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# Increased loss and decreased synthesis of hepatic glutathione after acute ethanol administration

## Turnover studies

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The effect of acute ethanol administration on rates of synthesis and utilization of hepatic glutathione (GSH) was studied in rats after a pulse of [$^{35}$S]cysteine. A 35% decrease in hepatic GSH content 5 h after administration of 4 g of ethanol/kg body wt. was accompanied by a 33% increase in the rate of GSH utilization. The decrease occurred without increases in hepatic oxidized glutathione (GSSG) or in the GSH/GSSG ratio. The rate of non-enzymic condensation of GSH with acetaldehyde could account for only 6% of the rate of hepatic GSH disappearance. The increased loss of [$^{35}$S]GSH induced by ethanol was not accompanied by an increased turnover; rather, a 30% inhibition of GSH synthesis balanced the increased rate of loss, leaving the turnover rate unchanged. The rate of acetaldehyde condensation with cysteine *in vitro* occurred at about one-third of the rate of GSH loss in ethanol-treated animals. However, ethanol induced only a minor decrease in liver cysteine content, which did not precede, but followed, the decrease in GSH. The characteristics of 2-methylthiazolidine-4-carboxylic acid, the condensation product between acetaldehyde and cysteine, were studied and methodologies were developed to determine its presence in tissues. It was not found in the liver of ethanol-treated animals. Ethanol administration led to a marked increase (47%) in plasma GSH in the post-hepatic inferior vena cava, but not in its pre-hepatic segment. Data suggest that an increased loss of GSH from the liver constitutes an important mechanism for the decrease in GSH induced by ethanol. In addition, an inhibition of GSH synthesis is observed.

GSH (γ-glutamylcysteinylglycine) plays a key role in the liver in several detoxification reactions and in the reduction of lipid peroxides, and appears to also play an important role in a variety of cell functions, including amino acid transport and the storage of thiol moieties (Kaplowitz, 1981; Meister, 1982).

Acute administration of ethanol, a known hepatotoxin, results in a marked decrease in GSH content in the liver (Estler & Ammon, 1966; MacDonald *et al.*, 1977; Guerri & Grisolia, 1980; Fernandez & Videla, 1981). The mechanism by which this effect is exerted is, however, not clear. Three general mechanisms have been suggested, namely (i) binding of GSH to acetaldehyde produced in the metabolism of ethanol (Viña *et al.*, 1980), (ii) oxidation of GSH by lipid peroxides produced by ethanol (Videla *et al.*, 1981; Videla & Valenzuela, 1982), and (iii) binding of acetaldehyde to cysteine, a precursor of GSH (Lieber, 1980), to form the 2-MTCA derivative (Cederbaum & Rubin, 1976; Nagasawa *et al.*, 1980).

GSH synthesis is thought to be regulated by end-product feedback inhibition (Richman & Meister, 1975). Since both the binding of GSH to acetaldehyde and its oxidation result in removal of GSH, either mechanism would be expected to increase the rate of turnover. Studies have shown that in the liver most of the GSH turnover is actually accounted for by its efflux from this organ (Adams *et al.*, 1983). An increased efflux of GSH

Abbreviations used: GSH, reduced glutathione; GSSG, oxidized glutathione; 2-MTCA, 2-methylthiazolidine-4-carboxylic acid.

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from the liver after ethanol administration would also result in an increased turnover. An increased disappearance of GSH from the liver by oxidative or non-oxidative mechanisms can be further differentiated by examining the hepatic GSH/GSSG ratio, whereas an increased efflux of GSH from the liver would result in an increase in GSH in post hepatic blood. On the other hand, if the mechanism of hepatic GSH depletion induced by ethanol involves a decreased synthesis rather than an increased utilization or loss, an increase in GSH turnover is not expected. In the present studies we have examined the effect of ethanol administration on hepatic GSH turnover after pulse-labelling of GSH with its radioactive precursor [$^{35}$S]cysteine. In addition, we have conducted studies *in vivo* and *in vitro* to assess the possible mechanisms implicated above.

## Materials and methods

### *Studies in vivo*

Female Sprague–Dawley rats (Charles River, St. Constant, Quebec, Canada) weighing 150–200g were starved for 18–20h before the experiments. Ethanol was administered by gastric intubation as a 20% (w/v) solution in water at a dose of 4g/kg body wt. Controls received an equivalent volume of water. Animals were decapitated, and their livers were quickly removed and frozen in liquid $N_2$ and kept at −70°C, until analysis, which was done within 1 week.

### *Turnover experiments*

Animals were injected intraperitoneally with 200$\mu$Ci of L-[$^{35}$S]cysteine (1 Ci/$\mu$mol; New England Nuclear, Montreal, Canada)/kg in a 0.9% NaCl solution containing 10mM-dithiothreitol. At 1h after the pulse administration of [$^{35}$S]cysteine (zero time), the animals were given either ethanol or water by gastric intubation and were killed after 1, 2, 3, 4 or 5h. This pulse method allows the estimation of the rate of synthesis of GSH by determining the rate of changes in the specific radioactivity of GSH, which reflects the dilution of the labelled compound by the newly synthesized unlabelled molecules.

### *Glutathione-efflux experiments*

Ethanol-treated and control animals were treated as above. At 3h the animals were anesthetized with 65mg of pentobarbital/kg. For the ethanol-treated animals the dose of pentobarbital was titrated to obtain an equal degree of surgical anaesthesia. At 3.5h a laparotomoy was performed and blood was sampled from the inferior vena cava, both below and above the point of confluence of hepatic-vein blood. The point below the hepatic vein was superior to the renal veins. The inferior vena cava above the liver was approached by inserting a curved needle (22-gauge) into the vein below the diaphragm. Blood was removed at a slow rate to avoid retrograde flux. Blood samples were immediately centrifuged in an Eppendorf model 5412 centrifuge. Plasma was separated and immediately deproteinized with 0.5M-$HClO_4$. The complete procedure from sample collection to the acidification of the plasma took 4–5min.

### *Analytical methods*

For the turnover experiments total radioactivity in [$^{35}$S]GSH and its specific radioactivity were determined after separation of GSH by h.p.l.c. in a $C_{18}$, 10$\mu$ Bondapak-RP column as described by Lauterburg *et al.* (1980). The eluting solvent was 0.05% (w/v) $H_3PO_4$, at a flow rate of 0.7ml/min. The $A_{220}$ of the effluent was monitored, GSH in the column effluent was determined spectrophotometrically at 412nm (Ellman, 1959), and its radioactivity was quantified by liquid-scintillation counting. GSH in total tissue was determined in neutralized 0.5M-$HClO_4$ supernatants, also as described by Ellman (1959). Hepatic GSSG was determined in $HClO_4$ supernatants by the NADPH–glutathione reductase method at 340nm (Sies & Summer, 1975). The term 'glutathione' is used for GSH, and 'total glutathione' refers to the sum of GSH and GSSG.

In the GSH-efflux experiments in which plasma GSH was determined, the method of Tietze (1969) was employed, which has a greater sensitivity than that of Ellman (1959) and because, as it allows the measurement of total glutathione, it avoids the possibility of GSH underestimation owing to its rapid auto-oxidation in the plasma. Hepatic cysteine was determined in 0.5M-$HClO_4$ supernatants by a scaled-down version of the method of Gaitonde (1967). We observed that, when livers were not frozen in liquid $N_2$, cysteine values were 20–30% lower than those in rapidly frozen livers. Freeze-clamping or rapid removal of the liver plus plunging into liquid $N_2$ gave identical results. When hepatic cysteine was assayed in the presence of added 2-MTCA, it was found that the condensate quantitatively decomposed to regenerate cysteine. Thus the method of Gaitonde (1967) yields the sum of free cysteine plus cysteine in the form of adduct, when both are present. Characterization of the stability of synthetic 2-MTCA showed that it is relatively stable in the pH range 10–11. We took advantage of the fact that, at an alkaline pH, free cysteine can be quickly oxidized to cystine, which does not interfere with the method of cysteine determination. To determine 2-MTCA in liver supernatants, $HClO_4$

extracts were rapidly titrated to pH 10.5 ± 0.3 with 4M-$K_2CO_3$ at 2°C. At this pH, $H_2O_2$ was added to a final concentration of 1mM to oxidize free cysteine, and after 5min the solution was re-acidified. After this procedure, cysteine measured under the conditions of Gaitonde's (1967) method is derived from 2-MTCA. Recovery of 2-MTCA, added at the time of homogenization to liquid-$N_2$-frozen liver powder, was 70%. 2-MTCA and free cysteine concentrations were calculated, with appropriate corrections for recovery, by assaying cysteine in $H_2O_2$-treated and untreated supernatants.

*Studies in vitro*

The rate of condensation of acetyldehyde with several thiol-containing molecules was determined by the change in $A_{340}$ for NADH induced by addition of GSH, cysteine or other analogues, to an alcohol dehydrogenase system in equilibrium, containing ethanol, $NAD^+$, acetaldehyde and NADH. After equilibrium (with an excess of enzyme), the initial rate at which NADH increases on addition of an acetaldehyde-trapping agent corresponds to the initial rate of acetaldehyde removal, and thus to the rate of adduct formation. Controls are performed to determine that the acetaldehyde-binding agent does not produce a drift in $A_{340}$. This was done (a) in a cuvette with a fixed [NADH]/[$NAD^+$] ratio in the absence of ethanol or alcohol dehydrogenase, and (b) in a cuvette in the presence of enzyme, NADH and ethanol but in the absence of $NAD^+$. It was also determined that the trapping agent was not a strong inhibitor of yeast alcohol dehydrogenase (EC 1.1.1.1). To ensure that the enzyme is not rate-limiting in the reaction, the rate of change in $A_{340}$ induced by addition of the compound after establishing the plateau for the first equilibrium should be a fraction (lower than 5%) of the rate at which the first equilibrium occurred. This method of assessing and quantifying the rate of acetaldehyde binding to different compounds has the advantage that steady-state concentrations of acetaldehyde, in physiologically relevant concentrations, can be easily attained by varying the concentrations of ethanol and $NAD^+$ in the cuvette. In the liver, concentrations of acetaldehyde of the order of 70μM are expected (Lindros, 1978). At equilibrium, at pH 7.35 (50mM-sodium phosphate buffer) and 37°C, a concentration of 70μM-acetaldehyde was attained by initial of 41.2mM-ethanol, 0.412mM-$NAD^+$ and 50 units of alcohol dehydrogenase/ml (1unit represents 1μmol of substrate transformed/min). The equilibrium constant ($K_1$) of $1.55 \times 10^{-11}$M obtained under these conditions is in line with a previous report of $1.3 \times 10^{-11}$M at 35°C (Sund & Theorell, 1963).

The equilibrium constant ($K_2$) for the adduct formation reaction between the different thiol compounds (X) and acetaldehyde was calculated from the alcohol dehydrogenase reaction at equilibrium, in the presence of known initial concentrations ($X_i$) of the thiol compound (0.2mM), $NAD^+$ (0.412mM) and ethanol (41.2mM), as follows.

At equilibrium at 37°C and pH 7.35:

$$CH_3\text{–}CH_2OH + NAD^+ \rightleftharpoons CH_3\text{–}CHO + NADH + H^+ \quad (1)$$

$$CH_3\text{–}CHO + X \rightleftharpoons (CH_3\text{–}CHO\cdot X) \quad (2)$$

$$K_1 = \frac{[CH_3\text{–}CHO][NADH][H^+]}{[CH_3\text{–}CH_2OH][NAD^+]} = 1.55 \times 10^{-11}\text{M}$$

$$K_2 = \frac{[CH_3\text{–}CHO\cdot X]}{[CH_3\text{–}CHO][X]}$$

$K_2$ can be calculated after solving for the equations below. The NADH concentration can be calculated from the $A_{340}$. Further:

$$[NADH] = [CH_3\text{–}CHO] + [CH_3\text{–}CHO\cdot X] \quad (a)$$

At equilibrium:

$$[CH_3\text{–}CHO] = \frac{K_1 \cdot [CH_3\text{–}CH_2OH][NAD^+]}{[NADH][H^+]} \quad (b)$$

The concentration of [$CH_3$–CHO·X] can be calculated by substituting [NADH] and [$CH_3$–CHO] in (*a*).

The concentrations of [X] and [NAD] at equilibrium are:

$$[X] = [X_i] - [CH_3\text{–}CHO\cdot X]$$

$$[NAD] = [NAD_i] - [NADH].$$

The above method allows the calculation of the adduct equilibrium constants for many acetaldehyde-binding agents, without requiring preparation and purification of the adduct formed.

The equilibrium constant derived by the alcohol dehydrogenase method above for 2-MTCA was compared with that derived from synthetic 2-MTCA prepared as described by Debey *et al.* (1958). A known concentration of 2-MTCA was incubated at 37°C in a sealed g.l.c. vial. Acetaldehyde released was measured by head-space g.l.c. (Giles & Meggiorini, 1983) at different times until equilibrium was attained.

Data are presented as means ± S.E.M. Significance was tested by Student's *t* test or by analysis of variance as required.

## Results

After a pulse administration of [$^{35}$S]cysteine to label the hepatic GSH pool, the decay of [$^{35}$S]GSH followed a first-order process ($r = 0.99$) (Fig. 1). Ethanol at a dose of 4g/kg significantly increased by 33% the first-order constant rate of [$^{35}$S]GSH disappearance from the liver. The estimated half life of [$^{35}$S]GSH was 3.10h for controls and 2.36h for ethanol-treated animals. The inset of Fig. 1 shows that ethanol administration caused a progressive decrease in hepatic GSH; at 5h the GSH content was 35% lower in the ethanol-treated animals than in controls.

Fig. 2 indicates that the decay in specific radioactivity of GSH was not altered by ethanol. This can occur if an inhibition in the synthesis of non-labelled GSH molecules of about the same order as that of the increased GSH utilization also occurs. An approximate estimate of the inhibition of GSH synthesis from 0 to 5h after ethanol treatment, of 30%, can be calculated from the product of the first-order rate constant and the concentration of GSH (assuming quasi-steady-state concentrations at hourly intervals from 0 to 5h).

Table 1 shows that the administration of ethanol did not result in increases in hepatic GSSG; rather a decrease was observed, which became significant 4h after ethanol administration. Moreover, the ratio GSH/GSSG was found not to be affected by

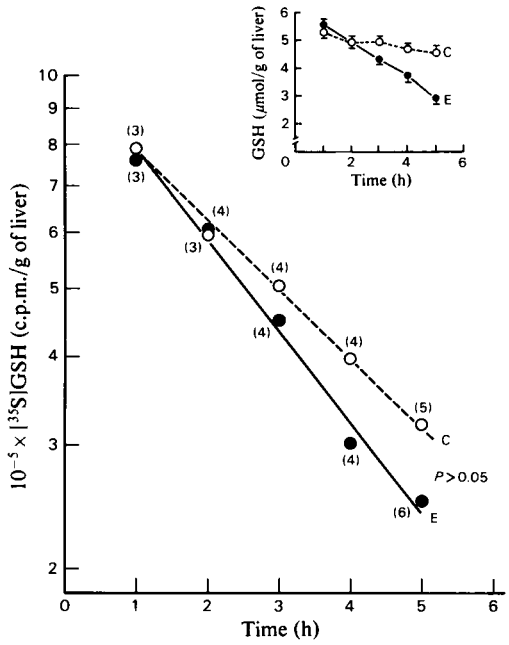


Fig. 1. *Effect of acute ethanol administration (4g/kg) on the rate of disappearance of [$^{35}$S]glutathione from the liver*
Equations for the lines were calculated by the least-squares method for log[$^{35}$S]glutathione versus time. The *P* value was calculated by analysis of variance for the individual values, with each animal providing one point at a single time. Numbers of animals at each point are shown in parentheses. The intercepts at zero time were not statistically different for the two groups. C and E represent control and ethanol-treated animals respectively.

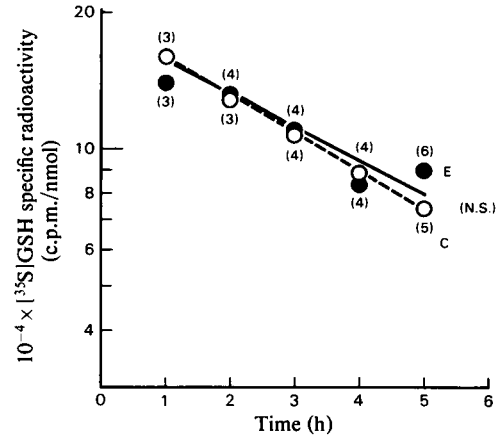


Fig. 2. *Changes in specific radioactivity of [$^{35}$S]glutathione versus time in controls and ethanol (4g/kg)-treated animals*
Numbers of animals at each time are shown in parentheses: N.S., not statistically different.

Table 1. *Effect of ethanol (4g/kg) on liver GSSG and GSH contents*
Hepatic GSH and GSSG (expressed as μmol/g of liver) were determined at 3, 4 or 5h after the oral administration of ethanol (E) or water (C). Each value represents the mean ± S.E.M. of seven to eight animals per group. *$P<0.05$; **$P<0.005$; ***$P<0.001$.

| | 3h | | 4h | | 5h | |
|---|---|---|---|---|---|---|
| | C | E | C | E | C | E |
| GSH | 4.94±0.34 | 5.25±0.22 | 4.68±0.14 | 3.76±0.14** | 5.05±0.18 | 3.28±0.21*** |
| GSSG | 0.410±0.038 | 0.377±0.025 | 0.420±0.090 | 0.257±0.020* | 0.338±0.038 | 0.238±0.025* |
| GSH/GSSG | 12.7±1.9 | 14.2±1.1 | 12.4±2.0 | 14.9±0.8 | 16.4±2.5 | 15.0±2.1 |

ethanol administration in conditions in which hepatic GSH contents were clearly depressed.

Experiments were conducted *in vitro* to determine whether the decrease in GSH synthesis, indicated by the turnover experiments, could be mediated by a decreased availability of cysteine resulting from adduct formation with acetaldehyde. Experiments were also conducted to determine whether the combination of acetaldehyde with GSH might be in part responsible for the observed increased utilization of GSH.

Table 2 shows that at, concentrations occurring *in vivo*, acetaldehyde (70 $\mu$M) condenses with cysteine (0.2 mM) at a rate of 2.64 nmol/min per ml. Such a rate of reaction is 5 times that of GSH condensation with acetaldehyde. When substitutions in the thiol group (*S*-methyl) or in the amino group (*N*-acetyl) of cysteine are introduced, the molecule is rendered inactive with regard to adduct formation. The latter might explain the low reactivity of GSH, in which the amino group of cysteine is substituted, towards acetaldehyde. Homocysteine, a precursor in the synthesis of cysteine, also reacted with acetaldehyde at a rate of about 60% of that of cysteine. The rates of reaction of these two thio-amino acids, cysteine and homocysteine, were comparable with the rate of reaction of acetaldehyde with penicillamine, a cysteine analogue known to react *in vivo* with the acetaldehyde formed in the metabolism of ethanol to yield the adduct, 2,5,5-trimethylthiazolidine-4-carboxylic acid (Nagasawa *et al.*, 1975).

Although the rate of the cysteine–acetaldehyde adduct formation at physiological concentrations is about one-third of the rate of depletion of liver GSH by ethanol, an accurate estimate of the relevance *in vivo* of the reaction of cysteine and homocysteine with acetaldehyde as a mechanism altering hepatic cysteine availability and thereby GSH contents is not possible. Actual hepatic cysteine contents were therefore determined. As indicated above, a new methodology based on the oxidation of the thiol group of cysteine was developed to determine cysteine in the presence of various concentrations of 2-MTCA. This method also allowed us to determine if 2-MTCA existed in the liver of animals treated with ethanol. The hepatic contents of cysteine were not affected 3–4 h after ethanol administration, times at which GSH contents were significantly decreased. Only at 5 h was a moderate but significant decrease (20%) in cysteine detected (Fig. 3). We found no evidence (detection limit 0.01 $\mu$mol/g) for the presence of 2-MTCA in the liver of ethanol-treated animals 1–5 h after the administration of ethanol.

If the cysteine–acetaldehyde adduct is formed in the liver, the possibility exists that it might quickly decompose back into its original constituents. Accordingly, the stability of the acetaldehyde adduct with cysteine was studied by determining the equilibrium reaction $K_E = [\text{Adduct}]/[\text{Acetaldehyde}] \times [\text{Cysteine}]$. This was also done for other analogues of cysteine, including penicillamine, for comparison purposes, since an acetaldehyde condensate with the latter analogue has been found to occur in the urine of animals given ethanol and penicillamine (Nagasawa *et al.*, 1975). Table 3 shows that the most stable acetaldehyde adduct was that formed with penicillamine, followed by those of homocysteine, cysteine

Table 2. *Rate of reaction of acetylaldehyde with glutathione, cysteine and cysteine analogues in vitro*

Thiol compounds at the concentrations indicated were incubated with 70 $\mu$M-acetaldehyde. Rates correspond to the initial velocities of the reaction. Data shown are means of two to three experiments which did not vary more than 5% from each other.

| | Concn. (mM) | Rate of reaction (nmol/min per ml) |
|---|---|---|
| Glutathione | 4.0 | 0.475 |
| Glutathione | 8.0 | 0.695 |
| Cysteine | 0.2 | 2.64 |
| Homocysteine | 0.2 | 1.66 |
| Penicillamine | 0.2 | 2.20 |
| *N*-Acetylcysteine | 0.2 | 0 |
| *S*-Methylcysteine | 0.2 | 0 |

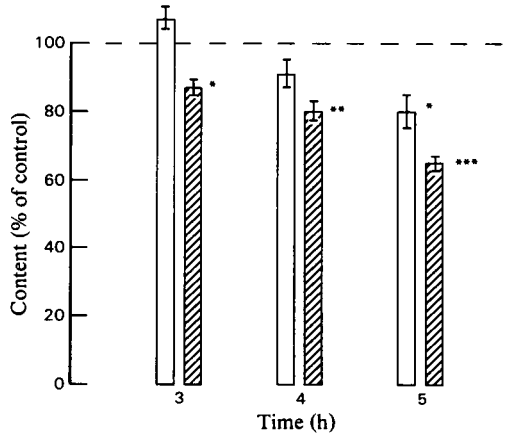


Fig. 3. *Effect of acute ethanol (4 g/kg) administration on liver cysteine* (□) *and GSH* (▨) *contents*

Values are presented as percentages of control values, ±S.E.M. Mean control cysteine values ($\mu$mol/g liver): 3 h, 0.278 ± 0.016; 4 h, 0.241 ± 0.021; 5 h, 0.257 ± 0.019). Each column represents the mean of four to seven animals per group. $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.001$.

and cysteine ethyl ester. The relative instability of the cysteine condensate was confirmed by incubating synthetic 2-MTCA for different times and determining the release of acetaldehyde. At 37°C, 2-MTCA (initial concn. 0.067mM) decomposed within 2.5h to yield a stable concentration of acetaldehyde of 50–55% of the maximal molar value. The equilibrium constant $*K_E$ ($26.60 \times 10^3 M^{-1}$), calculated by this direct method, in which the preparation, purification and characterization of the adduct is required, compares well with that ($32.97 \times 10^3 M^{-1}$) derived from the single spectrophotometric method in which such steps are obviated (see the Materials and methods section).

Fig. 4 shows that acute administration of ethanol resulted in a 47% increase in total plasma glutathione in the inferior vena cava above the liver. Plasma glutathione concentration in the inferior vena cava below the liver was only about one-third of that in the vessel above the point of confluence with hepatic blood. At this level of the inferior vena cava, no significant difference in total plasma glutathione concentrations was observed between the ethanol-treated and control animals.

Table 3. *Equilibrium constant ($K_E$) of the acetaldehyde condensate with different cysteine analogues*

$K_E$ = [Adduct] / [Acetaldehyde][Cysteine or analogue]. $*K_E$ determined by the alcohol dehydrogenase equilibrium method; $**K_E$ determined by acetaldehyde release by head-space g.l.c. Data shown are average of two experiments.

| | $10^3 \times K_E$ ($M^{-1}$) |
|---|---|
| Penicillamine* | 50.85 |
| Homocysteine* | 46.80 |
| Cysteine* | 32.97 |
| Cysteine ethyl ester* | 17.78 |
| Cysteine** | 26.60 |

## Discussion

There is abundant evidence in the literature that acute ethanol administration leads to a decrease in hepatic GSH (Estler & Ammon, 1966; MacDonald *et al.*, 1977; Guerri & Grisolia, 1980; Fernandez & Videla, 1981). Such a fall has been ascribed to decreased synthesis (Lauterburg *et al.*, 1983; Speisky *et al.*, 1983) or to an increased utilization of GSH, which has been postulated to result either from an increased lipoperoxidation (Videla & Valenzuela, 1982) or from the removal of liver GSH by binding to acetaldehyde (Viña *et al.*, 1980). Studies in which an increased utilization has been proposed are based on determinations of GSH concentrations, in which it is not possible to differentiate between changes caused by decreased synthesis and those resulting from an increased utilization.

Data in the present paper indicate that there is a significantly increased utilization or loss of GSH from the liver. Such a conclusion is derived from data showing a greater disappearance of [$^{35}$S]GSH from the liver in conditions in which the decay in

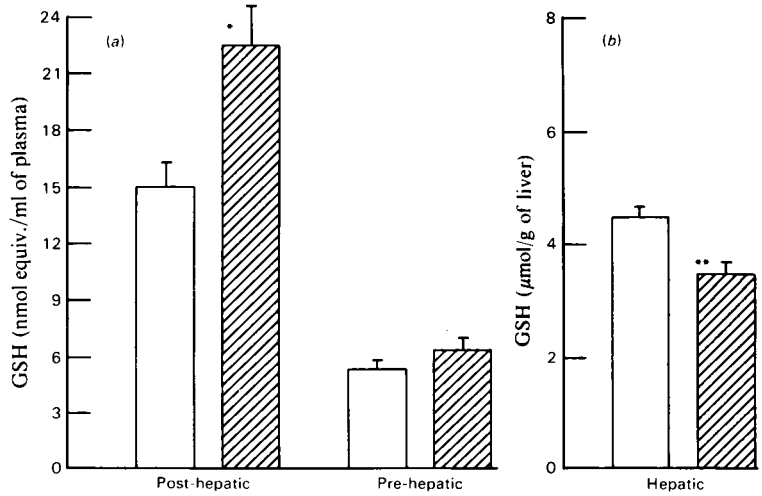


Fig. 4. *Effects of acute ethanol (4g/kg) administration on plasma and hepatic GSH contents*

(*a*) Total plasma glutathione from blood obtained from the inferior vena cava above (post-hepatic) and below (pre-hepatic) the liver; (*b*) hepatic content of glutathione. Each column represents the mean ±S.E.M. for ten animals per group: $*P<0.05$; $**P<0.005$. □, Control; ▨, ethanol-treated.

specific radioactivity of GSH was not altered by ethanol. Similar effects of ethanol are observed if [$^{35}$S]GSH contents are expressed per total liver (results not shown). Studies showing that ethanol-treated animals have increased plasma concentration of total glutathione in inferior-vena-cava blood that has received the hepatic outflow, but that such an increase is not observed in inferior-vena-cava blood before the point of confluence with hepatic blood, suggest that GSH is lost from the hepatocytes into the sinusoidal space. Such an increase in post-hepatic plasma GSH was observed in conditions in which a concomitant decrease in hepatic GSH was taking place.

Studies by Adams *et al.* (1983) have indicated that most of the turnover of GSH in the liver is due to efflux of hepatic GSH into the circulation. Our data in control animals are in agreement with such a postulate. Further, ethanol appears to accentuate the efflux of hepatic GSH, although studies should be conducted to determine a possible contribution of the non-hepatic splanchnic tissues to the increase in plasma GSH. From a liver blood-flow rate of 50ml/kg per min (Israel *et al.*, 1983), a 2.5-fold dilution of hepatic blood into the inferior vena cava (Bowman & Rand, 1980) and the concentrations of GSH found in the different segments of the inferior vena cava (from Fig. 4), we have calculated the increment in efflux of GSH into the inferior vena cava above the liver induced by ethanol to be 13.6nmol/g per min. This value should be compared with an average decrease in GSH from the liver of 10.8nmol/g per min, thus suggesting that an increased efflux of GSH, if maintained throughout the study, could quantitatively (126%) explain the observed decrease in hepatic GSH content induced by ethanol.

We have previously reported (Israel *et al.*, 1983) that acute ethanol administration leads to an increased liver blood flow. If GSH efflux from the liver is blood-flow dependent, an increase in GSH in the inferior vena cava could have resulted from an increased 'wash-out' of GSH from the liver. Further studies should be conducted to address this possibility. Our studies might also explain the observations by Fernandez *et al.* (1983), who found an ethanol-induced increase in plasma GSH from blood obtained by cardiac puncture in animals lightly anaesthetized with diethyl ether. We have confirmed an increase in arterial GSH after ethanol administration in animals anaesthetized with ether (results not shown). Fernandez *et al.* (1983), however, have suggested that the increases in plasma GSH were of erythrocyte origin. Our observation that the increase in plasma GSH after ethanol administration occurs in post-hepatic, but not in pre-hepatic, venous blood do not support the latter possibility.

The lack of an increase in hepatic GSSG or of changes in the GSH/GSSG ratio suggest that the decrease in hepatic GSH induced by ethanol can occur by mechanisms that do not involve an increased oxidative utilization of GSH. As we indicated previously (Bunout *et al.*, 1983), an increased lipoperoxidation is not found in our experimental conditions. Guerri & Grisolia (1980), who have also reported that acute ethanol administration resulted in decreased hepatic GSH, did not observe changes in the GSH/GSSG ratio. Similarly, Lauterburg *et al.* (1983) did not observe an increased biliary excretion of GSSG after acute ethanol administration in conditions which led to decreased hepatic GSH.

Although we have confirmed the occurrence of a non-enzymic condensation between acetaldehyde and GSH (Viña *et al.*, 1980), this mechanism does not appear to play a major role in GSH depletion. Our results indicate that the rate of this reaction would account for only 6% of the rate of disappearance of hepatic GSH after ethanol administration *in vivo* (0.5nmol/ml per min and 8nmol/g per min respectively). Although we cannot completely rule out the possibility of such a reaction occurring at a greater rate in the cell, the addition of purified GSH transferase had no effect in such a reaction (results not shown). Although Roark *et al.* (1982) have reported an apparently enzymic activity catalysing the reaction of acetaldehyde with GSH in liver supernatants, the possible contribution of such a reaction *in vivo* is not clear, since the reaction velocity was not reported and the $K_m$ for acetaldehyde was 1500$\mu$M.

The studies presented here suggest that the synthesis of GSH is also inhibited by ethanol. This is in line with a preliminary report by Lauterberg *et al.* (1983), who determined hepatic GSH turnover from the biliary excretion of the acetaminophen–GSH adduct. Our data, showing a mono-exponential decay of [$^{35}$S]GSH, suggest the existence of a single pool in the liver. This is in agreement with data by other investigators (Lauterburg *et al.*, 1980), who measured GSH turnover using different radioactive precursors ([$^{35}$S]cysteine, [$^{14}$C]glycine and [$^{14}$C]glutamate). In those studies the half-life was very close for the three precursors, which are known to have different metabolic fates, thus suggesting that re-incorporation of precursor [$^{35}$S]cysteine, used in our studies, after the initial pulse is minimal. Condensation of cysteine with acetaldehyde (2.64nmol/ml per min) could conceivably play a role in removing cysteine, thereby affecting GSH synthesis, since the intrahepatic concentrations of cysteine are similar to the $K_m$ value for $\gamma$-glutamylcysteine synthetase. However, we did not observe a decrease in cysteine contents at times (3

or 4h) at which GSH contents were already decreased. The inability of ethanol to decrease cysteine contents at these times may reflect a very large turnover of cysteine compared with that of GSH, such that the above condensation rate may be proportionally small as a mechanism to remove cysteine. Furthermore, we observed that the cysteine–acetaldehyde condensate is very unstable at physiological pH values, yielding back its original constituents. The latter might in part explain the inability of Nagasawa *et al.* (1975) to detect the cysteine–acetaldehyde adduct in the urine of animals given ethanol, while detecting the more stable penicillamine–acetaldehyde condensate. The small, although significant, decrease in hepatic cysteine found 5h after ethanol administration might be a consequence of the decrese in GSH content, since GSH has been suggested to act as a storage form for hepatic cysteine (Higashi *et al.*, 1977). An analysis of the possible significance of this decrease in cysteine, on the basis of the known $K_m$ of γ-glutamylcysteine synthetase for cysteine (0.35mM; Richman & Meister, 1975) and on the rate of GSH synthesis under steady-state conditions ($k \times$ concn. = 18.75nmol/min per g), indicates that a 20% decrease in cysteine could account for only 17% of the actual fall in liver GSH during the 4th and 5th hours. We have also determined that acute ethanol administration *in vivo*, or addition of ethanol (500mM) or acetaldehyde (500μM) *in vitro*, does not result in an inhibition of the activity of γ-glutamylcysteine synthetase, when measured at saturating substrate concentrations (results not shown).

In conclusion, the studies presented suggest that acute ethanol administration induces both a loss of GSH from the liver and an inhibition of GSH synthesis.

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