0026-895X/87/050506-07$02.00/0

MOLECULAR PHARMACOLOGY, 31:506-512

The Transport of S-Cysteine Conjugates in LLC-PK$_1$ Cells and Its Role in Toxicity

VAL H. SCHAEFFER and JAMES L. STEVENS

Division of Biochemistry and Biophysics, Center for Drugs and Biologics, Food and Drug Administration, Bethesda, Maryland 20892 and W. Alton Jones Cell Science Center, Lake Placid, New York 12946 (J.L.S.)

Received July 28, 1986; Accepted December 5, 1986

SUMMARY

The transport of S-cysteine conjugates was studied in the kidney cell line, LLC-PK$_1$, using the nephrotoxin, S-(1,2-dichlorovinyl)-L-cysteine (L-DCVC), as the model compound. The saturable uptake of this conjugate did not require sodium and was selectively inhibited by the amino acid transport system L-specific substrate, 2-amino-2-norbornane carboxylic acid, as well as a variety of other S-cysteine conjugates and neutral amino acids with large, nonpolar side chains. Kinetic studies suggested the existence of both low and high affinity transport systems with K_m values that differed by 25-fold. Although these uptake systems showed no discernible differences in substrate specificity, the low affinity transport was more sensitive to trans-stimulation. L-DCVC uptake in subconfluent cultures was about 3-fold that of confluent cells, suggesting either adaptive regulation to cell growth or polarization of transport to the basolateral membrane. L-DCVC toxicity in LLC-PK$_1$ cells was inhibited in the presence of nontoxic transport substrates but was potentiated when cells were preloaded with many of the same compounds, indicating that transport may be a rate-limiting factor in L-DCVC-induced toxicity under certain circumstances. The possible role of this system L-like uptake in the transport of S-cysteine conjugates *in vivo* is discussed.

A major metabolic pathway through which foreign chemicals are processed for excretion is conjugation with glutathione and the subsequent biosynthesis of mercapturic acids (1). Following their formation, glutathione S-conjugates are successively degraded by renal γ-glutamyltranspeptidase (5-glutamyl-peptide: amino acid 5-glutamyltranspeptidase, EC 2.3.2.2) and cysteinylglycine dipeptidase (L-cysteinyl-glycine hydrolase, EC 3.4.13.6) to yield the corresponding S-cysteine conjugates which are N-acetylated to form the mercapturic acids, the excretion product found in the urine and bile (1). This entire process is generally considered an important route for the detoxification of xenobiotics.

Recent evidence suggests that the S-glutathione and S-cysteine conjugates of several halogenated hydrocarbons are potent nephrotoxins (2). One such compound which has served as a model for the investigation of S-cysteine conjugate toxicity is L-DCVC, the cysteine conjugate of trichloroethylene. In the rat, this nephrotoxin produces acute renal failure characterized by proximal tubular necrosis (3). Its toxicity appears to be dependent on metabolism by cysteine conjugate β-lyase (EC 4.4.1.13) (4). Using L-DCVC as a substrate, this enzyme catalyzes a β-elimination reaction yielding pyruvate, ammonia, and a reactive sulfur-containing fragment capable of covalent binding to tissue macromolecules (5, 6). Therefore, mercapturate biosynthesis may participate in the activation of certain xenobiotics in addition to its well described role in detoxication.

The renal epithelial cell line, LLC-PK$_1$, grown on a solid substratum, possesses some functional and morphological characteristics of proximal tubular epithelium (7–9), the presumed site of S-cysteine conjugate toxicity. These cells have been shown previously to be sensitive to several nephrotoxic S-cysteine conjugates, including L-DCVC, and their S-glutathione analogs (10). The toxicity produced by L-DCVG appears to require the degradative enzymes of the mercapturate pathway since inhibition of γ-glutamyltransferase inhibits the toxicity of L-DCVG but not L-DCVC (10). Preliminary evidence indicated that several nontoxic S-cysteine conjugates inhibited the toxicity of L-DCVC, suggesting that the uptake of S-cysteine conjugates may be a rate-limiting factor for the toxicity of these compounds and their glutathione precursors.

The role of S-cysteine conjugate transport in proximal tubule toxicity has not been fully established; therefore, this study characterizes the transport of L-DCVC in LLC-PK$_1$ cells. This cell line with its established sensitivity to S-cysteine conjugate toxicity and proximal tubular transport systems provides a convenient model to investigate this area. The results indicate

ABBREVIATIONS: DCVC, S-(1,2-dichlorovinyl)-L-cysteine; DCVG, S-(1,2-dichlorovinyl)-L-glutathione; SMC, S-methyl-L-cysteine; SEC, S-ethyl-L-cysteine; SBC, S-benzyl-L-cysteine; AOA, aminooxyacetic acid; BCH, 2-amino-2-norbornane carboxylic acid; CTFC, S-(1-chloro-1,2,2-trifluoroethyl)-L-cysteine, HFPC, S-(1,1,2,3,3,3-hexafluoropropyl)-L-cysteine; HPLC, high performance liquid chromatography; EDTA, ethylenediaminetetraacetate; EBSS, Earle's balanced salts solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LDH, lactate dehydrogenase; AIB, α-aminoisobutyric acid.

that *S*-cysteine conjugates are primarily transported in LLC-PK_1 cells by a system that normally utilizes nonpolar, neutral amino acids and is probably located on the basolateral surface. In addition, the ability of several nontoxic *S*-cysteine conjugates and amino acids to modulate L-DCVC toxicity appears to be due to their manipulation of L-DCVC transport.

Materials and Methods

L-[^{14}C]Cysteine (310 mCi/mmol) and L-[^{35}S]cysteine (397 Ci/mmol) were obtained from New England Nuclear (Boston, MA). SMC, SEC, SBC, AOA, *p*-aminohippuric acid, and all amino acids were purchased from Sigma Chemical Co. (St. Louis, MO). BCH and trichloroethylene were supplied by Aldrich Chemical Co. (Milwaukee, WI). Chlorotrifluoroethylene and hexafluoropropylene were supplied by Matheson (Allentown, PA). All other chemicals were at least reagent grade and obtained locally.

The unlabeled L and D isomers of *S*-1,2-(dichlorovinyl)cysteine (DCVC) were synthesized from trichloroethylene and the appropriate isomer of cysteine by the method of McKinney *et al.* (11). L-[^{35}S]DCVC (8 μCi/μmol) and L-[^{14}C]DCVC (40 μCi/mmol) were synthesized from radiolabeled L-cysteine and purified as described previously (12), resulting in >98% radiochemical purity as judged by HPLC. CTFC and HFPC were also synthesized from chlorotrifluoroethylene and hexafluoropropylene, respectively, by previously published procedures (10).

Cell culture. LLC-PK_1 cells were obtained from The American Type Culture Collection (Rockville, MD). They were grown in 75-cm^2 plastic culture flasks, maintained in an atmosphere of 5% CO_2 at 37°, and fed with Dulbecco's modified Eagle's medium containing 25 mM glucose and supplemented with 2 mM glutamine and 10% fetal bovine serum. When cell growth reached saturation density, subcultures were prepared by treatment with 0.125% trypsin in Dulbecco's phosphate-buffered saline containing 1 mM EDTA. Experiments were conducted on cells between passage 206 and 230.

L-DCVC uptake. LLC-PK_1 cells were seeded in 12-well culture dishes (4.5 cm^2/well) at a density of 1×10^5 or in 24-well culture dishes (2.0 cm^2/well) at 5×10^4 and, unless otherwise indicated, were allowed to grow to confluence. At time of assay, cells were washed at least twice with 1 ml of EBSS, and L-[^{35}S]DCVC (0.1–0.7 μCi/ml) in 1 ml of 20 mM HEPES/Tris buffer, pH 7.4, containing 116.5 mM NaCl, 6.4 mM KCl, 1.8 mM $CaCl_2/2H_2O$, 0.8 mM $MgSO_4/7H_2O$, and 5.5 mM D-glucose (HEPES/Tris-EBSS), was added to each well and incubated at 25° for 5 min unless otherwise specified. The assay was terminated by rapid washing with unsupplemented HEPES/Tris-EBSS, the cells were solubilized in 0.1% deoxycholate, and uptake was determined by liquid scintillation spectrometry. Values were corrected for nonspecific processes by incubation in the presence of 10 mM unlabeled L-DCVC or the potent inhibitor SBC. Using 10 mM L-DCVC, cell-associated radioactivity did not change over a concentration range of 0.005–1 mM L-[^{35}S]DCVC. In experiments where >1 mM L-[^{35}S]DCVC was used, SBC 10 mM was found to be an effective inhibitor of uptake. Uptake buffer with equimolar replacement of choline chloride for sodium chloride was used for the Na^+-free incubations. Protein determinations were done on a small aliquot of the solubilized cells by the Bradford procedure (13). Kinetic parameters were determined using a curve-fitting program designed for analysis of nonlinear processes.

To determine the fraction of cell-associated radiolabel present as unmetabolized L-DCVC, cell protein was precipitated with 0.25 ml of 2.5 M perchloric acid after a 5-min uptake with L-[^{35}S]DCVC (0.1–0.2 μCi/ml). The supernatant resulting from neutralization with KOH in phosphate buffer and centrifugation for 5 min at 12,000 × *g* was analyzed by HPLC. Aliquots were spiked with 25 nmol of unlabeled L-DCVC and chromatographed using a reverse phase C_{18} μBondapak column (Waters Associates, Bedford, MA) eluted with 5% methanol in 0.1 M KH_2PO_4 (pH 3.0) at 1 ml/min as described elsewhere (12). One-ml fractions were collected and counted by liquid scintillation spectrometry. Co-chromatography of the radioactivity associated with added L-DCVC carrier, as measured by absorbance at 257 nm, was used to determine the fraction present as unmetabolized L-DCVC.

L-DCVC metabolism. Cysteine conjugate β-lyase activity with L-DCVC was determined in LLC-PK_1 cell homogenates by the extraction assay of Stevens *et al.* (10). Briefly, cells grown to confluence on 150-mm tissue culture dishes were homogenized in 0.02 M Tris-HCl, pH 7.5, containing 1 mM EDTA and 20 μg/ml leupeptin and dialyzed overnight against the same buffer. Incubation mixtures contained 0.25 ml of 1 mM L-[^{14}C]DCVC, test compound, and homogenate in the presence of 50 mM Tris-HCl, pH 8.0, at 37°. After 30 min, the reaction was terminated by the addition of 0.125 ml of 2 N HCl and extracted with 2 ml of ethyl acetate. The organic layer containing the product, [^{14}C]pyruvate (10), was analyzed by liquid scintillation spectrometry.

Measurement of cell death in LLC-PK_1. Cell death was measured by the release of the intracellular enzyme LDH. Confluent cell cultures grown on 24-well dishes were washed twice with EBSS followed by the addition of L-DCVC and/or test compound dissolved in the same medium. Aliquots of medium were removed at the appropriate time and LDH activity was assayed by following the oxidation of NADH (0.1 mM) at 340 nm in the presence of pyruvate (1 mM) and 0.2 M Tris-HCl, pH 7.3, in a 1-ml volume. Cell death was expressed as a percentage of LDH released from control cells treated with 0.2% Triton X-100.

Statistics. The Student's *t* test was used to test for significance ($p < 0.05$) when values were compared only to controls. In instances where it was desirable to compare among *S*-cysteine conjugates, the one-way analysis of variance was used to test for significance followed by the Duncan multiple range test to compare individual groups. In the case of toxicity data, where the variance among treatments was heterogenous as determined by the *F* test, the Kruskal-Wallis non-parametric analysis of variance and the distribution free multiple comparisons test were used to test for statistical significance among groups.

Results

Time course of L-DCVC uptake. Fig. 1 shows the accumulation of L-DCVC in confluent monolayers of LLC-PK_1 cells as a function of incubation time. The rate of uptake remained linear for 5 min and continued to increase in a nonlinear fashion approaching steady state by 30 min. Uptake over the first 5 min was linear at several concentrations between 5 and 500 μM. Replacement of sodium in the incubation medium with choline had no effect on the transport process suggesting that L-DCVC uptake was sodium independent.

DCVC uptake during growth in culture. Since it has been demonstrated that several transport processes are regulated by factors affecting cell growth and differentiation in LLC-PK_1 cells as well as other cultured cells (15, 16), it was necessary to examine saturable L-DCVC uptake as a function of LLC-PK_1 cell density and time in culture. This was done by determining uptake at 5 μM and 500 μM DCVC at various times following cell seeding (Fig. 2). These concentrations represent high and low affinity L-DCVC transport, as will be demonstrated in a subsequent section. Uptake at both concentrations was high (days 1–3) when the cells were subconfluent and undergoing rapid growth as indicated by the exponential rise in total cell protein per dish. This was followed by a rapid drop in the transport by 62% and 83% at the high and low concentrations of L-DCVC, respectively. This decline in the rate of uptake occurred when the cells appeared to have attained confluence by both microscopic examination and the decline in the rate of growth. In addition, cultures had begun to produce domes, indicating that occluding junctions are found (16). No differences were seen between Na^+-dependent and Na^+-independent L-DCVC transport in LLC-PK_1 cells over the 7 days following seeding (data not shown). All further experiments

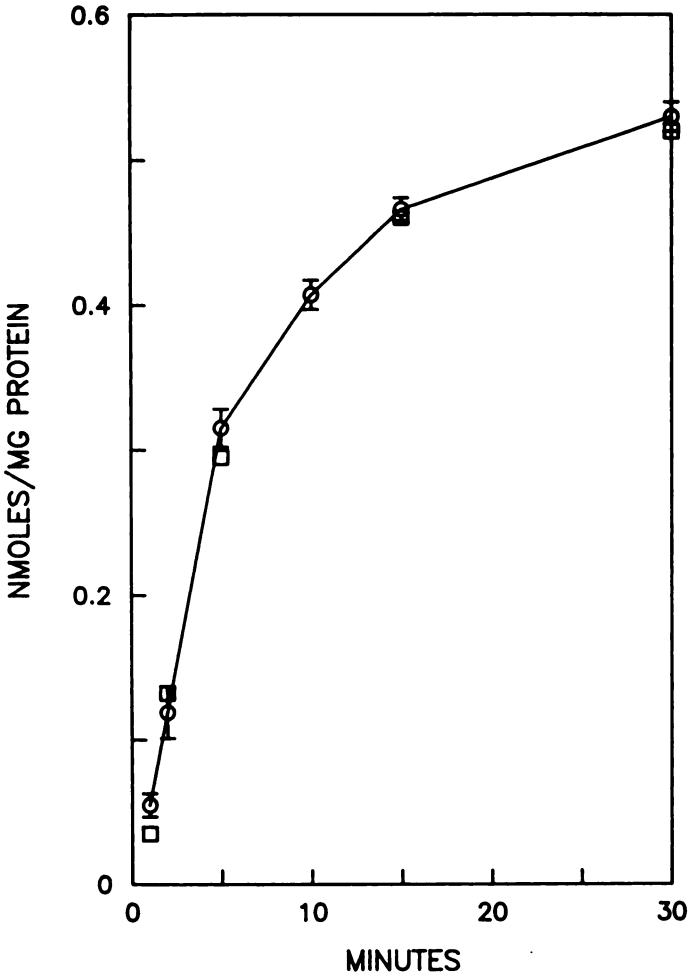

Fig. 1. Time course of L-DCVC uptake in LLC-PK$_1$. Confluent monolayers of LLC-PK$_1$ cells were incubated for the indicated times with 20 μM L-DCVC in the presence of 116.5 mM Na^+ (○) or buffer in which Na^+ was replaced by equimolar choline (□). Uptake assays were performed as described in Materials and Methods. Results are expressed as the mean ± SD (N = 3) except 10 min uptake (mean ± range, N = 2).

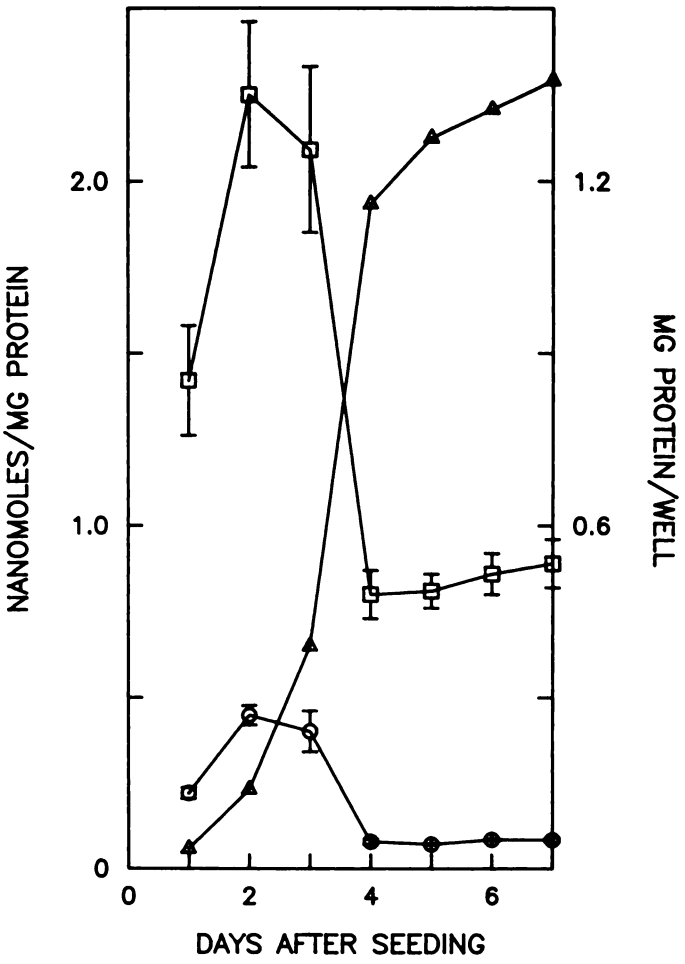

Fig. 2. L-DCVC uptake in LLC-PK$_1$ as a function of days in culture. LLC-PK$_1$ cells were seeded in 12-well dishes at a density of 1 × 10^3 cells/well. Five μM (○) and 500 μM (□) L-DCVC were incubated with cells at the indicated days after seeding and 5-min uptake assays were performed as described in Materials and Methods. Results are expressed as the mean ± SD of three independent observations. The mean cell protein content of three wells (△) was also determined at each day by the procedure outlined in Materials and Methods.

were performed on confluent monolayers (>5 days after seeding).

Concentration dependence of L-DCVC uptake. Initial uptake velocity was determined during a 5-min incubation over a 500-fold range of L-DCVC concentrations in the incubation medium (5 μM–2.5 mM). The characteristics of uptake suggest the presence of both saturable and nonsaturable processes (Fig. 3A). Kinetic analysis of the saturable uptake using an Eadie-Hofstee representation best fits a two-component model characterized by high (K_m = 13 μM, V_{max} = 47 pmol/min/mg of protein) and low (K_m = 336 μM, V_{max} = 168 pmol/min/mg of protein) affinity transport (Fig. 3B). Uptake measurements at several concentrations in the absence of sodium suggest that both these processes are sodium independent (Table 1).

Since L-DCVC can be metabolized by LLC-PK$_1$ cells, it was important to determine the extent of metabolism during the uptake procedure and whether this process influenced the kinetics of transport. HPLC analysis of intracellular L-[^{14}C]DCVC following 5 min uptake at a high (500 μM) and low (5 μM) concentration indicated that greater than 90% of the radioactivity was recovered as the unmetabolized S-cysteine conjugate (data not shown). In addition, pretreatment of LLC-PK$_1$ cells with AOA, which has been shown to inhibit L-DCVC metabolism *in situ* (10), did not alter transport at several concentrations of the S-cysteine conjugate (data not shown). These data suggest that metabolism does not influence the uptake of L-DCVC during the 5-min incubation period.

***cis*-Inhibition of L-DCVC uptake.** In order to assess the specificity of the agencies responsible for S-cysteine conjugate transport in LLC-PK$_1$ cells, L-DCVC uptake (20 μM) in the presence of a 1 mM concentration of several other S-cysteine conjugates, amino acids, and organic acids was examined (Table 2). Although all compounds tested showed some inhibition, the best inhibitors were S-cysteine conjugates and neutral amino acids with large nonpolar side chains. Histidine was considered a neutral amino acid since imidazole uncharged at pH 7.4. In contrast, most neutral amino acids with small side chains, charged amino acids, and mercapturates produced relatively weak to moderate inhibition.

The effect of several inhibitors on the high and low affinity transport systems was examined. From the kinetic parameters it is estimated that 85% of L-DCVC uptake at 5 μM should be mediated by the high affinity system, while 70% of the transport

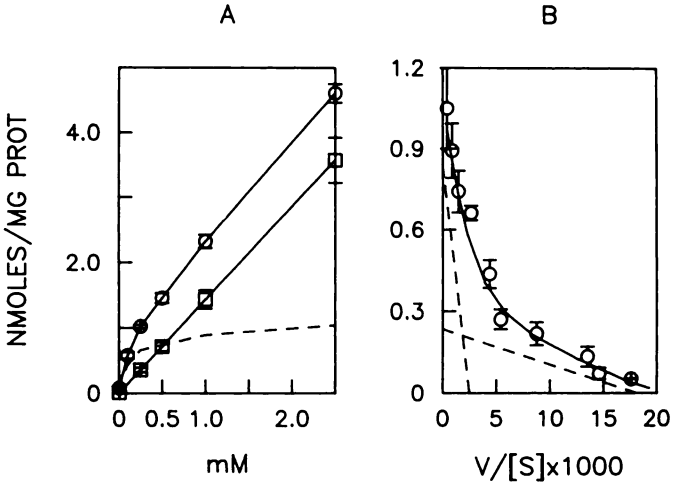

Fig. 3. A. Concentration dependence of L-DCVC uptake in LLC-PK$_1$. Confluent monolayers of LLC-PK$_1$ cells were incubated with the indicated concentrations of L-DCVC for 5 min. Uptake assays were conducted as outlined in Materials and Methods with total uptake (○) and nonsaturable entry measured in the presence of 10 mM unlabeled substrate (□). Subtracting the nonsaturable component from total uptake reveals the saturable component (– – –). Results are expressed as the mean ± SD of six experiments done in triplicate. B. Edie-Hofstee plot of L-DCVC uptake shown in A. Kinetic analysis of the saturable uptake revealed a high affinity component and low affinity component (– – –). Calculated kinetic parameters are: high, K_m = 13 ± 0.72 (μM), V_{max} = 47 ± 13 (pmol/min/mg of protein), and low, K_m = 336 ± 44 (μM), V_{max} = 168 ± 55 (pmol/min/mg of protein). These values are expressed as the mean ± SD of three independent plots.

TABLE 1

Na^+-dependent and -independent uptake at several concentrations of L-DCVC in LLC-PK$_1$ cells

Confluent monolayers of LLC-PK$_1$ cells were incubated with the indicated concentrations of L-DCVC in the presence of either 116.5 mM Na^+ or equimolar equivalents of choline (–Na^+). Five-min uptake assays were performed as described in Materials and Methods. Results are expressed as mean ± SD of six independent determinations. Uptake values in the presence and absence of Na^+ at each concentration were not significantly different ($p > 0.05$).

Concentration	Uptake	
	+Na^+	–Na^+
μM	*nmol/mg protein*	
5	0.059 ± 0.002	0.059 ± 0.002
25	0.27 ± 0.02	0.26 ± 0.01
100	0.49 ± 0.07	0.45 ± 0.05
500	0.79 ± 0.09	0.71 ± 0.07

at 500 μM should occur via the low affinity system. All of the *S*-cysteine conjugates were potent inhibitors of L-DCVC uptake at both concentrations (Table 3). Examination of the relative degrees of inhibition revealed that increasing hydrophobicity of the *S*-cysteine conjugates resulted in greater inhibition for the low affinity system (SBC > SEC > SMC). Inhibition of high affinity L-DCVC uptake by SBC was not significantly greater than that by SEC, probably due the very high degree of inhibition demonstrated by both compounds (>94%). D-DCVC was an effective inhibitor of L-DCVC uptake, suggesting that neither transport system was stereospecific with respect to *cis*-inhibition at these concentrations. Studies using amino acid analogs specific for several amino acid transport systems revealed that BCH, a system L-specific substrate, was a good inhibitor of both high and low affinity uptake of L-DCVC. In contrast, the system A substrates, AIB and the *N*-methylated analog of AIB, showed only moderate inhibition of L-DCVC uptake at both concentrations (14). Data from Tables 2 and 3 indicate that *S*-cysteine conjugates are predominantly transported in LLC-PK$_1$ cells by systems specific for nonpolar amino acids with hydrophobic side chains, characteristic of amino acid transport system L.

TABLE 2

Inhibition of L-DCVC uptake in LLC-PK$_1$ cells

Confluent monolayers of LLC-PK$_1$ cells were incubated with 20 μM L-[^{35}S]DCVC in the presence of 1 mM test compound and analyzed for uptake as described in Materials and Methods. Results are the mean ± SE of six independent observations. All values were significantly different from controls ($p < 0.05$).

Test compound (1 mM)	Percentage inhibition
S-Cysteine conjugates	
D-DCVC	88 ± 2
SMC	86 ± 3
SEC	95 ± 1
SBC	100 ± 1
CTFC	96 ± 1
HFPC	100 ± 1
Large, neutral amino acids	
Leucine	96 ± 2
Isoleucine	94 ± 2
Methionine	88 ± 1
Phenylalanine	100 ± 1
Histidine	88 ± 2
Small, neutral amino acids	
Alanine	25 ± 1
Serine	37 ± 2
Cysteine	38 ± 3
Charged amino acids	
Aspartic acid	19 ± 1
Arginine	24 ± 2
Organic anions	
N-acetyl DCVC	15 ± 2
p-Aminohippuric acid	14 ± 1

TABLE 3

Inhibition of 5 μM and 500 μM L-DCVC uptake in LLC-PK$_1$ cells

Confluent monolayers of LLC-PK$_1$ cells were incubated with the indicated concentrations of L-DCVC and test compounds during a 5-min uptake assay. The concentrations of test compounds employed were 1 mM and 10 mM with 5 μM and 500 μM L-DCVC incubations, respectively. Results are expressed as the mean ± SE of six independent observations. All values at both concentrations were different from controls ($p < 0.05$). When cysteine conjugates were compared with each other, SMC was different from SBC ($p < 0.05$) at 5 μM L-DCVC. However, all *S*-cysteine conjugates were different from each other ($p < 0.05$) with the exception of SEC and D-DCVC when 500 μM was used.

Test compound	Percentage inhibition	
	5 μM L-DCVC	500 μM L-DCVC
MeAIB[a]	40 ± 5	17 ± 1
AIB	34 ± 3	29 ± 3
SMC	88 ± 3	63 ± 2
BCH	87 ± 2	78 ± 2
SEC	94 ± 2	84 ± 1
D-DCVC	93 ± 2	88 ± 2
SBC	100 ± 2	100 ± 3

[a] MeAIB, α-(methylamino)isobutyric acid.

trans-Stimulation of L-DCVC uptake. To further confirm that system L is the predominant agency by which *S*-cysteine conjugates are transported in LLC-PK$_1$ cells, the ability to stimulate L-DCVC uptake by preloading the cells with other system L substrates was examined. This phenomenon, called *trans*-stimulation, is also a property of this amino acid transport system (17). *trans*-Stimulation of high (5 μM) and low (500 μM) affinity L-DCVC uptake was measured after preincubation with a 20-fold excess of various *S*-cysteine conjugates or amino acids and rinsing the cells prior to measuring

L-DCVC uptake (Table 4). The results show that SEC, SMC, D-DCVC, BCH, and histidine were able to *trans*-stimulate L-DCVC uptake. Unlike *cis*-inhibition, however, the ability of *S*-cysteine conjugates to *trans*-stimulate is *inversely* proportional to their hydrophobicity (SMC > SEC > SBC). In fact, SBC shows no tendency to *trans*-stimulate despite its potency as an inhibitor of L-DCVC transport. As expected, two amino acids that are not transported by system L, AIB and lysine, do not stimulate *S*-cysteine conjugate uptake.

It is also clear from Table 4 that L-DCVC uptake at the 500 μM concentration is significantly more sensitive to *trans*-stimulation than uptake at the 5 μM concentration. Examination of the concentration dependence of L-DCVC uptake in cells preloaded with a 20-fold excess of SMC revealed that the kinetics are best described by a 7-fold increase in the V_{max} of the low affinity uptake system with only a 3-fold increase in the V_{max} of the high affinity system (Table 5). Although *trans*-stimulation increases the K_m for both systems, this may be due to a small inhibitory contribution from residual SMC following preloading. The V_{max}/K_m values, which approximate the first order rate constant at low substrate concentration for the two systems, imply that low affinity uptake at low substrate concentrations may be preferentially enhanced by *trans*-stimulation. These data indicate that the major contribution to *trans*-stimulation of DCVC uptake is an increase in the V_{max} of the low affinity system.

Modulation of L-DCVC toxicity by nontoxic *S*-cysteine conjugates. Since *trans*-stimulation had a profound influence on the transport of L-DCVC, the effect of preloading LLC-PK_1 cells with the nontoxic *S*-cysteine conjugates and amino acids was examined. In this experiment, cells were preloaded with 10 mM test compound for 60 min prior to the administration of 0.5 mM L-DCVC. Cell death, as measured by the release of intracellular LDH into the media, was then assessed at a time when L-DCVC had just begun to produce death in cells that had not been preloaded (Table 6). Those *S*-cysteine conjugates and amino acids previously shown to *trans*-stimulate L-DCVC uptake enhanced the degree of intracellular LDH release, whereas AIB and SBC, both ineffective at stimulating uptake, did not potentiate L-DCVC toxicity.

The effect of the same nontoxic *S*-cysteine conjugates on the toxicity of L-DCVC in the absence of preloading was also examined. Cells were exposed to a concentration of L-DCVC (20 μM) previously shown to be toxic (10) in the presence or absence of a 1 mM concentration of several nontoxic *S*-cysteine conjugates (Table 7). SMC, SEC, SBC, D-DCVC, and BCH, all of which inhibited L-DCVC uptake, also blocked the toxicity caused by this *S*-cysteine conjugate. A correlation coefficient

TABLE 4

trans-Stimulation of L-DCVC uptake in LLC-PK_1 cells

Confluent monolayers of LLC-PK_1 cells were preloaded with the indicated test compounds by incubating for 60 min in the presence of a 20-fold greater concentration than that used to examine L-DCVC uptake. Cells were washed extensively before determining L-DCVC uptake at 5 and 500 μM as described in Materials and Methods. Results are reported as mean ± SE of six independent observations. When 5 μM L-DCVC was used, D-DCVC, BCH, L-DCVC, SEC, histidine, and SMC were different from control ($p < 0.05$). All *S*-cysteine conjugates were different ($p < 0.05$) from each other with the exception of L-DCVC and SEC. When 500 μM L-DCVC was used, all treatments with the exception of lysine and AIB were different from control ($p < 0.05$) and all *S*-cysteine conjugates were different from each other ($p < 0.05$).

Preloaded test compound	Percentage of control	
	5 μM L-DCVC	500 μM L-DCVC
None	100 ± 2	100 ± 6
SBC	98 ± 3	78 ± 3
Lysine	92 ± 3	98 ± 4
AIB	107 ± 2	94 ± 5
D-DCVC	115 ± 2	239 ± 6
BCH	125 ± 4	252 ± 6
L-DCVC	125 ± 4	260 ± 4
SEC	127 ± 3	291 ± 8
Histidine	141 ± 5	383 ± 7
SMC	137 ± 3	428 ± 15

TABLE 5

The effect of *trans*-stimulation on the kinetics of L-DCVC uptake in LLC-PK_1 cells

L-DCVC uptake assays at concentrations ranging from 0.0025–5 mM were done on confluent cultures of LLC-PK_1 cells that had been preincubated for 60 min in the presence (+) or absence (−) of a 20-fold excess of SMC in EBSS. The cells were extensively washed with unsupplemented EBSS before incubation with L-DCVC. Kinetic parameters were determined from the nonlinear curve fitting analysis described in Materials and Methods. Values are mean ± SD for at least two kinetic curves. K_m and V_{max}, under conditions of *trans*-stimulation were significantly different ($p < 0.05$) from their respective controls.

Uptake system	*trans*-Stimulation	K_m	V_{max}	V_{max}/K_m
		mM	nmol × min^{-1} × mg^{-1} protein	
High affinity	−	0.013 ± .002	0.047 ± 0.013	3.61
High affinity	+	0.034 ± 0.002	0.136 ± 0.023	4.00
Low affinity	−	0.336 ± 0.044	0.168 ± 0.055	0.50
Low affinity	+	1.00 ± 0.25	1.19 ± 0.23	1.19

TABLE 6

LLC-PK_1 cell toxicity produced by L-DCVC after preloading with nontoxic *S*-cysteine conjugates and amino acids

Confluent monolayers of LLC-PK_1 cells were initially preincubated with 10 mM test compound for 60 min followed by extensive washing with EBSS. Cells were then treated with 0.5 mM L-DCVC for 10 hr before the media were assayed for LDH. Toxicity due to preincubation with the test compounds was minimal (3–5% LDH release). Results are expressed as the mean ± SE of six independent observations. With the exception of AIB and SBC, all values are significantly greater than controls ($p < 0.05$). All *S*-cysteine conjugates were different ($p < 0.05$) from each other.

Test compound	Percentage of LDH release	Percentage of control
None	6.7 ± 1.0	100
AIB	5.6 ± 0.8	84
SBC	6.6 ± 1.7	98
D-DCVC	11.3 ± 2.9	169
BCH	14.3 ± 4.3	213
SEC	20.4 ± 4.7	304
Histidine	36.4 ± 6.3	543
SMC	41.2 ± 12.2	614

TABLE 7

LLC-PK_1 cell toxicity produced by L-DCVC in the presence of nontoxic *S*-cysteine conjugates and amino acids

Confluent monolayers of LLC-PK_1 cells were incubated with 20 μM L-DCVC in the presence of 1 mM test compounds dissolved in EBSS. Incubation media were assayed for LDH 14 hr following introduction of compounds as described in Materials and Methods. The toxicity due to test compounds themselves was minimal (3–5% LDH release). Results have been adjusted for LDH release contributed by the test compound alone and are expressed as the mean ± SE of six independent observations. With the exception of AIB, all values are different ($p < 0.05$) from control. All *S*-cysteine conjugates are different ($p < 0.05$) from each other except SEC and D-DCVC.

Test compound	Percentage of LDH release	Percentage inhibition
None	26.6 ± 2.0	0
Histidine	45.3 ± 4.6	−70
AIB	27.6 ± 5.3	−4
SMC	16.8 ± 1.9	37
BCH	11.0 ± 0.4	59
SEC	10.8 ± 0.5	56
D-DCVC	10.5 ± 0.4	61
SBC	5.3 ± 0.3	80

of 0.99 was obtained when percentage inhibition of uptake (abscissa) was plotted against percentage inhibition of toxicity for the *S*-cysteine conjugates (data not shown). Although histidine inhibited transport, it potentiated the toxicity of L-DCVC. AIB, which is not an inhibitor of L-DCVC uptake, does not alter the toxicity caused by this *S*-cysteine conjugate.

Since it had been shown that L-DCVC must be metabolized in order to express toxicity, it seemed possible that the nontoxic *S*-cysteine conjugates might interfere with DCVC metabolism, thus blocking the toxicity. Therefore, the effect of these conjugates on DCVC metabolism by LLC-PK_1 cell homogenates was examined (Table 8). The four compounds studied caused only mild inhibition of metabolism (20–37%). Even though the degree of inhibition among the *S*-cysteine conjugates was not significantly different, a correlation coefficient of −0.92 was obtained when percentage inhibition of metabolism (abscissa) was plotted against percentage inhibition of toxicity. In contrast, the previously established inhibitor of L-DCVC metabolism, AOA (4), caused substantial inhibition of this activity in LLC-PK_1 cell homogenates.

Discussion

The evidence presented here indicates that L-DCVC is taken up in LLC-PK_1 cells by an amino acid transport system which has several key features in common with the basolaterally located system L previously described in this cell line (18, 19). It is Na^+ independent and strongly inhibited by BCH, a system L-specific substrate, as well as large nonpolar amino acids and other *S*-cysteine conjugates. The weak inhibition by polar, acidic, and basic amino acids and mercapturates suggests that other transport systems do not significantly contribute to uptake. Although other amino acid transport systems show *trans*-stimulation, *S*-cysteine conjugate transport is *trans*-stimulated by substrates known to be effective with system L (20).

Kinetic analysis of L-DCVC uptake suggests the existence of a high affinity transport system and a low affinity system with about a 25-fold higher K_m. Although there are no discernible differences in substrate specificity between the two systems, the low affinity transport appears to be much more sensitive to *trans*-stimulation. The existence of multiple transport systems with system L-like properties is consistent with evidence suggesting considerable heterogeneity in sodium-independent neutral amino acid transport. This has been described in rat hepatocytes (21) and erythrocytes of several species including humans (22). It is currently believed that the classical system L is an amalgam of distinct transport components that possess similar characteristics (23).

It is clear that transport of LLC-PK_1 can play a key role in modifying the toxicity of nephrotoxic *S*-cysteine conjugates, such as L-DCVC. For example, toxicity was potentiated in cells preloaded with nontoxic transport substrates prior to incubation with L-DCVC in a manner which correlates with the ability of the substrates to *trans*-stimulate uptake. Conversely, incubating with a toxic dose of L-DCVC in the presence of a competing transport substrate significantly reduces toxicity. The one exception, histidine, suggests that modulation of toxicity using this protocol reflects a compound's ability to both *cis*-inhibit and *trans*-stimulate uptake.

Protection against toxicity by the nontoxic *S*-cysteine conjugates does not appear to involve alterations in L-DCVC metabolism. Although the inhibition of metabolism was modest and the degree of inhibition among the *S*-cysteine conjugates was not statistically different (Table 8), the data may suggest that the more hydrophilic conjugates (i.e., SMC) were the best inhibitors. This is in contrast to Tables 3 and 7 which show that the more hydrophobic conjugates (i.e., SBC) are the best inhibitors of uptake and toxicity, respectively.

It is interesting that the substrate requirements for *cis*-inhibition and *trans*-stimulation of uptake are not the same. The most potent inhibitors of L-DCVC uptake appear to be amino acids with bulky ring substituents such as SBC and phenylalanine, whereas substrates with less bulky side groups such as SMC are less effective. This is not the case with *trans*-stimulation. The more hydrophilic substrates such as SMC and histidine are better stimulators than their more hydrophobic counterparts. In fact, the most hydrophobic substrate and best inhibitor, SBC, does not appear to *trans*-stimulate at all. The explanation for this dichotomy is not known. Whereas *cis*-inhibition probably accurately reflects the affinity of a substrate for the primary binding site, *trans*-stimulation may be more complicated. This process is believed to represent an exchange diffusion in which the translocation rate of the substrate-loaded carrier is greater than that of the empty carrier (24). In this case the ability of the preloaded substrate to accumulate intracellularly would bring into play factors such as uptake by other transport systems, compartmentation within the cell, and intracellular metabolism.

Besides the processes of *cis*-inhibition and *trans*-stimulation, the transport of *S*-cysteine conjugates is also responsive to cell growth and maturation. Two factors may contribute to the lower transport in confluent cell cultures. The first is adaptive regulation of amino acid transport in response to the reduced growth state of the cells noted in several cell types (19, 25), including LLC-PK_1 cells (16). However, the sodium-independent amino acid transport system L is considered the least sensitive to this type of regulation. Despite this, it has been reported that leucine uptake by system L in another kidney cell line, MDCK, decreased about 3-fold when the cells reached confluence (26). A second explanation for this decreased uptake results from cell polarization. As they reach confluence, LLC-PK_1 cells attach via tight junctions after becoming closely apposed and subsequently develop membrane asymmetry (7). This could result in the polarization of the L transport systems to the basolateral membrane leading to restricted access of substrate and a reduction in uptake. Both autoradiographic studies (27) and uptake experiments with LLC-PK_1 cells grown

TABLE 8

Inhibition of L-DCVC metabolism in LLC-PK_1 cells

Homogenates from confluent monolayers of LLC-PK_1 cells were incubated with 1 mM L-[^{14}C]DCVC in the presence of the above inhibitors using the assay described previously. Specific activities are expressed as pmol/min/mg of protein and are presented as the mean ± SE of six determinations. AOA was preincubated with homogenates 15 min prior to assay. All values are different from control ($p < 0.01$); however, none of the *S*-cysteine conjugates are significantly different ($p > 0.05$) from each other.

Inhibitor	Specific activity	Percentage inhibition
None	166 ± 10	0
D-DCVC (10 mM)	119 ± 14	28
SMC (10 mM)	105 ± 9	37
SEC (10 mM)	109 ± 11	34
SBC (10 mM)	123 ± 3	20
AOA (0.1 mM)	19 ± 1	88

on a permeable support (19) indicate that neutral amino acid uptake is localized to the basolateral membrane in confluent cultures. This is consistent with localization of Na^+-independent uptake of neutral amino acids to the basolateral membrane (28).

Although the role of system L transport in *S*-cysteine conjugate uptake and toxicity is relatively straightforward in LLC-PK_1 cells, its function in the proximal tubule may be considerably more complex. Currently it is thought that *S*-glutathione conjugates are degraded by peptidases of the brush border of the proximal tubule, and the resulting *S*-cysteine conjugates are absorbed from the lumen (29), perhaps by an Na^+-dependent transport system (30). Inoue *et al.* (30, 31) have shown that, prior to renal excretion of the mercapturate, *S*-carbamidomethyl cysteine exists the kidney, presumably at the basolateral surface, and is *N*-acetylated by the hepatic *N*-acetyl transferase. Although the LLC-PK_1 cells demonstrate no Na^+-dependent uptake of *S*-cysteine conjugates under the conditions used in this study, the system L-like transport system described has several features which should be considered with regard to the basolateral flux of *S*-cysteine conjugates in the proximal tubule. First, the system is reversible and therefore could mediate the exodus, as well as the entry, of *S*-cysteine conjugates. Second, the low affinity component is *trans*-stimulated by certain amino acids which could result in substantial accumulation of toxic *S*-cysteine conjugates, a circumstance which, as we have shown, increases toxicity. Besides system L, it should be pointed out that other basolateral systems responsible for the transport of *S*-glutathione conjugates (32) and mercapturates (4, 33) may also play a role in *S*-cysteine conjugate toxicity *in vivo*.

In conclusion, *S*-cysteine conjugates can use existing amino acid transport systems for uptake in the proximal tubule cell line, LLC-PK_1. Furthermore, this transport joins metabolism of these compounds (4, 10) as a key determinant of toxicity. Uptake of *S*-cysteine conjugates *in vivo* appears to be more complex due to the hepatorenal cooperation utilized in mercapturate biosynthesis and excretion, necessitating the involvement of both multiple metabolic intermediates and transport systems. Discerning the importance of system L, as well as other modes of uptake, in the renal handling and nephrotoxicity of *S*-cysteine conjugates *in vivo*, will require characterization of transport in several other preparations such as membrane vesicles, isolated proximal tubule cells, and hepatocytes.

References

1. Chasseaud, L. F. Conjugation with glutathione and mercapturic acid excretion, in *Glutathione: Metabolism and Function* (W. B. Jakoby and I. M. Arias, eds). Raven Press, New York, 77–114 (1976).
2. Elfarra, A. A., and M. W. Anders. Renal processing of glutathione conjugates; role in nephrotoxicity. *Biochem. Pharmacol.* **33**:3729–3732 (1984).
3. Terracini, B., and V. H. Parker. A pathological study on the toxicity of *S*-dichlorovinyl-L-cysteine. *Food Cosmet. Toxicol.* **3**:67–74 (1965).
4. Elfarra, A. A., I. Jakobson, M. W. Anders. Mechanism of *S*-(1,2-dichlorovinyl)glutathione-induced nephrotoxicity. *Biochem. Pharmacol.* **35**:283–288 (1986).
5. Anderson, P. M., and M. O. Schultze. Interactions of *S*-(1,2-dichlorovinyl)-L-cysteine with proteins. *Arch. Biochem. Biophys.* **111**:593–602 (1965).
6. Jakoby, W. B., J. L. Stevens, M. W. Duffel, and R. A. Weisiger. The terminal enzymes of mercapturate formation and the thiomethyl shunt. *Rev. Biochem. Toxicol.* **5**:97–115 (1984).
7. Hull, R. N., W. R. Cherry, and G. W. Weaver. The origin and characteristics of a pig kidney cell strain LLC-PK_1. *In Vitro* Cell. Dev. Biol. **12**:670–677 (1976).
8. Mullin, J. M., and A. Kleinzeller. Sugar transport in the renal epithelial cell culture, in *Tissue Culture of Epithelial Cells* (M. Taub, ed.). Plenum Press, New York, 71–86 (1985).
9. Rabito, C. A. Occluding junctions in a renal cell line (LLC-PK_1) with characteristics of proximal tubule cells. *Am. J. Physiol.* **250**:F735–F743 (1986).
10. Stevens, J., P. Hayden, and G. Taylor. The role of glutathione conjugate metabolism and cysteine conjugate β-lyase in the mechanism of *S*-cysteine conjugate toxicity in LLC-PK_1 cells. *J. Biol. Chem.* **261**:3325–3332 (1986).
11. McKinney, L. L., J. C. Picken, R. B. Weakely, R. E. Eldridge, R. B. Campbell, J. C. Cowan, and H. E. Beister. Possible toxic factor of trichloroethylene-extracted soybean oil meal. *J. Am. Chem. Soc.* **81**:909–915 (1959).
12. Hayden, P., V. Schaeffer, G. Larsen, and J. L. Stevens. Radiolabelled *S*-cysteine conjugates. *Methods Enzymol.*, in press.
13. Bradford, M. M. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1976).
14. Amsler, K., C. Shaffer, and J. S. Cook. Growth-dependent AIB and meAIB uptake in LLC-PK_1 cells: effects of differentiation inducers and of TPA. *J. Cell. Physiol.* **114**:184–190 (1983).
15. Shotwell, M. A., M. S. Kilberg, and D. L. Oxender. The regulation of neutral amino acid transport in mammalian cells. *Biochim. Biophys. Acta.* **737**:267–284 (1983).
16. Lever, J. E. Cell culture models to study epithelial transport, in *Membranes and Transport* (A. N. Martonosi, ed.) Vol. 2. Plenum Press, New York, 231–236 (1982).
17. Christensen, H. N. Organic ion transport during seven decades: the amino acids. *Biochim. Biophys. Acta.* **779**:255–269 (1984).
18. Sepulveda, F. V., and J. D. Pearson. Characterization of neutral amino acid uptake by cultured epithelial cells from pig kidney. *J. Cell. Physiol.* **112**:182–188 (1982).
19. Rabito, C. A., and M. V. Karish. Polarized amino acid transport by an epithelial cell line of renal origin. *J. Biol. Chem.* **257**:6802–6808 (1982).
20. Oxender, D. L., and H. N. Christianson. Distinct mediating systems for the transport of neutral amino acids by the Ehrlich cell. *J. Biol. Chem.* **238**:3686–3699 (1963).
21. Weissbach, L., M. E. Handlogten, H. N. Christenson, and M. S. Kilberg. Evidence for two Na^+-independent neutral amino acid transport systems in primary cultures of rat hepatocytes. *J. Biol. Chem.* **257**:12006–12011 (1982).
22. Rosenberg, R., J. D. Young, and J. C. Ellory. L-Tryptophan transport in human red blood cells. *Biochim. Biophys. Acta.* **598**:375–384 (1980).
23. Vadgama, J. V., and H. N. Christensen. Discrimination of Na^+-independent transport systems L, T, and ASC in erythrocytes. *J. Biol. Chem.* **260**:2912–2921 (1985).
24. Harrison, L. I., and H. N. Christensen. Stimulation of differential effects on rates in membrane transport. *J. Theor. Biol.* **49**:439–459 (1975).
25. Guidotti, G. G., A. F. Borghetti, and G. C. Gazzola. The regulation of amino acid transport in animal cells. *Biochim. Biophys. Acta.* **515**:329–366 (1978).
26. Boerner, P., and M. H. Saier. Growth regulation and amino acid transport in epithelial cells: influence of culture conditions and transformation on A, ASC, and L transport activities. *J. Cell. Physiol.* **113**:240–246 (1982).
27. Sepulveda, F. V., and J. D. Pearson. Localization of alanine uptake by cultured renal epithelial cells to the basolateral membrane. *J. Cell. Physiol.* **118**:211–217 (1984).
28. Schafer, J. A., and D. W. Barfuss. Membrane mechanisms for transepithelial amino acid absorption and secretion. *Am. J. Physiol.* **238**:F335–F346 (1980).
29. Ullrich, K. J. Sugar amino acid and Na^+-cotransport in the proximal tubule. *Annu. Rev. Physiol.* **41**:181–195 (1979).
30. Inoue, M., K. Okajima, and Y. Morino. Metabolic coordination of liver and kidney in mercapturic acid biosynthesis *in vivo*. *Pathology* **2**:311–318 (1982).
31. Inoue, M., K. Okajima, and Y. Morino. Hepato-renal cooperation in biotransformation, membrane transport, and elimination of cysteine *S*-conjugates of xenobiotics. *J. Biochem.* **95**:247–254 (1984).
32. Lash, L. H., and D. P. Jones. Uptake of the conjugate *S*-(1,2-dichlorovinyl)glutathione by renal basal-lateral membrane vesicles and isolated kidney cells. *Mol. Pharmacol.* **28**:278–282 (1985).
33. Nash, J. A., L. J. King, E. A. Lock, and T. Green. The metabolism and disposition of hexachloro-1:3-butadiene in the rat and its relevance to nephrotoxicity. *Toxicol. Appl. Pharmacol.* **73**:124–137 (1984).

Send reprint requests to: Dr. James L. Stevens, W. Alton Jones Cell Science Center, 10 Old Barn Road, Lake Placid, NY 12946.