Maintenance of Glutathione Content in Isolated Hepatocytes

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1. During the standard procedure for the preparation of rat hepatocytes, about half of the cellular GSH (reduced glutathione) is lost. 2. This loss is prevented by the addition of 0.1 mM-EGTA (but not EDTA) to the perfusion medium. 3. On incubation with and without EGTA, isolated hepatocytes prepared in the presence of EGTA lose GSH. This loss is prevented by near-physiological concentrations of methionine or homocysteine, but not of cysteine. 4. Cysteine, at concentrations above 0.2 mM, causes a loss of GSH, probably by non-enzymic formation of a mixed disulphide. 5. Serine together with methionine or homocysteine increases GSH above the value in cells from starved rats *in vivo*. This is taken to suggest that cystathionine may be a cysteine donor in the synthesis of γ -glutamylcysteine, the precursor of GSH.

Isolated hepatocytes prepared by the principle of Berry & Friend (1969) (treatment of the isolated perfused liver with collagenase) have proved a valuable material in the study of liver metabolism. The cells, however, still have shortcomings. One is their inability to synthesize glycogen from glucose when the external concentration of glucose is of the order of, say, 10 mM, in contrast with the liver *in vivo*, which readily deposits glycogen at this glucose concentration. According to previous experiences, subnormal performance can be due to loss of low-molecular-weight cell constituents, such as glutamate (Cornell *et al.*, 1974) or methionine (Krebs *et al.*, 1976). We therefore decided to test systematically for loss of other low-molecular-weight cell constituents. The present paper is concerned with the intracellular concentrations of GSH. During the standard procedure for preparing hepatocytes in this laboratory (Krebs *et al.*, 1974) the concentration of GSH was found to decrease. This can be prevented by the addition of 0.1 mM-EGTA (but not of EDTA) to the perfusion medium. However, cells prepared in the presence of EGTA lose GSH when incubated in the presence of EGTA in the standard incubation medium. The present paper reports that physiological tissue concentrations of methionine (0.2 mM) enable hepatocytes to maintain their GSH.

(Oxoid Ltd., London S.E.1, U.K.). Fed rats had access to food *ad libitum*. Starved rats were without food for 48 h but had free access to water. All experiments were started between 09:00 and 10:00 h to avoid circadian variations of thiol concentrations (see Beck *et al.*, 1958).

Preparation of hepatocytes

Two procedures were used, based on the principle of Berry & Friend (1969), referred to below as methods 1 and 2. Method 1 was that described by Cornell *et al.* (1974) and Krebs *et al.* (1974). Method 2 was essentially the modification introduced by Seglen (1972). The liver was first perfused for 12 min with 150 ml of the Krebs–Henseleit (1932) medium containing no Ca^{2+} . After 12 min, collagenase (30 mg) and 0.11 M- CaCl_2 solution (2.5 ml) were added to the perfusion medium, and the perfusion was continued for another 12 min. The remainder of the procedure was as described by Krebs *et al.* (1974).

GSH was measured in the whole-cell suspension. Tests showed that the supernatant after rapid centrifugation did not contain any GSH. The measured GSH was therefore taken to be located within the hepatocytes.

Reagents

GSH, GSSG, collagenase and the enzymes and coenzymes used for analyses were obtained from Boehringer Corp. (London), Lewes, Sussex BN7 1LG, U.K. Amino acids, EGTA and EDTA were obtained from British Drug Houses, Poole, Dorset, U.K., and methylglyoxal was from Sigma (London) Chemical Co., Kingston upon Thames, Surrey KT2 7BH, U.K.

Materials and Methods

Animals

Female rats of the Wistar strain were fed on Oxoid pasteurized breeding diet for rats and mice

Abbreviations used: GSH, reduced glutathione; GSSG, oxidized glutathione.

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Analytical methods

GSH was measured by the glyoxalase method of Racker (1951). The conditions of the assay were those described by Bernt & Bergmeyer (1974), except that EDTA (0.3 mM final concentration) was added.

GSSG was measured by the decrease in A_{340} caused by the oxidation of NADPH by GSSG in the presence of glutathione reductase (Rall & Lehninger, 1952). The assay conditions were those of Bernt & Bergmeyer (1974). All measurements were made in duplicates, which agreed within 10%. The results are expressed as means $\pm$ S.E.M., with the numbers of observations in parentheses.

Results

Reduced and oxidized glutathione in freeze-clamped rat liver

The concentration of GSH in freeze-clamped livers (Wollenberger *et al.*, 1960) from fed rats was $5.31 \pm 0.15 \mu\text{mol/g}$ wet wt. of liver (six observations). In starved animals the concentration of GSH was $2.68 \pm 0.02 \mu\text{mol/g}$ (four observations). A similar decrease on starvation has been previously observed by Tateishi *et al.* (1974). Leaf & Neuberger (1947) observed a decrease in GSH concentration in liver when rats were fed with a low-protein diet.

The concentration of GSSG in freeze-clamped livers from fed rats was $0.27 \pm 0.05 \mu\text{mol/g}$ (six observations). This value is much higher than expected on the assumption that the glutathione reductase system is near equilibrium *in vivo*. Given the equilibrium constant of glutathione reductase (EC 1.6.4.2)

$$\frac{[\text{NADP}^+][\text{GSH}]^2}{[\text{NADPH}][\text{GSSG}][\text{H}^+]} = 9.8 \times 10^8$$

(Scott *et al.*, 1963) and the ratio [free NADP⁺]/[free NADPH] of 10^{-2} at pH 7.0 (Veech *et al.*, 1969), the expected equilibrium concentration of GSSG at pH 7 would be below 10 nM. The discrepancy between the expected and found values, of four orders of magnitude, raises the question whether the high GSSG concentrations found are the result of an artifact, namely oxidation of GSH *post mortem* (see Veech *et al.*, 1969). The following tests make this unlikely. Storing the EDTA-containing HClO₄ extract of freeze-clamped rat liver at 0°C or at room temperature (22°C) did not change the GSH or GSSG content. Storing the extract after neutralization with K₂HPO₄ to pH 7.0 caused a slow linear increase in the GSSG concentration of about 50% in 100 min. As the assay of GSSG was done within less than 5 min, errors due to post-neutralization gain were less than 2.5%, i.e. negligible. No loss is expected during freeze-clamping (which took place within

10 s of the death of the animal) and during the extraction with HClO₄. This leads to the conclusion that the glutathione reductase system is not at equilibrium *in vivo*. This may be explained by the relatively low maximum capacity of the enzyme (only 2.5% of lactate dehydrogenase, 5% of glutamate dehydrogenase and 28% of isocitrate dehydrogenase in rat liver, according to Veech *et al.*, 1969) and to the relatively high K_m , 50 μM (Mize & Langdon, 1962), which is several orders of magnitude above the equilibrium concentration in the liver.

GSH in isolated hepatocytes

In isolated hepatocytes from fed rats prepared by method 1 the concentration of GSH was $3.30 \pm 0.12 \mu\text{mol/g}$ wet wt. (four observations). After 60 min incubation, the concentration of GSH had fallen to $2.47 \mu\text{mol/g}$ wet wt. (mean of two values). Addition of lactate (10 mM), oleate (1 mM) or glutamine (5 mM), with or without EDTA (0.1 mM), had no effect on this fall. The presence of EDTA (0.1 mM) in the perfusion medium was also without effect. Nor had EDTA any effect when method 2 was used. But when cells were prepared by method 2 with EGTA (0.1 mM final concentration) the initial concentration of GSH was only slightly lower than that in freeze-clamped liver, $4.70 \pm 0.18 \mu\text{mol/g}$ wet wt. (20 observations). However, cells failed to maintain the GSH concentration on incubation in the presence of EGTA. Addition of glucose (5 or 10 mM), glutamine (10 mM), ethanol (10 mM), dithiothreitol (0.2 mM), lysine (2 mM) or histidine (2 mM) did not prevent a loss.

Table 1. Effect of methionine on the maintenance of GSH concentration in isolated hepatocytes from fed rats after 60 min incubation

The cells were prepared by method 2 with 0.1 mM-EGTA added. The initial concentration of GSH was $4.38 \pm 0.32 \mu\text{mol/g}$ wet wt. The significance test refers to the comparison of GSH concentration in the absence and presence of methionine. All values are means $\pm$ S.E.M. for three observations.

[Methionine] added (mM)	[GSH] after 60 min incubation (mM)	Significance by Student's <i>t</i> test (<i>P</i> for paired samples)	Percentage of initial [GSH] left after 60 min incubation
1.0	4.10 ± 0.65	<0.0025	95
0.50	3.80 ± 0.51	<0.01	87
0.20	3.87 ± 0.40	<0.05	89
0.10	2.94 ± 0.24	<0.10	67
0.050	2.56 ± 0.42	<0.20	59
0.025	2.65 ± 0.48	<0.15	61
0	2.46 ± 0.33		57

Methionine and homocysteine were the only substances that maintained the concentration of GSH during an incubation period of 60min. Ethionine was inactive. Methionine was effective at concentrations of 0.2mM or above (Table 1). The normal concentration of methionine in the plasma is about 0.04mM and in the liver about 0.1mM (Schimassek & Gerok, 1965). Homocysteine was effective at somewhat lower concentrations, 0.12mM being sufficient to maintain the initial GSH concentration (Table 2).

Effect of cysteine on GSH concentration. Tateishi *et al.* (1974) suggested that cysteine is a limiting factor in the synthesis of GSH *in vivo*. It was therefore expected that the addition of cysteine might raise the GSH concentration in the cells. However, contrary to the expectation, addition of cysteine to the

medium, even at 0.25mM, the approximate tissue concentration of cysteine (Tateishi *et al.*, 1974), depleted the cells of GSH. At the plasma concentration of about 0.02mM, cysteine had no effect (Table 3). According to Jocelyn (1967), mixed disulphides are readily formed non-enzymically when solutions of GSSG and cysteine or GSH and cystine are mixed at pH 7.4 and 37°C. As some of the added cysteine is likely to be oxidized to cystine, both types of interaction may occur on addition of cysteine. This would account for the loss of GSH.

GSH in hepatocytes of starved rats. As mentioned above, the concentration of GSH in freeze-clamped liver from 48h-starved rats is about half that in livers from fed rats (2.68 compared with 5.32 $\mu\text{mol/g}$). A similar difference was found in isolated hepatocytes, where the respective values were 2.17 and 4.70 $\mu\text{mol/g}$. After 60min incubation, the GSH concentration in the hepatocytes had fallen to 1.46 $\mu\text{mol/g}$. As shown in Table 4, addition of 0.2mM-methionine prevented this fall. Attempts to restore GSH concentration to the value in fed cells by the addition of precursors of GSH showed that methionine plus serine increased GSH significantly, but not to the value in fed cells (Table 4). Cysteine caused a fall in GSH concentration, presumably by the formation of mixed disulphides.

Glycine and glutamine had no effect on GSH concentration (not shown in Table 4). Glutamine rather than glutamate was added because glutamine enters the hepatocytes more readily than glutamate and is converted into the latter.

Relations between metabolic performance and GSH content of hepatocytes. The rates of gluconeogenesis from lactate and of ketone-body formation from oleate were not affected by the addition of methionine plus EGTA, although GSH was maintained at higher concentrations. Nor was the stability of

Table 2. *Effect of homocysteine on the maintenance of the concentration of GSH in isolated hepatocytes from fed rats after 60min incubation*

The cells were prepared by method 2, with 0.1 mM-EGTA added. The initial GSH concentration was $4.43 \pm 0.24 \mu\text{mol/g}$ wet wt. The significance test refers to the comparison of GSH concentration in the absence and presence of homocysteine. All measurements are means $\pm$ S.E.M. for three observations.

[Homocysteine] added (mM)	[GSH] after 60min incubation (mM)	Significance by Student's <i>t</i> test (<i>P</i> for paired samples)	Percentage of initial [GSH] left after 60min incubation
1.000	3.86 ± 0.08	<0.01	87
0.500	3.93 ± 0.30	<0.05	88
0.250	3.67 ± 0.08	<0.0025	82
0.120	3.56 ± 0.52	<0.10	80
0.062	3.47 ± 0.57	<0.15	78
0.031	2.93 ± 0.09	<0.20	66
0	2.77 ± 0.14		62

Table 3. *Effect of cysteine on GSH concentration in isolated hepatocytes from fed rats after 60min incubation*

The cells were prepared by method 2, with 0.1 mM-EGTA added. The initial GSH concentration in the cells was 4.30mM. Results of a representative experiment are shown.

[Cysteine] (mM)	[GSH] after 60min incubation (mM)	Percentage of initial [GSH] left after 60min incubation
4	0	0
2	0.87	20
1	1.75	41
0.5	2.10	49
0.25	2.24	52
0	3.00	70

Table 4. *Effect of methionine and serine on GSH concentration in liver cells from starved rats after 60min incubation*

The cells were prepared by method 2, with 0.1 mM-EGTA added. The initial GSH concentration was 2.17 ± 0.11 (eight observations) $\mu\text{mol/g}$ wet wt.

Substrate added	[GSH] ($\mu\text{mol/g}$ wet wt.)	Percentage of initial [GSH] after 60min incubation
None	1.46 ± 0.10 (7)	67
Serine (2mM)	1.64 ± 0.08 (3)	75
Methionine (0.2mM)	2.24 ± 0.08 (5)	103
Methionine (2mM)	2.32 ± 0.12 (7)	106
Methionine (2mM) + serine (2mM)	3.29 ± 0.09 (3)	151
Methionine (0.2mM) + serine (2mM)	2.68 ± 0.09 (3)	123

glycogen affected by higher GSH concentration. The percentage of cells permeable to succinate (Mapes & Harris, 1975) or to Trypan Blue was also the same in the presence and absence of methionine plus EGTA.

Discussion

Cause of loss of GSH during the preparation of isolated hepatocytes

The loss of GSH on perfusion of the liver or on incubation of hepatocytes in the suspension medium may be explained on the basis of the finding by Sies *et al.* (1972) and Sies & Summer (1975) that, on peroxidation of GSH in the perfused liver, GSSG diffuses into the medium. This indicates that hepatocytes cannot retain GSSG, in contrast with GSH. The fact that the loss of GSH is prevented by EGTA, but not by EDTA, can be explained by differences in the stability constant of the magnesium complexes of the two chelating agents. The concentration of these agents was 0.1 mM and the concentration of total Mg was 1 mM. It can be calculated that under these conditions virtually all EDTA is complexed to Mg, although there is sufficient free EGTA to complex the heavy metals (Fe, Cu, Mn) that catalyse the oxidation of GSH by O₂. The stabilizing effect of EGTA has been reported by Högborg & Kristoferson (1977).

Nature of the effects of methionine, homocysteine and serine

The relevant facts are: (1) cysteine does not promote the maintenance of GSH, in contrast with methionine and homocysteine; (2) homocysteine is slightly more effective than methionine; (3) serine increases the effect of methionine or homocysteine. These facts may be taken to suggest that cystathionine may be a more effective cysteine donor than free cysteine in the synthesis of γ -glutamylcysteine (catalysed by γ -glutamylcysteine synthetase).

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