

## Effect of oral glutathione on hepatic glutathione levels in rats and mice

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(Received 16 March 1989 – Accepted 3 July 1989)

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Administration of oral glutathione (GSH) increases hepatic GSH levels in fasted rats, in mice treated with GSH depletors such as diethyl maleate and in mice treated with high doses of paracetamol. An increase in hepatic GSH levels after administration of oral GSH does not occur in animals treated with buthionine sulphoximine, an inhibitor of GSH synthesis. Administration of oral GSH leads to an increase in the concentration of L-cysteine, a precursor of GSH, in portal blood plasma. Oral administration of L-methionine produced a significant decrease of hepatic ATP in fasted rats, but not in fed rats. Administration of N-acetylcysteine or GSH did not affect the hepatic ATP levels. The results show that the oral intake of GSH is a safe and efficient form of administration of its constituent amino acids in cases when GSH synthesis is required to replete hepatic GSH levels.

**Glutathione: Liver: Starvation: Mice: Rats**

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Glutathione (GSH) is the most abundant non-protein thiol in most cells (Fahey & Newton, 1983) and plays several important roles in cell physiology (Kosower & Kosower, 1978; Meister & Anderson, 1983; Viña *et al.* 1986). However, the levels of GSH in cells decrease in a variety of circumstances such as fasting (Tateishi *et al.* 1974), overdosage of paracetamol (Mitchell *et al.* 1974) or the process of ageing (Hazelton & Lang, 1980).

The availability of L-cysteine is the rate-limiting factor for GSH synthesis (Tateishi *et al.* 1974). Attempts to increase the hepatic levels of GSH have focused on the administration of L-cysteine or L-methionine (Vale *et al.* 1981), N-acetylcysteine (Prescott *et al.* 1977), or 2-oxothiazolidine carboxylate (Williamson & Meister, 1981) which are all precursors of L-cysteine. However, in some circumstances these substances have undesirable side effects. L-Cysteine itself may be toxic (Olney *et al.* 1972; Viña *et al.* 1983 *b*) and L-methionine can also promote depletion of hepatic ATP levels (Hardwick *et al.* 1970). Parenteral GSH cannot be used to increase intracellular GSH levels because free GSH does not enter the cells (Hahn *et al.* 1978).

GSH levels may be increased by administration of liposomally entrapped GSH (Wendel, 1983) or of GSH monoethyl ester (Puri & Meister, 1983). These procedures are difficult and not readily available. In contrast with parenteral GSH, dietary GSH has proved effective in reversing the age-associated decline in immune response in mice (Furukawa *et al.* 1987). However, direct evidence that oral GSH, which is a natural and non-toxic substance

(Sakamoto *et al.* 1983), serves to increase the intracellular levels of GSH in cells was lacking.

To address this question, we have studied the ability of oral GSH to replenish hepatic GSH in three different circumstances known to deplete the levels of hepatic GSH: fasting (Tateishi *et al.* 1974; Viña *et al.* 1987), pretreatment with diethyl maleate, which promotes a depletion of hepatic GSH but which does not cause cell necrosis (Boyland & Chasseaud, 1967), and paracetamol overdose, which promotes a depletion of hepatic levels of GSH that is followed by hepatic centrilobular necrosis (Mitchell *et al.* 1974). Oral administration of GSH promotes an increase in hepatic GSH levels in all the cases studied. The biochemical mechanism of this effect is also discussed.

## MATERIALS AND METHODS

### *Animals*

Wistar rats, 4–5 months of age, or Swiss mice, 2–3 months of age, were used. All animals were maintained on a 12 h light–12 h dark cycle and were fed *ad lib.* on a standard diet for rats and mice (Prasa, Vara de Quart, Valencia, Spain) which contains (g/kg) carbohydrate 530, lipid 36 and protein 146. Unless otherwise stated they had free access to food and water.

For experiments on fasting, rats were used because fasting decreases the levels of hepatic GSH in rats (Tateishi *et al.* 1974). For experiments with diethyl maleate or paracetamol, mice were used because they are a better model than rats to study the effects of paracetamol intoxication (Miners *et al.* 1984).

Some mice were injected intraperitoneally with diethyl maleate (1 g (5 mmol)/kg body-weight) or with paracetamol (0.9 g/kg body-weight.)

The precursors for hepatic GSH synthesis used were methionine (1 g (6.7 mmol)/kg body-weight, *N*-acetylcysteine (1 g (6.1 mmoles)/kg body-weight or GSH (1 g (3.26 mmol)/kg body-weight). These precursors were given orally through a cannula under light diethyl ether anaesthesia. We verified that this procedure does not affect the hepatic and blood variables measured.

### *Determination of metabolites*

Metabolites were always measured in freeze-clamped tissues. GSH was determined by the glyoxalase (*EC* 4.4.1.5) method of Racker (1951). ATP was measured by the hexokinase (*EC* 2.7.1.1) – glucose-6-phosphate dehydrogenase (*EC* 1.1.1.49) method (Lamprecht & Trautschold, 1979) and glycogen by the amyloglucosidase (*EC* 3.2.1.3) – hexokinase – glucose-6-phosphate dehydrogenase method of Keppler & Decker (1979). L-Cysteine was measured as described by Gaitonde (1967).

### *Statistics*

Statistical analyses were performed as recently reported (Viña *et al.* 1987). Essentially, we used the least significant difference test which consists of two steps. First an analysis of variance was performed and the null hypothesis was accepted for all numbers of those sets in which *F* was not significant at the level  $P < 0.05$ . Second, the sets of data in which *F* was significant were examined by the modified *t* test with  $P < 0.05$  as the critical limit.

## RESULTS

*Effect of oral GSH on the levels of hepatic GSH in 48 h-fasted rats*

Fasting for 48 h decreased the levels of hepatic GSH (Tateishi *et al.* 1974). The concentration of GSH found in fed rats was 5.7 (SE 0.4)  $\mu\text{mol/g}$  (fresh weight) ( $n$  6) and this value fell to 3.6 (SE 1.4)  $\mu\text{mol/g}$  fresh weight ( $n$  8) in fasted animals. Table 1 shows that oral administration of GSH significantly increased the levels of hepatic GSH of 48 h fasted rats to values similar to those found in fed controls.

Table 2 shows that oral administration of L-methionine induced a depletion of hepatic ATP only in fasted rats. Since L-methionine is an excellent GSH precursor (Viña *et al.* 1978), a small dose of methionine might be enough to promote GSH synthesis without depleting ATP. It should be pointed out that the same amount of methionine and GSH in terms of g/kg body-weight was used but that this represents a twofold molar excess of methionine over GSH (see methods). In contrast with methionine, oral GSH did not cause a significant depletion of hepatic ATP either in fed or in starved rats except 24 h after the administration of GSH to fed rats. In this case an ATP depletion was observed. We cannot explain this particular result.

*Effects of oral GSH on the levels of hepatic GSH after the administration of diethylmaleate*

Table 3 shows that oral administration of GSH, methionine or *N*-acetylcysteine promotes GSH synthesis in liver after depletion with diethyl maleate. GSH replenishment is slower when GSH is given as a precursor than with the other two compounds used. This is probably due to the fact that oral GSH gives rise to cysteine slowly (see Discussion).

Apart from a decrease in GSH, no other hepatotoxic effects of diethyl maleate were observed. Hepatic ATP levels and blood alanine aminotransferase (*EC* 2.6.1.2) were similar in controls, in mice injected with diethyl maleate and in mice injected with diethyl maleate plus GSH (values not shown).

*Protection against paracetamol hepatotoxicity by oral GSH*

Table 4 shows that oral GSH partially prevented the fall in GSH levels after treatment of mice with paracetamol.

It was reported that paracetamol overdose promotes glycogen depletion (Hinson *et al.* 1983). We have confirmed this observation and found that when paracetamol was administered together with oral GSH the hepatic glycogen levels were always higher than those found in animals treated with paracetamol alone. A good indication of hepatic cellular damage is the leakage of cytosolic enzymes to blood plasma. Table 4 shows that oral GSH prevented the increase in alanine aminotransferase activity in plasma subsequent to paracetamol administration. Furthermore, 24 h after administration of paracetamol all the six mice had died. However, when paracetamol was given together with oral GSH 67% of the mice survived. *N*-acetylcysteine and methionine had similar protective effects against paracetamol overdose. It is worthwhile emphasizing that no animals died except in the group treated 24 h with paracetamol alone.

*Requirement of GSH synthesis to explain the increase in hepatic GSH after oral administration of GSH*

The effect of oral GSH on the hepatic concentration of GSH could be due to: (1) a direct absorption of the tripeptide from the intestine which could be transported via the portal vein and taken up by the liver or (2) a degradation to the constituent amino acids by the combined action of  $\gamma$ -glutamyl transpeptidase (present in intestinal brush border cells) and

Table 1. *Effect of oral methionine, N-acetylcysteine (N-Ac-cys) or glutathione (GSH) on the concentrations of hepatic GSH ( $\mu\text{mol/g}$  fresh wt) in rats fasted for 48 h*  
(Mean values with their standard errors; no. of observations in parentheses)

Oral treatment... Period after treatment (h)	Saline† (control) (6)		Methionine (5)		N-Ac-cys (5)		GSH (4)	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
2.5	3.1	0.2	5.9*	0.3	3.4	0.4	5.8*	0.2
10	3.0	0.1	4.9*	0.3	5.4*	0.3	4.2*	0.3
24	3.1	0.2	5.2*	0.2	5.9*	0.3	7.0*	0.3

The animals were killed at the indicated period after treatment. Hepatic GSH concentration in fed rats was 5.7 (SE 0.4) ( $n$  6)  $\mu\text{mol/g}$ .

Mean values were significantly different from control values: \* $P < 0.05$ .

† 9 g Sodium chloride/l.

Table 2. *Effect of oral methionine and glutathione (GSH) on hepatic ATP concentrations ( $\mu\text{mol/g}$  fresh wt) in fed rats and rats fasted for 48 h*  
(Mean values with their standard errors; no. of observations in parentheses. The animals were killed at the indicated period after treatment)

Oral treatment... Period after treatment (h)	Saline† (control)		Methionine		GSH	
	Mean	SE	Mean	SE	Mean	SE
Fed rats						
2.5	1.9	0.1 (3)	1.7	0.1 (6)	2.7*	0.1 (6)
10	2.0	0.1 (9)	1.6*	0.1 (4)	2.1	0.2 (5)
24	1.7	0.1 (6)	—	—	1.1*	0.2 (5)
Fasted rats						
2.5	1.2	0.1 (5)	0.7*	0.1 (5)	1.3	0.2 (4)
10	1.0	0.2 (6)	0.7	0.1 (5)	1.3	0.3 (4)
24	1.4	0.2 (4)	0.6*	0.1 (4)	1.6	0.2 (4)

Mean values were significantly different from control values: \* $P < 0.05$ .

† 9 g Sodium chloride/l.

Table 3. *Effects of oral glutathione (GSH), methionine or N-acetyl-cysteine (N-Ac-cys) on hepatic GSH concentrations ( $\mu\text{mol/g}$  fresh wt) in mice treated with diethyl maleate (DEM)*  
(Mean values with their standard errors; no. of observations in parentheses. The animals were killed at the indicated period after treatment)

Oral treatment... Period after treatment (h)	DEM		DEM + methionine		DEM + N-Ac-cys		DEM + GSH	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
2.5	1.4	0.1 (8)	5.8*	0.3 (6)	6.4*	0.2 (4)	2.3*	0.1 (6)
10	2.7	0.2 (6)	7.5*	0.5 (6)	7.7*	0.2 (5)	7.0*	0.2 (5)
24	4.4	0.2 (4)	6.6*	0.2 (5)	4.4*	0.2 (5)	5.2*	0.1 (6)

Mean values were significantly different from control values: \* $P < 0.05$ .

Table 4. Protection against paracetamol toxicity (900 mg/kg body-weight) afforded by glutathione (GSH), methionine or N-acetylcysteine (1 g/kg body-weight)

(Mean values with their standard errors; no. of observations in parentheses. The animals were killed at the indicated period after treatment)

Period after treatment (h)	GSH ( $\mu\text{mol/g}$ fresh wt)		Glycogen ( $\mu\text{mol}$ of glucose equivalent/g fresh wt)		AAT (i.u.)	
	Mean	SE	Mean	SE	Mean	SE
Paracetamol						
2.5	0.9	0.1 (16)	43	3 (11)	27	2 (5)
10	1.7	0.2 (6)	4	1 (4)	1503	123 (5)
24	100% of the mice injected had died (6)					
Paracetamol + GSH						
2.5	1.4*	0.1 (11)	16*	3 (6)	14*	1 (5)
10	5.6*	0.3 (6)	46*	9 (6)	21*	1 (5)
24	5.7	0.3 (6)	86	9 (5)	28	4 (5)
Paracetamol + N-acetylcysteine						
2.5	2.5*	0.2 (6)	4*	1 (6)	19**	3 (5)
10	5.8*	0.2 (6)	88*	9 (6)	49*	16 (5)
24	7.0	0.4 (4)	132	14 (4)	21	31 (5)
Paracetamol + methionine						
2.5	2.9*	0.2 (6)	38	3 (6)	18*	2 (4)
10	4.4*	0.2 (7)	27*	2 (7)	34*	3 (3)
24	7.8	0.3 (5)	118	14 (4)	33	6 (4)

AAT, alanine aminotransferase (EC 2.6.1.2).

Mean values were significantly different from control (paracetamol alone) values: \* $P < 0.05$ , \*\* $P < 0.01$ .

The values found in untreated mice were: GSH: 5.7 (SE 0.4), (n 6); glycogen: 99 (SE 17), (n 6) and AAT: 28 (SE 3), (n 6).

intestinal dipeptidases. In this case the free amino acids could be absorbed from the intestine and taken up by the liver. This requires active GSH synthesis by the liver.

The first hypothesis is unlikely because GSH itself does not enter the hepatocytes (Hahn *et al.* 1978). Thus, we tested the second hypothesis, i.e. that GSH is converted to its constituent amino acids in the intestine and that these are transported to the liver where active GSH synthesis is promoted. Since the availability of cysteine is a rate-limiting step for GSH synthesis in liver (Tateishi *et al.* 1974), the concentration of cysteine was measured in portal blood plasma of 48 h fasted rats and a value of 0.09 (SE 0.04)  $\mu\text{mol/ml}$  (n 10) was found. However, in fasted rats, 1 h after the administration of oral GSH (1 g (3.26 mmol)/kg body-weight), the concentration of cysteine in their portal blood plasma was 0.21 (SE 0.05) (n 14). When rats were treated with diethyl maleate their hepatic GSH level fell to 1.6 (SE 0.6)  $\mu\text{mol/g}$  (n 4), but when rats pretreated with diethyl maleate were given oral GSH (3.26 mmol/kg body-weight.) their hepatic GSH concentration was increased, as expected, to a value of 3.8 (SE 1.0)  $\mu\text{mol/g}$  (n 4). When rats treated with diethyl maleate were given 3.26 mmol/kg body-weight of oral GSH and 5  $\mu\text{mol/kg}$  body-weight of buthionine sulphoximine as an inhibitor of GSH synthesis (Griffith & Meister, 1979), their hepatic GSH concentration was 0.21 (SE 0.15)  $\mu\text{mol/g}$  (n 4). The fact that in the presence of an inhibitor of GSH synthesis administration of oral GSH does not serve to increase the levels of hepatic GSH, shows that the increase in hepatic GSH levels after the administration of oral GSH is dependent on active GSH synthesis.

## DISCUSSION

*Ability of oral GSH to serve as a precursor for hepatic GSH. Comparison with other precursors*

Intracellular GSH protects the cells against several agents that are potentially harmful, such as hydroperoxides (Chance *et al.* 1979), xenobiotics (Orrenius & Moldéus, 1984) or ionizing radiations (Révész & Edgren, 1982). In many cases, cellular GSH levels are lowered and to restore these levels may be very important for cell survival. This is the case in fasting which decreases the hepatic levels of GSH. Fasted animals are more susceptible to hepatic damage by drug overdosage than fed animals. A major reason for this susceptibility is the fall in GSH levels. Here we show that administration of oral GSH increases the hepatic levels of GSH in fasted rats.

In liver, L-cysteine availability is the limiting factor for GSH synthesis (Tateishi *et al.* 1974); therefore, the supply of this amino acid is essential to restore the physiological levels of GSH. However, administration of free L-cysteine is dangerous because of its toxicity. The toxicity of this amino acid has been demonstrated in several types of cells and organs such as brain (Olney *et al.* 1972; Viña *et al.* 1983a) and liver (Viña *et al.* 1980). In isolated hepatocytes, we observed that incubation with L-cysteine promotes a decrease in GSH levels (Viña *et al.* 1978) and several other signs of cytotoxicity. These side effects are due to very rapid auto-oxidation (Viña *et al.* 1983b) which gives rise to free radicals (Saez *et al.* 1982). The toxic effects of L-cysteine do not occur when the oxidation rate of the amino acid is maintained very low (Beatty & Reed, 1980); therefore, it is important that L-cysteine reaches the liver slowly as is the case after the administration of oral GSH. All these factors explain why the cell accumulates free thiols as GSH, which acts as a reservoir of L-cysteine (Tateishi *et al.* 1977).

The rate of auto-oxidation of *N*-acetylcysteine is much lower than that of free L-cysteine. Thus, *N*-acetylcysteine is much less harmful than L-cysteine. Intraperitoneal administration of *N*-acetylcysteine may induce GSH depletion (Estrela *et al.* 1983) but oral *N*-acetyl-L-cysteine will not. Oral, but not intraperitoneal, *N*-acetyl-L-cysteine protects against paracetamol intoxication in rats (Lauterburg *et al.* 1983). The use of oral *N*-acetylcysteine in the treatment of paracetamol overdose in humans has been very successful (Smilkstein *et al.* 1988). This is due to the fact that the acid gastric pH maintains *N*-acetylcysteine in the reduced state. Then it is absorbed and reaches the liver slowly via the portal vein, promoting GSH synthesis. As with L-cysteine, if a large amount of *N*-acetyl-L-cysteine reaches the liver it may cause GSH depletion. This may be the case with intraperitoneal *N*-acetyl-L-cysteine but not with oral *N*-acetyl-L-cysteine (Estrela *et al.* 1983).

L-Methionine is very effective in promoting GSH synthesis in isolated hepatocytes (Viña *et al.* 1978) and protects the liver against paracetamol overdosage (Vale *et al.* 1981), but may be toxic. Hardwick *et al.* (1970) and Harper *et al.* (1970) reviewed the toxic effects of L-methionine and reported that administration of this amino acid may lead to ATP depletion in the liver. We have observed that administration of large doses of L-methionine promotes a depletion in hepatic ATP levels in fasted rats.

Free GSH does not enter the cells (Hahn *et al.* 1978). The levels of GSH in cells may be increased, however, by the administration of liposomally entrapped GSH. This procedure is effective but liposomally entrapped GSH is not readily available and requires intravenous administration. Recently, it was reported that GSH monoethyl ester enters the cells. The ester bond is readily hydrolysed within the hepatocytes yielding free GSH (Anderson *et al.* 1985). We have synthesized the ester and observed that incubation of isolated hepatocytes with this substance results in an elevation of the intracellular GSH levels (V. Catalán,

J. F. Llopis & J. Viña, unpublished results). However, to our knowledge, this ester is not commercially available and its safety has not been proved.

*The mechanism of the effect of oral GSH on hepatic GSH*

To study the mechanism of the increase in hepatic GSH after oral administration of GSH, the portal levels of L-cysteine in control rats and in rats after the administration of GSH were measured and in the latter case they were found to increase. Thus, an explanation for the effectiveness of oral GSH in increasing the hepatic levels of GSH is that oral GSH is converted to its constituent amino acids by the concerted action of  $\gamma$ -glutamyl transferase and intestinal dipeptidases. The increase in hepatic GSH after oral GSH administration requires active GSH synthesis in the liver. Indeed, when rats were given oral GSH and buthionine sulfoximine (an inhibitor of hepatic GSH synthesis), oral GSH did not promote an increase in hepatic GSH levels (see Results).

Thus, oral GSH can be considered as a safe precursor of L-cysteine, because it generates the amino acid slowly through the combined action of intestinal  $\gamma$ -glutamyl transpeptidase and  $\alpha$ -dipeptidases. 2-Oxothiazolidine carboxylate is another safe precursor for GSH synthesis (Williamson & Meister, 1981) because it generates L-cysteine slowly, i.e. through the enzymic action of 5-oxoprolinase (EC 3.5.2.9).

The main fact reported here, i.e. that oral administration of GSH increases the intracellular levels of GSH, may explain in part some results by other authors who have observed that dietary GSH supplementation reverses the age-associated decline in immune response in mice (Furukawa *et al.* 1987). Furthermore, since oral GSH may be used to protect the liver against, for instance, paracetamol overdose, the facts reported here may have practical importance.

The authors thank Mrs Juana Belloch and Miss E. Gascó for their excellent technical assistance. This work was supported by grants of the Fondo de Investigaciones Sanitarias de la Seguridad Social (FISS 87/1415) and from the Comisión Asesora de Investigación Científica y Técnica (DEP 88-483) to J.V. and from the Comisión Asesora Científica y Técnica (CAICYT-Ref. 0888-84) to J.R.V.

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