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Changes in Glutathione in Acute and Chronic Alcohol Intoxication

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GUERRI, C. AND S. GRISOLÍA. *Changes in glutathione in acute and chronic alcohol intoxication.* PHARMAC. BIOCHEM. BEHAV. 13: Suppl. 1, 53–61, 1980.—After 5 hours of acute ethanol intoxication, the levels of reduced glutathione of liver, heart, kidney and brain decreased to 60, 71, 70 and 83% of controls, respectively. The decrease was less when animals were pretreated with pyrazole. Pretreatment with disulfiram potentiated the effect of ethanol. Levels of GSSG were not altered by these treatments. Chronic ethanol administration, in a liquid diet, also produced a decrease in GSH levels. The lowest levels were observed at 4 weeks in liver (65%). However, there was a slow recovery to 75% and 80%, respectively, at 10 and 14 weeks of treatment. Withdrawal from alcohol administration after 10 and 14 weeks intoxication resulted in a complete recovery in GSH levels within 24 hours. Similar changes were seen in other tissues, although they were less marked than in liver, and no changes were observed in GSSG. To clarify the changes in glutathione levels, we measured the main enzymes connected with the synthesis (γ-glutamylcysteine synthetase + glutathione synthetase) and utilization (γ-glutamyl transpeptidase) of glutathione. In liver, the activity of the glutathione synthesizing system was unchanged after 4 weeks, but increased to 140% within 10 weeks; γ-glutamyl transpeptidase was slightly elevated (120%) at 4, 10 and 14 weeks. Similar changes in these enzymes were observed in kidney and brain. γ-Glutamyl transpeptidase was elevated in serum (120%) at 4 weeks, but reached 140% within 10 weeks. The levels of the glutathione synthesizing system returned to normal in all tissues within 2 days after alcohol withdrawal; however, γ-glutamyl transpeptidase levels did not revert to normal until one week after withdrawal.

Acute ethanol intoxication Chronic ethanol intoxication Glutathione

REDUCED glutathione (GSH) is the most abundant non-protein thiol in the cell. It has several known important roles, e.g. as a coenzyme for several reactions [8, 26, 43], in detoxication mechanisms [32], as a protector of SH-enzymes [17], in maintaining integrity of erythrocyte membranes [21], and for transparency of the lens [18]. In addition, various membrane functions, including ion and sugar transport, release of neurotransmitters and the action of hormones, are apparently dependent upon a suitable thiol-disulphide balance, a balance which can be seriously affected by changes in the GSH status of the cell or system [22].

We found a marked decrease in liver GSH after methanol and formaldehyde intoxication of mice [14]. We could also protect the animals from these intoxications by SH-reagents [15]. Congruent with these results, a decrease in liver GSH after acute ethanol intoxication has been shown. This work also demonstrated that some sulphydryl compounds significantly increase the survival of mice given a lethal dose of ethanol [29].

Further, SH-reagents protected enzymes from inactivation by acetaldehyde or formaldehyde "in vitro" [12]. These aldehydes may play an important role in the intoxications by their respective alcohols. Indeed, acetaldehyde was shown to be responsible for the lowered GSH levels found after addition of alcohol to hepatocytes [48]. It may also be involved in most of the typical manifestations of alcohol abuse, including dependence, hepatotoxicity, and cardiomyopathy as well as in producing alcohol aversion [25].

In this study we report the effect of chronic and of acute ethanol intoxication on the GSH levels of liver and of other tissues. Since glutathione levels reflect a balance between glutathione utilization and biosynthesis, to further elucidate changes in these levels in alcoholism we also studied the activities of enzymes involved in glutathione synthesis (γ-glutamylcysteine synthetase + glutathione synthetase) and degradation (γ-glutamyl transpeptidase) in both chronic ethanol consumption and in the alcohol withdrawal syndrome.

METHOD

Chemicals

L-γ-glutamyl-p-nitroaniline, glycylglycine, GSH, GSSG, L-glutamate, L-cysteine, dithiothreitol, glycine, EDTA, disulfiram and glutathione reductase (E.C.1.6.4.2) were from Sigma Chemical Co., and Pyrazole was from Fluka A.G. Other reagents used were of the highest purity available.

Acute Ethanol Intoxication

Male mice weighing approximately 30 g were pre-treated with disulfiram (60 mg/100 g body weight), given in saline

TABLE 1

GSH LEVELS IN MOUSE TISSUE AFTER ACUTE ETHANOL ADMINISTRATION. EFFECTS OF DISULFIRAM AND PYRAZOLE*

Tissue	Treatment					
	None (10)	Ethanol (7)	Pyrazole (5)	Disulfiram (5)	Ethanol + Pyrazole (7)	Ethanol + Disulfiram (7)
Liver	2.3 ± 0.3	1.6 ± 0.3	2.4 ± 0.4	2.05 ± 0.1	2.0 ± 0.06	0.95 ± 0.1
Heart	0.7 ± 0.1	0.5 ± 0.03	0.72 ± 0.2	0.78 ± 0.08	0.62 ± 0.06	0.46 ± 0.04
Kidney	1.28 ± 0.1	0.9 ± 0.1	1.27 ± 0.3	1.1 ± 0.08	1.1 ± 0.1	0.85 ± 0.1
Brain	0.76 ± 0.03	0.64 ± 0.04	0.73 ± 0.04	0.7 ± 0.05	0.71 ± 0.08	0.62 ± 0.06

Mice received disulfiram (60 mg/100 g, orally) or pyrazole (50 mg/100 g, intraperitoneally) 16 hr before ethanol injection (6 g/kg, IP). The mice were killed 5 hr after ethanol injection. Results are expressed as mg/g wet tissue.
*Number of animals used is shown in parenthesis.

orally, or pyrazole (50 mg/100 g body weight) in saline intraperitoneally, 16 hr prior to ethanol. Ethanol (6 g/kg body weight) as a 30% solution in saline was administered intraperitoneally, and animals were sacrificed 5 hr later. Tissues were removed, and GSH [2] and GSSG levels [2] were measured.

Chronic Ethanol Intoxication and Withdrawal

Male Sprague-Dawley rats with an average initial body weight of 150 g were fed for 4, 10 and 14 weeks a liquid diet [23] in which ethanol provided 36% of total calories, 16% protein and 35% fat. Pair-fed control rats were given a similar diet for the same period, except that carbohydrates replaced ethanol isocalorically. For withdrawal, the ethanol liquid diet was replaced by the control liquid diet. During the experimental period, the rats were housed in plastic boxes, 2 or 3 animals per box, at 22°C, 60% humidity, on a 12 hr light/12 hr dark cycle.

Tissue Preparations

Rats were killed by decapitation and their tissues were quickly excised, removed, and homogenized in 10 parts (w/v) of 0.25 M cold sucrose with a Super Dispox Tissumizer homogenizer at full speed with cooling by immersion in an ice bath. A portion of the homogenate was then mixed with 1 volume of cold 10% trichloroacetic acid containing 2 mM EDTA and the mixture was centrifuged to separate the "acid supernatants." This acid supernatant was used for determination of GSH [2], GSSG [2] and the total non-protein thiol groups [9]. Another portion of the tissue homogenate was centrifuged to prepare cytosol as described by Tateiski *et al.* [46]. This cytosol fraction was used to measure glutathione synthesis.

Enzyme Assays and Protein Determination

Glutathione synthesis (γ-glutamylcysteine synthetase (E.C. 6.3.2.2) + glutathione synthetase (E.C. 6.3.2.2)) was assayed according to Teteishi *et al.* [46].

γ-Glutamyltranspeptidase (E.C. 2.3.2.2) was assayed in 5% (w/v) homogenates (0.01–0.1 ml) [46] using glutamyl-p-nitroanilide as substrate and glycylglycine as the γ-glutamyl group acceptor [33]. Incubations were for 60 min at 25°C, except for kidney when the incubations were for only 5 min.

Protein was determined by the method of Lowry *et al.* [27] and by the biuret method [38]. Bovine serum albumin was used as a standard.

RESULTS

Effect of Acute and Chronic Ethanol Administration and Withdrawal on GSH Tissue Levels

Table 1 shows lowered levels of non-protein thiol groups (GSH accounts for 95% of sulphydryl groups in the cell) in various mouse tissues 5 hours after an acute dose of ethanol. The decrease of GSH levels was more marked in liver than in other tissues. A similar ethanol depletion of GSH in liver has been found by others [29].

In order to clarify whether acetaldehyde or ethanol was responsible for this effect, we pre-treated a group of animals with disulfiram, an inhibitor of aldehyde dehydrogenase, to raise the level of acetaldehyde after alcohol administration and another group with pyrazole, an inhibitor of alcohol dehydrogenase, to decrease the rate of ethanol metabolism [6]. As shown in Table 1, we found a further decrease in GSH levels in all tissues investigated, and particularly in the liver of animals pre-treated with disulfiram and then intoxicated with ethanol, over those receiving only ethanol. Pyrazole produced the opposite effect; after pretreatment with this drug, alcohol was less effective in reducing GSH levels. These results indicate that acetaldehyde is responsible for the decrease in GSH levels found in animals treated with alcohol.

It has been postulated that in alcoholic individuals alcohol consumption induces higher levels of acetaldehyde than in non-alcoholics [20] and particularly in those subjects whose genetic make-up shows a predisposition [34]. It thus seemed of interest to investigate the levels of GSH in tissues of rats ingesting alcohol for extended periods of time.

As illustrated in Figs. 1 and 3, we found a reduction in levels of reduced glutathione in all tissues tested following alcohol ingestion for 4, 10 and 14 weeks. Maximal effect was seen in liver after 4 weeks of alcohol; thereafter, GSH levels increased to reach a plateau at 80% of the normal values after ca. 10 weeks. It must be stressed that in this experiment we used a liquid diet and the alcohol intake was quite high (9-10 g alcohol/kg/day). When a similar experiment was done with 20% alcohol in the drinking water (6-7 g/kg/day), similar GSH results in liver were seen only after 9 weeks.

Withdrawal of alcohol after 10 and 14 weeks (Figs. 2, 4) of alcohol intake in the liquid diet, produced a sharp restoration

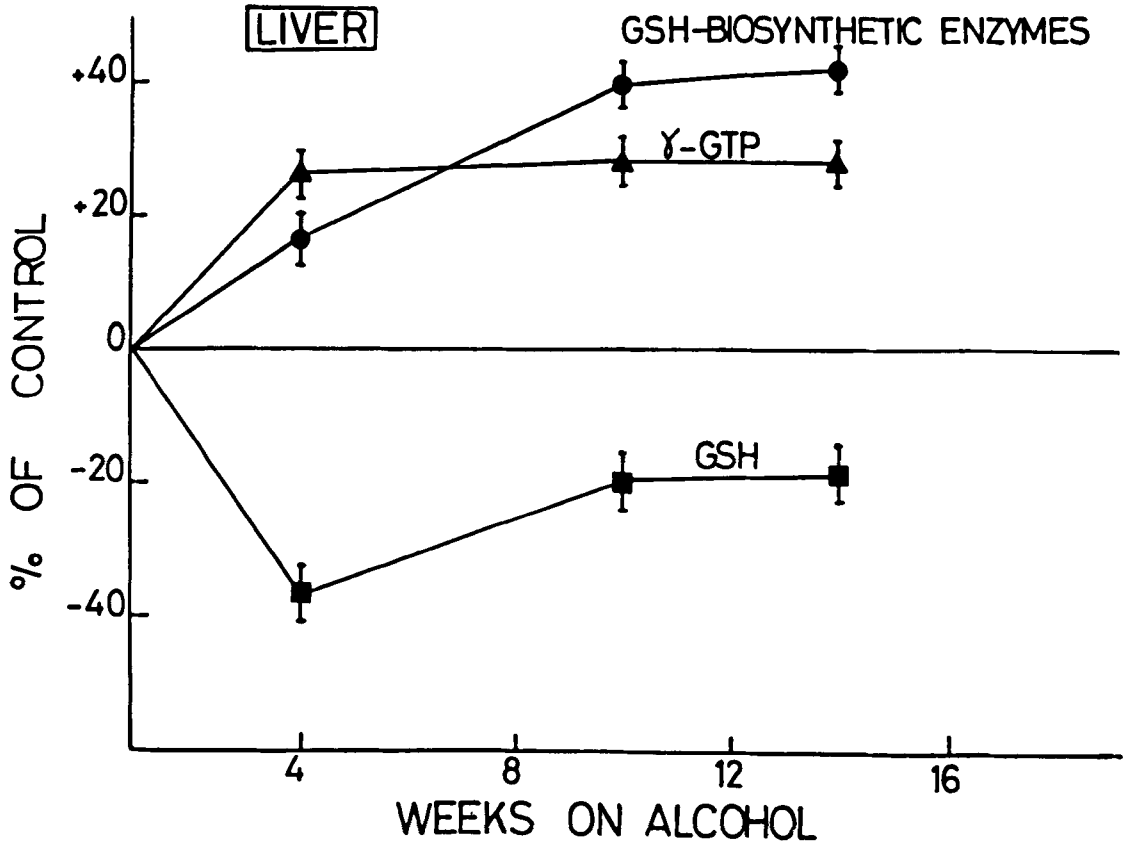

FIG. 1. Levels of GSH, glutathione biosynthetic enzymes and γ-glutamyl transpeptidase of rat liver during chronic ethanol intoxication. Rats were fed ethanol liquid diet or control liquid diet for different time intervals. After 4, 10 and 14 weeks on ethanol, the animals were sacrificed and GSH, GSSG, glutathione biosynthetic enzymes and γ-glutamyl transpeptidase (γ-GTP) were determined, as described in Method. Each point represents the average (±SD) obtained from 5–6 animals. Values are given as a percentage of control.

of the thiol levels; 100% of control value was reached 24 hr after withdrawal in all tissues investigated.

It should be noted that the ratio of reduced/oxidized glutathione did not change in any of these cases. Thus, alcohol only affected total GSH/GSSG levels, which could be the result of changes in either its synthesis or degradation. We therefore measured the levels of the enzymes involved in biosynthesis and in degradation of glutathione in both normal and chronically alcoholic rats.

Effect of Chronic Ethanol Ingestion and of Withdrawal on the Glutathione Synthesizing System and γ-Glutamyl-transpeptidase

The "glutathione synthesizing system" (γ-glutamyl-cysteine synthetase + glutathione synthetase) was assayed by measuring P_i release in the presence of three constituent amino acids (glutamate, cysteine, and glycine), since the two reactions are ATP-dependent. No attempt was made to assay each enzyme separately. We measured GSH degradation by using γ-glutamyl-transpeptidase which seems to be involved in GSH degradation.

Levels of these enzymes in several tissues from control rats are presented in Table 2.

TABLE 2

VALUES OF GLUTATHIONE SYNTHESIZING ENZYME SYSTEM AND OF γ-GLUTAMYL TRANSPEPTIDASE IN CONTROL RATS

Tissues	Glutathione Biosynthetic Enzymes*	γ-Glutamyl Transpeptidase†
Liver	31 ± 1.6	7.0 ± 0.8
Heart	—	3.0 ± 0.1
Kidney	480 ± 18	882.0 ± 32
Brain	85 ± 0.3	46.0 ± 3.2
Muscle	—	6.4 ± 0.08

*(nmoles P_i/min/mg cytosol prot.)
†(μmoles hydrolyzed p-nitroaniline/hr/g tissue at 25°C.)
Values represent mean ± S.D.

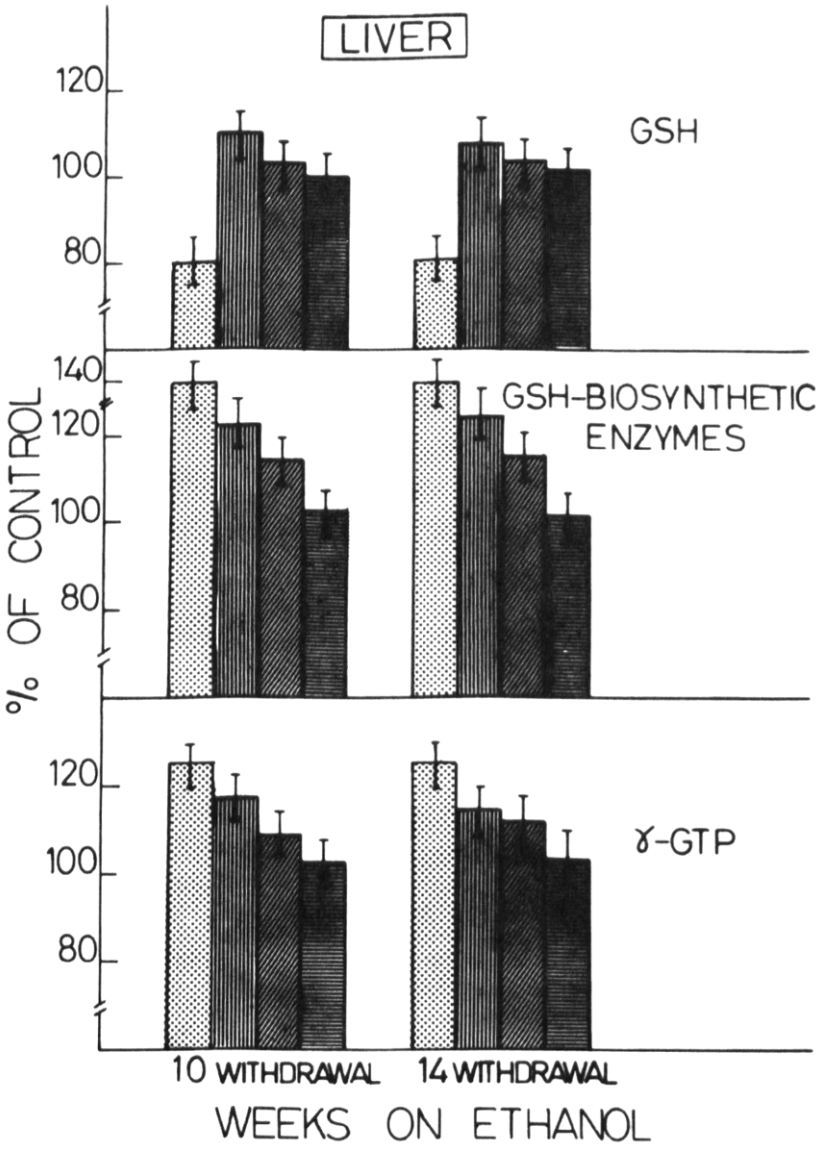

FIG. 2. Levels of GSH, glutathione biosynthetic enzymes and γ-glutamyl transpeptidase of liver from rats during ethanol withdrawal after 10 and 14 weeks of chronic ethanol ingestion. Rats were fed the liquid diet for 10 and 14 weeks; the ethanol liquid diet was then replaced by the control liquid diet. Rats were sacrificed at intervals during withdrawal (1, 2 and 7 days) and GSH, GSSG and the enzymes indicated were determined (see Method). Each value represents the average (±SD) from 4–5 animals. Value at the end of chronic ethanol treatment, stippled bar. Ethanol withdrawal was after 1 day—vertical stripes; 2 days—diagonal stripes; or 7 days—horizontal stripes.

As shown in Fig. 1, the "glutathione synthesizing system" in rat liver increases progressively with time of alcohol consumption, to reach a plateau (140% of controls) after 10 and 14 weeks. Similar changes, but less marked, are seen in other tissues tested, such as kidney and brain (Fig. 3).

It is especially interesting that the increase found in the "glutathione synthesizing system" after long treatment with alcohol (10 and 14 weeks) corresponds to the small recovery found in the GSH levels during the same periods of time (Figs. 1 and 3).

In the case of γ-glutamyl transpeptidase (γ-GTP), short and prolonged treatments with ethanol produced an increase (20%) in the enzyme level of brain and kidney, while the activity in liver was enhanced ~25%. This increase appears to be due, in part, to the proliferation of smooth endoplasmic reticulum which is associated with chronic alcohol consumption [40], rather than simply a direct effect of alcohol on the enzyme. Serum γ-GTP, however, shows a marked increase which perhaps correlates with the enhancement of hepatic activity (see below).

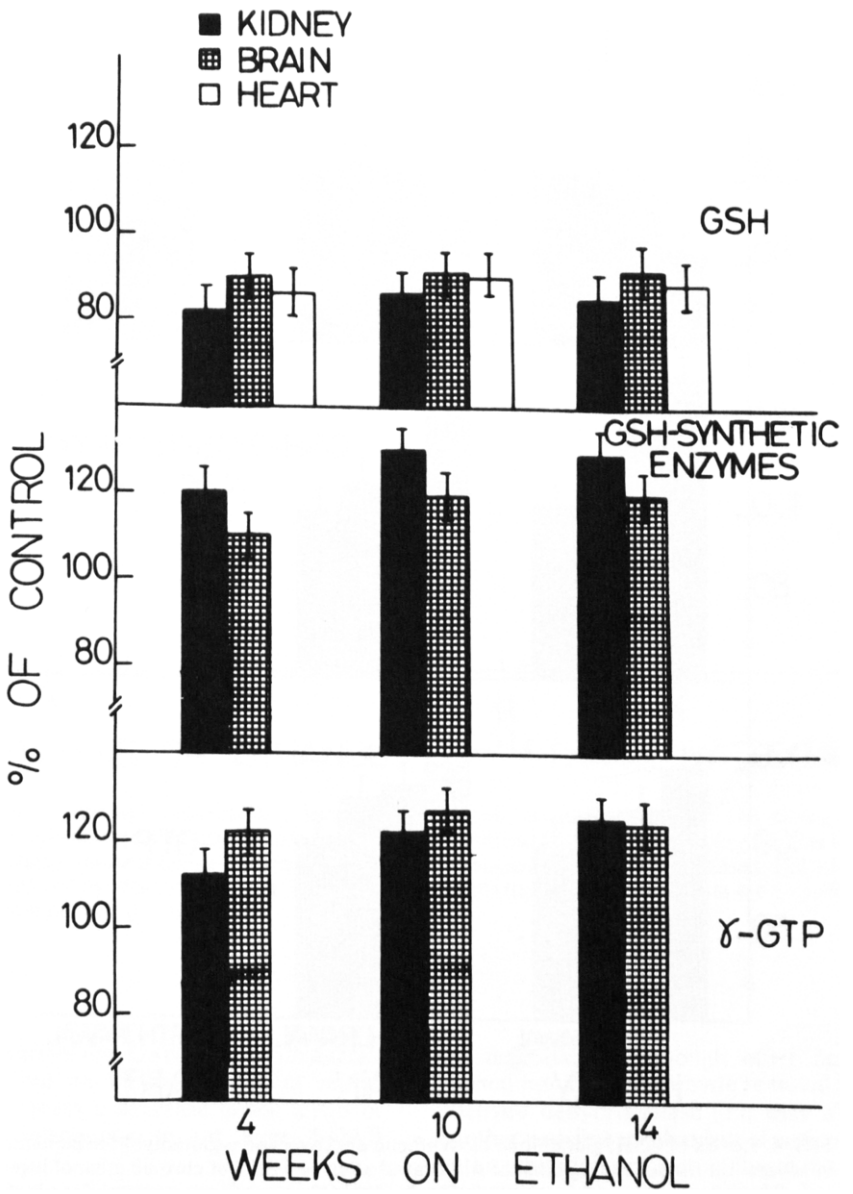

FIG. 3. Levels of GSH, glutathione biosynthetic enzymes and γ-glutamyl transpeptidase of several rat tissues after different periods of ethanol ingestion. The procedure was the same as in Fig. 1, except the measurements were done in the indicated tissues. Each value represents the average (±SD) from 5–6 animals, and is given as a percentage of control.

We also measured the activity of these enzymes during ethanol withdrawal for different periods of time (1, 2 and 7 days) after 10 and 14 weeks of chronic ethanol intake.

Figure 2 shows that after 24 hours of ethanol deprivation, the glutathione biosynthetic enzymes are still at high levels which may be responsible for the recovery of tissue GSH levels after ethanol withdrawal. The levels of the liver glutathione biosynthetic enzymes were normal at 48 hours, and after 1 week of ethanol deprivation, all the parameters measured were normal.

The recovery of the enzymes in alcohol withdrawal in other tissues followed the same pattern as the liver enzymes (Fig. 4). We illustrate only the data of ethanol withdrawal after 14 weeks of chronic ethanol consumption, since the results after 10 weeks were quite similar.

In the case of γ-glutamyl-transpeptidase (in all cases), the levels were still maintained 48 hr after withdrawal and reached normal levels 1 week later. This was especially marked for the plasma enzyme (Fig. 5).

DISCUSSION

Glutathione is the most abundant and important intracellular sulfhydryl compound. Both reduced (GSH) and

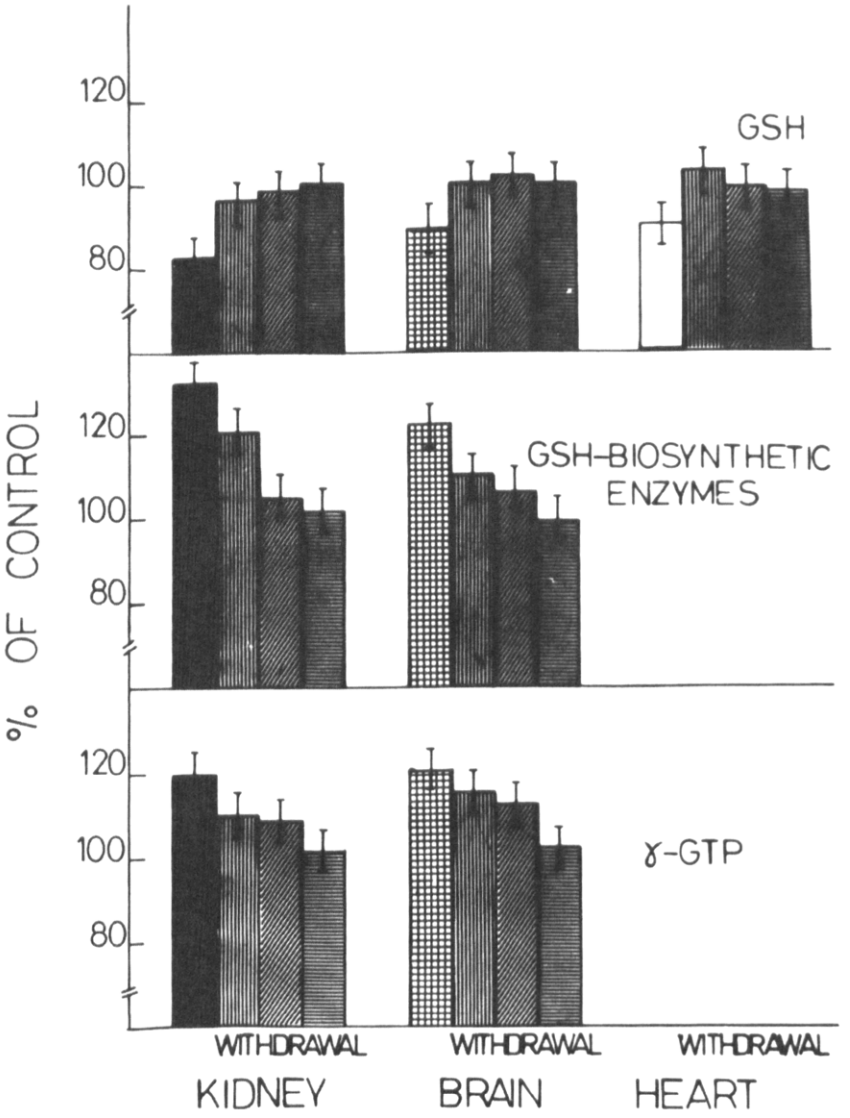

FIG. 4. Levels of GSH, glutathione biosynthetic enzymes and γ-glutamyl transpeptidase in several rat tissues during ethanol withdrawal after 14 weeks of chronic ethanol ingestion. The procedure was the same as in Fig. 2, except the measurements were done in the indicated tissues. Each value represents the average (±SD) from 4–5 animals, and is given as a percentage of control. Value at the end of chronic ethanol treatment, solid bar. Ethanol withdrawal after 1 day—vertical stripes; 2 days—diagonal stripes; or 7 days—horizontal stripes.

oxidized (GSSG) glutathione are related to several structural and functional processes of the cell and are involved in the protective mechanism against the deleterious effects of several agents and/or their metabolites [22], e.g. the hepatoxicity of many drugs is preceded by GSH depletion [5].

Ethanol may produce cellular damage of hepatocytes [24]. The biochemical mechanisms leading to cell damage, necrosis and liver cirrhosis are still unknown, although several factors have been proposed and acetaldehyde has often been implicated as playing an important role in ethanol toxicity [25]. In this study, we have shown a marked decrease in the glutathione pool (with no net oxidation of GSH to GSSG) in many tissues after both acute and chronic ethanol intoxication. We have also shown that acetaldehyde may play an important role in this decrease following acute ethanol intoxication, since pyrazole pretreatment of animals prevents the decrease of GSH levels, while disulfiram pretreated animals undergo a further decrease in GSH levels.

In addition, it has been reported [29] that some sulphydryl compounds are protective against acute ethanol toxicity. Congruent with these results, we showed decreases in GSH after methanol and formaldehyde administration and demonstrated a marked protection against these intoxications by SH-reagents, such as 2,3 dimercaptopropanol and cysteine [15].

The decrease in glutathione could result via several

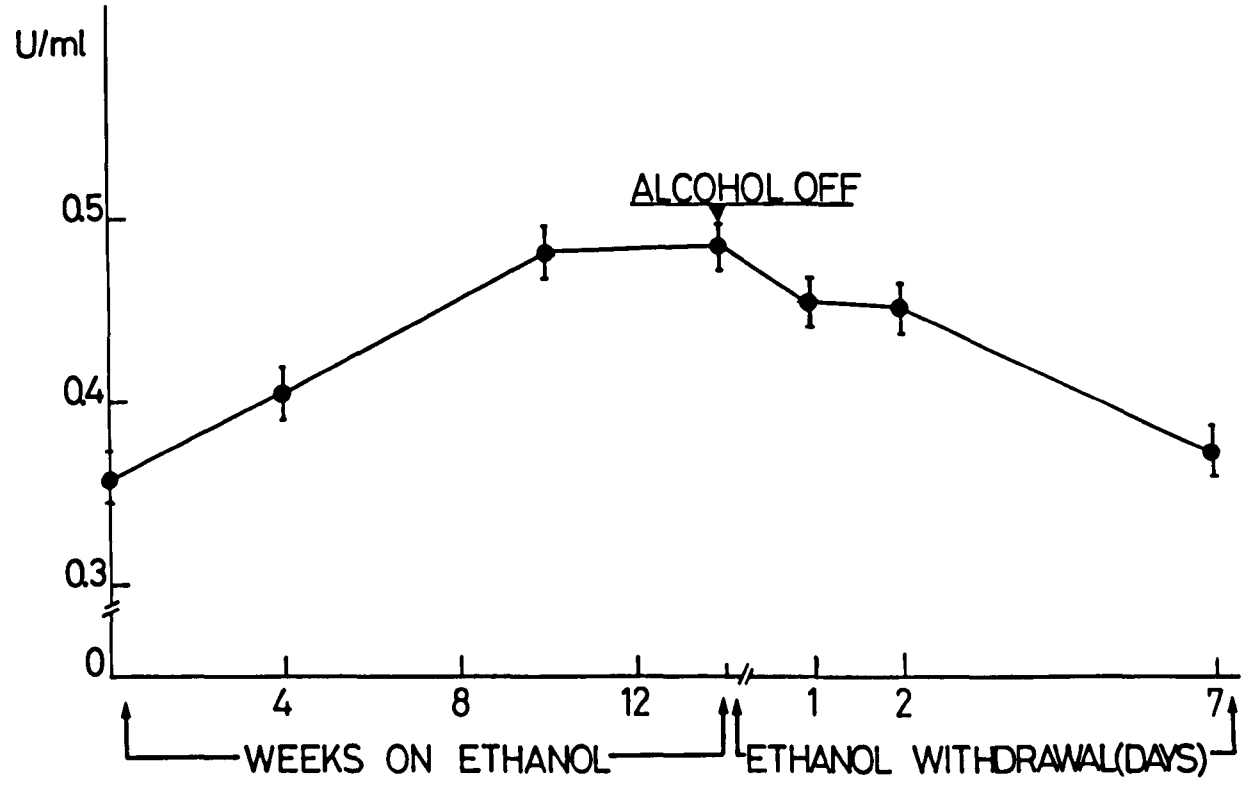

FIG. 5. Levels of plasma γ-glutamyl transpeptidase levels during chronic ethanol intoxication and during different withdrawal periods. Rats were fed the ethanol liquid diet or the control liquid diet for different time intervals. After the time indicated, the animals were sacrificed and γ-glutamyl transpeptidase was measured in plasma obtained from the hepatic blood. For ethanol withdrawal, the ethanol liquid diet was replaced by control liquid diet. Each value represents the average (±SD) from 4–6 animals. Values are given as μmoles p-nitroaniline/hr/ml plasma at 25°C.

mechanisms. It has been shown that GSH reacts with acetaldehyde "in vitro" [48] and an interesting recent report shows that acetaldehyde causes a decrease in the levels of non-protein sulphydryl compounds in rat liver 4-DAB hepatoma [41].

Another possible mechanism is based on the antioxidant properties of GSH [11,16]. It has been shown that acute and chronic ethanol intoxications result in alcohol and acetaldehyde-mediated [7] free radical increase and lipid peroxide formation [35,47]. Reduced glutathione, as a substrate of glutathione peroxidase, reacts with lipid peroxides which are potentially toxic to normal cell constituents [3,10]. This process increases the GSSG concentrations which are released from the tissues to the bile [1]. In fact, an increased biliary GSSG release in chronically ethanol treated rats has been shown [42]. These two processes may be responsible for the rapid decrease of the levels of glutathione found shortly after acute administration of ethanol or other alcohols.

Long term changes in glutathione levels may result from changes in the rate of its synthesis and/or degradation. Increased levels of γ-glutamyl-transpeptidase could be related with lower levels of glutathione. It is of interest that, corresponding with the maximal increase of the glutathione synthesizing system reached after 10 weeks, there is a partial recovery of the liver glutathione levels. This may correspond to the establishment of a new steady state between synthesis and degradation, although other factors, such as an increased rate of acetaldehyde removal by an induced low-Km aldehyde dehydrogenase [13] may also be of importance. Another possible mechanism of action of acetaldehyde could be the inhibition or inactivation of the glutathione synthesizing system. Although inactivation by acetaldehyde has been observed with certain enzymes [12,14], normal or increased levels of the synthesizing system has been shown at times when the glutathione concentrations were already decreased. Rapidly reversible inactivation or inhibition of this enzyme system by acetaldehyde has not been ruled out as yet.

The enzyme γ-glutamyl-transpeptidase (γ-GTP) catalyzes the transfer of the γ-glutamyl group from γ-glutamyl peptides (E.C.2.2.3.2) to amino acids (E.C.2.3.2.2) [39]. It also catalyzes the hydrolysis of γ-glutamyl compounds and the autotranspeptidation reaction [45]. The enzyme appears to be located in the membrane of cells with high secretory or absorptive capacities [30]. It has been postulated that it might be useful in the differential diagnosis of liver diseases [28]. In fact, high serum γ-GTP activity is commonly found in alcoholics with histological abnormalities of the liver [4]. This increase has been attributed in part to the proliferation of liver smooth endoplasmic reticulum, which occurs in chronic alcohol ingestion [40], since this enzyme is located primarily in the microsomal fraction of hepatocytes [19].

Although there is an effective feedback mechanism of

control of glutathione biosynthesis [31], it is believed that the major function of γ-glutamyl-transpeptidase is to regulate the intracellular concentration of glutathione by degrading it [30,31]. In the present study, we have found increases in activity of this enzyme in liver, kidney, serum and brain of chronically alcoholic rats.

Recently, it has been demonstrated that there is membrane bound γ-GTP in brain tissue [36]. A comparison of the γ-GTP levels between long-sleep and short-sleep mice [37] has provided evidence that the enzyme may be involved in determining an animal's sensitivity to alcohol.

Although alcohol can possibly induce proliferation of the smooth endoplasmic reticulum in extrahepatic tissues, alcohol induced changes of the structure of membranes [44] may also explain the generalized increases of γ-glutamyl-transpeptidase activity since this enzyme is membrane bound [30]. Enzyme release from various tissues could possibly contribute to the increased serum levels [4].

Finally, it seems clear that GSH levels and their regulation are very important for the normal functioning of liver and other tissues and, therefore, any change in GSH levels may be etiologically important in the changes observed in acute and chronic alcoholism.

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