Hepatic Glutathione Concentrations Linked to Ethionine Toxicity in Rats

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(Received 18 August 1967)

1. The effect of injected ethionine on liver GSH concentrations was studied in male and female rats. 2. Liver GSH concentrations were markedly and consistently decreased in 5 hr. and elevated within 24–44 hr. By 72 hr. the amount of liver GSH of ethionine-poisoned rats had the same values as saline-injected control rats. At no time was erythrocyte GSH concentration affected by ethionine. 3. The concentrations of non-protein thiol compounds, glycogen and ATP were also significantly decreased when the rats were killed 5 hr. after ethionine injection. 4. ATP, adenine or methionine did not prevent the decrease of liver GSH produced by ethionine. From these and other observations we conclude that ethionine is not an antagonist of methionine with respect to GSH metabolism. 5. The metabolic relationship between ethionine toxicity and liver GSH concentration is briefly discussed.

Studies on the action of ethionine in rat liver have shown that this ethyl analogue of methionine produces a number of adverse effects including an inhibition of RNA and protein synthesis (Villa-Trevino, Shull & Farber, 1963, 1966), a decrease in glycogen formation (Lupu & Farber, 1954), a decrease in phosphorylase activity and ATP concentration (Shull, 1962), and an accumulation of triglycerides (Jensen, Chaikoff & Tarver, 1951). Since each of these effects can be counteracted by methionine or by ATP it is generally believed that ethionine is acting as a specific antagonist of methionine. Early studies by Calcutt (1961) indicated that the feeding of DL-ethionine to female rats resulted in a prolonged elevation of the thiol concentration in liver but not in kidney. Hsu & Geller (1967) have reported that ethionine administration increased the concentration of hepatic GSH, a tripeptide that requires 1 mol. of ATP in each of its two stages of synthesis (Snoke & Bloch, 1952). Further, simultaneous supplementation of the stock diet with methionine and ethionine did not prevent the increase in GSH, and the methionine supplement alone caused an increase in GSH. These findings suggest that ethionine and methionine are undergoing the same reactions and producing similar effects rather than antagonistic ones, and that ethionine also acts as a 'toxic agent'. Additional evidence is presented here indicating the existence of a metabolic relationship between ethionine toxicity by injection and hepatic GSH concentrations.

MATERIALS AND METHODS

Materials

Animals. Rats of the McCollum strain from our own colony weighing 180–270 g. for females and 250–280 g. for males were chosen for most of the studies. In two experiments female albino Sprague-Dawley rats weighing 200–250 g. were obtained from a commercial source. In each individual experiment the control group consisted of five or six rats matched with the experimental groups for age, sex, weight and origin. Before the experiment the animals were given Purina Laboratory Chow. Water was available at all times. Food was removed overnight before the first administration of the compounds. In certain studies animals were deprived of food for 18 hr. before the termination of an experiment. All animals were killed by decapitation. The number of rats used in each experiment and time of death are given in the legends to the Tables.

Chemicals. DL-Ethionine was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. From the results of thin-layer chromatography with butan-1-ol-acetic acid-water (4:1:1, by vol.) its purity was greater than 99%. The ethionine was dissolved in 0.9% NaCl and neutralized with NaOH. The unanaesthetized rats were injected intraperitoneally with 0.9% NaCl solution containing 25 mg. or 15 mg. of ethionine/ml. The dose was 100 mg./100 g. body wt. as used in previous studies (Shull, 1962; Slater & Sawyer, 1966). Control rats received injections of 0.9% NaCl solution corresponding to the volumes administered with ethionine.

GSH and GSSG were obtained from Schwarz BioResearch Inc., New York, N.Y., U.S.A. Adenine, ATP, NADP⁺, NADPH, glucose 6-phosphate (disodium salt) and 6-phosphogluconate (sodium salt) were obtained from Sigma

Chemical Co., St Louis, Mo., U.S.A. All other chemicals were the best reagent grades available from commercial sources.

KOH, precipitated by 95% ethanol and determined by the method of Seifter, Dayton, Novie & Muntwyler (1950).

Methods

GSH assay. GSH in erythrocytes and liver was measured by the method of Patterson & Lazarow (1955). The reaction of GSH with alloxan was used to produce a compound with E_{max} at 305 m μ . Blood was prepared for assay as indicated by Patterson & Lazarow (1955) except that the precipitated proteins were removed from the haemolysate by filtration instead of by centrifugation. Liver was homogenized in 30–40 vol. of ice-cold 5% metaphosphoric acid in a Potter-Elvehjem homogenizer. The homogenate was saturated with NaCl and kept in the cold for 1 hr., shaken occasionally and filtered through Whatman no. 2 paper. The DL-ethionine added to liver homogenates in concentrations up to 0.02 M did not affect the measurement of GSH.

Enzyme assay. Fresh liver (400–500 mg. wet wt.) was rapidly excised, and a 10% (w/v) tissue homogenate was prepared in 0.25 M-sucrose–1 mM-EDTA by using a motor-driven Teflon-pestle tissue grinder. The supernatant fluid obtained by centrifuging the homogenate at 105 000g for 20 min. at 0° in a Spinco model L ultracentrifuge was assayed for glutathione reductase by the spectrophotometric method of Manso & Wróblewski (1958). The activities of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in the supernatant fluid were assayed by the method of Glock & McLean (1953) as modified by Fitch, Hill & Chaikoff (1959).

Extinctions for GSH and enzyme assays were measured at 25° in a Beckman model DU spectrophotometer equipped with thermospacers. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) with crystalline bovine plasma albumin as the standard.

Other determinations. Liver non-protein thiol concentrations were determined by the procedure of Grunert & Phillips (1951). The intensity of the red colour of non-protein thiol and nitroprusside dissolved in metaphosphoric acid was measured with a Klett–Summerson colorimeter with a 560 m μ green filter. ATP was estimated on a piece of frozen liver by the luciferin–luciferase method (Strehler & McElory, 1957). Liver glycogen was extracted with hot

RESULTS AND DISCUSSION

Table 1 gives the concentrations of GSH in erythrocytes and liver after administration of ethionine. Liver GSH concentrations were markedly decreased in 5 hr. and partially restored in 18 hr. By 24 hr. the GSH concentration in the liver of ethionine-injected rats was 60% greater than the normal value. Unlike liver, erythrocyte GSH concentrations were unaffected by ethionine. Similar changes in the concentration of liver GSH during the 5 and 24 hr. periods were also observed in male rats after the injection of ethionine (Table 2).

Since the measurement of GSH in tissue by the alloxan method is not yet completely satisfactory (Patterson & Lazarow, 1952), and since GSH occupies 90% or more of non-protein thiol compounds, the latter was measured by the nitroprusside method. Table 3 shows that the effect of ethionine on the concentrations of liver non-protein thiol compounds was similar to the changes of hepatic GSH contents, except that the increase of non-protein thiol compounds and GSH did not occur until 44 hr. later. After 72 hr., the amount of liver GSH of ethionine-poisoned rats had the same values as saline-injected animals. It is not clear why Sprague–Dawley rats required a longer period of time for increase of liver GSH than McCollum rats (Table 1).

Table 3 shows that liver glycogen was decreased simultaneously in rats killed 5 hr. after ethionine injection and was gradually increased later.

Table 4 indicates that a drastic decrease in hepatic ATP occurred 5 hr. after the administration of ethionine. This decrease corresponded favourably with that previously reported for the effect of

Table 1. *Effect of ethionine on liver GSH concentrations at various times*

‘+’ Refers to ethionine-treated rats. Total dose of 1 mg. of DL-ethionine/g. body wt. was injected intraperitoneally. Half of the dose was given at zero time and half 1 hr. later. ‘–’ Refers to control rats receiving an injection of 0.9% NaCl solution. The results are expressed as means $\pm$ s.d.

Ethionine	Time after injection (hr.)	No. of female rats	Concn. of GSH	
			Erythrocytes (μ moles/100 ml.)	Liver (μ moles/100 g.)
–	2	6	169 $\pm$ 3	398 $\pm$ 11
+	2	5	175 $\pm$ 10	354 $\pm$ 12
–	5	6	168 $\pm$ 7	315 $\pm$ 22
+	5	6	164 $\pm$ 6	181 $\pm$ 6
–	18	5	167 $\pm$ 9	397 $\pm$ 10
+	18	5	178 $\pm$ 10	291 $\pm$ 30
–	24	6	171 $\pm$ 5	394 $\pm$ 14
+	24	6	168 $\pm$ 7	621 $\pm$ 34

Table 2. *Effect of ethionine on liver GSH concentrations in male rats*

Ethionine was administered as described in Table 1. The results are expressed as means $\pm$ s.d.

Ethionine	Time after injection (hr.)	No. of male rats	Concn. of GSH	
			Erythrocytes (μ moles/100 ml.)	Liver (μ moles/100 g.)
—	5	6	180 $\pm$ 15	338 $\pm$ 29
+	5	5	195 $\pm$ 21	258 $\pm$ 11
—	24	5	193 $\pm$ 19	404 $\pm$ 13
+	24	5	220 $\pm$ 28	677 $\pm$ 25

Table 3. *Effect of ethionine on liver non-protein thiol compounds, GSH and glycogen concentrations at various times in Sprague-Dawley rats*

Ethionine was administered as described in Table 1. The results are expressed as means $\pm$ s.d.

Ethionine	Time after injection (hr.)	No. of female rats	Non-protein SH compounds (μ moles/100 g.)	GSH (μ moles/100 g.)	Glycogen (%)
—	5	6	416 $\pm$ 79	355 $\pm$ 22	0.320 $\pm$ 0.091
+	5	6	285 $\pm$ 52	243 $\pm$ 25	0.026 $\pm$ 0.002
—	18	5	437 $\pm$ 53	379 $\pm$ 22	—
+	18	5	206 $\pm$ 89	290 $\pm$ 50	—
—	24	6	476 $\pm$ 195	410 $\pm$ 59	0.308 $\pm$ 0.071
+	24	6	337 $\pm$ 145	406 $\pm$ 98	0.051 $\pm$ 0.005
—	44	5	428 $\pm$ 26	393 $\pm$ 19	0.385 $\pm$ 0.039
+	44	5	1042 $\pm$ 180	657 $\pm$ 88	0.203 $\pm$ 0.077
—	73	6	—	375 $\pm$ 143	0.538 $\pm$ 0.084
+	73	7	—	505 $\pm$ 135	0.579 $\pm$ 0.132

Table 4. *Effect of ethionine on liver ATP concentrations*

Ethionine was administered as described in Table 1. All animals were killed 5 hr. after the first dose of ethionine or 0.9% NaCl solution. The results are expressed as means $\pm$ s.d.

Ethionine	No. of female rats	Concn. of ATP (μ mole/g. wet wt.)	Relative concn. of ATP
—	5	0.280 $\pm$ 0.003	(100)
+	6	0.058 $\pm$ 0.015	21

ethionine on ATP concentrations of the liver (Shull, 1962).

If the decreased GSH in the liver of ethionine-treated rats was due to ATP deficiency, it might be expected that repletion with this compound would correct this defect. To test this possibility, ATP or adenine was injected into a group of ethionine-treated rats and liver GSH content was determined. The results of these experiments (Table 5) show that the animals treated with ethionine plus ATP or adenine showed no increase in liver GSH as compared with those receiving ethionine alone. In

fact, the administration of 0.64m-mole of ATP further significantly decreased liver GSH of ethionine-treated rats and also normal animals. The unexpected findings suggest that the role of ATP lies not only in the synthesis of this tripeptide but also in its utilization or degradation.

Table 6 shows that the administration of an equimolar concentration of methionine is incapable of preventing the effect of ethionine on liver GSH concentrations. Its ineffectiveness was again demonstrated when the dose was increased to twice the concentration of the administered ethionine, which, however, restores the glycogen content (Table 7).

The activities of the enzymes glutathione reductase, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase affected by ethionine are given in Table 8. No significant difference was found between the two groups.

Under the experimental conditions reported, neither ATP nor adenine was effective in preventing the changes in liver GSH, which indicated that an ATP trapping effect of *S*-adenosylethionine (Farber, Shull, Villa-Trevino, Lombardi & Thomas, 1964) was not limiting GSH metabolism.

Since the changes of liver GSH concentration

Table 5. *Effect of ATP and adenine on liver GSH concentrations of ethionine-treated and control rats*

Ethionine was administered as described in Table 1. Animals were killed 5 hr. after receiving the first dose. Total dose of 0.32 m-mole of ATP or adenine was given subcutaneously, half of the dose given after 2 hr. and half given after 4.5 hr. ATP $\times 2$ refers to 0.64 m-mole of ATP. The results are expressed as means $\pm$ s.d.

Ethionine	Other treatments	No. of female rats	Concn. of GSH	
			Erythrocytes (μ moles/100 ml.)	Liver (μ moles/100 g.)
—	None	6	161 $\pm$ 5	349 $\pm$ 7
+	None	6	158 $\pm$ 7	175 $\pm$ 10
—	None	4	184 $\pm$ 5	341 $\pm$ 7
+	None	5	164 $\pm$ 20	226 $\pm$ 8
+	ATP	6	187 $\pm$ 10	224 $\pm$ 22
—	None	6	183 $\pm$ 18	408 $\pm$ 17
+	None	6	160 $\pm$ 4	268 $\pm$ 12
+	ATP $\times 2$	6	203 $\pm$ 10	197 $\pm$ 15
—	None	5	192 $\pm$ 10	361 $\pm$ 5
+	None	5	189 $\pm$ 7	249 $\pm$ 16
+	Adenine	5	158 $\pm$ 12	239 $\pm$ 15
—	None	5	194 $\pm$ 4	426 $\pm$ 24
—	ATP $\times 2$	5	225 $\pm$ 6	358 $\pm$ 14

Table 6. *Ineffectiveness of methionine in normalizing the change of liver GSH concentrations induced by ethionine*

'+' Refers to ethionine- or methionine-treated rats. Total dose of 1 mg. of DL-ethionine or DL-methionine/g. body wt. was injected intraperitoneally. Half of the dose was given at zero time and half 1 hr. later. Animals were killed 5 and 24 hr. after receiving the first dose. '—' Refers to control rats receiving injection of 0.9% NaCl solution. The results are expressed as means $\pm$ s.d.

Ethionine	Methionine	Time after injection (hr.)	No. of female rats	Concn. of GSH	
				Erythrocytes (μ moles/100 ml.)	Liver (μ moles/100 g.)
—	—	5	5	182 $\pm$ 12	382 $\pm$ 19
+	—	5	5	193 $\pm$ 31	248 $\pm$ 17
+	+	5	5	165 $\pm$ 18	291 $\pm$ 18
—	—	24	5	186 $\pm$ 9	329 $\pm$ 14
+	—	24	5	182 $\pm$ 8	535 $\pm$ 22
+	+	24	4	213 $\pm$ 5	489 $\pm$ 15

Table 7. *Effect of ethionine and ethionine + methionine on liver GSH and liver glycogen concentrations 5 hr. after injection*

Animals received 2 mg. of methionine/g. body wt. or 1 mg. of ethionine/g. body wt. or both as described in Table 1. Controls received appropriate volumes of 0.9% NaCl solution. The results are expressed as means $\pm$ s.d. Figures in parentheses represent the number of rats used for each assay.

Ethionine	Methionine	Concn. of GSH (μ moles/100 g. wet wt.)	Concn. of glycogen (g./100 g. wet wt.)
—	—	348 $\pm$ 23 (6)	0.320 $\pm$ 0.091 (6)
+	—	232 $\pm$ 22 (6)	0.017 $\pm$ 0.002 (6)
+	+	238 $\pm$ 31 (6)	0.254 $\pm$ 0.068 (6)

were not reversed by methionine, one must accept that there are at least two types of ethionine-induced biochemical lesions in the liver. One is completely counteracted by methionine and this compound is therefore considered to be a specific antagonist of methionine. The other type comprises lesions due to a toxic effect. The extent of the reversal of these lesions is dictated by the success in removal of the toxic agent. It is known that GSH in the liver participates in the formation of a variety of conjugated organic compounds that are excreted in the bile or urine or both. This process is one of the hepatic mechanisms for metabolizing or disposing of a variety of toxic substances. One example is the formation of a GSH conjugate with

Table 8. *Glutathione reductase, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase concentrations in ethionine-treated Sprague-Dawley rats*

Ethionine was administered as described in Table 1. Enzyme activities are expressed as μ moles of NADPH formed or oxidized/min./g. of protein. The results are expressed as means $\pm$ s.d. Figures in parentheses represent the number of rats used for each assay.

Ethionine	Time after injection (hr.)	Concn. of GSH (μ moles/100 g.)	Enzyme activities		
			Glutathione reductase	Glucose 6-phosphate dehydrogenase	6-Phosphogluconate dehydrogenase
—	5	365 $\pm$ 19 (6)	30 $\pm$ 2.9 (5)	28 $\pm$ 10.1 (3)	39 $\pm$ 8.9 (3)
+	5	227 $\pm$ 9 (6)	31 $\pm$ 2.9 (5)	18 $\pm$ 2.2 (3)	29 $\pm$ 2.0 (3)
—	44	393 $\pm$ 15 (5)	23 $\pm$ 3.3 (2)	42 $\pm$ 3.0 (2)	35 $\pm$ 0.8 (2)
+	44	649 $\pm$ 8.0 (5)	30 $\pm$ 0.8 (5)	48 $\pm$ 1.5 (2)	43 $\pm$ 0.6 (2)

Bromosulphthalein in rat bile as reported by Combes & Stakelum (1960). Further evidence for GSH conjugation in the detoxification of foreign substances can be found in the studies of mercapturic acid formation. Stekol (1940) found that administration of *S-p*-bromobenzyleysteine and *S-p*-bromobenzylglutathione to rats resulted in excretion of the same mercapturic acid. Booth, Boyland & Sims (1960) found that naphthalene, which is excreted as a mercapturic acid (Boyland & Sims, 1958), forms a GSH conjugate when incubated with rat liver homogenate. It would thus appear that the mercapturic acids formed in the urine can be traced to GSH-linked precursors in the liver.

The possibility of GSH conjugation with ethionine or its derivatives, however, remains to be studied. It is possible that the decreased GSH concentration at 5 hr. is caused by damage of the synthesizing mechanism as well as by enhanced GSH catabolism. When the toxic concentration decreases, the hepatic cells become more active with respect to GSH metabolism. The increased hepatic GSH probably reflects the time-interval during which ethionine concentration equals the stimulatory amount encountered in low-level chronic administration (Calcutt, 1961). Whether the increased GSH in the liver of ethionine-injected rats is due to the increased precursors such as glutamic acid, glycine and cysteine as reported by Wu & Bollman (1954) is not clear. Additional isotope work is needed to clarify this possibility. Nevertheless, it is certain that the effects of ethionine toxicity, such as those of the hepatic GSH concentrations, are time-dependent.

The authors thank Miss Florence Hill and Mr Louis Lange, III, for their excellent technical assistance.

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