

Hypokalemic Nephropathy in the Rat

Role of Ammonia in Chronic Tubular Injury

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Abstract

Chronic potassium deficiency results in progressive tubulointerstitial injury, associated with augmented renal ammoniogenesis. We investigated the role of elevated renal ammonia levels and the interaction of ammonia with the complement system in this injury. Potassium deficiency was induced in rats by feeding a low potassium diet. Experimental animals received 150 mM NaHCO₃ or equimolar NaCl, as drinking water. After 3 wk, NaHCO₃ supplemented rats demonstrated decreased ammonia production, less renal hypertrophy, less histologic evidence of injury, and less proteinuria. In vitro studies on normal cortical tubular fragments, the addition of ammonia to serum in concentrations comparable to renal cortical levels in potassium-deficient animals significantly increased tubular deposition of C3 as quantitated by a radiolabeled antibody binding technique. Thus, alkali supplementation reduced chronic tubulointerstitial disease in a rat model of hypokalemic nephropathy. We propose that increased cortical ammonia levels contribute to hypokalemic nephropathy through ammonia-mediated activation of the alternative complement pathway.

Introduction

Chronic potassium deficiency in the rat results in enlarged kidneys with prominent tubular and interstitial lesions (1–4). If prolonged, irreversible damage will occur with progression to end-stage renal disease despite subsequent correction of the potassium depletion (5). Functional lesions include the inability to concentrate the urine with resultant polyuria; mild decreases in glomerular filtration rate (GFR)¹ and renal plasma flow; and proteinuria due at least in part to decreased reabsorption of filtered protein by the proximal tubule (1, 3, 6). Potassium deple-

tion is associated with metabolic changes that stimulate ammoniogenesis in a manner similar to chronic metabolic acidosis, and result in increased renal ammonia production and excretion (7). Normally, the proximal tubule is responsible for the bulk of renal NH₃ production (8). Ammoniogenesis in this segment is suppressible by NaHCO₃ induced metabolic alkalosis, most likely due to the decrease in renal extraction of glutamine and intramitochondrial glutaminase activity induced by alkali feeding (1, 7–12). Besides serving as a buffer effecting urinary acid excretion, ammonia has been proposed as a local toxin in several experimental models of renal injury (13–16). In the present study we examined the effect of modulation of ammoniogenesis on renal injury in a rat model of chronic potassium deficiency. The possible role of NH₃-mediated complement activation was examined by immunofluorescence studies and by antibody binding assay, a new technique for quantitative determination of complement deposition on cortical tubular fragments.

Methods

Our investigation consisted of two studies. Study I was designed to compare the effect of dietary supplementation with NaHCO₃ versus NaCl in a rat model of chronic potassium (K) deficiency. In study II we used a radiolabeled antibody binding assay to investigate the modulating effects of NH₃ on complement deposition on cortical tubular fragments, in vitro.

Study I

Renal function, morphology, and immunofluorescence in chronically K deficient rats

In vivo, renal functional studies. Male Sprague-Dawley rats, 250–275 g, were maintained on a K-deficient diet (ICN Nutritional Biochemicals, Cleveland, Ohio) for 3 wk. Experimental animals were given either 150 mM NaHCO₃ (KDEF + HCO₃) or 150 mM NaCl (KDEF) as drinking water. Control animals received K-sufficient diet (ICN Nutritional Biochemicals) and equimolar NaCl as drinking water. The animals had free access to food and water. At the end of the study period, animals were housed separately in metabolic cages and urine output was collected for 24 h. Total urinary protein excretion was assayed by the Coomassie dye method (Bio-Rad Protein Assay; Bio-Rad Laboratories, Richmond, CA). Low molecular weight protein excretion was quantified by passing urine samples through a PM-10 membrane in an Amicon ultrafiltration cell (Amicon Corp., Danvers, MA), allowing assay of that portion of urinary protein with a molecular weight < 10,000 by the Coomassie method. Urinary potassium excretion was determined by flame photometry (Radiometer Corp., Copenhagen, Denmark). Total urinary ammonia excretion was measured by the Bertholet reaction method (17).

Animals subsequently underwent clearance studies and determination of renal NH₃ production rates. A tracheostomy was performed after Inactin anesthesia (100 mg/kg of body weight i.p.). Polyethylene (PE-50) catheters were inserted into two femoral or internal jugular veins for infusion. A PE-50 catheter was inserted into the left femoral artery for blood sampling and blood pressure monitoring (BPM-1; Stentor Engineering Corp., Kansas City, MO). The left renal vein was cannulated with a 23-gauge needle for blood sampling. The left ureter was catheterized with PE-10 tubing. Urine was collected under oil in calibrated glass tubes. To determine glomerular filtration rate, a solution of normal saline con-

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1. *Abbreviations used in this paper:* GFR, glomerular filtration rate; GHBS, Hanks' balanced salt solution-0.1% gel; KDEF, K deficient; KRB, Krebs-Ringer bicarbonate; NRS, normal rat serum; RBF, renal blood flow; RPF, renal plasma flow; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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taining [*methoxy*-³H]inulin (10 μ Ci/ml) was infused at a rate of 1.2 ml/min after a priming dose of 0.5 ml over 5 min. Normal rat plasma was given at 0.5 ml/h after a priming dose of 1% of body weight given over 20 min and average urinary losses were replaced with normal saline (\sim 2 ml/h) to maintain euvoemia. After a 30-min equilibration period, two clearance collections were performed. Hematocrit (Hct) was determined by the hematocrit reader (Micro-Capillary, International Equipment Co., Needham Heights, MA) and total plasma protein concentration was determined by refractometry. Radioactivity in urine and plasma was quantitated by liquid scintillation counting. GFR was calculated by the standard formula. Renal plasma flow was determined by utilizing the renal extraction of inulin, and renal blood flow (RBF) by dividing RPF by 1 – Hct.

After the completion of clearance studies a timed urine collection was performed for determination of urinary NH₃ excretion rate. A sample of renal vein blood was drawn for measurement of renal vein total ammonia concentration. The sample was slowly drawn into an iced, heparinized syringe and immediately centrifuged at 4°C. The plasma was stored at –70°C until analyzed. A sample of arterial blood was collected and processed in an identical fashion for determination of arterial plasma total NH₃ concentration. Arterial and renal venous plasma samples were analyzed for NH₃ concentration by enzymatic assay (glutamate dehydrogenase, Sigma Diagnostics, St. Louis, MO). This technique measures total ammonia concentration, or the sum of NH₃ and NH₄⁺ moieties. Therefore, NH₃ levels reported here represent total ammonia concentrations (NH₃ + NH₄⁺) rather than free ammonia (NH₃) concentration. Renal ammonia production was calculated as the product of the RBF and the renal venous, arterial plasma difference in ammonia concentration, plus the urinary excretion of ammonia. A final sample of arterial blood was collected for determination of plasma potassium by flame photometry and analyzed for pH and PCO₂ and derived bicarbonate on an Instrumentation Laboratory blood gas analyzer (Instrumentation Laboratory Inc., Waltham, MA). The left kidney was weighed after stripping the capsule and removing hilar blood vessels. A sample of rectus abdominus muscle was desiccated to constant dry weight, digested in concentrated nitric acid, and analyzed for potassium content by flame photometry.

Morphologic studies. A separate cohort of animals was prepared in an identical fashion to that described above. After 21 d on experimental or control diet and drinking water, animals were anesthetized with Inactin as above. The right kidney was removed, sectioned, and snap-frozen in isopentane on dry ice. Samples were stored in isopentane at –70°C until processed for immunofluorescence studies. 4- μ m sections were cut on a cryostat at –20°C, fixed in acetone, and stained by a double antibody sandwich technique for C3 and albumin. Appropriate dilutions of IgG fractions of goat anti-rat C3 and rabbit anti-rat albumin (Cappel Laboratories, Malvern, PA) were applied to tissue sections, incubated for 60 min at 20°C in a humidified chamber, and washed three times with phosphate-buffered saline (PBS). Secondary antibodies, applied in the same fashion, included fluorescein isothiocyanate conjugates of rabbit anti-goat IgG and goat anti-rabbit IgG (Cappel Laboratories, Cochranville, PA). The left kidney was perfusion fixed in situ with 1.25% glutaraldehyde in 0.17 M cacodylate buffer, by cannulation of the abdominal aorta and retrograde perfusion at ambient systemic arterial blood pressure. Tissue for histopathology was sectioned at 5 μ m, and stained with hematoxylin and eosin. Sections were evaluated in a coded, blinded manner with regard to tubular dilatation, tubular atrophy, intratubular cast formation and interstitial infiltrate using a scale of 0 to +3, corresponding to absent to severe lesions.

Study II

Modulating effects of NH₃ on complement deposition on cortical tubule fragments in vitro; quantitation by labeled antibody binding

PURIFICATION AND LABELING OF GOAT, ANTI-RAT C3

Preparation of affinity column with rat C3. 1 ml of normal rat serum (NRS) was thawed from storage at –70°C and chelated with 10 mM EDTA at 4°C for 60 min to prevent complement activation. Chelated

NRS was then passed over a 5-ml column consisting of the IgG fraction of commercially available goat, anti-rat C3 (Cappel Laboratories) which had been coupled to CNBr Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) according to manufacturers instructions. Rat C3 was selectively eluted with 3 M KSCN, dialyzed against 0.1 M NaCl, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (18). Gel electrophoresis revealed intact alpha and beta chains migrating at 115 and 75 kD, respectively, as major protein bands. Additional bands migrated at 145 and 80 kD. Rat C3 was lyophilized then dialyzed against 0.1 M NaHCO₃/0.5 M NaCl, pH 8.0 and then coupled to CNBr-Sepharose 4B. A small aliquot of rat C3 reserved from this preparation was reactive with goat anti-rat C3 on Ouchterlony double immunodiffusion.

AFFINITY PURIFICATION OF GOAT ANTI-RAT C3

The IgG fraction of commercially available goat anti-rat C3 (Cappel Laboratories) was isolated after caprylic acid precipitation (19). After exhaustive dialysis against PBS to remove excess caprylic acid, antibodies specific for rat C3 were isolated by affinity chromatography on the rat C3 column described above. Specific antibody containing fractions were eluted with 3 M KSCN, pooled, dialyzed against PBS, and concentrated by dialysis against polyethylene glycol (compound 20 M, Union Carbide, S. Charleston, WV).

RADIOLABELING OF AFFINITY PURIFIED GOAT ANTI-RAT C3

Affinity purified goat, anti-rat C3 was labeled by reductive methylation with [³H]NaBH₄ (22 Ci/mmol; Research Products International, Prospect Park, IL), according to the method of Tack et al. (20). The resulting preparation had a specific activity of 90 Ci/mmol, which is well within the range of activity established by Tack and co-workers in their report.

ZYMOSAN MODEL SYSTEM

Zymosan particles were employed as a model system because of their well described ability to activate the alternative complement pathway with resultant attachment of C3 to the particle surface ("opsonized" zymosan), after incubation in normal serum (21). Zymosan (Sigma Chemical Co., St. Louis, MO) was added to Hanks' balanced salt solution – 0.1% gel (GHBS) at 10 mg/ml and boiled at 100°C for 60 min. The sample was centrifuged and resuspended in GHBS at a concentration of 50 mg/ml. One part of zymosan particle suspension (150 μ l) was incubated with three parts of NRS (450 μ l) or PBS (450 μ l), for 15 min at 37°C; and then centrifuged, washed, and resuspended in PBS (600 μ l). Qualitative immunofluorescence studies of opsonized and nonopsonized zymosan were performed by incubation with an appropriate dilution of affinity purified goat, anti-rat C3, followed by fluorescein isothiocyanate conjugated rabbit, anti-goat IgG (Cappel Laboratories).

Quantitative analysis of C3 deposition on zymosan was performed by determination of binding of affinity purified ³H-goat, anti-rat C3. Opsonized and nonopsonized zymosan particle suspensions (50 μ l) were incubated with ³H-goat, anti-rat C3 (2 μ g), unlabeled antibody (28 μ g) and GHBS in a final volume of 200 μ l for 1 h at 37°C on a rotating rack. 30- μ l aliquots were deposited on filter paper disks (#934-aH, Whatman, Inc., Clifton, NJ) and copiously washed with PBS using a cell harvester apparatus (Titertek Cell-Harvester, model 550, Flow Laboratories, Rockville, MD). The discs were solubilized in Aquasol-2 (New England Nuclear Research Products; Boston, MA) overnight and counted by liquid scintillation to determine bound label.

IN VITRO STUDIES OF COMPLEMENT DEPOSITION ON CORTICAL TUBULE FRAGMENTS

Preparation of cortical tubule fragments. Cortical tubule fragments were prepared by a modification of the method of Kumar et al. (22). After Inactin anesthesia of normal Sprague-Dawley rats, a midline abdominal incision was made and the aorta cannulated below the renal arteries. The kidneys were perfused in situ with modified Krebs-Ringer bicarbonate buffer (KRB:NaCl 120 mM, KCl 4.82 mM, glucose 10 mM, KH₂PO₄ 1.31 mM, CaCl₂·2H₂O 1.22 mM, Mg SO₄ 1.33 mM, NaHCO₃ 24.2 mM pH 7.4) at ambient systemic pressure. The kidneys were removed, the capsule stripped, and the kidneys placed in iced KRB. The cortex was

dissected free and the cortical slices pooled, rinsed in cold KRB, and then suspended in 20 ml of KRB containing 1.95 mg/ml collagenase (194 U/mg, Cooper Biomedical Corp., Malvern, PA) and gassed with 95% O₂/5% CO₂. The suspension was incubated at room temperature for 1 h with gentle mixing by magnetic stirrer. The cortical tubules were dispersed by passing twice through a short length of PE-190 tubing and twice through PE-90 tubing. The suspension was gently centrifuged for 1 min, the supernatant discarded and the pellet resuspended in 10 ml of iced KRB. The suspension was filtered through a nylon mesh (250 μ m) and the filtrate gently centrifuged for 1 min. After two more washings, the pellet was suspended in KRB at a final concentration of 100 μ g/ml, wet weight of tubules. The viability of proximal tubule segments prepared by this method has been previously documented (22). Visual microscopic inspection consistently revealed less than two glomeruli per five high power fields.

BINDING OF LABELED, AFFINITY PURIFIED GOAT, ANTI-RAT C3 TO CORTICAL TUBULE FRAGMENTS

To quantitate C3 deposition on tubule fragments, we determined binding of labeled, affinity purified goat anti-rat C3 to tubule fragments in vitro. Aliquots of tubule suspension (\sim 5.0 mg of tubules, wet weight) were incubated with ³H-anti-rat C3 (2 μ g) alone, or with a 100-fold excess of unlabeled antibody. The final volume was adjusted to 500 μ l with KRB and incubated for 1 h at 37°C on a rotating rack. Aliquots of each reaction mixture (100 μ l) were deposited on filter paper disks, washed on a cell harvester and counted by liquid scintillation as described above. Specific binding was considered to be the difference between bound counts with and without excess unlabeled antibody. Specific binding of ³H-anti-rat C3 to cortical tubule fragments on which complement components had been previously deposited by incubation with NRS and NH₃ (500 μ M) was determined using progressively increasing amounts of labeled antibody (Fig. 1). Saturation of specific binding sites was demonstrated at levels $>$ 2 μ g of ³H-goat, anti-rat C3 and this quantity of antibody was therefore used in all subsequent experiments.

MODULATING EFFECTS ON NH₃ ON CORTICAL TUBULE COMPLEMENT DEPOSITION

Normal rat cortical tubular fragments were prepared as described above. Cortical tubule fragments (30 mg wet weight) were incubated with aliquots of NRS to which 0–15 μ l of 10 mM NH₄Cl, in distilled water, was added to yield final concentrations of 150 μ M NH₃ (NRS alone), 300 μ M NH₃ and 500 μ M NH₃, as determined by enzymatic assay. To establish a baseline level of C3 deposition, tubules were incubated with KRB and NH₃ (333 μ M) but without NRS, and therefore without an exogenous source of complement. C3 detected on these tubules would presumably reflect levels present under normal conditions in the rat. The total volume for the incubation mixture was 300 μ l in all cases. Samples were incubated for 2.5 h at 37°C on a rotating rack and then centrifuged, washed, and

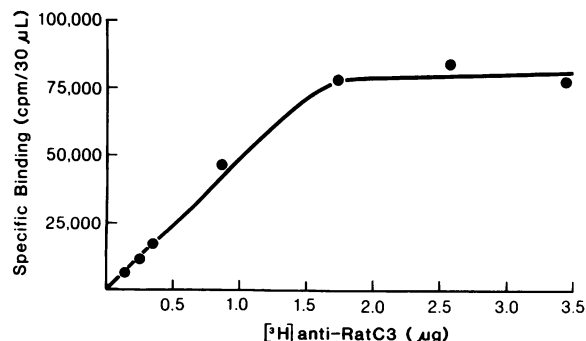


Figure 1. Specific binding of ³H-anti-rat C3 to cortical tubule fragments previously incubated with NRS and NH₃ (500 μ M). Binding determined with progressively increasing amounts of labeled antibody (0.0–3.5 μ g). Each point represents the mean for duplicate experiments.

resuspended in 300 μ l KRB. Aliquots from each reaction vessel were assayed for ³H-anti-rat C3 binding as described above. Addition of NH₄Cl in these quantities did not alter the pH of NRS. Tubule preparations from two rats were combined and the same preparation used for the various reaction mixtures each time the assay was performed. Antibody binding was determined for control tubules and tubules incubated with each level of NH₃ in triplicate, with replicate experiments done on different days. Deposition of C3 on cortical tubule fragments was also analyzed by qualitative immunofluorescence microscopy as described above.

Statistics

Data are presented as means \pm SD. Statistical significance of results was assessed by analysis of variance and subsequent Bonferroni's T-test. Differences were considered statistically significant for $P < 0.05$.

Results

Study I

In vivo renal studies. General physical and chemical parameters of the three groups of animals are shown in Table I. A similar degree of hypokalemia was found in both K-deficient groups (KDEF) after three weeks of dietary manipulation with a mean plasma K measured at 2.4 meq/liter. Both experimental groups showed a 28% reduction in muscle potassium content as compared with control. Final body weights did not differ between KDEF and KDEF + HCO₃; however, neither group gained weight normally. Potassium deficient NaCl supplemented rats demonstrated the expected renal hypertrophy with a marked increase in kidney weight in KDEF as compared with control (1.86 \pm 0.25 vs. 1.10 \pm 0.05 g, $P < 0.001$). However, NaHCO₃ supplementation blunted this renal hypertrophic response as reflected in a significant reduction in kidney weight in KDEF + HCO₃ as compared with KDEF (1.47 \pm 0.20 vs. 1.86 \pm 0.25, $P < 0.01$). NaHCO₃ supplemented animals manifested a moderate metabolic alkalosis, as reflected in a significantly increased arterial pH and derived HCO₃ as compared with KDEF. Thus, NaHCO₃ induced metabolic alkalosis was associated with a blunting of the renal hypertrophic response to chronic K deficiency.

Table I. General Features of Rats in Study I

| | KDEF | KDEF + HCO ₃ | Control |
|---|--------------------------|---------------------------------------|-------------------------|
| Plasma K ⁺ (meq/liter) | 2.4* \pm 1.6 (8)* | 2.4* \pm 0.9 (5) | 4.5 \pm 1.1 (8) |
| Muscle K (meq/100 g) | 26.4* \pm 2.1 (7) | 26.5* \pm 3.2 (7) | 36.4 \pm 6.8 (8) |
| Body weight (g) | 294* \pm 25 (12) | 276* \pm 20 (15) | 331 \pm 18 (12) |
| Kidney weight (g) | 1.86* \pm 0.25 (12) | 1.47* [‡] \pm 0.20 (15) | 1.10 \pm 0.05 (12) |
| Arterial pH | 7.44 \pm 0.05 (4) | 7.58 [‡] \pm 0.05 (6) | ND |
| Arterial [HCO ₃] (meq/liter) | 21.7 \pm 4.9 (4) | 34.7 [‡] \pm 7.5 (6) | ND |

* $P < 0.05$ vs. control.

[‡] n

[‡] $P < 0.05$ KDEF + HCO₃ vs. KDEF.

Renal functional parameters are tabulated in Table II. Potassium deficient rats demonstrated marked polyuria. 24-h urine volumes were higher in NaCl-supplemented rats than in NaHCO₃-supplemented rats (83.3±18.6 vs. 46.4±15.0 ml, *P* < 0.05), presumably reflecting increased intake. Total proteinuria was significantly reduced in KDEF + HCO₃, with a mean excretion rate measured at 20±6 mg/24 h as compared with 39±21 mg/24 h in KDEF (*P* < 0.05). Similarly, urinary excretion of low molecular weight protein (mol wt < 10,000) was significantly reduced in KDEF + HCO₃ as compared to KDEF (4±2 vs. 7±2 mg/24 h, *P* < 0.05). Thus, protein excretion rates suggested less renal injury in NaHCO₃-supplemented rats as compared with NaCl-supplemented rats. The decreased urinary excretion of low molecular weight protein suggested preservation of tubular functional capacity to reabsorb filtered protein. Both K-deficient groups demonstrated diminished GFR as compared with controls. NaHCO₃-supplemented rats had a tendency to higher GFRs than NaCl-supplemented, K deficient rats (0.91±0.29 vs. 0.74±0.28 ml/min, *P* = NS); whereas renal plasma flows were essentially identical in both experimental groups.

NaHCO₃ supplemented, K-deficient rats had a markedly reduced renal venous ammonia concentration, 214±28 μM, as compared with NaCl supplemented, K-deficient rats, 392±110 μM (*P* < 0.05), (Table III). The level of renal vein ammonia in NaHCO₃-treated rats was not significantly different from control levels, 179±57 μM (*P* = NS). Because of the rapid diffusion equilibrium of NH₃ in the renal cortex, renal venous NH₃ concentration is thought to reflect ambient cortical tissue levels (24). Thus, metabolic alkalosis induced by alkali feeding was associated with decreased ambient cortical tissue NH₃ levels. As can be seen, this decrease in cortical NH₃ levels was a reflection of significantly reduced renal NH₃ production in NaHCO₃ supplemented K-deficient animals. Ammonia production rates in KDEF + HCO₃, 1.33±0.47 μmol/min, were significantly less than rates in KDEF, 3.11±1.30 (*P* < 0.05) and not significantly different from control, 1.84±0.56 (*P* = NS).

Morphologic studies. Analysis by light microscopy revealed focal areas of tubulointerstitial injury in the potassium-deficient

Table III. Renal NH₃ Metabolism of Rats in Study I

| | KDEF | KDEF + HCO ₃ | Control |
|---|-------------------------------|--------------------------------|-------------------|
| Renal vein NH ₃ (μM) | 392* ±110 (6) [‡] | 214 [§] ±28 (6) | 179 ±57 (8) |
| Urinary NH ₃ excretion rate (μmol/min) | 2.29* ±0.70 (6) | 0.89 [§] ±0.29 (6) | 1.49 ±0.49 (8) |
| NH ₃ production rate (μmol/min) | 3.11* ±1.30 (6) | 1.33 [§] ±0.47 (6) | 1.84 ±0.56 (8) |

* *P* < 0.05 vs. control.

[‡] *n*

[§] *P* < 0.05, KDEF + HCO₃ vs. KDEF.

groups. Semiquantitative histopathological scoring showed more tubulointerstitial injury in KDEF than KDEF + HCO₃ or control, when samples were analyzed for tubular dilation and atrophy, intratubular cast formation and interstitial infiltrate (Table IV and Fig. 2). Hence, more severe histologic evidence of tubulointerstitial injury was found in agreement with functional studies.

Immunofluorescent studies (Fig. 3) revealed focal, circumferential, peritubular deposition of C3 in potassium deficient, NaCl-supplemented rats. We observed that deposition of C3 was most prominent in tubules immediately adjacent to renal venules. This peritubular immunofluorescence was qualitatively reduced in NaHCO₃-supplemented rats and essentially absent in control rats. Albumin was not demonstrable in a peritubular pattern in either group of K-deficient animals. Therefore, peritubular deposition of C3 appeared unique and not a consequence of nonspecific deposition of macromolecules. While immunofluorescent microscopy demonstrated the peritubular presence of C3 in both groups of K-deficient rats, we found that this technique was not precise enough to make reliable quantitative comparisons of the extent of complement deposition.

Study II

Zymosan model system. Qualitative immunofluorescence studies were performed, using the affinity purified, anti-rat C3, in both opsonized and nonopsonized zymosan particles. Opsonized zymosan particles demonstrated bright, linear fluorescence. In contrast, nonopsonized particles showed no fluorescence. Thus, the affinity purified, anti-rat C3 bound specifically to particles

Table II. Renal Functional Parameters of Rats in Study I

| | KDEF | KDEF + HCO ₃ | Control |
|---|-----------------------------|----------------------------|--------------------|
| Urine output (ml/24 h) | 83* ±19 (8) [‡] | 46 [§] ±15 (8) | 29 ±9 (8) |
| Urinary total protein excretion (mg/24 h) | 39* ±21 (15) | 20 [§] ±6 (15) | 17 ±7 (16) |
| Urinary low molecular weight protein (<10,000 mol wt) excretion (mg/24 h) | 7* ±2 (12) | 4* [§] ±2 (11) | 2 ±1 (12) |
| GFR (ml/min) | 0.74* ±0.28 (10) | 0.91* ±0.29 (8) | 1.27 ±0.14 (10) |
| Renal plasma flow (ml/min) | 4.67 ±2.31 (10) | 4.69 ±2.06 (8) | 4.89 ±0.67 (10) |

* *P* < 0.05 vs. control.

[‡] *n*

[§] *P* < 0.05, KDEF + HCO₃ vs. KDEF.

Table IV. Histopathological Scoring for Tubulointerstitial Injury in Rats in Study I*

| | KDEF <i>n</i> = 4 | KDEF + HCO ₃ <i>n</i> = 4 | Control <i>n</i> = 8 |
|-------------------------|----------------------|---|-------------------------|
| Tubular dilatation | 1.75 | 0.88 | 1.33 |
| Tubular atrophy | 1.00 | 0.25 | 0.33 |
| Intratubular casts | 1.75 | 0.63 | 1.00 |
| Interstitial infiltrate | 1.50 | 0.00 | 0.00 |
| Total score | 6.00 [‡] | 2.00 [§] | 2.66 |

* Scored 0–3+, corresponding to absent–severe lesions.

[‡] *P* < 0.05 vs. control.

[§] *P* < 0.05 KDEF + HCO₃ vs. KDEF.

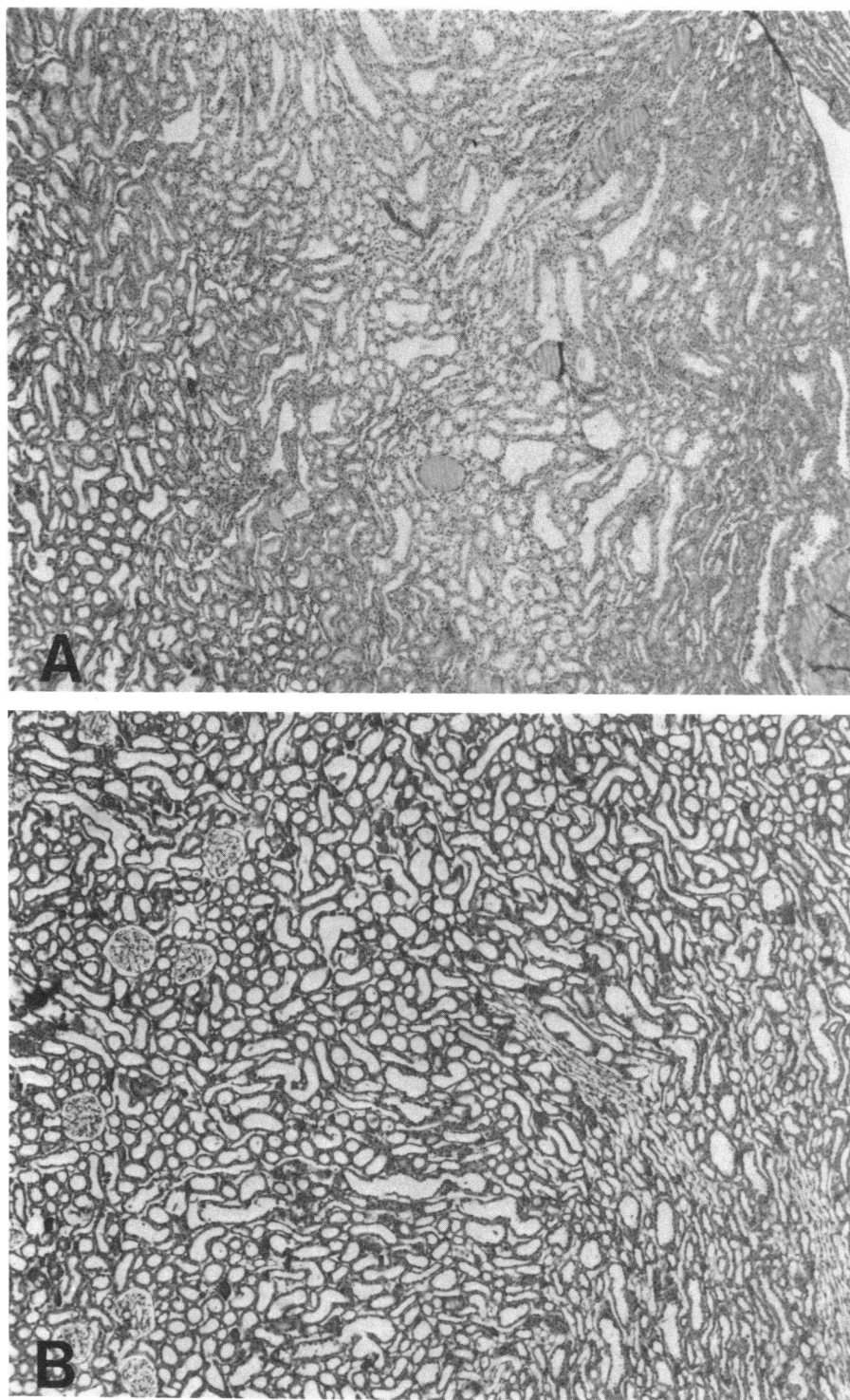


Figure 2. Sections of renal cortex of K-deficient rats, examined by light microscopy. NaHCO₃-supplemented rats. $\times 120$ (B), and $\times 300$ (D); NaCl-supplemented rats, $\times 120$ (A), and $\times 300$ (C). NaHCO₃-supplemented rats demonstrated less tubular dilatation and atrophy, less intratubular cast formation and less interstitial infiltrate than NaCl-supplemented rats.

on which complement components had been deposited. Opsonized zymosan demonstrated markedly increased binding of ^3H -anti-rat C3 (Fig. 4) with a mean value measured at $45,330 \pm 2249$ cpm/ $30\ \mu\text{l}$ as compared to 4812 ± 632 cpm/ $30\ \mu\text{l}$ in the non-opsonized zymosan ($P < 0.001$). Thus, in the model system of complement deposition on zymosan particles, analysis by labeled antibody binding gave quantitative results that were entirely consistent with the qualitative immunohistochemical studies.

In vitro studies: complement deposition on cortical tubular fragments. Immunofluorescence studies of normal rat cortical

tubules using affinity purified, anti-rat C3 demonstrated markedly increased C3 deposition on tubules incubated with NRS and NH_3 at a final concentration of $300\ \mu\text{M}$ (Fig. 5 A), and to a less striking degree on tubules incubated with NRS at a final NH_3 concentration of $150\ \mu\text{M}$ (Fig. 5 B); as compared with control tubules incubated with buffer and $300\ \mu\text{M}$ NH_3 in the absence of NRS and thus with no exogenous source of C3 available for reaction with NH_3 (Fig. 5 C). The differences in C3 deposition between tubules incubated with NRS at final NH_3 concentrations of 150 , 300 , and $500\ \mu\text{M}$ could not be distin-

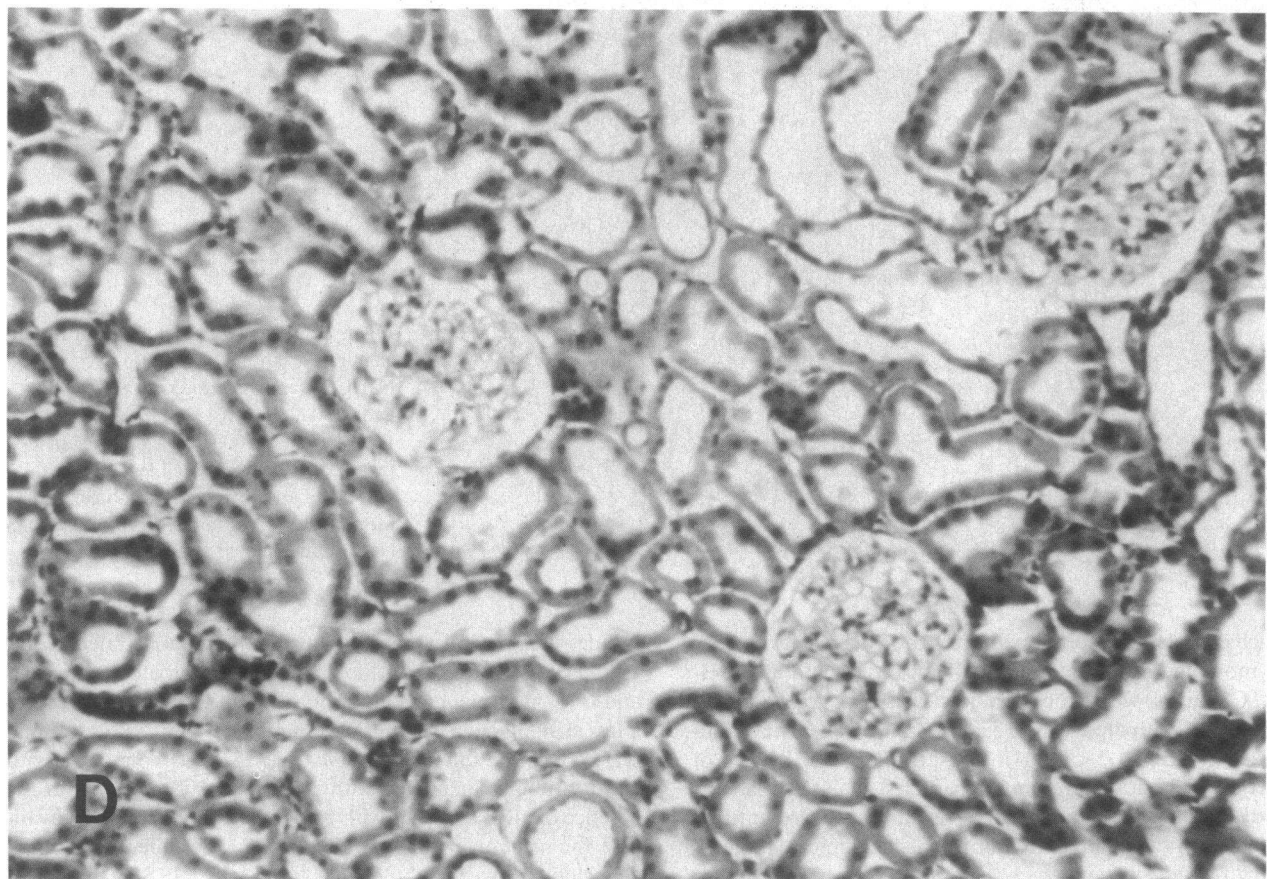
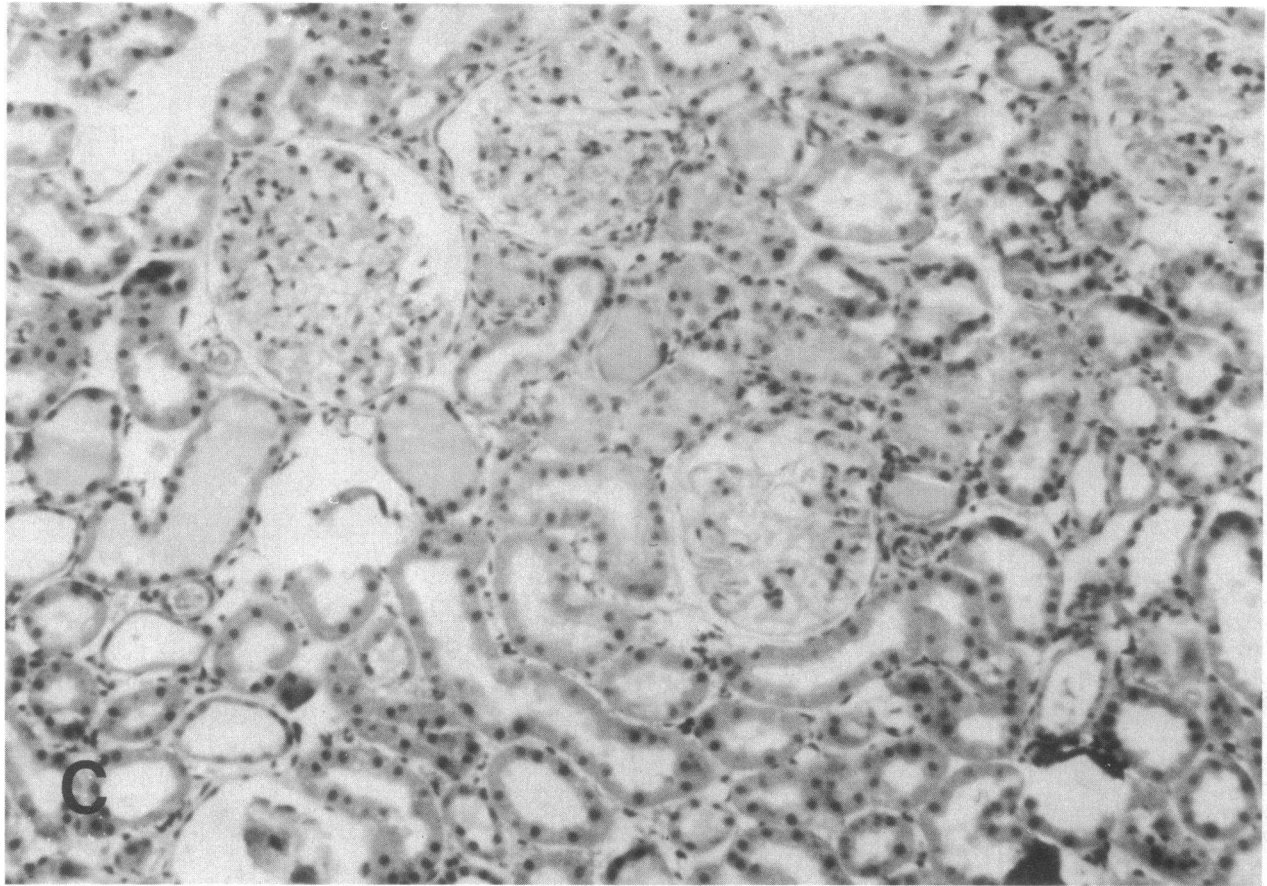


Figure 2 (Continued)

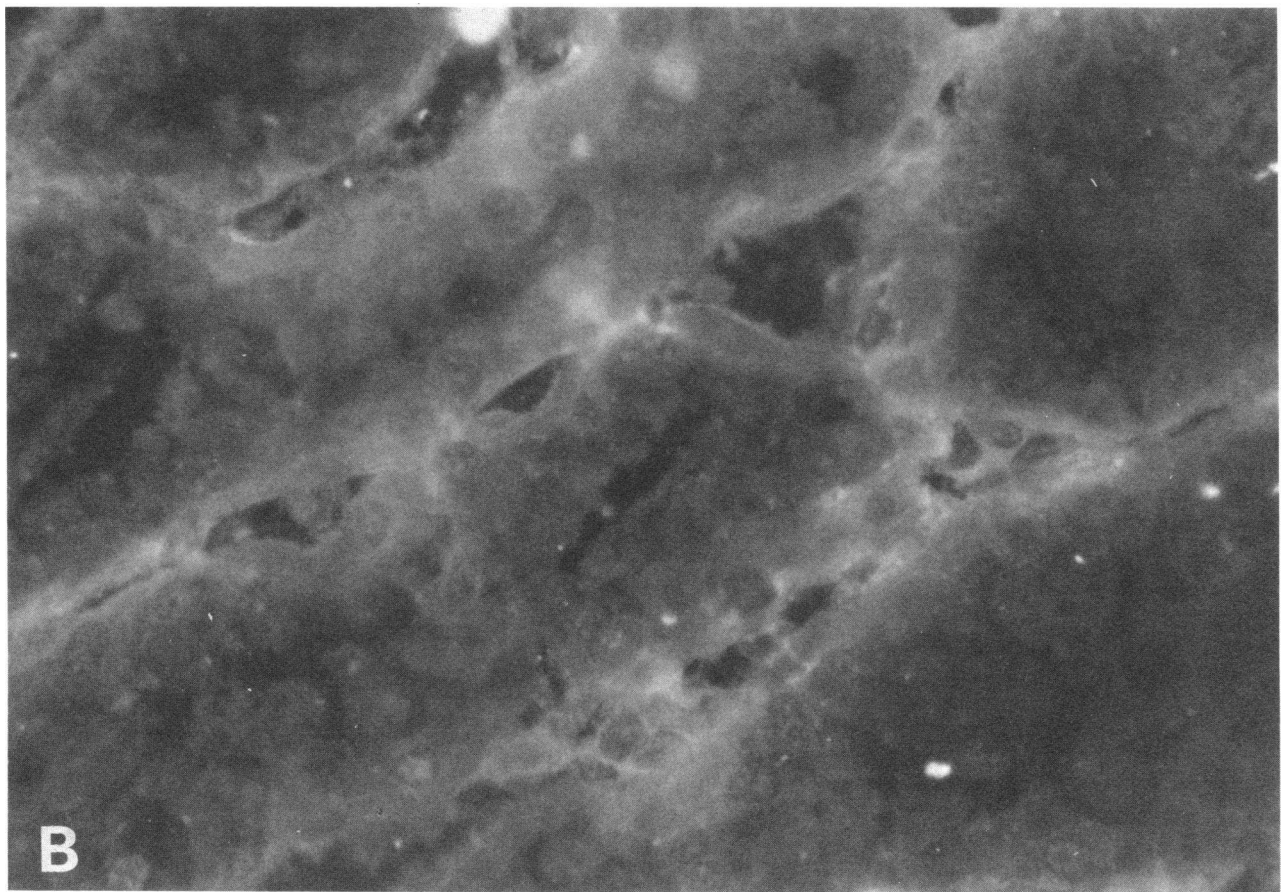
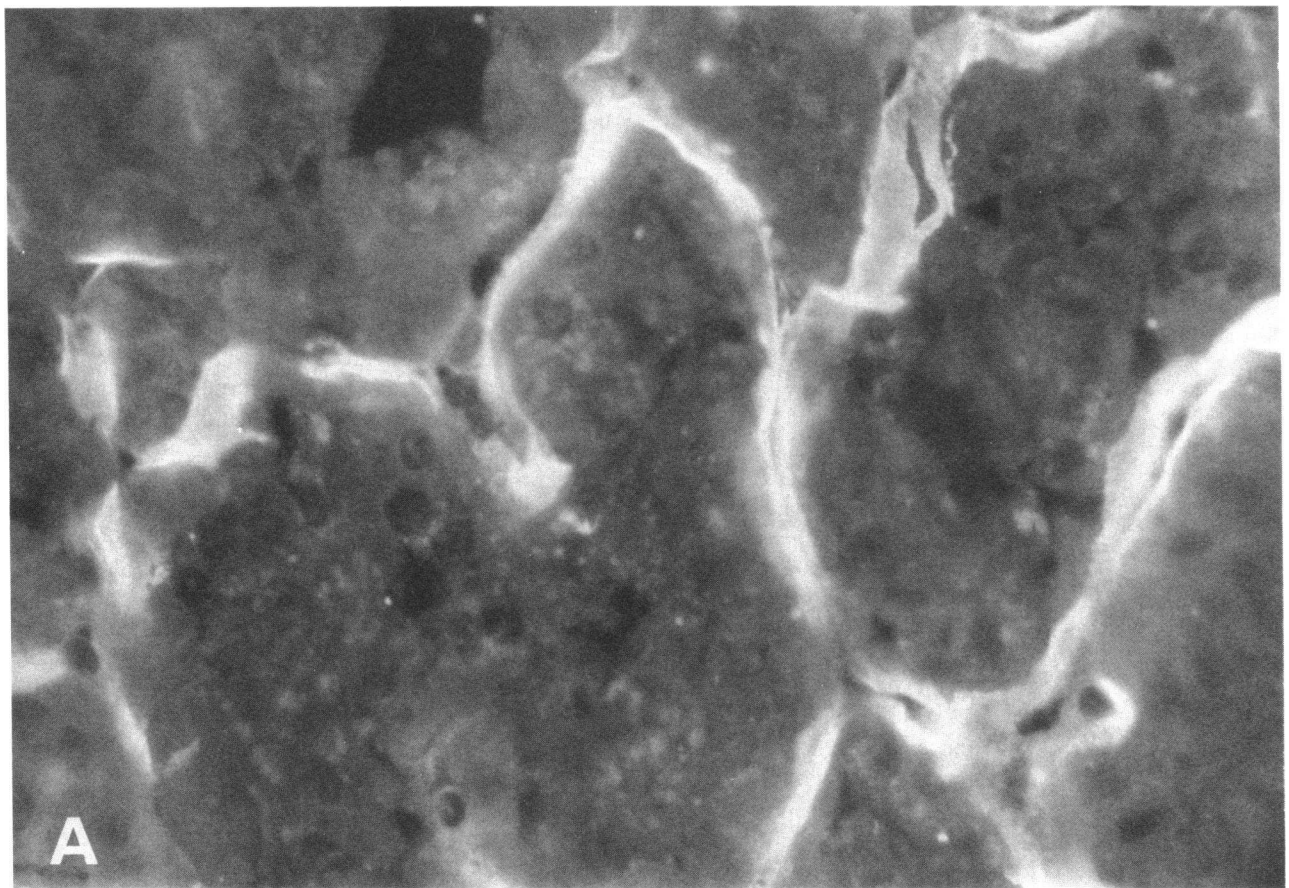


Figure 3. Sections of renal cortex of K-deficient rats examined by immunofluorescence for C3. (A) cortical tubules from NaCl-supplemented rat, $\times 400$. (B) cortical tubules from NaHCO_3 -supplemented rat, $\times 400$.

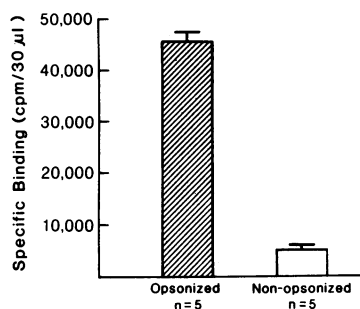


Figure 4. Specific binding of affinity purified ^3H -anti-rat C3 to zymosan. Opsonized zymosan (striped bar) demonstrates markedly increased binding as compared with nonopsonized zymosan (open bar, $P < 0.001$).

guished using semiquantitative visual scoring of tubular immunofluorescence. However, quantitative analysis of C3 deposition by labeled antibody binding (Fig. 6) showed significantly increased deposition of C3 on cortical tubule fragments incubated with NRS at $300 \mu\text{M}$ NH_3 as compared with controls ($79,202 \pm 7,694$ cpm/50 μl vs. $50,362 \pm 4,811$ cpm/50 μl , $P < 0.05$). When compared to controls, tubules incubated with NRS and NH_3 at $150 \mu\text{M}$ and $500 \mu\text{M}$, showed a tendency to increased C3 deposition ($69,040 \pm 6,798$ and $64,004 \pm 9,551$, cpm/50 μl , respectively) but this difference was not statistically significant. Thus, NH_3 in a concentration similar to the pathologically elevated levels found in the renal venous plasma of K-deficient animals in vivo ($392 \pm 110 \mu\text{M}$), significantly enhanced tubular deposition of the complement component, C3, present in normal rat serum. The enhancement of complement deposition by pathologically relevant concentrations of NH_3 was clearly demonstrated by quantitative, labeled antibody binding studies. Immunofluorescence microscopy, while providing striking visual evidence of complement deposition on tubules incubated with serum and NH_3 could not resolve the differences in deposition between the various ammonia levels, reflecting the essentially nonquantitative nature of this technique. The biphasic nature of this curve, with a decrease in C3 deposition as NH_3 concentrations increase beyond $300 \mu\text{M}$, is consistent with the amidation of all potentially active C3 substrate at higher NH_3 concentrations. C3 deposition is reduced in this situation because amidated C3 cannot be enzymatically activated for covalent binding to cell surfaces (13).

Discussion

Chronic potassium depletion leads to progressive renal injury in experimental models and humans (1–5). The tubulointerstitial compartment bears the brunt of injury as reflected by morphologic and functional parameters. In neither rat nor man are significant histologic lesions of glomeruli or blood vessels found (3). However, decreases in GFR and RPF (1), and proteinuria (6), albeit mild, have been reported. Although the association of increased renal ammoniogenesis and potassium deficiency is well described (7), the exact stimulus to the cortical tubular epithelium is unclear. The possible biochemical effector mechanisms responsible for the ammonia/potassium relationship are discussed in detail by Tannen (7). In the rat and man, potassium depletion is associated with a normal to increased plasma bicarbonate level and, therefore, changes in systemic acid-base status alone are unlikely to be responsible for the changes in ammonia metabolism. This lack of responsiveness of ammoniogenic mechanisms to changes in systemic pH has also been demonstrated in chronic respiratory acidosis in the rat (24). The

similarity in biochemical responses to chronic metabolic acidosis and chronic potassium depletion suggests that both may be a result of intracellular acidosis, a condition associated with both states. It is also possible that decreased intracellular potassium concentration alone may be the primary stimulus to accelerated ammoniogenesis, although there is little evidence supporting this hypothesis (7). In the current study we have demonstrated that oral NaHCO_3 supplementation effectively suppressed renal ammonia production in chronically K-deficient rats without altering the degree of hypokalemia or tissue K-depletion. This new observation lends support to the hypothesis that the control of renal ammoniogenesis is not primarily dependent on intracellular potassium levels but rather on intracellular pH and the requirements of the organism to maintain hydrogen ion homeostasis.

Renal hypertrophy has been described in both rat and man in response to chronic potassium depletion (3). Our data demonstrates a marked effect of NaHCO_3 supplementation with a significant blunting of renal hypertrophy despite continued K depletion. The mechanism responsible for this surprising and fascinating result is unclear. Renal hypertrophy and accelerated ammoniogenesis are associated in several other experimental models including partial ablation of renal mass and/or institution of a high protein diet (25–28) and chronic metabolic acidosis (29). Studies of sea urchin eggs after fertilization (30) and mammalian cell culture systems exposed to growth factors (31, 32) have demonstrated that rapid influxes of Na^+ , due to activation of a Na^+/H^+ exchanger, and subsequent changes in intracellular pH are intimately related to stimulation of cell growth and division. Seifter and Harris (33) reported increased Na^+/H^+ exchange in rat cortical brush border membrane vesicles, in association with renal cortical growth, in chronically K-depleted rats. We speculate that NaHCO_3 supplementation, through direct changes in intracellular pH, or via changes in NH_3 production and subsequent changes in Na^+ flux across cortical tubular epithelial cell membranes, blocks the stimulus to cortical growth found in chronic K^+ depletion.

NaHCO_3 supplemented, K-deficient rats demonstrated less tubulointerstitial disease as evidenced by decreased urinary protein excretion rates and improved renal histology. The decrease in total urinary protein excretion rate demonstrated by NaHCO_3 supplemented K-deficient rats could reflect diminished glomerular injury, consistent with the tendency toward improved GFRs in KDEF + HCO_3^- as compared with KDEF. However, we contend that the differences in urinary protein excretion reflect, at least in a large part, differences in proximal tubular catabolism of filtered protein, a contention supported by lower excretion rates of low molecular weight proteins in KDEF + HCO_3^- . The preservation of proximal tubular ability to reabsorb and catabolize filtered protein, with subsequent decreased urinary protein excretion rate, indicates less tubular epithelial injury in NaHCO_3 treated animals. Histologic analysis revealed less tubular dilatation and atrophy, less intratubular cast formation and less interstitial infiltrate in NaHCO_3 supplemented K-deficient rats, in agreement with functional studies.

We propose that suppression of ammoniogenesis by NaHCO_3 supplementation and protection from the expected tubulointerstitial injury in chronic potassium deficiency are causally related. The biochemical reaction of nucleophiles with the third component of complement, C3, has recently been described in detail (34–36). Nucleophilic amines, such as ammonia, attack a reactive thiolester bond contained within the alpha chain of C3, breaking the bond and resulting in the formation of an amide



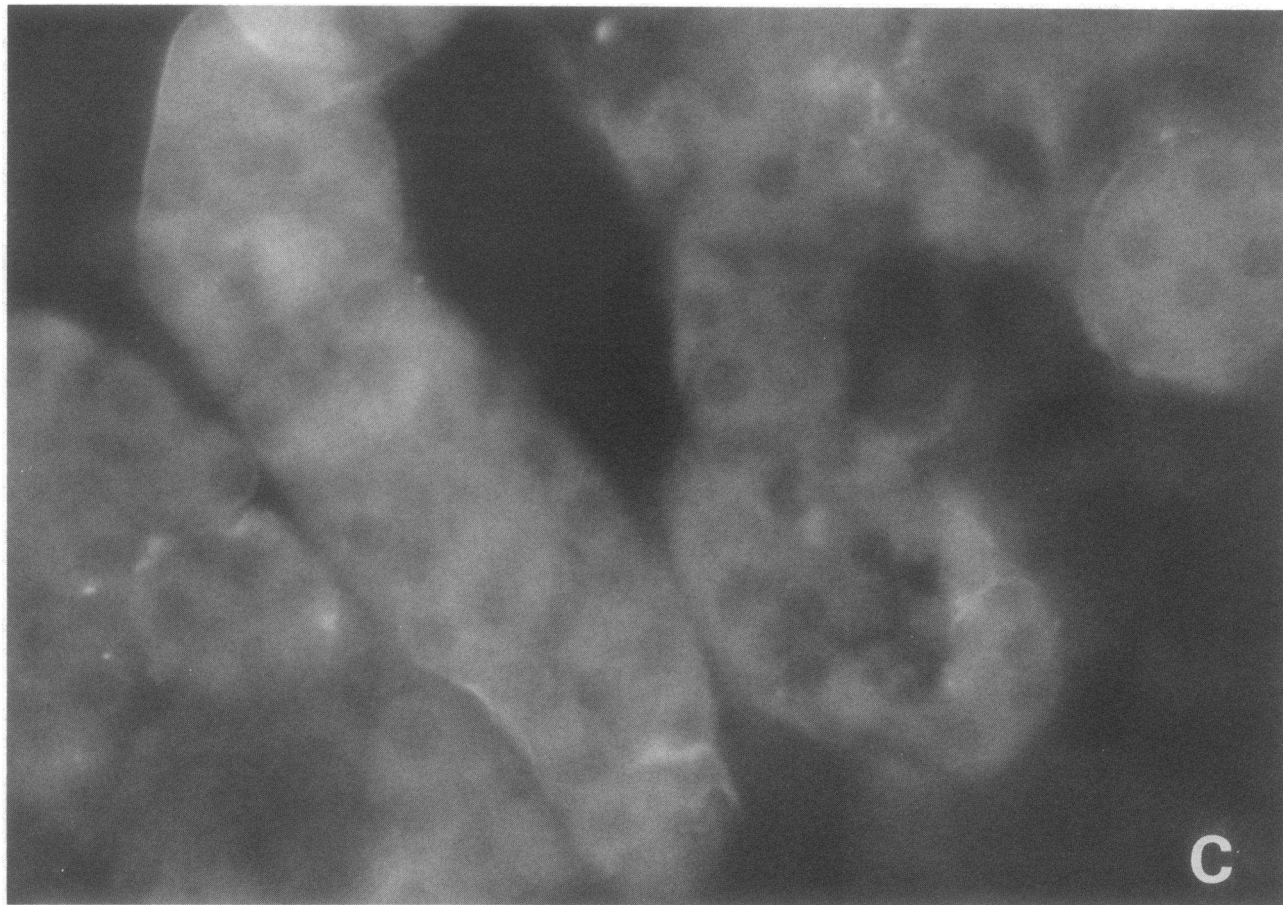


Figure 5 (Continued)

linkage at the reactive carbonyl group of the thiolester glutamyl. Concomitant with the binding of the nucleophile, C3 undergoes conformational changes and acquires functional properties similar to C3b (37, 38), including the ability to form the initial fluid-phase convertase of the alternative complement pathway (39, 40) and to react with cellular C3b receptors (41). Ammonia-treated C3, or amidated C3, has the identical properties, but has also been shown to stimulate oxidative metabolism of polymorphonuclear leukocytes and monocytes (42).

Beeson and Rowley (43) first proposed that the interaction of renal ammonia production and the complement system was important in the pathogenesis of renal disease. Antibody-induced activation of both the classical and alternative complement systems is believed to be injurious in experimental and human glomerulonephritis, based on measurements of serum complement concentrations and immunofluorescence studies showing glomerular deposition of both early and late complement proteins (44). However, in many instances only C3 and the later complement components (C5–C9) are found, without out immunoglobulin or early complement components, suggesting nonimmune activation of the alternative complement pathway (45, 46). Falk and co-workers (47) observed peritubular deposition of complement proteins in a variety of nonnephritic

chronic human renal diseases. The mechanism by which complement proteins are deposited and their pathogenetic significance in nonnephritic renal disease is unknown. Nath, Hostetter, and Hostetter (13) recently observed that reduction of renal ammoniogenesis by reduced dietary acid load was associated with decreased evidence of renal injury in the remnant kidney model of chronic renal failure in the rat. They demonstrated decreased peritubular deposition of C3 and C5b-9 in animals with suppressed renal ammoniogenesis and postulated that the protective effect was related to reduced reaction of cortical ammonia with C3 and, therefore, less activation of the alternative complement pathway.

That a similar mechanism is possible in the tubulointerstitial injury of chronic potassium deficiency is supported by two lines of evidence. Firstly, immunofluorescence microscopy demonstrated focal peritubular deposition of C3 in K-deficient rats: a nonnephritic model of chronic tubulointerstitial injury in which there is no evidence for antibody mediated activation of the complement system. The accentuation of complement activation, and subsequent deposition of C3 on tubules in proximity to vascular spaces could reflect local increases in NH_3 concentration in the interlobular artery or afferent arteriole, perhaps related to concomitant cortical "trapping" of NH_3 by diffusion

Figure 5. Binding of affinity purified anti-rat C3 to cortical tubule fragments analyzed by immunofluorescence microscopy ($\times 100$). Tubules incubated with NRS and NH_3 at a concentration of 300 μM (A) and 150 μM (B), demonstrate increased fluorescence for C3 as compared with control tubules incubated with buffer and 300 μM NH_3 in the absence of NRS (C).

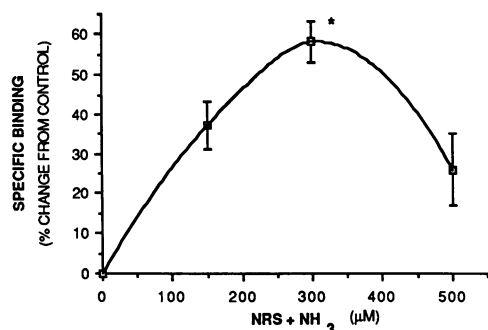


Figure 6. Specific binding of affinity purified ^3H -anti-rat C3 to cortical tubule fragments incubated with NRS and NH_3 . Binding observed on cortical tubule fragments incubated with buffer and NH_3 serves as baseline control. * $P < 0.05$ vs. baseline control value.

from the renal venule (48). Immunofluorescence microscopy suggested decreased peritubular C3 deposition in NaHCO_3 supplemented rats when renal ammoniogenesis was suppressed. We must consider this line of evidence as suggestive, but not conclusive, due to the subjective nature of immunofluorescence microscopy and the inability of this technique to resolve significant but modest differences: a limitation noted by other investigators (49). Secondly, quantitative, in vitro studies clearly demonstrated that addition of NH_3 to normal serum in concentrations similar to those found in the renal vein of K-deficient rats, will lead to significantly increased deposition of complement on cortical tubular fragments, presumably due to the biochemical reaction of nucleophilic ammonia with the third component of complement and subsequent activation of the alternative complement pathway. Nath et al. (13) using a standard assay for the hemolysis of rabbit erythrocytes by the alternative complement pathway also observed an increase in alternative pathway activity after the addition of ammonia to human serum in concentrations similar to those found in the renal vein of K-deficient animals.

Our studies of supplementation with NaHCO_3 in chronically K-deficient rats demonstrate lower cortical levels of NH_3 and less evidence of tubulointerstitial injury as determined by both functional and morphological analysis. In vitro studies using a new technique for quantitating complement deposition in tissues by labeled antibody binding provide evidence for a direct acti-

vation by NH_3 , at pathologically important concentrations, of the alternative complement system with subsequent deposition of complement components on cortical tubules. We therefore propose (Fig. 7) that chronic potassium deficiency induces increased renal NH_3 production and therefore increased intrarenal NH_3 levels. Subsequent ammonia-mediated complement activation then engenders progressive, tubulointerstitial injury. By suppressing ammoniogenesis, NaHCO_3 supplementation prevents this injurious cascade.

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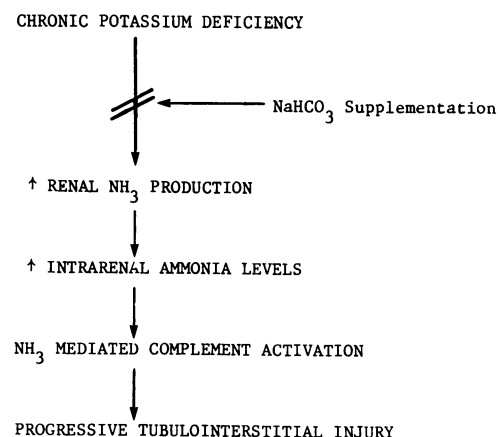


Figure 7. Proposed mechanism linking NH_3 , complement, and progressive tubulointerstitial injury in hypokalemic nephropathy.

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