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S C Lu, ... , J Kuhlenkamp, N Kaplowitz

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### Research Article

In cultured rat hepatocytes, cystine led to an inhibition of GSH efflux by lowering the  $V_{max}$  by approximately 35% without affecting the  $K_m$ . The cystine-mediated inhibition of GSH efflux was rapid in onset (< 1 h), with near maximum effect at 0.1 mM. Inhibition was still observed when cystine uptake was prevented. Cystine and sulfobromophthalein-GSH, a selective inhibitor of sinusoidal transport of GSH, did not exhibit additive inhibitory effects on GSH efflux. Depletion of ATP or membrane depolarization after cystine treatment were excluded as potential mechanisms. DTT not only reversed the cystine-mediated inhibition of GSH efflux, it stimulated GSH efflux up to 400-500%. The DTT effect was immediate in onset, reaching maximum after 30 min, and was partially reversed by cystine, suggesting that the two share a common site(s) of action. DTT treatment did not alter cellular ATP levels or change the membrane potential. In cultured hepatocytes, DTT treatment increased the  $V_{max}$  of GSH efflux by approximately 500% without affecting the  $K_m$ . Inhibition of microtubular function and vesicular acidification did not affect basal or DTT stimulated efflux. Both cystine and DTT effects on sinusoidal GSH efflux were confirmed in perfused livers. In summary, the capacity of the sinusoidal GSH transporter is markedly influenced by thiol-disulfide status.

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# Thiol-Disulfide Effects on Hepatic Glutathione Transport

## Studies in Cultured Rat Hepatocytes and Perfused Livers

Shelly C. Lu, Jun-Li Ge, Hai-Ying Huang, John Kuhlenkamp, and Neil Kaplowitz

Division of Gastrointestinal and Liver Diseases, Department of Medicine, University of Southern California School of Medicine, Los Angeles, California 90033

### Abstract

In cultured rat hepatocytes, cystine led to an inhibition of GSH efflux by lowering the  $V_{max}$  by  $\sim 35\%$  without affecting the  $K_m$ . The cystine-mediated inhibition of GSH efflux was rapid in onset ( $< 1$  h), with near maximum effect at 0.1 mM. Inhibition was still observed when cystine uptake was prevented. Cystine and sulfobromophthalein-GSH, a selective inhibitor of sinusoidal transport of GSH, did not exhibit additive inhibitory effects on GSH efflux. Depletion of ATP or membrane depolarization after cystine treatment were excluded as potential mechanisms. DTT not only reversed the cystine-mediated inhibition of GSH efflux, it stimulated GSH efflux up to 400–500%. The DTT effect was immediate in onset, reaching maximum after 30 min, and was partially reversed by cystine, suggesting that the two share a common site(s) of action. DTT treatment did not alter cellular ATP levels or change the membrane potential. In cultured hepatocytes, DTT treatment increased the  $V_{max}$  of GSH efflux by  $\sim 500\%$  without affecting the  $K_m$ . Inhibition of microtubular function and vesicular acidification did not affect basal or DTT stimulated efflux. Both cystine and DTT effects on sinusoidal GSH efflux were confirmed in perfused livers. In summary, the capacity of the sinusoidal GSH transporter is markedly influenced by thiol-disulfide status. (*J. Clin. Invest.* 1993. 92:1188–1197.) Key words: cystine • DTT • BSP-GSH • sinusoidal GSH efflux • sulfhydryls

### Introduction

GSH is a tripeptide that is found in all mammalian cells, with especially high levels in the liver. GSH has important protective and metabolic functions (1, 2). The liver plays a central role in a complex interorgan homeostasis of GSH by being the predominant source of plasma GSH (3). Within the hepatocyte, there are several GSH transport systems. The sinusoidal GSH transport system, located in the basolateral membrane of the hepatocyte, releases GSH into blood. A distinct GSH transport system localized in the canalicular membrane translocates GSH into bile (1), and an intracellular GSH transport system localized in the inner mitochondrial membrane mediates the transport of GSH from cytosol into this organelle (4). Our

Address correspondence to Shelly C. Lu, Division of Gastrointestinal and Liver Diseases, LAC 11-221, Department of Medicine, University of Southern California School of Medicine, 2025 Zonal Avenue, Los Angeles, CA 90033.

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laboratory has studied GSH transport extensively using different experimental models. The efflux of GSH from the perfused liver and isolated hepatocytes is a saturable process (5, 6) that can be competitively inhibited by organic anions, bilirubin (7), and methionine (8). Furthermore, we have shown that membrane potential is a driving force for the transport of GSH, so that hyperpolarization increases and depolarization decreases the release of GSH (9). GSH efflux is also under hormonal control, both in primary cultures of rat hepatocytes and in perfused livers; cAMP-dependent hormones stimulated sinusoidal GSH release by hyperpolarization of the plasma membrane potential (10). Recently, our laboratory demonstrated bidirectional GSH transport by a sinusoidal GSH transport system with low affinity for both efflux and uptake (11, 12). However, the sinusoidal 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. In our previous studies, we have noted a lower capacity for GSH efflux in cultured hepatocytes compared with freshly isolated cells (10). In the present studies, examining culture conditions initially revealed the novel finding that cells cultured in standard medium (containing  $\sim 0.2$  mM cystine) exhibited lower efflux than cells cultured without cystine. This led us to a detailed examination of the effects of disulfides and thiols on GSH export in cultured hepatocytes and the intact liver.

### Methods

**Materials.** GSH, oxidized glutathione (GSSG),<sup>1</sup> collagenase (type IV), BSA, L-methionine, L-cystine, NADPH, 5,5'-dithiobis-(2-nitrobenzoic acid), sodium EDTA, GSSG reductase, hydrocortisone, insulin, DL-DTT, L-DTT, dithioerythritol (DTE), cysteamine, cystamine, taurocholic acid, ouabain, L-(S-S)- $\alpha$ -amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid (acivicin), serine, Hepes, colchicine, lumicolchicine, chloroquine, nocodazole, monensin, and calmidazolium were purchased from Sigma Chemical Co. (St. Louis, MO). Staurosporin was purchased from Boehringer Mannheim Corp. (Indianapolis, IN). Custom-made sulfur amino acid-free DME/F-12 medium (SAF) was 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). Thiolyte MQ was purchased from Calbiochem Corp. (San Diego, CA). [<sup>3</sup>H]Alanine (85 Ci/mmol) and [<sup>14</sup>C]sucrose (0.25 mCi/mmol) were purchased from New England Nuclear (DuPont, Boston, MA). BSP-GSH was enzymatically synthesized from the GSH S-transferase (GST) catalyzed reaction between GSH and sulfobromophthalein (BSP) under alkaline conditions as previously described (7). BSP-GSH was separated from unreacted BSP by acetone extraction and

1. *Abbreviations used in this paper:* ANOVA, analysis of variance; BSP, sulfobromophthalein; CySSG, cysteine-GSH mixed disulfide; DTE, dithioerythritol; GSSG, oxidized glutathione; GST, GSH S-transferase; SAF, sulfur amino acid-free DME/F-12 medium.

assayed at 580 nm under alkaline conditions. The conjugate was collected, lyophilized, and stored at  $-20^{\circ}\text{C}$  until use. Cysteine-GSH mixed disulfide (CySSG) was synthesized according to the method of Eriksson and Eriksson (13).

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

**Cell culture preparation.** Hepatocytes were isolated aseptically according to the method of Moldeus et al. (14). Initial cell viability as determined by 0.2% trypan blue exclusion was  $\geq 90\%$ . The control medium used was SAF, containing high glucose (3,151 mg/liter), 10% fetal bovine serum, insulin (1  $\mu\text{g}/\text{ml}$ ), hydrocortisone (50 nM), and supplemented with methionine (1 mM).  $1.5\text{--}2 \times 10^6$  cells in 5 ml control medium were plated on  $60 \times 15\text{-mm}$  dishes precoated with rat tail collagen and incubated at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$ , 95% air. In some experiments, to achieve very high levels of cell GSH, low numbers of cells ( $0.5 \times 10^6$  cells) were plated. This has been previously demonstrated not to affect GSH efflux kinetics (10). Medium was changed 2–3 h after plating to remove dead, unattached cells. On the average, plating efficiency was  $\sim 60\%$ . The replacement medium was the same as control medium except for omission of serum and addition of varying concentrations of cystine (for overnight cystine time course and dose-response studies). In studies of more acute time course, after medium change, the agent of interest was added to medium the next morning (18 h after plating) for various time periods.

**GSH efflux in cultured cells.** All experiments were carried out 20–24 h after plating. The medium was aspirated, cells were washed twice with Krebs-Henseleit buffer, supplemented with 12.5 mM Hepes (pH 7.4), and then were incubated with 5 ml Krebs at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$  for 90 min. To determine the effect of temperature, in some experiments cells were washed with ice-cold Krebs and then incubated with 5 ml Krebs on ice for 90 min. In experiments assessing the effect of BSP-GSH (40  $\mu\text{M}$ ), it was added back in Krebs buffer during the 90-min incubation. Aliquots of supernatant (500  $\mu\text{l}$ ) were removed at 5, 30, 60, and 90 min for GSH determination by the method of Tietze (15) or for determination of mass and molecular form of GSH by HPLC according to the method of Fariss and Reed (16). GSH values obtained by these two methods agreed closely, generally within 15%. The presence of BSP-GSH (40  $\mu\text{M}$ ) in the buffer did not affect the recycling assay (15) under our conditions, as the recovery of added GSH standard (10–50  $\mu\text{M}$ ) was the same with or without it. The accumulation of GSH in the supernatant was linear over 90 min as we previously reported (10); thus efflux rates were calculated by linear regression. In parallel experiments, cell lysis during the course of incubation was estimated by measuring the release of GST from the cytosol into the supernatant as a fraction of total cellular enzyme activity using 1-chloro-2,4-dinitrobenzoic acid and GSH as substrates (17). Cell lysis was  $< 5\%$  and did not increase during the 90-min incubation for controls as well as any of the treatments. At the end of the experiments, cells were detached by trypsin-EDTA (0.05%, 0.02%, respectively) for cell counting by both Coulter counter and hemocytometer and analysis of viability, which was  $\geq 90\%$  as determined by trypan blue exclusion. In addition, cell diameter was estimated by the Channelyzer (Coulter counter). Cellular GSH was extracted with 10% TCA solution and, after centrifugation at 13,000  $g$  in a microfuge (Beckman Instruments, Inc., Fullerton, CA) for 1 min to remove the denatured proteins, total GSH was measured by the method of Tietze (15) in the supernatant samples. In other experiments, the above supernatant samples after centrifugation were derivatized for HPLC analysis (16). To measure cellular GST content, cells were scraped off the plates with a rubber policeman and treated with 10% Triton X-100 in PBS (1:1, vol/vol). After centrifugation in a microfuge, enzyme activity was measured as previously described.

**GSH efflux in *in situ* liver perfusions.** The design, method, and apparatus for *in situ* liver perfusions were as previously described (5). For each *in situ* liver perfusion, the bile duct was cannulated with PE-10 tubing (Clay Adams, Parsippany, NJ) and bile was collected after a retrograde biliary infusion of 20  $\mu\text{mol}/\text{kg}$  acivicin (in 200  $\mu\text{l}$

volume), an irreversible inhibitor of  $\gamma$ -glutamyl transpeptidase. During perfusions (45–55 min), bile flow was maintained by adding taurocholate (15  $\mu\text{M}$ ) to the Krebs-Ringer buffer. The perfusion rates using oxygenated Krebs-Ringer bicarbonate buffer were  $4.23 \pm 0.15$  ml/min per g liver, pressure generated by the perfusion of liver was  $4.0 \pm 0.3$  cm  $\text{H}_2\text{O}$ , and the  $\text{O}_2$  uptakes were  $2.3 \pm 0.1$   $\mu\text{mol}/\text{min}$  per g liver during the control period (mean  $\pm$  SEM,  $n = 13$ ). These values did not change significantly during treatment periods and they compare closely to those reported previously (5, 10). Perfusate samples were taken at the end of 5-min intervals, whereas bile samples were collected continuously at 10-min intervals. Perfusate and bile GSH were measured by the method of Tietze (15) (during control periods) and by HPLC. For each perfusion there was a control period (10 min) followed by treatment with the agent of interest (15–30 min) and a recovery period (15 min). Bile flow rates were determined gravimetrically. Cell lysis was monitored by the measurement of GST in the perfusates, and no cumulative lysis was detected up to 55 min. GSH level was measured at the beginning and end of the experiment in liver biopsies (0.2 g) by both the method of Tietze (15) and HPLC.

**Measurement of paracellular permeability in *in situ* liver perfusions.** Paracellular permeability was measured by using steady state infusions of tracer [ $^{14}\text{C}$ ]sucrose ( $\sim 0.1$   $\mu\text{Ci}/\text{min}$  per g), a commonly used marker of paracellular transport (18, 19). The protocol for liver perfusions was the same as above except for the omission of acivicin pretreatment. Perfusate samples were collected at the end of 5-min intervals while bile samples were collected continuously over the same period. The steady state ratios of bile-to-perfusate  $^{14}\text{C}$  concentrations were used as an index of paracellular permeability. This was determined for control (15 min) and treatment (15 min) periods.

**Alanine uptake by cultured rat hepatocytes.** Alanine uptake was measured according to the method described by Van Dyke and Scharschmidt (20). The medium used for washing and stopping uptake was a balanced electrolyte solution with the following composition (mM): 130 NaCl, 5 KCl, 0.8  $\text{MgSO}_4$ , 1.2  $\text{CaSO}_4$ , 0.8  $\text{Na}_2\text{HPO}_4$ , 5  $\text{NaHCO}_3$ , 10 Hepes (adjusted to pH 7.4 with NaOH), and 5 glucose. Transport medium was the same medium supplemented with nonradioactive alanine (0.5 mM), [ $^3\text{H}$ ]Alanine (1  $\mu\text{Ci}/\text{ml}$ ), and aminooxyacetate (2.5 mM) to inhibit alanine metabolism. Cultured cells were washed twice (2 ml each) with prewarmed buffer and transport was initiated by addition of 1 ml transport medium. After incubation at  $37^{\circ}\text{C}$  for the required time, uptake was terminated by washing five times (2 ml each) with ice-cold wash buffer. Cells were then dissolved with 0.5 ml of 0.5 N NaOH; an aliquot was used for determining the radioactivity and another for protein assay by reagent (Bio-Rad Laboratories, Richmond, CA). To estimate trapping, uptake at  $4^{\circ}\text{C}$  (on ice) was studied in parallel. Duplicate plates were used for each time point and condition. The difference between  $37$  and  $4^{\circ}\text{C}$  uptake values represented true uptake. Uptake was expressed as nmol alanine/mg protein per min.

**Cellular ATP content.** Cultured hepatocytes were plated under different media conditions and cellular ATP levels were determined by HPLC according to the method of Jones (21).

**Kinetic analyses.** The kinetic model used to estimate the apparent  $V_{\text{max}}$  and the  $K_m$ , as before (5, 6, 10), was the Hill equation represented by:  $V = V_{\text{max}} (\text{GSH})^n / [K_m^n + (\text{GSH})^n]$ , where  $n$  is the number of binding/transport sites. Nonlinear least-squares fitting was done with the SAAM program (22) 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 (5, 10).

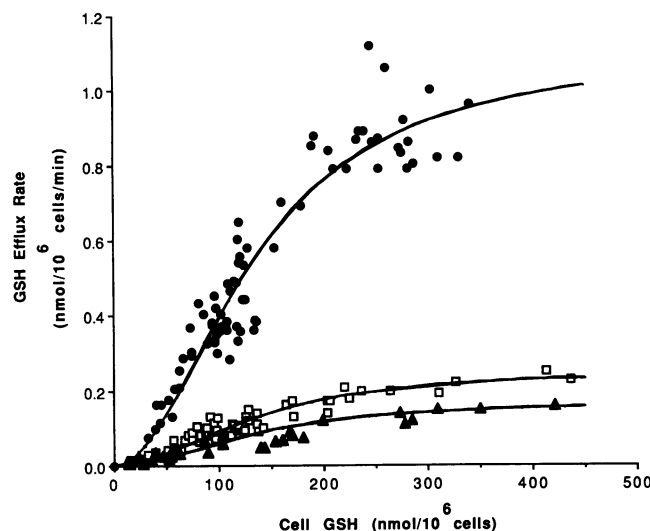
**Statistical analysis.** For cultured cells, each cell prep was derived from one animal and triplicate plates were used for each condition as well as time points except in the case of alanine uptake (duplicate plates were used). The mean of each triplicate (or duplicate) from one experiment was considered  $n = 1$  and the means of multiple experiments were compared by paired Student's  $t$  test in the case of two comparisons or one-way analysis of variance (ANOVA) followed by Fisher's test for multiple comparisons. Whenever percentage data were compared, they were transformed with the arcsin square root transformation before

ANOVA analysis. For perfused livers, treatment (mean of three highest or lowest values after the start of treatment) and recovery values (mean of last three values after termination of treatment) were compared with control (mean of three determinations during the control period) by ANOVA followed by Fisher's test. For comparison of kinetic parameters, unpaired *t* test was used where *n* = number of cell preps that included the entire range of cell GSH. Two-tailed *t* tests were used unless otherwise noted.

## Results

### Effects of cystine on GSH efflux in cultured rat hepatocytes

**Kinetics.** In preliminary studies not shown, we observed lower GSH efflux from cultured hepatocytes after overnight incubation in DME/F-12 supplemented with methionine versus SAF medium supplemented with methionine. Since the only difference in these two conditions is cystine present in DME/F-12, the effect of cystine on the kinetics of GSH efflux was assessed by culturing rat hepatocytes in the control medium (SAF, containing insulin, hydrocortisone, and 1 mM methionine) versus control medium plus 0.18 mM cystine (to simulate standard DME/F-12 medium) for 20–24 h. Efflux was then determined in amino acid-free buffer. Fig. 1 shows the relationship of cell GSH and GSH efflux rate. Cell GSH represents the sum of supernatant and cell GSH at the end of



**Figure 1.** GSH efflux from hepatocytes cultured in control medium (SAF supplemented with 1 mM methionine) ( $\square$ ) compared with control medium plus 0.18 mM cystine ( $\blacktriangle$ ) and pretreatment with 2 mM DTT for 30 min ( $\bullet$ ). Data pooled from 13 different cell preps are represented. Cell GSH (the sum of cell and supernatant GSH at the end of efflux experiments) was varied by pretreating cells with varying doses of diethyl maleate (0.03–0.3 mM for 30 min). In addition, very high GSH values were obtained under low cell density conditions (see Methods). The solid lines represent nonlinear least squares computer fits of the Hill equation to the data using the SAAM program. *n*, the number of binding/transport sites, had to be constrained to 2 to avoid systematic deviations between the fitted curves and the data.  $V_{\max}$  and  $K_m$  were determined by the program. The  $V_{\max}$  values (nmol/10<sup>6</sup> cells per min) were control medium = 0.24±0.01, plus cystine = 0.16±0.01, plus DTT = 1.20±0.06. The  $K_m$  values (nmol/10<sup>6</sup> cells) were control medium = 124±5.8, plus cystine = 124±6.8, plus DTT = 146±8.7. Values are mean±SD. *P* < 0.05 between  $V_{\max}$  values of cystine and DTT treated vs. control medium by unpaired *t* test. The  $K_m$  values did not differ significantly.

the efflux experiment (equivalent to cell GSH at the start of efflux measurements) since there is no GSH synthesis during the 90-min incubation in Krebs-Henseleit buffer in the absence of precursor amino acids (10). From the data in Fig. 1 the following kinetic parameters for GSH efflux (mean±SD) were calculated:  $V_{\max}$  = 0.16±0.01 nmol/10<sup>6</sup> cells per min and  $K_m$  = 124±5.8 nmol/10<sup>6</sup> cells for cells cultured in control medium plus 0.18 mM cystine;  $V_{\max}$  = 0.24±0.01 nmol/10<sup>6</sup> cells per min and  $K_m$  = 124±6.8 nmol/10<sup>6</sup> cells for cells cultured in control medium. The difference in  $V_{\max}$  was significant (*P* < 0.05, unpaired *t* test). The medium pH, cell diameters, and cell viability were unaffected by the addition of cystine (data not shown).

**Dose dependence and time course.** Table I shows the dose effect of cystine on GSH efflux from cultured hepatocytes after overnight incubation. There was a near maximal, ~ 50%, inhibition in GSH efflux when cells were incubated in the presence of 0.1 mM cystine overnight; a concentration of cystine that approximates that found in rat plasma (23). In terms of the time course of the cystine inhibitory effect on GSH efflux, we noted maximum inhibition after 1 h of pretreatment of cells with 0.5 mM cystine without recovery for 90 min after cystine removal (1 h pretreatment: control cell GSH = 113±9.7 nmol/10<sup>6</sup> cells, GSH efflux rate = 0.12±0.01 nmol/10<sup>6</sup> cells per min; cystine-treated cell GSH = 102±8.8, GSH efflux rate = 0.05±0.003 or ~ 43% of control rates; results are expressed as mean±SEM from four cell preparations and GSH efflux rates are significantly different from controls; *P* < 0.001 by paired *t* test). With shorter duration of exposure (15 min), there was inhibition of GSH efflux initially (up to 30 min after cystine removal), which subsequently recovered to that of control efflux rates (data not shown).

**Relationship of cystine transport and the ability of cystine to inhibit GSH efflux.** We next examined whether cystine uptake is required for the inhibition of GSH efflux. We as well as others have shown previously that cystine uptake is induced (via the  $X_c^-$  system and inhibited by glutamate) in cultured rat hepatocytes, especially in the presence of insulin and hydrocor-

**Table I.** Dose Effect of Cystine on GSH Efflux from Cultured Hepatocytes after Overnight Incubation

Dose	Cell GSH	GSH efflux	Fractional GSH efflux*
mM	nmol/10 <sup>6</sup> cells	nmol/min per 10 <sup>6</sup> cells	% total GSH effluxed/h
0	100±17	0.11±0.01	6.8±0.8
0.02	96±20	0.08±0.01 <sup>‡</sup>	5.0±0.6
0.1	92±17	0.05±0.01 <sup>‡</sup>	3.7±0.5 <sup>‡</sup>
0.5	89±18	0.04±0.01 <sup>‡</sup>	3.1±0.5 <sup>‡</sup>

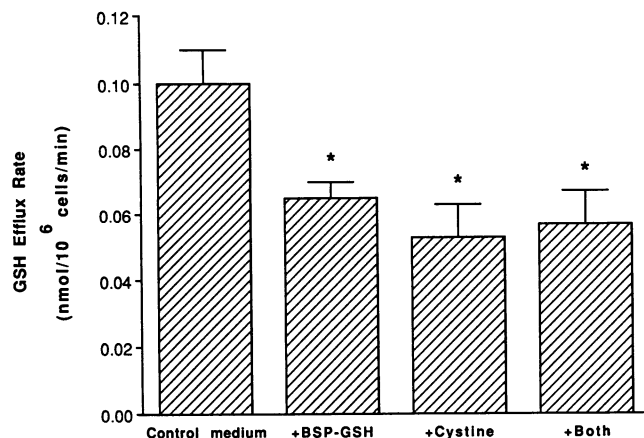
Results are expressed as mean±SEM from five cell preparations. Cells were cultured in control medium (SAF supplemented with 1 mM methionine, see Methods) with varying concentrations of cystine added. After an overnight incubation, medium was aspirated and cells were washed free of cystine and GSH efflux was determined in Krebs buffer alone (see Methods for details). Cell GSH refers to the sum of cell and supernatant GSH at the end of efflux experiments. \* Fractional GSH efflux = % of total GSH (cell + supernatant) that appeared in the supernatant per hour. <sup>‡</sup> *P* < 0.05 vs. control by ANOVA followed by Fisher's test.

tisone (24, 25). Using conditions (coincubation of cells with 0.1 mM cystine and 2.5 mM glutamate for 1 h) where cystine uptake would be negligible as we have previously shown (25), we saw no change in the inhibitory effect of cystine on GSH efflux (control cell GSH =  $81 \pm 2$  nmol/ $10^6$  cells, GSH efflux rate =  $0.09 \pm 0.02$  nmol/ $10^6$  cells per min; cystine plus glutamate-treated cell GSH =  $83 \pm 2$ , GSH efflux rate =  $0.05 \pm 0.02$ ; results are expressed as mean  $\pm$  SEM from three cell preparations and GSH efflux rates are significantly different;  $P < 0.01$  by paired *t* test). Treatment with glutamate alone had no influence on GSH efflux (data not shown).

To further address the temporal relationship of cystine transport and the ability of cystine to inhibit GSH transport in cultured hepatocytes, we studied both at 2 h after plating. As early as 2 h after plating, treatment of cultured cells with cystine (0.5 mM for 1 h, treatment began 1 h after plating) also significantly inhibited GSH efflux (control cell GSH =  $86 \pm 1$ , cystine treated =  $85 \pm 2$  nmol/ $10^6$  cells; control GSH efflux =  $0.17 \pm 0.01$ , cystine treated =  $0.10 \pm 0.01$  nmol/ $10^6$  cells per min; results are expressed as mean  $\pm$  SEM from three cell preparations;  $P < 0.05$  between the two GSH efflux rates). At the same time, there was no glutamate-inhibitable cystine uptake (data not shown). This is in agreement with findings of Takada and Bannai (24), who noted induction of cystine transport via the glutamate-inhibitable  $X_c^-$  system in culture required a time lag of 12 h. Thus, these results strongly suggest that there is dissociation between cystine transport and its ability to inhibit GSH efflux and that cystine exerts its inhibitory effect from outside the cell.

**Polarity.** To assess the polarity of the cystine effect in cultured hepatocytes, we studied the effect of BSP-GSH, a selective sinusoidal GSH transport inhibitor (7), on cystine-mediated inhibition of GSH efflux. As shown in Fig. 2, when cultured hepatocytes were incubated with BSP-GSH (40  $\mu$ M), total GSH efflux was inhibited by  $\sim 40\%$ , similar to that observed previously (7). Cystine (0.5 mM) coincubation did not result in further inhibition of GSH efflux. Furthermore, BSP-GSH exerted no additional inhibition after cells were pretreated with cystine first (GSH efflux rates in nmol/ $10^6$  cells per min: control =  $0.10 \pm 0.02$ , cystine [0.5 mM, 1-h pretreatment] =  $0.04 \pm 0.01$ , BSP-GSH [40  $\mu$ M, 1-h pretreatment] =  $0.05 \pm 0.003$ , cystine treatment first followed by its removal and BSP-GSH treatment =  $0.04 \pm 0.01$ ; results are expressed as mean  $\pm$  SEM from four cell preparations;  $P < 0.05$  between each treatment group and control by ANOVA. Cell GSH values [ $104 \pm 12.2$  nmol/ $10^6$  cells] were similar among these groups). Thus, the BSP-GSH-insensitive transport, presumably the canalicular equivalent, was not affected by cystine, suggesting that cystine inhibits sinusoidal GSH transport. The inhibitory effect of BSP-GSH was rapidly reversible since no inhibition was seen when BSP-GSH was not present in the Krebs buffer during the 90-min efflux experiment (data not shown). This rapid reversibility also excludes a nonspecific toxic effect of BSP-GSH on cultured hepatocytes.

**Possible mechanisms.** In examining the potential mechanisms of cystine-mediated inhibition of GSH efflux, we systematically examined the effect of cystine on possible or known driving forces of GSH efflux, namely cellular ATP and membrane potential (9, 10). The former is especially important since cystine loading (0.5 mM) of proximal tubule cells has been shown to inhibit bicarbonate and glucose transport by depleting cellular ATP ( $\sim 50\%$  depletion) (26, 27). However,



**Figure 2.** The effect of BSP-GSH on GSH efflux and cystine-mediated inhibition of GSH efflux from cultured hepatocytes. Cells were cultured overnight in the control medium with either BSP-GSH (40  $\mu$ M), cystine (0.5 mM), or both. The next day, the medium was aspirated and the cells were washed twice. GSH efflux studies were carried out in Krebs buffer alone after overnight incubation in control medium  $\pm$  cystine or in Krebs buffer with BSP-GSH (40  $\mu$ M) added back after overnight incubation in control medium + BSP-GSH. Results are expressed as mean  $\pm$  SEM from four cell preparations. Cell GSH (the sum of cell and supernatant GSH at the end of efflux experiments) did not differ significantly among the different groups (control medium =  $121 \pm 11$ , +BSP-GSH =  $140 \pm 12$ , +cystine =  $111 \pm 12$ , +both =  $133 \pm 11$  nmol/ $10^6$  cells). \* $P < 0.05$  vs. control medium by ANOVA followed by Fisher's test.

we found no difference in cellular ATP levels after overnight incubation of cultured hepatocytes with 0.5 mM cystine (control =  $15.1 \pm 0.99$ , cystine treated =  $17.1 \pm 1.09$  nmol/ $10^6$  cells,  $n = 3$  cell preparations).

We used alanine uptake as an indirect measure of changes in membrane potential since its initial rate of uptake is known to be influenced by membrane potential and is inhibited by ouabain (28). Like others, we also found alanine uptake to be linear to 2 min; thus all studies were done at 2 min of incubation. When cells were pretreated with ouabain (1 mM) for 30 min, alanine uptake was inhibited by 25%, whereas cystine (0.5 mM) pretreatment had no effect on alanine uptake (control =  $0.58 \pm 0.11$ , cystine pretreated =  $0.54 \pm 0.14$ , ouabain pretreated =  $0.43 \pm 0.09$  nmol/mg protein per min; results are expressed as mean  $\pm$  SEM from four cell preparations;  $P < 0.05$  between ouabain pretreated and control by ANOVA). Thus, it is unlikely that cystine inhibited GSH efflux by depolarizing the membrane potential.

Since the recycling assay (15) used to measure GSH does not detect CySSG, one possible explanation for the apparent cystine-mediated inhibition of GSH efflux is that, after cells are loaded with cystine (minimum pretreatment time of 1 h was used in above studies), there may be a slow release of cystine into the supernatant during the efflux experiment (90 min) even though incubation media were washed free of cystine before determining GSH efflux. Supernatant (90 min) and cell (after TCA precipitation, see Methods) samples therefore were processed for HPLC to determine the molecular form of GSH. We found no supernatant or intracellular CySSG under these conditions. Furthermore, 99% of the GSH in both supernatant and cells was present as the reduced form. After cystine pretreatment (0.5 mM for 1 h) the amount of GSH (in nmol/ $10^6$

cells) was decreased in the 90-min supernatant sample (control =  $18.1 \pm 2.1$ , cystine =  $12 \pm 1.9$ ) but increased inside the cell (control =  $75.8 \pm 7.5$ , cystine =  $87.3 \pm 6.5$ ) whereas total GSH was unchanged (control =  $93.3 \pm 7.6$ , cystine =  $97.6 \pm 7.4$ ) compared with controls (results are expressed as mean  $\pm$  SEM from six cell preparations,  $P < 0.05$  vs. control by paired *t* test in the cases of cell and supernatant GSH), reflecting the inhibition of efflux.

Finally, to see if the effect of cystine on GSH efflux is mediated by formation of disulfides, we treated cultured cells overnight with cystine (0.5 mM) followed by treatment with DTT (2 mM) for 30 min to reduce disulfides before efflux experiments. Table II shows that DTT not only completely reversed the inhibitory effect of cystine, but unexpectedly stimulated GSH efflux. A potential error is the assay used to measure GSH, which relies on 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) (15). DTT also reacts with DTNB and could give falsely elevated values (even though DTT was washed off before experiments, it is possible that DTT that had accumulated in cells was slowly released during efflux studies). However, we confirmed the DTT-induced increase in GSH efflux by performing HPLC analysis, which clearly differentiates GSH from DTT (data not shown).

**Contribution of CySSG to inhibition of GSH efflux.** Even though there was no CySSG formed during efflux experiments after removal of cystine, it is well known that, in the presence of cystine, the efflux of GSH results in the formation of CySSG in the medium, which in fact has been used by some investigators to estimate GSH efflux (29). To address the question of whether it was cystine or CySSG, or the combination that inhibited GSH efflux, the amount of CySSG formed during cystine incubation was quantitated by HPLC in some experiments and in others by measuring the amount of GSH effluxed into the medium in the controls (thus, maximum amount of CySSG that could have formed). Cell GSH was varied by pretreating cultured cells with diethyl maleate (0–0.5 mM) for 20 min to have different amounts of CySSG present during cystine (0.5 mM) incubation (30 min). GSH efflux was then measured in

Krebs buffer alone. GSH efflux was still inhibited by cystine ( $\sim 60 \pm 3\%$  of controls) after exposure to formed CySSG varying from 0.1 to 1.8  $\mu$ M (results are from seven cell preparations). The degree of inhibition did not correlate with the concentration of CySSG present at the end of the cystine treatment period. Finally, the effect of CySSG on GSH efflux in cultured cells was directly examined by treating cultured cells with 2.0  $\mu$ M of CySSG for 1 h. CySSG had no effect on GSH efflux at this concentration (cell GSH: control =  $143 \pm 3$ , CySSG =  $136 \pm 13$ , cystine control (0.5 mM) =  $135 \pm 9$  nmol/ $10^6$  cells; GSH efflux: control =  $0.11 \pm 0.01$ , CySSG =  $0.10 \pm 0.01$ , and cystine =  $0.06 \pm 0.01$  nmol/ $10^6$  cells per min; results are mean  $\pm$  SE from two cell preparations). Of note, cystine also had no effect at 2  $\mu$ M concentration ( $n = 6$  cell preparations). Therefore, the inhibition on GSH efflux was clearly attributable to cystine rather than CySSG in these experiments.

**Other disulfides or thiol-reacting agent.** Table III shows the effects of other disulfides or thiol-reacting agent on GSH efflux in cultured rat hepatocytes. All of the disulfides examined inhibited GSH efflux to a similar degree as cystine. Also, combination of two different disulfides, cystine and GSSG, exerted no additional inhibition. Of particular interest is Thiolyte MQ, which forms covalent fluorescent adducts with exposed thiol groups on the outer membrane surface of intact cells (Calbiochem Corp., La Jolla, CA). When Thiolyte MQ-treated cells were exposed to DTT (2 mM) for 30 min, GSH efflux was restored to the same high rate seen after DTT treatment of control cells (data not shown). Thus, the inhibition by Thiolyte MQ provides additional evidence to support the suggestion that a critical thiol(s) of the transporter protein and/or an adjacent regulatory protein exposed to the external environment undergoes mixed disulfide formation with disulfides in the medium and/or protein disulfide formation leading to inhibition of GSH efflux.

#### Effects of thiol-reducing agents on GSH efflux in cultured rat hepatocytes

**Possible liberation of intracellular or extracellular GSH.** One possible explanation for the DTT effect is that it reduced intra-

Table II. Effect of DTT on Cystine-mediated Inhibition of GSH Efflux from Cultured Hepatocytes

Condition	Cell GSH nmol/ $10^6$ cells	GSH efflux nmol/min per $10^6$ cells	Fractional GSH efflux
			% of total GSH effluxed/h
Control	138 $\pm$ 26	0.11 $\pm$ 0.01	5.2 $\pm$ 0.67
+Cystine	140 $\pm$ 26	0.06 $\pm$ 0.02*	2.4 $\pm$ 0.31*
+DTT	115 $\pm$ 10	0.45 $\pm$ 0.05 <sup>‡</sup>	24 $\pm$ 1.7 <sup>‡</sup>
+DTT and cystine	155 $\pm$ 34	0.68 $\pm$ 0.18*	26 $\pm$ 1.8 <sup>‡</sup>

Results are expressed as mean  $\pm$  SEM from six cell preparations. Cells were cultured in control medium (control, SAF supplemented with 1 mM methionine), plus cystine (0.5 mM) or vehicle overnight. The next day cells were treated with DTT (2 mM added to culture medium) or vehicle for 30 min before efflux experiment. Before efflux experiment, both agents were removed and cells were washed twice and GSH efflux was determined in Krebs buffer alone. Cell GSH refers to the sum of cell and supernatant GSH at the end of efflux experiments. \*  $P < 0.05$ , <sup>‡</sup>  $P < 0.01$  vs. control by ANOVA followed by Fisher's test.

Table III. Effect of Disulfides on GSH Efflux from Cultured Hepatocytes

Treatment	Percent of control		<i>n</i>
	Cell GSH	GSH efflux	
Cystine (0.5 mM)	101 $\pm$ 2.4	54 $\pm$ 3.9*	9
GSSG (0.5 mM)	100 $\pm$ 2.9	57 $\pm$ 3.2*	9
Cystamine (0.5 mM)	95 $\pm$ 3.8	68 $\pm$ 6.7*	5
Thiolyte MQ (0.5 mM)	93 $\pm$ 1.2	67 $\pm$ 4.8*	5
Cystine (0.5 mM) + GSSG (0.5 mM)	102 $\pm$ 3.5	51 $\pm$ 4.5*	4

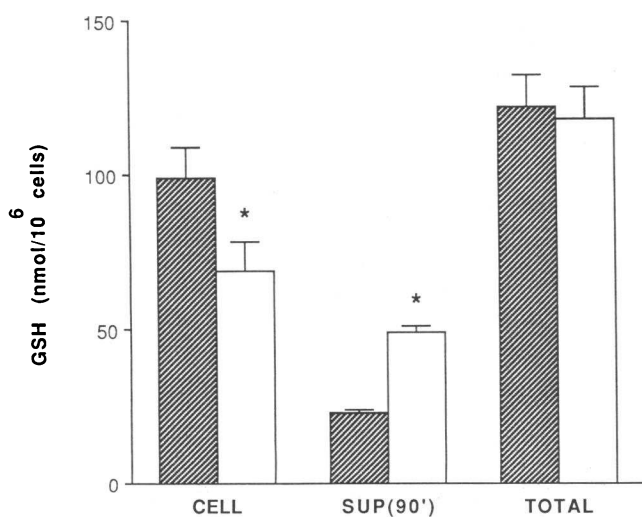
Results are expressed as percent of control (mean  $\pm$  SEM). *n* refers to the number of cell preps. Cells were cultured in the control medium overnight. Effects of these agents were examined by pretreating cells for 1 h. Cells were washed free of these agents during the efflux experiments. Control values were cell GSH (the sum of cell and supernatant GSH at the end of efflux experiments),  $124 \pm 14$  nmol/ $10^6$  cells and GSH efflux,  $0.13 \pm 0.01$  nmol/ $10^6$  cells per min. \*  $P < 0.05$  vs. control by ANOVA followed by Fisher's test.

cellular protein-GSH disulfides, thus raising cell GSH, resulting in increased GSH efflux. Fig. 3 shows the effect of DTT pretreatment (2 mM for 30 min) on cell, supernatant, and total GSH at the end of the 90-min efflux experiment. After DTT pretreatment, cell GSH fell significantly at the end of the efflux experiment whereas supernatant GSH increased, so that the total GSH is unchanged compared with controls. Thus, the effect of DTT cannot be explained by raising cell GSH available for efflux by reduction of protein GSH-mixed disulfides. The increased efflux is reflected in a quantitatively lower cell GSH.

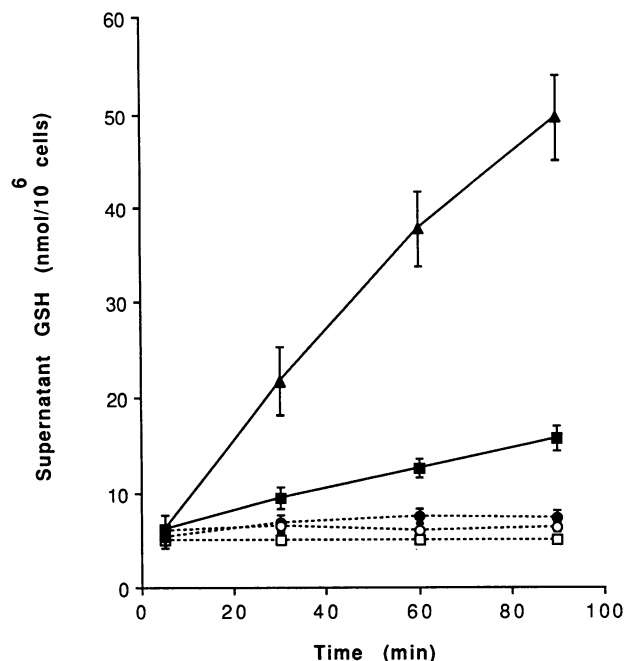
Another potential explanation for the DTT effect is that ectosurface-bound GSH is liberated by the thiol-reducing action of DTT rather than a true increase in the transport of GSH. To examine this, we compared GSH efflux at 37 and 4°C after DTT pretreatment (2 mM for 30 min at 37 or 4°C) with controls. Fig. 4 shows that, after cells were pretreated with DTT at either temperature, no GSH efflux occurred at 4°C, as in controls. Thus, it is unlikely that the DTT effect is due to release of surface-bound GSH since this would have occurred at either temperature, but rather it is most likely due to stimulation of GSH transport.

**Kinetics.** Fig. 1 illustrates the effect of DTT on GSH efflux kinetics in cultured rat hepatocytes. The data in Fig. 1 were fitted by the Hill model using the SAAM program (22) and showed that DTT stimulated GSH efflux by increasing significantly the  $V_{max}$  from  $0.24 \pm 0.01$  to  $1.20 \pm 0.06$  nmol/10<sup>6</sup> cells per min ( $P < 0.05$ , unpaired  $t$  test). The  $K_m$  did not change significantly after DTT treatment (controls =  $124 \pm 6.8$ , DTT treated =  $146 \pm 8.7$  nmol/10<sup>6</sup> cells).

**Time course and dose dependence.** Fig. 5 shows the time course and dose of DTT effect (2 mM). The onset of the effect of DTT was immediate, but required  $\geq 30$  min to reach maximal effect. The magnitude of the increase in GSH efflux was related to the dose as well as the duration of treatment. At 0.1



**Figure 3.** The effect of DTT on cell and supernatant GSH. Cells were plated in the control medium overnight, the effect of DTT was examined by pretreating cells with 2 mM DTT (□) compared with vehicle control (▨) for 30 min. After washing cells free of DTT, GSH efflux experiments were carried out over 90 min in precursor-free Krebs buffer. Cell, supernatant, and total GSH values at the end of the 90 min are shown. Results are expressed as mean  $\pm$  SEM from seven cell preparations. \* $P < 0.001$  vs. control by paired  $t$  test.

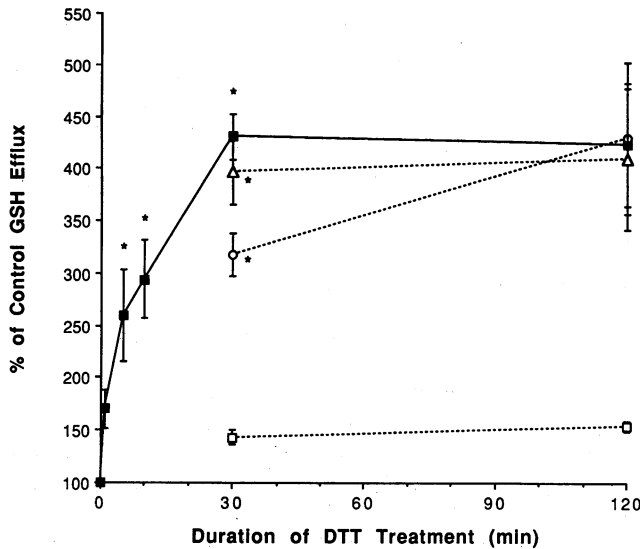


**Figure 4.** The effect of temperature on DTT-mediated increase in GSH efflux. GSH efflux at 37°C from controls (■) or DTT (2 mM, 30 min) pretreated at 37°C (▲) vs. GSH efflux at 4°C from controls (□), DTT pretreated at 37°C (●), or 4°C (○). Results are expressed as mean  $\pm$  SEM from three to five experiments. No measurable GSH efflux occurred at 4°C from controls or DTT pretreated cells (at 4 or 37°C). Cell GSH (the sum of cell and supernatant GSH at the end of efflux experiments) did not differ significantly among the different groups ( $84.2 \pm 10.1$  nmol/10<sup>6</sup> cells). GSH efflux rates (mean  $\pm$  SEM) at 37°C for control was  $0.11 \pm 0.01$  and for DTT was  $0.49 \pm 0.04$  nmol/10<sup>6</sup> cells per min.

mM DTT, regardless of the duration of treatment (30 min vs. 2 h), GSH efflux increased only  $\sim 50\%$ ; at 0.5 mM DTT, GSH efflux rates increased further with 2 h of pretreatment to maximum DTT stimulation. Thus, at 0.1 mM DTT, DTT was limiting (perhaps because of oxidation), at 0.5 mM DTT, the magnitude of effect depended on duration of exposure; and at  $\geq 1.0$  mM the effect was maximal after 30 min.

**Potential mechanisms. Effects on ATP and membrane potential.** DTT pretreatment did not alter cellular ATP levels (controls =  $15.13 \pm 0.99$ , DTT =  $13.47 \pm 1.54$  nmol/10<sup>6</sup> cells; results are expressed as mean  $\pm$  SEM from three cell preparations) or alanine uptake. In fact, GSH efflux was inhibited by 33% after 1 mM ouabain pretreatment for 30 min, but when cells were pretreated with both ouabain and DTT, the ouabain inhibition on GSH efflux was overcome (see Table IV), despite the fact that alanine uptake was still inhibited by ouabain in the presence of DTT. Therefore, the effect of DTT is not mediated by an increase in cellular ATP or membrane hyperpolarization, specifically, via an increase in the Na/K-ATPase activity. Moreover, since DTT did not stimulate alanine uptake, another mechanism for hyperpolarization, such as K<sup>+</sup> conductance, can also be excluded.

**Inhibition of DTT effect.** Next we examined if the stimulatory effect of DTT could be reversed. Table V shows the effects of cystine (0.5 mM) on DTT-stimulated GSH efflux. Cells were treated initially with DTT (2 mM, 30 min), and then medium was changed to DTT-free containing either cystine



**Figure 5.** Time course and dose of DTT effect on GSH efflux. Cells were pretreated with 0.1 (□), 0.5 (○), 1 (△), and 2 mM (■) DTT for varying duration and DTT was removed before efflux experiment. GSH efflux experiments were carried out over 90 min in precursor-free Krebs buffer. Results are expressed as percent mean±SEM of control GSH efflux from 3 to 13 cell preparations. Note that maximal stimulation of GSH efflux occurred after pretreatment of cells with 1 mM DTT for 30 min. Cell GSH (the sum of cell and supernatant GSH at the end of efflux experiments) did not differ significantly among the groups. Control cell GSH = 74±12 nmol/10<sup>6</sup> cells, GSH efflux rate = 0.07±0.01 nmol/10<sup>6</sup> cells per min. \**P* < 0.05 vs. control by ANOVA followed by Fisher's test.

(0.5 mM) or vehicle for an additional 1 h. The results show that cystine partially reversed the effect of DTT (~ 50% reversal). These results suggest that there is at least overlap in the site(s) of action of cystine and DTT and that both probably act via modification of the thiol-disulfide status of the GSH transporter.

**Recruitment of intracellular transporters.** To examine if the mechanism of DTT-stimulated GSH efflux involves recruitment of intracellular transporters via vesicular pathway, we

**Table IV.** Effect of Ouabain and DTT on GSH Efflux and Alanine Uptake from Cultured Hepatocytes

Condition	Cell GSH	GSH efflux	Alanine uptake
	nmol/10 <sup>6</sup> cells	nmol/min per 10 <sup>6</sup> cells	nmol/mg protein per min
Control	117±11.6	0.10±0.01	0.59±0.10
+Ouabain	117±12.3	0.07±0.01*	0.43±0.08*
+DTT	110±7.6	0.42±0.03*	0.59±0.12
+DTT and ouabain	107±8.8	0.40±0.03*	0.44±0.11*

Results are expressed as mean±SEM from five cell preparations. Cells were treated with ouabain (1 mM), DTT (2 mM), or both for 30 min before experiments. GSH efflux and alanine uptake were studied as described in Methods. Cell GSH refers to the sum of cell and supernatant GSH at the end of efflux experiments. \**P* < 0.05 vs. control by ANOVA followed by Fisher's test.

studied the effects of inhibitors of microtubular function and vesicular acidification (30, 31) on DTT-stimulated GSH efflux. However, pretreatment of cells with colchicine (100 μM), chloroquine (1 mM), monensin (10 μM), and nocodazole (10 μg/ml) for 2 to 3 h had no effect on basal or DTT-stimulated GSH efflux (data not shown). This suggests that the effect of DTT is not likely to be recruitment of GSH transporters from an intracellular pool by vesicular pathway.

**Involvement of signal transduction pathways.** We previously showed that cAMP-dependent hormones stimulated GSH efflux by a ouabain-sensitive mechanism, presumably via stimulation of Na/K-ATPase activity (10). We have already shown that ouabain did not inhibit the DTT-mediated stimulation of GSH efflux (Table IV). To see if other signal transduction mechanisms, specifically protein kinase C and calmodulin-Ca<sup>2+</sup> complex, may be involved in mediating the DTT effect, we studied the effect of inhibitors of these pathways, staurosporin and calmidazolium, respectively, on DTT-mediated stimulation of GSH efflux. We have previously used these inhibitors to prevent Ca<sup>2+</sup> and protein kinase C-dependent hormones from inhibiting GSH synthesis in cultured rat hepatocytes (32). Treatment of cultured rat hepatocytes with staurosporin (1 μM) or calmidazolium (5 μM) for 30 min did not alter the basal GSH efflux rates or prevent the DTT-mediated stimulation of GSH efflux (GSH efflux rates in nmol/10<sup>6</sup> cells per min: control = 0.09±0.01, DTT = 0.38±0.02, staurosporin = 0.09±0.01, calmidazolium = 0.10±0.02, staurosporin followed by DTT [2 mM, 30 min] = 0.42±0.01, calmidazolium followed by DTT = 0.39±0.01; results are expressed as mean±SEM from three cell preparations, *P* < 0.05 between DTT, staurosporin-DTT, calmidazolium-DTT, and control by ANOVA. Cell GSH levels [83±7 nmol/10<sup>6</sup> cells] did not differ significantly among the different groups). Thus, the effect of DTT is unlikely to be mediated by any of these signal transduction mechanisms.

**Effects of other thiols on GSH efflux in cultured hepatocytes.** We examined the effect of a number of thiol-reducing agents on GSH efflux. Table VI shows that L-DTT and DTE were as effective as DTT (DL-DTT) in stimulating GSH efflux. Of the other thiol agents examined, cysteamine (4 mM) exerted a minimal stimulation and N-acetylcysteine had no effect. Total cell thiols increased to a similar extent with all these agents (data not shown), suggesting that the ineffectiveness was not due to limited cellular uptake, but rather due to the structural differences and potency of the thiol-reducing effect.

#### Effects of cystine and DTT on GSH efflux in perfused livers

We used the perfused liver model to verify the physiological significance of these findings in cultured cells and to better define the polarity of the effects. Fig. 6 shows the effect of cystine on GSH efflux in the perfused liver. Immediately after cystine perfusion, HPLC analysis showed that almost all the GSH released from the liver into perfusate had reacted with cystine, forming CySSG. Thus, the GSH mass refers to the total of CySSG and GSH. Cystine, at physiological concentration (0.1 mM), inhibited sinusoidal GSH efflux with maximum inhibition occurring after 10 min of treatment. This inhibition was reversible, as sinusoidal GSH efflux rates returned to control level 10 min after termination of treatment. Biliary total GSH (GSH + GSSG) efflux and bile flow were unaffected. It should be noted that cystine is not taken up by the intact liver therefore its action must be external. Fig. 7 illustrates the effect



Table V. Effect of Cystine on DTT-mediated Stimulation of GSH Efflux from Cultured Hepatocytes

Pretreatments		Cell GSH nmol/10 <sup>6</sup> cells	GSH efflux nmol/min per 10 <sup>6</sup> cells	Fractional GSH efflux % of total GSH effluxed/h
0–30 min	30–90 min			
Vehicle	None	141±21.4	0.15±0.01	7.1±1.01
DTT	None	135±20.8	0.55±0.05*	26.0±2.3*
DTT	Control medium	135±19.4	0.55±0.05*	25.0±1.7*
DTT	Control medium + cystine	131±19.8	0.27±0.03‡	13.6±3.1*

Results are expressed as mean±SEM from five cell preparations. Cells were cultured in control medium overnight. The next day, cells were either treated with DTT (2 mM, 30 min) or vehicle followed immediately with efflux experiments or had medium changed to SAF + methionine (control medium) alone or plus cystine (0.5 mM) for 1 additional hour. Efflux experiments were done in Krebs buffer alone. Cell GSH refers to the sum of cell and supernatant GSH at the end of efflux experiments. \*  $P < 0.05$  vs. control, ‡  $P < 0.05$  vs. control, DTT, and DTT-control medium by ANOVA followed by Fisher's test.

of DTT (1 mM) on GSH efflux in the perfused liver. Depending on the duration of DTT treatment, 400–500% increase in sinusoidal GSH efflux rates were noted. The stimulatory effect of DTT was immediate but required 10–15 min before reaching its maximum. With shorter duration of treatment, there was a trend toward reversibility not seen with longer exposure to DTT. Biliary total GSH (GSH + GSSG) efflux and bile flow were unaffected by DTT treatment.

To exclude changes in paracellular permeability as an explanation for the increased appearance of GSH in the effluent perfusate during DTT treatment, we measured the steady state ratios of bile to perfusate [<sup>14</sup>C]sucrose concentrations. There was no change in the ratio during DTT treatment (control period = 0.30±0.02, DTT = 0.35±0.03; results are expressed as mean±SEM from four animals). These values agree closely with reported values (18, 19). Bile flow did not change during DTT perfusion (control = 0.97±0.03, DTT = 0.93±0.08 μl/min per g).

## Discussion

While studying the effect of culture conditions on efflux of GSH from rat hepatocytes, we noted lower rates in the presence

Table VI. Effect of Thiol Reagents on GSH Efflux from Cultured Hepatocytes

Treatment	Percent of control		n
	Cell GSH	GSH efflux	
DTT (2 mM)	100±2.9	346±21*	7
L-DTT (2 mM)	111±8.8	323±30*	4
DTE (2 mM)	104±5.2	381±12*	5
Cysteamine (4 mM)	101±3.1	133±4.1*	5
N-acetylcysteine (4 mM)	90±5.3	80±5.4	5

Results are expressed as percent of control (mean±SEM). n refers to the number of cell preparations. Cells were cultured in the control medium overnight. Effects of thiols were examined by pretreating cells with thiols for 30 min. Cells were washed free of these agents during the efflux experiments. Control values were cell GSH (the sum of cell and supernatant GSH at the end of efflux experiments), 108±17.4 nmol/10<sup>6</sup> cells and GSH efflux, 0.15±0.01 nmol/10<sup>6</sup> cells per min. \*  $P < 0.05$  vs. control by ANOVA followed by Fisher's test.

of cystine. In pursuing this initial observation in greater detail, we found that, when cystine was deleted from the culture media, the  $V_{max}$  for GSH efflux was 50% greater whereas the  $K_m$  was unaltered. The cystine-mediated inhibition of GSH efflux occurred rapidly, with as little as 0.02 mM cystine and had near maximum effect (55% inhibition) at 0.1 mM cystine concentration, which is the level found in rat plasma (23). The inhibi-

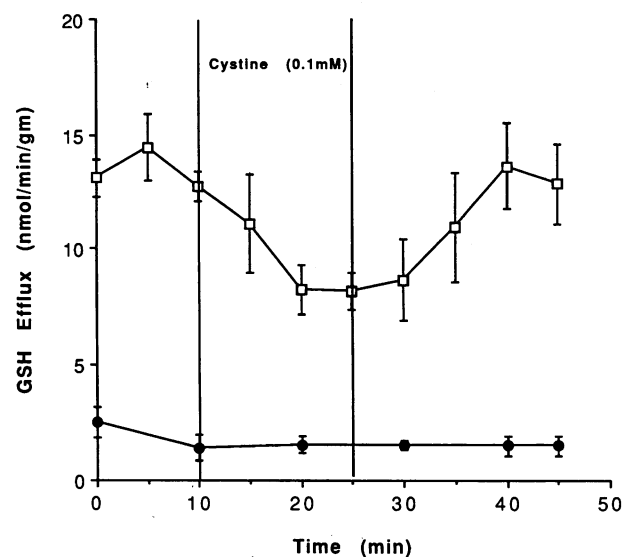
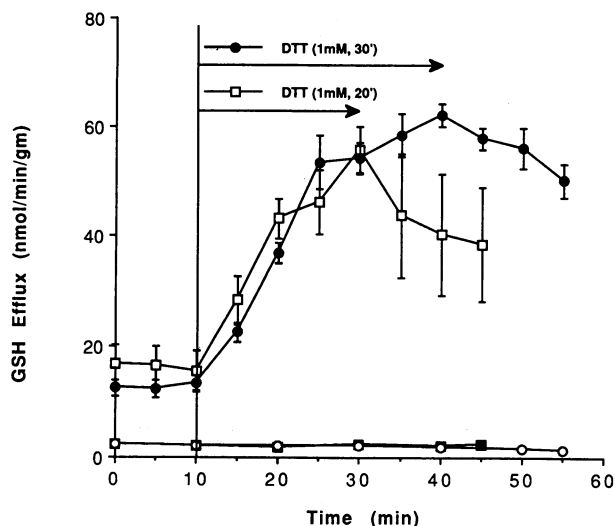


Figure 6. Effect of cystine (0.1 mM) on GSH efflux in the single-pass perfused rat liver. Results are expressed as mean±SEM from five animals. Sinusoidal GSH efflux (□) was determined by HPLC (see Methods) and recovered almost completely as a mixed disulfide with cysteine. GSH efflux was inhibited maximally after 10 min of perfusion with cystine and reversed 10 min after termination of cystine perfusion. Biliary total GSH (GSH + GSSG) efflux (●) was unaffected. Sinusoidal GSH efflux values from control period (average of three determinations per rat and mean±SEM from five rats) = 13.43±0.92, GSH efflux during cystine perfusion (average of three lowest values after start of cystine perfusion) = 7.72±1.07, mean recovery period (last three values after termination of cystine) = 11.98±1.65 nmol/min per g.  $P < 0.05$  between sinusoidal GSH efflux values during cystine perfusion and controls by ANOVA followed by Fisher's test. Bile flow (μl/min per g) did not change significantly during perfusion (control = 1.18, cystine = 1.05). Liver GSH values (μmol/g) were starting = 5.75±0.51, end = 5.07±0.47, amount lost due to efflux = 0.76±0.07.



**Figure 7.** Effect of DTT (1 mM) on GSH efflux in the perfused liver. Results are expressed as mean $\pm$ SEM from four animals for both 20- and 30-min DTT perfusions. Sinusoidal GSH efflux ( $\square$ ,  $\bullet$ ) was stimulated immediately after starting DTT but did not reach maximal stimulation until 15 min exposure to DTT. Sinusoidal GSH efflux values (nmol/min per g) from control period (average of three determinations per rat and mean $\pm$ SEM from four rats) = 16.33 $\pm$ 3.5 for 20-min DTT perfusions and 12.81 $\pm$ 1.4 for 30-min DTT perfusions, mean DTT values (average of three highest values after start of DTT perfusion) = 53.61 $\pm$ 5.1 for 20-min DTT perfusions and 60.56 $\pm$ 2.2 for 30-min DTT perfusions, mean recovery period (last three determinations after termination of DTT) = 40.87 $\pm$ 10.8 for 20-min DTT perfusions and 54.58 $\pm$ 2.7 for 30-min DTT perfusions.  $P < 0.05$  between sinusoidal GSH efflux values during DTT perfusion (both 20 and 30 min) and controls by ANOVA followed by Fisher's test. Biliary total GSH (GSH + GSSG) efflux ( $\blacksquare$ ,  $\circ$ ) was unaffected by DTT. Bile flow ( $\mu$ l/min per g) did not change significantly during perfusion (control = 1.16, DTT = 1.23). Liver GSH values ( $\mu$ mol/g) were starting = 6.23 $\pm$ 0.26 and 5.87 $\pm$ 0.24, end = 4.23 $\pm$ 0.35 and 3.68 $\pm$ 0.20, amount lost due to efflux = 1.70 $\pm$ 0.16 and 1.80 $\pm$ 0.09 for 20- and 30-min DTT perfusions, respectively.

tory effect of cystine appeared to be exerted from outside of the cell since it occurred under conditions in which cystine uptake was either inhibited or had not been induced in culture and was observed in perfused liver, which does not take up cystine. The inhibitory effect was exerted at the sinusoidal pole since it was not additive with inhibition by the selective sinusoidal GSH transport inhibitor BSP-GSH and was directly confirmed in the perfused liver. The mechanism involved was not ATP depletion or membrane depolarization since cellular ATP levels and alanine uptake were unaffected by cystine treatment. We previously found that 15-min incubation of isolated hepatocytes with GSSG *trans*-stimulated GSH efflux (11). In the present report, inhibition of GSH efflux was seen after 1 h of preincubation with GSSG and its removal. Thus, the predominant effect of extracellular GSSG on GSH efflux will depend upon duration of exposure and presence of GSSG. Other disulfides, including cystamine and Thiolyte MQ also inhibited GSH efflux. The effect of the latter is especially revealing since it is not membrane permeant. Thus, the findings are most consistent with soluble disulfides acting through disulfide exchange with membrane ectosurface thiol(s) closely associated with the function of the GSH transporter.

One previous report suggested that cystine resulting from the rapid oxidation of cysteine in the recirculating rat liver perfusion model inhibited GSH efflux (33). However, we had previously discounted this work as not being interpretable (1) since the methods used could not account for extracellular CySSG formation, which indeed is shown to be the exclusive molecular form of GSH after efflux in the presence of excess cystine. However, despite this masking of GSH, using appropriate HPLC techniques we now show conclusively that cystine inhibits sinusoidal GSH efflux and it does so by decreasing  $V_{\max}$  for GSH efflux. Since this effect is seen at physiological concentrations of cystine, we assume that the transport system is under some degree of constant inhibitory tone. Whether this tone varies *in vivo*, because of changes in plasma thiols and disulfides, remains to be determined. However, we speculate that this could be a factor in interorgan homeostasis. For example, increased availability of cystine in plasma (because of dietary intake or extrahepatic breakdown of GSH to cysteine and its autoxidation to cystine) could signal the liver through this mechanism to decrease release of GSH since the need for GSH to supply cysteine/cystine is reduced. However, it remains to be seen how responsive this system is to regulation through changes in extracellular thiol redox conditions that occur *in vivo* under normal or pathophysiological circumstances.

DTT not only reversed the effect of cystine and Thiolyte MQ, it stimulated GSH efflux by 400–500%. Although stimulation of GSH efflux by DTT was suggested by Hahn and Oberrauch (34) in the perfused liver in 1978, their findings were inconclusive because of lack of appropriate methodology to measure GSH in the presence of DTT, which has now been overcome by the use of derivatization and HPLC. The effect of DTT on GSH efflux was not seen at 4°C, which supports the view that its effect is on the carrier. We have previously observed that extracellular GSH *trans*-stimulates efflux of cell GSH and vice versa (11). Our present findings with DTT, however, raise the possibility that stimulation of fractional efflux of cell GSH in the presence of extracellular GSH is due to thiol-reducing action of the latter on the transporter as opposed to true *trans*-stimulation. Several points argue against this possibility: (a) we previously showed that extracellular GSH maximally *trans*-stimulates cell GSH efflux at extracellular GSH concentrations considerably lower than intracellular; cell GSH did not fall in that case, indicating one-for-one exchange (11). If the effect of GSH were through a thiol action, as with DTT, a fall in cell GSH would be expected when the extracellular GSH concentration is lower than intracellular; (b) in contrast to dithiols, monothiols other than GSH exerted little or no stimulatory effect on GSH transport; (c) *trans*-stimulation of cell GSH efflux by extracellular non-thiol-containing analogues of GSH, such as ophthalmic acid and BSP-GSH, was previously observed by us; and (d) the effect of DTT on GSH efflux differs from that of GSH in that the stimulation persists after removal of DTT whereas the stimulation of efflux immediately reverses after removal of GSH (11). Thus, we would conclude that DTT exerts a stimulatory effect through a thiol-reducing action on the carrier whereas GSH and its analogues exert a stimulatory effect mainly by serving as substrates for bidirectional transport. However, it is not possible for us to exclude the possibility that some portion of the stimulatory effect of GSH on the carrier is due to a thiol action.

DTT markedly increased the  $V_{\max}$  without altering the driving forces for GSH transport. The effect of DTT is unlikely to

be mediated by cAMP, protein kinase C, or calmodulin-Ca<sup>2+</sup> signal transduction pathways. Several explanations are possible, including activation of membrane transporters that exist in an inactive disulfide form or mobilization of an intracellular pool of transporters through fusion of intracellular vesicles with the plasma membrane. The latter possibility is unlikely on the basis of our findings that colchicine, chloroquine, monensin, and nocodazole did not prevent the stimulation of GSH efflux by DTT.

As noted above, not all thiols stimulated GSH efflux. All the dithiols studied exerted a stimulatory effect whereas monothiols such as cysteamine and *N*-acetylcysteine had little or no effect. It is unclear if these differences reflect the chemical-reducing properties of dithiols or the structural properties required to gain access to the critical disulfide that is being reduced. This difference in action of mono- and dithiols is well known in reducing protein vicinal thiols (35).

In summary, our results show that the capacity of the sinusoidal GSH transporter is affected by the thiol-disulfide status of certain critical sulfhydryl group(s). These findings suggest that there may be critical cysteine residue(s) present on the GSH transporter whose thiol status determine the activity of the transporter. Elucidation of the exact molecular mechanisms will require cloning and sequencing of the sinusoidal GSH transporter.

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## References

- Kaplowitz, N., T. K. Aw, and M. Ookhtens. 1985. The regulation of hepatic glutathione. *Annu. Rev. Pharmacol. Toxicol.* 25:715-744.
- Meister, A., and M. E. Anderson. 1983. Glutathione. *Annu. Rev. Biochem.* 52:711-760.
- Lauterburg, B. H., J. D. Adams, and J. R. Mitchell. 1984. Hepatic glutathione homeostasis in the rat: efflux accounts for glutathione turnover. *Hepatology* 4:586-590.
- Fernandez-Checa, J. C., C. Garcia-Ruiz, M. Ookhtens, and N. Kaplowitz. 1991. Impaired uptake of glutathione by hepatic mitochondria from chronic ethanol-fed rats. *J. Clin. Invest.* 87:397-405.
- Ookhtens, M., K. Hobdy, M. C. Corvasce, T. Y. Aw, and N. Kaplowitz. 1985. Sinusoidal efflux of glutathione in the perfused rat liver: evidence for a carrier-mediated process. *J. Clin. Invest.* 75:258-265.
- Aw, T. K., M. Ookhtens, C. Ren, and N. Kaplowitz. 1986. Kinetics of glutathione efflux from isolated rat hepatocytes. *Am. J. Physiol.* 250:G236-G243.
- Ookhtens, M., I. Lyon, J. Fernandez-Checa, and N. Kaplowitz. 1988. Inhibition of glutathione efflux in the perfused rat liver and isolated hepatocytes by organic anions and bilirubin. Kinetics, sidedness, and molecular forms. *J. Clin. Invest.* 82:608-616.
- Aw, T. K., M. Ookhtens, and N. Kaplowitz. 1984. Inhibition of glutathione efflux from isolated rat hepatocytes by methionine. *J. Biol. Chem.* 259:9355-9358.
- Fernandez-Checa, J. C., C. Ren, T. K. Aw, M. Ookhtens, and N. Kaplowitz. 1988. Effect of membrane potential and cellular ATP on glutathione efflux from isolated rat hepatocytes. *Am. J. Physiol.* 255:G403-G408.
- Lu, S., C. Garcia-Ruiz, J. Kuhlenkamp, M. Ookhtens, M. Salas-Prato, and N. Kaplowitz. 1990. Hormonal regulation of GSH efflux. *J. Biol. Chem.* 265:16088-16095.
- Garcia-Ruiz, C., J. C. Fernandez-Checa, and N. Kaplowitz. 1992. Bidirectional plasma membrane transport of reduced glutathione in intact hepatocytes and membrane vesicles. *J. Biol. Chem.* 267:22256-22264.
- Fernandez-Checa, J. C., Y. R. Yi, C. Garcia-Ruiz, Z. Knezic, S. M. Tahara, and N. Kaplowitz. 1993. Expression of rat liver sinusoidal and canalicular GSH transport systems in *Xenopus laevis* oocytes. *J. Biol. Chem.* 268:2324-2328.
- Eriksson, B., and S. A. Eriksson. 1967. Synthesis and characterization of the L-cysteine-glutathione mixed disulfide. *Acta. Chem. Scand.* 21:1304-1312.
- Moldeus, P., J. Hogberg, and S. Orrenius. 1978. Isolation and use of liver cells. *Methods Enzymol.* 51:60-70.
- Tietze, F. 1969. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. *Anal. Biochem.* 27:502-522.
- Fariss, M. W., and D. J. Reed. 1987. High-performance liquid chromatography of thiols and disulfides: dinitrophenol derivatives. *Methods Enzymol.* 143:101-109.
- Booth, J. B., E. Boyland, and P. Sims. 1961. An enzyme from rat liver catalyzing conjugations with glutathione. *Biochem. J.* 79:516-524.
- Ballatori, N., and A. T. Truong. 1989. Altered hepatic junctional permeability, bile acid excretion and glutathione efflux during oxidant challenge. *J. Pharmacol. Exp. Ther.* 251:1069-1075.
- Ookhtens, M., and T. Maddatu. 1991. Mechanism of changes in hepatic sinusoidal and biliary glutathione efflux with age in rats. *Am. J. Physiol.* 261:G648-G656.
- Van Dyke, R. W., and B. F. Scharschmidt. 1983. (Na,K)-ATPase-mediated cation pumping in cultured rat hepatocytes. *J. Biol. Chem.* 258:12912-12919.
- Jones, D. P. 1981. Determination of pyridine nucleotides in cell extracts by high-performance liquid chromatography. *J. Chromatogr.* 225:446-449.
- Berman, M., and M. F. Weiss. 1977. SAAM Manual. Publication No. 76-730. Department of Health, Education and Welfare, National Institutes of Health, Government Printing Office, Washington, DC.
- Lash, L. H., and D. P. Jones. 1985. Distribution of oxidized and reduced forms of glutathione and cysteine in rat plasma. *Arch. Biochem. Biophys.* 240:583-592.
- Takada, A., and S. Bannai. 1984. Transport of cystine in isolated rat hepatocytes in primary culture. *J. Biol. Chem.* 259:2441-2445.
- Lu, S. C., J. Ge, J. Kuhlenkamp, and N. Kaplowitz. 1992. Insulin and glucocorticoid dependence of hepatic g-glutamylcysteine synthetase and glutathione synthesis in the rat. *J. Clin. Invest.* 90:524-532.
- Salmon, R. F., and M. Baum. 1990. Intracellular cystine loading inhibits transport in the rabbit proximal convoluted tubule. *J. Clin. Invest.* 85:340-344.
- Coor, C., R. F. Salmon, R. Quigley, D. Marver, and M. Baum. 1991. Role of adenosine triphosphate (ATP) and NAK ATPase in the inhibition of proximal tubule transport with intracellular cystine loading. *J. Clin. Invest.* 87:955-961.
- Moule, S. K., N. M. Bradford, and J. D. McGivan. 1987. Short-term stimulation of Na<sup>+</sup>-dependent amino acid transport by dibutyryl cyclic AMP in hepatocytes. *Biochem. J.* 241:737-743.
- Fariss, M. W., and D. J. Reed. 1983. Measurement of glutathione and glutathione disulfide efflux from isolated rat hepatocytes. In *Isolation, Characterization, and Use of Hepatocytes*. R. A. Harris and N. W. Cornell, editors. Elsevier Science Publishing Co. 349-355.
- McAbee, D. D., M. C. Lear, and P. H. Weigel. 1991. Total cellular activity and distribution of a subpopulation of galactosyl receptors in isolated rat hepatocytes are differentially affected by microtubule drugs, monensin, low temperature, and chloroquine. *J. Cell. Biochem.* 45:59-68.
- Eilers, U., J. Klumperman, and H. Hauri. 1989. Nocodazole, a microtubule-active drug, interferes with apical protein delivery in cultured intestinal epithelial cells (Caco-2). *J. Cell Biol.* 108:13-22.
- Lu, S., J. Kuhlenkamp, C. Garcia-Ruiz, and N. Kaplowitz. 1991. Hormone-mediated down-regulation of hepatic glutathione synthesis in the rat. *J. Clin. Invest.* 88:260-269.
- Glazenburg, E. J., J. E. Bruggink, K. Wolters-Keulemans, and G. J. Mulder. 1984. Inhibition of glutathione efflux in the recirculating rat liver perfusion by cysteine but not by oxothiazolidine carboxylate, an intracellular cysteine precursor. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 178:271-274.
- Hahn, R., and W. Oberrauch. 1978. Unidirectional transport of reduced glutathione in rat liver and its metabolization in the extracellular space. In *Functions of Glutathione in Liver and Kidney*. H. Sies and A. Wendel, editors. Springer-Verlag, Berlin. 32-40.
- Berleth, E. S., J. Li, J. A. Braunscheidel, and C. M. Pickart. 1992. A reactive nucleophile proximal to vicinal thiols is an evolutionarily conserved feature in the mechanism of arg aminoacyl-tRNA protein transferase. *Arch. Biochem. Biophys.* 298:498-504.