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Pathways of Glutathione Metabolism and Transport in Isolated Proximal Tubular Cells from Rat Kidney

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ABSTRACT. Cellular uptake and metabolism of exogenous glutathione (GSH) in freshly isolated proximal tubular (PT) cells from rat kidney were examined in the absence and presence of inhibitors of GSH turnover [acivicin, L-buthionine-*S*,*R*-sulfoximine (BSO)] to quantify and assess the role of different pathways in the handling of GSH in this renal cell population. Incubation of PT cells with 2 or 5 mM GSH in the presence of acivicin/BSO produced 3- to 4-fold increases in intracellular GSH within 10–15 min. These significantly higher intracellular concentrations were maintained for up to 60 min. At lower concentrations of extracellular GSH, an initial increase in intracellular GSH concentrations was observed, but this was not maintained for the 60-min time course. In the absence of inhibitors, intracellular concentrations of GSH increased to levels that were 2- to 3-fold higher than initial values in the first 10–15 min, but these dropped below initial levels thereafter. In both the absence and presence of acivicin/BSO, PT cells catalyzed oxidation of GSH to glutathione disulfide (GSSG) and degradation of GSH to glutamate and cyst(e)ine. Exogenous *tert*-butyl hydroperoxide oxidized intracellular GSH to GSSG in a concentration-dependent manner and extracellular GSSG was transported into PT cells, but limited intracellular reduction of GSSG to GSH occurred. Furthermore, incubation of cells with precursor amino acids produced little intracellular synthesis of GSH, suggesting that PT cells have limited biosynthetic capacity for GSH under these conditions. Hence, direct uptake of GSH, rather than reduction of GSSG or resynthesis from precursors, may be the primary mechanism to maintain intracellular thiol redox status under toxicological conditions. Since PT cells are a primary target for toxicants, the ability of these cells to rapidly take up and metabolize GSH may serve as a defensive mechanism to protect against chemical injury. BIOCHEM PHARMACOL 52;2:259–272, 1996.

KEY WORDS. glutathione; kidney; proximal tubule; nephron; metabolism; transport

Intracellular GSH‡ is an essential component of cellular defense against oxidants and various other types of toxic and pathologic stresses. Maintenance of adequate concentrations of GSH and the reduced thiol-disulfide status within cells is necessary for proper function. Cells of the kidney, in particular those of the PT region, are susceptible to agents that alter cellular redox status. This is due to a high basal metabolic rate and a high rate of metabolite transport. The ability of the kidney to utilize thiols, particularly GSH, may play an important role in cellular defense against various toxic chemicals and in the degree of cellular injury after exposure to toxicants [1, 2].

GSH status within renal PT cells is determined by intracellular synthesis, redox reactions, and the use of extracellular GSH [3]. Renal PT cells are exposed to extracellular GSH in two ways: GSH in plasma, derived primarily from efflux from the liver, either undergoes glomerular filtration, enters the lumen, and reaches the brush-border membrane, or it enters the peritubular plasma and reaches the basal-lateral membrane. GSH in the lumen can be degraded then to its component amino acids by GGT (EC 2.3.2.2) and dipeptidases on the brush-border plasma membrane, and the component amino acids can be transported into the renal cell for resynthesis of GSH. GSH in the peritubular plasma can be transported across the basal-lateral membrane into the cells by an Na^+-dependent system [4, 5], or it can be oxidized to GSSG by the copper-containing thiol oxidase (EC 1.8.4.2) and the GSSG may be transported then into the renal cell [5, 6].

In previous studies on the susceptibility of renal cell populations derived from different regions of the nephron to various chemical toxicants, we observed that freshly isolated cells from the DT region of the nephron exhibit sig-

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‡ *Abbreviations:* acivicin, L-(αS,5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid; BSO, L-buthionine-*S*,*R*-sulfoximine; CySH, cysteine; CyS_2, cystine; DT, distal tubular; GCS, γ-glutamylcysteine synthetase; GGT, γ-glutamyltransferase; Glut, L-glutamate; GSH, glutathione; GSSG, glutathione disulfide; Mes, 2-(*N*-morpholino)ethanesulfonic acid; PAH, *p*-aminohippurate; PT, proximal tubular; and tBH, *tert*-butyl hydroperoxide.

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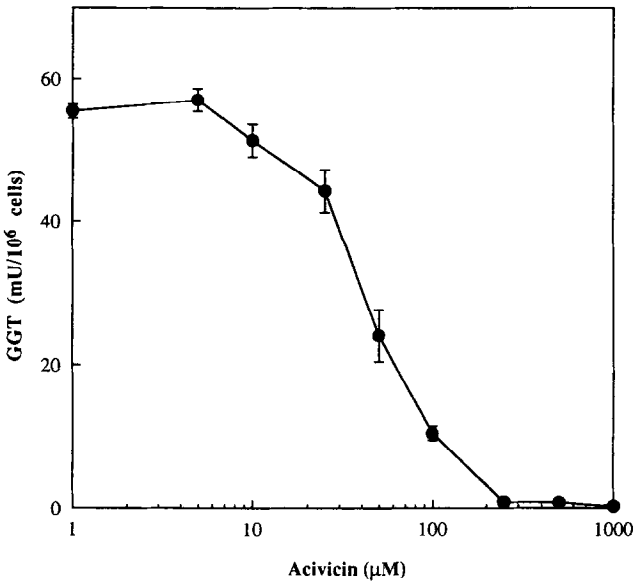

FIG. 1. Inhibition of GGT activity in renal PT cells by acivicin. Isolated PT cells (2–3 x 10^6 cells/mL) were incubated at 37° with either buffer or from 1 μM to 1 mM acivicin for 15 min. GGT activity was measured spectrophotometrically at 37° with glycylglycine and γ-glutamyl-*p*-nitroanilide as substrates by determining the formation of *p*-nitroanilide at 410 nm. Results are means ± SEM of measurements from 4 separate cell preparations. Semi-log plots were constructed because the acivicin concentrations used spanned three orders of magnitude. Control GGT activity ([acivicin] = 0) was 68.9 ± 2.4 mU/10^6 cells. GGT activities at all concentrations of acivicin were significantly ($P < 0.05$) less than the control activity.

nificantly more injury than cells from the PT region to oxidants, such as peroxides and redox-cycling quinones [7], and to alkylating agents that bind GSH and protein sulfhydryl groups, such as methyl vinyl ketone and allyl alcohol [8]. Measurement of the activities of several GSH-dependent and other relevant drug metabolism enzymes showed that PT cells have significantly higher activities than DT cells of GSH peroxidase (EC 1.11.1.9), GSSG reductase (EC 1.6.4.2), catalase (EC 1.11.1.6), and NADPH:quinone oxidoreductase (EC 1.6.99.2) [7], which is consistent with the known heterogeneity of the distribution of GSH and GSH-dependent enzymes along the nephron [9, 10]. This suggested that the relative resistance of PT cells to oxidative stress may be due to their greater ability to utilize GSH for cellular defense mechanisms. Intracellular concentrations of GSH in PT cells are only modestly (i.e. approximately 25%) greater than those in DT cells [7, 9, 11], and the activity of GCS (EC 6.3.2.2) is similar in the two cell populations [12]. Hence, it does not appear to be the amount of intracellular GSH in the two cell populations that is critical for cellular function and susceptibility to chemical injury, but rather, it is differences in how the two cell types metabolize and utilize GSH that appear to be determining factors.

Based on the findings described above, we chose in the present study to modulate intracellular GSH in renal PT cells, by altering metabolism and redox status and by measurement of uptake and handling of extracellular GSH, to assess the roles of these processes in regulation of GSH status in these cells. These processes are involved in the handling of extracellular GSH (transport, degradation, and oxidation), and these were measured at several GSH concentrations. The results demonstrated that renal PT cells can actively accumulate GSH from the extracellular medium to concentrations that are several-fold higher than initial GSH concentrations. High activities of oxidation and degradation were also found, indicating that degradation, uptake of constituent amino acids, and intracellular resynthesis of GSH may also be a mechanism for preservation of intracellular thiol-disulfide redox status, although intracellular resynthesis of GSH was not an efficient means to increase intracellular GSH content. GSSG can be reduced intracellularly after treatment of PT cells with tBH or after uptake of GSSG, but restoration of intracellular GSH status by uptake of exogenous GSH was also a more efficient mechanism to restore intracellular redox status during oxidative stress.

MATERIALS AND METHODS

Materials

Collagenase (type I), Percoll, bovine serum albumin (fraction V), BSO, tBH, PAH, acivicin, L-γ-glutamyl-L-

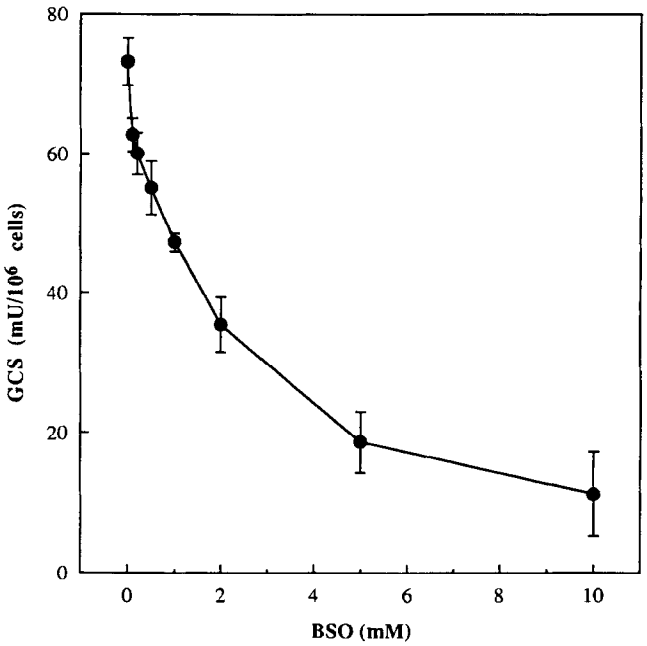

FIG. 2. Inhibition of GCS activity in renal PT cells by BSO. Isolated PT cells (2–3 x 10^6 cells/mL) were incubated at 37° with either buffer or 0.1 to 10 mM BSO for 15 min. GCS activity was measured spectrophotometrically at 37° by coupling formation of L-γ-glutamyl-L-α-aminobutyric acid and ADP to pyruvate kinase and lactate dehydrogenase, followed by determination of NADH oxidation at 340 nm. Results are the means ± SEM of measurements from 4 separate cell preparations. BSO concentrations above 0.2 mM produced significant ($P < 0.05$) inhibition of enzyme activity with respect to control cells.

glutamate, and γ-glutamyl-*p*-nitroanilide were purchased from the Sigma Chemical Co. (St. Louis, MO). Silicone oil (high temperature, n_D = 1.4950 at 20°, *d* = 1.050) and mineral oil (white, light paraffin oil, n_D = 1.4760 at 20°, *d* = 0.862) were purchased from the Aldrich Chemical Co. (Milwaukee, WI). All other chemicals were of the highest purity available and were obtained from commercial sources.

Isolation of Renal PT Cells

Isolated renal cortical cells were obtained by collagenase perfusion [13] from male Fischer 344 rats (200–300 g; Charles River Laboratories, Wilmington, MA). Animals were housed in the Wayne State University vivarium, were allowed access to food and water *ad lib.*, and were kept in a room on a 12-hr light–dark cycle. Prior to surgery, rats were anesthetized with intraperitoneal injections of sodium pentobarbital (50 mg/kg body weight). To obtain an enriched preparation of PT cells, cortical cells were subjected to density gradient centrifugation in Percoll, as previously described [11]. Briefly, cortical cells (5 mL, 5–8 × 10^6 cells/mL) were layered on 35 mL of 45% (v/v) isosmotic Percoll solution in 50-mL polycarbonate centrifuge tubes and were centrifuged at 4° for 30 min at 20,000 *g* in a Sorvall RC2B centrifuge in an SS34 rotor. PT cells were obtained by pooling fractions from the Percoll gradient representing the top quarter (i.e. lowest density) of the gradient. Marker enzyme activities and functional assays were used to confirm identity and purity of the PT cells [11, 14]. Based on these criteria, the PT cell preparation was estimated to have a purity of 97% [11].

Cell concentrations were determined in the presence of 0.2% (w/v) trypan blue on a hemacytometer, and viability was estimated by the number of cells that excluded the dye. Greater than 90% of cells excluded trypan blue at the time of isolation, and the cell preparation maintained greater than 80% viability during the time course of the experiments. All incubations for transport and metabolism studies were performed in 25-mL polyethylene Erlenmeyer flasks at 37° on a Dubnoff metabolic shaking water bath (60 cycles/min).

Processing of Samples for Measurement of GSH and Related Metabolites

Before incubations with GSH, cells were preincubated for 15 min at 37° with either buffer, 0.25 mM acivicin, or 0.25 mM acivicin + 2 mM BSO. Aliquots of preincubated cells were then incubated with the indicated concentration of GSH or GSSG and processed for analysis of either intracellular GSH and GSSG or extracellular GSH and related metabolites as described below.

For measurement of intracellular GSH and GSSG content, aliquots of cell suspensions (0.5 mL) were layered on 1.0 mL of 20% (v/v) Percoll in saline (0.9%, w/v, NaCl) in 1.5-mL plastic microcentrifuge tubes and were centrifuged for 30 sec. Supernatants were removed with a Pasteur pipette, tubes were drained dry, and cell pellets were resus-

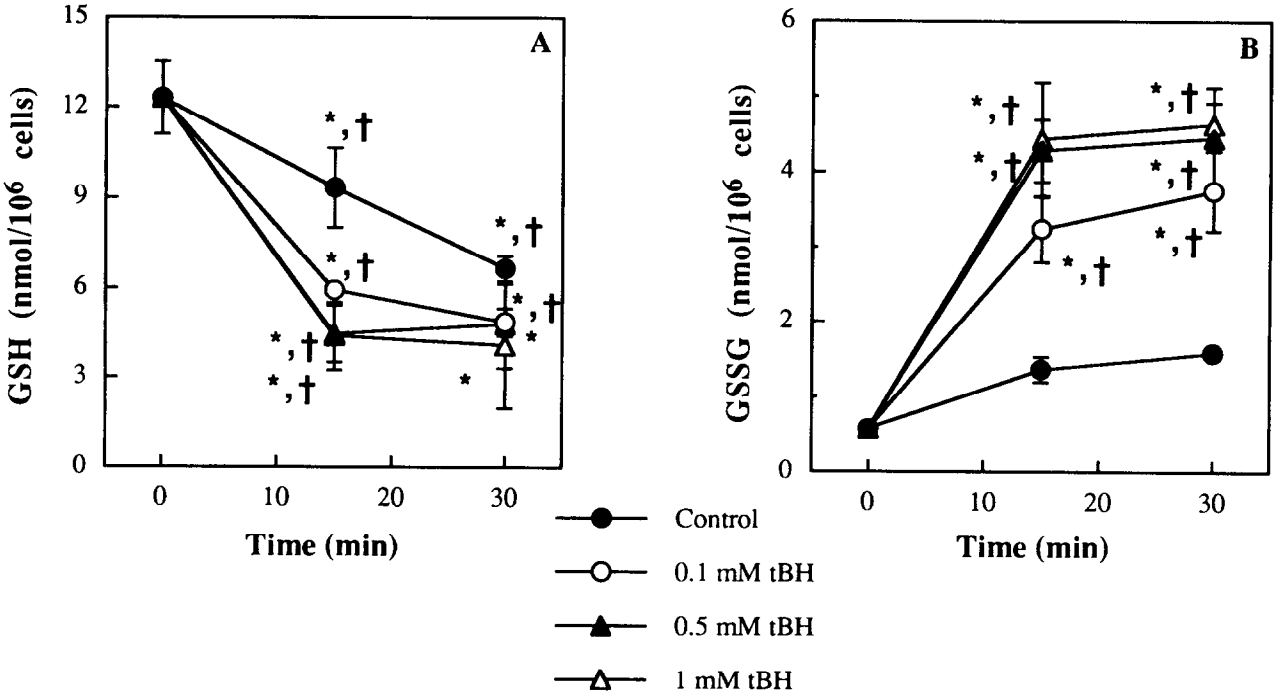

FIG. 3. Oxidation of intracellular GSH by tBH. Isolated PT cells (2–3 × 10^6 cells/mL) were preincubated for 15 min with 5 mM GSH and 0.25 mM acivicin at 37° and were then incubated with 0, 0.1, 0.5, or 1 mM tBH. At the indicated times, intracellular contents of GSH (A) and GSSG (B) were measured by HPLC. Results are the means ± SEM of measurements from 3 separate cell preparations. Key: (*) significantly different ($P < 0.05$) from the corresponding time 0 sample; and (†) significantly different ($P < 0.05$) from control samples at the same time point.

pended in 0.5 mL of saline. Perchloric acid (70%, v/v, 0.1 mL), 0.05 mL of 1.5 mM L-γ-glutamyl-L-glutamate (internal standard), and 0.05 mL of 15 mM bathophenanthroline disulfonate (BPDS) (to prevent auto-oxidation of GSH) were added, and tubes were centrifuged at 10,000 *g* for 2 min. Acid-soluble supernatants were derivatized for analysis by HPLC (see below).

For measurement of extracellular GSH and related metabolites, aliquots of cell suspensions (0.5 mL) were mixed with the top layer in tubes prepared in the following manner. Microcentrifuge tubes (1.5 mL capacity) were layered from the bottom with 0.25 mL of 40% (v/v) glycerol, 0.5 mL of silicone oil:paraffin oil (6:1), and 0.1 mL of Mes buffer (19.8 mM Mes, pH 7.4, containing 19.8 mM EGTA, 19.8 mM EDTA, and 0.25 M mannitol). Tubes were centrifuged at 10,000 *g* for 30 sec, 0.5-mL aliquots from the top layer were removed, and aliquots were treated with perchloric acid, internal standard, and BPDS as described above. Acid-soluble supernatants were derivatized for analysis by HPLC (see below).

HPLC Analysis of GSH and Related Metabolites

Contents of GSH and CySH in acid-soluble extracts were measured as *S*-carboxymethyl-*N*-dinitrophenyl derivatives, those of Glut were measured as *N*-dinitrophenyl derivatives, and those of GSSG and CyS_2 were measured as *N,N*-bis-dinitrophenyl derivatives by HPLC according to the method of Fariss and Reed [15]. Separations were achieved with a reversed-phase, μBondapak amine 10-mm cartridge (8 mm × 10 cm) (Waters Associates, Milford, MA) with a Waters model 600E multisolvent delivery system using an ion-exchange method with a methanol-acetate mobile phase and gradient elution. Derivatives were detected at 365 nm and were quantified with respect to standards using a Waters model 745B data module. To control for variations in sample volumes, peak areas were normalized to the internal standard.

Enzyme Assays

GGT activity was measured at 410 nm as *p*-nitroanilide formation with γ-glutamyl-*p*-nitroanilide and glycylglycine as substrates [16]. GCS activity was measured with L-α-aminobutyrate as an analogue of L-cysteine. The assay was performed by coupling formation of L-γ-glutamyl-L-α-aminobutyrate and ADP to pyruvate kinase (EC 2.7.1.40), lactate dehydrogenase (EC 1.1.1.27), and ultimately, NADH oxidation and the consequent decrease in absorbance at 340 nm [17]. Rates of NADH oxidation in the absence of added substrates (i.e. ATP, phosphoenolpyruvate, Glut, L-α-aminobutyrate), which was usually less than

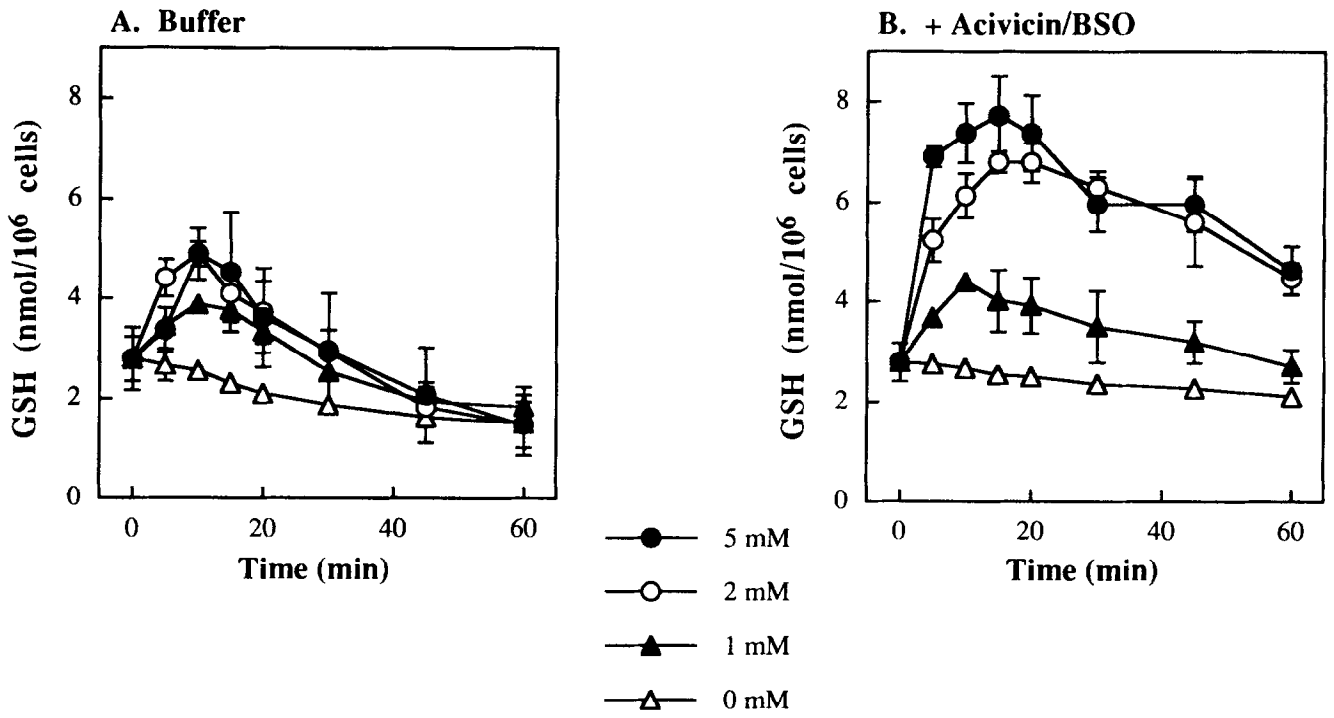

FIG. 4. Time and concentration dependence of GSH uptake in PT cells in the absence (A) and presence (B) of acivicin/BSO. Isolated PT cells (5–10 × 10^6 cells/mL) were incubated at 37° with either Krebs-Henseleit buffer or 0.25 mM acivicin and 2 mM BSO for 15 min. Cells were then incubated for up to 60 min with 0, 1, 2, or 5 mM GSH. At the indicated times, intracellular GSH content was measured by HPLC. Results are the means ± SEM of measurements from 3 separate cell preparations. In the absence of inhibitors, the following time points were significantly different ($P < 0.05$) from corresponding values at time 0: *0 mM:* 30, 45, and 60 min; *1 mM:* all except 30 min; *2 mM:* 5, 10, 15, and 60 min; *5 mM:* 10, 15, and 60 min. In the presence of acivicin/BSO, the following time points were significantly different ($P < 0.05$) from the corresponding values at time 0: *0 mM:* 45 and 60 min; *1 mM:* 5, 10, 15, and 30 min; *2 mM:* all; *5 mM:* all.

10% of rates in the presence of added substrates, were subtracted from all measurements to give values that were due specifically to formation of L-γ-glutamyl-L-α-aminobutyrate.

Data Analysis

All values are means ± SEM, unless otherwise indicated, of measurements from the indicated number of separate cell preparations. Significant differences between means were first assessed by a one-way analysis of variance. When significant "F-values" were obtained with the analysis of variance, the Fisher's protected least significant difference *t*-test was performed to determine which means were significantly different from one another with two-tail probabilities of less than 0.05 considered significant. Significant differences between time points or treatment groups are indicated on figures unless placement of symbols makes the figures unclear; in those cases, statistical results are given in figure legends.

RESULTS

Modulation of Intracellular GSH Status by Metabolic Inhibitors and Oxidants

To confirm and compare the ability of acivicin to inhibit GGT activity, PT cells were incubated for 15 min with between 1 μM and 1 mM acivicin before measurement of GGT activity (Fig. 1). A concentration-dependent sensitivity to inhibition of GGT activity by acivicin was observed, and a half-maximal inhibition of activity was obtained with between 10 and 30 μM acivicin. Above 100 μM acivicin, nearly complete (i.e. >95%) inhibition of GGT activity occurred. Based on these results and on previous studies [5, 18] that showed that acivicin is a nonspecific alkylating agent and may be cytotoxic at higher concentrations (generally ≥1 mM), we used 0.25 mM acivicin in all subsequent studies where inhibition of GGT was required.

To confirm the ability of BSO, a specific inhibitor of GCS [19], to inhibit GSH synthesis successfully, PT cells were incubated for 15 min at 37° with between 0.1 and 10 mM BSO before measurement of GCS activity (Fig. 2). A concentration-dependent inhibition of GCS activity was observed with a maximum of 85% inhibition with 10 mM BSO. A concentration of 2 mM, which inhibited GSH synthesis by approximately 50%, was chosen for subsequent experiments, however, due to potential cytotoxicity with use of higher BSO concentrations.

Intracellular GSH redox status was directly modulated by titration of cells with tBH (Fig. 3). Isolated PT cells were first preincubated with 0.25 mM acivicin and 5 mM GSH to load them with GSH, and then these cells were incu-

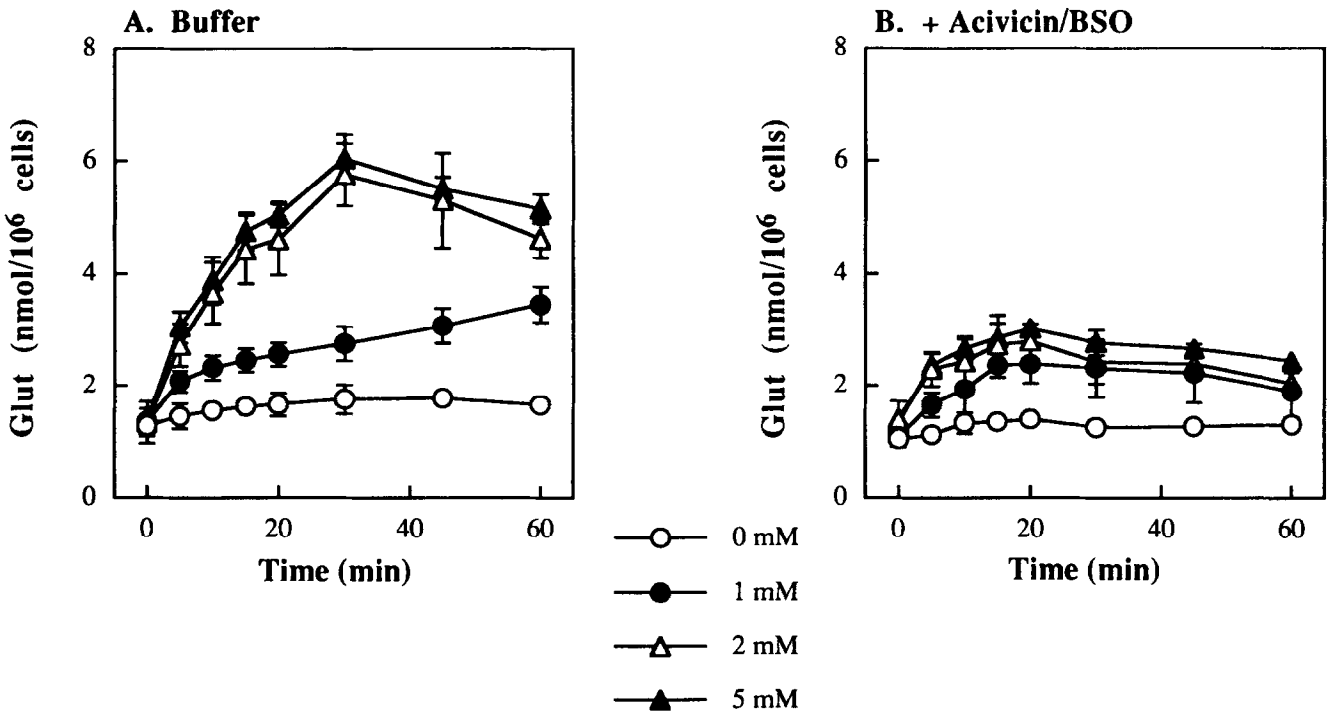

FIG. 5. Formation of intracellular Glut after incubation of PT cells with GSH. Isolated PT cells (2–3 × 10^6 cells/mL) were preincubated with either buffer (A) or 0.25 mM acivicin + 2 mM BSO (B) for 15 min at 37° and then were incubated with either 0, 1, 2, or 5 mM GSH. At the indicated times, the intracellular content of Glut was measured by HPLC. Results are the means ± SEM of measurements from 3 separate cell preparations. In the absence of inhibitors, the following time points were significantly different ($P < 0.05$) from corresponding values at time 0: *0 mM:* 30 and 45 min; *1 mM:* all; *2 mM:* all; *5 mM:* all. In the presence of acivicin/BSO, the following time points were significantly different ($P < 0.05$) from the corresponding values at time 0: *0 mM:* none; *1 mM:* all; *2 mM:* all except 60 min; *5 mM:* all.

bated with 0, 0.1, 0.5, or 1 mM tBH for up to 30 min. These concentrations of tBH were chosen based on a previous cytotoxicity study in PT cells [7] that showed progressive cellular injury over this concentration range, with 0.1 mM producing minimal detectable cytotoxicity and 1 mM producing nearly maximal cytotoxicity. The intracellular content of GSH increasingly declined over time as the concentration of tBH to which the PT cells were exposed increased (Fig. 3A). The maximal rate of loss of GSH (approximately 8 nmol/15 min per 10^6 cells) appeared to occur with approximately 1 mM tBH, although 0.1 mM tBH produced nearly 70% of the maximal response. A similar, although inverted, pattern in the time- and tBH concentration-dependent formation of GSSG was observed (Fig. 3B), with the GSH loss being nearly completely accounted for by GSSG formation in all incubations.

Alterations in Intracellular GSH and Metabolites by Incubation of PT Cells with GSH

To study the GSH transport capacity of PT cells, intracellular accumulation of GSH was measured during incubations with extracellular GSH (0, 1, 2, and 5 mM), both in the absence (Fig. 4A) and the presence (Fig. 4B) of 0.25 mM acivicin + 2 mM BSO. In the absence of inhibitors, PT cells exhibited up to a 75% increase in intracellular content of GSH within 10 min (Fig. 4A). These increased concentrations, however, were not maintained for the entire time course, but declined continuously, after reaching maximal levels at 10 min, to levels that were moderately lower than initial GSH concentrations. PT cells incubated with buffer in the absence of added GSH exhibited modest decreases in intracellular GSH content to 67% of initial levels after 30-min and to 54% of initial levels after 60-min incubations.

In the presence of acivicin and BSO, PT cells exhibited much larger increases in intracellular GSH content (Fig. 4B). After incubation with 1, 2, or 5 mM extracellular GSH, intracellular GSH content increased within 10–15 min to maximal levels that were 1.6-, 2.4-, and 2.8-fold higher, respectively, than initial levels. Intracellular GSH content remained at these significantly higher levels throughout the 60-min incubation with either 2 or 5 mM extracellular GSH, whereas cells incubated with 1 mM GSH did not maintain higher intracellular GSH content throughout the time course. PT cells incubated with buffer in the absence of added GSH exhibited modest decreases in intracellular GSH content (84% of initial levels at 30 min; 76% of initial levels at 60 min), which were less than the decreases that occurred in PT cells without acivicin/BSO. These results demonstrate that renal PT cells, when exposed to concentrations of GSH greater than 1 mM, can

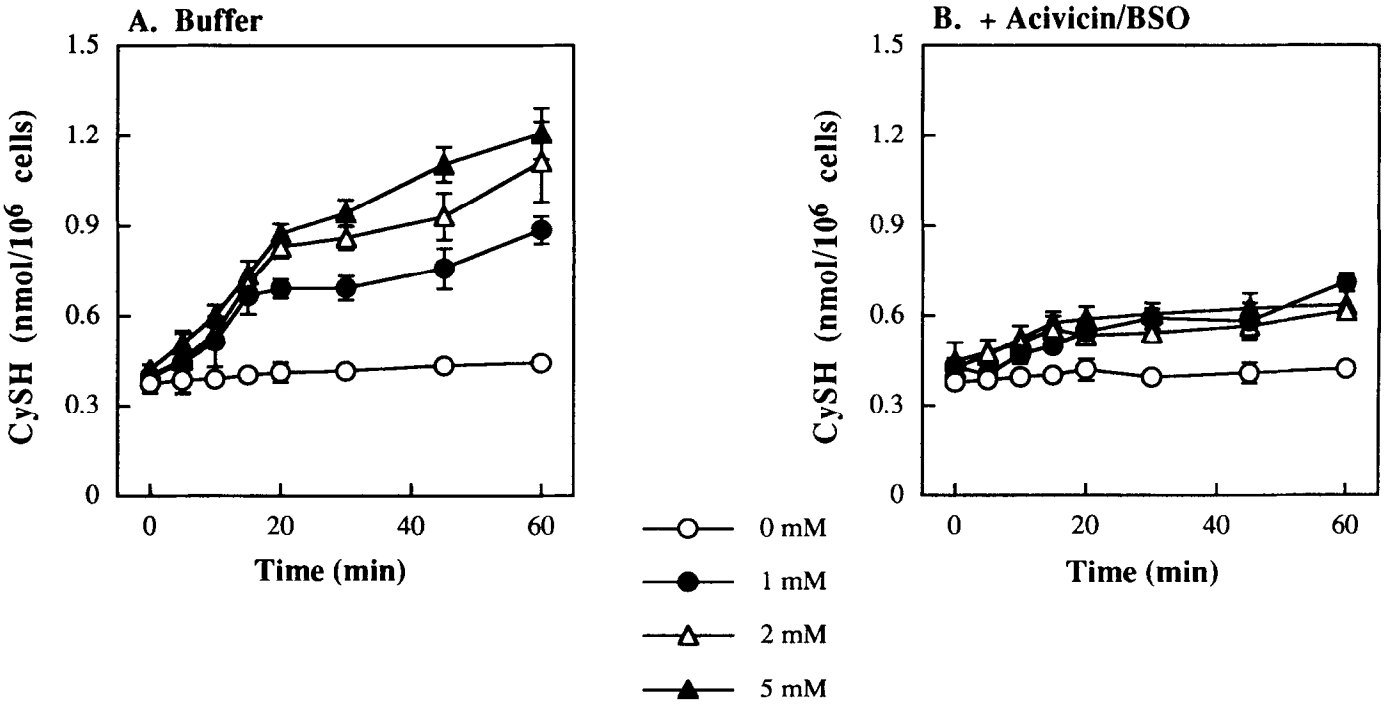

FIG. 6. Formation of intracellular CySH after incubation of PT cells with GSH. Isolated PT cells (2–3 × 10^6 cells/mL) were preincubated with either buffer (A) or 0.25 mM acivicin + 2 mM BSO (B) for 15 min at 37° and then were incubated with either 0, 1, 2, or 5 mM GSH. At the indicated times, the intracellular content of CySH was measured by HPLC. Results are the means ± SEM of measurements from 3 separate cell preparations. In the absence of inhibitors, the following time points were significantly different ($P < 0.05$) from corresponding values at time 0: *0 mM:* 45 and 60 min; *1 mM:* all except 5 and 10 min; *2 mM:* all except 5 min; *5 mM:* all. In the presence of acivicin/BSO, the following time points were significantly different ($P < 0.05$) from the corresponding values at time 0: *0 mM:* 60 min; *1 mM:* all except 5 and 10 min; *2 mM:* 45 and 60 min; *5 mM:* all except 5 and 10 min.

transport GSH into the cell and, in the presence of inhibitors of GSH turnover, retain significantly elevated intracellular GSH content.

Intracellular contents of Glut (Fig. 5), CySH (Fig. 6), and CyS_2 (Fig. 7) in PT cells preincubated with either buffer or acivicin/BSO and then incubated with 0, 1, 2, or 5 mM GSH were also followed over time. Intracellular contents of all three compounds were markedly higher in PT cells preincubated without inhibitors of GSH turnover, showing that most of the increase in intracellular amino acids is due to the catalytic action of GGT. In the absence of acivicin/BSO pretreatment, intracellular accumulation of GSH breakdown products exceeded that of GSH by at least 2-fold, indicating the predominance of degradation and uptake of amino acids in the handling of extracellular GSH under these conditions. In contrast, in the presence of acivicin/BSO pretreatment, intracellular accumulation of GSH exceeded that of the degradation products by 2- to 5-fold (depending on initial concentration of extracellular GSH), indicating the predominance of uptake of intact tripeptide in the handling of extracellular GSH under these conditions.

To demonstrate further the role of carrier-mediated transport across plasma membranes of renal PT cells in modulation of intracellular GSH status, the effect of 10 mM PAH on intracellular accumulation of 5 mM GSH in PT cells preincubated with either buffer or acivicin/BSO was studied (Fig. 8). PAH is a well-characterized inhibitor of GSH uptake across the renal PT cell basal-lateral membrane [5]. Both without (Fig. 8A) and with (Fig. 8B) inhibitors of GSH turnover, PAH substantially inhibited intracellular uptake and accumulation of GSH (89 and 74% inhibition of initial uptake rate, respectively), supporting the conclusion that carrier-mediated transport was occurring.

Intracellular Reduction and Biosynthetic Capacity for GSH

Uptake and intracellular accumulation of GSSG in the absence and presence of acivicin (Fig. 9, A and B) were measured to assess the capability of PT cells to transport GSSG. As shown previously [5], GSSG can be transported across basal-lateral membranes into renal PT cells. As with GSH uptake and accumulation, those for GSSG were approximately 2-fold higher if GGT activity was inhibited by acivicin prior to incubation than if GGT activity was not inhibited.

During measurements of GSSG uptake, we also followed changes in intracellular content of GSH, in the absence and presence of acivicin (Fig. 9, C and D), to assess the intracellular reducing capacity of PT cells. Higher amounts of intracellular GSH were recovered in cells pretreated with acivicin, as would be expected since degradation is a com-

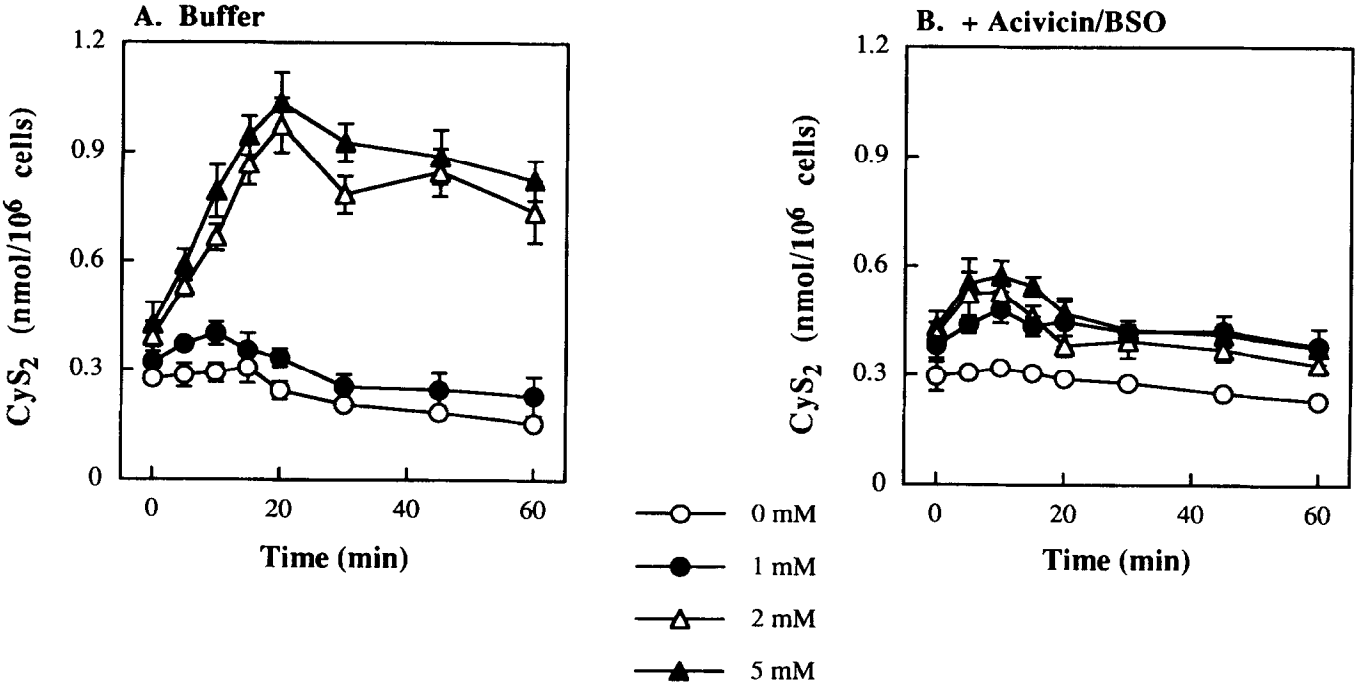

FIG. 7. Formation of intracellular CyS_2 after incubation of PT cells with GSH. Isolated PT cells (2–3 × 10^6 cells/mL) were preincubated with either buffer (A) or 0.25 mM acivicin + 2 mM BSO (B) for 15 min at 37° and then were incubated with either 0, 1, 2, or 5 mM GSH. At the indicated times, the intracellular content of CyS_2 was measured by HPLC. Results are the means ± SEM of measurements from 3 separate cell preparations. In the absence of inhibitors, the following time points were significantly different ($P < 0.05$) from corresponding values at time 0: *0 mM:* 30, 45, and 60 min; *1 mM:* 5, 10, and 60 min; *2 mM:* all; *5 mM:* all. In the presence of acivicin/BSO, the following time points were significantly different ($P < 0.05$) from the corresponding values at time 0: *0 mM:* none; *1 mM:* 10 min; *2 mM:* 10 and 60 min; *5 mM:* 5, 10, and 15 min.

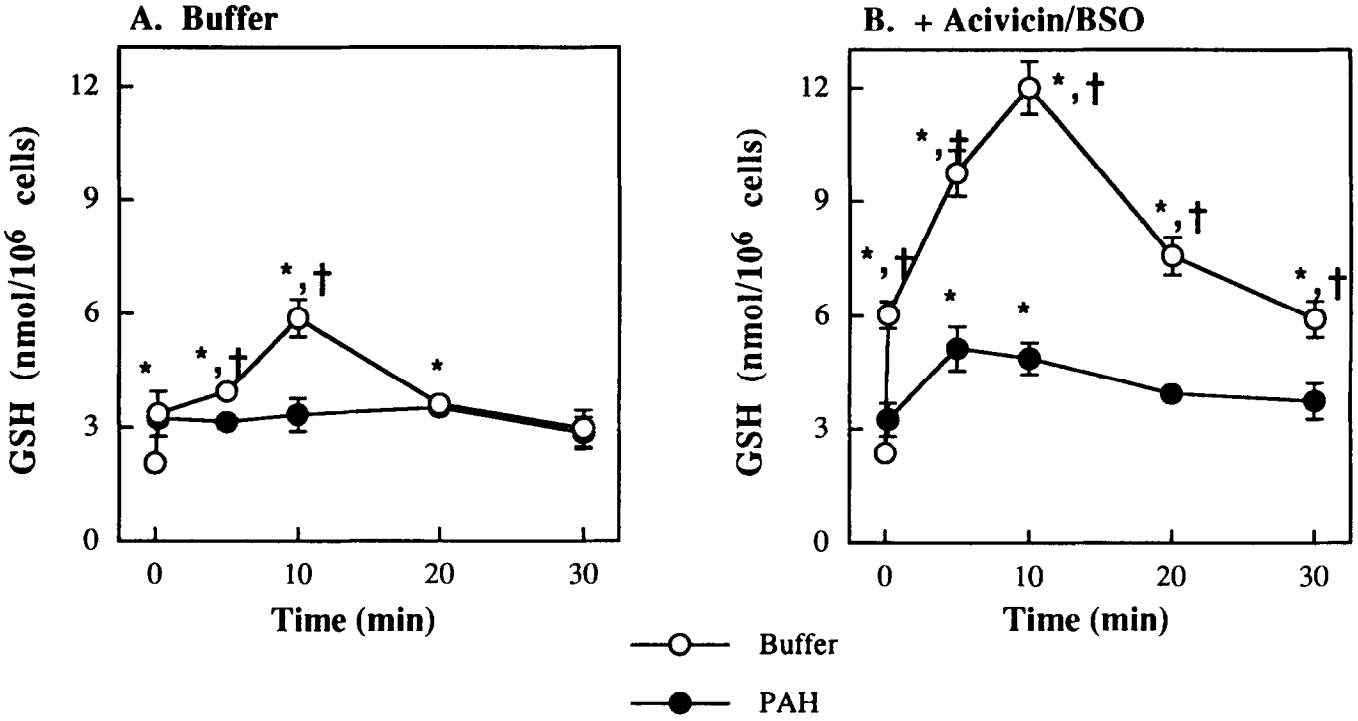

FIG. 8. Effect of PAH on GSH uptake in PT cells. Isolated PT cells (2–3 x 10^6 cells/mL) were preincubated with either buffer (A) or 0.25 mM acivicin + 2 mM BSO (B) for 15 min at 37° and then were incubated with 5 mM GSH in the absence or presence of 10 mM PAH. At the indicated times, the intracellular GSH content was measured by HPLC. Results are the means ± SEM of measurements from 3 separate cell preparations. Key: (*) significantly different ($P < 0.05$) from the corresponding time 0 sample; and (†) significantly different ($P < 0.05$) from PAH-treated samples at the same time point.

peting process. Using 2.5 mM GSSG uptake data to compare reduction capability with transport capability for 5 mM GSH (i.e. comparing data with the same concentration of GSH-equivalents; cf. Fig. 4), we found that in the absence of inhibitors of GSH turnover, the net rate of 5 mM GSH uptake was 0.210 ± 0.019 nmol/min per 10^6 cells and that of intracellular reduction of GSSG to GSH was 0.024 ± 0.003 nmol/min per 10^6 cells. In the presence of acivicin, GSH uptake rate was 0.459 ± 0.038 nmol/min per 10^6 cells while that of intracellular reduction of GSSG was 0.070 ± 0.006 nmol/min per 10^6 cells. Hence, intracellular reduction of GSSG to GSH is a much slower mechanism of increasing intracellular GSH than is incubation of cells with exogenous GSH.

Another potential way to increase intracellular GSH content, besides incubation of cells with GSH or GSSG, is to incubate PT cells with precursor amino acids (Glut, CySH, and glycine) and measure amino acid uptake and GSH synthesis (Fig. 10). PT cells were preincubated with either buffer or acivicin, and the two groups were then incubated with either buffer or a 5 mM concentration of each of the three precursor amino acids. Intracellular accumulation of Glut was significantly greater in the absence of acivicin whereas that of CySH was not altered by inhibition of GGT. In contrast, accumulation of CyS_2 was higher in acivicin-treated cells. No significant changes in intracellular GSH content under any conditions were observed, indicating no detectable net synthesis of GSH (data not shown).

Alterations in Extracellular GSH and Metabolites by Incubation of PT Cells with GSH

Since several key steps in renal GSH metabolism occur at the plasma membrane surface in extracellular medium, assessments of changes in medium composition for cells incubated with GSH can provide information about cellular handling of GSH. Besides transport into the cell, extracellular GSH may undergo two additional fates: degradation to its constituent amino acids and oxidation to GSSG. Time courses of GSH loss and formation of GSSG, CySH, CyS_2, and Glut in medium from PT cells incubated with various concentrations of extracellular GSH ± acivicin/BSO were determined (Fig. 11).

With 5 mM GSH, the extracellular GSH concentration decreased much more rapidly in the absence than in the presence of inhibitors (Fig. 11A). Extracellular content of GSH in the PT incubation solutions decreased by 53 and 30%, without and with inhibitors, respectively, during 60-min incubations. These decreases were accompanied by the formation of GSSG (Fig. 11B), CySH (Fig. 11C), CyS_2 (Fig. 11D), and Glut (Fig. 11E). In the absence of acivicin/BSO, GSSG, CySH, CyS_2, and Glut concentrations in the extracellular medium increased much more rapidly than in

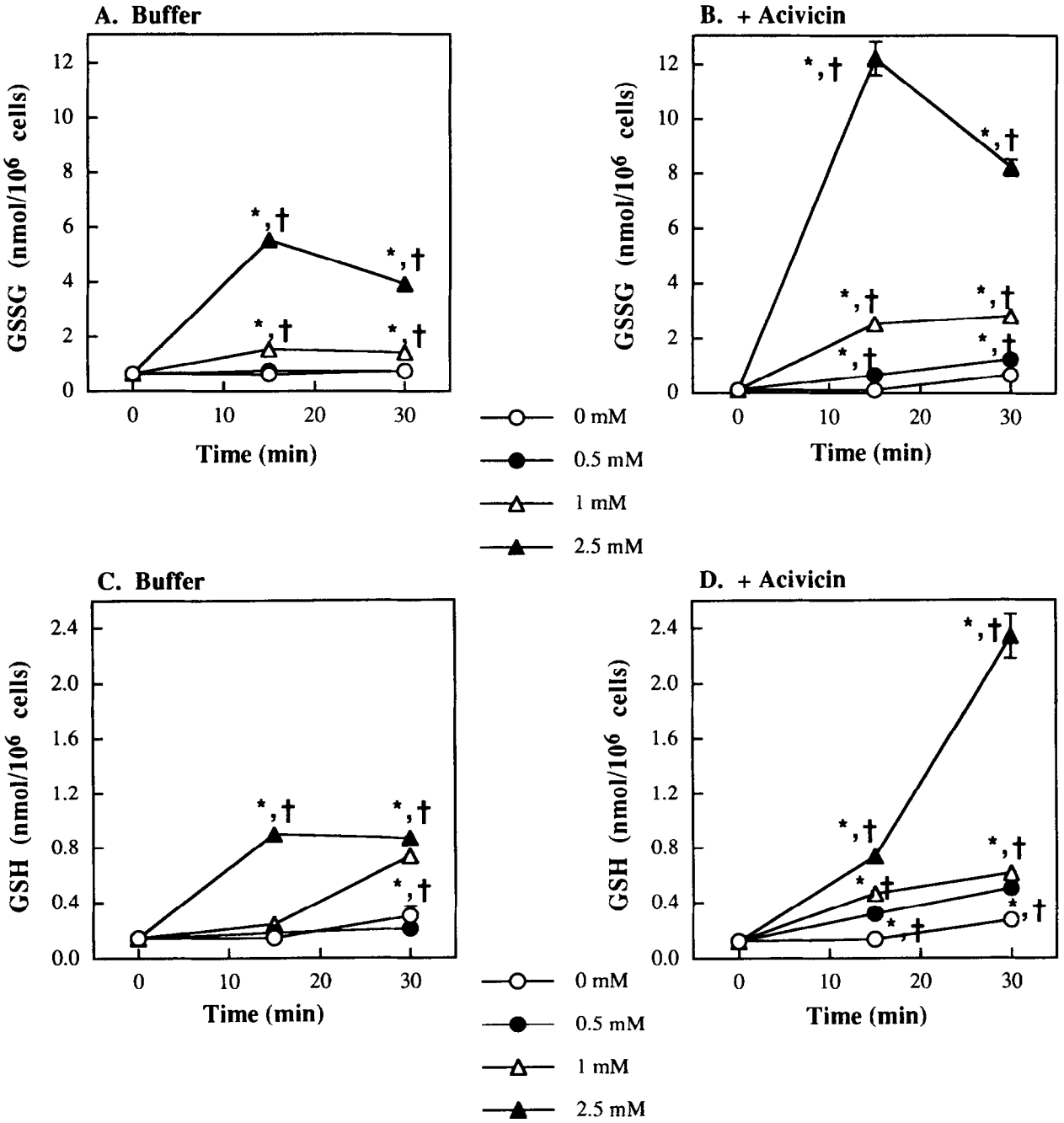

FIG. 9. Uptake of GSSG and intracellular reduction to GSH in renal PT cells. Isolated PT cells (2–3 × 10^6 cells/mL) were preincubated with either buffer (A and C) or 0.25 mM acivicin + 2 mM BSO (B and D) for 15 min at 37° and then were incubated with the indicated concentrations of GSSG. At the indicated times, intracellular contents of GSSG (A and B) and GSH (C and D) were measured by HPLC. Results are the means ± SEM of measurements from 3 separate cell preparations. Key: (*) significantly different ($P < 0.05$) from the corresponding time 0 sample; and (†) significantly different ($P < 0.05$) from control samples (i.e. 0 mM) at the same time point.

cells incubated in the presence of these inhibitors. Extracellular CyS_2 was only detected in medium from cells incubated without acivicin/BSO. The time-dependent pattern of change in CyS_2 concentrations was similar to that of CySH, with concentrations increasing throughout the 60-min time course.

The patterns of time-dependent change in the extracellular concentrations of GSH and its oxidation or degradation products were very similar for the first 20–30 min of the incubations of PT cells with 2, 1, and 0.5 mM GSH (data not shown) as compared with those described above for incubations with 5 mM GSH. Increasing fractions of initial GSH were lost from the medium in both the absence and presence of acivicin/BSO, as the initial concentration

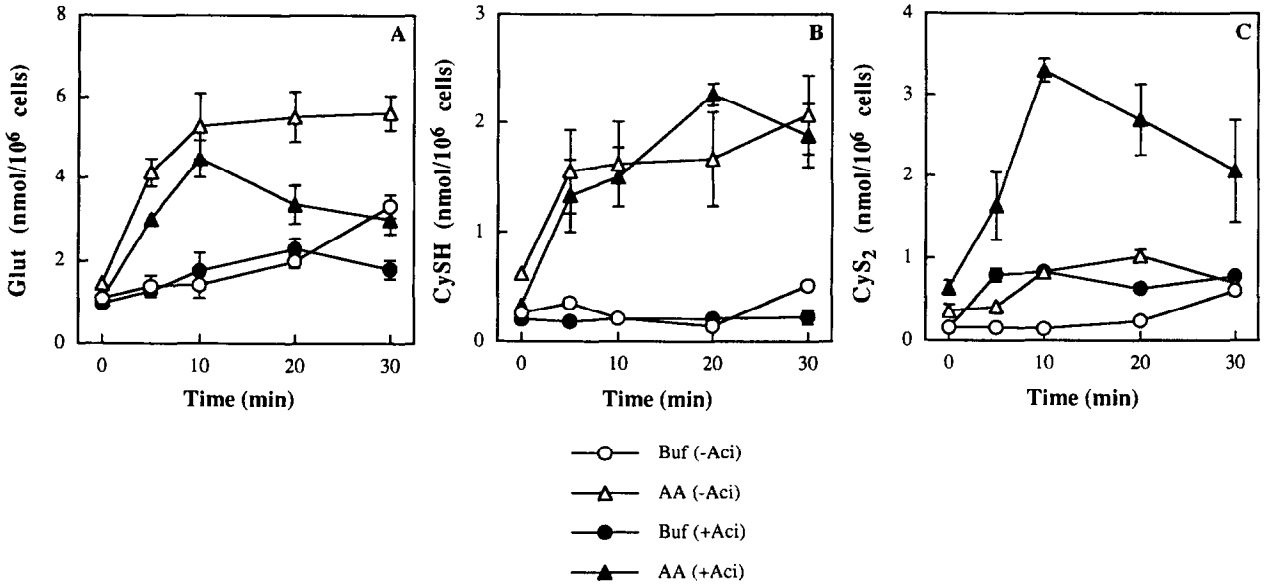

FIG. 10. **Uptake and intracellular accumulation of precursor amino acids for GSH in renal PT cells. Isolated PT cells (2–3 × 10^6 cells/mL) were preincubated with either buffer (open symbols) or 0.25 mM acivicin (filled symbols) for 15 min at 37° and then were incubated with 5 mM each of Glut, CySH, and glycine. At the indicated times, intracellular contents of Glut (A), CySH (B), and CyS_2 (C) were measured by HPLC. Results are the means ± SEM of measurements from 3 separate cell preparations. Statistically significant ($P < 0.05$) differences: (A) 1. vs corresponding sample at time 0 (*Buffer-Aci:* 20 and 30 min; *Buffer + Aci:* 10, 20, and 30 min; *Amino Acids - Aci:* all; *Amino Acids + Aci:* all); 2. vs corresponding samples - Aci at same time point (*Buffer:* 30 min; *Amino Acids:* 5, 20, and 30 min); 3. vs corresponding samples incubated with buffer at the same time point (*-Aci:* all; *+Aci:* all except 0 min). (B) 1. vs corresponding sample at time 0 (*Buffer - Aci:* 20 and 30 min; *Buffer + Aci:* none; *Amino Acids - Aci:* all; *Amino Acids + Aci:* all); 2. vs corresponding samples - Aci at the same time point (*Buffer:* 5 and 30 min; *Amino Acids:* 0 min); 3. vs corresponding samples incubated with buffer at the same time point (*-Aci:* all; *+ Aci:* all). (C) 1. vs corresponding sample at time 0 (*Buffer - Aci:* 20 and 30 min; *Buffer + Aci:* all, *Amino Acids - Aci:* all except 0 min; *Amino Acids + Aci:* all); 2. vs corresponding samples - Aci at the same time point (*Buffer:* all *Amino Acids:* all); 3. vs corresponding samples incubated with buffer at the same time point (*- Aci:* all except 30 min; *+ Aci:* all).**

of GSH with which cells were incubated was lowered. Hence, in the absence of inhibitors, the fraction of initial extracellular GSH lost during 30 and 60 min of incubation, respectively, was 36 and 53%, 63 and 77%, 55 and 80%, or 87 and 93% in the PT extracellular medium having initial GSH concentrations of 5, 2, 1, or 0.5 mM, respectively. In the presence of acivicin/BSO, the fraction of extracellular GSH lost was significantly lower in all corresponding cases, but it increased similarly with decreasing concentrations of initial extracellular GSH, and was 13 and 27%, 22 and 36%, 34 and 49%, or 32 and 49% in PT cells incubated with 5, 2, 1, or 0.5 mM GSH, respectively.

Differences in formation of GSSG over time between cells incubated in the absence or presence of inhibitors diminished with decreasing initial concentrations of GSH. The general pattern of change in CySH formation over time at 2 mM extracellular GSH was similar to that of the change observed at 5 mM extracellular GSH. At 1 and 0.5 mM extracellular GSH, however, minimal differences between cells in the absence or presence of inhibitors were observed. Formation of CyS_2 in incubations of cells with 2 and 1 mM GSH was only detected in the absence of inhibitors, and the patterns were similar to those at 5 mM extracellular GSH.

DISCUSSION

Renal cellular handling of GSH involves primarily three processes: membrane transport, oxidation, and degradation. Since the enzymes responsible for GSH degradation are on the brush-border plasma membrane and have their active sites facing the extracellular fluid, a secondary component of renal GSH handling is uptake of the constituent amino acids followed by intracellular resynthesis of GSH. Enzymes that catalyze GSH oxidation are present on the basal-lateral plasma membrane [6] and within the cell, so that another secondary component of renal GSH handling is uptake of GSSG and intracellular reduction back to GSH. Maintenance of sufficient content of GSH and proper GSH/GSSG redox state are critical to normal cellular function.

In toxicological or pathological situations, knowledge of the mechanisms by which a cell handles GSH can be exploited to protect the tissue from injury. For example, protection of renal PT cells from tBH-induced oxidative injury by exogenous GSH is associated with the ability of these cells to transport intact GSH across the basal-lateral plasma membrane [4, 5, 20], thereby increasing intracellular GSH content. Other studies have also demonstrated that extra-

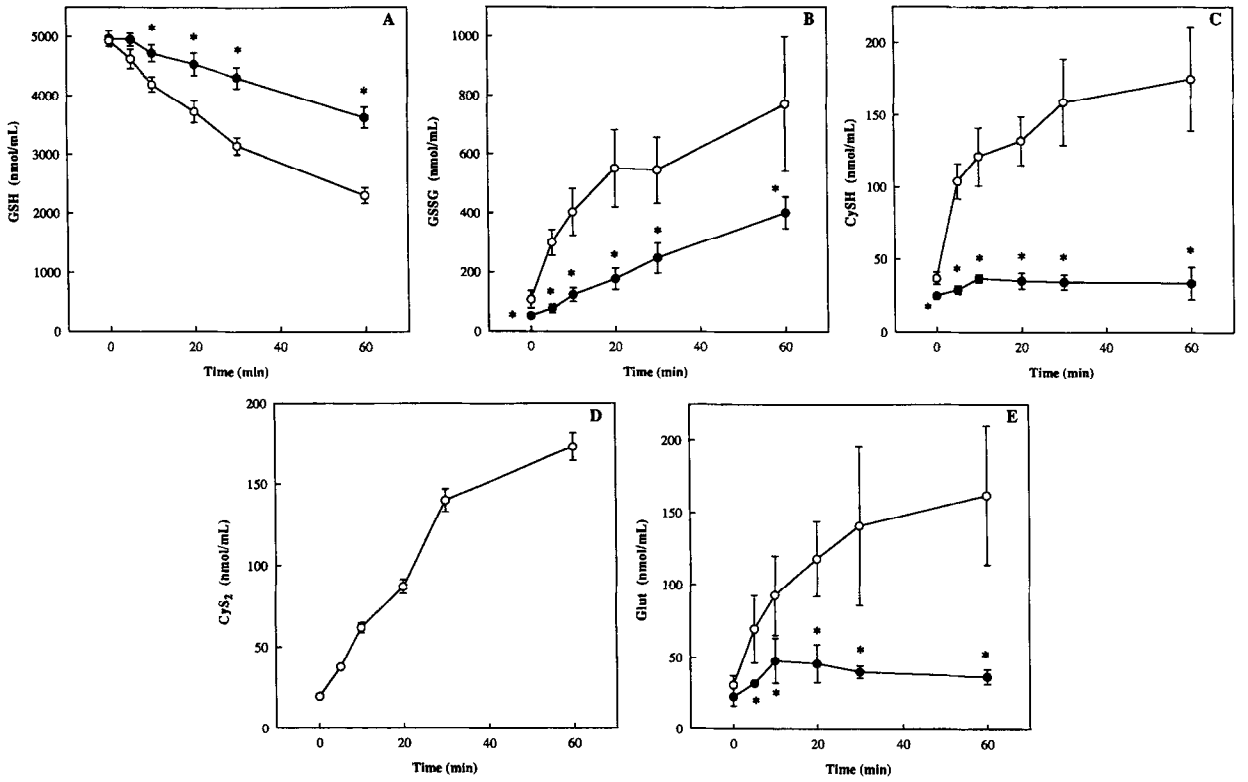

FIG. 11. Loss of extracellular GSH and formation of extracellular metabolites during incubation of PT cells with GSH. Isolated PT cells (5–10 x 10^6 cells/mL) were incubated at 37° with either buffer (open circles) or 0.25 mM acivicin + 2 mM BSO (filled circles) for 15 min. Then cells were incubated for up to 60 min with 5 mM GSH. At the indicated times, 0.5-mL aliquots of cells were removed and extracellular medium was analyzed by HPLC for derivatives of GSH (A), GSSG (B), CySH (C), CyS_2 (D), and Glut (E). Results are the means ± SEM of measurements from 3–4 separate cell preparations Key: (*) Significantly ($P < 0.05$) different from amount in medium from cells preincubated with buffer and incubated for the same time.

cellular GSH protects renal PT cells from oxidative injury [7, 21]. Hence, addition of GSH to extracellular medium may be an important method to prevent or counteract oxidative damage to renal PT cells. While previous studies have characterized GSH turnover [22, 23] and transport [20] in renal cortical cells, which are predominantly of PT origin, a quantitative assessment of the relative roles of the various processes that account for renal PT cellular handling of intracellular and extracellular GSH has not been performed. Therefore, the present study was undertaken to quantitate the various steps involved in the handling of GSH by PT cells to determine the relative importance of each in the regulation of cellular GSH status.

We initially looked at modulation of intracellular GSH status with inhibitors of GSH degradation and synthesis and by addition of an oxidant whose metabolism leads to GSH oxidation. Quantitation of inhibition of GSH degradation with acivicin and GSH synthesis with BSO was important because these two agents are necessary tools in the study of renal handling of GSH. Although we can readily obtain nearly complete inhibition of GGT with acivicin at concentrations that are not cytotoxic to renal cells [24], the residual amount of activity, which may be less than 1% of the uninhibited level, may still be high enough to produce significant degradation of extracellular GSH. This is because renal PT cells contain extremely high activities of GGT. Regarding BSO-induced inhibition of GSH synthesis, we could only obtain approximately 50% inhibition of GCS activity with non-cytotoxic concentrations of BSO. Addition of increasing concentrations of tBH produced a time- and concentration-dependent oxidation of GSH. At the lowest concentration of tBH used (i.e. 0.1 mM), significant GSH oxidation occurred, but previous studies [7] showed that no cytotoxicity occurs under these conditions. Hence, renal PT cells can exhibit marked GSH oxidation without cellular injury.

Next GSH transport and metabolism rates were measured in both the absence and presence of inhibitors of GSH turnover to compare these two processes under two experimental conditions at which transport is measurable [4, 5, 20]. The use of inhibitors of GSH turnover, which is an artificial condition, is necessitated in cells that possess high levels of GGT activity. Since suspensions of isolated PT cells are the experimental model, extracellular GSH is exposed to both the luminal and basal-lateral membrane surfaces. In the absence of acivicin, it is difficult to distinguish transport from degradation. BSO has been included in this and in prior studies of GSH transport in renal cells [20]

TABLE 1. Rates of GSH metabolism and balance sheet of disposition of GSH in renal PT cells in the absence of acivicin/BSO

Process measured -Acivicin/BSO	5 mM GSH	2 mM GSH	1 mM GSH	0.5 mM GSH
GSH loss	−59.6 ± 4.2	−42.3 ± 6.6	−19.0 ± 2.8	−14.7 ± 0.3
GSH uptake*	1.13 ± 0.24	2.66 ± 0.81	1.13 ± 0.24	—†
GSH oxidation (2 GSSG)	24.0 ± 7.4	10.7 ± 2.0	7.10 ± 1.20	−0.66 ± 0.28
GSH degradation				
$([Glut + CySH + 2CyS_2]/2)_e$	7.87 ± 2.86	5.15 ± 1.06	3.54 ± 0.77	4.51 ± 1.47
$([Glut + CySH + 2CyS_2]/2)_i$	0.83 ± 0.07	0.76 ± 0.08	−0.32 ± 0.03	—†
Balance	−25.8	−23.0	−7.55	−10.9‡

Data from Fig. 11 (5 mM GSH) and other data not shown (2, 1, and 0.5 mM GSH) for changes over the first 30 min of incubations were converted to rates of change per minute. GSH uptake values from Fig. 4 and amino acid uptake values from Figs. 5–7 were converted to amounts transported into cells per mL of medium and are included to obtain a balance sheet. Rates are means ± SEM, are expressed as nmol/min per mL, and are from 3 experiments for all measurements except uptake, which are from 3–5 experiments.

* Rates of GSH uptake were converted from nmol/min per 10^6 cells by multiplying the mean cell concentration (8.05×10^6 cells/mL) used in uptake experiments to obtain rates expressed as nmol/min per ml.

† Not measured.

‡ Balance for incubation with 0.5 mM GSH does not include uptake measurements, since these were not determined.

to minimize the possibility that measured GSH uptake is actually due to degradation, uptake of the constituent amino acids, and intracellular resynthesis, although this may be somewhat redundant with efficient inhibition of GGT activity. Hence, due to the inherent properties of this *in vitro* model, these experimental manipulations were necessary to enable determination of the various processes involved in the regulation of renal PT cellular GSH status.

GSH uptake into renal PT cells was demonstrated both with and without acivicin, although intracellular accumulation was significantly higher and was sustained for a longer period of time in the presence of the GGT inhibitor. Function of the previously described basal-lateral plasma membrane transporter was shown by the marked inhibition of intracellular accumulation by PAH. Approximately 2- to 3-fold higher amounts of Glut and CySH + CyS_2 were found in PT cells incubated without acivicin treatment as compared with those pretreated with acivicin (cf. Figs. 5–7), showing that GSH degradation had occurred.

Since rates of change of the contents of GSH and its metabolites in PT cells appeared to be approximately linear over the first 30 min of incubation (cf. Fig. 11), changes in concentrations of GSH, GSSG, CySH, CyS_2, and Glut in extracellular medium between 0 and 30 min were converted to rates of change per minute for each initial extracellular GSH concentration for PT cells incubated in the absence or presence of acivicin/BSO (Tables 1 and 2). GSH uptake was also factored into the fate of extracellular GSH by converting rates per 10^6 cells to rates per mL, and a balance sheet was prepared to determine if the observed GSH loss at each concentration of initial extracellular GSH could be accounted for by the three processes mea-

TABLE 2. Rates of GSH metabolism and balance sheet of disposition of GSH in renal PT cells in the presence of acivicin/BSO

Process measured + Acivicin/BSO	5 mM GSH	2 mM GSH	1 mM GSH	0.5 mM GSH
GSH loss	−22.2 ± 3.7	−14.4 ± 0.6	−13.5 ± 3.8	−5.26 ± 0.11
GSH uptake*	6.12 ± 0.89	3.78 ± 0.64	1.53 ± 0.08	—†
GSH oxidation (2 GSSG)	13.1 ± 3.0	8.12 ± 1.92	6.02 ± 1.46	3.22 ± 0.78
GSH degradation				
$([Glut + CySH + 2CyS_2]/2)_e$	0.57 ± 0.25	0.45 ± 0.27	2.57 ± 0.94	0.01 ± 0.30
$([Glut + CySH + 2CyS_2]/2)_i$	0.22 ± 0.02	0.14 ± 0.02	0.19 ± 0.02	—†
Balance	−2.19	−1.91	−3.19	−2.03‡

Data from Fig. 11 (5 mM GSH) and other data not shown (2, 1, and 0.5 mM GSH) for changes over the first 30 min of incubations were converted to rates of change per minute. GSH uptake values from Fig. 4 and amino acid uptake values from Figs. 5–7 were converted to amounts transported into cells per mL of medium and are included to obtain a balance sheet. Rates are means ± SEM, are expressed as nmol/min per mL, and are from 3 experiments for all measurements except uptake, which are from 3–5 experiments.

* Rates of GSH uptake were converted from nmol/min per 10^6 cells by multiplying the mean cell concentration (8.05×10^6 cells/mL) used in uptake experiments to obtain rates expressed as nmol/min per ml.

† Not measured.

‡ Balance for incubation with 0.5 mM GSH does not include uptake measurements, since these were not determined.

sured (i.e. GSH uptake, GSH oxidation [2 GSSG], and GSH degradation [{Glut + CySH + 2 CyS_2}/2]. Both intracellular and extracellular oxidation and degradation products were factored into these assessments.

In both the absence and presence of acivicin/BSO, the total GSH loss from the extracellular medium could not be accounted for completely by these processes. In the absence of acivicin/BSO (Table 1), uptake, oxidation, and degradation accounted for only 43, 54, or 40% of the total GSH loss with initial extracellular GSH concentrations of 5, 2, or 1 mM, respectively. In contrast, in the presence of acivicin/BSO (Table 2), uptake, oxidation, and degradation accounted for 90, 87, or 76% of the total GSH loss with initial extracellular GSH concentrations of 5, 2, or 1 mM, respectively. Hence, other processes, such as covalent binding to proteins or other molecules or utilization of the constituent amino acids for protein synthesis, must be considered to potentially account for the total GSH loss from the extracellular medium particularly in the absence of inhibitors of GSH turnover.

A scheme of cellular handling of GSH is shown (Fig. 12) that summarizes the relationships between transport, oxidation, and degradation. In the absence of acivicin/BSO, degradation and oxidation are the major processes occurring at the plasma membrane surface, with uptake accounting for only 6% or less of the total loss of GSH. After preincubation with the inhibitors, however, uptake into the cell played a major role in the disposition of exogenous GSH, accounting for nearly 30% of the total GSH consumption. Comparison of reductive and biosynthetic capacity with transport capacity shows that uptake of exogenous GSH is by far the most effective means to increase intracellular GSH for protecting PT cells from oxidative stress or other forms of toxic injury. Hence, under toxicological conditions such as oxidative stress, renal PT cells can only partially regenerate GSH from GSSG, and they have a negligible capacity to resynthesize GSH from the precursor amino acids.

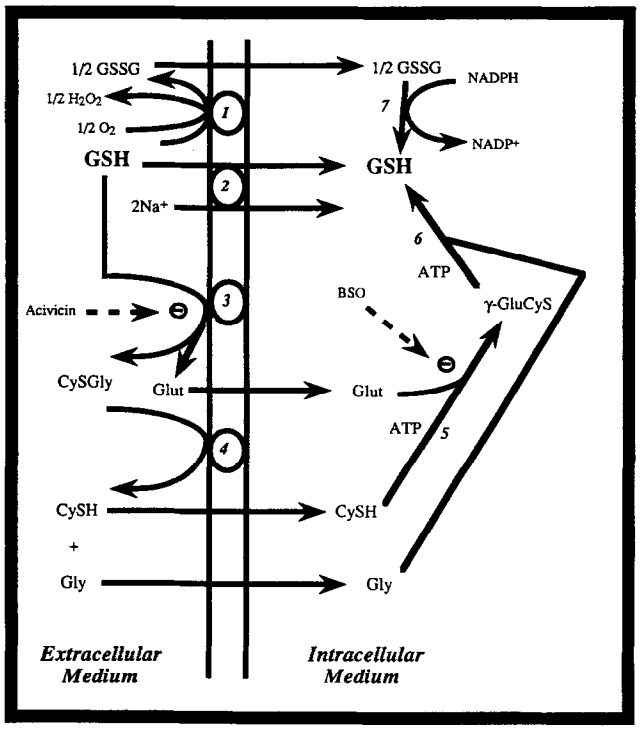

FIG. 12. Pathways of GSH metabolism and transport in renal PT cells. Scheme showing fates of extracellular GSH in suspensions of isolated renal PT cells. Enzymatic processes: (1) copper-containing thiol oxidase localized to basal-lateral membrane; (2) Na^+-coupled transport of GSH localized to basal-lateral membrane; (3) GGT localized primarily to brush-border membrane; (4) cysteinylglycine dipeptidase activity; (5) GCS; (6) GSH synthetase; and (7) GSSG reductase.

In conclusion, this study has defined and quantified several of the steps involved in the renal PT cell handling of GSH. Although additional study is required to completely account for the consumption of GSH in extracellular medium, the relative importance of several key steps can be assessed under physiological, toxicological, and pathological conditions. Degradation predominates in the absence of inhibitors of GSH turnover, but GSH uptake can play a quantitatively significant role that is greater than that of reduction or biosynthesis, particularly under toxicological or pathological conditions where intracellular GSH may be depleted.

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