitations of this technique are that only certain accessible sites may be examined directly, that peritubular and luminal factors are difficult to control, and that total ammonia production rates for a particular segment cannot be derived directly from luminal fluid ammonia measurements.

To examine total ammonia production in defined nephron segments that are actively transporting, we have combined the technique of perfusing isolated nephron segments in vitro with

a technique for measuring the small amounts of ammonia produced by an individual nephron segment. The technique of perfusing isolated nephron segments in vitro has provided important insight into the transport functions of specific portions of the nephron (12, 13). The advantages of this technique are that the regional origin of the tubule being perfused is known, that tubules examined using this procedure are actively transporting solutes and fluid, and that perfusion and bath conditions may be altered in a controlled way. The ultramicroassay for ammonia used in this study is based on a series of enzymatic reactions coupled with a bioluminescent enzymatic reaction that permits detection of the picomole amounts of ammonia produced by an individual proximal tubule segment.

This study was designed to determine the effect of perfusion on ammonia production by isolated proximal tubule segments from mice. We studied mice because, like the rat, they excrete ammonia into the urine under normal circumstances. The major advantage of using the mouse is that mouse proximal tubule segments, in contrast to those of the rat, may be dissected without collagenase treatment, thus permitting perfusion in vitro.

## Methods

*Animals.* Male Swiss-Webster mice (Hilltop Lab Animals, Inc., Chatsworth, CA) weighing 25–35 g were used in these studies. The mice consumed Purina Rodent Chow (Ralston Purina Co., St. Louis, MO) and water ad libitum.

*Isolation and perfusion of proximal tubules.* Isolation and in vitro perfusion of proximal tubules were accomplished using the method originally described by Burg and colleagues (14) with some modifications. Mice were sacrificed by cervical dislocation, and the left kidney was removed. 1-mm thick slices were made along the cortico-medullary axis and placed in ice cold Krebs-Ringer bicarbonate (KRB)[1] buffer (composition, 125 mM NaCl, 25 mM $NaHCO_3$, 5 mM KCl, 1.2 mM sodium phosphate, 1.0 mM magnesium sulfate, and 1.0 mM calcium chloride) previously gassed with a mixture of 95% $O_2$ and 5% $CO_2$, pH 7.4, at 37°C. Segments composing the late convoluted and early straight portion of the superficial proximal tubules (0.5–1.2 mm in length) were removed from slices by freehand dissection under direct microscopic visualization. A dissected segment was then transferred to a perfusion chamber containing KRB buffer. The perfusion chamber was slightly modified so that the bath volume could be reduced to 200 $\mu$l. One end of the tubule was cannulated and perfused with KRB buffer. While being perfused continuously, the tubule was washed by adding and withdrawing 100-$\mu$l aliquots of KRB buffer containing 0.5 mM L-glutamine and 1 mM sodium acetate equilibrated at 37°C with a 95% $O_2$ and 5% $CO_2$ gas mixture and then incubated in a volume of ~150–200 $\mu$l. The bath medium was covered with mineral oil pregassed at 37°C with a mixture of 95% $O_2$:5% $CO_2$. The temperature of the oil and sample were maintained at 37°C, and mixing of the bath medium was accomplished by continuous bubbling of the oil above the sample with 95% $O_2$:5% $CO_2$ gas mixture (10). Bubbling of the oil above the medium containing the cannulated tubule resulted in vigorous vibration of the tubule in the medium. After a preincubation period of 20 min, the bath medium was exchanged with fresh KRB buffer containing L-glutamine and sodium acetate and then incubated for 20–30 min. The fluid exchange was accomplished by first adding and then removing 150 $\mu$l of buffer several times. At the end of the incubation period, 100 $\mu$l of incubation medium was transferred to a 300-$\mu$l microfuge tube, and when necessary, replaced by 100 $\mu$l of fresh KRB buffer with glutamine and sodium acetate for a second

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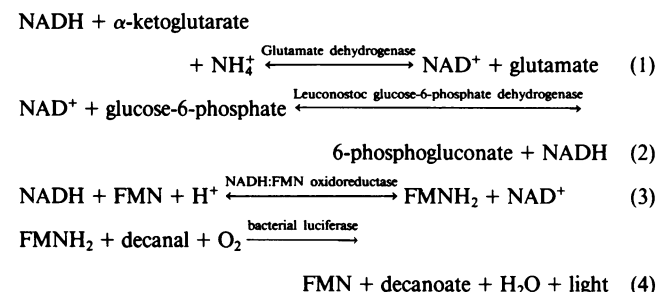1. *Abbreviations used in this paper:* KRB, Krebs-Ringer bicarbonate.

experimental period of 20–30 min. During the second incubation period, the incubation condition was changed. The exact bath volume was determined by measuring the dilution of known amounts of trypan blue in KRB added to the bath. Tubules were examined under a variety of conditions, as described in detail in Results, including the unperfused state, perfused with and without 0.5 mM L-glutamine in the perfusate, perfused at different perfusion flow rates, and perfused with low sodium perfusate (KRB with NaCl isoosmotically replaced with mannitol) containing 1.0 mM amiloride HCl (Merck, Sharp & Dohme, West Point, PA).

The 100-$\mu$l bath medium samples were treated with 25 $\mu$l of ice-cold 10% perchloric acid, neutralized with 2 M potassium bicarbonate, and centrifuged for 1 min in a microfuge (Beckman Instruments, Inc., Fullerton, CA). All steps were performed at 4°C. The supernatants were then analyzed by the ultramicroassay for total ammonia as described below. Blanks consisting of the same buffer as sample without incubation with tubule were treated in parallel with the experimental samples.

In some experiments, tubular luminal fluid samples were obtained by collecting the fluid emanating from the tubule with a collection pipette. Luminal fluid collected under oil was diluted in 100 $\mu$l perfusate and treated with perchloric acid and potassium bicarbonate for analysis of ammonia as described above. Because of the larger bath volumes required when using a collecting pipette, we were unable to measure accurately the bath concentration of ammonia simultaneously with collected perfusion fluid ammonia concentration.

In some proximal tubules, perfusion flow rate was determined by adding to the perfusate extensively dialyzed ³H-methoxy-inulin (New England Nuclear, Boston, MA) and measuring the rate at which this radioactive marker left the distal end of the perfused segment. Fluid reabsorption by some proximal tubules was measured as described previously (15).

*Ultramicroassay for total ammonia.* Since this method of assaying total ammonia has not been reported, it will be described in detail. The method uses a series of enzymatic reactions that are coupled with a bacterial luciferase bioluminescence reaction as outlined below:

$$NADH + \alpha\text{-ketoglutarate}$$
$$+ NH_4^+ \xleftrightarrow{\text{Glutamate dehydrogenase}} NAD^+ + glutamate \quad (1)$$
$$NAD^+ + glucose\text{-}6\text{-phosphate} \xleftrightarrow{\text{Leuconostoc glucose-6-phosphate dehydrogenase}}$$
$$6\text{-phosphogluconate} + NADH \quad (2)$$
$$NADH + FMN + H^+ \xleftarrow{\text{NADH:FMN oxidoreductase}} FMNH_2 + NAD^+ \quad (3)$$
$$FMNH_2 + decanal + O_2 \xrightarrow{\text{bacterial luciferase}}$$
$$FMN + decanoate + H_2O + light \quad (4)$$

Ammonia present in the sample or standard reacts with $\alpha$-ketoglutarate and NADH in the presence of glutamate dehydrogenase to form glutamate and $NAD^+$ (reaction 1). Because $\alpha$-ketoglutarate and NADH are present in excess, the amount of $NAD^+$ formed is equal to the amount of total ammonia ($NH_4^+$ and $NH_3$) originally present in the sample. The excess NADH is destroyed selectively by heating with acid. Then the $NAD^+$ is measured using a modified method of Brolin et al. (16), which incorporates an NAD-specific bacterial luciferase system (Bactilight I; Analytical Luminescence Laboratory, San Diego, CA) as depicted in reactions 2–4. The plateau rate of light emission is proportional to the concentration of ammonia present in the original sample.

60 $\mu$l of the supernatants of the samples, blanks, and standards treated with perchloric acid and potassium bicarbonate were added to 300 $\mu$l microfuge tubes. 20 $\mu$l of a 0.5-M Tris buffer, pH 7.6, containing 0.12 mM NADH and 5 mM $\alpha$-ketoglutarate and 20 $\mu$l of 0.1 M potassium phosphate, pH 7.6, in 50% glycerol containing 1 mg/ml bovine liver glutamate dehydrogenase (Sigma Chemical Co., St. Louis, MO) were added to each sample, and the mixture was incubated at room temperature for 30 min. The reaction was terminated by the

addition of 25 μl of 0.5 M sodium sulfate in 0.02 N sulfuric acid. The samples were then heated to 60°C for 45 min to destroy the excess NADH. After cooling the samples to room temperature, 90 μl of sample were mixed with 300 μl of commercially available 0.1 M phosphate buffer containing decanal and flavin mononucleotide (Bactilight buffer, Aldehyde reagent, and flavin mononucleotide; Analytical Luminescence Laboratory) followed by 20 μl of the Leuconostoc glucose-6-phosphate dehydrogenase solution [50 U/ml of lyophilized Leuconostoc glucose-6-phosphate dehydrogenase (Sigma Chemical Co.) in 5 mM glycine buffer, pH 7.8], 40 μl of 25-mM glucose-6-phosphate in 1.0 M TES (N-tris[Hydroxymethyl]-methyl-2-aminoethane-sulfonic acid) buffer (pH 7.7), and 90 μl of NAD-specific bacterial luciferase reagent (Bactilight I in Bactilight buffer; Analytical Luminescence Laboratory). Peak light emission and plateau rate of light emission were measured in a photometer (Model 20; Turner Designs, Mountain View, CA). Standard solutions were analyzed in parallel with samples and blanks each time assay was performed.

We found that the difference between the steady-state light emission of standards and that of the blank was linearly related to the concentration of total ammonia in the sample. This linear relationship was maintained for samples containing 0–4 μM (pmol/μl) of ammonia as shown in Fig. 1. The coefficient of variation for standards containing 1.0 μM total ammonia was ±0.11 ($n = 4$), and for standards containing 2.0 μM, was ±0.07 ($n = 4$). Most of the experimentally obtained samples contained 1.0 to 2.0 μM total ammonia. Samples containing 2.00 μM total ammonia in incubation medium were analyzed after being incubated under the experimental conditions. They contained 2.01±0.06 μM at the end of the incubation ($n = 4$).

All chemicals used in this study were of analytical grade or of the highest purity commercially available to minimize contamination with ammonia.

*Calculations and data analysis.* In experiments in which one end of the perfused segment was left open, luminal fluid emanating from the segment mixed with the bath medium. Therefore, under these conditions, the rate of ammonia accumulated in the bath represented total ammonia production by the perfused segment and was expressed as picomoles of NH₃ per millimeter per minute. Thus, the total ammonia production rate (picomoles per millimeter per minute) equaled: [Ammonia concentration (picomoles per microliter) × bath volume (microliters)]/[tubule length (millimeters) × incubation time (minutes)].

Luminal ammonia output was equal to the rate of ammonia appearance in fluid collected from the end of a perfused segment and was expressed as picomoles per millimeter per minute.

Data are expressed as mean±SE and are analyzed by paired or unpaired Student's $t$ test as appropriate.

## Results

Blood bicarbonate was 20.1±0.4 meq/liter and urine total ammonia excretion was 45.9±6.4 μmol/mg creatinine in 20 mice consuming a diet identical to those used in this study.

The rate of ammonia production from a single perfused superficial proximal tubule segment with a perfusion flow rate of 22 nl/min was constant for 90 min. Ammonia production rate for unperfused tubules was also constant for 90 min. All tubules excluded trypan blue added to the bath at the end of the experimental periods. In addition, fluid reabsorption by proximal segments perfused under experimental conditions remained constant for 90 min (1.09±0.26 nl/mm per minute; $n = 5$). The measurements reported in the remainder of this paper were all obtained within 90 min after beginning the incubation of the tubule.

Total ammonia production rates were measured in unperfused proximal tubule segments and segments perfused with KRB buffer with and without glutamine (Fig. 2). Unperfused segments were treated in the same way as perfused segments except that after cannulation perfusion was stopped. The perfusion flow rate measured in tubules perfused at a pressure of 35–40 cm H₂O was 22.7±3.4 nl/min ($n = 5$). The rate of total ammonia production measured in seven unperfused segments was 6.0±0.2 pmol/mm per minute. When proximal segments were perfused with KRB buffer containing 0.5 mM glutamine, the rate of ammonia production was 31.9±2.5
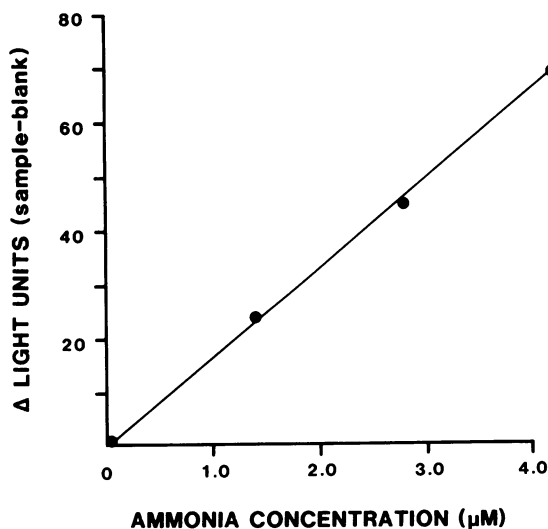


*Figure 1.* Standard curve of ammonia assay. Results of a representative assay showing a linear relationship between ammonia content of standard samples and the difference between plateau light emission of blank samples. Each closed circle represents the mean of two determinations.
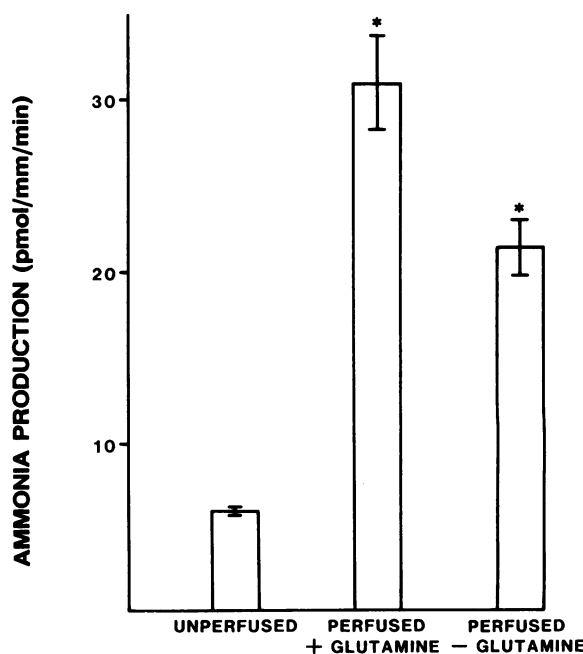


*Figure 2.* The ammonia production rates by unperfused proximal tubule segments, and segments perfused with KRB buffer containing glutamine (+ glutamine) and without glutamine (− glutamine). Values are mean±SE. *Significant difference between the means of segments perfused + and − glutamine and mean of unperfused segment, $P < 0.001$. Difference between means of segments perfused + glutamine and − glutamine was also significant, $P < 0.01$.

pmol/mm per minute ($n = 12$). When glutamine was removed from the perfusate, the rate of ammonia production perfused segments was $21.5\pm1.4$ ($n = 18$). The total ammonia production rate for unperfused segments was significantly less than observed in tubules perfused in the presence or absence of glutamine ($P < 0.001$). Segments perfused with glutamine in the perfusate produced ammonia at a significantly higher rate than segments perfused without glutamine in the perfusate ($P < 0.01$).

To determine the contribution of luminal output of ammonia to total ammonia production, we made timed collections of the fluid emanating from the end of five segments perfused without glutamine in the perfusate at perfusion pressures of 35–40 cm $H_2O$ and a mean perfusion flow rate of $22.0\pm2.7$ nl/min. The luminal output of ammonia was $13.7\pm1.8$ pmol/mm per minute. The difference in the mean ammonia production rates observed in unperfused segments and segments perfused with KRB buffer without glutamine obtained in experiments shown in Fig. 2 was $15.5\pm1.4$ pmol/mm per minute. Thus, 91% of the increase in ammonia production rate observed with perfusion was accounted for by ammonia emanating from the lumen. Furthermore, a greater proportion of the total ammonia released from the perfused segment came from the ammonia leaving the distal end of the segment than from the peritubular portion of the segment, which was calculated from the total ammonia output minus the luminal output of ammonia (ratio of luminal vs. peritubular ammonia output = 1.8:1.0).

To explore the mechanisms by which perfusion increased total ammonia production, we examined the effect of changes in flow rate on ammonia production by isolated perfused proximal tubule segments. The flow rates were altered by two mechanisms. In one series of experiments, the perfusion flow rate was changed by changing the perfusion pressure. As noted above, when tubules were perfused with 35–40 cm $H_2O$ pressure the flow rate was $22.7\pm3.4$ nl/min. When the perfusion pressure was reduced to 5 cm $H_2O$ the flow rate fell to $3.7\pm0.1$ nl/min ($n = 4$). Experiments on the effect of changing the perfusion flow rate on ammonia production were performed with and without glutamine in the perfusate. Each tubule examined was perfused at high and low pressure. When perfused at a high flow rate (22.7 nl/min), proximal tubules

produced ammonia more rapidly than when perfused at a low flow rate (3.7 nl/min), with a mean difference of $14.9\pm3.7$ pmol/mm per minute in the segments perfused with glutamine in perfusate ($P < 0.02$) and a mean difference of $7.8\pm0.9$ in segments perfused without glutamine in the perfusate ($P < 0.001$) (Fig. 3).

In another series of experiments, the perfusion flow rate was altered by constricting the end of the perfused tubule so that flow was reduced at a constant perfusion pressure. The flow rates changed from a base line of $22.0\pm2.7$ nl/min to $7.8\pm1.4$ nl/min when the constriction was imposed. Because of structural limitations of the perfusion chamber, only luminal output of ammonia could be determined under these experimental conditions. When proximal segments were perfused at reduced luminal flow rates, their luminal output of ammonia was $6.6\pm0.9$ pmol/mm per minute ($n = 5$), and when segments perfused at the higher flow rate, luminal output was $13.7\pm1.8$ pmol/mm per minute ($n = 5$). The difference in luminal ammonia output between segments perfused at low and high flow rates was significant ($P < 0.01$).

To ascertain whether flow-induced changes in ammonia production depended upon the flow-induced changes in the rate of fluid reabsorption observed in the proximal tubule (17), we perfused proximal segments with perfusate containing 25 mM sodium (sodium chloride isoosmotically replaced by mannitol) and 1.0 mM amiloride hydrochloride, a condition that has been shown to reduce fluid reabsorption in the rat proximal tubule (18). We found that fluid reabsorption by the mouse proximal tubule fell to $-0.11\pm0.36$ nl/mm per minute ($n = 3$) from a control value $1.09\pm0.26$ nl/mm per minute. In contrast, total ammonia production rate observed with tubules perfused with the low sodium solution containing amiloride was $22.4\pm1.4$ pmol/mm per minute, which did not differ significantly from controls perfused at similar flow rates.

## Discussion

By using a highly sensitive bioluminescence assay to measure picomole quantities of ammonia, we were able to determine directly ammonia production by individual isolated mouse proximal tubule segments perfused in vitro. Our results indicate that perfusion of the lumen of proximal tubule segments
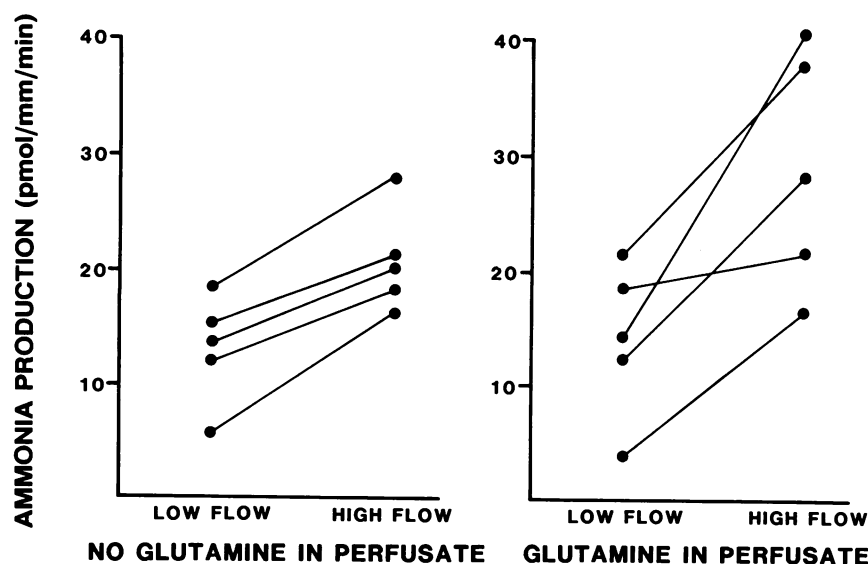


*Figure 3.* Total ammonia production by proximal segments perfused with (*right*) and without (*left*) glutamine in the perfusate at low and high perfusion flow rates ($3.7\pm0.1$ and $22.7\pm3.4$ nl/min, respectively). Each closed circle represents the mean of two determinations and the lines connecting data points indicates data obtained from a single nephron segment. Significant difference between low and high flow when perfused without glutamine, $P < 0.001$. Significant difference between low and high flow when perfused with glutamine, $P < 0.02$.

significantly increases total ammonia production; that is, ammonia released into the peritubular fluid and into the luminal fluid. Our data on ammonia production in unperfused mouse proximal segments are comparable with those observed in corresponding unperfused rat proximal tubule segments (10). Perfusion of the tubular lumen with buffer containing glutamine resulted in significantly higher rates of ammonia production than perfusion without glutamine in the perfusate. The difference in the total ammonia production between tubules perfused with glutamine and tubules perfused without glutamine suggests that the delivery of glutamine, the main substrate for ammonia production (19, 20), to the tubular lumen directly enhances total ammonia production. Such data are consistent with observations made in the intact dog, which indicate that glutamine filtered at the glomerulus and reabsorbed along the nephron accounts for a major portion of renal glutamine extraction and that glutamine extraction is proportional to ammonia production (21).

Even in the absence of glutamine in the perfusate, the rate of total ammonia production was greater in perfused segments compared with unperfused segments. This observation suggests that in addition to luminal glutamine delivery, luminal perfusion in itself increases the rate of ammonia production by proximal segments.

To characterize the mechanism underlying perfusion-dependent enhancement of total ammonia production by mouse proximal tubules, we first analyzed the amounts of ammonia appearing in the luminal fluid. We found that the major portion (91%) of the increment in total ammonia production observed with perfusion was accounted for by luminal ammonia output. Next, we examined the effects of changes in perfusion flow rate on ammonia production by segments. As illustrated in Fig. 3, increasing the perfusion flow rate by raising the perfusion pressure resulted in a significant rise in total ammonia production in the presence or absence of glutamine in the perfusate. When the luminal flow was decreased at a constant perfusion pressure by constricting the end of the perfused segment with a second pipette, luminal ammonia output was reduced. Unfortunately, accurate simultaneous measurements of luminal ammonia output and bath medium ammonia accumulation could not be obtained because substantially larger bath volumes were needed when both perfusion and collecting pipettes were used. Nevertheless, since we showed that a major portion of total ammonia production with perfusion originates from luminal ammonia output, luminal ammonia output may qualitatively reflect total ammonia production under the present experimental conditions. Perfusion of segments at a constant pressure but diminished flow rate resulted in a significant fall in luminal ammonia output, suggesting that reduced flow rather than reduced pressure led to the fall in ammonia production by perfused segments.

Our observations of flow-enhanced luminal entry of ammonia in the mouse proximal tubule are consistent with observations in rat proximal tubules. Free-flow micropuncture studies in rats indicate a linear relationship between luminal entry of ammonia and tubule fluid flow rate (22). Yet, conclusions about ammonia production in proximal tubules could not be made from the micropuncture studies since the relationship between luminal entry of ammonia and tubular ammonia production could not be ascertained under micropuncture conditions.

Since total ammonia production by isolated proximal segments changed in response to perfusion and altered flow

rate, the machinery for producing ammonia within the cell was not operating at a fixed rate. In other words, ammonia produced within the tubular cell was not merely being shunted for release from peritubular to luminal sides of the tubular membrane as may occur with acute changes in urinary pH (1).

The mechanism by which perfusion flow rates affect ammonia production in perfused segments is not known. Halperin and associates (23, 24) have suggested that changes in tubular transport function may have a great impact on the regulation of ammonia production. They have proposed that ATP, which is produced in the process of forming ammonia from glutamine, may be a limiting factor in ammonia production. For example, increasing ATP utilization would tend to increase the rate of ammonia production. Their initial studies indicated a linear correlation between ammonia production and sodium reabsorption, suggesting that ATP utilization by the nephron in transporting sodium may promote the formation of ammonia (23). In contrast, in our experiments we lowered perfusate sodium and added amiloride to the perfusate to reduce transcellular sodium flux, and our results suggested that the increased ammonia production observed with perfusion of proximal segments did not depend upon normal net transepithelial sodium transport. Caution should be exercised when attempting to compare our data with those of Halperin and associates (23, 24), because 1) the data were obtained from different species; 2) their data were derived from whole kidneys in vivo, whereas our data were obtained in isolated nephron segments perfused in vitro; and 3) different experimental conditions were applied to alter sodium transport in the two studies. Nevertheless, the advantages of the isolated perfused nephron model are that peritubular (bath), luminal (perfusate), and flow conditions may be controlled, making interpretation of results easier.

The enhanced rate of ammonia production observed with perfusion and increased flow rates may have resulted from enhanced flow-dependent diffusion of ammonia into the lumen accompanied by accelerated ammonia formation within the proximal tubule. Our measurements of total ammonia production and luminal output of ammonia with perfusion indicate that ammonia enters the luminal fluid at least 1.8 times as fast as it enters into the peritubular fluid. The relatively high luminal output of ammonia observed with perfusion is remarkable given the high gradient of ammonia from cell to bath compared with that from cell to lumen. Proximal tubules perfused at 22.7 nl/min without glutamine in the perfusate produced ammonia at a rate of 21.5±1.4 pmol/mm per minute so that a 0.8-mm segment perfused under similar conditions in a bath volume of 170 $\mu$l would be expected to yield a bath concentration of 2 $\mu$M ammonia within 20 min. In contrast, the luminal ammonia output of proximal tubules perfused under similar flow rates (22.0±2.7 nl/min) is 13.7±1.8 pmol/ mm per minute, so that the luminal concentration of ammonia is 13.7:22.0 or 0.6 mM. As a result, the lumen-to-bath concentration ratio is 300:1.

The high luminal contribution to total ammonia production and high total ammonia gradient observed with perfusion is also surprising if the high proximal permeability of ammonia observed in rabbits applies to mice (25). Preferential luminal trapping of ammonia as ammonium does not seem to apply in the proximal tubule, especially under the high flow perfusion conditions used in these experiments, in which luminal bicarbonate and pH would not be expected to fall to a degree

sufficient to explain the large lumen to bath concentration gradient. The observed preferential movement of total ammonia from cell to lumen in perfused mouse segments indicates enhanced ammonia/ammonium transport or permeability at the brush border compared with peritubular membrane. A brush border ammonium transporter could facilitate the transport of ammonium into the lumen. Ammonium has been shown to interact with the brush border sodium-hydrogen exchanger of the rabbit (26). Nevertheless, our studies using a combination of low luminal sodium and amiloride to reduce the rates of net fluid reabsorption and sodium-hydrogen exchange did not reveal an effect on total ammonia production.

The mechanism by which flow enhances ammonia production remains unclear and requires further exploration. Removal of ammonia or other inhibitory end products may enchance ammonia production. High intracellular ammonia levels may reduce the deamination of glutamate by glutamate dehydrogenase (27), and accumulation of glutamate may inhibit glutaminase activity (28, 29). As a result, removal of intracellular ammonia would promote deamination of glutamate, which in turn would promote deamidation of glutamine.

In conclusion, the technique of measuring ammonia production by isolated perfused nephron segments provides a means for examining ammonia metabolism in defined individual functioning nephron segments. This technique taken with whole kidney and micropuncture studies will provide important insight into ammonia production and handling by the nephron. This study demonstrates that luminal perfusion, luminal substrate delivery, and perfusion flow rate affect ammonia production in mouse proximal tubule segments. The mechanism of perfusion-induced changes in ammonia production seems to be independent of normal luminal sodium concentrations and sodium hydrogen exchange rate.

## Acknowledgments

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