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Specificity and Directionality of Thiol Effects on Sinusoidal Glutathione Transport in Rat Liver

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SUMMARY

In rats the sinusoidal glutathione (GSH) carrier transports GSH bidirectionally, and its activity is influenced by the thiol-disulfide status; the V_{max} of sinusoidal GSH efflux was increased by dithiothreitol (DTT) and decreased by cystine. In the present work we examined the specificity and directionality of the thiol effect. Using *in situ* perfused livers, we found that 1 mM DTT and other dithiols, including 1,2-ethanedithiol, 1,3-propanedithiol, and 1,4-butanedithiol, stimulated sinusoidal GSH efflux by 200–500% but dihydrothioctic acid, which is negatively charged, had no effect. Uncharged or positively charged monothiols (2 mM), such as dimercaprol, monothioglycerol, 2-mercaptoethanol, 3-mercapto-2-butanol, 1-mercapto-2-propanol, and cysteamine, also exerted a stimulatory effect on sinusoidal GSH efflux. In contrast, monothiols containing a negatively charged substituent, such as penicillamine, captopril, *N*-acetylcysteine, mercaptopropionylglycine, mercaptoethanesulfonic acid, mercaptoacetic acid, and mercaptopropionic acid, had no effect. The thiol moiety was essential for activity, inasmuch as ethanol, propanol, propanediol, and glycerol had no effect on sinusoidal GSH efflux. The effect of DTT or cystine pretreatment (2 mM or 0.5 mM, respectively, for 30 min) on GSH uptake was then examined using cultured rat hepatocytes. The linear rate of [^{35}S]GSH uptake and the concentration dependence were measured after cells were pretreated with acivicin (0.5 mM, for 15 min) and buthionine sulfoximine (10 mM, 15 min), to prevent breakdown and resynthesis of GSH from precursors, respectively. Uptake buffer also contained 20 mM α-(methylamino)isobutyric acid and 20 mM threonine (inhibitors of amino acid transport systems A and ASC, respectively), to prevent uptake of cysteine. Pretreatment with DTT decreased the V_{max} of GSH uptake by ~50% (control V_{max} value, 24 nmol/10^6 cells/30 min), whereas the K_m remained unaffected (~8 mM). Cystine pretreatment had no influence on GSH uptake but inhibited efflux. In conclusion, the presence of at least one thiol group and the absence of negative charge are required to stimulate sinusoidal GSH efflux. The direction of GSH transport is modulated by the thiol-disulfide status, so that thiol reduction changes the GSH transporter from a bidirectional GSH transporter into a preferentially unidirectional (outward) transporter by inhibiting uptake while stimulating efflux and thiol oxidation favors inward transport by inhibiting only efflux.

GSH is the main non-protein thiol within cells; it plays a key role in defense and serves as a reservoir for cysteine (1, 2). The liver plays a central role in the complex interorgan homeostasis of GSH by being the predominant source of plasma GSH (3). Our laboratory has extensively studied the transport of GSH using different experimental models. We have shown that, in rat hepatocytes, Hep G2 cells, and *Xenopus laevis* oocytes injected with rat liver poly(A)$^+$ RNA, transport of GSH occurs bidirectionally (4–6). The affinities for both uptake and efflux are low, so that the GSH transporter operates as a net efflux pump under normal physiological conditions, because the intracellular GSH concentration is several orders of magnitude greater than the extracellular GSH concentration. Recently we also discovered that the activity of the sinusoidal GSH transporter in rats is modulated by the thiol-disulfide status (7). In both cultured rat hepatocytes and perfused rat liver, DTT (1 mM) stimulated sinusoidal GSH efflux by 400%, whereas cystine (0.5 mM) inhibited sinusoidal GSH efflux by ~50% without affecting biliary GSH efflux. Kinetic analysis revealed that the V_{max} of GSH efflux was increased by DTT and decreased by cystine treatment. The present work was aimed at delineating the structural requirements and the directionality of the thiol effect on the sinusoidal GSH transporter.

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Experimental Procedures

Materials

GSH, GSSG, collagenase (type IV), bovine serum albumin, L-methionine, serine, L-cystine, NADPH, 5,5′-dithiobis-(2-nitrobenzoic acid),

ABBREVIATIONS: GSH, reduced glutathione; GSSG, oxidized glutathione; DTT, DL-dithiothreitol; BSO, DL-buthionine-(*S,R*)-sulfoximine; HPLC, high performance liquid chromatography; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TCA, trichloroacetic acid; DME/F-12, Dulbecco's modified Eagle's medium/Ham's F-12 medium (1:1 mixture); SAF, sulfur amino acid-free Dulbecco's modified Eagle's medium/Ham's F-12 medium (1:1 mixture); ANOVA, analysis of variance.

sodium EDTA, GSSG reductase, hydrocortisone, insulin, DTT, *trans*-4,5-dihydroxy-1,2-dithiane (oxidized DTT), cysteamine, L-(5*S*)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid (acivicin), HEPES, BSO, α-(methylamino)isobutyric acid, threonine, dimercaprol, *N*-acetylcysteine, penicillamine, DL-6,8-thioctic acid (reduced thioctic acid or dihydrothioctic acid), ethanol, glycerol, propanol, 1,2-propanediol, and captopril were purchased from Sigma Chemical Co. (St. Louis, MO). 1,2-Ethanedithiol, 1,3-propanedithiol, 1,4-butanedithiol, monothiolglycerol, 2-mercaptoethanol, 3-mercapto-2-butanol, 1-mercapto-2-propanol, mercaptopropionylglycine, mercaptoethanesulfonic acid, mercaptoacetic acid, and mercaptopropionic acid were purchased from Aldrich (Milwaukee, WI). DME/F-12 and custom-made SAF were purchased from Irvine Scientific (Irvine, CA). Fetal bovine serum was purchased from Gemini Bio-Products, Inc. (Calabasas, CA). HPLC-grade methanol was purchased from Fisher Scientific Co. (Springfield, NJ). [^{35}S]GSH (267 Ci/mmol) and L-[^{35}S]methionine (>1000 Ci/mmol) were purchased from New England Nuclear-DuPont (Boston, MA).

Animals

Male Sprague-Dawley rats (Harlan Laboratory Animals, Inc., Indianapolis, IN, or Hilltop Lab Animals, Inc., Scottdale, PA) weighing 260–320 g were maintained with Purina rodent chow (Ralston Purina Co., St. Louis, MO) and water available *ad libitum*.

Cell Culture Preparation

Hepatocytes were isolated aseptically according to the method of Moldeus *et al.* (8). Initial cell viability, as determined by 0.2% trypan blue exclusion, was ≥90%. The basic medium used was DME/F-12 containing high glucose levels (3151 mg/liter), 10% fetal bovine serum, 1 μg/ml insulin, and 50 nM hydrocortisone and supplemented with 1 mM methionine. Cells (1.5–2 × 10^6) in 5 ml of basic medium were plated on 60- × 15-mm dishes precoated with rat tail collagen and were incubated at 37° in 5% CO_2/95% air. Medium was changed 2–3 hr after plating to remove dead unattached cells. On average, plating efficiency was ~60%. The replacement medium was the same as the basic medium except for the omission of serum.

Bidirectional GSH Transport in Cultured Rat Hepatocytes

GSH efflux as determined by the release of prelabeled [^{35}S] GSH. Cultured cells were plated using the basic medium as described above. The following day, cell GSH was depleted by treating cells with diethyl maleate (0.5 mM) for 20 min. Cells were then washed twice with culture medium and replenished with fresh SAF supplemented with 1 mM methionine and serine plus L-[^{35}S]methionine (3–5 μCi/ml, 2 ml/60- × 15-mm plate) to label cell GSH. After 2 hr, cells were washed five times with Krebs-Henseleit buffer to remove labeled methionine and cells were incubated with 1 ml of Krebs buffer at 37° in the incubator. At the end of a 30-min incubation, cells were washed five times with ice-cold Krebs buffer and detached with trypsin-EDTA, the cell number was determined with a Coulter counter, and cell GSH was extracted with 10% TCA and processed along with medium (collected at the end of 30 min) for radio-HPLC analysis, by the method of Fariss and Reed (9), for GSH mass, molecular form, and cpm. Cell GSH levels were also determined by the method of Tietze (10). Cell GSH levels determined by HPLC and by the method of Tietze agreed closely, generally within 15%. The effect of GSH and DTT on release of prelabeled cell GSH was determined by adding these agents to the Krebs buffer during efflux experiments. Results are expressed as percentage of total labeled GSH (in cpm) released into the supernatant in 30 min.

GSH uptake. The technique used for measuring GSH uptake was as described previously (5). Cells were pretreated with acivicin (0.5 mM, for 15 min) and BSO (10 mM, for 15 min) to prevent breakdown of GSH and resynthesis of GSH from precursors, respectively. This protocol was previously verified by radio-HPLC. Specifically, the molecular form of radioisotope was mostly GSH if cells were incubated with labeled cysteine in the absence of BSO treatment but was mostly sulfate breakdown product of cysteine, with no detectable incorporation into GSH, if cells were pretreated with BSO (5).

The medium used for washing and stopping uptake was 135 mM NaCl, 1.2 mM $MgCl_2$, 0.81 mM $MgSO_4$, 27.8 mM glucose, 2.5 mM $CaCl_2$, 25 mM HEPES, adjusted to pH 7.2 with NaOH. Uptake medium was the same medium containing [^{35}S]GSH (4–6 μCi/ml, 1 ml/plate) and nonradioactive GSH (0.5–30 mM). Cells were washed once (2 ml) with prewarmed buffer and transport was initiated by addition of 1 ml of uptake medium. After incubation at 37° for the required time, uptake was terminated by washing twice (2 ml each) with ice-cold buffer, followed by incubation for another 5 min at 4° with 1.5 ml of 5% bovine serum albumin mixed in buffer, to displace surface-bound radioactivity; this was followed by three more washes at 4° (2 ml each). The washing procedure was validated by counting dpm in the supernatant after each wash. After the last wash, no radioactivity was recovered in the supernatant. Cells were then detached with trypsin-EDTA, an aliquot was used for scintillation counting, another was used for cell counting, and the remainder was treated with 10% TCA. Cell GSH levels were determined in the TCA-precipitated supernatant by the method of Tietze (10), and in some experiments the TCA-precipitated supernatant was also processed for radio-HPLC analysis by the method of Fariss and Reed (9). To estimate trapping, uptake at 4° (on ice) was studied in parallel. Duplicate plates were used for each time point and condition. The counts at 4° did not change with different concentrations of GSH (0.5–30 mM) and represented ~45% of total counts. The difference between 37° and 4° uptake values represented true uptake.

GSH (10 mM) uptake was linear up to 45 min; thus, all subsequent studies evaluating the kinetics of GSH uptake and the effects of DTT and cystine pretreatment were done using 30-min incubations. DTT or cystine treatment did not alter the 4° counts. Uptake was expressed as nanomoles of GSH/milligram of protein/30 min.

In the case of cystine pretreatment, cells were plated using SAF supplemented with methionine (1 mM), instead of DME/F-12. Before measurement of GSH uptake, in addition to pretreatment with acivicin and BSO cells were also treated with cystine (0.5 mM) or vehicle for 30 min and then washed five times. The adequacy of this washing was confirmed by radio-HPLC of the uptake buffer, which showed no GSH-cysteine mixed disulfide, which would have formed had there been any cystine present in the uptake medium.

GSH Efflux in Cultured Rat Hepatocytes

The protocol for GSH efflux in cultured cells was as described previously (7, 11). The only modification was shortening of the efflux time from 90 min to 60 min. After cultured cells were plated in basic medium (DME/F-12 plus methionine) overnight, the effect of thiols on GSH efflux was determined by preincubating cells with 2 mM (DTT) or 4 mM (monothiols) thiol in Krebs buffer for 30 min. This was followed by washing cells five times with prewarmed Krebs buffer before determination of GSH efflux. To ensure uptake of thiols, in parallel experiments cells were washed with ice-cold Krebs buffer five times at the end of a 30-min incubation with thiols, and total nonprotein thiol was determined in the TCA-precipitated supernatant by the Ellman assay (12). The number of washings was based on knowing that this was adequate for cystine treatment (see above).

Effects of Thiols on Sinusoidal GSH Efflux During *In Situ* Liver Perfusion

The design, method, and apparatus for *in situ* liver perfusion were as described previously (7, 13). To evaluate the effect of thiols on sinusoidal GSH efflux, after a base-line period of 10 min thiol agents or their alcohol controls (1 mM for dithiols and 2 mM for monothiols and alcohol controls) were added in the perfusion buffer for 20 min, they were then removed, and perfusate samples were collected every 5 min for another 30 min (total experiment time, 60 min). The perfusion rates using oxygenated Krebs-Ringer bicarbonate buffer were 4.27 ± 0.05 ml/min/g of liver, the pressure drop across the lines was 4.03 ± 0.18 cm of H_2O, and the O_2 uptake was 2.16 ± 0.05 μmol/min/g of liver during the control period (mean ± standard error, n = 78). These values compare closely with those reported previously (7, 13) and did not change significantly during treatment periods, except for treatment

with ethanedithiol, propanedithiol, and ethanol. The O_2 uptake increased 16%, 18%, and 21%, respectively, during perfusion with these three agents and reversed on their removal. Perfusate GSH levels were measured by the method of Tietze (10) (during control periods) and by HPLC. Cell lysis was monitored by the measurement of glutathione-*S*-transferase activity in the perfusate as a fraction of total liver enzyme activity, using 1-chloro-2,4-dinitrobenzoic acid and GSH as substrates (14); lysis was not cumulative and remained <0.05% during the 60-min perfusion. GSH levels were measured at the beginning and end of the experiments in liver biopsies (0.2 g) by the method of Tietze (10).

Kinetic Analyses

The kinetic model used to estimate the apparent V_{max} and the K_m, as before (11, 13, 15), was the Hill equation, represented by $V = V_{max}[GSH]^n/(K_m^n + [GSH]^n)$, where n is the number of binding/transport sites. Nonlinear least-squares fitting was done with the SAAM program (16) on an IBM 3090 computer. A detailed discussion regarding the selection of the appropriate kinetic model and criteria used for goodness of fit has been presented previously (11, 13).

Statistical Analyses

For cultured cells, each cell preparation was derived from one animal and duplicate plates were used for each condition and time point. The mean of each duplicate from one experiment was considered $n = 1$ and the means of multiple experiments were compared by paired Student's t test (two comparisons) or ANOVA (more than two comparisons). For perfused livers, treatment values (mean of three highest values or all values after the start of treatment if there was no effect) and recovery values (mean of last three values after termination of treatment) were compared with control values (mean of three determinations during the control period) by ANOVA followed by Fisher's test. For comparison of kinetic parameters, the unpaired t test was used, where n is the number of cell preparations that included the entire range of cell GSH levels. Two-tailed t tests were used unless otherwise noted.

Results

Structural Specificity of the Thiol Effect on Sinusoidal GSH Efflux

Studies in perfused liver. We previously showed that DTT, dithioerythritol, and L-DTT all stimulated GSH efflux in cultured rat hepatocytes and DTT stimulated sinusoidal GSH efflux in perfused liver (7). We now show that, in addition to DTT, dimercaprol also stimulated sinusoidal GSH efflux (Table 1). All of these agents are dithiols with adjacent hydroxyl group(s). To determine whether this relationship is critical for the stimulatory effect to occur, we studied dithiols without hydroxyl groups, such as 1,2-ethanedithiol, 1,3-propanedithiol, and 1,4-butanedithiol. As Table 1 shows, all three are potent stimulatory agents, with 1,3-propanedithiol being the most potent (520% over base-line). Surprisingly, dihydrothioctic acid, which is a dithiol with a negative charge, had no effect. Note that the degree of persistence of enhanced sinusoidal GSH efflux after removal of the exogenous thiol was less in those situations where tissue GSH levels exhibited a greater decline. This was seen with 1,2-ethanedithiol and 1,3-propanedithiol, which exhibited the greatest stimulation of efflux and, as expected, the greatest fall in liver GSH levels, which would explain the decline in the post-thiol recovery efflux rates. When monothiols were then examined, as Table 2 shows, uncharged monothiols such as monothioglycerol, 2-mercaptoethanol, 3-mercapto-2-butanol, and 1-mercapto-2-propanol and the positively charged monothiol cysteamine all stimulated sinusoidal GSH efflux, but monothiols containing a negatively charged substituent with or without an amino group, such as penicillamine, *N*-acetylcysteine, mercaptopropionylglycine, mercaptoethanesulfonic acid, mercaptoacetic acid, mercaptopropionic acid, and captopril, all had no effect. The thiol moiety was essential for this stimulatory effect to occur, because respective alcohol controls, such as glycerol, 1-propanol, 1,2-propanediol, and ethanol, had no effect on sinusoidal GSH efflux (Table 3). In all perfusions with thiols, the concentration of the thiol agent in the perfusion buffer and the effluent was checked using the Ellman assay (12). There was no detectable difference in the concentrations of these thiol agents in the inflow and outflow solutions, thus excluding limitation in the availability of these thiol agents to the more perivenous hepatocytes, as a consequence of high efficiency extraction by more proximally perfused hepatocytes, or differences due to different rates of auto-oxidation of thiols (data not shown). Thus, it appears that both the absence of a negatively charged substituent and the presence of at least one sulfhydryl group are necessary to stimulate the efflux of hepatic GSH via the sinusoidal GSH transporter.

Although the concentrations of the thiol agents in the perfusion buffer and the effluent were not appreciably different, there may still have been oxidation (≤10%) of these thiols agents during the 20-min perfusion. To see what effect a mixture of reduced and oxidized thiol agent would have on sinusoidal GSH efflux, we studied sinusoidal GSH efflux in response to 1 mM reduced DTT plus 0.25 mM oxidized DTT.

TABLE 1

Effect of dithiols on sinusoidal GSH efflux in perfused liver

Results are expressed as mean ± standard error from three or four perfusions for all agents. After a 10-min base-line period, the effects of 1 mM dithiols were examined by adding these agents to the perfusion buffer for 20 min, and then recovery was examined for another 30 min after removal of the agents. Base-line values are the average of three basal values, peak values are the average of three consecutive highest values after infusion of agent, and recovery values are the average of the last three values. See Experimental Procedures for details.

Dithiol	Base-line efflux	Thiol-induced peak efflux	Post-thiol recovery efflux	Liver GSH levels	
				Beginning	End
	nmol/g/min	nmol/g/min	nmol/g/min	μmol/g	
DTT	16.33 ± 3.50	53.61 ± 5.10[a]	40.87 ± 10.80[b]	6.23 ± 0.26	4.23 ± 0.35
Dimercaprol	11.85 ± 1.09	30.14 ± 0.76[a]	22.20 ± 2.34	6.48 ± 0.13	4.47 ± 0.34
1,2-Ethanedithiol	13.62 ± 1.26	63.97 ± 6.12[a]	26.83 ± 1.06[a]	6.33 ± 0.41	3.36 ± 0.14
1,3-Propanedithiol	15.80 ± 1.57	97.91 ± 7.55[a]	32.98 ± 7.09	6.16 ± 0.30	2.32 ± 0.49
1,4-Butanedithiol	11.49 ± 1.52	57.32 ± 1.92[a]	40.98 ± 0.98[a]	6.45 ± 0.18	3.85 ± 0.20
Dihydrothioctic	10.30 ± 1.29	10.66 ± 0.33	11.18 ± 1.98	5.06 ± 0.33	4.82 ± 0.14

[a] $p < 0.01$ versus basal rates, by ANOVA.
[b] $p < 0.05$.

TABLE 2

Effect of monothiols on sinusoidal GSH efflux in perfused liver

Results are expressed as mean ± standard error from three perfusions for all agents. After a 10-min base-line period, the effects of 2 mM monothiols were examined by adding these agents to the perfusion buffer for 20 min, and then recovery was examined for another 30 min after removal of the agents. Base-line values are the average of three basal values, peak values are the average of three consecutive highest values after infusion of agent and recovery values are the average of the last three values. See Experimental Procedures for details.

Monothiol	Base-line efflux	Thiol-induced peak efflux	Post-thiol recovery efflux	Liver GSH levels	
				Beginning	End
	nmol/g/min	nmol/g/min	nmol/g/min	μmol/g	
Monothioglycerol	10.12 ± 1.53	19.47 ± 1.67[a]	14.84 ± 1.90[b]	6.85 ± 0.68	4.95 ± 0.98
2-Mercaptoethanol	12.54 ± 1.13	46.15 ± 7.32[b]	18.05 ± 1.57[b]	8.03 ± 0.35	6.71 ± 0.18
3-Mercapto-2-butanol	11.47 ± 1.01	22.71 ± 2.98[b]	15.54 ± 1.33[a]	6.80 ± 0.15	5.89 ± 0.29
1-Mercapto-2-propanol	12.64 ± 0.77	26.05 ± 1.32[a]	22.82 ± 1.24[b]	5.48 ± 0.12	4.27 ± 0.16
Cysteamine	11.40 ± 1.0	22.88 ± 3.07[b]	19.37 ± 2.16[b]	7.11 ± 0.07	5.54 ± 0.36
Penicillamine	13.48 ± 0.62	11.55 ± 0.61	11.01 ± 0.49	5.51 ± 0.20	4.16 ± 0.02
N-Acetylcysteine	14.15 ± 0.97	14.53 ± 1.95	15.49 ± 2.38	6.32 ± 0.29	5.90 ± 0.64
Mercaptopropionylglycine	8.74 ± 1.11	7.53 ± 1.70	8.38 ± 0.38	5.88 ± 0.32	5.75 ± 0.18
Mercaptoethanesulfonic acid	10.45 ± 0.68	10.90 ± 0.42	8.53 ± 0.61	5.40 ± 0.37	5.28 ± 0.27
Mercaptoacetic acid	11.41 ± 1.40	12.01 ± 0.63	9.31 ± 1.27	7.07 ± 0.09	6.55 ± 0.07
Mercaptopropionic acid	10.10 ± 0.84	13.92 ± 0.77	6.29 ± 0.39	6.26 ± 0.21	4.87 ± 0.22
Captopril	15.18 ± 2.05	14.04 ± 1.97	13.33 ± 1.57	7.35 ± 0.26	6.91 ± 0.30

[a] $p < 0.01$ versus basal rates, by ANOVA.
[b] $p < 0.05$.

TABLE 3

Effect of alcohols on sinusoidal GSH efflux in perfused liver

Results are expressed as mean ± standard error from three perfusions for all agents. After a 10-min base-line period, the effects of these alcohols were examined by adding these agents (1 mM) to the perfusion buffer for 20 min, and then recovery was examined for another 30 min after removal of the agents. Base-line values are the average of three basal values, peak values are the average of all values during infusion, and recovery values are the average of the last three values. See Experimental Procedures for details.

Alcohol	Base-line efflux	Peak efflux	Recovery efflux	Liver GSH levels	
				Beginning	End
	nmol/g/min	nmol/g/min	nmol/g/min	μmol/g	
Glycerol	12.59 ± 1.23	7.98 ± 0.70[a]	9.39 ± 0.98	5.43 ± 0.23	5.22 ± 0.11
1-Propanol	11.17 ± 0.35	11.45 ± 1.34	12.51 ± 1.52	6.88 ± 0.12	6.81 ± 0.13
1,2-propanediol	13.32 ± 1.15	11.61 ± 0.75	11.97 ± 0.98	6.32 ± 0.33	5.80 ± 0.47
Ethanol	13.57 ± 0.25	12.1 ± 0.75	11.73 ± 0.27	7.16 ± 0.06	6.45 ± 0.11

[a] $p < 0.05$ versus basal rates, by ANOVA.

Sinusoidal GSH efflux increased from a basal rate of 11.71 ± 0.86 nmol/g/min to a peak of 64.11 ± 3.84 nmol/g/min, with a recovery rate of 47.04 ± 2.99 nmol/g/min (protocol and definitions as described for in situ liver perfusions were used; results are expressed as mean ± standard error from four animals). Liver GSH levels fell from 6.41 ± 0.36 to 3.36 ± 0.11 μmol/g. The stimulation of sinusoidal GSH efflux was similar to that seen with 1 mM DTT alone. Thus, even with 20% of total thiols being present in the oxidized form, the overall effect was still that of the reduced thiol. Furthermore, the concentrations of thiol agents used (1 mM for dithiols and 2 mM for monothiols) were maximal concentrations, so a slight decline in the concentration of the reduced form is not likely to alter the overall effect.

Studies in cultured rat hepatocytes. Although the availability of thiol agents was not limiting in the perfused liver studies, whether the lack of effect of thiols containing a negatively charged substituent was due to lack of uptake was not addressed. To address the uptake of thiol agents as well as to confirm the charge requirement, we studied the effects of DTT, penicillamine, cysteamine, and *N*-acetylcysteine, compared with controls, on total non-protein thiol levels and GSH efflux. Cultured cells were treated with these agents (2 mM for DTT and 4 mM for all monothiols) in Krebs buffer for 30 min and then washed five times with either warm Krebs buffer (for subsequent efflux experiments) or ice-cold Krebs buffer (for subsequent determination of total non-protein thiols). Treatment with all thiol agents increased total non-protein thiols, compared with controls (control, 101 ± 7; DTT, 129 ± 9; penicillamine, 149 ± 6; cysteamine, 146 ± 3, *N*-acetylcysteine, 145 ± 9 nmol/10^6 cells; results are expressed as mean ± standard error from four experiments; all values are significantly different from control, with $p < 0.05$, by ANOVA). Although all thiol agents raised the total cell non-protein thiol level, only DTT and cysteamine significantly stimulated GSH efflux, as we previously observed (7); penicillamine and *N*-acetylcysteine had no effect (data not shown). Thus, it is unlikely that the lack of effect of penicillamine or *N*-acetylcysteine on GSH efflux was due to lack of uptake.

Directionality of the Thiol-Disulfide Effect on GSH Transport in Cultured Rat Hepatocytes

Studies comparing efflux and uptake of GSH in parallel. To evaluate the effect of DTT on bidirectional GSH transport, both GSH efflux and GSH uptake were examined in parallel with each cell preparation. As shown in Fig. 1A, in the presence of DTT the release of prelabeled cell GSH was enhanced, as predicted. In freshly isolated rat hepatocytes, extracellular GSH *trans*-stimulated GSH efflux (4). Here, both 1 and 10 mM extracellular GSH exhibited a trend to increase efflux. The combination of DTT and GSH stimulated efflux similarly to DTT alone. When cell GSH levels were measured

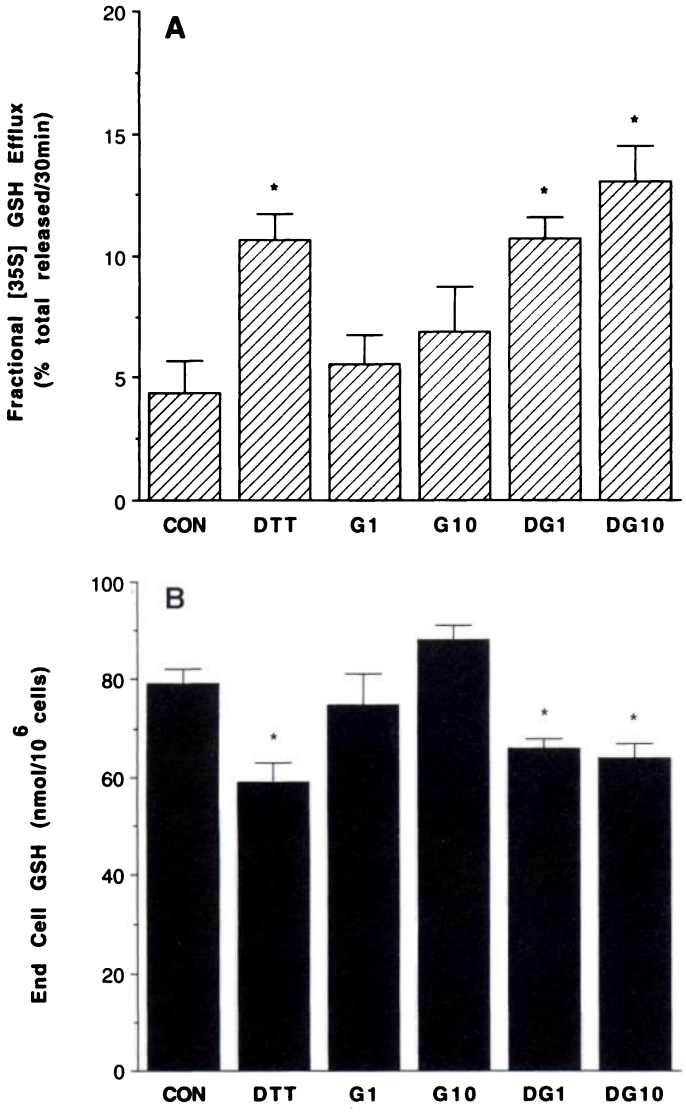

Fig. 1. A, Effect of extracellular GSH and DTT on GSH efflux. Cells were cultured in basic medium overnight. The following morning, cell GSH was depleted with diethyl maleate (0.5 mM, for 20 min) and cells were then incubated for 2 hr with [^{35}S]methionine (3–5 μCi/ml, 2 ml/plate) supplemented with unlabeled methionine (1 mM) and serine (1 mM), in culture medium. Cells were then washed free of these agents and efflux experiments were performed using Krebs buffer with DTT (2 mM), GSH (1 or 10 mM) (*G1* or *G10*), both DTT and GSH (*DG1* or *DG10*), or vehicle (*CON*) added. At the end of the 30-min incubation, cell and supernatant samples were processed for HPLC (see Experimental Procedures for details). Results are expressed as mean ± standard error from four cell preparations. GSH efflux is expressed as percentage of total [^{35}S]GSH that was released in 30 min. B, Effect of extracellular GSH and DTT on cell GSH levels at the end of the efflux experiment. Results are expressed as mean ± standard error from four cell preparations. At the end of the efflux experiment, cells were detached and processed for GSH analysis by both the Tietze method (10) and radio-HPLC. Cell GSH specific activities were as follows: control, 3853 ± 743; DTT, 3843 ± 830; 1 mM GSH, 3855 ± 720; 10 mM GSH, 3403 ± 728; DTT plus 1 mM GSH, 3748 ± 731; DTT plus 10 mM GSH, 3334 ± 671 cpm/nmol; the percentages of total dose incorporated as [^{35}S]GSH/10^6 cells were as follows: control, 2.17 ± 0.32; DTT, 1.73 ± 0.34; 1 mM GSH, 2.03 ± 0.19; 10 mM GSH, 2.09 ± 0.26; DTT plus 1 mM GSH, 1.86 ± 0.21; DTT plus 10 mM GSH, 1.69 ± 0.31% (all of these values were not significantly different from control). *, $p < 0.05$ versus control, by ANOVA.

at the end of the 30-min experiment, as shown in Fig. 1B, the presence of DTT led to lower cell GSH levels, due to loss of intracellular GSH. High extracellular GSH levels (10 mM) tended to increase cell GSH levels, but cell GSH levels with the combination of DTT and GSH were significantly lower than vehicle control or the respective GSH controls. Because cell GSH levels fell after DTT treatment despite the presence of 10 mM GSH in the medium, the enhanced efflux was not matched by uptake to maintain cell GSH levels. This suggests that the effect of DTT on the GSH transporter is to enhance outward transport while inhibiting inward transport. In fact, when GSH uptake was studied in parallel, as shown in Fig. 2A, the presence of DTT lowered GSH uptake ($p < 0.05$ versus 1 mM GSH, $p = 0.1$ versus 10 mM GSH). One of the four experiments showed discrepant results comparing 10 mM GSH with DTT plus 10 mM GSH; otherwise, the degree of inhibition would be much more pronounced. Again, Fig. 2B shows that the final cell GSH levels were significantly lower in the presence of DTT. Note that the cell GSH levels are different in the efflux and uptake counterparts because the protocol for efflux included depletion of intracellular GSH with diethyl maleate, followed by repletion with radiolabeled methionine.

Effect of DTT on kinetics of GSH uptake. We previously showed that DTT pretreatment (2 mM, for 30 min) increased the V_{max} of GSH efflux from 0.24 to 1.2 nmol/10^6 cells/min, while not significantly affecting the K_m (~125 nmol/10^6 cells) (7). To determine the influence of DTT on the kinetics of GSH uptake, we first measured GSH uptake over a wide concentration range (0.5–30 mM). GSH uptake was linear for 45 min (data not shown), so that all experiments were done with 30-min incubations. The effect of DTT was evaluated by pretreating cells with DTT (2 mM) for 30 min. This is different from the studies described above, where DTT was added in the buffer during uptake experiments. We chose to pretreat cells with DTT and remove it for the kinetic analysis because our earlier work on GSH efflux kinetics was done with pretreatment. Furthermore, removing DTT would avoid concerns about the possibility that DTT is a competitive substrate for the transporter. However, pretreatment with DTT may result in lowered cell GSH levels, due to increased GSH efflux. To prevent a significant difference in the starting cell GSH levels for uptake experiments, pretreatment with DTT was done in culture medium in the presence of 10 mM GSH. Pretreatment with 10 mM GSH did not result in alteration of GSH transport activity after its removal (4). Note that this appears to be different from the results shown in Fig. 1A, where the cell GSH level was lowered with DTT plus 10 mM GSH, in comparison with control. The explanation is that in Fig. 1A the cell GSH values represent values at the end of 30 min of efflux for cells incubated with either GSH or DTT, or both, in Krebs buffer. Thus, GSH fell because there was increased GSH efflux but no GSH synthesis to compensate for the loss. This is in contrast to pretreatment of cells with DTT plus GSH in culture medium containing excess precursors for GSH synthesis. Fig. 3 shows the effect of DTT on the kinetics of GSH uptake in cultured rat hepatocytes. In cultured rat hepatocytes DTT pretreatment significantly decreased the V_{max} of GSH uptake, from a control value of 24.2 ± 2.7 to 11.0 ± 2.1 nmol/10^6 cells/30 min, whereas the K_m remained unaltered (control, 7.7 ± 2.2; DTT-pretreated, 8.6 ± 4.1 mM). The free fit revealed an n value (transport or binding sites) of 1.2, which is suggestive of more than one

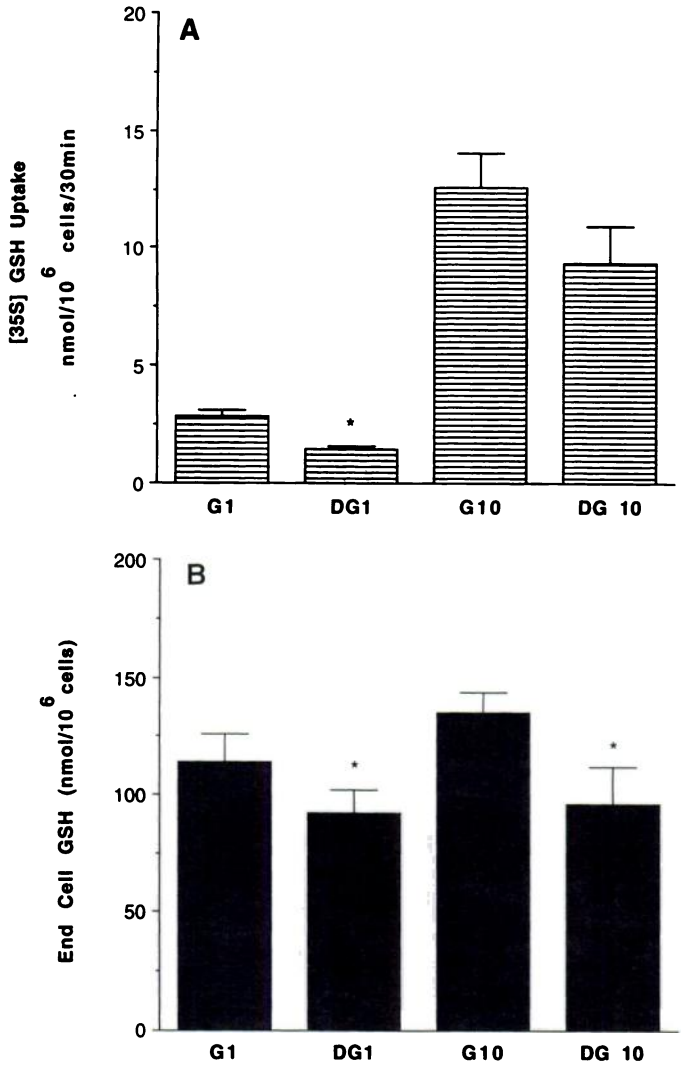

Fig. 2. A, Effect of DTT on GSH uptake. The effect of DTT on uptake of GSH was assayed in parallel with studies on GSH efflux. The same cell preparations described in Fig. 1 were used. After overnight culture in basic medium, cells were pretreated with acivicin (0.5 mM) and BSO (10 mM) for 15 min. GSH uptake was measured as described in Experimental Procedures, in buffer containing [^{35}S]GSH (4–5 μCi/ml) plus 1 or 10 mM GSH (*G1* or *G10*). The effect of DTT was assessed by adding DTT (2 mM) to the aforementioned buffer (*DG1* or *DG10*). GSH uptake represents net uptake values, with 4° blank values subtracted. Cell GSH values represent values measured at the end of the 30-min incubation. Results are expressed as mean ± standard error from four cell preparations. B, Cell GSH values at the end of uptake experiments. Results are expressed as mean ± standard error from four cell preparations. *, $p < 0.05$ versus the respective control (either 1 or 10 mM GSH), by paired Student's *t* test.

interactive transport site, as we have observed for GSH uptake in Hep G2 cells (5). Indeed, if n was fixed at 2.0 the fit at high GSH concentrations (≥15 mM) was further improved but the fit at lower GSH concentrations worsened somewhat (data not shown). The cell GSH levels are shown in Fig. 4. The starting cell GSH levels were the same in control and DTT- plus GSH-pretreated cells. At the end of the 30-min uptake experiment, cell GSH levels increased in the controls incubated with GSH concentrations of ≥5 mM (except for 15 mM) but decreased in the DTT-pretreated cases (except for 10 mM, where $p = 0.1$), in comparison with starting cell GSH levels. At the end of the 30-min uptake, all cell GSH levels for the DTT-pretreated cases were significantly lower than the respective GSH controls. This is because of increased GSH efflux, without compensating GSH synthesis, and decreased GSH uptake.

Effect of cystine pretreatment on GSH uptake. In contrast to DTT, cystine pretreatment was previously reported to decrease the V_{max} of GSH efflux, from 0.24 to 0.16 nmol/10^6 cells/min, while not significantly affecting the K_m (~125 nmol/10^6 cells) (7). To examine the effect of cystine pretreatment on GSH uptake, cells were cultured using SAF supplemented with methionine. SAF was used instead of DME/F-12 because the latter contains ~0.2 mM cystine. GSH uptake was measured as described previously, after pretreatment with cystine (0.5 mM, for 30 min) or vehicle. As shown in Table 4, cystine pretreatment did not inhibit GSH uptake; in fact, there was a trend of increased GSH uptake ($p = 0.1$).

Discussion

GSH undergoes an interorgan homeostasis, with the liver releasing GSH into plasma and bile and GSH being subsequently removed in extrahepatic loci (1, 2). Indeed, the liver is the major source of plasma GSH (3). GSH in plasma may serve to scavenge free radicals, maintain the thiol redox state, be taken up directly by extrahepatic organs, or undergo hydrolysis initiated by γ-glutamyl transpeptidase. The latter appears to be the predominant mechanism of disposal in adult rats, supporting the view that GSH is a vehicle for stabilizing, detoxifying, and transferring cysteine. Thus, the release of GSH into sinusoidal plasma may be a critical step in the interorgan homeostasis of defense. Both sinusoidal and canalicular GSH effluxes are mediated by carrier mechanisms involving the participation of specific gene products (6).

We have been interested in the effects of various factors that might influence the regulation of the sinusoidal transporter or that might lead to pharmacological manipulation to increase the delivery of GSH to plasma. Dysregulation of GSH homeostasis might play a role in diseases or aging, and pathophysiological circumstances and mechanisms by which the transporter is inhibited would be of potential value in understanding disease consequences. It was along the latter lines that we initially explored the influence of the thiol-disulfide status of the extracellular environment on the function of the GSH transporter. We observed previously that DTT markedly enhanced sinusoidal GSH efflux, whereas cystine inhibited it (7). The purpose of the present studies was to further explore the structural specificity and mechanism of this effect.

Our previous impression, based on the effect of DTT versus the lack of stimulation by *N*-acetylcysteine, was that stimulation required dithiols to reduce vicinal disulfides in the transporter polypeptide. It now seems clear that the effect is seen with a broad range of mono- and dithiols, mostly exhibiting no charge, although cysteamine (a cation) also exerted a modest stimulatory effect. However, all of the compounds tested that contained a negative charge, with (penicillamine) or without (*N*-acetylcysteine, dihydrothioctic acid, etc.) a positive charge, were without effect. We ruled out the possibility that the lack of effect of these compounds carrying a negative charge was due to differences in intracellular or zonal availability. Thus, similar increases in cell thiol levels were observed with these compounds, compared with DTT. In addition, sufficiently high concentrations were used in perfused liver studies to ensure that the concentrations leaving the liver were near those enter-

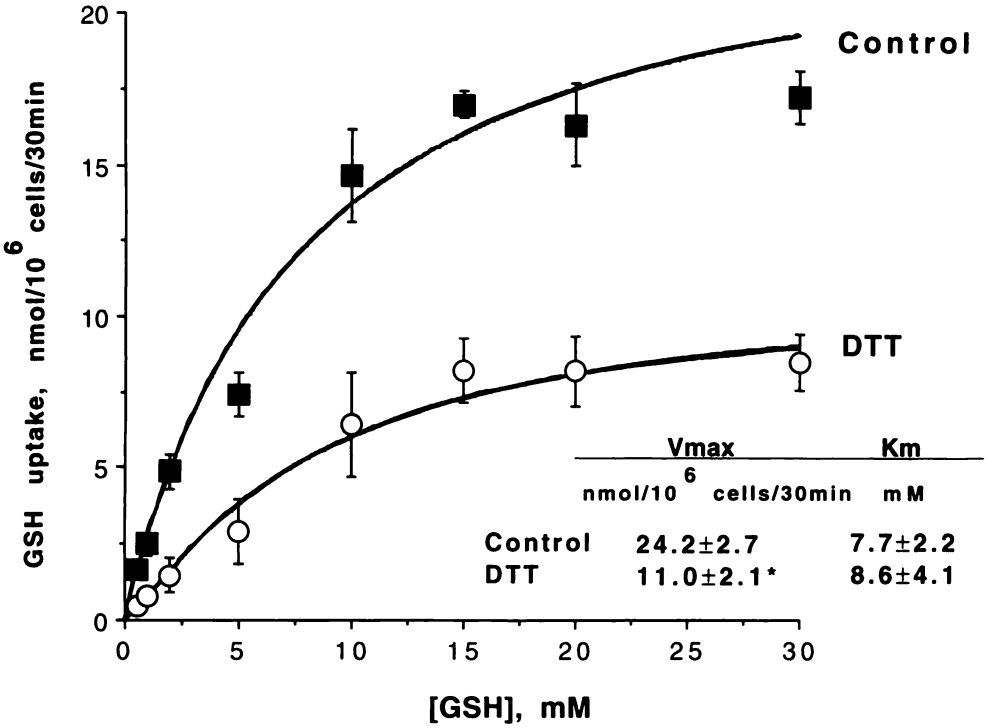

Fig. 3. Effect of DTT pretreatment on the kinetics of GSH uptake in cultured rat hepatocytes. After overnight culture in basic medium, cells were pretreated with acivicin (0.5 mM) and BSO (10 mM) for 15 min. Cells were also pretreated with DTT (2 mM) plus GSH (10 mM) or vehicle for 30 min. All cells were washed five times after this pretreatment. GSH uptake was then measured as described in Experimental Procedures, in buffer containing [^{35}S]GSH (4–5 μCi/ml) plus 0.5–30 mM GSH. Results are expressed as mean ± standard error from four to eight cell preparations. *Solid lines*, nonlinear least-squares computer fits of the Michaelis-Menten equation to the data, using the SAAM program. V_{max} and K_m values were determined by the program. The V_{max} for GSH uptake decreased from 24.2 ± 2.7 to 11.0 ± 2.1 nmol/10^6 cells/30 min after DTT pretreatment, whereas the K_m did not significantly change (control, 7.7 ± 2.2; DTT-pretreated, 8.6 ± 4.1 mM). Values are mean ± standard deviation. *, $p < 0.05$ versus control, by unpaired *t* test.

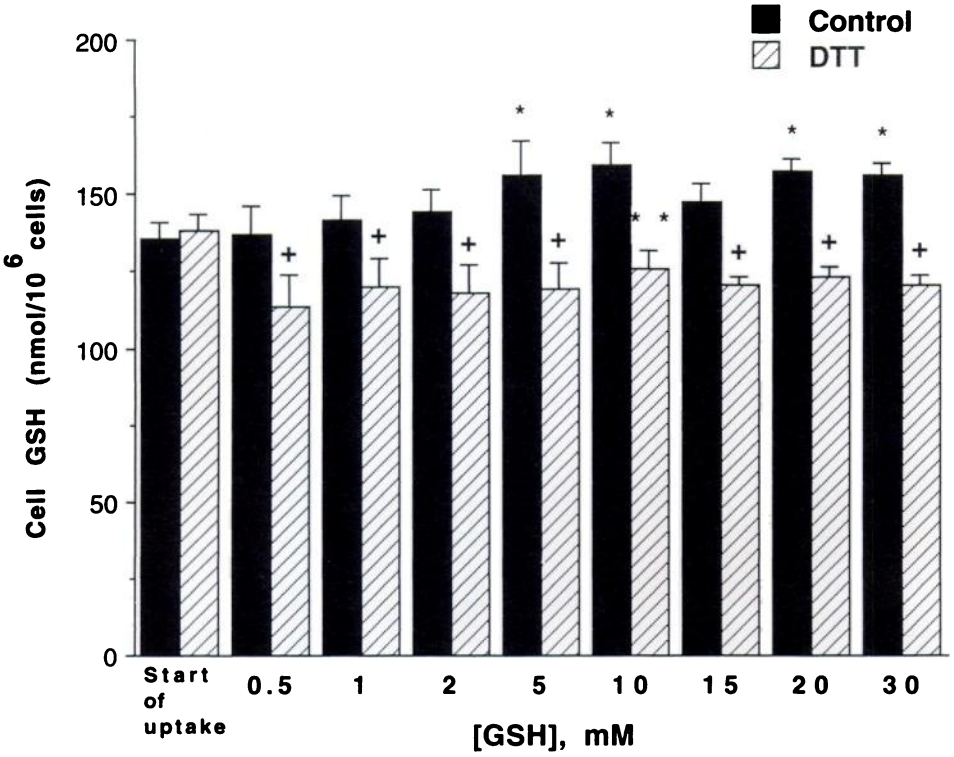

Fig. 4. Effect of DTT pretreatment on cell GSH values at the end of 30-min uptake experiments. Results are expressed as mean ± standard error from four to eight cell preparations. For each uptake experiment, duplicate plates for control and DTT-plus GSH-pretreated cases were saved for cell GSH measurement (cell GSH levels at the start of uptake). At the end of the 30-min uptake experiment, cell GSH levels increased in the controls for GSH concentrations of ≥5 mM (except for 15 mM) but decreased in all DTT-pretreated cases (except for 10 mM; $p = 0.1$). All of the cell GSH values in the DTT-pretreated cases were lower than the respective GSH controls. *, $p < 0.05$ versus control starting values; +, $p < 0.05$ versus DTT starting values as well as the respective GSH counterpart; **, $p < 0.05$ versus 10 mM GSH control, by ANOVA.

ing the liver. It is not certain whether the stimulatory thiol compounds exert their effects on the outside of the cell, within the membrane, or inside the cell. However, the converse effect, namely inhibition by disulfides, was exerted on the outside (7), suggesting that this is likely to be the case with the thiols as well.

Our previous work ruled out an effect of the thiol-disulfide status on the driving force (membrane potential) or affinity of the transporter for GSH. We can only speculate that the negative charge effect is to limit access to the specific structural milieu where disulfide reduction occurs. Despite the recent advances in characterization of the structure of the GSH transporters (17, 18), we understand very little about the mechanism of transporter function. Although manipulation of the thiol-disulfide status suggests critical thiol groups in the transporter, future studies combining these manipulations with site-directed mutagenesis may provide the necessary details to understand how these agents exert their effects.

We have shown in rat hepatocytes that extracellular GSH *trans*-stimulates efflux of cell GSH (4). When the putative

TABLE 4

Effect of cystine pretreatment on GSH uptake in cultured rat hepatocytes

Results are expressed as mean ± standard error from three cell preparations. Cells were plated using SAF supplemented with methionine (1 mM) instead of DME/F-12. Before measurement of GSH uptake, in addition to pretreatment with acivicin and BSO cells were also treated with cystine (0.5 mM) or vehicle for 30 min and then washed five times. The adequacy of this washing was confirmed by radio-HPLC of the uptake buffer, which showed no GSH-cysteine mixed disulfide, which would have formed had there been any cystine present in the uptake medium. GSH (1 mM) uptake was measured as described in Experimental Procedures.

Condition	GSH uptake	Final cell GSH levels
	nmol/10^6 cells/30 min	*nmol/10^6 cells*
Control	1.24 ± 0.07	147 ± 13
Cystine pretreated	1.50 ± 0.04	148 ± 13

sinusoidal and canalicular GSH transporters are expressed in *Xenopus* oocytes, either uptake or efflux is seen (6). Thus, the transporters appear to be facilitative and bidirectional but transport is not perfectly symmetrical, in that kinetics of uptake differ from kinetics of efflux (7) and inhibitors exert sided effects (6, 19, 20). Furthermore, the effect of DTT on the kinetics is to raise the V_{max} for efflux while lowering the V_{max} for uptake. Thus, the transporter favors efflux and depletes cell GSH even in the presence of high extracellular GSH levels. Therefore, DTT and other thiols force the transporter to operate more unidirectionally out. In contrast, cystine inhibits efflux while increasing uptake. Thus, the effect of a more oxidized extracellular environment on bidirectional GSH transport would be to retain GSH in the liver. This could be a pathophysiologically important phenomenon in impairing extrahepatic defense. More work will be required to assess the importance of increased plasma disulfides and consequent decreased GSH efflux. Furthermore, the utility of stimulating or unmasking sinusoidal GSH efflux capacity for protection of the liver sinusoids and other organs remains to be evaluated in various models. The ideal choice of thiol stimulant, as well as dose, route of administration, etc., remains to be established. Candidates for use *in vivo* include cysteamine and dimercaprol. Disease models that should be evaluated include ischemia/reperfusion of liver or other organs, aging, and GSH homeostasis in advanced experimental cirrhosis.

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