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### Research Article

We reported that glucagon and phenylephrine decrease hepatocyte GSH by inhibiting gamma-glutamylcysteine synthetase (GCS), the rate-limiting enzyme in GSH synthesis (Lu, S.C., J. Kuhlenkamp, C. Garcia-Ruiz, and N. Kaplowitz. 1991. *J. Clin. Invest.* 88:260-269). In contrast, we have found that insulin (In, 1 microgram/ml) and hydrocortisone (HC, 50 nM) increased GSH of cultured hepatocytes up to 50-70% (earliest significant change at 6 h) with either methionine or cystine alone as the sole sulfur amino acid in the medium. The effect of In occurred independent of glucose concentration in the medium. Changes in steady-state cellular cysteine levels, cell volume, GSH efflux, or expression of gamma-glutamyl transpeptidase were excluded as possible mechanisms. Both hormones are known to induce cystine/glutamate transport, but this was excluded as the predominant mechanism since the induction in cystine uptake required a lag period of greater than 6 h, and the increase in cell GSH still occurred when cystine uptake was blocked. Assay of GSH synthesis in extracts of detergent-treated cells revealed that In and HC increased the activity of GCS by 45-65% (earliest significant change at 4 h) but not GSH synthetase. In and HC treatment increased the Vmax of GCS by 31-43% with no change in Km. Both the hormone-mediated increase in cell GSH and GCS activity were blocked with either cycloheximide or actinomycin D. Finally, [...]

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# Insulin and Glucocorticoid Dependence of Hepatic $\gamma$ -Glutamylcysteine Synthetase and Glutathione Synthesis in the Rat

## Studies in Cultured Hepatocytes and In Vivo

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### Abstract

We reported that glucagon and phenylephrine decrease hepatocyte GSH by inhibiting  $\gamma$ -glutamylcysteine synthetase (GCS), the rate-limiting enzyme in GSH synthesis (Lu, S. C., J. Kuhlenskamp, C. Garcia-Ruiz, and N. Kaplowitz. 1991. *J. Clin. Invest.* 88:260–269). In contrast, we have found that insulin (In, 1  $\mu$ g/ml) and hydrocortisone (HC, 50 nM) increased GSH of cultured hepatocytes up to 50–70% (earliest significant change at 6 h) with either methionine or cystine alone as the sole sulfur amino acid in the medium. The effect of In occurred independent of glucose concentration in the medium. Changes in steady-state cellular cysteine levels, cell volume, GSH efflux, or expression of  $\gamma$ -glutamyl transpeptidase were excluded as possible mechanisms. Both hormones are known to induce cystine/glutamate transport, but this was excluded as the predominant mechanism since the induction in cystine uptake required a lag period of > 6 h, and the increase in cell GSH still occurred when cystine uptake was blocked. Assay of GSH synthesis in extracts of detergent-treated cells revealed that In and HC increased the activity of GCS by 45–65% (earliest significant change at 4 h) but not GSH synthetase. In and HC treatment increased the  $V_{max}$  of GCS by 31–43% with no change in  $K_m$ . Both the hormone-mediated increase in cell GSH and GCS activity were blocked with either cycloheximide or actinomycin D. Finally, when studied in vivo, streptozotocin-treated diabetic and adrenalectomized rats exhibited lower hepatic GSH levels and GCS activities than respective controls. Both of these abnormalities were prevented with hormone replacement. Thus, both in vitro and in vivo, In and glucocorticoids are required for normal expression of GCS. (*J. Clin. Invest.* 1992. 90:524–532.) Key words: cultured hepatocytes • diabetes mellitus • streptozotocin • adrenalectomy • dexamethasone

### Introduction

Hepatic GSH plays a vital defensive role against toxins and free radicals, and in the storage and transfer of cysteine (1, 2). The synthesis of GSH from its constituent amino acids involves two

ATP-requiring enzymatic steps: the formation of  $\gamma$ -glutamylcysteine from glutamate and cysteine, and formation of GSH from  $\gamma$ -glutamylcysteine and glycine. The first step of GSH biosynthesis is rate-limiting and catalyzed by  $\gamma$ -glutamylcysteine synthetase (GCS)<sup>1</sup> which is regulated physiologically by feedback competitive inhibition by GSH and the availability of cysteine (1, 3, 4). We have recently demonstrated that the synthesis of hepatic GSH is also regulated by various hormones. Specifically, cAMP, Ca<sup>2+</sup>, and C-kinase-dependent hormones downregulate hepatic GSH synthesis acutely by inhibiting GCS, presumably through enzyme phosphorylation (5). The possibility of hormone-mediated upregulation of hepatic GSH synthesis has not been explored. We examined insulin (In) and hydrocortisone (HC) since they are commonly used as growth factors in culturing hepatocytes but their effects on GSH are unknown. We found that both In and HC increase hepatocyte GSH. This report delineated the mechanisms of their stimulatory effects and demonstrated the physiologic importance of these hormones in hepatic GSH homeostasis using in vivo models.

### Methods

#### Materials

GSH, collagenase (type IV), BSA, L-methionine, L-cysteine, L-cystine, DTT, diethyl maleate (DEM), NADPH, 5,5'-dithiobis (2-nitrobenzoic acid), sodium EDTA, GSH reductase, hydrocortisone, insulin, glutathione-S-transferases, dexamethasone, streptozotocin, digitonin, fetal bovine serum (FBS),  $\alpha$ -amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid (acivicin), cycloheximide, actinomycin D, and HEPES were purchased from Sigma Chemical Co. (St. Louis, MO). DL-Buthionine-S-R-sulfoximine was obtained from Schweizerhall (South Plainfield, NJ). DME/F12 medium was purchased from Gibco Laboratories (Grand Island, NY). Sulfur amino acid-free (SAF) DME/F12 medium was custom made by Irvine Scientific (Santa Ana, CA). DME low glucose (1,000 mg/liter) was from Irvine Scientific. Monochlorobimane was purchased from Molecular Probes, Inc. (Eugene, OR).  $\gamma$ -(bis) Glutamylcysteine was prepared by enzymatic hydrolysis of oxidized glutathione (6) and reduced with DTT before use. The purity of  $\gamma$ -(bis) glutamylcysteine was confirmed by HPLC. L-[<sup>35</sup>S]methionine (1,120 Ci/mmol) and L-[<sup>35</sup>S]cystine (193 mCi/mmol) were obtained from Amersham Corp. (Arlington Heights, IL). All other reagents were of analytical grade and were obtained from commercial sources.

#### Animals

Male Sprague-Dawley rats (Harlan Laboratory Animals, Inc., Indianapolis, IN), weighing 260–320 g for preparation of cell cultures and in

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1. Abbreviations used in this paper: ADX, adrenalectomy; DEM, diethyl maleate; Dex, dexamethasone; FBS, fetal bovine serum; GCS,  $\gamma$ -glutamylcysteine synthetase; HC, hydrocortisone; In, insulin; mBC1, monochlorobimane; SAF, sulfur amino acid-free; STZ, streptozotocin.

vivo experiments, were maintained on Purina rodent chow (Ralston Purina Co., St. Louis, MO) and water ad lib. Adrenalectomized rats received 0.9% NaCl instead of water.

### *Cell culture preparation*

Isolation of hepatocytes was done aseptically according to the method of Moldeus et al. (7). Initial cell viability was  $\geq 85\%$  as determined by 0.2% trypan blue exclusion. For most experiments, the plating medium was DME/F12, high glucose (3,151 mg/liter), containing 10% FBS, and supplemented with excess methionine (1 mM). For experiments designed to examine the effects of sulfur amino acid precursors, SAF medium supplemented with either methionine (1 mM) or cystine (0.5 mM) was used instead of DME/F12 as plating medium.  $1.5\text{--}2 \times 10^6$  cells in 5-ml plating medium were plated on  $60 \times 15\text{-mm}$  dishes pre-coated with rat tail collagen, incubated at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$ , 95% air. 2–3 h after plating, medium was changed to omit serum and to remove dead, unattached cells. Cell attachment averaged  $\sim 50\%$ . The continued exposure to serum led to decreased cell GSH, an effect which was not further studied.

### *Measurement of GSH and cysteine*

Cultured cells were detached by trypsin-EDTA (0.05%, 0.02%, respectively). Cultured cells, liver homogenates, or liver cytosols were treated with 10% trichloroacetic acid to extract cellular GSH. The mixture was centrifuged at 13,000 *g* in a microfuge (Beckman Instruments, Inc., Palo Alto, CA) for 1 min to remove the denatured proteins and GSH was measured in the supernatant by the recycling method of Tietze (8).

To measure cysteine concentrations from cultured hepatocytes, cells ( $\sim 10^6$ ) were collected by detaching cells with trypsin-EDTA, and centrifuged in a microfuge for 30 s to remove the trypsin-EDTA solution. Cells were then treated with 5% perchloric acid to precipitate the proteins, centrifuged for 1 min in a microfuge, and supernatant cysteine levels were determined according to the method of Gaitonde (9). Cysteine levels were measured in liver homogenates according to the method of Gaitonde (9).

### *Measurement of enzyme activities of GSH synthesis using monochlorobimane*

Monochlorobimane (mBCl) can be used to measure activities of enzymes involved in GSH synthesis (5, 10). Briefly, mBCl forms a fluorescent adduct with GSH in a reaction catalyzed by GSH S-transferase. GSH synthesis rates measured by the rate of adduct formation fluorimetrically agree with synthesis rates determined by the method of Tietze (10).

To measure GCS and GSH synthetase activities in cultured hepatocytes, cells were first incubated in DME/F12 medium supplemented with In (1  $\mu\text{g}/\text{ml}$ ) or HC (50 nM) or vehicle for various time periods. Some plates from each condition were used for cell GSH measurement by the method of Tietze (8) and cell counting by both Coulter counter (Coulter Electronics, Hialeah, FL) and hemocytometer. The rest of the plates from each condition were treated with DEM (0.3 mM) for 30 min to deplete cell GSH. This is to avoid high background with mBCl and to eliminate feedback inhibition exerted by preexisting GSH. At the end of 30 min incubation with DEM, cells were washed and treated with digitonin (50  $\mu\text{M}$ ) for 20 min to permeabilize cell membrane. Digitonin-treated cell-free protein extract was obtained by pooling scraped cells from several plates and centrifuging at 1,500 *g* for 20 min. The cell-free extract gave similar glutathione S-transferase activity (11) as compared to 10% Triton-treated cells (data not shown), thus confirming presence of all cytosolic components in the extract.

The cytosol fraction of rat liver was obtained by centrifuging the homogenate (33% wt/vol in 0.01 M sodium phosphate, 0.25 M sucrose, pH 7.4) at 100,000 *g* for 60 min. To decrease the GSH present, cytosol was dialyzed overnight using molecularporous membrane tubing (mol wt cutoff: 12,000–14,000; Spectrum Medical Industries, Inc., Los Angeles, CA) in  $100\times$  vol of 0.01 M sodium phosphate, pH 7.4. After overnight dialysis, cytosolic GSH concentration was decreased by 99–99.5%.

The GSH synthesis rate was measured in cell-free conditions by adding digitonin-treated cultured cell extract (1–2 mg protein as measured by assay; Bio-Rad Laboratories, Richmond, CA) or liver cytosol (2–3.5 mg protein) to the cuvette containing: 100 mM Tris-HCl, 150 mM KCl, 20 mM  $\text{MgCl}_2$ , 2 mM EDTA pH 7.3, glutamate (10 mM), glycine (10 mM), ATP (3 mM), cysteine + DTT (0.025–0.5 mM) plus 100  $\mu\text{M}$  mBCl in a final volume of 2.5 ml at  $37^\circ\text{C}$ . The difference in initial rate of linear increase in fluorescence over 6–8 min with or without pretreatment with buthionine sulfoximine (5 mM for 5 min at  $37^\circ\text{C}$ ) is equivalent to the rate of GSH synthesis. The change in fluorescence is converted to GSH concentration units by using standard curves. Specifically, formation of fluorescent adducts was monitored by adding mBCl (100  $\mu\text{M}$ ) and glutathione S-transferase (0.1 U/ml) to the cuvette containing GSH standards. Standard curves were then generated by applying linear regression to the relationship between fluorescence and GSH concentration. This method assesses the formation of GSH as an end product from two steps: the formation of  $\gamma$ -glutamylcysteine from cysteine and glutamate (catalyzed by GCS) and the formation of GSH from  $\gamma$ -glutamylcysteine and glycine (catalyzed by GSH synthetase). To assess only the second step in GSH synthesis, cultured cell extract or liver cytosol was added to the cuvette containing 100 mM Tris-HCl, 150 mM KCl, 2 mM EDTA, pH 7.3, substrates  $\gamma$ -glutamylcysteine + DTT (0.2 mM), glycine (10 mM), cofactors ATP (3 mM) and  $\text{Mg}^{2+}$  (20 mM), plus 100  $\mu\text{M}$  mBCl in a final vol of 2.5 ml at  $37^\circ\text{C}$ . The difference in initial rate of linear increase in fluorescence over 8–10 min in the presence of all precursors and cofactors for the second step of GSH synthesis versus only  $\gamma$ -glutamylcysteine + DTT is equivalent to the rate of GSH synthesis catalyzed by GSH synthetase.

### *Uptake of methionine and cystine by cultured rat hepatocytes*

Cultured hepatocytes were plated in DME/F12 containing high glucose, 10% FBS, and excess methionine (1 mM). 2 h after plating medium was changed to omit FBS and In (1  $\mu\text{g}/\text{ml}$ ) or HC (50 nM) or vehicle was added. Most uptake studies were done 16 h after this medium change. For shorter time incubations, medium was changed to control medium (no FBS, In, or HC) 2 h after plating. 16 h after plating medium was changed to that supplemented with hormone or vehicle. Uptake studies were carried out 6 h after this second medium change.

The technique used for measuring sulfur amino acid uptake was adapted from Takada and Bannai (12). The medium used for washing and stopping uptake was 10 mM PBS, pH 7.4, with 0.01%  $\text{CaCl}_2$ , 0.01%  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , and 0.1% glucose. Transport medium was the same medium supplemented with nonradioactive methionine (1 mM) and cystine (0.18 mM) to simulate the concentrations present in the culture medium (DME/F12, supplemented with 1 mM methionine). Cultured cells were washed twice (2 ml each) with prewarmed buffer and transport was initiated by addition of 1.5 ml transport medium containing either  $^{35}\text{S}$ -methionine (0.5–1.0  $\mu\text{Ci}$ ) or  $^{35}\text{S}$ -cystine (0.17  $\mu\text{Ci}$ ). 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. The number of washes was determined by counting dpm in the supernatant after each wash. After four, five, and six washes, 0.02%, 0.004%, and 0.001% of total dpm was recovered in the supernatant, respectively. 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. 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  $37^\circ\text{C}$  and  $4^\circ\text{C}$  uptake values represented true uptake. Uptake was expressed as nanomoles of methionine or cystine per milligram protein per minute.

In some experiments cystine uptake was measured by adding tracer doses of cystine (0.5  $\mu\text{Ci}$ ) directly to culture medium (5 ml) at the end of 16 h incubation of cells with In (1  $\mu\text{g}/\text{ml}$ ) or HC (50 nM) or vehicle with or without glutamate (2.5 mM). This was to confirm that treatment of cells with glutamate (2.5 mM) for 16 h still blocked the entry of cystine.

## In vivo experiments

All in vivo experiments were carried out on fed rats.

**Effects of streptozotocin treatment.** Rats were treated with streptozotocin (80 mg/ml in 0.05 M citric acid- $\text{Na}_2\text{HPO}_4$  mixture) intraperitoneally (60 mg/kg). Control rats received vehicle. Moderate diabetes (glucose 250–350 mg/dl) was achieved within 12–24 h as determined by measuring blood glucose by Chemstrip bG with Accu-Check II (Boehringer Mannheim Diagnostics, Indianapolis, IN). One day after streptozotocin treatment diabetic rats were randomized to receive insulin (NPH; Eli Lilly and Co., Indianapolis, IN) 2–3 U in a.m. and 4–5 U in p.m. subcutaneously to control the glucose levels within 100–200 mg/dl. Control and untreated diabetic rats received saline subcutaneously. Rats were killed 4 d after streptozotocin treatment. GSH and cysteine levels were determined in liver homogenates and GCS activities were determined in liver cytosols which had been dialyzed overnight as described above.

**Effects of bilateral adrenalectomy (ADX) and dexamethasone (Dex) treatment.** Adrenalectomized and sham-operated rats were purchased from Harlan Laboratories. They were matched for weight on the day of surgery and recuperated for 6 d postoperatively before they were shipped. Half the number of rats in each group were randomized to receive Dex (5  $\mu\text{g}/100$  g body weight intraperitoneally) each day for 4 d starting from postoperative day 11–13. The other half received vehicle. Rats that had ADX procedure received 0.9% NaCl as drinking water. All rats were killed on postoperative day 15–17. Completeness of adrenalectomy was confirmed by examination during autopsy. Hepatic GSH and cysteine levels and GCS activity were measured as described above. Serum samples were also assayed for glucose (Chemstrip bG with Accu-Check II), creatinine, and urea nitrogen (Sigma Diagnostic Kits; Sigma Chemical Co., St. Louis, MO).

## Statistical analysis

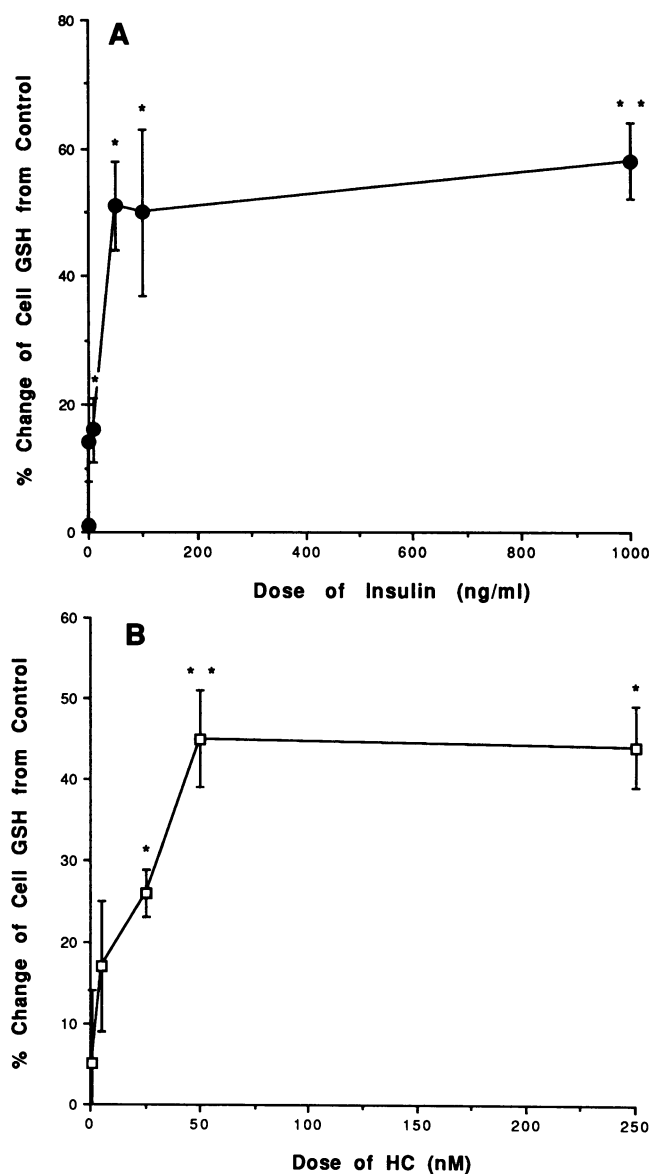
For cultured cells, each cell prep was derived from one animal and triplicate plates were used for each condition as well as time points (control vs. treatment) except in the case of uptake studies (duplicate plates were used). The mean of each triplicate (or duplicate) from one experiment was considered  $n = 1$  and the means of multiple experiments were compared by paired Student's  $t$  test with the Bonferroni correction or ANOVA when appropriate. One-tailed test was used unless otherwise noted. For in vivo experiments, unpaired Student's  $t$  test (in the case of only two comparisons) or ANOVA (for multiple comparisons) was used.

Kinetic data were fitted with the Michaelis-Menten equation using the Simulation, Analysis, and Modeling program to extract the  $V_{\text{max}}$  and  $K_m$  values. The standard errors of the mean values at each concentration of the substrate were submitted as statistical weights (proportional to the inverse of standard errors). The significance of the differences in kinetics parameters were determined by paired Student's  $t$  test.

## Results

**Effects of In and HC on GSH in cultured hepatocytes.** The effects of In and HC were evaluated after overnight incubation. Addition of In (1  $\mu\text{g}/\text{ml}$ ) or HC (50 nM) increased cell GSH by 50–70% (controls,  $106 \pm 14$ ; In,  $173 \pm 17$ ; and HC,  $160 \pm 20$  nmol/ $10^6$  cells; results are expressed as mean  $\pm$  SEM from four to six cell preps, both In and HC are significantly different from controls with  $P < 0.05$  by two-tailed paired Student's  $t$  test).

Dose response of In and HC on cell GSH was examined after overnight incubation. Fig. 1, A and B, showed that the threshold dose (lowest dose examined that resulted in significantly higher cell GSH levels in comparison to controls) was 10 ng/ml for In and 25 nM for HC. Time course study using standard DME/F12 + methionine (1 mM) medium showed that cell GSH level was significantly higher after 6 h of incuba-



**Figure 1.** Dose response of In (A) and HC (B) on cell GSH in cultured hepatocytes. Cultured hepatocytes were plated in the usual plating medium and at 2 h changed to DME/F12 with varying doses of In or HC or vehicle (control). Cell GSH levels were measured 16 h later. Results from four to seven cell preps are shown as percent change of cell GSH from control. Threshold doses of In and HC were 10 ng/ml and 25 nM, respectively. Control GSH values were  $78 \pm 13$  nmol/ $10^6$  cells (mean  $\pm$  SEM). \* $P < 0.05$ ; \*\* $P < 0.01$  vs. control by paired Student's  $t$  test.

tion with both hormones, although maximum effects were seen after 8 h (no further increase after 12h) (Table I).

Both In and HC significantly increased cell GSH after 6 h of incubation when cells were plated in SAF medium supplemented with either methionine (1 mM) or cystine (0.5 mM) as the sole sulfur amino acid precursor (SAF + methionine controls,  $75 \pm 2.0$ ; In,  $96 \pm 4.0$ ; HC,  $98 \pm 2.6$  nmol/ $10^6$  cells; results are expressed as mean  $\pm$  SEM from four cells preps,  $P < 0.05$  for both In and HC vs. controls; SAF + cystine controls,  $56 \pm 2.9$ ; In,  $67 \pm 2.8$ ; HC,  $75 \pm 2.9$  nmol/ $10^6$  cells; results are expressed as mean  $\pm$  SEM from three cell preps,  $P < 0.05$  for both In and HC vs. controls).

**Table I. Time Course of Insulin- and Hydrocortisone-Mediated Increase of GSH in Cultured Hepatocytes**

Incubation	Control	Insulin	Hydrocortisone	
<i>h</i>	Cell GSH, nmol/10 <sup>6</sup> cells			<i>n</i>
4	87±14	84±17 (95)	89±15 (101)	3
6	62±10	76±10 (127)*	74±12 (119)*	5
8	63±8	80± 8 (126)*	87±10 (139)*	5
12	74±20	115±13 (153)*	109±11 (144)*	3
16	98±15	166±17 (169)*	146±20 (151)*	6

Results are expressed as mean±SEM for the number of cell preps (*n*). Values in parentheses refer to percentage of control. Cultured hepatocytes were treated with In (1 µg/ml) or HC (50 nM) or vehicle (control) and cell GSH levels were measured after variable incubation time periods. \* *P* < 0.03 vs. control by paired *t* test.

To evaluate the effect of glucose concentration in the medium on the In-mediated increase in cell GSH, cells were plated in DME medium containing low glucose (1,000 mg/liter) or high glucose (3,151 mg/liter) with or without In (1 µg/ml). Cell GSH was significantly higher in the presence of In regardless of the medium glucose concentration (low glucose control, 34±2.8; with In, 54±3.6; high glucose control, 36±1.1; with In, 58±3.5 nmol/10<sup>6</sup> cells; results are expressed as mean±SEM from four cell preps, *P* < 0.05 for In vs. its respective control by ANOVA).

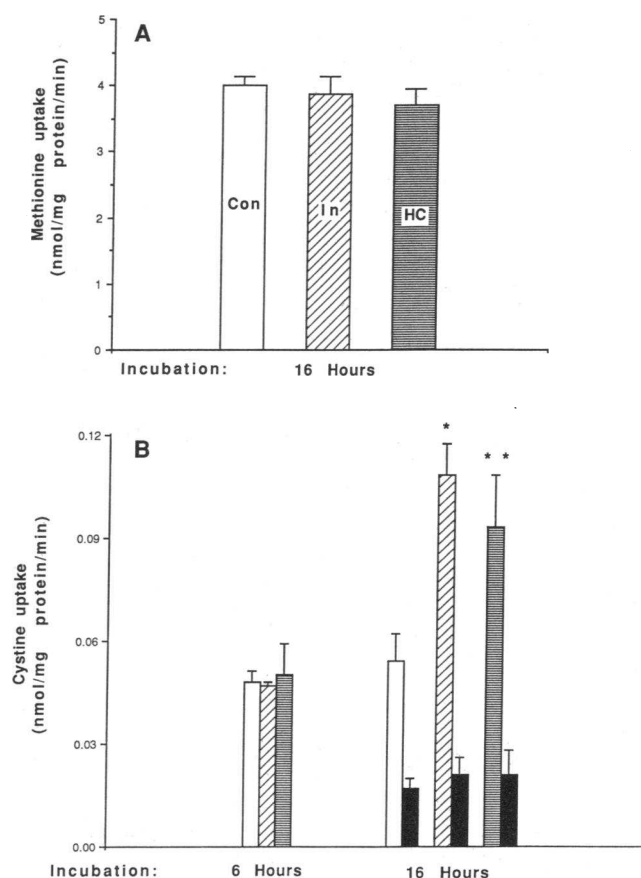
The stimulatory effects of In and HC on hepatocyte GSH level occurred independent of cell plating density (data not shown).

**Mechanisms of In and HC-mediated stimulation of cell GSH.** Potential mechanisms for the hormone-mediated increase in cell GSH included: (a) Changes in cell volume, that is, if cell volume increases with In or HC treatment than intracellular GSH concentration would fall which would exert less feedback inhibition on GCS. This in turn may lead to increased GSH synthesis and more GSH per cell. (b) Decreased GSH efflux. (c) Increased expression of γ-glutamyl transpeptidase which would increase breakdown of GSH released by the cell and hence the availability of the rate limiting precursor, cysteine. (d) Increased cysteine availability, either through increased uptake of sulfur amino acids, increased activity of *trans*-sulfuration, or decreased activities of cysteine catabolic pathways. (e) Increased activities of enzymes involved in GSH synthesis, namely, GCS and GSH synthetase.

We excluded the following potential mechanisms for the hormone-mediated stimulation of hepatocyte GSH level: changes in cell volume as estimated by channelyzer (Coulter counter, Coulter Electronics, data not shown), GSH efflux (13), or changes in the expression of γ-glutamyl transpeptidase. The latter was excluded by finding that incubation of cultured hepatocytes with acivicin (0.25 mM) for 16 h did not abolish the stimulatory effects of either In or HC (data not shown). In terms of GSH efflux, the relationship between GSH efflux rate and cell GSH after In or HC treatment did not diverge from the normal curve (13). Thus, although the GSH efflux rates were higher, they were appropriate for the cell GSH level.

Increased cysteine availability can occur by increased transport of sulfur amino acid precursors, which in the case of cul-

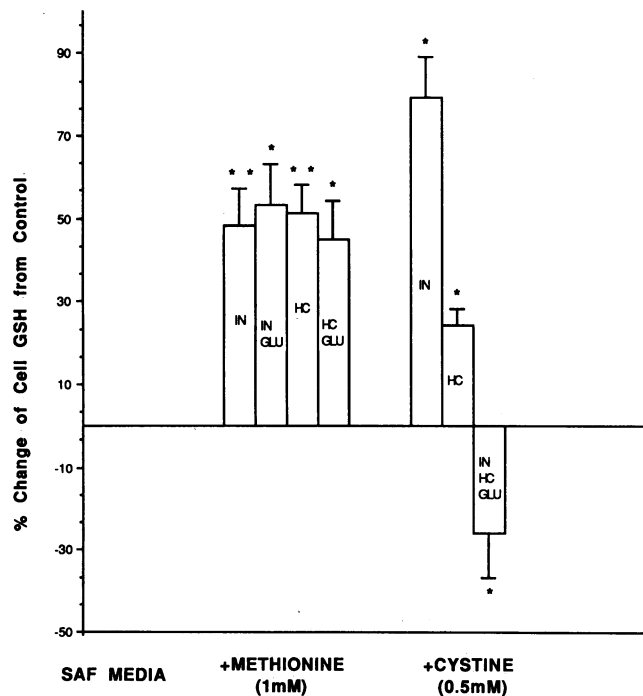
tured hepatocytes would be methionine and cystine since almost all cysteine is autoxidized to cystine in culture media (14, 15). Both In and HC have been shown to increase cystine/glutamate uptake (χ<sub>c</sub>-system) in cultured hepatocytes (12). Uptake of methionine was rapid and linear over 1 min whereas uptake of cystine was much slower and remained linear over 10 min (data not shown). Subsequent uptake studies were performed by measuring the uptake value at 40 s for methionine and 5 min for cystine. Fig. 2 shows uptake of methionine and cystine with or without hormone treatment. Methionine uptake was not influenced by the addition of In or HC. Cystine uptake was significantly increased by In or HC treatment. This increase in cystine uptake was glutamate (2.5 mM)-inhibitable as previously described (12) and required a lag period of > 6 h.



**Figure 2.** Uptake of methionine (A) and cystine (B) after treatment with In (▨, 1 µg/ml), or HC (■, 50 nM), or vehicle control (□). Cultured hepatocytes were treated with In or HC for 6 h (cystine) or 16 h (both methionine and cystine). Tracer amounts of [<sup>35</sup>S]-methionine or [<sup>35</sup>S]-cystine were added to uptake medium that contained 1 mM methionine and 0.18 mM cystine to approximate concentrations normally present in culture medium (see Methods for details). Results from four cell preps are shown as mean±SEM for methionine uptake and no difference was noted with In or HC treatment. Control values were 4.0±0.13; In, 3.88±0.26; HC, 3.71±0.24 nmol/mg protein per min. On the other hand, cystine uptake was significantly increased after 16 h treatment either In or HC (results from five cell preps are shown as mean±SEM). Control values were 0.05±0.01; In, 0.11±0.01; HC, 0.09±0.01 nmol/mg protein per min. This hormone-mediated increase in cystine uptake was glutamate (■, 2.5 mM)-inhibitable and was not evident after 6 h of hormone treatment. \**P* < 0.001; \*\**P* < 0.01 vs. control by paired *t* test.

Therefore, the increase in cell GSH at 6 h for both In and HC cannot be explained by increased cystine uptake.

To see if the increase in cystine uptake could be responsible for the increase in cell GSH after overnight treatment with In or HC, effects of the hormones were examined using SAF media supplemented with either 1 mM methionine or 0.5 mM cystine and sufficient glutamate (2.5 mM) to block the entry of cystine. This point was confirmed by measuring cystine uptake after cultured hepatocytes were incubated with In or HC or vehicle with or without glutamate (2.5 mM) overnight (data not shown). Fig. 3 shows that both hormones significantly increased cell GSH regardless of the type of sulfur amino acid precursor present in media. Inhibiting cystine uptake did not abolish the stimulatory effects of In and HC on cell GSH when media contained methionine. Therefore, this excludes the possibility that methionine conversion to cysteine intracellularly leads to liberation of cysteine into the medium with its accumulation as cystine which is then available for reuptake by an induced cystine transporter. As expected, glutamate treatment lowered the cell GSH when media contained only cystine verifying the efficacy of glutamate in inhibiting cystine uptake. Thus, hormone-induced amino acid transport cannot explain



**Figure 3.** Effects of sulfur amino acid precursors on In- and HC-mediated increase in cell GSH. Cultured hepatocytes were plated using SAF media supplemented with either 1 mM methionine or 0.5 mM cystine. In (1  $\mu\text{g}/\text{ml}$ ) or HC (50 nM) and in some cases, glutamate (GLU, 2.5 mM) was added to medium. Cell GSH levels were determined 16 h later. Results from four to seven preps are shown as mean  $\pm$  SEM in percent change of cell GSH from control. Both In and HC significantly increased cell GSH regardless of the type of sulfur amino acid precursor present in media. Glutamate had no influence on In- or HC-mediated increase in cell GSH when medium contained only methionine but it lowered the cell GSH levels when medium contained only cystine. Control cell GSH for SAF + methionine,  $67 \pm 6.6$ ; SAF + cystine,  $45 \pm 7.9$  nmol/ $10^6$  cells. \* $P < 0.05$ ; \*\* $P < 0.02$  vs. control by paired  $t$  test.

the increase in cell GSH when methionine is employed as the sole sulfur amino acid precursor.

We also examined steady-state cysteine levels in cultured hepatocytes. When cultured hepatocytes were incubated with In (1  $\mu\text{g}/\text{ml}$ ) or HC (50 nM) overnight in DME/F12 containing excess methionine (1 mM), cysteine levels were unchanged from that of controls (control cysteine,  $3.2 \pm 0.06$ ; In,  $3.4 \pm 0.06$ ; HC,  $3.8 \pm 0.06$  nmol/ $10^6$  cells) despite significant increases in cell GSH levels (control GSH,  $62.3 \pm 18.1$ ; In,  $96.8 \pm 23.8$ ; HC,  $78.5 \pm 23.5$  nmol/ $10^6$  cells; data expressed as mean  $\pm$  SEM from six cell preps,  $P < 0.05$  vs. control by paired  $t$  test). No significant differences in steady-state cysteine levels were seen with hormone treatment at earlier time points or in different culture media (data not shown).

To see if increases in GSH synthetic enzyme activities may be responsible for the increase in cell GSH, we measured enzyme activities in digitonin extracts after hormone treatment of cultured cells for variable periods. Table II shows that cell GSH levels were significantly increased after 16 h incubation with In (1  $\mu\text{g}/\text{ml}$ ) or HC (50 nM), and depleted to 11–13% of original values after 30 min of DEM (0.3 mM) treatment. GSH synthesis rates from substrates for both steps in GSH synthesis (cysteine 0.1 mM, ATP 3 mM, glutamate 10 mM, and glycine 10 mM) were increased by 45–65% after treatment of cultured cells with In or HC. In contrast, GSH synthesis rates from substrates for the second step in GSH synthesis ( $\gamma$ -glutamylcysteine 0.2 mM, glycine 10 mM, ATP 3 mM, and  $\text{Mg}^{2+}$ ) were not significantly different among the three groups. This increase in GCS activity was evident after 4 h of incubation with In or HC (control,  $0.34 \pm 0.05$ ; In,  $0.66 \pm 0.11$ ; HC,  $0.56 \pm 0.11$  nmol GSH synthesized/mg per min; results are expressed as mean  $\pm$  SE from four cell preps, both In and HC are significantly different from controls at  $P < 0.05$  by paired  $t$  test) but not after 2 h (control,  $0.32 \pm 0.05$ ; In,  $0.33 \pm 0.02$ ; HC,  $0.31 \pm 0.05$  nmol/mg per min;  $n = 3$ ). The amount of protein in the cell-free extract per  $10^6$  cells were similar in the three groups. It should be noted that the protein extracts contained cytosol and other solubilized cell constituents. Thus the specific enzyme activities of GCS in these extracts are lower than that of cytosol due to the presence of a greater fraction of total cellular protein in the digitonin extracts.

To ascertain whether In and HC treatment affect the  $K_m$  or  $V_{\text{max}}$  or both of GCS, GSH synthesis rates in cell-free protein extracts were measured while increasing the concentration of cysteine from 0.025 to 0.5 mM. Fig. 4 shows results from four cell preps after overnight incubation with In (1  $\mu\text{g}/\text{ml}$ ) or HC (50 nM). With increasing cysteine concentration, all three conditions showed saturation in GSH synthesis rates.  $V_{\text{max}}$  and  $K_m$  values were estimated by fitting the Michaelis-Menten equation to the data using the SAAM program. Mean data from four cell preps were weighed with their variances. Both In and HC increased the  $V_{\text{max}}$  of GCS without affecting the  $K_m$  significantly.

To see if In- or HC-mediated increase of cell GSH and GCS activity requires RNA or protein synthesis, the effects of cycloheximide (5  $\mu\text{M}$ ) or actinomycin D (1  $\mu\text{g}/\text{ml}$ ) were assessed by measuring cell GCS activity after 4 h or GSH levels after 6 h of incubation with either In (1  $\mu\text{g}/\text{ml}$ ) or HC (50 nM). Table III shows that both In and HC increased GCS activity and cell GSH levels after 4 and 6 h of incubation, respectively, and both cycloheximide and actinomycin D abolished their stimulatory effects.

Table II. Effects of Insulin and Hydrocortisone on GSH and GSH Synthesis Rates in Cell-Free Protein Extracts from Cultured Hepatocytes

	Cell GSH		GSH synthesis rate		
	Before DEM	After DEM	Both steps	Second step	Protein in extract
	nmol/10 <sup>6</sup> cells		nmol/mg protein per min		mg/10 <sup>6</sup> cells
Con	73.6±18.1	10.0±2.4	0.38±0.04	2.11±0.30	1.34±0.22
In	110±24*	12.8±4.3	0.63±0.04*	2.36±0.38	1.34±0.20
HC	93.2±23.5 <sup>‡</sup>	11.4±2.4	0.55±0.05*	2.84±0.54	1.11±0.17

Results are expressed as mean±SE from five to nine cell preps ( $n = 9$  for GSH synthesis measurement from both steps,  $n = 5$  for GSH synthesis measurement from second step only). Cultured hepatocytes were incubated with In ( $1 \mu\text{g/ml}$ ) or HC ( $50 \text{ nM}$ ) for 16 h. For each condition, cell GSH was measured before and after 30 min of DEM ( $0.3 \text{ mM}$ ) treatment. GSH synthesis rates were measured in digitonin-treated cell-free protein extracts fluorometrically with mBCl (see Methods). \*  $P < 0.01$ ; <sup>‡</sup>  $P < 0.05$  vs. control by paired Student's  $t$  test.

*Effects of removal of insulin and glucocorticoids on hepatic GSH metabolism in vivo.* We next investigated the influences of insulin or glucocorticoid deficiency on hepatic GSH metabolism in vivo. We used streptozotocin (STZ)-induced diabetic or bilaterally ADX rats as our in vivo models of insulin or glucocorticoid deficiency, respectively (16, 17). Tables IV and V show that STZ-treated diabetic and ADX rats exhibited lower hepatic GSH levels as well as GCS activities than respective controls. On the other hand, activity of GSH synthetase (GSH synthesis rates from  $\gamma$ -glutamylcysteine and glycine) was unaffected by these treatments (controls for diabetic rats:  $7.73 \pm 0.37$ ; STZ,  $7.65 \pm 0.72$ ; sham-operated controls for ADX rats,  $7.76 \pm 0.51$ ; ADX,  $7.83 \pm 0.32 \text{ nmol/mg per min}$ ). Cysteine levels were also unaffected by these treatments. Both the decreased hepatic GSH levels and GCS activities were pre-

vented with insulin or Dex replacement. Dex treatment did not influence hepatic GSH levels or GCS activities in sham-operated controls.

Serum glucose levels (mean±SE, in mg/dl) were not affected by ADX or Dex (sham controls,  $116 \pm 6.9$ ; sham + Dex,  $138 \pm 9.9$ ; ADX,  $114 \pm 14$ ; ADX + Dex,  $134 \pm 19$ ). Serum creatinine levels (mean±SE, in mg/dl) and serum urea nitrogen levels (mean±SE, in mg/dl) were also similar among the four groups (creatinine: sham,  $0.61 \pm 0.06$ ; sham + Dex,  $0.60 \pm 0.04$ ; ADX,  $0.61 \pm 0.05$ ; ADX + Dex,  $0.52 \pm 0.02$ ; urea nitrogen: sham,  $21.0 \pm 0.4$ ; sham + Dex,  $22.0 \pm 1.1$ ; ADX,  $22.4 \pm 2.4$ ; ADX + Dex,  $21.1 \pm 0.5$ ; ratio of urea nitrogen to creatinine: sham,  $36 \pm 3$ ; sham + Dex,  $37 \pm 2$ ; ADX,  $37 \pm 4$ ; ADX + Dex,  $40 \pm 1$ ). These values are within the normal range reported (18). This excludes significant dehydration in these animals.

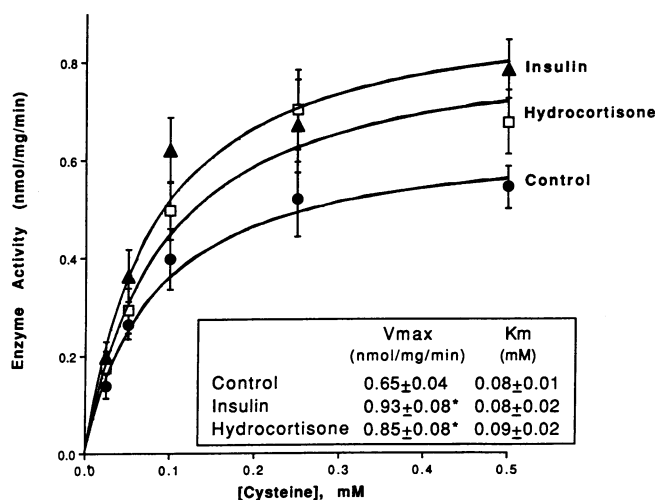


Figure 4. Effects of In and HC on kinetics of GSH synthesis in cultured hepatocytes. Results from four cell preps after overnight incubation with In ( $1 \mu\text{g/ml}$ ) or HC ( $50 \text{ nM}$ ) are shown. GSH synthesis rates in cell-free protein extracts were measured while increasing the concentration of cysteine from 0.025 to 0.5 mM. With increasing cysteine concentration, all three conditions showed saturation in GSH synthesis rates.  $V_{\text{max}}$  and  $K_m$  values were estimated by fitting the Michaelis-Menten equation to the data using the SAAM program. Mean data were weighed with their variances. Both In and HC increased the  $V_{\text{max}}$  of GCS without affecting the  $K_m$  significantly. \*  $P < 0.05$  vs. control by paired  $t$  test.

Table III. Effects of Cycloheximide and Actinomycin D on Insulin- and Hydrocortisone-mediated Increase of GSH and GCS Activity in Cultured Hepatocytes

	Cell GSH	GCS Activity
	nmol/10 <sup>6</sup> cells	nmol GSH synthesized/min per mg protein
Control	$78 \pm 2.1$	$0.34 \pm 0.05$
+ Insulin	$92 \pm 4.9^*$	$0.66 \pm 0.11^*$
+ Hydrocortisone	$96 \pm 6.2^*$	$0.56 \pm 0.11^*$
Cycloheximide control	$78 \pm 3.0$	$0.32 \pm 0.06$
+ Insulin	$75 \pm 3.4$	$0.32 \pm 0.09$
+ Hydrocortisone	$78 \pm 3.6$	$0.27 \pm 0.03$
Actinomycin D control	$78 \pm 5.7$	$0.29 \pm 0.04$
+ Insulin	$76 \pm 4.8$	$0.35 \pm 0.02$
+ Hydrocortisone	$73 \pm 6.5$	$0.30 \pm 0.03$

Results are expressed as mean±SEM from four cell preps. Cultured hepatocytes were incubated with In ( $1 \mu\text{g/ml}$ ) or HC ( $50 \text{ nM}$ ) for four (GCS activity) or 6 h (cell GSH levels). To assess the effects of cycloheximide ( $5 \mu\text{M}$ ) and actinomycin D ( $1 \mu\text{g/ml}$ ), cells were coincubated with these agents for 4 or 6 h. Cell GCS activity was determined in digitonin-treated protein extracts as nmol of GSH synthesized from precursors for both steps of GSH synthesis (cysteine,  $0.1 \text{ mM}$ ; glycine,  $10 \text{ mM}$ ; glutamate,  $10 \text{ mM}$ ; ATP,  $3 \text{ mM}$ ) per mg protein per min was determined by mBCl technique (see Methods for details). \*  $P < 0.05$  vs. control by paired Student's  $t$  test.



Table IV. Effects of Insulin Replacement on Hepatic Glutathione, Cysteine Levels, and GCS Activity in Diabetic Rats

	GSH	Cysteine	GCS activity*
	$\mu\text{mol/gm}$ liver	$\mu\text{mol/g}$ liver	<i>nmol GSH</i> <i>synthesized/min</i> <i>per mg</i> <i>cytosol protein</i>
Control	6.84±0.17	0.14±0.02	2.83±0.15
STZ	5.20±0.45 <sup>‡</sup>	0.18±0.02	1.75±0.15 <sup>§</sup>
STZ + Insulin	6.48±0.15	0.20±0.02	2.62±0.09

Results are expressed as mean±SE from six to ten animals for each group. Rats were given streptozotocin (STZ, 60 mg/kg intraperitoneally) to induce diabetes mellitus or received vehicle control. Diabetic rats were then randomized to receive insulin (NPH, 2–3 U every a.m., 4–5 U every p.m. subcutaneously to maintain glucose around 100–200 mg/dl) or saline. Liver GSH, cysteine levels, and GCS activity were measured 4 d after STZ treatment (see Methods for details). Body weights were similar at the beginning of treatment (288±1.6 g), but the untreated diabetic rats lost 10% of body weight by day 4. The weight loss in diabetic untreated rats is commonly observed despite similar amount of food intake (29, 30). Liver weights also decreased in the untreated diabetic rats in proportion to the decrease in body weights. The amount of cytosolic protein/g liver was similar in all three groups (38±1.5 mg/g). Therefore, expression of GCS activity per gram liver showed the same differences. \* GCS activity, nmol of GSH synthesized per min per mg cytosol protein from precursors (cysteine, 0.1 mM; glycine, 10 mM; glutamate, 10 mM; ATP, 3 mM) as determined by mBCl technique (see Methods). <sup>‡</sup>  $P < 0.05$  vs. control and insulin-treated diabetic rats (STZ + Insulin); <sup>§</sup>  $P < 0.01$  vs. control and STZ + Insulin rats by ANOVA.

## Discussion

Hepatic GSH is vital for defense against toxins and interorgan homeostasis of GSH. The synthesis of hepatic GSH is a key step in the process and our work has been aimed at understanding its regulation. We began the present studies by investigating the influence of In and HC on GSH levels in cultured hepatocytes. We chose to examine these two hormones because of their known importance in regulation of numerous proteins and enzymes in hepatocytes (19–26). We observed that the lowest doses of In and HC which increased GSH were in the known physiologic range of each hormone (27, 28). Thus, it is possible that physiologic changes in In and HC regulate GSH synthesis. However, detailed *in vivo* studies will be required to prove this hypothesis. Of importance, the effect of In on stimulation of cell GSH occurred independent of the glucose concentration in the medium. Thus, it is unlikely that “hyperglycemia” alone had a negative impact on GSH synthesis which was corrected with In. Furthermore, hepatic GSH and GCS activities were lower in the ADX rats despite no change in serum glucose levels from sham controls.

Many factors can potentially influence steady-state cell GSH levels. We therefore set out to systematically examine these to define the mechanism(s) accounting for the In- and HC-induced increases in hepatocyte GSH. A number of factors were excluded, namely, changes in cell volume, GSH efflux, and expression of  $\gamma$ -glutamyl transpeptidase. The two remaining major possibilities were then studied in detail: availability of cysteine and activities of GSH synthetic enzymes.

Availability of cysteine can be increased in cultured hepatocytes by increased uptake of sulfur amino acids, namely, methionine and cystine, increased activity of transsulfuration pathway, or decreased activity of cysteine catabolic pathways. The increase in cell GSH induced by In or HC occurred when only methionine or cystine was the sole source of sulfur amino acid, and as early as 6 h after hormone treatment. Methionine uptake was unaffected by In or HC. Increased activity of transsulfuration also could not account for the increase in cell GSH since it occurred with cystine alone. In contrast to methionine, cystine transport is known to be induced by In and glucocorticoids in culture (12). We confirmed this finding. However, we could dissociate the increase in cell GSH from the increase in cystine uptake in time course studies; the former preceded the latter. These results do not exclude a contributory role for induction of cystine transport in increasing cell GSH (especially at the 16 h time point) but demonstrate that another mechanism makes a major contribution.

The other mechanism we considered was increase in GSH synthetic enzymes. Both In and HC increased the activity of GCS but not GSH synthetase. Kinetic analysis revealed an increased  $V_{\text{max}}$  for GCS but no change in  $K_m$ , suggesting increased amount of enzyme. The induction of GCS became significant after a lag of 4 h and was blocked by exposure of cells to actinomycin D and cycloheximide during this interval. These findings strongly suggest that In and HC increase GCS levels by an RNA and protein synthesis dependent mechanism. Possible mechanisms include increased transcription of the GCS gene, stabilization of the GCS mRNA, increased transla-

Table V. Effects of Adrenalectomy and Dexamethasone on Hepatic GSH, Cysteine Levels, and GCS Activity

	GSH	Cysteine	GCS activity
	$\mu\text{mol/g}$ liver	$\mu\text{mol/g}$ liver	<i>nmol GSH</i> <i>synthesized/min/mg</i> <i>cytosol protein</i>
Sham control	6.40±0.23	0.15±0.01	2.86±0.07
Sham + Dex	6.41±0.14	0.11±0.01	2.82±0.10
ADX	4.08±0.25*	0.15±0.02	2.40±0.14*
ADX + Dex	6.13±0.35	0.13±0.02	2.66±0.08

Results are expressed as mean±SE from 5–11 animals for each group. Bilaterally ADX and sham-operated rats were matched for weight. Rats were randomized to receive dexamethasone (Dex, 5  $\mu\text{g}/100$  g body weight intraperitoneally) or vehicle each day for 4 d starting from postoperative day 11–13. Rats that had ADX procedure received 0.9% NaCl as drinking water. All rats were killed on postoperative day 15–17. Completeness of adrenalectomy was confirmed by examination during autopsy. GSH and cysteine levels were determined from liver homogenates and GCS activity measured as described above. Body weights of the ADX groups were significantly lower as compared to sham-operated groups at the time of death (sham = 305±1.6; sham + Dex = 286±3.8; ADX = 266±4.5; ADX + Dex = 273±2.5). Percent body weight accounted for by liver in the ADX group without Dex treatment was significantly lower (2.96±0.1 vs. sham with or without Dex groups of 3.7±0.1%), and was more than corrected after Dex replacement (4.2±0.1). However, the amount of cytosolic protein per gram liver was similar in all groups (39.6±1.4 mg/g). Therefore, results were not affected by expressing GCS per gram liver. \*  $P < 0.05$  vs. sham control and ADX + Dex by ANOVA.



tion of the GCS mRNA, or decreased degradation (stabilization) of GCS. The inhibition by actinomycin D favors the former. However, further work at the molecular level will be required to delineate the mechanisms of In and HC.

The simplest in vivo counterpart to the induction of GCS by In and HC in cell culture is to examine the influence of deficiency of these hormones on hepatic GSH and GCS as well as the effect of replacement of the respective deficient hormone. Indeed, both STZ-induced diabetic rats and ADX rats exhibited lower hepatic GSH levels and specific activities of GCS in hepatic cytosol compared to controls. Both effects were reversed by hormone replacement which excludes other mechanisms for the changes related to the STZ or surgery rather than hormone deficiency. The specific activities of GSH synthetase were unaffected by STZ or adrenalectomy. The magnitude of fall in liver GSH level did not correlate with GCS activity in the two in vivo models (diabetic rats had more profound fall in GCS activity but less fall in liver GSH level in comparison to the ADX untreated rats). This points to the complexities of in vivo models, so that other factors probably contribute to these differences. For example, ADX rats may have been more "stressed" and had increased sinusoidal GSH efflux and hence much lower hepatic GSH levels (13); or hormones such as glucagon (which may be increased in diabetes) may further downregulate GCS activity in untreated diabetic rats (5). Regardless of other factors that may have been involved, deficiency of In or glucocorticoids was associated with decreased GCS which undoubtedly played a contributory role in the fall in hepatic GSH and appropriate replacement of the hormones corrected the abnormalities.

When calculations are made to compare the GCS specific activities of cultured cells to that of liver cytosols from diabetic or ADX rats with or without hormonal replacement (based on  $10^8$  hepatocytes per gram liver), the results are in close agreement. Thus, the hormone-deficient cultured hepatocyte model closely mimics the in vivo deficiency models. In and HC play an important role in the basal maintenance of hepatic GSH indicating hormone dependence. As noted above, it remains to be seen if changes in these hormones within the physiologic range exert a regulatory role.

There are conflicting data in the literature regarding the effect of diabetes on hepatic GSH levels (29, 30). Loven and colleagues reported lower hepatic GSH concentration in diabetic rats (29) which was corrected with insulin replacement. No mechanism was provided in their work, however. McLennan et al. on the other hand, did not find lower hepatic GSH level in diabetic rats but reported significantly increased hepatic  $\gamma$ -glutamyl *trans*-peptidase activity (30). This resulted in almost undetectable canalicular GSH efflux, presumably due to increased breakdown of GSH. This in fact may have masked a lower hepatic GSH since the increased activity of the hepatic  $\gamma$ -glutamyl cycle could increase the availability of cysteine for GSH synthesis by its reuptake in the canaliculus or bile duct (cholehepatic shunting). Our data are in agreement with that of Loven and colleagues, and suggest that one potential mechanism for lower hepatic GSH levels in untreated diabetic rats is lower hepatic GCS activity. This may have significance concerning the pathophysiology of complications of diabetes mellitus. GSH depletion in erythrocytes and lens have been reported in diabetes and may contribute to increased susceptibility to oxidant stress and cataract formation, respectively (31, 32). Since the liver is the source of plasma GSH and cysteine

(1, 2, 33), decreased capacity for the liver to synthesize GSH may translate into decreased availability of GSH and cysteine to the rest of the body. This may in turn predispose the animal to complications from diminished GSH and sulfhydryls. These possibilities are worthy of further study.

In summary, both insulin and glucocorticoids increased GSH levels in cultured hepatocytes by induction of GCS activity. The importance of these findings was confirmed using in vivo models. In fact, results from in vivo experiments showed that insulin and glucocorticoids are important in "maintaining" normal hepatic GSH levels and expression of GCS activities.

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