Biochemical Pharmacology, Vol. 35, No. 9, pp. 1533–1537, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00

INCREASED HEPATIC EFFLUX OF GLUTATHIONE AFTER CHRONIC ETHANOL FEEDING*,†

JEFFREY L. PIERSON and MACK C. MITCHELL‡
Department of Medicine and The Alcohol Research Center, The Johns Hopkins University School of Medicine, Baltimore, Maryland, U.S.A.

(*Received* 1 *July* 1985; *accepted* 18 *October* 1985)

Abstract—Chronic ethanol feeding increases hepatotoxicity of drugs, such as acetaminophen, which form electrophilic metabolites. Availability of glutathione (GSH) is important in preventing liver damage from reactive metabolites. Chronic ethanol feeding has been reported to increase turnover of hepatic GSH in rats. The results of the present study show that the total hepatic efflux of GSH was increased from 5.95 ± 0.42 nmoles/min/g liver (control) to 9.96 ± 0.57 nmoles/min/g ($P < 0.001$) in isolated perfused livers from rats 24 hr after withdrawal from chronic ethanol feeding. The increase in total efflux of GSH was due to a significant increase in sinusoidal GSH efflux from 4.76 ± 0.49 nmoles/min/g liver in control rats to 9.07 ± 0.47 nmoles/min/g ($P < 0.001$) in ethanol-fed rats, while biliary efflux decreased slightly, 1.20 ± 0.11 (control) vs 0.89 ± 0.31 (ethanol). The increase in cellular efflux of GSH was similar in magnitude to the increase in hepatic GSH turnover that we reported previously. Biliary GSSG was similar in both groups of animals. Hepatic GGT activity was increased slightly, but not significantly, whereas renal GGT activity was similar in ethanol-fed rats. Hepatic GSH and GSSG levels were also similar. The increase in turnover of hepatic GSH in rats withdrawn from chronic ethanol feeding was most likely due to increased cellular efflux of GSH. This finding suggests that chronic ethanol feeding may increase cellular requirements for GSH, although the mechanism remains unknown. This alteration in GSH turnover may have important consequences for detoxification of xenobiotics or their metabolites by the liver.

Glutathione (GSH) a tripeptide found in high concentrations in the liver, is important in protection of cells from reactive electrophiles, free radicals and reactive oxygen intermediates formed during drug metabolism [1, 2]. Previous studies have suggested that availability of GSH is an important determinant of drug-induced hepatotoxicity. The steady-state GSH concentration [3–5], the rate of *de novo* GSH synthesis [6–8], and the rate of GSH turnover [9] all determine availability of GSH. For example, fasting decreases steady-state GSH concentrations, yet increases GSH turnover and hepatic efflux of GSH [10]. In the basal state, GSH turnover can be accounted for almost entirely on the basis of hepatic efflux of GSH [10]. During fasting, plasma clearance of GSH is increased and plasma GSH concentrations are decreased [10]. This decreased plasma GSH concentration may stimulate increased hepatic GSH efflux as a mechanism of providing cysteine to extrahepatic tissues [10].

Chronic ethanol consumption increases hepatotoxicity of several drugs in animals [11–13] and possibly in man [14, 15]. The effects of ethanol ingestion upon glutathione metabolism are important in understanding the possible mechanism by which ethanol may enhance drug-induced liver damage and perhaps the hepatotoxicity of ethanol itself.

Previous studies have reported conflicting results on the effects of chronic ethanol consumption on steady-state GSH concentration [9, 16–18]. We have shown that chronic ethanol consumption increases the hepatic turnover of GSH in rats, although the mechanism for this effect is uncertain [9]. Therefore, we measured the sinusoidal efflux of GSH from isolated perfused livers of chronically ethanol-fed and pair-fed control rats, to determine whether hepatic efflux of GSH might account for the observed increase in the turnover of hepatic GSH.

MATERIALS AND METHODS

Animals. Male Fischer 344 rats weighing 175–225 g were pair-fed liquid diets (Bioserve, Rahway, NJ) containing 36% of calories as ethanol or an isocaloric mixture with maltose-dextrins substituted for ethanol. Animals were housed in individual cages and pair-fed for 6 weeks. Twenty-four hours prior to experimentation, each rat was given an equal amount of control diet to minimize short-term nutritional effects and to eliminate the effects of acute ethanol consumption superimposed on the chronic effects.

Liver perfusion. Rats were anesthetized with pentobarbital (50 mg/kg, i.p.) and heparinized (500 units i.p.) 5 min prior to laparotomy. A cannula (PE-10 tubing) was placed in the common bile duct and bile was collected into 5% metaphosphoric acid at 10-min intervals for 2 hr. The portal vein was cannulated with a 16 gauge Teflon i.v. catheter, and the liver was

* This study was presented at the annual meeting of the American Federation for Clinical Research and published in abstract form in *Clin. Res.* **33**, 325A (1985).

† This work was supported by the Alcoholic Beverage Medical Research Foundation.

‡ Author to whom all correspondence should be addressed: Mack C. Mitchell, M.D., Division of Gastroenterology, The Johns Hopkins Hospital, 600 North Wolfe St., Baltimore, MD 21205.

Table 1. Effect of chronic ethanol on hepatic GSH and GSSG content

	Liver weight (g liver/100 g body wt)	Hepatic GSH (μmoles/g liver)	Hepatic GSSG* (μmoles/g liver)
Control	2.98 ± 0.04	7.45 ± 0.47	0.43 ± 0.05
Ethanol	3.10 ± 0.13	7.57 ± 0.87	0.26 ± 0.04
	NS	NS	($P < 0.05$)

Results shown are mean ± S.E. for five animals in each group. NS = not significant.
* Expressed as GSH equivalents.

perfused with Kreb's bicarbonate buffer (pH 7.4) containing pyruvate (0.3 mM) and lactate (2.1 mM) at 3.5 to 4.5 ml/min/g at 38°. Perfusate was collected in 10-min samples for 2 hr.

Assays. Reduced glutathione (GSH) was measured in bile and perfusate by the method of Hissin and Hilf [19]. A portion of the liver was homogenized in 15% trichloroacetic acid containing 0.01 N HCl immediately after the termination of perfusion. Total GSH and glutathione disulfide (GSSG) in liver were measured by the method of Tietze [20]. GSSG in bile was measured by the GSSG-reductase catalyzed oxidation of NADPH to NADP as described [21]. GSSG is expressed as equivalents of GSH.

Kidneys were harvested immediately after initiation of liver perfusion and homogenized in 10 vol. of ice-cold 50 mM Tris–HCl, pH 8.2. A portion of the liver was homogenized in the same buffer at the termination of perfusion for assay of gamma-glutamyltransferase. Activity of gamma-glutamyltransferase (GGT) in kidney and liver was determined by the release of *p*-nitroaniline from L-glutamyl-*p*-nitroanilide as described [22].

Statistics. All results are expressed as mean ± standard error unless otherwise specified. Student's *t*-test (two-tailed) was used to compare means of the ethanol and control groups. A value of $P < 0.05$ was considered the minimum level of statistical significance.

RESULTS

Weight gain was similar in both control and ethanol-fed rats over the 6-week course of pair-feeding, averaging 13.9 ± 8.2% of the initial body weight. Liver weight as a percentage of body weight was unchanged in ethanol-fed rats (Table 1). Hepatic GSH levels measured at the completion of each perfusion were similar in both groups, while GSSG levels were slightly lower in ethanol-fed rats (Table 1).

Within 10–15 min after cannulation of the portal vein, bile flow and glutathione efflux stabilized and remained constant for the next 80 min. After this time, there was a slow decline in bile flow and both sinusoidal and biliary efflux of GSH. For this reason, only those values between 20 and 100 min were used to calculate GSH and GSSG efflux in each experiment. Average values were then pooled to determine the mean for each group of animals. Efflux was calculated from the concentration of GSH times the perfusate or bile flow rate for sinusoidal and biliary efflux respectively. Bile flow was similar in ethanol-fed (0.59 ± 0.21 μl/min/g liver) and control (0.75 ± 0.10 μl/min/g) rats.

Sinusoidal efflux of GSH nearly doubled in ethanol-fed rats, from 4.76 ± 0.49 nmoles/min/g liver to 9.07 ± 0.47 nmoles/min/g liver ($P < 0.001$) (Table 2). Although biliary efflux of GSH was higher in the control group, 1.20 ± 0.11 nmoles/min/g liver vs 0.89 ± 0.31 nmoles/min/g liver (Table 2), total hepatic efflux of GSH (sinusoidal + biliary) was significantly greater in the ethanol-fed rats, 9.96 ± 0.57 nmoles/min/g liver vs 5.95 ± 0.42 nmoles/min/g liver. There was no difference in biliary GSSG output, 0.86 ± 0.04 nmoles/min/g liver (control) vs 0.86 ± 0.19 nmoles/min/g liver (ethanol) (Table 2).

Since glutathione can be degraded into component amino acids via the gamma-glutamyl cycle, we measured the activity of gamma-glutamyltransferase, the rate-limiting step in GSH degradation, in both liver and kidney homogenates from each animal. As shown in Table 3, renal GGT activity was 100-fold greater than hepatic GGT activity in both groups. Hepatic GGT activity was slightly higher in ethanol-fed animals, but this difference was not statistically significant ($P = 0.15$) (Table 3). This activity of GGT in livers after 2 hr of perfusion

Table 2. Effects of chronic ethanol on hepatic efflux of GSH and GSSG

	GSH efflux (nmoles/min/g)		GSSH efflux (nmoles/min/g)
	Sinusoidal	Biliary	Biliary
Control	4.76 ± 0.49	1.20 ± 0.11	0.86 ± 0.04
Ethanol	9.07 ± 0.47	0.89 ± 0.31	0.86 ± 0.19
	$P < 0.001$	NS	NS

Results shown are mean ± S.E. for five animals in each group. NS = not significant.

Table 3. Effect of chronic ethanol on hepatic and renal GGT activity

	GGT Activity (nmoles/min/mg protein)	
	Hepatic	Renal
Control	1.95 ± 0.81	304 ± 17.7
Ethanol	3.20 ± 1.28	286 ± 27.9
	NS	NS

Results shown are mean ± S.E. for five animals in each group. NS = not significant.

was similar to that found in the livers of animals immediately after sacrifice (data not shown). Renal GGT activity was similar in both groups.

DISCUSSION

The results of this study show that chronic ethanol consumption significantly increased hepatic efflux of glutathione. We have shown previously that the turnover of hepatic GSH is more than doubled after chronic ethanol consumption, with a fractional turnover rate of $0.121 \pm 0.444\,hr^{-1}$ in controls vs $0.298 \pm 0.051\,hr^{-1}$ in ethanol-fed rats [9]. The increase in sinusoidal efflux of GSH (Table 2) in ethanol-fed rats was similar in magnitude to the increase in turnover rate previously reported [9]. These observations are consistent and suggest that hepatic efflux of GSH is an important determinant of GSH turnover under a variety of experimental conditions.

The initial step in glutathione turnover is believed to be release from cells. Release of GSH from the liver into the plasma is part of the interorgan metabolism of glutathione [23]. GSH is taken up by the kidney and degraded by GGT, the rate-limiting enzyme in glutathione catabolism [2]. Our results show that the activity of renal GGT was similar in both ethanol-fed and control rats. Although it is possible that there is increased renal clearance of GSH, it is unlikely that a change in renal GGT activity provides the stimulus for the increase in turnover previously observed or the increase in hepatic efflux after chronic ethanol feeding. Chronic ethanol feeding causes an increase in serum and hepatic GGT activity in both rodents [24, 25] and in patients [26, 27] with alcohol-induced fatty liver. We also observed an increase in hepatic GGT activity. However, the observed increase was not sufficient to account for the larger changes seen in hepatic efflux or turnover of GSH. Thus, the stimulus for increased efflux and turnover remains uncertain.

Lauterburg and co-workers showed that, in fasted rats, there is a marked increase in hepatic efflux of glutathione as well as an increase in plasma clearance of glutathione [10]. They speculated that, during fasting, a decrease in availability of cysteine to extrahepatic tissue stimulates increased hepatic efflux of GSH, a potential reservoir for cysteine. During fasting, cysteine may be synthesized in the liver by transsulfuration of methionine and incorporated into glutathione (Fig. 1). Whether a similar effect may occur during chronic ethanol feeding remains unknown. During fasting, steady-state GSH levels are decreased, whereas they are increased or unchanged 24 hr after withdrawal from chronic ethanol feeding. In the present study, GSH levels were similar in both groups at the termination of the perfusion. Taking into account the increased rate of efflux of GSH in ethanol-fed rats, it is possible that preperfusion GSH levels were increased compared to controls. One could speculate that synthesis of GSH is also increased in these animals, an hypothesis which is consistent with our previous observations [9] and those of Vendemiale and coworkers [28]. Of interest is the fact that hepatic GGT activity is increased during fasting as well as after chronic ethanol consumption [10]. Both increases may occur in response to increases in GSH efflux although there is no direct evidence for this. Chronic ethanol feeding has been shown to increase dietary methionine requirements in rats [29]. Furthermore, dietary supplementation with methionine or choline can partially reverse alcohol-induced fatty liver [30–32]. Chronic ethanol feeding increases the activity of *S*-adenosylmethionine synthase, cystathionine synthase and betaine-homocysteine methyltransferase, whereas methyltetrahydrofolate-homocysteine methyltransferase activity decreases [33] (Fig. 1). These changes would favor increased conversion of methionine to cysteine. Further evidence in support of increased transsulfuration of methionine is provided by the observation that plasma and hepatic concentrations of α-amino-*N*-butyric acid (AANB) are increased by chronic ethanol feeding [34]. Since AANB is in equilibrium with α-ketobutyrate, which is formed during synthesis of cysteine via transsulfuration, the increase in AANB is evidence of increased transsulfuration of methionine. An increased requirement for GSH might be expected to increase transsulfuration of methionine to cysteine, particularly during cysteine deprivation.

Acetaldehyde, the major metabolite of ethanol, may react with glutathione to form a stable adduct. Large doses of ethanol acutely deplete hepatic glutathione stores and may decrease GSH synthesis [16, 17, 35–37]. Cysteine protects against acetaldehyde-related inhibition of mitochondrial respiration, possibly through adduct formation [38]. Since chronic ethanol consumption in man results in increased acetaldehyde levels [39], it is tempting to speculate that, during ethanol consumption, acetaldehyde increases the requirement for glutathione which results in an overall increase in its turnover. Conflicting results have been reported on the effects of chronic ethanol feeding on steady-state GSH concentrations [9, 16–18]. In studies in which decreased steady-state glutathione concentrations were observed, animals had been fed ethanol until they were killed. Thus, the effects of recent ethanol intake are superimposed on the long-term effects and may account for the decreased GSH concentrations observed. Our results suggest that steady-state GSH concentrations are similar, or possibly increased, after withdrawal from chronic ethanol consumption. Plasma GSH concentrations and plasma clearance of glutathione during chronic ethanol consumption

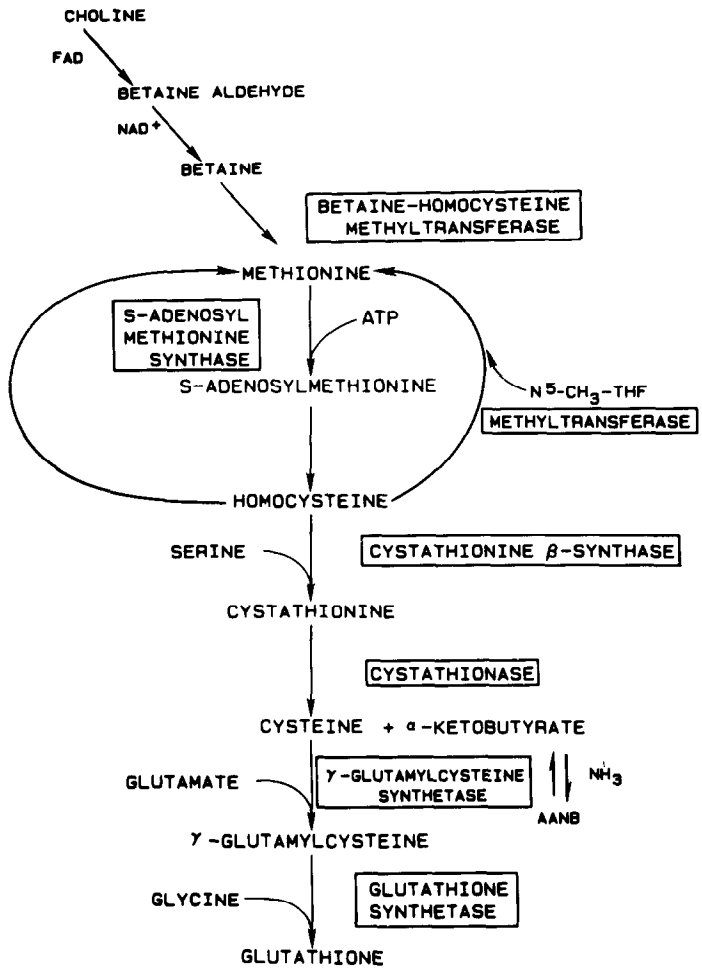

Fig. 1. Hepatic transsulfuration pathway. The enzymatic conversion of methionine to cysteine and glutathione is diagrammed. Enzymes involved in this pathway are indicated in boxes.

and after withdrawal have not been reported, but would be of interest.

In summary, we have shown that chronic ethanol consumption significantly increased hepatic efflux of glutathione. This increased efflux of glutathione is consistent with our previous observation that hepatic GSH turnover is increased in ethanol-fed rats. The actual stimulus for increased turnover and efflux of hepatic GSH during ethanol feeding remains unknown. The observed increases in turnover and hepatic efflux of GSH could have important toxicological consequences. Further work is needed to define the effects of having long-term ethanol consumption on cellular mechanisms of detoxification and protection against chemical insults to the liver.

Acknowledgements—The authors would like to thank Ms. Laura S. Wacker for her expert technical assistance and Ms. Judith Hariton for assistance in preparing this manuscript.

REFERENCES

1. N. D. Kosower and E. M. Kosower, *Int. Rev. Cytol.* **54**, 109 (1978).
2. A. Meister and M. E. Anderson, *Science* **220**, 472 (1983).
3. J. R. Mitchell, D. J. Jollow, W. Z. Potter, J. R. Gillette and B. B. Brodie, *J. Pharmac. exp. Ther.* **187**, 211 (1973).
4. J. R. Mitchell, S. S. Thorgeirsson, W. Z. Potter, D. J. Jollow and H. Keiser, *Clin. Pharmac. Ther.* **16**, 676 (1974).
5. B. H. Lauterburg and J. R. Mitchell, *Hepatology* **2**, 8 (1982).
6. T. Suga, I. Ohata and M. J. Akagi, *J. Biochem., Tokyo* **59**, 209 (1966).
7. N. D. Zampaglione, D. J. Jollow, J. R. Mitchell, B. Stripp, M. Hamrick and J. R. Gillette, *J. Pharmac. exp. Ther.* **187**, 218 (1973).
8. B. H. Lauterburg, Y. Vaishnav, W. H. Stillwell and J. R. Mitchell, *J. Pharmac. exp. Ther.* **213**, 54 (1980).
9. S. B. Morton and M. C. Mitchell, *Biochem. Pharmac.* **34**, 1559 (1985).
10. B. H. Lauterburg, J. D. Adams and J. R. Mitchell, *Hepatology* **4**, 586 (1984).
11. O. Strubelt, *Biochem. Pharmac.* **29**, 1445 (1980).
12. C. Sato, U. Matsuda and C. S. Lieber, *Gastroenterology* **80**, 140 (1981).
13. A. C. Smith, R. W. Freeman and R. D. Harbison, *Biochem. Pharmac.* **30**, 453 (1981).

14. D. J. Emby and B. N. Fraser, *S. Afr. med. J.* **51**, 208 (1977).
15. C. J. McClain, J. P. Kromhout, F. J. Peterson and J. L. Holtzman, *J. Am. med. Ass.* **244**, 251 (1980).
16. C. Guerri and S. Grisolia, *Pharmac. Biochem. Behav.* **13**, 53 (1980).
17. V. Fernandez and L. A. Videla, *Experientia* **37**, 392 (1981).
18. C. Hetu, L. Yelle and J. G. Joly, *Drug Metab. Dispos.* **10**, 245 (1982).
19. P. J. Hissin and R. Hilf, *Analyt. Biochem.* **74**, 214 (1976).
20. F. Tietze, *Analyt. Biochem.* **27**, 502 (1969).
21. T. P. M. Akerboom and H. Sies, *Meth. Enzym.* **17**, 373 (1981).
22. G. Szacz, *Clin. Chem.* **15**, 124 (1969).
23. N. Kaplowitz, T. Y. Aw and M. Ookhtens, *A. Rev. Pharmac. Toxic.* **25**, 715 (1985).
24. R. Teschke and A. S. Petrides, *Biochem. Pharmac.* **31**, 375 (1982).
25. G. Gadeholt, J. Aarbake, E. Dybing, M. Sjoblom and J. Morland, *J. Pharmac. exp. Ther.* **213**, 196 (1980).
26. R. Teschke, M. Neuefeind, M. Nishimura and G. Strohmeyer, *Gut.* **24**, 625 (1983).
27. E. Ivanov, D. Adjarov, M. Etarska, T. Stankushev, K. Burmbarov and M. Keimova, *Enzyme* **25**, 304 (1980).
28. G. Vendemiale, E. Jaytilleke, S. Shaw and C. S. Lieber, *Life Sci.* **34**, 1065 (1984).
29. G. Klatskin, W. A. Krehl and H. O. Conn, *J. exp. Med.* **100**, 605 (1954).
30. C. H. Best, W. S. Hartroft, C. C. Lucas and J. H. Ridout, *Br. med. J.* **2**, 1001 (1949).
31. C. S. Lieber and L. M. Decarli, *Gastroenterology* **50**, 316 (1966).
32. W. O. Dobbins, III, E. L. Rollins, S. G. Brooks and H. J. Fallon, *Gastroenterology* **62**, 1020 (1972).
33. J. D. Finkelstein, J. P. Cello and W. E. Kyle, *Biochem. biophys. Res. Commun.* **61**, 525 (1974).
34. S. Shaw and C. S. Lieber, *Gastroenterology* **78**, 108 (1980).
35. C. M. MacDonald, J. Dow and M. R. Moore, *Biochem. Pharmac.* **26**, 1529 (1977).
36. B. H. Lauterburg, S. Davies and J. R. Mitchell, *J. Pharmac. exp. Ther.* **230**, 7 (1984).
37. H. Speisky, A. MacDonald, G. Giles, H. Orrego and Y. Israel, *Biochem. J.* **25**, 715 (1985).
38. C. Hirayama, Y. Kishimoto, T. Wakushima and Y. Murawaki, *Biochem. Pharmac.* **32**, 321 (1983).
39. M. A. Korsten, S. Matsvzaki, L. Feinman and C. S. Lieber, *New Engl J. Med.* **292**, 386 (1975).