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Research Article

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Transport of Ammonia in the Rabbit Cortical Collecting Tubule

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Abstract

Nonionic diffusion and diffusion equilibrium of ammonia have been generally accepted as the mechanism of urinary ammonium excretion. However, these characteristics have not been examined directly *in vitro*. In the present studies, nonionic diffusion and diffusion equilibrium of ammonia were examined in rabbit cortical collecting tubules perfused *in vitro*. Collected fluid ammonium and pH were measured in tubules exposed to chemical gradients of NH₃/NH₄⁺. In tubules perfused with an acid perfusate free of ammonia and bathed with solutions containing NH₄Cl, collected fluid ammonia failed to equilibrate across the epithelium except at slow flow rates. The estimated apparent permeability coefficient to NH₃ was $\sim 5 \times 10^{-3}$ cm/s. Predominant nonionic diffusion of NH₃, rather than transport of NH₄⁺, was indicated by alkalinization of luminal fluid in tubules exposed to peritubular NH₄Cl and by the relative influence of peritubular NH₄⁺ and NH₃ on ammonia entry. In tubules perfused with an acid solution containing NH₄Cl, little loss of ammonium was detectable, indicating a low permeability to NH₄⁺.

In contrast to the restricted diffusion of NH₃ in cortical collecting tubules, proximal convoluted tubules exhibited a much higher apparent permeability to NH₃. In conclusion, nonionic diffusion of NH₃ accounted for most ammonium transport in the proximal convoluted tubule and in the cortical collecting tubule. However, there was relatively restricted diffusion in the collecting tubules; this may account for the failure of whole kidney ammonium excretion to obey quantitatively the predictions of nonionic diffusion and diffusion equilibrium of ammonia.

Introduction

Urinary excretion of ammonium has long been held to occur by nonionic diffusion of ammonia (NH₃). NH₃ has also been held to be in diffusion equilibrium throughout the renal cortex (1–3), resulting in distribution of ammonium (NH₄⁺) according to pH. Also, ionic NH₄⁺ has been thought to be relatively impermeable in tubular epithelia, resulting in so-called “trapping” of ammonia in acidic tubular fluid. However, the changes in urinary excretion of ammonium with changes in urine pH and urine flow rate are not always quantitatively those predicted by nonionic diffusion (4, 5). This fact has been

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attributed to limitation of production of ammonia by the kidney and/or to back diffusion of NH₄⁺ out of the tubular lumen. Finite permeability to NH₄⁺ has recently been found in a variety of epithelial tissues. In fact, in the fish gill, transport of NH₄⁺ predominates over transport of NH₃ (6). Recently in the turtle bladder, a model epithelium for the collecting tubule, the permeability to NH₄⁺ has been found to be 50 times smaller than the permeability to NH₃ (7). Although this permeability difference is large, the permeability to NH₄⁺ was hypothesized to account for the limitation of excretion of ammonium in an acid urine. Similar permeability characteristics of the mammalian collecting tubule have not been studied *in vitro* where control and manipulation of both the luminal and peritubular environment can be accomplished.

Transport of ammonia or ammonium across the collecting tubule has recently gained added importance because of studies defining the segmental handling of ammonia *in vivo*. Most of renal ammonia production can probably be accounted for by production in the proximal convoluted tubule (8–11). However, luminal ammonia in the superficial distal convoluted tubule is insufficient to account for all of urinary ammonia excretion (9–11). That is, ammonia appears to be lost between the late proximal superficial tubule and the early distal convoluted tubule. Also, other studies have directly demonstrated ammonia addition into the collecting tubule (11–13). These studies, however, have not defined the mechanism of ammonia entry into the collecting tubule. Ammonia entry might be by nonionic diffusion as has been widely held. However, ammonia might also be entering by transport of NH₄⁺ (14), as has recently been postulated for the turtle bladder (15) and for the thick ascending limb (16).

The present studies were designed to examine ammonia transport in the isolated perfused rabbit cortical collecting tubule. Specifically, ammonia transport in the presence of transepithelial gradients of ammonia is examined. In this fashion the relative permeability to NH₃ and NH₄⁺ can be determined. As expected, the apparent permeability to ionic NH₄⁺ is relatively low. Unexpectedly, however, the apparent permeability to nonionic NH₃ is sufficiently low that NH₃ fails to reach diffusion equilibrium except at low flow rates. Such a diffusion barrier for NH₃ equilibration may account for certain *in vivo* observations that differ from the predictions of nonionic diffusion and diffusion equilibrium of NH₃. The findings in the cortical collecting tubule were compared with those of studies in the proximal convoluted tubule of the rabbit studied in a similar fashion. The proximal convoluted tubules exhibited a much higher apparent permeability to NH₃.

Methods

Standard techniques of *in vitro* microperfusion of isolated cortical collecting tubules and proximal convoluted tubules were used (17). The cortical collecting tubules and nonsurface proximal convoluted

tubules (probably S2 segment predominantly in that glomeruli were not attached) were dissected from kidneys harvested from normal female New Zealand white rabbits. Tubules were perfused between glass pipettes bathed in a chamber kept at 37°C. Transepithelial potential was measured between calomel electrodes by using agarose bridges (containing solutions similar to the tubule bathing solutions), which contacted the perfusate and bathing solution. Exhaustively dialyzed [*methoxy-³H*]inulin (New England Nuclear, Boston, MA) was used in the perfusate as a volume marker and to check for bath leaks into the collected fluid. J_V (fluid reabsorption) was $<0.1 \text{ nl mm}^{-1} \text{ min}^{-1}$ in the collecting tubules. Calculated J_V 's in the proximal tubules were variable because of the fast flow rates, small collection pipettes, and minimal bath protein; however, the ratio of perfusion rate to collecting rate was 1.00 ± 0.01 (SE, $n = 21$ collections).

The bathing solutions contained 20 mM NaHCO₃, 5 mM KCl, 8 mM Na₂HPO₄, 2 mM NaH₂PO₄, 1 mM MgSO₄, 5 mM alanine, 5 mM sodium lactate, 1.2 mM CaCl₂, 8.3 mM glucose, 5 vol % fetal calf serum, and sufficient sodium chloride to adjust the final osmolality to 300 ± 5 mosmol. 0–10 mM ammonium chloride was added to the bathing solutions as specified below in replacement for an equivalent amount of sodium chloride. The perfusion solutions contained 5 mM KCl, 8 mM NaH₂PO₄, 2 mM Na₂HPO₄, 1 mM MgSO₄, 8.3 mM glucose, 5 mM sodium lactate, 1.2 mM CaCl₂, and sufficient NaCl to adjust the final osmolality to 300 ± 5 mosmol. The high phosphate concentrations serve to buffer pH changes and to lessen unstirred layer effects (7). In a single series of experiments, as described below, 10 mM NH₄Cl was added to the perfusate in substitution for an equivalent amount of sodium chloride. All solutions were gassed with 95% O₂/5% CO₂ at 37°C. (In a single group of experiments, NaCl substituted for the NaHCO₃ in the bath, and both the bath and perfusate were bubbled with 100% O₂.) Final bath pH was approximately 7.4 and perfusate, pH 6.1. In a single series of experiments, the bath pH was raised to approximately 7.64 by increasing the sodium bicarbonate concentration to 40 mM and bubbling with 95% O₂/5% CO₂. In those solutions containing ammonium chloride, ammonium chloride was added after gassing the solution.

The pH and PCO₂ of all final bulk solutions were measured by using an Instrumentation Laboratory, Inc. pH/Blood Gas Analyzer 813 (Lexington, MA). The pH of the perfusate and collected fluid was measured in some experiments by using single-barrel glass membrane pH microelectrodes as previously described (18). The pH electrodes were calibrated with two buffers located by the perfusion apparatus and kept at 37°C. After a constant perfusion rate was obtained, the pH electrode was quickly inserted down the collection pipette into the small reservoir of collected fluid near the tubule. The circuit was completed with an agarose bridge in the bathing solution. A Ling electrode was used similarly to correct for any voltage difference between the bath and the reservoir of collected fluid.

The concentration of ammonium in the perfusate and collected fluid was measured using a coulometric microtitration technique (19). The accuracy of this technique has been previously demonstrated (10). For each experiment, ammonium concentration was measured in duplicate in (a) the fluid effluxing from the distal end of the perfused tubule into a collection pipette ("collected fluid") and (b) the perfusate instilled into mock collection pipettes from the perfusion pipette ("perfusate"). (As shown in Fig. 2, the ammonium concentration measured in the perfusate was low [0.8 mM]—compared with that in the collected fluid—but was greater than zero. Because the perfusate is nominally free of any ammonia, the measured perfusate "ammonium" serves as a "blank" control. Therefore, except in Fig. 2 and in those experiments in which NH₄Cl was added to the perfusate, "collected fluid" ammonium concentration refers to the [NH₄⁺] measured in the collected fluid minus the mean perfusate [NH₄⁺] for that experiment.) Tubular flow rate was assessed by measuring the time required to fill constant volume pipettes (10–30 nl). Perfusion flow rate was varied by varying the height of a fluid reservoir connected to the perfusion pipette. With each experiment, collections were made at several flow rates. During experimentation when flow rate was changed, a reequi-

libration time of 15–20 min was allowed before collecting fluid for analysis of ammonium or pH.

Calculations. The apparent permeability coefficient to NH₃ was calculated as $P_{\text{NH}_3} = J_{\text{NH}_3}/([{\text{NH}_3}]_B - [\text{NH}_3]_L)^{-1}$, where P_{NH_3} is in centimeters per second and $[{\text{NH}_3}]_B$ is the calculated bath NH₃ concentration in millimolar. $[\text{NH}_3]_L$ is the mean luminal [NH₃] calculated as the arithmetic mean of the nominal perfusate [NH₃] (equal to 0) and the collected fluid [NH₃] calculated using $\text{NH}_3 = (10)^{\text{pH}-9} \times [\text{NH}_4^+]$, where pH and [NH₄⁺] are the values measured in the collected fluid. This analysis does not consider any contribution of acid or alkaline disequilibrium pH's (see Discussion); our pH measurements should represent equilibrium values. Using the logarithmic mean resulted in little difference. J_{NH_3} is the influx of NH₃ calculated as $V_0 \times [\text{NH}_4^+]/(\pi dL)$, where V_0 is the collection flow rate in nanoliters per minute, d is the tubule diameter (assumed equal to 20 micrometers), L is the tubule length measured by eyepiece micrometer in the perfusion chamber, and $[\text{NH}_4^+]$ is the collected fluid NH₄⁺ concentration. Estimates of NH₄⁺ permeability were calculated from experiments examining ammonium exit from the lumen (see below): $P_{\text{NH}_4^+} = J_{\text{NH}_4^+}/[{\text{NH}_4^+}L - (VF/RT)(NH_4^+L + NH_4^+B)/2]^{-1}$, where V is the measured transepithelial voltage, and F , R , and T have their usual meanings. NH₄⁺*L* is the luminal NH₄⁺ concentration (10 mM) and NH₄⁺*B* is the bath NH₄⁺ concentration (0 mM). Other calculations are discussed in the Appendix.

Statistics. Results are expressed as means \pm SE except for pH values which are given as the pH of the mean [H⁺]. Such results were compared by Student's *t* test. Linear regression lines were calculated by the method of least squares and compared by analysis of variance of the slopes and intercepts. Where indicated, results were calculated after logarithmic transformation of data. Statistical significance was taken as $P < 0.05$.

Results

Collected fluid ammonia without ammonium chloride addition. Fig. 1 illustrates the collected fluid NH₄⁺ concentration in 17 cortical collecting tubules, which were bathed with no ammonium chloride added to the bathing solution. The mean collected fluid ammonium concentration was only 0.72 ± 0.24 mM. The collected fluid ammonium concentration was >1.25 mM in only three collections from these 17 tubules. Not shown in Fig. 1 are 13 collections from six proximal convoluted tubules bathed in solutions free of ammonium chloride. The mean collected fluid NH₄⁺ was only 0.72 ± 0.41 mM (mean

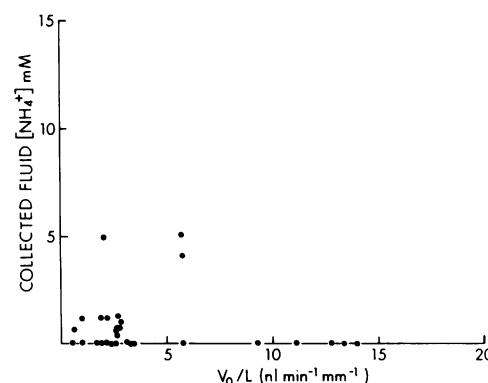


Figure 1. Collected fluid NH₄⁺ concentration (in millimolar) as a function of flow rate, factored for tubule length (in nanoliters/minute per millimeter) in cortical collecting tubules bathed in solutions free of added NH₄Cl. Perfusion pH is 6. 17 tubules. In this and all subsequent figures, points represent individual collections.

flow rate $17 \pm 7 \text{ nl min}^{-1} \text{ mm}^{-1}$). Hence, in the conditions of our study, collected fluid ammonium did not represent significant production of ammonia.

Ammonia entry into cortical collecting tubules bathed with 10 mM NH₄Cl. Figs. 2 and 3 show the collected fluid ammonium concentration from six cortical collecting tubules bathed with 10 mM ammonium chloride. Collected fluid ammonium concentration increased in a curvilinear fashion as perfusion rate was slowed (Fig. 2). At the slowest flow rates, the collected fluid NH₄⁺ was ~15 mM, which is higher than the bath NH₄⁺ concentration. However, at flow rates above ~5 nl mm⁻¹ min⁻¹, the collected fluid NH₄⁺ concentration failed to reach the levels of NH₄⁺ in the bath. Considering the buffer capacity of the perfusing solutions (see Appendix) and the collected fluid pH's (see below), this means that NH₃ had not reached diffusion equilibrium at the higher flow rates.

To ease comparison with subsequent data, Fig. 3 illustrates the same data as in Fig. 2 except that the horizontal scale is logarithmic. It is apparent that there was an inverse correlation between collected fluid NH₄⁺ and the log of the flow rate ($r = 0.94$, $P < 0.01$). This is the expected relationship for diffusive processes in tubular structures.

If all of the collected fluid NH₄⁺ is due to nonionic diffusion of NH₃ from the bath, the permeability of these tubules to NH₃ can be estimated. The mean apparent permeability coefficient to NH₃ in these cortical collecting tubules was $7 \times 10^{-3} \text{ cm/s}$. The NH₃ concentration gradient used to calculate the permeability coefficient included the luminal NH₃ concentration, which is a function of measured [NH₄⁺] and pH. The pH values used are those discussed below. If the pH values used in the calculations were falsely low and/or the calculated luminal [NH₃] low, then the permeability estimate is lower than the true permeability.

Collected fluid pH. Fig. 4 shows the collected fluid pH as a function of flow rate in the same cortical collecting tubules bathed in 10 mM NH₄Cl. Collected fluid pH rose in a curvilinear fashion as the flow rate was lowered. When the cortical collecting tubules were bathed with no ammonium chloride in the bath, the collected fluid pH remained acidic as indicated by the standard error cross in the lower left corner of Fig. 4. The alkalinization of the luminal fluid in the tubules

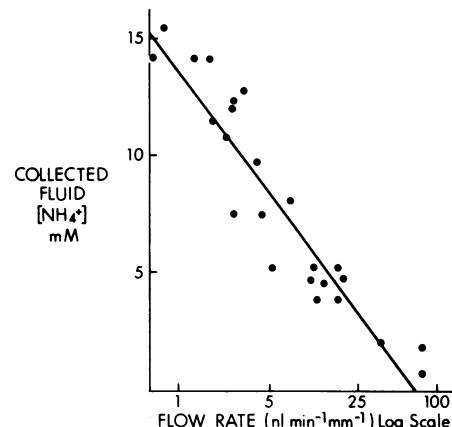


Figure 3. Same data as in Fig. 2 except that collected fluid ammonium concentration is shown as a function of the log of perfusate flow rate and collected fluid NH₄⁺ concentration is corrected for the mean perfusate NH₄⁺ concentration from the same experiment. The solid line represents the linear regression line calculated by the method of least squares.

bathed in ammonium chloride is consistent with entry of predominantly nonionized NH₃, a weak base.

Entry of a given amount of NH₃ (which then forms NH₄⁺) will produce an elevation in luminal pH, which can be calculated by knowing the initial pH and buffer content. (The assumptions in these calculations are given in the Appendix.) As shown in Fig. 4, the measured pH values were similar to the values expected from calculations that used the measured collected fluid [NH₄⁺]. Hence, the measured collected fluid pH and [NH₄⁺] values were in accord with predominant luminal entry of NH₃.

Ammonia exit from the cortical collecting tubule. To examine lumen-to-bath efflux of ammonia from the cortical collecting tubule, 10 mM NH₄Cl was added to the perfusate

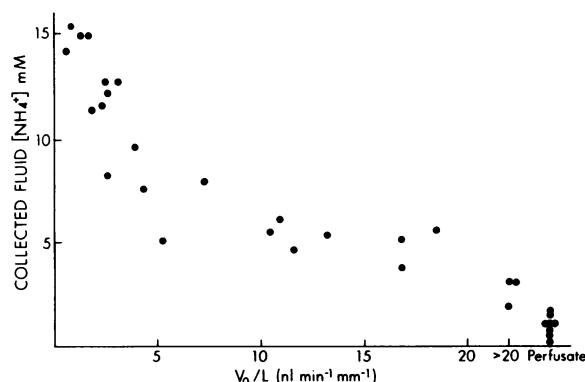


Figure 2. Collected fluid NH₄⁺ concentration (in millimolar) as a function of perfusate flow rate (in nanoliters/minute per millimeter) in six cortical collecting tubules bathed in 10 mM ammonium chloride at pH 7.4. Perfusate pH was 6; measured perfusate NH₄⁺ concentration was 0.8 mM.

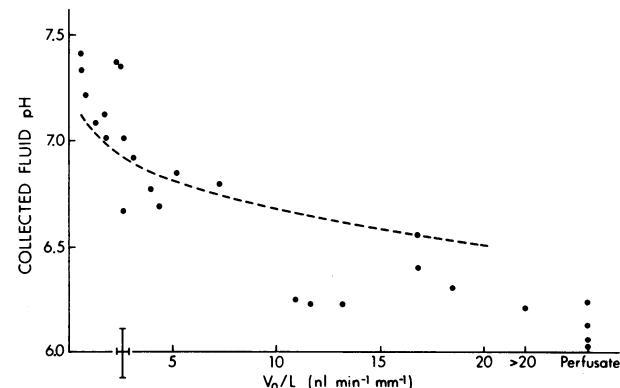


Figure 4. Collected fluid pH in the six cortical collecting tubules of Fig. 2. Bathing solution is pH 7.38 and contains 10 mM ammonium chloride. The mean perfusate pH measured in mock collection pipettes was 6.10 ± 0.04 . The standard error cross on the horizontal axis represents the collected fluid pH in six measurements in tubules bathed in no ammonium chloride. The dashed line represents the expected collected fluid pH if all the collected NH₄⁺ in Fig. 2 was a result of NH₃ transport. (The linear regression line of Fig. 3 was used for these calculations.) See text and Appendix.

in substitution for an equivalent amount of sodium chloride. As before, the perfuse pH was 6. In these experiments, no ammonium chloride was added to the bathing solutions. Fig. 5 illustrates that the collected fluid ammonium concentration in these experiments was nearly the same as that in the perfusate. The mean collected fluid ammonium concentration (11.24 ± 0.40 mM) was not statistically different from the mean perfuse ammonium concentration (11.52 ± 0.51 mM). This indicates little passive movement of ionic NH_4^+ across the cortical collecting tubule from lumen to bath; the permeability to NH_4^+ was too low to measure in our experiments.

Collected fluid ammonium with varying peritubular $\text{NH}_3/\text{NH}_4^+$. The alkalinization of collected fluid with peritubular ammonium chloride and the low apparent permeability (lumen-to-bath) to NH_4^+ are consistent with predominant transport of NH_3 , not NH_4^+ , with these particular experimental conditions. Predominant transport of NH_3 (passive bath-to-lumen) would predict that luminal entry of ammonia would depend predominantly on peritubular NH_3 concentration, not NH_4^+ concentration. To examine this prediction, additional cortical collecting tubules were studied at varying concentrations of NH_3 and NH_4^+ in the bathing solutions and compared with the prior data presented in Fig. 3. Table I summarizes the bathing solution compositions. NH_4^+ concentrations are, of course, approximately equal to the added total concentration at these pH values.

Fig. 3 illustrates the relationship between collected fluid ammonium concentration and flow rate (factored for tubular length) in those tubules bathed in 10 mM ammonium chloride at pH 7.38. This relationship is $\text{collected fluid } \text{NH}_4^+ = [13.6 \pm 0.6] - [3.2 \pm 0.3] \times \ln V_0/L$. V_0/L represents flow rate factored for tubule length. Fig. 6 illustrates collected fluid ammonium concentration as a function of flow rate in those tubules bathed with 5 mM ammonium chloride at a measured mean bath pH of 7.37. In these tubules both the NH_4^+ concentration and the NH_3 concentration were one-half that in the previous group. In Fig. 6, $\text{NH}_4^+ = [6.8 \pm 0.5] - [2.4 \pm 0.5] \times \ln V_0/L$ ($r = 0.80$, $P < 0.01$). The intercept but not the slope of this relationship was statistically different from the data with 10 mM ammonium chloride. The mean apparent

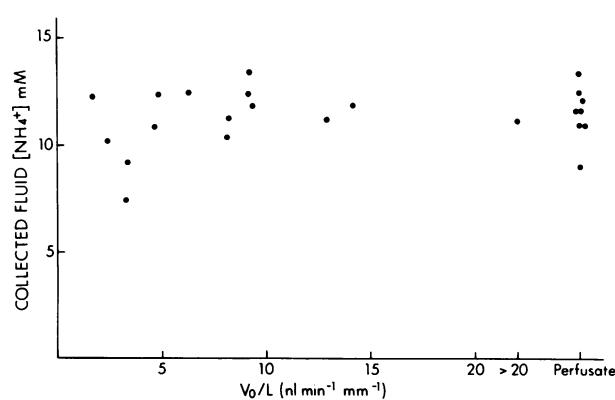


Figure 5. Perfuse and collected fluid NH_4^+ concentration (in millimolar) as a function of perfuse flow rate in four cortical collecting tubules perfused with a solution at pH 6 containing 10 mM NH_4^+ . Mean perfuse and collected fluid NH_4^+ concentration were not statistically different (11.52 ± 0.51 mM and 11.24 ± 0.40 mM, respectively).

Table I. Bathing Solution Composition

NH_4Cl	Measured mean pH	Calculated NH_3 concentration*
mM		mM
10	7.38	0.24
5	7.37	0.12
5	7.64	0.22

* Using $\text{pK}_a = 9.0$.

permeability coefficient to NH_3 calculated as above in these tubules was 4×10^{-3} cm/s. Also, the collected fluid pH was measured in four tubules bathed with 5 mM NH_4Cl . The pH values again demonstrated alkalinization of luminal fluid at slower flow rates although the magnitude was less than with 10 mM NH_4Cl in the bath. The maximum collected fluid pH values were 6.6–6.75 at flow rates of $1\text{--}2 \text{ nl mm}^{-1} \text{ min}^{-1}$. The correlation with the predicted pH (based on the collected fluid NH_4^+) was similar to that of Fig. 4.

Fig. 7 illustrates the collected fluid ammonium concentration in tubules bathed with 5 mM ammonium chloride at a bath pH of 7.64. $\text{NH}_4^+ = [11.3 \pm 0.9] - [2.7 \pm 0.6] \times \ln V_0/L$ ($r = 0.75$, $P < 0.01$). The intercept is significantly different from both of the prior two groups. Note, however, that the difference between the two groups bathed in 5 mM ammonium chloride is nearly twofold greater than the difference between the two groups bathed with a similar NH_3 concentration. Therefore, qualitatively ammonia entry appears to be predominantly dependent on bath NH_3 , not NH_4^+ .

Note that the data in both Figs. 6 and 7 indicate that collected fluid NH_4^+ concentration again fails to reach the level in the bathing solution at faster flow rates. If only NH_3 equilibrated across the tubules and no other mechanisms of a change in luminal pH occurred (see Appendix), the predicted equilibrium luminal NH_4^+ values are 16.6, 12.1, and 15.9 mM for the data of Figs. 3, 6, and 7, respectively.

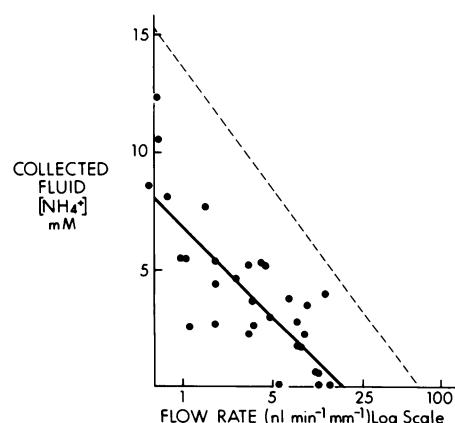


Figure 6. Collected fluid NH_4^+ concentration (in millimolar) as a function of flow rate in eight cortical collecting tubules bathed with 5 mM ammonium chloride at pH 7.4. The solid line is the linear regression line. The dotted line represents the linear regression from Fig. 3 for comparison. Details of the two lines are in the text. Initial perfuse pH was 6 in both cases.

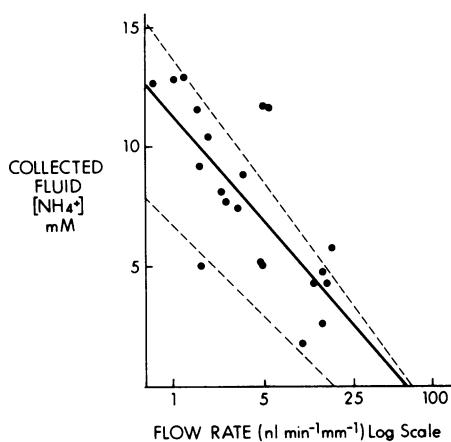


Figure 7. Collected fluid NH_4^+ concentration (in millimolar) vs. flow rate in five cortical collecting tubules bathed with 5 mM NH_4Cl at pH 7.64. The solid line represents the linear regression line calculated from least squares, the top dashed line represents the linear regression line from Fig. 3 and the bottom dashed line is from Fig. 6. Details of these lines are in the text. Perfusion pH was again 6.

Ammonia entry into cortical collecting tubules in the absence of $\text{HCO}_3^-/\text{CO}_2$. Nonionic diffusion of NH_3 into the tubular lumen will consume protons, H^+ ; the extent of the rise in luminal pH will depend on luminal buffers. Also, because the luminal pH will determine the $\text{NH}_3/\text{NH}_4^+$ ratio, diffusion of NH_3 down a chemical gradient from bath to lumen will depend on luminal buffers. Fig. 8 shows collected fluid ammonium concentration in cortical collecting tubules perfused and bathed in solutions nominally free of HCO_3^- and CO_2 . The bath was pH 7.19 and contained 10 mM NH_4Cl ; the perfusate was pH 6.2 and contained no NH_4Cl . In the $\text{HCO}_3^-/\text{CO}_2$ -free solutions, collected fluid NH_4^+ (as a function of flow rate) was less than in HCO_3^- -containing solutions (Fig. 3) with the same concentration of NH_4Cl in the bath—as predicted with nonionic

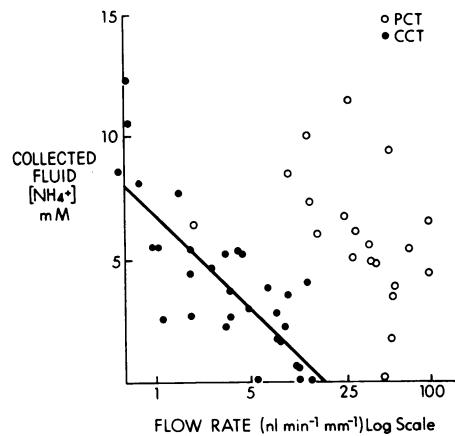


Figure 9. Comparison of collected fluid NH_4^+ concentration in millimolar as a function of flow rate (log scale) in cortical collecting tubules (data of Fig. 6) and five proximal convoluted tubules under the same conditions. Both sets of tubules were bathed with 5 mM NH_4Cl at pH 7.4. Perfusion pH was 6 in both cases.

diffusion. The calculated apparent permeability to NH_3 (with the same assumptions as before) was 2×10^{-3} cm/s. And the collected fluid NH_4^+ again failed to reach the level in the bathing solution at most flow rates. (The predicted equilibrium luminal NH_4^+ value was 6.3 mM.) Therefore, these experiments support nonionic diffusion of NH_3 and are consistent with restricted diffusion of NH_3 , even in the absence of $\text{HCO}_3^-/\text{CO}_2$.

Ammonia entry into proximal convoluted tubules bathed with 5 mM ammonium chloride. Fig. 9 compares collected fluid ammonium concentration in proximal convoluted tubules and cortical collecting tubules bathed with 5 mM ammonium chloride at a mean pH of 7.36. (5 mM NH_4Cl and high flow rates were used in the proximal convoluted tubule because preliminary experiments using 10 mM NH_4Cl in the bath produced collected fluid $[\text{NH}_4^+]$ of 13.9 ± 1.9 mM at flow rates <20 nl min^{-1} mm^{-1} .) Both sets of tubules in Fig. 9 were

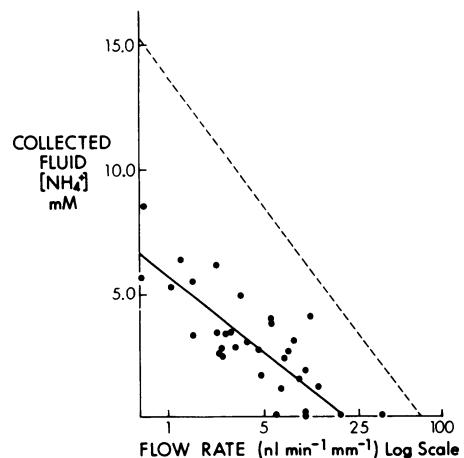


Figure 8. Collected fluid NH_4^+ concentration (in millimolar) vs. flow rate in six cortical collecting tubules bathed with 10 mM NH_4Cl in the absence of $\text{HCO}_3^-/\text{CO}_2$ in the solutions. The solid line represents the linear regression line calculated from least squares. $\text{NH}_4^+ = [5.5 \pm 0.4] - [1.9 \pm 0.3] \times \ln \frac{V_0}{L}$ ($r = 0.77$, $P < 0.01$). The dashed line is for comparison with the data in Fig. 3.

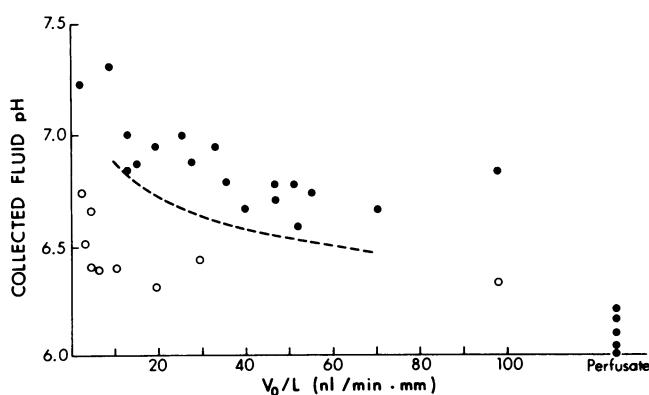


Figure 10. Collected fluid pH in six proximal convoluted tubules. Bathing solution is pH 7.36 and contains 5 mM NH_4Cl (●). Collected fluid pH when no ammonium chloride was in the bath (○). The mean perfusate pH in mock collection pipettes was 6.11 ± 0.04 . The dashed line represents the expected collected fluid pH if the collected fluid NH_4^+ and pH were solely the result of NH_3 transport. The individual values of Fig. 9 (in the range shown) were used to calculate the expected collected fluid pH values from which the dashed line was derived by the method of least squares.

perfused with the acidic perfusate. Despite the much higher flow rates used in the proximal convoluted tubules, the collected fluid ammonium concentrations were comparable or higher than in the cortical collecting tubules. Therefore, the proximal convoluted tubule exhibited a much higher apparent permeability to ammonia than the cortical collecting tubule.

Collected fluid pH in these proximal convoluted tubules (Fig. 10) showed a similar relationship with flow rate (alkalinization with slower flow rates) as that shown in Fig. 4 for cortical collecting tubules except that the points are shifted up and to the right, consistent with a higher NH₃ influx. And the collected fluid pH in those proximal convoluted tubules bathed with NH₄Cl was higher than in those proximal convoluted tubules bathed without added ammonium chloride. This is again consistent with predominant entry of NH₃ into the proximal convoluted tubule. That the measured collected fluid pH was generally higher than that predicted from the measured NH₄⁺ concentration may be secondary to passive pH equilibration across the tubule, due to HCO₃⁻ and H⁺ permeability (18).

Discussion

These studies provide the first direct evaluation of nonionic diffusion of ammonia across the mammalian cortical collecting tubule *in vitro*. All studies were done using gradients of ammonia across the tubular epithelia, to examine passive permeability properties. These particular studies were not designed to exclude active transport of NH₄⁺. All of our results are consistent with predominant nonionic diffusion of NH₃ with little, if any, transport of NH₄⁺. However, our results contrast with previous concepts regarding ammonia transport; namely, equilibration of ammonia across the rabbit cortical collecting tubule was flow rate-dependent and ammonia did not appear to equilibrate except at slow flow rates. Ammonia has previously been thought to be in diffusion equilibrium in the entire renal cortex independent of luminal flow rates (1). Our results also unexpectedly indicate that all tubular epithelia are not equally highly permeable to NH₃. Rabbit proximal convoluted tubules and cortical collecting tubules were found to differ markedly in their apparent permeability characteristics to ammonia.

Nonionic diffusion of NH₃, diffusion equilibrium of NH₃ in all renal cortical structures, and relative impermeability of ionic NH₄⁺ have been the principles of our understanding of urinary ammonia excretion (5). In recent years, *in vivo* studies have greatly enhanced the understanding of segmental ammonium handling by the kidney. However, these studies in general have not evaluated the above principles, which predict that total ammonia concentration (predominantly NH₄⁺ at physiologic pH values) will depend only upon pH gradients. For example, an intraluminal fluid at a pH of 6.4 would be predicted to have an NH₄⁺ concentration 10-fold higher than an adjacent peritubular fluid having a pH of 7.4, NH₃ concentration being the same in both compartments. On a whole kidney level this would imply that urinary excretion of ammonium would increase linearly with both urine flow rate and urinary hydrogen ion concentration (increase exponentially with a fall in urine pH). Quantitatively, these changes in urinary ammonium excretion are not always found with changes in urine flow rate and pH (4, 5). One explanation that is usually discounted is possible inequality of NH₃ concentration in adjacent structures. (Medullary NH₃ has however been found to be higher than cortical NH₃.) However, recent in

vivo data suggest that NH₃ is lower in the early distal tubule than in the late proximal tubule of superficial nephrons of the rat (20). The present data provide an explanation for both the whole kidney data and the recent *in vivo* micropuncture data of Simon et al. (20): restricted diffusion of NH₃.¹

The results of all of the protocols in the cortical collecting tubule were consistent with disequilibrium of NH₃ across this epithelium at physiologic to near-physiologic luminal flow rates. NH₃ disequilibrium is based on calculated values from the measured collected fluid NH₄⁺ and pH. Only if the luminal pH were significantly higher than the measured values, would the calculated luminal NH₃ concentration equal that in the bath. Figs. 2, 6, and 7 illustrate that the collected fluid NH₄⁺ did not reach the predicted equilibrium values (16.6, 12.1, and 15.9 mM, respectively) at flow rates >2–5 nl min⁻¹ mm⁻¹. (See Appendix for explanation of “predicted equilibrium values.”) And the predicted NH₄⁺ concentration would be even higher if net H⁺ secretion or HCO₃⁻ reabsorption were considered. Because the physiologic flow rate *in vivo* in this segment has been estimated to be at least 2–5 nl min⁻¹ (21), our data are relevant to the *in vivo* circumstance.

Restricted diffusion of NH₃ is contrary to most prior assumptions regarding ammonia excretion. However, as recently pointed out by Goldstein et al. (6), NH₃ is not readily lipid-soluble and therefore may not be readily permeant in all epithelia. In the turtle bladder, Arruda et al. (15), but not Schwartz and Tripolone (7), have found diffusion of NH₃ is less than that predicted for an equivalent layer of water, i.e., restricted diffusion. In the collecting tubule, an alkaline disequilibrium pH might cause some component of the apparent restriction to NH₃ diffusion. This alkaline disequilibrium pH could result from NH₃ entry into the tubular lumen, which lacks carbonic anhydrase. The apparent NH₃ permeability would then underestimate the true NH₃ permeability because the luminal NH₃ concentration would be higher than that calculated from the collected fluid [NH₄⁺] and measured equilibrium pH. (Knepper et al. (22) have demonstrated the opposite: an acid disequilibrium pH from H⁺ secretion that drives ammonia secretion in cortical collecting tubules from deoxycorticosterone-treated rabbits.) However, our experiments in the absence of HCO₃⁻/CO₂ are evidence that other factors probably also contribute to the restricted diffusion of NH₃. In the nominal absence of HCO₃⁻/CO₂, any disequilibrium pH should be small. In any case, restricted diffusion of NH₃, whatever the mechanism, is apparent in our experiments.

Although the present studies demonstrated restricted diffusion of NH₃ in the cortical collecting tubule, nonionic diffusion of NH₃ as a primary mechanism of ammonia transport in this segment was supported. Supporting evidence included the predominant dependence of ammonia entry on bath NH₃, not NH₄⁺ (Figs. 6 and 7), and the alkalinization of luminal fluid commensurate with higher NH₄⁺ concentrations (Fig. 4). The collected fluid did not become more alkaline in the absence of bath NH₄Cl, consistent with relatively low HCO₃⁻ and H⁺ permeabilities in this segment (23, 24). If NH₄⁺ was the species predominantly transported into the lumen, the

1. These data only address how a NH₃ concentration difference is maintained between adjacent tubules and do not address how [NH₃] is lowered between the end proximal tubule and the distal tubule. The loss of ammonia has been postulated to occur by nonionic diffusion from the descending limb of Henle's loop (5) and/or by transport of NH₄⁺ in the thick ascending limb (16).

luminal fluid should have become more acid. The present studies were not designed to exclude some mechanisms of NH_4^+ transport. Specifically, small amounts of active NH_4^+ transport were not excluded. Such transport has been found in the turtle bladder (15) and other nonmammalian epithelia. However, active transport of NH_4^+ in the turtle bladder, a model epithelium for the collecting tubule, has been disputed (7). Transport of NH_4^+ (via electrical driving forces) has been found in the rat thick ascending limb (16) but not in the collecting tubule (22). Thus, nonionic diffusion of NH_3 , albeit somewhat restricted, appears to be the primary mechanism of ammonia transport in the cortical collecting tubule.

Passive NH_4^+ transport was also examined in the present studies. Little loss of intraluminal NH_4^+ occurred in tubules perfused with NH_4Cl and bathed with solutions free of NH_4^+ (Fig. 5). Also bath-to-lumen movement of NH_4^+ was probably much less than NH_3 flux based on the collected fluid pH values (Fig. 4) and the relative influence of bath NH_4^+ and NH_3 on collected fluid NH_4^+ in Figs. 6 and 7. Near-doubling of NH_3 concentration with constant NH_4^+ in the bath increased collected fluid NH_4^+ approximately twofold (compare Figs. 6 and 7). Also decreasing NH_4^+ concentration in half while keeping NH_3 nearly constant had little effect on collected fluid NH_4^+ , (Fig. 7).² The present data did not exclude some bath-to-lumen entry of NH_4^+ . However, the ratio of the calculated permeabilities of NH_3 to NH_4^+ was more than was found in the turtle bladder. (Although the apparent NH_4^+ permeability was too low to measure in our "ammonia exit" experiments (see Fig. 5), a maximum NH_4^+ permeability can be estimated and a ratio of $\text{NH}_3/\text{NH}_4^+$ permeability of >100:1 can be obtained.) All of this suggests that luminal entry of NH_4^+ per se is less important than NH_3 entry, and that NH_4^+ is "trapped" in acidic luminal solutions. Loss of luminal NH_4^+ in this segment (if applicable to other animal species) is an unlikely explanation for the failure of whole-kidney ammonium excretion to quantitatively obey the predictions of nonionic diffusion.³

Proximal convoluted tubules exhibited a much higher apparent permeability to ammonia than the cortical collecting

tubule. Transport of NH_3 rather than NH_4^+ was again suggested by the alkalinization of the collected fluid by exposure of the tubules to peritubular NH_4Cl . The apparent permeability coefficient to NH_3 ($\sim 6 \times 10^{-2} \text{ cm/s}$ if all the collected fluid NH_4^+ were due to luminal NH_3 entry) was approximately the same as the CO_2 permeability in the same segment (25) and was actually lower than the apparent permeability coefficient to H^+ (18). If the permeability to NH_4^+ were of the same magnitude as to other ions ($2-5 \times 10^{-5} \text{ cm/s}$), then passive NH_3 will exceed passive NH_4^+ transport at pH values > 6. The findings in the proximal tubules were more compatible with diffusion equilibrium of ammonia than the studies with collecting tubules. However, if ammonia produced is added directly to the luminal fluid as suggested by recent studies (26), then relative delivery of total ammonia to the more distal nephron and to the peritubular solution will depend on the ratio of ammonia production and luminal flow rate, i.e., the resulting luminal concentration. For instance, if flow rate were doubled with constant ammonia production, then luminal ammonia concentration would be reduced in half, and diffusion of NH_3 out of the lumen would be reduced in half, assuming equal luminal pH.

In summary, these studies demonstrate that entry of ammonium into the tubular lumen of rabbit cortical collecting tubules was flow rate-dependent and that ammonia did not equilibrate across the epithelium except at slow flow rates. However, transport of ammonium in the collecting tubule occurred predominantly by nonionic diffusion of NH_3 . The finding of restricted diffusion of NH_3 may partially explain the failure of urinary ammonium excretion to quantitatively conform to the predictions of nonionic diffusion. With acidic perfusate in the cortical collecting tubule, little loss of luminal NH_4^+ was detectable. In contrast to the collecting tubule, the apparent permeability to NH_3 in the proximal convoluted tubule was much higher (~ 10 times that in the cortical collecting tubule). The results of the present study, however, must be interpreted with recognition of the fact that the rabbit produces less ammonia than other species (rat, dog, and man) (e.g., ref. 27).⁴ In addition, future studies in other nephron segments with the techniques described herein will be necessary to complete the on-going modeling of whole kidney ammonia handling.

Appendix

With the most simple model of nonionic diffusion of NH_3 across the cortical collecting tubule, the equilibrium values of luminal $[\text{NH}_4^+]$ and pH can be easily calculated. By equilibrium values, we mean the values that would be reached with diffusion of NH_3 at infinitely slow luminal flow rates. These values will be independent of the tubule permeability to NH_3 if the following conditions are assumed for this simplified model: (a) no active or passive transport of NH_4^+ per se; (b) no active transport of NH_3 per se; (c) no transport of H^+ , HCO_3^- , or other buffers that would change luminal pH independent of NH_3 movement; and (d) equilibration of CO_2 across the tubule at these slow flow rates. (The third assumption is known to be wrong; HCO_3^- can be reabsorbed or secreted by the cortical collecting tubule. However, in these studies, the rates of ammonia entry are in general higher than the rates of HCO_3^- transport reported by others.) In our system of only

4. The rabbit can acidify its urine and has been the animal model studied most in vitro, with general applicability to other species. The limitation in ammonia excretion in the rabbit is probably attributable to decreased production (via phosphate-dependent glutaminase) rather than to limited transport (28). However, as always, extrapolation between species should be cautious.

2. Some of the differences between the linear regressions of the 10 mM NH_4Cl group and the 5 mM NH_4Cl at pH 7.64 group (Fig. 7) may be due to the small difference in NH_3 concentration (0.24 vs. 0.22 mM respectively). Also, unstirred layer-type effects could theoretically contribute to the difference: (a) the higher total ammonia concentration in the first group might provide a higher NH_3 concentration at the peritubular surface in the face of continuing ammonia loss from a peritubular unstirred layer; and (b) any HCO_3^- entry into an unstirred peritubular solution will raise the pH (and hence NH_3 concentration) relatively more in the lower pH solution (the 10 mM NH_4Cl group). The tubular cells per se could constitute such an unstirred layer if the apical membrane were the limiting barrier to NH_3 diffusion and if the intracellular pH and ammonia reflected the peritubular solution. Of course, some bath-to-lumen entry of NH_4^+ could also have caused the small difference in the two groups of studies.

3. The flux ratios of NH_3/NH_4 will depend not only on the permeability ratio but also on the concentration ratio, which will be a function of pH. At pH 7.4, 6.8, and 5, for example, the ratios of $\text{NH}_4^+/\text{NH}_3$ are 40:1, 158:1, and 10,000:1, respectively. If the permeability ratio were exactly 100:1, NH_4^+ flux would equal NH_3 flux at pH 7. However, NH_4^+ flux is not increasing significantly as pH is lowered; NH_3 flux is falling. Also, a lumen-negative transepithelial voltage will lower lumen-to-bath NH_4^+ flux. Hence, the implications above that NH_4^+ is "trapped" in acidic fluids should still be valid.

phosphate and $\text{HCO}_3^-/\text{CO}_2$ buffers, all NH_3 entry will titrate the pH higher and raise HPO_4^{2-} and HCO_3^- concentrations. At infinitely slow flow rates, luminal $[\text{NH}_3]$ will equal peritubular $[\text{NH}_3]$. Luminal pH will satisfy Eqs. 1–4.

Bicarbonate:

$$\text{pH} = 6.1 + \log \frac{[\text{HCO}_3^-]}{0.03 \times \text{PCO}_2}; \quad (1)$$

Phosphate:

$$\text{pH} = 6.8 + \log \frac{[\text{HPO}_4^{2-}]}{[\text{H}_2\text{PO}_4^{-}]}; \quad (2)$$

and because total phosphate is 10 mM,

$$\text{pH} = 6.8 + \log \frac{[\text{HPO}_4^{2-}]}{10 - [\text{HPO}_4^{2-}]}; \quad (3)$$

Ammonia:

$$\text{pH} = 9.0 + \log \frac{\text{NH}_3}{\text{NH}_4^+}; \quad (4)$$

and because each H^+ used to form NH_4^+ from the entry of NH_3 will titrate CO_2 (H_2CO_3) or $\text{H}_2\text{PO}_4^{-1}$ to produce additional HCO_3^- or HPO_4^{2-} ,

$$\text{NH}_4^+ = (\text{HCO}_3^- - \text{HCO}_3i^-) + (\text{HPO}_4^{2-} - \text{HPO}_4i^{-2}), \quad (5)$$

where HCO_3i^- and HPO_4i^{-2} represent the initial concentrations before the entry of NH_3 . Inasmuch as we assumed luminal PCO_2 and NH_3 are equal to bath PCO_2 and NH_3 , respectively, at these infinitely slow perfusion rates, luminal PCO_2 and NH_3 are known. This leaves four equations (1 and 3–5) and four unknowns (pH, HCO_3^- , HPO_4^{2-} , and NH_4^+). Hence, equilibrium values can be calculated. For our measured bath pH, PCO_2 , and calculated NH_3 concentrations, the calculated luminal equilibrium values are pH = 7.16 and $[\text{NH}_4^+] = 16.6$ mM for the 10 mM NH_4Cl bath; pH = 7.00 and $[\text{NH}_4^+] = 12.1$ mM for the 5 mM NH_4Cl bath (bath pH 7.37).

With a similar approach that uses the above assumptions, the expected collected fluid pH for a measured collection fluid $[\text{NH}_4^+]$ can be calculated if we assume all NH_4^+ resulted from NH_3 diffusion. In this case PCO_2 is assumed to be in equilibrium across the tubule, but NH_3 is not. However, now NH_4^+ is a measured value and the equations can be solved for pH. These values are discussed earlier in Results.

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