

Heterogeneity of glutathione synthesis and secretion in the proximal tubule of the rabbit

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Parks, Lisa D., Rudolfs K. Zalups, and Delon W. Barfuss. Heterogeneity of glutathione synthesis and secretion in the proximal tubule of the rabbit. *Am. J. Physiol. Renal Physiol.* 43: F924–F931, 1998.—This study was designed to examine the synthesis and possible secretion of glutathione (GSH) in the S₁, S₂, and S₃ segments of the rabbit proximal tubule. GSH synthesis and secretion rates were measured in the three segments of the proximal tubule, using the isolated perfused renal tubule technique. Tritiated (³H) glycine was perfused into segments and synthesized [³H]GSH (³H on the glycine residue) was measured in the bathing solution, collectate, and tubule extract. In the S₁ segments, GSH was synthesized at the rate of 8.65 ± 0.88 fmol·min⁻¹·mm⁻¹ tubule length and preferentially secreted into the lumen at the rate of 7.28 ± 0.74 fmol·min⁻¹·mm⁻¹. The difference between synthesis and secretion appeared in the bathing solution. The S₂ segment synthesized GSH at the rate of 3.88 ± 0.82 and secreted GSH at the rate of 2.78 ± 0.57 fmol·min⁻¹·mm⁻¹. GSH synthesis and secretion rates in the S₃ segment were 5.45 ± 1.19 and 4.22 ± 1.16 fmol·min⁻¹·mm⁻¹, respectively. Cellular concentrations of [³H]GSH increased along the length of the proximal tubule, with the highest concentrations in the S₃ segment. The respective GSH cellular concentrations in the S₁, S₂, and S₃ segments were 35.89 ± 10.51, 49.65 ± 9.32, and 116.90 ± 15.76 μM. These findings indicate that there is heterogeneity of GSH synthesis along the proximal tubule and that synthesized GSH is secreted preferentially into the lumen.

kidney; renal transport; luminal membrane; basolateral membrane

RENAL GLUTATHIONE (GSH) metabolism has been studied extensively (8, 9, 11, 16, 22, 23, 27–29). GSH is synthesized intracellularly from glutamate, cysteine, and glycine. Its synthesis is catalyzed by γ-glutamylcysteine synthetase, followed by GSH synthetase. The first step in GSH synthesis (γ-glutamylcysteine formed by γ-glutamylcysteine synthetase and ATP) is controlled through feedback inhibition by GSH (22). This feedback determines the upper concentration limit for intracellular GSH. The second synthetic step, where glycine bonds to γ-glutamylcysteine, requires one molecule of ATP and the activity of the enzyme GSH synthetase. After being synthesized in proximal tubular cells, GSH can remain in the cell or be transported into the tubular lumen across the luminal membrane or into the blood across the basolateral membrane. In the cell, it has been shown that 72% of GSH is in the mitochondrial pool and that the remaining 28% is located in the cytoplasm (28). It has also been shown that GSH transferases, found in several organs, including the cytosol of the proximal tubular cells, are considered important components in the detoxification of xenobiot-

ics (8, 9, 27). If transported into the luminal fluid, GSH could prevent the toxic effects of free radicals and/or toxicants that may have been filtered at the glomerulus or secreted by the proximal tubular cells. Under normal conditions, GSH secreted into the lumen is degraded rapidly into its constituent amino acids, first by γ-glutamyltransferase (γ-GT) into glutamate and cysteinyl-glycine (Cys-Gly) and then by luminal membrane dipeptidases (Cys-Gly to Cys and Gly). These amino acids are then absorbed by proximal tubular cells. Certain drugs are very effective at inhibiting specific enzymes required in the synthesis and degradation of GSH. α-Amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid (acivicin) is one such compound. It inhibits γ-GT by alkylation, which prevents the extracellular degradation of GSH (12, 13, 17, 30).

Several studies have shown that a decrease in intracellular concentrations of GSH in the kidneys increases the sensitivity of these organs to oxidative injury and susceptibility to cellular disruption induced by heavy metals (34). GSH has been shown to provide protection against cellular injury induced by *t*-butylhydroperoxide (16). Addition of GSH to the culture medium has also been shown to eliminate cellular injury in primary cultures of rat proximal tubular epithelial cells, induced by 60 min of anoxia and 30 min of reoxygenation (23). Finally, in isolated perfused proximal tubular segments, addition of GSH (80 μM) to a perfusate containing 18.4 μM inorganic mercury provided complete protection from the toxic effects of mercury (34).

A major question in the renal handling of GSH has been whether it is secreted preferentially into the lumen, where it could provide protection to the extracellular surface of the luminal membrane. According to *in vivo* studies by Griffith (11), GSH transport occurs in renal proximal tubular cells, but the mechanism involved in this transport is unknown. The only other mention of GSH secretion in the kidney was in a study by Scott and Curthoys (31). They proposed that apical and basolateral secretion occurs in LLC-PK₁ cells. The only other known peptide/protein demonstrated to be synthesized and then preferentially secreted into the lumen along the nephron is the Tamm-Horsfall protein, which is secreted in the thick ascending limb and is proposed to function protectively to prevent renal calculi formation (29). More recently, angiotensin II has been shown to be secreted into the lumen of the proximal tubule of rats (5). However, angiotensin II is not synthesized by proximal tubular cells.

Despite the current body of literature, very little is known about the purported secretion of GSH in the proximal tubule. The primary aim of the present study was to confirm and measure the relative rates of

secretion across the luminal and basolateral membranes of intracellularly synthesized GSH in S_1 , S_2 , and S_3 segments of the rabbit proximal tubule.

MATERIALS AND METHODS

Materials

Perfusing solutions containing [^3H]glycine and [^{14}C]polyethylene glycol ([^{14}C]PEG) were prepared fresh for each experiment. All isotopes were purchased from NEN (Boston, MA). The [^3H]glycine had a specific activity of 35.1 Ci/mmol and was stored in ethanol-water (2:98, vol/vol) at 5°C. For perfusion experiments, 20 μl of the [^3H]glycine stock solution were dried down under nitrogen together with 10 μl of [^{14}C]PEG. The dried contents were then reconstituted in 50 μl of perfusion solution. The final perfusate concentration of [^3H]glycine was 11.2 μM . The [^{14}C]PEG (mol wt 2,000) was used as a volume marker in all perfusion experiments. It had a specific activity of 11.0 mCi/g and a perfusate concentration of 2.3 mM. All other chemicals were purchased from Sigma Chemical (St. Louis, MO).

Solutions

Perfusion solution. The basic perfusion and bathing solutions [artificial perfusion media (APM)] contained (in mM) 145 Na^+ , 140 Cl^- , 5.0 K^+ , 2.5 Ca^{2+} , 1.2 Mg^{2+} , 1.2 SO_4^{2-} , 2.0 HPO_4^{2-} - H_2PO_4^- , 0.5 L-glutamate, 1.0 D-glucose, and 10 μM L-cysteine. Osmolality was adjusted to 290 mosmol/kg H_2O , and pH was adjusted to 7.4. [^3H]glycine (11.2 μM), [^{14}C]PEG (2.3 mM), and the vital dye FD & C Green (250 nM) were added to the perfusion solution only. When acivicin was used, it was added to the perfusion solution at 1 mM.

Dissecting solution. A phosphate/sucrose buffer solution was used to store kidney slices until dissected to obtain isolated proximal tubular segments. It contained 69 mM HPO_4^{2-} - H_2PO_4^- and 140 mM sucrose. Osmolality was adjusted to 290 mosmol/kg H_2O , and pH was adjusted to 7.4.

Animals

Female New Zealand White, specific pathogen-free rabbits were purchased from Myrtle's Rabbit Farm (Thompson Station, TN). All rabbits were maintained on regular rabbit chow and given water ad libitum. Rabbits were anesthetized with ketamine (33 mg/kg body wt) and xylazine (33 mg/kg body wt) purchased from Butler Chemical (Bedford, OH). All experiments were conducted according to the NIH "Guide for the Care and Use of Laboratory Animals."

Methods

Isolated perfused-tubule technique. Methods used for the identification, dissection, and perfusion of the three segments of the rabbit proximal tubule have been described previously (2, 3). To obtain tubular segments, rabbits were first anesthetized, and then the kidneys were quickly removed. The kidneys were then cut into thin (~1 mm) coronal sections, which were stored in cold (4°C) phosphate-sucrose buffer solution. These slices could be used for the next 8–12 h. Tubules were dissected manually from the individual slices under a stereomicroscope. Individual segments were identified as described in Barfuss et al. (2) while bathed in the phosphate-sucrose buffer solution. In brief, S_1 segments were obtained from the cortical surface of the slices. They had a larger diameter than the rest of the tubular segments and had a convoluted shape. The S_2 segments were straight, slightly smaller in diameter than the S_1 segments, and

spanned the length of the cortex. The S_3 segments were the last 1 mm of the proximal tubule, identified by their attachment to the much thinner descending thin limb of Henle.

Tubules were transferred to a Lucite chamber that was mounted on the stage of an inverted microscope and perfused in vitro by techniques modified by our laboratory and others (2, 4). Briefly, tubules were suspended between two sets of pipettes, one set to perfuse and the other to collect perfused fluid. Perfused tubular segments were allowed to warm to 37°C (~10 min), and observations were begun after an additional 15-min equilibration period. The perfusion rate was maintained at ~6 nl/min by hydrostatic pressure, and the perfused solution was collected in a constant volume pipette (40–80 nl). These collections were timed to determine the collection rate (nl/min) for each sample. During all experiments, the bathing fluid was pumped to the bathing chamber (0.5 ml) at 0.26 ml/min and was continually aspirated and collected directly into a scintillation vial at 5-min intervals.

TRITIATED GLUTATHIONE EXTRACTION FROM TUBULE. At the end of each experiment, the tubule was harvested to extract the cytoplasmic contents, and these contents were analyzed for [^3H]GSH, [^3H]glycine, [^3H]Cys-Gly, and ^3H -labeled oxidized glutathione (GSSG). Harvesting was accomplished by grabbing the tubule with fine forceps, pulling it free from the perfusion pipettes, and quickly (<1 s) placing it in 10 μl of 3% TCA solution. The TCA solution instantly disrupts the cell membrane, releasing the cytoplasmic contents. The tubule turns white in appearance and becomes rigid. These cytoplasmic contents were collected as the cellular extract and analyzed for total ^3H and for ^3H associated with each ^3H -labeled compound.

The following description provides technical features of the isolated perfused-tubule techniques that were unique to the present study.

SAMPLES. Five samples of perfusate, collectate, and bathing fluid samples were collected from each perfused segment. Two were used for high-performance liquid chromatography (HPLC) analysis, while the remaining three were analyzed for total amount of ^3H (Brinkmann 5108 scintillation counter). The single sample of cellular extract per tubule was split in half. One half was used for HPLC analysis, and the other half was used for total ^3H analysis.

Steady state. After an individual tubule was perfused and warmed, a period of 15 min was allowed for the attainment of a steady state for [^3H]GSH synthesis, cell-to-lumen secretion, and cell-to-bath transport and the transepithelial transport of [^3H]glycine. Steady-state conditions were confirmed by taking samples from 0 to 20 min after warm-up time and checking for constant values of all parameters. For each tubule, samples were collected during the following 20–30 min.

Perfusion rate. The average perfusion rate in all experiments was maintained at ~6 nl/min (Table 1). To determine whether perfusion rate affected the rates of synthesis and/or degradation of [^3H]GSH, several tubular segments (S_1 , S_2 , and S_3) were perfused at higher rates (20–50 nl/min). In this range of perfusion rates, there was no significant difference in the rate of [^3H]GSH synthesis when compared with tubules perfused at 6 nl/min.

Effective concentration of acivicin. Because [^3H]GSH secreted into the lumen can be degraded rapidly to [^3H]Cys-Gly by γ -GT with subsequent degradation of [^3H]Cys-Gly to [^3H]glycine and cysteine by dipeptidases (thus underestimating the rate of [^3H]GSH secretion), acivicin was added to the perfusion solution to inhibit the activity of luminal γ -GT. Several different concentrations of acivicin were coperfused

Table 1. Summary of cellular synthesis rates, secretion rates into the tubule lumen, appearance rates in the bathing solution, cellular concentrations, and MLC of [³H]GSH in the S₁, S₂, and S₃ segments of rabbit proximal tubule

Segment	Synthesis, fmol·min ⁻¹ ·mm ⁻¹	Secretion, fmol·min ⁻¹ ·mm ⁻¹	Appearance in Bath, fmol·min ⁻¹ ·mm ⁻¹	[GSH] in Cells, μM	MLC, μM	Perfusion Rate, nl/min
S ₁ (-)	4.36 ± 1.39	2.86 ± 0.82	1.49 ± 0.59	35.89 ± 10.51	1.84 ± 0.30	5.83 ± 0.54
S ₁ (+)	8.65 ± 0.88	7.28 ± 0.74	1.37 ± 0.16	25.70 ± 1.91	3.36 ± 0.08	6.17 ± 0.32
S ₂ (-)	3.00 ± 0.71	2.24 ± 0.57	0.75 ± 0.19	49.65 ± 9.32	1.34 ± 0.21	6.06 ± 0.62
S ₂ (+)	3.88 ± 0.82	2.78 ± 0.57	1.10 ± 0.30	27.80 ± 5.70	2.56 ± 0.18	6.19 ± 0.52
S ₃ (-)	3.89 ± 0.64	2.73 ± 0.57	1.16 ± 0.27	116.90 ± 15.79	0.98 ± 0.18	6.07 ± 0.58
S ₃ (+)	5.45 ± 1.19	4.22 ± 1.16	1.23 ± 0.30	93.34 ± 9.35	1.95 ± 0.33	6.64 ± 0.60

Values are means ± SE for each group of ≥5 tubules. [GSH], GSH concentration; MLC, [³H]GSH mean luminal concentrations. [³H]GSH was synthesized from 11.2 μM [³H]glycine being perfused through the tubular lumen. Notations (-) and (+) refer to the absence or presence of 1.0 mM acivicin in the luminal fluid.

with 4.6 μM [³H]GSH to determine the most effective concentration. A maximum inhibitory effect on γ-GT, coupled with a minimum of cellular damage and/or nonspecific alterations in transport mechanisms, was necessary. Initially, 0.25 mM acivicin and [³H]GSH were perfused, with no resulting tubular damage. However, HPLC analysis of the collectate showed that, with this acivicin concentration, there was some level of degradation of GSH. This was shown by 36.7 ± 1.1% of the total collectate radioactivity (³H) being associated with the glycine chromatographic peak. Another 1.44 ± 0.05% of the total radioactivity was associated with Cys-Gly. Perfusing with 2.5 mM acivicin and [³H]GSH decreased the radioactivity in the glycine chromatographic peak to 0.74 ± 0.74% of total ³H in the collectate; however, cellular swelling and vital dye uptake occurred at the perfusion end of the tubule after 40 min. It was likely that the high concentration of acivicin in the lumen was affecting cellular integrity. Indeed, lumen-to-bath transport of [³H]glycine was inhibited significantly when the perfusate contained 2.5 mM acivicin. An acivicin concentration of 1.0 mM in the lumen was most effective at preventing degradation of GSH while preserving tubular integrity. The radioactivity associated with the glycine chromatographic peak in the collectate samples was 2.56 ± 0.76%, with no measurable ³H activity above background associated with the Cys-Gly peak, whereas the remaining ³H was associated with GSH. Tubular integrity appeared to be preserved throughout the experiments. There was no evidence of cellular blebbing or swelling or uptake of the vital dye. In addition, lumen-to-bath transport rates of [³H]glycine were measured and compared in the absence of acivicin and found to be similar in each segment type. Thus it appeared that acivicin did not have a deleterious effect on amino acid transport.

Calculations

Measurement of tubular (intercellular) leak. The pericellular leak of luminal fluid (J_L , nl·min⁻¹·mm⁻¹) into the bathing fluid was measured by the appearance of the volume marker [¹⁴C]PEG in the bathing solution and was calculated using the following equation

$$J_L = [^{14}\text{C}]\text{PEG}_{\text{cpm}} \div ([^{14}\text{C}]\text{PEG}_{\text{MLC}} \times T \times L)$$

where PEG_{cpm} represents the counts/min of ¹⁴C appearing in the bathing solution in T minutes. [¹⁴C]PEG_{MLC} was the mean luminal [¹⁴C]PEG concentration (in cpm/nl), and L was the tubule length (in mm). Any experiment with an average leak >0.2 nl·min⁻¹·mm⁻¹ was excluded from further analysis.

Cellular concentration calculations. The cellular concentration ([³H]GSH_{cell}, in μM) of [³H]GSH was calculated by

$$[{}^3\text{H}]\text{GSH}_{\text{cell}} = ([{}^3\text{H}]\text{GSH}_{\text{cpm}} \div \text{SA}_{\text{GLY}}) \div V_{\text{cell}}$$

where [³H]GSH_{cpm} represents cpm of the [³H]GSH in the TCA-soluble fraction of the perfused tubule, which we have generically referred to as the tubular extract. SA_{Gly} is the specific activity (cpm/mol) of [³H]glycine, and V_{cell} is the cellular volume for that tubule segment.

V_{cell} (in femtoliters) was calculated for each tubular segment using the equation

$$V_{\text{cell}} = \pi (r_o^2 - r_i^2) \times L \times 0.7$$

where r_o and r_i are the outer and inner radii (in μm), respectively, L is the tubular length (in μm), and 0.7 accounts for the fraction of cellular volume that is assumed to be water.

Cellular concentrations of [³H]GSH were determined from total amount of ³H extracted from the tubular cells and corrected for contamination of ³H-labeled compounds ([³H]glycine, [³H]cysteinyl-glycine, and others). Radioactivity of the volume marker in the cellular extract was used to determine extracellular ³H contamination (this contamination was never >1%). HPLC analysis was used to determine the fraction of the total extracted ³H associated with GSH.

Calculation of rates for luminal secretion of [³H]GSH. Rates of secretion of cellularly synthesized [³H]GSH into the lumen from the proximal tubular epithelial cells (J_{C-L} , fmol·min⁻¹·mm⁻¹) was calculated, using the following equation

$$J_{C-L} = ([{}^3\text{H}]\text{GSH}_C \times \text{SA}_{\text{Gly}} \times V_C) \div L$$

where [³H]GSH_C was the concentration of [³H]GSH in the collectate (cpm/nl), V_C was the collection rate from the lumen (nl/min), SA_{Gly} was the [³H]glycine specific activity (fmol/cpm), and L was tubule length (in mm). [³H]GSH_C was calculated by

$$[{}^3\text{H}]\text{GSH}_C = {}^3\text{H}_C \times [{}^3\text{H}]\text{GSH}_F$$

³H_C was the total ³H (cpm/nl) in a particular collectate sample, and [³H]GSH_F was the fraction of the total ³H in that same sample associated with GSH determined by HPLC analysis.

Calculation for rates of basolateral secretion of [³H]GSH. The secretion rate of cellularly synthesized [³H]GSH into the bathing solution from the cells (J_{C-B} , fmol·min⁻¹·mm⁻¹) was calculated by

$$J_{C-B} = ({}^3\text{H}_B \times [{}^3\text{H}]\text{GSH}_F \times \text{SA}_{\text{Gly}}) \div (T \times L)$$

³H_B represents the total ³H appearing in the bathing solution (cpm), and [³H]GSH_F was the fraction of total bath ³H_B determined to be associated with GSH by HPLC analysis. T

was the duration (in min) over which the bath sample was collected, and L the tubule length (in mm).

Rate of [^3H]GSH synthesis. Rate of synthesis of [^3H]GSH ($[\text{^3H}]\text{GSH}_{\text{syn}}$, $\text{fmol}\cdot\text{min}^{-1}\cdot\text{mm}^{-1}$) was calculated by the combined rates of [^3H]GSH secretion into the luminal ($J_{\text{C}\rightarrow\text{L}}$) and the bathing fluid ($J_{\text{C}\rightarrow\text{B}}$)

$$[\text{^3H}]\text{GSH}_{\text{syn}} = J_{\text{C}\rightarrow\text{L}} + J_{\text{C}\rightarrow\text{B}}$$

High-Performance Liquid Chromatography

Reverse-phase HPLC was used to determine the fraction of ^3H in each sample associated with the chromatographic peaks of GSH, GSSG, glycine, Cys-Gly, and other unknown compounds. This analysis was done on perfusate, collectate, bathing solution, and cellular extract samples.

A Waters (Milford, MA) HPLC system equipped with two model 510 pumps, a 420-AC fluorescence detector, a systems interface module device, and a Bio-Rad AS-100 refrigerated automatic injector with a 1-ml injector loop. The data acquisition software was Maxima 820, loaded into an IBM-XT computer.

Several protocols exist for the dansylation of compounds possessing primary amines (GSH, GSSG, Cys-Gly, and glycine) (1, 7, 15, 20, 21, 25, 26, 33), and these were consulted for development of the procedures used for the precolumn derivatization of all samples used in this study. Samples generated from perfusion experiments were placed in microcentrifuge tubes with 800 μl of APM and 100 μl of a standard solution containing 1.3 mM of GSH, GSSG, glycine, and Cys-Gly, dissolved in 40 mM Li_2CO_3 at pH 9.5. This addition generated a sufficient signal resulting in a chromatogram that clearly showed the peaks for GSH, GSSG, glycine, and Cys-Gly. In addition, 180 μl of 40 mM iodoacetic acid was added to stabilize the free sulfhydryl groups to prevent oxidation of GSH to GSSG. In perfusate, bath, and collectate samples, 8 μl of saturated KOH was added. To the TCA cellular extract of the tubule, 15 μl of saturated KOH were added. Samples were vortexed briefly (30 s), incubated at room temperature for 20 min, and stored at 4°C.

Dansyl chloride (DNS-Cl) was added to acetone in a 1.5 mg-to-1 ml ratio to give a 5.6 mM stock solution. The DNS-Cl solution (500 μl) was added to every sample, shaken gently for 2 min, and allowed to incubate in the dark for 55–60 min. This reaction was pH sensitive and most completely reacted at pH 9.0–9.2.

To stop the reaction, 2 ml of chloroform were added and mixed well. This mixture was centrifuged for 5 min to separate the chloroform layer from the aqueous sample. A 1-ml aliquot from the top aqueous layer containing the dansylated products was loaded into the HPLC injector for analysis.

The mobile phase was made of *eluent A*, a 0.05 M sodium acetate solution at pH 4.6, and *eluent B*, 100% HPLC-grade methanol. DNS-glycine, DNS-GSH, DNS-GSSG, and DNS-Cys-Gly were separated, using the following gradient. The gradient program was a linear gradient from 0 to 36% *solvent B* in 6 min. From 6 to 15 min, *solvent B* continued to run at 36%. From 15 to 21 min, there was a linear gradient from 36 to 60% *eluent B* that remained constant until *minute 22*. From 22 to 32 min, the gradient was again linear from 60 to 100% *eluent B*. Then a continuation of 100% *eluent B* for 5 min to clean the column, followed by a 10-min equilibration period with 100% *eluent A* to restore initial conditions before a new sample was injected.

Purchased dansylated standards of glycine, cysteine, and glutamate eluted at the same time ± 10 s as the dansylated samples prepared in the laboratory. When tested for yield of

dansylated [^3H]glycine stock isotope, 96–98% of the ^3H was recovered in the chromatographic peak associated with commercially available DNS-glycine. Dansylated standards for GSH, GSSG, and Cys-Gly were not commercially available and were made and tested in the laboratory. When [^3H]GSH was dansylated in the lab, 85–90% of the ^3H was associated with the DNS-GSH peak, and 5–10% was associated with the DNS-GSSG peak. The peak associated with Cys-Gly was not tested with ^3H , but, in HPLC runs of collectate, bath, and tubule extracts, no significant ^3H was associated with that peak.

For the mathematical analysis of chromatographic data, after the peak collections for GSH, GSSG, Cys-Gly, and glycine were counted for ^3H , the fraction of total ^3H associated with each peak was calculated. Rates of flux and cellular concentrations were calculated from HPLC data obtained from samples from each tubule (perfusate, collectate, bathing fluid, and tubular extract).

Statistics

To determine the transport rates for each of the three proximal tubular segments, a minimum of five tubules was perfused for each experimental condition. Three or more flux measurements per tubule were made and averaged. The mean values from individual tubules were used to compute an overall mean and standard error for each segment and for each experimental condition. Chromatographic samples were analyzed in duplicate. A two-way analysis of variance (ANOVA) and Tukey's honest significant difference post hoc test were used to assess differences between means.

RESULTS

Table 1 provides a summary of data shown in Figs. 1–5.

[^3H]GSH Synthesis

The rate of [^3H]GSH synthesis in S_1 segments was greater than the rate in the S_2 and S_3 segments (Fig. 1). The apparent rate of synthesis was maximized, following inhibition of γ -GT. In the absence of acivicin, the

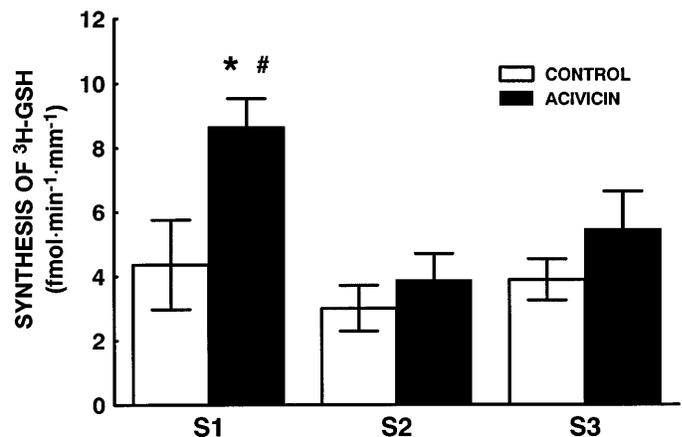


Fig. 1. Rates of cellular synthesis of [^3H]GSH in the S_1 , S_2 , and S_3 segments of rabbit proximal tubules perfused with and without 1.0 mM acivicin. Perfusate contained 11.2 μM [^3H]glycine. Each bar is mean \pm SE of 5 or more tubules. ANOVA F values are 5.483, 8.601, and 1.849 for each segment, respectively. $P < 0.05$: * significantly different from corresponding mean of same segment not treated with acivicin; # significantly different from corresponding means for the S_2 and S_3 segments treated with acivicin.

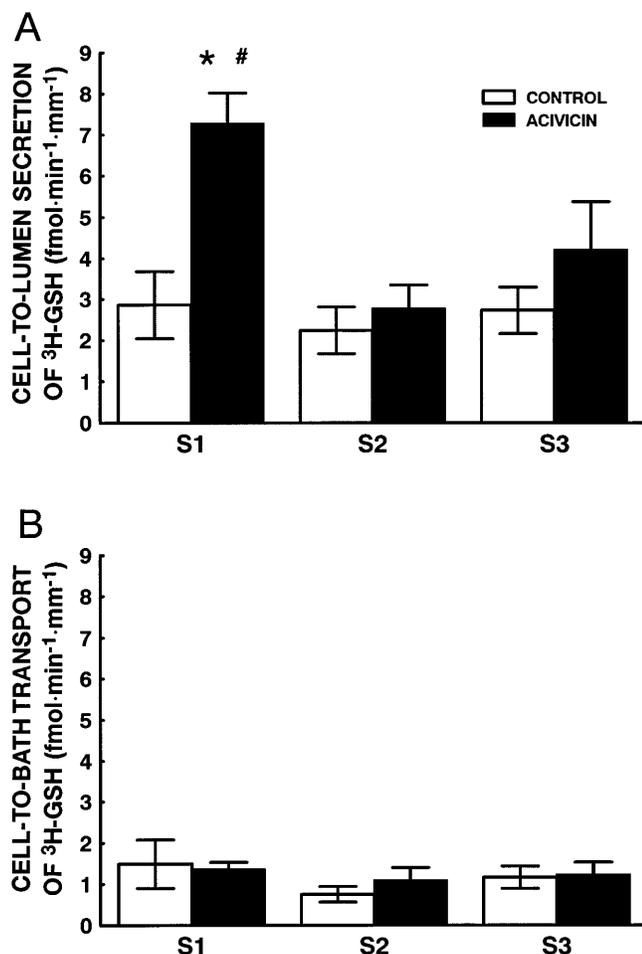


Fig. 2. *A*: cell-to-lumen secretion rates of synthesized [^3H]GSH between the S_1 , S_2 , and S_3 segments of rabbit proximal tubules perfused with and without 1.0 mM acivicin. Perfusate contained 11.2 μM [^3H]glycine. Each bar is mean \pm SE of 5 or more tubules. ANOVA F values are 6.273, 12.856, and 3.789 for each segment, respectively. $P < 0.05$: *significantly different from corresponding mean of the same segment not treated with acivicin; #significantly different from the corresponding means for the S_2 and S_3 segments treated with acivicin. *B*: cell-to-bath transport rates of synthesized [^3H]GSH of isolated perfused S_1 , S_2 , and S_3 segments of rabbit proximal tubules perfused with and without acivicin. Perfusate contained 11.2 μM [^3H]glycine. There was no significant difference among segments or within segments perfused with or without 1 mM acivicin. Each bar is mean \pm SE of 5 or more tubules.

calculated rate of synthesis of [^3H]GSH was significantly lower in the S_1 segment and tended to be lower in the S_2 and S_3 than in tubules perfused with acivicin. This was presumably because of the enzymatic degradation of luminal GSH by γ -GT.

Secretion of [^3H]GSH

Cell-to-lumen secretion rates of cellularly synthesized [^3H]GSH (Fig. 2*A*) were heterogeneous along the proximal tubule. The greatest rate occurred in the S_1 segment, whereas, in the S_2 and S_3 segments, the rates were similar. These greater secretion rates in the S_1 , S_2 , and S_3 segments were manifested only when γ -GT was inhibited. When γ -GT was not inhibited, the apparent rates of [^3H]GSH secretion were not different among

the tubular segments. However, small amounts of [^3H]GSH were still present in the luminal fluid.

Appearance of [^3H]GSH in Bathing Solution

The appearance of synthesized [^3H]GSH in the bathing solution (indicating cell-to-bath transport) did not display any axial heterogeneity along the proximal tubule, all rates being about the same at ~ 1 $\text{fmol}\cdot\text{min}^{-1}\cdot\text{mm}^{-1}$. Co-perfusion with 1.0 mM acivicin did not affect the rates of cell-to-bath transport. These data are shown in Fig. 2*B*.

Cellular Concentration of [^3H]GSH

[^3H]GSH synthesized within the tubular epithelial cells was present in the TCA-soluble tubular extract (presumably the cytoplasm) of the tubule from the S_1 , S_2 , and S_3 segments. These data are shown in Fig. 3. The presence of acivicin in the luminal fluid did not affect these values. There was axial heterogeneity in the cellular [^3H]GSH concentration among the proximal tubule segments. The cellular concentration of [^3H]GSH in the S_1 and S_2 segments was ~ 30 μM , whereas the [^3H]GSH concentration in the S_3 segment was much higher (~ 100 μM).

Mean Luminal Levels of [^3H]GSH

The mean concentration of [^3H]GSH in the luminal fluid showed axial heterogeneity along the proximal tubule in the presence of acivicin. The greatest concentration was in the S_1 segment and progressively decreased to the S_3 segment. The absence of 1.0 mM acivicin in the lumen eliminated this axial heterogeneity but did not eliminate the presence of [^3H]GSH in the lumen (collectate). These data are presented in Fig. 4.

DISCUSSION

The data collected during this study indicate that all segments (S_1 , S_2 , and S_3) of the proximal tubule of the

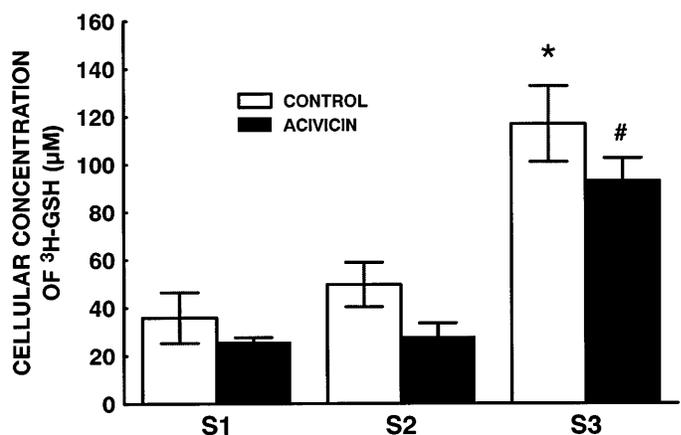


Fig. 3. Cellular concentrations of synthesized [^3H]GSH in the S_1 , S_2 , and S_3 segments of rabbit proximal tubules perfused with and without 1.0 mM acivicin. Perfusate contained 11.2 μM [^3H]glycine. Each bar is mean \pm SE of 5 tubules. ANOVA F values are 34.083, 5.378, and 0.270 for each segment, respectively. $P < 0.05$: *significantly different from corresponding control means for S_1 and S_2 segments; #significantly different from corresponding means of acivicin-treated S_1 and S_2 segments.

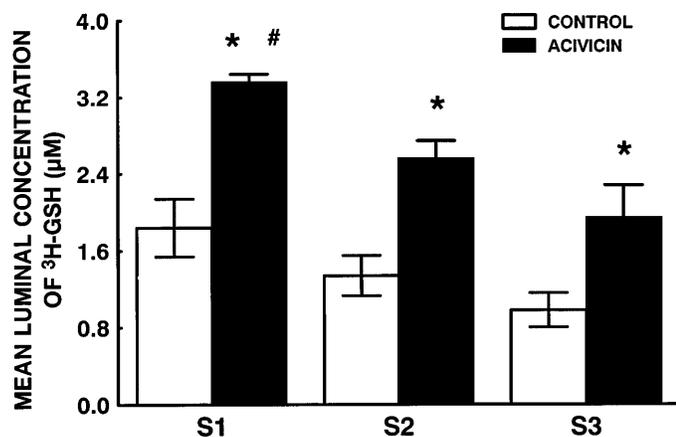


Fig. 4. Mean luminal concentrations of synthesized [³H]GSH in S₁, S₂, and S₃ segments of rabbit proximal tubules perfused with and without 1.0 mM acivicin. Perfusate contained 11.2 µM [³H]glycine. Each bar is mean ± SE of 5 or more tubules. ANOVA *F* values are 13.323, 48.482, and 0.798 for each segment, respectively. *P* < 0.05: *significantly different from corresponding mean of the same segment not treated with acivicin; #significantly different from corresponding means for the S₁ and S₂ segments treated with acivicin.

rabbit nephron synthesize GSH. In addition, a portion of the synthesized GSH is transported bidirectionally out of the tubular epithelial cells, with it being preferentially secreted across luminal membrane into the luminal fluid. Also, GSH synthesis and secretion display axial heterogeneity along the proximal tubule, with the greater rates for both synthesis and secretion occurring in the S₁ segment. The fact that GSH can be transported across the luminal and basolateral membranes of the proximal tubule as an intact tripeptide has been documented (14, 17, 19), but preferential secretion into the lumen has not been clearly established, and nothing has been known about axial heterogeneity of GSH synthesis and secretion.

There is some evidence from previous work that GSH can be secreted into the lumen of the proximal tubule. Scott and Curthoys (31) studied GSH transport in LLC-PK₁ cells grown on filter supports in the presence and absence of acivicin in the cultural medium. The rates of accumulation of GSH from the apical and basolateral membranes pretreated with acivicin were 22 and 34 nmol · mg protein⁻¹ · h⁻¹, respectively (31). In addition, *in vivo* studies showed that the urinary excretion of GSH in rats exceeded the filtered load when γ-GT was inhibited by acivicin (31). These data indicate some level of secretion of GSH by the kidney. To date, these are the only studies that provide evidence for the secretion of GSH at the luminal and basolateral membranes of the proximal tubule.

Axial Heterogeneity of Cell-to-Lumen Secretion

Our data indicate that preferential cell-to-lumen secretion of GSH, and the rate of this secretion decreases in proximal tubular segments as one moves distally from the beginning of the proximal tubule (Fig. 2, A and B). To observe the maximum rates of cell-to-lumen secretion of synthesized [³H]GSH, luminal γ-GT had to be inhibited. Inhibition of luminal γ-GT with

acivicin prevented secreted [³H]GSH from being degraded, which permitted a more accurate measurement of the total amount of [³H]GSH secreted into the luminal fluid. Inhibiting γ-GT significantly increased the measured rate of secretion of [³H]GSH in the S₁ segment only. According to these data, it appears the activity of this enzyme is measurably lower in S₂ and S₃ segments.

Preferential Cell-to-Lumen Transport

In all segments of the proximal tubule, the rate of cell-to-lumen secretion of synthesized [³H]GSH was greater than the corresponding rate of cell-to-bath transport (Fig. 2, A and B). This clearly indicates that GSH is preferentially transported (secreted) into the luminal fluid of the proximal tubule. Because the kidney removes numerous toxicants from body fluids by glomerular filtration and/or secretion, the luminal membrane of the proximal tubule and other segments of the nephron can be exposed to high concentrations of these toxic compounds. In addition, the concentration of these compounds is increased by volume absorption along the proximal tubule. We suggest that secretion of GSH into the luminal fluid helps to provide protection to the luminal membrane of the proximal tubule and other segments of the nephron from the harmful effects of exogenous and endogenous toxins and toxicants. If there are no toxic substances in the luminal fluid to which GSH can bind, then the GSH is rapidly degraded into its constituent amino acids, which are then reabsorbed avidly.

In vitro evidence from renal basolateral membrane vesicles indicates that GSH can potentially be transported intact into the proximal tubular cells via the GSH export pump (16, 19). If this transport mechanism were sequestering some or all of the [³H]GSH transported into the bathing solution, the observed preferential secretion of synthesized [³H]GSH into the lumen would be overestimated. Although this transport is probably occurring, we assume that the rate is very

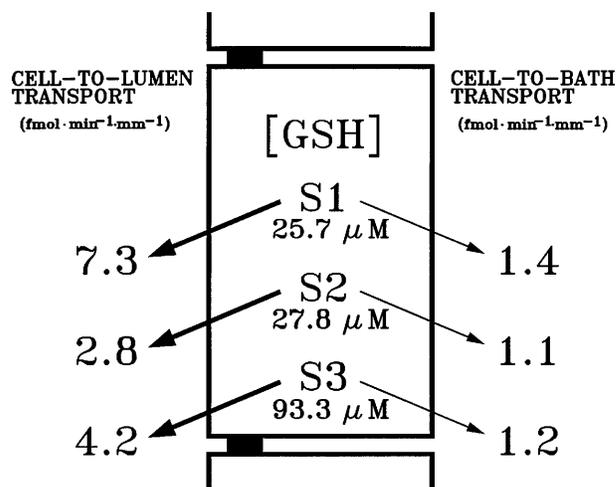


Fig. 5. A composite model of the S₁, S₂, and S₃ segments of the rabbit proximal tubule, illustrating axial heterogeneity of synthesis and preferential secretion of [³H]GSH. [³H]GSH synthesis rate is the sum of the cell-to-bath and cell-to-lumen transport.

minimal in this system. The reasoning for this is as follows. First, the bathing solution was constantly replenished with GSH-free APM solution at the rate of 0.26 ml/min. Second, the amount of secreted [^3H]GSH per minute (femtomolar range) was significantly diluted in the 0.5-ml bathing chamber. As a result of these two conditions, the concentration of [^3H]GSH in the bathing solution was likely very low (<1 pM). Consequently, uptake of [^3H]GSH at the basolateral membrane into the cell was probably minimal. Thus we conclude that the preferential secretion of synthesized GSH into the luminal fluid is not an over estimate.

Axial Heterogeneity of GSH Synthesis

Rates of cellular synthesis of [^3H]GSH displayed the same pattern of axial heterogeneity as did the rates of cell-to-lumen secretion of ^3H -GSH (Figs. 1 and 5). The increased measured rate of GSH synthesis in the S_1 segment in the presence of acivicin is likely related to the inhibition of luminal γ -GT, since the rate of GSH synthesis was calculated as the sum of cell-to-lumen and cell-to-bath transport of [^3H]GSH. The greater rates of synthesis and cell-to-lumen secretion of GSH in the S_1 segment of the proximal tubule may reflect the greater demands required of this segment. The S_1 segment is the first segment of the nephron arising from the glomerulus, thus potentially exposing it to greater amounts of filtered toxins and/or toxicants. Adequate secretion of GSH by the S_1 segment could potentially detoxify the glomerular ultrafiltrate, which could provide protection to the more distal segments of the nephron. This might decrease the necessity of the S_2 and S_3 segments secreting as much GSH. Axial heterogeneity of cell-to-lumen secretion of [^3H]GSH is also manifested by the greater mean luminal concentration of [^3H]GSH in proximal tubular segments that are closest to the glomerulus (Fig. 4, Table 1).

Mechanism for GSH Transport

Brehe et al. (6) suggested that the early straight segment of the proximal tubule (S_2) had the highest cellular concentrations of GSH. However, GSH was measured in regions of the kidney that were identified as containing primarily one type of segment. Without dissecting out individual segments, it is difficult to identify a region as having only one segment type and therefore difficult to extrapolate to individual segments. Our data are the first line of direct evidence for axial heterogeneity of cellular synthesis and preferential secretion of GSH along the proximal tubule of the nephron.

In the present study, the intracellular concentration of [^3H]GSH is, at a minimum, ten times greater than the mean luminal concentration in all segments of the proximal tubule under the conditions they were perfused (with or without acivicin present in the perfusate). Thus cell-to-lumen and cell-to-bath transport processes under the conditions of these studies were dissipative, down a chemical potential gradient.

Minimal Estimates of Measurements

It needs to be emphasized that the rates of synthesis, cell-to-lumen secretion, cell-to-bath transport, and the cellular concentration of [^3H]GSH reported in this study may be underestimates of the true values for these parameters. This is due to the necessity of using the specific activity (disintegrations \cdot s $^{-1}$ \cdot mg $^{-1}$) of [^3H]glycine to calculate the values of these parameters. If the cells of the perfused segments were completely depleted of all nonlabeled glycine and GSH during the warm-up period of perfusion so that the only glycine available for GSH synthesis was [^3H]glycine, then the values we report for these parameters are accurate. However, if there were substantial nonlabeled glycine and/or GSH present in the cytoplasm (lowering the specific activity of the [^3H]glycine and [^3H]GSH), then these values are minimal estimates.

Unfortunately, there are no analytical techniques that can reliably quantitate nonradiolabeled glycine or GSH at the low levels in a single isolated tubule. The important issue is that the relative rates of cell-to-lumen and cell-to-bath GSH transport greatly favors the cell-to-lumen transport into the luminal fluid of the proximal tubule. The actual secretion rates of cellular GSH into the luminal fluid could be much greater than the levels we measured. This indicates the potential importance of luminal GSH as a protectant of the luminal membrane against filtered and/or secreted toxins and toxicants.

In summary, we conclude that GSH can be synthesized in all segments of the proximal tubule of the rabbit. The greatest rate of GSH synthesis and cell-to-lumen transport occurs in the S_1 segment, and both rates decrease progressively in the segments of the proximal tubule furthest from the glomerulus. In addition, our findings indicate that glycine absorbed from the luminal fluid can be utilized for the synthesis of GSH. We also conclude that synthesized GSH in proximal tubular cells is preferentially transported into the luminal fluid, presumably to detoxify the forming urine, thus providing protection to the luminal membrane and cells of the various segments of the nephron. There was no evidence for active cell-to-lumen or cell-to-bath transport of GSH. This summary is illustrated in Fig. 5.

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REFERENCES

1. **Alpert, A. J., and H. F. Gilbert.** Detection of oxidized and reduced glutathione with a recycling postcolumn reaction. *Anal. Biochem.* 144: 553–562, 1985.
2. **Barfuss, D. W., J. M. Mays, and J. A. Schafer.** Peritubular uptake and transepithelial transport of glycine in isolated proximal tubules. *Am. J. Physiol.* 238 (*Renal Fluid Electrolyte Physiol.* 7): F324–F333, 1980.
3. **Barfuss, D. W., W. P. McCann, and R. E. Katholi.** Axial heterogeneity of adenosine transport and metabolism in the rabbit proximal tubule. *Kidney Int.* 41: 1143–1149, 1992.
4. **Barfuss, D. W., and J. A. Schafer.** Active amino acid absorption by proximal convoluted and proximal straight tubules. *Am. J.*

- Physiol.* 236 (*Renal Fluid Electrolyte Physiol.* 5): F149–F162, 1979.
5. **Braam, B., K. D. Mitchell, J. Fox, and L. G. Navar.** Proximal tubular secretion of angiotensin II in rats. *Am. J. Physiol.* 264 (*Renal Fluid Electrolyte Physiol.* 33): F891–F898, 1993.
 6. **Brehe, J. E., A. W. K. Chan, R. Avery, and H. B. Burch.** Effect of methionine sulfoximine on glutathione and amino acid levels in the nephron. *Am. J. Physiol.* 231: 1536–1540, 1976.
 7. **Buckpitt, A. R., D. E. Rollins, S. D. Nelson, R. B. Franklin, and J. R. Mitchell.** Quantitative determination of the glutathione, cysteine, and N-acetyl cysteine conjugates of acetaminophen by high-pressure liquid chromatography. *Anal. Biochem.* 83: 168–177, 1977.
 8. **Campbell, J. A. H., N. M. Bass, and R. E. Kirsch.** Immunohistological localization of ligandin in human tissues. *Cancer* 45: 503–510, 1980.
 9. **Campbell, J. A. H., A. V. Corrigan, A. Guy, and R. E. Kirsch.** Immunohistological localization of alpha, mu, and pi class glutathione S-transferases in human tissues. *Cancer* 67: 1608–1613, 1991.
 10. **Foulkes, E. C.** Tubular reabsorption delay of amino acids in the rabbit kidney. *Am. J. Physiol.* 249 (*Renal Fluid Electrolyte Physiol.* 18): F878–F883, 1985.
 11. **Griffith, O. W.** The role of glutathione turnover in the apparent renal secretion of cystine. *J. Biol. Chem.* 256: 12263–12268, 1981.
 12. **Griffith, O. W.** Mechanism of action, metabolism, and toxicity of buthionine sulfoximine and its higher homologs, potent inhibitors of glutathione synthesis. *J. Biol. Chem.* 257: 13704–13712, 1982.
 13. **Griffith, O. W., and A. Meister.** Selective inhibition of γ -glutamyl cycle enzymes by substrate analogs. *Proc. Natl. Acad. Sci. USA* 74: 3330–3334, 1977.
 14. **Hagen, T. M., and D. P. Jones.** Transport of glutathione in intestine, kidney and lung. *Adv. Biosci.* 65: 107–114, 1987.
 15. **Jones, D. P., E. Maellaro, S. Jiang, A. F. Slater, and S. Orrenius.** Effects of N-acetyl-L-cysteine on T-cell apoptosis are not mediated by increased cellular glutathione. *Immunol. Lett.* 45: 203–209, 1995.
 16. **Jones, D. P., P. Moldeus, A. H. Stead, K. Ormstad, H. Jornvall, and S. Orrenius.** Metabolism of glutathione and a glutathione conjugate by isolated kidney cells. *J. Biol. Chem.* 254: 2787–2792, 1979.
 17. **Lash, L. H., and D. P. Jones.** Transport of glutathione by renal basal-lateral membrane vesicles. *Biochem. Biophys. Res. Commun.* 112: 55–60, 1983.
 18. **Lash, L. H., and D. P. Jones.** Distribution of oxidized and reduced forms of glutathione and cysteine in rat plasma. *Arch. Biochem. Biophys.* 240: 583–592, 1985.
 19. **Lash, L. H., and D. P. Jones.** Uptake of the glutathione conjugate S-(1,2-dichlorovinyl)glutathione by renal basal-lateral membrane vesicles and isolated kidney cells. *Mol. Pharmacol.* 28: 278–282, 1985.
 20. **Ling, L., C. Dewaele, and W. R. G. Baeyens.** Micro liquid chromatography with fluorescence detection of thiols and disulfides. *J. Chromatogr. Sci.* 553: 433–439, 1991.
 21. **Martin, J., and I. N. H. White.** Fluorometric determination of oxidized and reduced glutathione in cells and tissues by high-performance liquid chromatography following derivitization with dansyl chloride. *J. Chromatogr. Sci.* 568: 219–225, 1991.
 22. **Meister, A.** Glutathione: metabolism and function via the γ -glutamyl cycle. *Life Sci.* 15: 177–190, 1974.
 23. **Meister, A., and M. E. Anderson.** Glutathione. *Ann. Rev. Biochem.* 52: 711–751, 1983.
 24. **Reed, D., J. Babson, P. Beatty, A. Brodie, W. Ellis, and D. Potter.** High-performance liquid chromatography analysis of nanomole levels of glutathione, glutathione disulfide, and related thiols and disulfides. *Anal. Biochem.* 106: 55–62, 1980.
 25. **Reitsma, B., and E. Yeung.** Optical activity and ultraviolet absorbance detection of dansyl L-amino acids separated by gradient liquid chromatography. *Anal. Chem.* 59: 1059–1061, 1987.
 26. **Richie, J. P., and C. A. Lang.** The determination of glutathione, cyst(e)ine, and other thiols and disulfides in biological samples using high-performance liquid chromatography with dual electrochemical detection. *Anal. Biochem.* 163: 9–15, 1987.
 27. **Rozell, B., H. A. Hansson, C. Guthenberg, M. Kalim Tahir, and B. Mannervik.** Glutathione transferases of classes alpha, mu, and pi show selective expression in different regions of rat kidney. *Xenobiotica* 23: 835–849, 1993.
 28. **Schnellmann, R. G., S. M. Gilchrist, and L. Mandel.** Intracellular distribution and depletion of glutathione in rabbit renal proximal tubules. *Kidney Int.* 34: 229–233, 1988.
 29. **Schnellmann, R. G., and L. J. Mandel.** Intracellular compartmentation of glutathione in rabbit renal proximal tubules. *Biochem. Biophys. Res. Commun.* 133: 1001–1005, 1985.
 30. **Schoffner, J. M., A. S. Voljavec, J. Dixon, A. Kaufman, D. C. Wallace, and W. E. Mitch.** Renal amino acid transport in adults with oxidative phosphorylation diseases. *Kidney Int.* 47: 1101–1107, 1995.
 31. **Scott, R. D., and N. P. Curthoys.** Renal clearance of glutathione measured in rats pretreated with inhibitors of glutathione metabolism. *Am. J. Physiol.* 252 (*Renal Fluid Electrolyte Physiol.* 21): F877–F882, 1987.
 32. **Silbernagl, S.** Tubular transport of amino acids and small peptides. In: *Handbook of Physiology. Renal Physiology.* Bethesda, MD: Am. Physiol. Soc., 1992, vol. II, sect. 8, chapt. 41, p. 1937–1976.
 33. **Tapuhi, Y., D. Schmidt, W. Lindner, and B. Karger.** Dansylation of amino acids for high-performance liquid chromatography analysis. *Anal. Biochem.* 115: 123–129, 1981.
 34. **Zalups, R. K., M. K. Robinson, and D. W. Barfuss.** Factors affecting inorganic mercury transport and toxicity in the isolated perfused proximal tubule. *J. Am. Soc. Nephrol.* 2: 866–878, 1991.