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Uptake of the Glutathione Conjugate *S*-(1,2-Dichlorovinyl)glutathione by Renal Basal-Lateral Membrane Vesicles and Isolated Kidney Cells

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SUMMARY

Transport of the glutathione *S*-conjugate, *S*-(1,2-dichlorovinyl)glutathione (DCVG), was studied in renal basal-lateral membrane vesicles and isolated rat kidney cells. The time course of *S*-(1,2-dichlorovinyl)glutathione uptake in membrane vesicles exhibited an overshoot in the presence of sodium, indicating transport against a concentration gradient. The initial rate of uptake with membrane potential clamped at 0 mV was stimulated 2.5-fold by an inwardly directed gradient of 100 mM sodium chloride. Hyperpolarization of the membrane potential to −60 mV in the presence of sodium stimulated uptake another 2.7-fold, indicating that cotransport of sodium and *S*-(1,2-dichlorovinyl)glutathione is electrogenic. Sodium-dependent DCVG uptake was inhibited by glutathione, glutathione disulfide, and γ-glutamylglutamate, but not by the corresponding cysteine *S*-conjugate, *S*-(1,2-dichlorovinyl)cysteine, indicating that the transport system is specific for the γ-glutamyl moiety. Probenecid was also a potent inhibitor of sodium-dependent uptake. *S*-(1,2-dichlorovinyl)glutathione inhibited sodium-dependent uptake of glutathione in a concentration-dependent manner. Thus, these results show that uptake of DCVG and glutathione is mediated by the same sodium-coupled system. Uptake of *S*-(1,2-dichlorovinyl)glutathione was also demonstrated in isolated kidney cells; in the presence of sodium, cells accumulated approximately 4-fold more DCVG than in the absence of sodium. This basal-lateral membrane transport system can enable efficient delivery of circulating *S*-(1,2-dichlorovinyl)glutathione to kidney cells and may, therefore, contribute to its potent and selective nephrotoxicity. In addition, it suggests that renal clearance of glutathione conjugates may include transport from the blood through epithelial cells into the lumen as well as direct filtration through the glomerulus.

INTRODUCTION

An important mechanism for the detoxication of a variety of xenobiotics is their conjugation with GSH.[3] Formation of GSH *S*-conjugates is catalyzed by the GSH *S*-transferases (RX:glutathione *R*-transferase, EC 2.5.1.18), a group of enzymes which are most abundant in the liver but are found in many mammalian cell types (1). Because of differential tissue distribution of enzymes involved in detoxication, subsequent conversion of GSH *S*-conjugates to the ultimate excretion products, mercapturic acids, requires transport of the conjugates from the liver into plasma and bile (2, 3). The GSH *S*-conjugates are then metabolized to the corresponding cysteine *S*-conjugates in the kidney (4), and to a lesser extent, in the small intestine (5), by the brush-border membrane enzymes, γ-glutamyltransferase ((5-glutamyl)-peptide:amino-acid 5-glutamyltransferase, EC 2.3.2.2) and cysteinylglycine dipeptidase (L-cysteinyl-glycine hydrolase, EC 3.4.13.6). Cysteine *S*-conjugate *N*-acetyltransferase (cysteine thioether *N*-acetyltransferase, EC 2.3.1.80) catalyzes the final step in mercapturic acid formation (6).

Although GSH conjugation is generally associated with detoxication, conjugation of a variety of chemicals, including many halogenated hydrocarbons, is associated with nephrotoxicity (7). Among these is the GSH *S*-conjugate of trichloroethylene, DCVG, which is a potent nephrotoxin and produces large increases in blood urea nitrogen concentrations and urinary glucose excretion rates (7, 8).

A major determinant of a compound's toxicity is the manner in which it gains access to its target. Knowledge of the means of access is important not only in elucidat-

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[3] The abbreviations used are: GSH, reduced glutathione; GSSG, glutathione disulfide; DCVG, *S*-(1,2-dichlorovinyl)glutathione; DCVC, *S*-(1,2-dichlorovinyl)cysteine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; AT-125, L-(αS,5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid; HPLC, high-performance liquid chromatography.

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ing the mechanism of toxicity but also in development of therapeutic agents to counteract the toxicity. One mechanism for delivery of DCVG and other GSH *S*-conjugates to the kidneys is glomerular filtration. However, during a single pass through the renal circulation, only about 25% of the plasma conjugate is removed by this process. A significant amount of circulating GSH is also removed by a nonfiltration mechanism (9) which involves transport into renal cells across the basal-lateral membrane (10–12). We have characterized an electrogenic, Na^+-coupled, and probenecid-sensitive transport system for uptake of intact GSH in renal basal-lateral membrane vesicles (11,12). This system may account for the ability of the kidneys to extract most of the GSH passing through the renal circulation. Three facts suggest that a similar mechanism for delivery of GSH *S*-conjugates to the kidneys, in addition to glomerular filtration, may be functioning: GSH and GSH *S*-conjugates undergo a similar pattern of interorgan metabolism (2, 3, 7); the same enzymes are responsible for the conversion of GSH and GSH *S*-conjugates to cysteine and cysteine *S*-conjugates, respectively (4); the basal-lateral membrane GSH transport system exhibits a broad substrate specificity for γ-glutamyl compounds (12).

In the present study, DCVG was employed as a model compound to test whether GSH *S*-conjugates are transported by the renal basal-lateral membrane, and if so, whether transport is mediated by the GSH system. The results demonstrate that DCVG transport occurs and is Na^+-dependent, electrogenic, and probenecid-sensitive. These properties and mutual inhibition between DCVG and GSH for uptake indicate that the two compounds are transported by the same system. Furthermore, Na^+-dependent uptake of intact DCVG was demonstrated in isolated kidney cells. *In vivo*, this transport system may normally function to increase renal clearance of drug conjugates. However, the enhanced delivery of DCVG and possibly that of other GSH *S*-conjugates to the kidneys may contribute to their selective nephrotoxicity.

MATERIALS AND METHODS

GSH, GSSG, phenylmethylsulfonyl fluoride, valinomycin, 1-fluoro-2,4-dinitrobenzene, Percoll, probenecid, γ-glutamylglutamate, and collagenase (type IV) were purchased from Sigma. [*glycine*-2-^{3}H]GSH (specific activity, 5000 Ci/mol) was purchased from New England Nuclear. Nitrocellulose filters (0.45 μm pore size) were purchased from Gelman (Ann Arbor, MI). AT-125 was a gift from Dr. Donald J. Reed, Oregon State University. DCVG and DCVC were gifts from Dr. Adnan A. Elfarra, University of Rochester. All other chemicals were of reagent grade and were purchased locally.

Male white rats (Sprague-Dawley derived, King Animal Laboratories, Oregon, WI, 200–300 g) were anesthetized with diethyl ether and were killed by cutting through the diaphragm. To prepare membrane vesicles, the kidneys were immediately removed and placed in ice-cold Na^+-free 10 mM Tris-Hepes buffer, pH 7.6, containing 250 mM sucrose and 0.1 mM phenylmethylsulfonyl fluoride to inhibit proteolysis. The brush-border and basal-lateral regions of the plasma membrane were prepared according to the Percoll density-gradient centrifugation method of Scalera *et al.* (13) with the modification in buffer described above. Membrane fractions were identified by the use of marker enzymes as previously described (14). For use in transport studies, fractions from the Percoll gradient corresponding to the basal-lateral or brush-border regions of the plasma membrane were pooled and concentrated as previously described (11). Protein concentration was determined by the method of Bradford (15) with the dye-reagent concentrate from Bio-Rad and bovine serum albumin as standard. Use of bovine serum albumin as standard for this assay overestimates protein concentration. Accordingly, the present results must be adjusted to allow absolute rates to be directly compared to studies in which other protein standards or assays were used.

Isolated kidney cells were prepared by the collagenase perfusion method of Jones *et al.* (16). Cell viability and concentration were estimated in a hemacytometer in the presence of 0.2% (w/v) trypan blue. Cell viability was typically 75–90%.

DCVG content of samples was measured by HPLC by a modification of the method of Reed *et al.* (17). DCVG was converted to the *N*-dinitrophenyl derivative by addition of 0.5 ml of 1.5% (v/v) 1-fluoro-2,4-dinitrobenzene in absolute ethanol to 0.5 ml of sample and incubation for 4 h in the dark. K_2CO_3 (1 M, approximately 0.1 ml) was added until yellow color appeared. The *N*-dinitrophenyl derivative of DCVG was separated on a 10-μ Ultrasil-NH_2 column (4.6 mm × 25 cm; Beckman Instruments). Solvent A was 80% methanol and solvent B was 2 M sodium acetate, pH 4.5, containing 64% methanol. Chromatographic runs were performed at room temperature at a flow rate of 1.5 ml/min with 25% B for 20 min. When DCVG content of cells was measured, an additional 10-min run at 100% B was employed to wash out any remaining compounds bound to the column. Because excess 1-fluoro-2,4-dinitrobenzene was used in the derivatization procedure, amino groups on the column were also derivatized, thus decreasing the number of available positively charged groups for ion exchange. To compensate for the decrease in amino groups and the consequent decrease in retention time as the column aged, the program was altered by decreasing the acetate concentration in the solvent. Samples were detected by measuring absorbance at 365 nm and were quantitated with respect to standards by integration with a Hewlett-Packard 3390A integrator (Hewlett-Packard, Atlanta, GA). The assay was linear in the range of 0.05–5 nmol of DCVG.

Before transport measurements were made, both membrane vesicles and cells were pretreated for 15 min with 0.25 mM AT-125 to inhibit γ-glutamyltransferase activity (18, 19) unless otherwise indicated. DCVG uptake by renal basal-lateral membrane vesicles was measured by incubating 50 μl of the membrane suspension containing 0.10–0.15 mg of protein at room temperature with 450 μl of uptake solution containing 5 mM DCVG. Membrane potential was set at the indicated values by preincubating vesicles with the K^+-ionophore, valinomycin (20 μg/ml), and subsequently measuring uptake in the presence of various intravesicular/extravesicular ratios of K^+ (12). Composition of intra- and extravesicular solutions is detailed in the figure legends. At indicated times, 100-μl aliquots of the incubation mixture were filtered on 0.45-μm nitrocellulose filters. Filters were washed with 4 ml of ice-cold 250 mM NaCl and were placed in 0.5 ml of distilled water for 1 h to release the intravesicular contents. Filters were then removed, and the solutions were derivatized and analyzed by HPLC as described above. Zero time measurements, determined as previously described (12), were subtracted from all determinations. Uptake of radiolabeled GSH by basal-lateral membrane vesicles was measured with membrane potential clamped at 0 mV (11, 12).

Uptake of DCVG by isolated kidney cells, suspended in Krebs-Henseleit buffer, pH 7.4, supplemented with 25 mM Hepes and 2% (w/v) bovine serum albumin, was measured at 37° on a shaking water bath by incubating 1.6 × 10^6 cells/ml with 1 mM DCVG. For Na^+-free incubations, cells were suspended in Krebs-Henseleit buffer containing choline·Cl replacing NaCl and $KHCO_3$ replacing $NaHCO_3$. At indicated times, 0.5-ml aliquots were layered on 1 ml of 20% (v/v) Percoll in Krebs-Henseleit buffer and were centrifuged for 1 min in a Microfuge. The cell pellet was resuspended in 0.5 ml of Krebs-Heneseleit buffer, 0.1 ml of 30% (w/v) trichloroacetic acid was added, and the mixture was centrifuged for 2 min in a Microfuge. Acid extracts (0.5 ml) were derivatized and analyzed by HPLC as described above. Uptake at zero time was subtracted for all determinations.

RESULTS

Basal-lateral membrane vesicles were pretreated for 15 min with 0.25 mM AT-125 to inhibit γ-glutamyltransferase and minimize artifacts in uptake measurements due to degradation of DCVG. DCVG uptake was measured with membrane potential clamped at 0 mV in the presence and absence of Na^+ and at −60 mV in the presence of Na^+ (Fig. 1). Time-dependent uptake of the intact conjugate was observed, and the initial rate of uptake at 0 mV was stimulated 2.5-fold by the presence of an inwardly directed gradient of 100 mM NaCl. An overshoot, typical of Na^+-solute cotransport processes, occurred, indicating transport of DCVG against a concentration gradient. Hyperpolarization of the membrane potential to −60 mV in the presence of Na^+ stimulated uptake rate another 2.7-fold, indicating that Na^+-DCVG cotransport is electrogenic and is driven by the Na^+ gradient and the membrane potential. No Na^+-dependent uptake of DCVG was observed in isolated brush-border membrane vesicles.

The specificity of the system mediating DCVG uptake in basal-lateral membrane vesicles was studied by examining the effects of a 5-fold excess of various compounds on uptake (Table 1). Membrane potential was clamped at 0 mV to prevent simple charge effects due to the presence of other anions in the incubation mixture. None of the compounds tested affected Na^+-independent DCVG uptake. However, GSH, GSSG, and γ-glutamylglutamate substantially inhibited Na^+-dependent DCVG uptake. In contrast, the cysteine *S*-conjugate of trichloroethylene, DCVC, did not inhibit DCVG uptake; this negative result along with the above results indicate a requirement for a γ-glutamyl moiety for transport by this system. In addition, 0.1 mM probenecid inhibited 66% of the Na^+-dependent uptake of 5 mM DCVG, suggesting that the acidic nature of the DCVG molecule is involved in the transport mechanism.

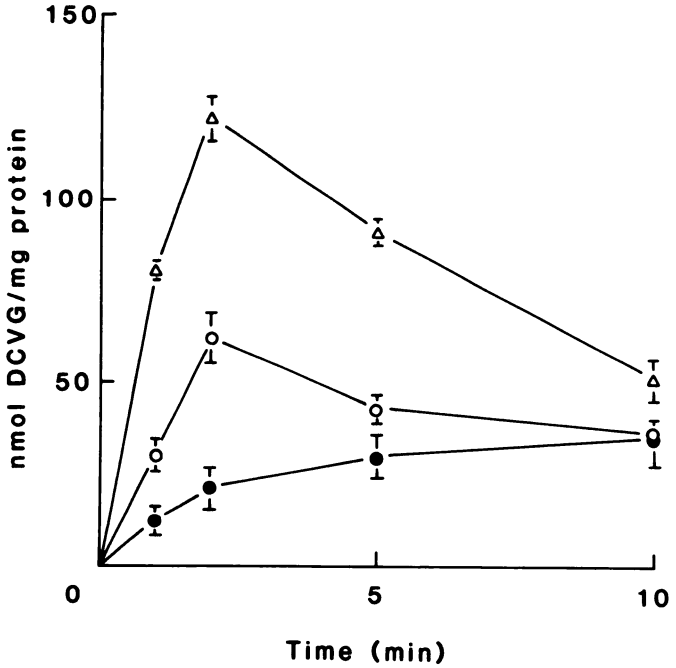

FIG. 1. *Effect of Na^+ and membrane potential on uptake of DCVG by renal basal-lateral membrane vesicles*

Uptake of 5 mM DCVG at the indicated times was measured by HPLC as described under Materials and Methods. The calculated Nernst K^+-diffusion potential was varied using valinomycin in the presence of various intra- to extravesicular KCl gradients. Under all conditions, solutions contained 10 mM Tris-Hepes, pH 7.6. Intravesicular solutions contained 100 mM sucrose, 50 mM KCl, and 100 mM Tris-Cl (adjusted to pH 7.6 with Tris base). For measurement of uptake at 0 mV in the absence of Na^+ (●), extravesicular solution contained 100 mM sucrose, 50 mM KCl, and 100 mM Tris-Cl. For measurement of uptake in the presence of Na^+ at 0 mV (○), extravesicular solution contained 100 mM NaCl, 50 mM KCl, and 50 mM Tris-Cl. For measurement of uptake in the presence of Na^+ at −60 mV (△), extravesicular solution contained 100 mM NaCl, 5 mM KCl, and 95 mM Tris-Cl. Results are the mean ± SE of 3 preparations.

The Na^+ and membrane potential dependence of DCVG uptake and the specificity observed suggested that the same system responsible for uptake of GSH and other γ-glutamyl compounds also mediates uptake of the GSH *S*-conjugate, DCVG. To confirm this, the effect of DCVG on the initial rate of uptake of 1 mM GSH was examined with membrane potential clamped at 0 mV (Fig. 2). DCVG inhibited the Na^+-dependent uptake of GSH in a concentration-dependent manner, with 50% inhibition occurring at approximately 1 mM DCVG. The mutual inhibition of transport by DCVG and GSH shows that the two compounds share the same transport system. The concentration of DCVG which produced 50% inhibition of GSH uptake suggests that the affinity of the transporter for the two substrates is nearly the same.

Na^+-dependent DCVG uptake was also demonstrated in isolated kidney cells (Fig. 3). Four-fold more DCVG was accumulated by the cells after 30 min of incubation in the presence of Na^+ (18 nmol/10^6 cells) than when Na^+ was replaced by choline and K^+ in the incubation medium (4.6 nmol/10^6 cells). In the absence of AT-125, uptake of the intact conjugate was still observed, but the amount transported was about 50% of that in the presence of AT-125, indicating that some of the DCVG was degraded by γ-glutamyltransferase. In addition, 5 mM GSH inhibited uptake of 1 mM DCVG in kidney cells at 5 min by 85% (data not shown), confirming the results obtained with isolated membrane vesicles.

TABLE 1

Inhibition of DCVG uptake

Uptake of 5 mM DCVG in renal basal-lateral membrane vesicles was measured after incubation for 2 min in the presence and absence of 100 mM NaCl by HPLC as described under Materials and Methods. Membrane potential was clamped at 0 mV as described in the legend to Fig. 1. Results are the mean ± SE of 3 preparations.

Addition	Na^+-dependent uptake	Control
	nmol DCVG/min/mg protein	%
None	22.6 ± 3.2	100
25 mM GSH	5.2 ± 0.7	23.0
25 mM GSSG	1.6 ± 1.3	7.1
25 mM γ-Glutamylglutamate	6.6 ± 2.0	29.0
25 mM DCVC	21.6 ± 2.4	95.4
0.1 mM Probenecid	7.7 ± 1.1	33.8

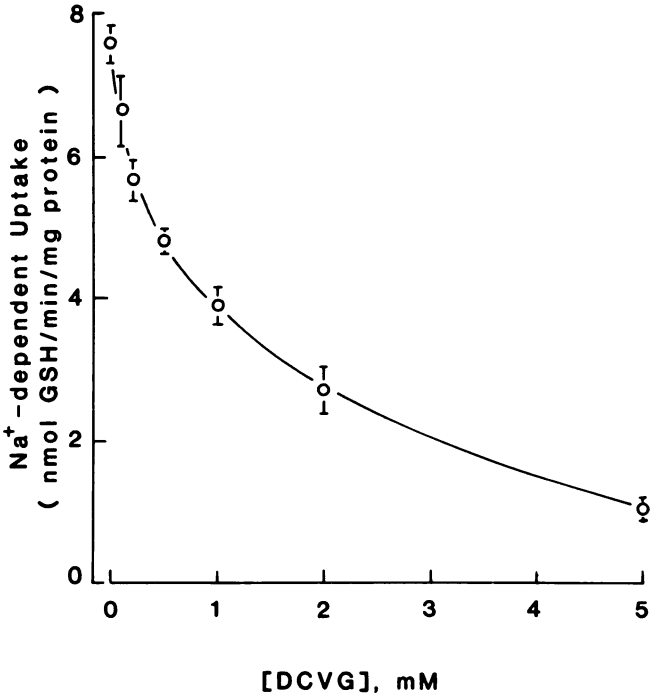

FIG. 2. *Effect of DCVG on GSH uptake by renal basal-lateral membrane vesicles*

The initial rate of uptake of 1 mM GSH was measured with radiolabeled GSH in the presence and absence of 100 mM NaCl and at the indicated concentrations of DCVG by filtration after 1 min of incubation as described under Materials and Methods. Membrane potential was clamped at 0 mV with valinomycin and equimolar concentrations of KCl on the two sides of the membrane. The composition of intra- and extravesicular solutions was as described in the legend of Fig. 1. Results are corrected for uptake in the absence of Na^+ and are the mean ± SE of 3 preparations.

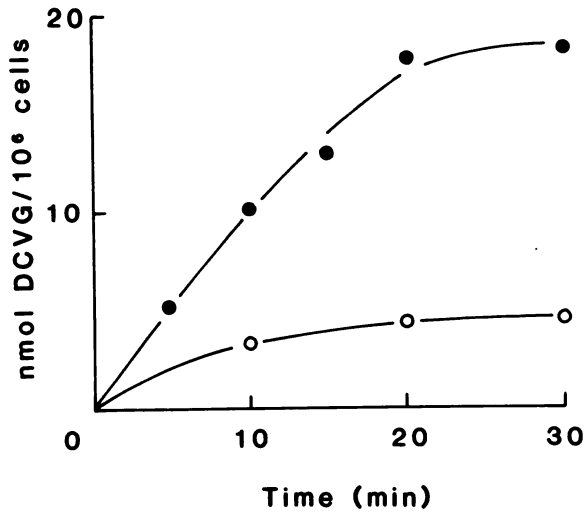

FIG. 3. *Uptake of DCVG by isolated kidney cells*

Cells (1.6×10^6/ml) were pretreated with 0.25 mM AT-125 and were incubated at 37° on a shaking water bath in the presence (●) and absence (○) of Na^+ with 1 mM DCVG. At the indicated times, 0.5-ml aliquots were centrifuged through 20% (v/v) Percoll. The acid extract of the cell pellet was derivatized with 1-fluoro-2,4-dinitrobenzene and analyzed by HPLC as described under Materials and Methods. Results are the mean of 3 preparations for measurements in the presence of Na^+ and are from 1 preparation for measurements in the absence of Na^+.

DISCUSSION

The findings presented in this study demonstrate for the first time cellular uptake of an intact GSH *S*-conjugate. DCVG was chosen as a model compound because it has been identified as a potent and specific nephrotoxin (7, 8). It is, therefore, of interest to determine the mechanism by which DCVG gains access to target sites in the renal proximal tubule.

Transport processes for GSH *S*-conjugates have been described in other tissues. Efflux of GSH *S*-conjugates across the liver sinusoidal membrane into plasma is the first step in the hepatorenal processing of these compounds (2, 20). In addition, efflux across the bile canalicular membrane occurs (21–24), enabling subsequent metabolism of the conjugates by the small intestine (2, 3, 5). Transport processes for GSH *S*-conjugates have been described in the isolated perfused rat heart (25) and in human erythrocytes (26), indicating that many tissues have the capacity to transport these compounds. However, the system described in the present study is the first demonstration of uptake of a GSH *S*-conjugate.

DCVG uptake across the renal basal-lateral membrane was coupled to Na^+. This Na^+-dependent transport was electrogenic and was inhibited by γ-glutamyl compounds and probenecid. These properties are the same as those described for uptake of GSH by the renal basal-lateral membrane (11, 12). Mutual inhibition of transport between GSH and DCVG confirmed that the two compounds are transported by the same system. Uptake of DCVG by intact kidney cells provided further evidence that this system may function *in vivo* to deliver DCVG and possibly other GSH *S*-conjugate to the kidney for further processing.

A schematic summary of renal metabolism of DCVG is shown in Fig. 4. DCVG can gain access to the kidney by two mechanisms, glomerular filtration and uptake from the peritubular plasma by transport across the basal-lateral membrane. The filtered DCVG is converted on the brush-border membrane in a two-step process to the cysteine *S*-conjugate, DCVC. Similarly, the DCVG which enters the cell via transport across the basal-lateral membrane will presumably be transported to the active site of γ-glutamyltransferase on the brush-border membrane by a system similar or identical to the recently described GSH transport system in renal brush-border membranes (27). DCVG can then be converted to DCVC by the same processing as occurs for filtered DCVG. Evidence for this process occurring is the ability of AT-125 to partially protect against the renal toxicity of DCVG (7, 8). DCVC formed in the lumen is transported into the cell where it can have two fates: it can be *N*-acetylated by the cysteine *S*-conjugate *N*-acetyltransferase to form the mercapturic acid (6) or it can be cleaved by the cysteine conjugate β-lyase (28; EC 4.4.1.13) to pyruvate, ammonia, and a reactive thiol, which is presumably dichlorovinylmercaptan (8). In the former case, the mercapturic acid is secreted into the lumen and excreted in the urine. In the latter case, conversion of DCVC to a reactive thiol is thought to be responsible for the nephrotoxicity of DCVG and DCVC (7, 8).

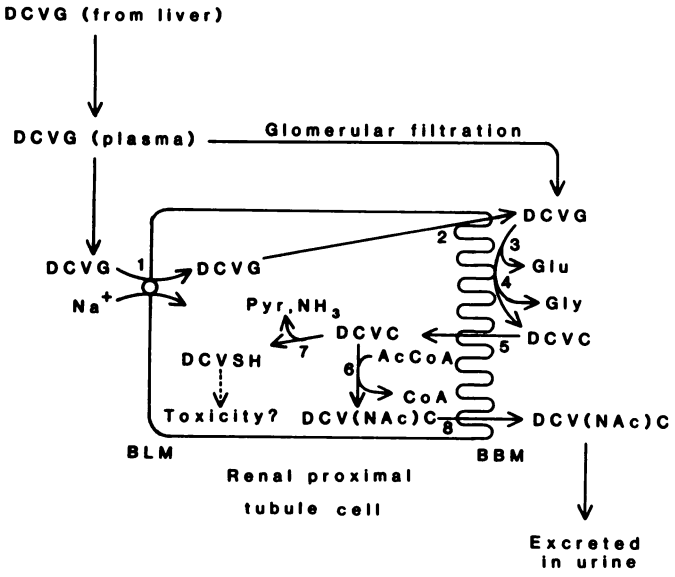

FIG. 4. *Renal metabolism of DCVG*

1, Na^+-DCVG cotransport across the basal-lateral membrane (*BLM*); *2*, efflux of DCVG across the brush-border membrane (*BBM*) into the lumen; *3*, removal of glutamyl residue by γ-glutamyltransferase; *4*, removal of glycyl residue by cysteinylglycine dipeptidase; *5*, uptake of DCVC across the brush-border membrane; *6*, acetylation of DCVC by cysteine *S*-conjugate *N*-acetyltransferase; *7*, cleavage of DCVC by cysteine conjugate β-lyase; *8*, efflux of mercapturic acid of DCVC across brush-border membranes into lumen. Abbreviations: *Glu*, glutamate; *Gly*, glycine; *DCV(NAc)C*, *S*-(1,2-dichlorovinyl)-*N*-acetylcysteine; *AcCoA*, acetyl-CoA; *Pyr*, pyruvate; *DCVSH*, dichlorovinylmercaptan.

Transport across the basal-lateral membrane provides an alternate route of entry into kidney cells and enhances delivery of circulating DCVG to the kidneys. Thus, DCVG and its toxic metabolites may accumulate to higher concentrations in kidney than in other tissues. In this manner, the transport system may contribute to the selective nephrotoxicity of GSH *S*-conjugates of various halogenated hydrocarbons. Since transport by this Na^+-dependent system is inhibited by probenecid, it may also be possible to block nephrotoxicity by treatment with this compound, especially in combination with AT-125.

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