JCI The Journal of Clinical Investigation

Effect of chronic ethanol feeding on rat hepatocytic glutathione. Compartmentation, efflux, and response to incubation with ethanol.

J C Fernandez-Checa, M Ookhtens, N Kaplowitz

J Clin Invest. 1987;80(1):57-62. https://doi.org/10.1172/JCI113063.

Research Article

Hepatocytes from rats that were fed ethanol chronically for 6-8 wk were found to have a modest decrease in cytosolic GSH (24%) and a marked decrease in mitochondrial GSH (65%) as compared with pair-fed controls. Incubation of hepatocytes from ethanol-fed rats for 4 h in modified Fisher's medium revealed a greater absolute and fractional GSH efflux rate than controls with maintenance of constant cellular GSH, indicating increased net GSH synthesis. Inhibition of gamma-glutamyltransferase had no effect on these results, which indicates that no degradation of GSH had occurred during these studies. Enhanced fractional efflux was also noted in the perfused livers from ethanol-fed rats. Incubation of hepatocytes in medium containing up to 50 mM ethanol had no effect on cellular GSH, accumulation of GSH in the medium, or cell viability. Thus, chronic ethanol feeding causes a modest fall in cytosolic and a marked fall in mitochondrial GSH. Fractional GSH efflux and therefore synthesis are increased under basal conditions by chronic ethanol feeding, whereas the cellular concentration of GSH drops to a lower steady state level. Incubation of hepatocytes with ethanol indicates that it has no direct, acute effect on hepatic GSH homeostasis.

Effect of Chronic Ethanol Feeding on Rat Hepatocytic Glutathione

Compartmentation, Efflux, and Response to Incubation with Ethanol

Jose C. Fernandez-Checa, Murad Ookhtens, and Neil Kaplowitz
Liver Research Laboratory, Wadsworth Veterans Administration Hospital Center, Los Angeles, California 90073; and University of California at Los Angeles School of Medicine, Los Angeles, California 90073

Abstract

Hepatocytes from rats that were fed ethanol chronically for 6–8 wk were found to have a modest decrease in cytosolic GSH (24%) and a marked decrease in mitochondrial GSH (65%) as compared with pair-fed controls. Incubation of hepatocytes from ethanol-fed rats for 4 h in modified Fisher's medium revealed a greater absolute and fractional GSH efflux rate than controls with maintenance of constant cellular GSH, indicating increased net GSH synthesis. Inhibition of γ-glutamyltransferase had no effect on these results, which indicates that no degradation of GSH had occurred during these studies. Enhanced fractional efflux was also noted in the perfused livers from ethanol-fed rats. Incubation of hepatocytes in medium containing up to 50 mM ethanol had no effect on cellular GSH, accumulation of GSH in the medium, or cell viability. Thus, chronic ethanol feeding causes a modest fall in cytosolic and a marked fall in mitochondrial GSH. Fractional GSH efflux and therefore synthesis are increased under basal conditions by chronic ethanol feeding, whereas the cellular concentration of GSH drops to a lower steady state level. Incubation of hepatocytes with ethanol indicates that it has no direct, acute effect on hepatic GSH homeostasis.

Introduction

Glutathione (GSH) is a tripeptide that plays a very important role in cell defense (1). Cellular GSH exists as two metabolically independent pools, the bulk of which (85–90%) is localized in the cytoplasm, whereas the remainder is compartmentalized in the mitochondria (2). During aerobic metabolism, mitochondria consume molecular oxygen producing some oxygen-free radicals and hydrogen peroxide (H_2O_2) (3, 4). GSH is critical in reducing H_2O_2 as well as organic peroxides—breaking the chain of reactions leading from superoxide anion to the active hydroxyl radical through intermediate H_2O_2—by the action of glutathione redox cycle (5). When mitochondrial GSH (6, 7) is severely depleted, cell viability may be lost, although as to how general a phenomenon this may be remains controversial (8).

The status of hepatic GSH after acute or chronic exposure to ethanol has been a matter of intense investigation. Large doses of ethanol deplete hepatic GSH in vivo (9) and in vitro (10) by mechanisms that are not yet clear. It has been suggested that acute GSH depletion by ethanol is caused by an acetaldehyde adduct (10) or by impairing GSH synthesis (11, 12). The reports of the effect of chronic ethanol exposure on hepatic GSH have been inconsistent with decreases, increases, or no changes observed in GSH levels (13–15). No work has been done to define the effect of ethanol on the small but vital mitochondrial pool of GSH. Since mitochondria are an important target for ethanol toxicity (16, 17), it is important to know if selective depletion of mitochondrial GSH occurs with acute or chronic ethanol exposure.

The aims of the current studies were to determine the effect of chronic ethanol feeding on the compartmentation of hepatocytic GSH and efflux of cytosolic GSH under steady state conditions, the contribution of degradation by γ-glutamyltransferase (γ-GT)[1] to GSH levels in cells and medium, and the effect of incubations in the presence of ethanol.

Methods

Materials. L-alanine, L-arginine, L-asparagine, L-aspartate, L-hydroxyproline, L-glutamine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, L-taurine, L-valine, collagenase (type IV), GSH, and oxidized glutathione (GSSG) were purchased from Sigma Chemical Co. (St. Louis, MO). L-(αS-5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid (AT-125) was a gift from Ruth Davis, National Cancer Institute, Bethesda, MD. NADPH was obtained from United States Biochemical Corp. (Cleveland, OH). The incubation medium was a modified Fisher's medium (18) as follows: 50.0 mg/liter L-alanine, 60.0 mg/liter L-aspartic acid, 50.0 mg/liter L-glycine, 10.0 mg/liter L-hydroxyproline, 40.0 mg/liter L-proline, 20.0 mg/liter L-ornithine, and 23.7 mg/liter L-taurine were added, whereas phenol red, methionine, cystine, penicillin, streptomycin, and horse serum were omitted. The medium was supplemented before use with 1.125 g $NaHCO_3$/liter and 20 mM Hepes, gassed with 95% O_2 and 5% CO_2, and the pH adjusted to 7.4.

Animals. Male Sprague-Dawley rats weighing 160–180 g were pair fed liquid diets (19) (Bioserv, Rahway, NJ) containing 36% of calories as ethanol or an isocaloric mixture with maltose dextrin substituted for ethanol. Animals were housed in individual cages and pair fed for 6 wk from 7 p.m. to 7 a.m. The amount of ethanol consumed by the ethanol-treated group was 25.3 g ethanol per kg/d. The weight gain was similar in both groups (final weight 360±19 vs. 385±25 g).

Preparation of mitochondria from whole liver. Two livers from pair-fed control and two from ethanol-fed rats were homogenized (25% wt/vol) in iced-cold buffer consisting of 70 mM sucrose, 220 mM D-mannitol, and 2.0 mM Hepes, pH 7.4. Debris and nuclei were pelleted at 2,450 *g* for 10 min. The mitochondrial pellet was obtained after centrifugation at 7,000 *g* for 15 min and washed three times. The crude homogenate and mitochondrial fractions were assayed for lactic dehydrogenase (LDH)

Address all correspondence and reprint requests to Dr. Kaplowitz, W151N Liver Research Lab, Wadsworth Veterans Administration Hospital, Los Angeles, CA 90073.

Received for publication 13 November 1986 and in revised form 11 February 1987.

1. *Abbreviations used in this paper:* AT-125, L-(αS-5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid; γ-GT, γ-glutamyltransferase; GSSG, oxidized glutathione; LDH, lactic dehydrogenase; SDH, succinic dehydrogenase.

and succinic dehydrogenase (SDH). The recovery of SDH and LDH in the mitochondrial pellet averaged 10.4% and 0.23%, respectively, from pair-fed controls and 9.7 and 0.25%, respectively, from ethanol-fed liver. Thus, correction for contamination with cytosolic GSH could be neglected and the nearly identical recovery of mitochondria in the two groups validates correcting the recovered mitochondrial GSH for mitochondrial recovery.

Cell isolation and incubation. Hepatocytes were isolated according to the method of Moldeus et al. (20). The viability of the cells was ascertained by the trypan blue exclusion: routinely, 94–97% of the cells excluded 0.2% trypan blue. The cells were resuspended in modified Fisher's medium and maintained at 20°C in a rotating round-bottom flask without loss of viability. Cells were incubated at 37°C in 25-ml Erlenmeyer flasks at concentrations of 2×10^6 to 3×10^6 cells/ml in an orbital-shaker bath under the atmosphere of 95% O_2/5% CO_2 for 4 h. At hourly intervals, 0.5-ml aliquots were removed for fractionation of cells and for GSH determination in the cytosol, mitochondria, and medium.

In some incubations, ethanol in vitro at concentrations of 10, 20, and 50 mM was included in the medium and the incubation continued for 4 h as described above.

Separation of cytosol and particulate fractions. At various times of incubations plasma membrane of cells was selectively disrupted by digitonin according to Andersson et al. (21). Briefly, in 1.5-ml Eppendorf tubes containing from the bottom: 0.1 ml 40% glycerol; 0.5 ml silicone–mineral oil mixture; and a 0.1-ml top layer of 19.8 mM EDTA, 250 mM mannitol in 17.0 mM Hepes, pH 7.4, a 0.5-ml aliquot of the cell suspension was mixed with the top layer and centrifuged at 13,000 *g* in a microfuge at room temperature for 3 min. Separation of cytosolic and mitochondrial compartments was achieved by including 0.24 mg/ml digitonin in the Hepes buffer. A sample from the supernatant was withdrawn and kept to measure enzyme activities; oil mixture was carefully removed and discarded without perturbing the glycerol layer. 0.4 ml Krebs–Henseleit buffer and 0.1 ml 10% Triton X-100 were added to the glycerol layer followed by 15–20 s of sonication. Samples from the supernatant and glycerol layer were assayed spectrophotometrically for LDH and SDH activities as described by Veeger et al. (22) and compared with whole cells lysed with Triton X-100.

Liver perfusion. Rats were anesthetized with 50 mg/kg pentobarbitol i.p. and given 50 IU heparin i.v. Liver of both controls and ethanol-treated rats were perfused in situ, single pass with Krebs–Ringer bicarbonate buffer gassed to equilibrium with 95% O_2 and 5% CO_2 of pH 7.4 at 37°C as previously described (23, 24). We continuously monitored the O_2 tension in the inflow and outflow lines to determine the rate of O_2 uptake by the liver. GSH was measured in the effluent samples.

Table I. Fractionation of Hepatocytes from Control and Ethanol-treated Rats

Group	Digitonin	LDH	SDH
		%	%
Control			
Supernatant	−	8.95±0.5	1.97±0.4
Pellet	−	89.1±2.3	94.0±1.5
Supernatant	+	98.4±0.3	2.9±0.6
Pellet	+	1.3±0.1	91.6±2.2
Ethanol			
Supernatant	−	7.7±0.1	1.3±0.1
Pellet	−	91.2±2.4	86.3±3.4
Supernatant	+	98.7±0.1	3.1±0.4
Pellet	+	1.3±0.1	94.3±3.4

All values are mean±SD, $n = 8$.

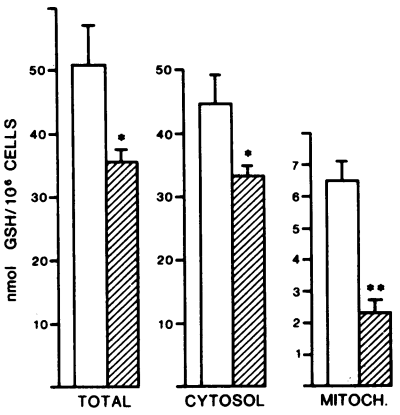

Figure 1. Effects of chronic ethanol administration on total hepatocellular, cytoplasmic, and mitochondrial GSH. Cells from control (□) and ethanol-treated (▨) rats immediately upon isolation underwent disruption of plasma membrane by digitonin to separate cytosolic and particulate fractions as described in Methods. Values are mean±SD, $n = 8$. * and ** indicate $P < 0.01$ and $P < 0.001$, respectively.

Gas chromatography of ethanol and acetaldehyde. Head space aliquots (25) liberated from the incubations of cells with ethanol in vitro were run on a Sulpeco 80/120 carbopack B column (5% carbowax, 20 M) obtained from Supelco Inc., Bellefonte, PA. Column temperature ranged from 70° to 170°C at 5°C/min; flow rate was 20 ml/min. The values were checked against standards. Ethanol and acetaldehyde had retention times of 5.1 and 1.4 min, respectively.

Inhibition of γ-GT. Inhibition of γ-GT was achieved by preincubating hepatocytes from both control and ethanol-treated rats with 0.25 mM AT-125 for 15 min at 37°C. Then the hepatocytes were washed twice with Fisher's modified medium and resuspended at 2×10^6 cells/ml for continuation of the experiment. The activity of γ-GT was measured as the release of *p*-nitroanilide (26).

Assays. Total cellular or mitochondrial GSH plus GSSG content was determined after extraction with 10% trichloroacetic acid (1:1 vol/vol) by using the method of Tietze (27). Some samples were spot checked for GSH and GSSG by using the high performance liquid chromatography (HPLC) method of Reed et al. (28).

Statistical analysis. Data were expressed as the mean and standard deviation. Statistical evaluations between sample means were made by the two-tailed Student's *t* test. $P < 0.05$ was used as the criterion of significance.

Results

Fractionation of hepatocytes by digitonin. A rapid separation of mitochondria from soluble fraction was achieved by treatment of the cell suspension with digitonin, followed by immediate centrifugation. By using 0.24 mg/ml of digitonin, > 98% of the cytosolic marker, LDH, was recovered in the supernatant and 1.3% in the pellet (glycerol layer) for both control and ethanol-treated cells (Table I). This shows that there was almost complete disruption of plasma membrane and virtually no cross-contamination. Since only 3% of the mitochondrial marker, SDH, was released into the supernatant for both control and ethanol-treated hepatocytes, mitochondria were recovered intact in the glycerol layer.

Effect of chronic ethanol administration on hepatic GSH. Cells from both control and ethanol-treated rats were used immediately after isolation for total cellular GSH determination and for disruption of plasma membrane by digitonin to measure the cytosolic and mitochondrial GSH content. Results in Fig. 1 show that chronic ethanol administration significantly decreased the GSH content of whole cells, cytosol, and mitochondria with

Table II. Hepatocellular GSH Content and Efflux Rate from Control and Ethanol-treated Rats

	Accumulation of GSH (nmol/10^6 cells)							
	Medium GSH		Total GSH*		GSH efflux rate (nmol/10^6 cells per min)		Fractional GSH efflux rate (h^{-1})‡	
Time	Control	Chronic ETOH	Control	Chronic ETOH	Control	Chronic ETOH	Control	Chronic ETOH
h								
0–1	6.8±2.1	16.6±3.4§	9.5±1.8	19.0±4.5§	0.113±0.01	0.276±0.03§	0.132±0.03	0.416±0.03‖
1–4	9.7±1.7	10.5±2.9	16.5±2.5	18.0±2.3	0.053±0.01	0.057±0.01	0.058±0.01	0.079±0.02§
0–4	16.5±3.2	27.1±3.7§	26.0±3.1	37.0±4.3§	0.068±0.01	0.113±0.02§	0.073±0.01	0.152±0.02§

All values are mean±SD, $n = 8$. * Total represents accumulation of GSH in cells plus medium during the time intervals. ‡ Fractional GSH efflux rate was calculated as the fraction of cellular GSH found in the medium as nmol/10^6 cells/min at each time interval. § $P < 0.01$ vs. control. ‖ $P < 0.001$ vs. control.

31, 24, and 65% decreases, respectively, in the ethanol-treated rats vs. control. More than 90% of glutathione in cytosol and mitochondria was in the reduced form.

To verify that the mitochondrial GSH depletion in freshly isolated cells from ethanol-fed rats was not an artifact of cell isolation, we determined GSH in the mitochondrial pellet and cytosol prepared from whole liver of two ethanol-fed and two pair-fed rats. The cytosolic GSH in ethanol-fed rats was decreased by 30%, whereas the mitochondrial GSH was decreased by 63% vs. control, confirming the findings in isolated cells.

GSH in cells and medium during 4-h incubations. Hepatocytes from control and ethanol-treated rats maintained total GSH levels (Fig. 2) as well as relative mitochondrial and cytosolic levels (not shown) nearly constant throughout 4 h of incubation in medium without sulfur amino acids. GSH continued to accumulate extracellularly during the experiments. During the 1st h the absolute amount of GSH accumulated in the medium was significantly greater with ethanol-treated cells despite a somewhat lower cellular GSH (Table II). After the 1st h the rate of accumulation was parallel in both groups. Since GSH in cells remained nearly constant while medium GSH increased, the total GSH increase in the system (cells plus medium) represented a minimum estimate of net synthesis (not accounting for degradation). Thus, the absolute and fractional increase in total (cell plus medium) GSH was significantly greater in incubations of the cells from ethanol-treated rats, largely due to the accumulation in medium (Table II). Even without adding precursor amino acids, it is apparent that sufficient precursor must have been available to maintain cellular GSH near steady state while supporting greater net synthesis in the cells from chronic ethanol-treated rats.

Inhibition of γ-GT. In normal liver it is well known that efflux of GSH at steady state nearly matches total turnover and thus synthesis. However, we considered the possibility that ethanol might induce cell surface or intracellular γ-GT that might lead to hydrolysis of GSH and underestimation of its accumulation in cells or medium.

We found no differences in total cellular γ-GT in hepatocytes from control and ethanol-fed rats. Preincubation for 15 min with AT-125 lead to nearly complete loss of enzyme activity (not shown). AT-125 pretreatment had no effect on the levels of GSH in cells or medium from either group over the course of 4-h incubation (Table III). Thus, we could be confident that no degradation of GSH had occurred in our system. Therefore, GSH efflux represented net synthesis at near steady cellular GSH.

GSH efflux from the perfused liver. To verify the increased

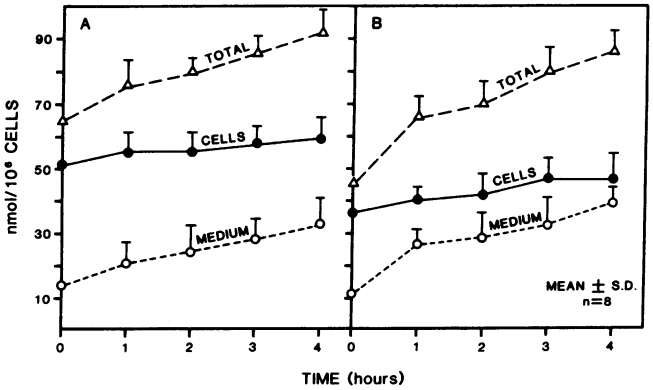

Figure 2. Changes in hepatocellular and medium GSH over time. Cells were incubated for 4 h in Fisher's modified medium at 37°C. At the indicated times, an aliquot of cell suspension (2×10^6 cells/ml) was taken and the medium rapidly separated from cells by centrifugation. GSH was determined in cells and medium. Total refers to the sum of cellular and medium GSH at each time. Values are mean±SD, $n = 8$. (*A*) control. (*B*) chronic ethanol-treated.

Table III. Effect of AT-125 on Cellular and Medium GSH from Control and Ethanol-treated Cells

Time	Group	Addition	Cellular*	Medium*
h				
0	Control	—	51.0±6.0	14.9±2.1
	Ethanol	—	35.8±2.0	10.7±1.4
2	Control	None	54.7±6.1	24.9±5.3
	Control	AT-125	57.7±2.9	25.3±3.4
	Ethanol	None	42.3±6.7	28.4±3.4
	Ethanol	AT-125	45.3±3.7	30.3±2.5
4	Control	None	60.2±7.7	32.9±6.1
	Control	AT-125	64.3±3.6	33.8±0.4
	Ethanol	None	46.0±4.9	38.0±4.1
	Ethanol	AT-125	47.2±5.2	40.9±7.9

All values are mean±SD, $n = 3$. *In nmol/10^6 cells of GSH.

Table IV. Sinusoidal GSH Efflux from Control and Ethanol-treated Liver in the Absence or Presence of 50 mM Ethanol

Group	Hepatic GSH	O_2 uptake	Sinusoidal GSH efflux rate			
			0 mM ETOH		50 mM ETOH	
			Mass	Fractional	Mass	Fractional
	μmol/g liver	*μmol·min⁻¹·g⁻¹*	*nmol/min per g*	*h⁻¹*	*nmol/min per g*	*h⁻¹*
Control	5.96±0.48	2.3±0.05	18.7±2.9	0.18±0.03	19.6±3.2	0.19±0.01
Ethanol	3.70±0.33*	2.2±0.07	25.9±3.9*	0.42±0.03‡	25.0±0.9*	0.40±0.02‡

All values are mean±SD, $n = 3$. * $P < 0.01$ vs. control. ‡ $P < 0.001$ vs. control.

efflux seen in the 1st h of incubation of cells from chronic alcohol-fed rats, we examined the steady state efflux in a more physiologic organ model (i.e., the isolated, perfused liver). A significant increase in efflux was observed from the chronic ethanol-fed rat liver that was even more pronounced as a fractional rate (Table IV). The addition of 50 mM ethanol to the perfusion, to our surprise, had absolutely no effect on the release of GSH into the perfusate. This finding prompted a detailed examination of the effects of ethanol added to the incubation medium of isolated hepatocytes from control and ethanol-fed rats.

Effect of incubation of cells with ethanol. When hepatocytes were incubated with 10, 20, or 50 mM ethanol over 4 h, no effect on cell viability (Fig. 3), cellular GSH, or medium GSH (Fig. 4) was observed either in controls or after chronic ethanol feeding. Moreover, hepatocytes from chow-fed rats exhibited no change in GSH when incubated with 50 mM ethanol or 0.1 mM acetaldehyde plus 0.1 mM cyanamide (data not shown).

To insure that the cells were metabolizing ethanol, we examined the ethanol and acetaldehyde concentrations in incubations with or without cells. In the suspending buffer alone there was negligible evaporation throughout the 4-h incubations. In the presence of cells, however, there was a drop in ethanol concentrations in the incubations (Fig. 5). With each of the ethanol concentrations studied, a constant amount of acetaldehyde (~ 100 μM) was observed in the incubation medium throughout the incubation, as well as the accumulation of acetate at 10 mM at 4 h.

Discussion

Chronic ethanol feeding resulted in a modest fall in cytosolic GSH similar to some but not all previous reports. However, we have shown for the first time that mitochondrial GSH was dramatically diminished in hepatocytes from chronic ethanol-fed rats. Ethanol is well known to exert somewhat selective toxic effects on mitochondria (29, 30). Since lipid peroxidation may be an important factor in mediating the hepatotoxicity of ethanol (31, 32), the depletion of mitochondrial GSH may markedly affect the susceptibility of hepatic mitochondria to oxidant stress. Whether this is an important factor in the pathogenesis of alcoholic liver disease remains to be determined.

We have confirmed the findings of Pierson et al. (33) that chronic ethanol feeding enhances hepatic GSH efflux from the perfused liver. Current evidence suggests that GSH efflux is a carrier-mediated process (24). It is uncertain if enhanced efflux despite lower cytosolic GSH is due to induction of a carrier protein as a constituent of the plasma membrane, a change in the lipid milieu of the carrier, or a change in the driving forces for transport.

We could not identify a direct, acute effect of ethanol or its metabolites in isolated hepatocytes on the concentration of cellular GSH or on the accumulation of GSH in the medium. In a previous report (10), a decreased GSH is observed with acute ethanol administration to hepatocytes. According to those data, the potentiation of the acetaldehyde effect by using an inhibitor of aldehyde dehydrogenase (disulfiram) showed that the effect

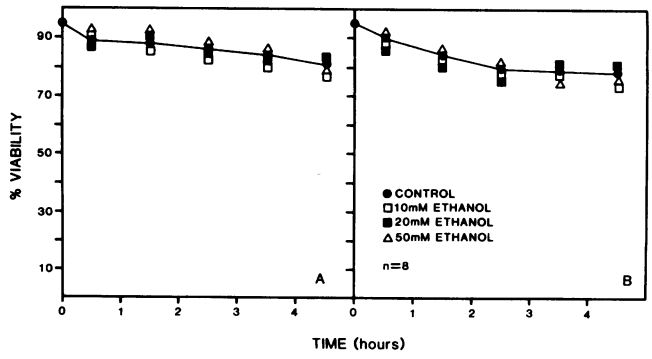

Figure 3. The viability of hepatocytes from control (*A*) and ethanol-treated (*B*) cells was determined by trypan blue exclusion in the absence (●) or presence of 10 (□), 20 (■), and 50 mM (△) ethanol incubated for 4 h at 37°C in Fisher's modified medium. Values are mean±SD, $n = 8$.

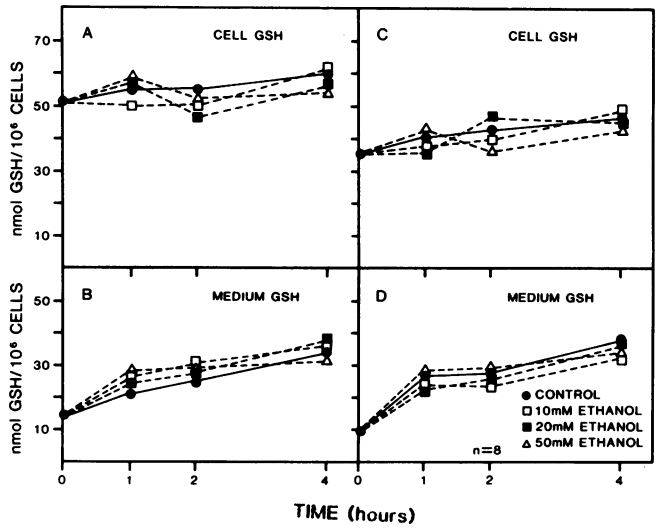

Figure 4. Variations of cellular and medium GSH with time in the presence of different ethanol concentrations. Cells (2×10^6/ml) from control (*A* and *B*) and ethanol-treated (*C* and *D*) rats were incubated in the absence (●) and presence of 10 (□), 20 (■), and 50 mM (△) ethanol concentrations. Values are mean±SD, $n = 8$.

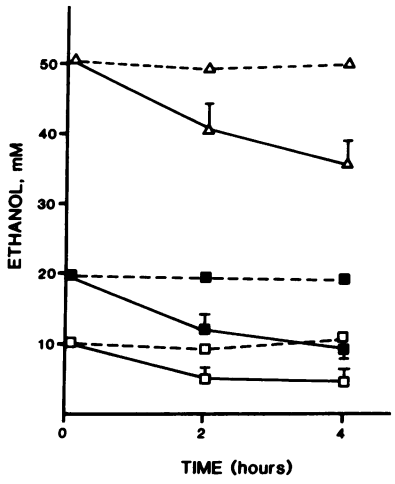

Figure 5. Metabolism of 10 (□), 20 (■), and 50 mM (△) ethanol by hepatocytes (2×10^6 cells/ml) (——) at 37°C in modified Fisher's medium in an orbital-shaker bath. Evaporation of ethanol in the absence of cells (- - -) was negligible at all ethanol concentrations during the course of incubation. Values are mean±SD, $n = 5$.

of ethanol is due to its conversion to acetaldehyde. However, disulfiram inhibits the low-Michaelis constant aldehyde dehydrogenase by reacting with sulfydryl groups (34). Potentially, disulfiram has the ability to perturb the sulfydryl group of GSH and cause misleading results.

Since AT-125 did not affect cellular or medium GSH, no significant degradation of GSH had occurred during our incubations. Furthermore, since cellular GSH was maintained nearly constant while GSH accumulated in the medium, GSH efflux must have closely approximated total synthesis. Thus, synthesis was neither impaired by chronic ethanol treatment nor was it inhibited by up to 50 mM ethanol. In vivo studies have suggested marked depletion of hepatic GSH and decreased synthesis with massive doses of ethanol (12). Our findings suggest that these in vivo effects are not due to the direct action of ethanol or acetaldehyde on the hepatocyte, but could be due to either nonspecific systemic toxicity or other modulators of GSH turnover, such as hormones, present in vivo but not in vitro.

In our experimental approach, we have not included sulfur amino acids in the medium to simplify the assay of glutathione. Clearly, sufficient intracellular precursors were available to sustain net synthesis of GSH and to maintain steady state cell levels of GSH. GSH synthesis (efflux) was greater in hepatocytes from ethanol-fed rats. The lower steady state concentration of cellular GSH in ethanol-fed liver may be due, at least partially, to the greater efflux rate. Thus, it is possible that the hepatocytes cannot compensate for the elevated efflux by maintaining normal GSH levels, but rather, a new and lower steady state level of hepatic GSH is established when synthesis matches efflux. Future work will need to examine the effect of optimizing precursor amino acid concentrations in the medium to determine the capacity to maintain steady state synthesis (efflux) and cellular concentrations of GSH.

Acknowledgments

This work was supported by Veterans Administration Medical Research Funds. Dr. Fernandez-Checa was the recipient of an American Liver Foundation postdoctoral fellowship award.

References

1. Kaplowitz, N., T. Y. Aw, and M. Ookhtens. 1985. The regulation of hepatic glutathione. *Annu. Rev. Pharmacol. Toxicol.* 25:715–744.

2. Wahllander, A., A. Soboll, and H. Sies. 1975. Hepatic mitochondrial and cytosolic glutathione content and the subcellular distribution of GSH-S-transferases. *FEBS (Fed. Eur. Biochem. Soc.). Lett.* 97:138–140.

3. Oshino, N., B. Chance, H. Sies, and T. Bucher. 1973. The role of H_2O_2 generation in perfused rat liver and the reaction of catalase compound I and hydrogen donors. *Arch. Biochem. Biophys.* 154:117–131.

4. Fridovich, S. 1986. Biological effects of the superoxide radical. *Arch. Biochem. Biophys.* 247:1–11.

5. Reed, D. J. 1986. Regulation of reductive processes by glutathione. *Biochem. Pharmacol.* 35:7–13.

6. Meredith, M. J., and D. J. Reed. 1982. Status of the mitochondrial pool of glutathione in the isolated hepatocyte. *J. Biol. Chem.* 257:3747–3753.

7. Meredith, M. J., and D. J. Reed. 1983. Depletion in vitro of mitochondrial glutathione in rat hepatocytes and enhancement of lipid peroxidation by adriamicin and 1,3-bis (2-chloroethyl)-1-nitrosourea (BCNU). *Biochem. Pharmacol.* 32:1383–1388.

8. Ku, R. H. and R. E. Billings. 1986. The role of mitochondrial glutathione and cellular protein sulfhydryls in formaldehyde toxicity in glutathione-depleted rat hepatocytes. *Arch. Biochem. Biophys.* 247:183–189.

9. Videla, L. A., V. Fernandez, G. Ugante, and A. Valenzuela. 1980. Effect of acute ethanol intoxication on the content of reduced glutathione of the liver in relation to its lipoperoxidative capacity in the rat. *FEBS (Fed. Eur. Biochem. Soc.). Lett.* 111:6–10.

10. Vina, J., J. M. Estrela, C. Guerri, and F. Romero. 1980. Effect of ethanol on glutathione concentration in isolated hepatocytes. *Biochem. J.* 188:549–552.

11. Speisky, H., A. McDonald, G. Giles, H. Orrego, and Y. Israel. 1985. Increased loss and decreased synthesis of hepatic glutathione after acute ethanol administration. *Biochem. J.* 225:565–572.

12. Lauterburg, B. H., S. Davies, and J. R. Mitchell. 1984. Ethanol suppresses hepatic glutathione synthesis in rats in vivo. *J. Pharm. Exp. Ther.* 230:7–11.

13. Shaw, S., E. Tayatilleke, W. A. Ross, E. R. Gordon, and C. S. Lieber. 1981. Ethanol-induced lipid peroxidation: potentiation by long-term alcohol feeding and attenuation by methionine. *J. Lab. Clin. Med.* 98:417–424.

14. Venderviale, G., E. Tayetilleke, S. Shaw, and C. S. Lieber. 1984. Suppression of biliary glutathione excretion by chronic ethanol feeding in the rat. *Life Sci.* 34:1065–1073.

15. Moldeus, P., B. Andersson, A. Northing, and J. Ormstad. 1980. Effect of chronic ethanol administration on drug metabolism in isolated hepatocytes with emphasis on parecetanol activation. *Biochem. Pharmacol.* 29:1741–1745.

16. Thayer, W. S., and E. Rubin. 1981. Molecular alterations in the respiratory chain of rat liver after chronic ethanol consumption. *J. Biol. Chem.* 256:6090–6097.

17. Waring, A. J., H. Rottenberg, T. Ohnishi, and E. Rubin. 1982. The effect of chronic ethanol consumption on temperature-dependent physical properties of liver mitochondrial membranes. *Arch. Biochem. Biophys.* 216:51–61.

18. Fisher, G. A., and A. C. Sartorelli. 1964. Development, maintenance and assay of drug resistance. *Methods Med. Res.* 10:247–262.

19. DeCarli, L. M., and C. S. Lieber. 1967. Fatty liver in the rat after prolonged intake of ethanol with a nutritionally adequate new liquid diet. *J. Nutr.* 91:131–138.

20. Moldeus, P., T. Hogberg, and S. Orrehnius. 1978. Isolation and use of liver cells. *Methods Enzymol.* 51:60–70.

21. Andersson, B. S., and D. P. Jones. 1985. Use of digitonin fractionation to determine mitochondrial transmembrane ion distribution in cells during anoxia. *Anal. Biochem.* 146:164–172.

22. Veeger, C., D. V. DerVartanian, and W. P. Zeylemaker. 1969. Succinate dehydrogenase. *Methods Enzymol.* 13:81–90.

23. Sies, H. 1978. The use of perfusion of liver and other organs for the study of microsomal electron transport and cytochrome P-450. *Methods Enzymol.* 51:48–59.

24. 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.

25. Duritz, T. H., and A. J. Truitteb Jr. 1964. A rapid method for the simultaneous determination of acetaldehyde and ethanol in blood using gas chromatography. *Q. J. Stud. Alcohol.* 25:498–510.

26. Szasz, G. 1968. A kinetic photometric method for serum-glutamyl transpeptidase. *Clin. Chem.* 15:124–136.

27. Tietze, F. 1969. Enzymatic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. *Anal. Biochem.* 27:502–522.

28. Reed, D. J., T. R. Babson, D. W. Beetty, A. E. Brodie, W. W. Ellis, and D. W. Potter. 1980. High-performance liquid chromatography analysis of nanomole levels of glutathione, glutathione disulfide, and selected thiols and disulfides. *Anal. Biochem.* 106:55–62.

29. Rottenberg, H., D. E. Robertson, and E. Rubin. 1980. The effect of ethanol on the temperature dependence of respiration and ATPase activities of rat liver mitochondria. *Lab. Invest.* 42:318–326.

30. Thayer, W. S., and E. Rubin. 1979. Effects of chronic ethanol intoxication on oxidative phosphorylation in rat liver submitochondrial particles. *J. Biol. Chem.* 254:7717–7723.

31. Diluzio, N. R., and A. D. Hartman. 1967. Role of lipid peroxidation in the pathogenesis of the ethanol-induced fatty liver. *Fed. Proc.* 26:1436–1442.

32. Muller, A., and H. Sies. 1982. Role of alcohol dehydrogenase activity and of acetaldehyde in ethanol-induced ethane and pentane production by isolated perfused rat liver. *Biochem. J.* 206:153–156.

33. Pierson, J. L., and M. C. Mitchell. 1986. Increased hepatic efflux of glutathione after chronic ethanol feeding. *Biochem. Pharmacol.* 35: 1533–1537.

34. Kitson, T. M. 1977. The disulfiram-ethanol reaction: a review. *J. Stud. Alcohol.* 38:96–113.