Measurement of Oxidized Glutathione and Total Glutathione in the Perfused Rat Heart

By P. L. WENDELL

Department of Biochemistry, University of Bristol, University Walk, Bristol BS8 1TD, U.K.

(Received 12 January 1970)

1. A method was developed for the assay of GSSG in heart tissue. 2. GSSG and total glutathione were measured in rat hearts perfused under a variety of conditions. About 2% of the total glutathione is present as GSSG. The concentrations of GSSG and GSH remained constant under all the conditions tested. 3. These results are discussed with reference to the equilibrium and rate of the glutathione reductase reaction in the cell. It is concluded that the enzyme reaction does not lie near equilibrium.

The tripeptide GSH is known to be widely distributed among living cells, where it is present at concentrations up to 10 mM (Bhattacharya, Robson & Stewart, 1955). Although a number of reactions involving GSH have been described, no convincing evidence has been provided of any metabolic role sufficient to justify this distribution. It is known that glutathione reductase (EC 1.6.4.2), which is also widely distributed, maintains glutathione largely in its reduced form, but no accurate determination of the tissue concentration of GSSG had previously been made, so that the value of the [GSSG]/[GSH] ratio was unknown. It was therefore impossible to judge whether the glutathione reductase reaction is close to or far removed from equilibrium in the cell.

It was therefore decided to measure the concentrations of GSSG and of GSH in an intact tissue, and to calculate the [GSSG]/[GSH] ratio. It was also decided to see whether changes in [GSSG]/[GSH] could be correlated with changes in any other metabolic parameter, in particular the [NADP⁺]/[NADPH] ratio (since NADPH is the reductant in the glutathione reductase reaction).

A number of reliable methods are available for assaying the relatively high concentrations of GSH in tissues. Estimates of GSSG concentrations, however, have been variable, and have indicated that only a small percentage of the total glutathione may exist in this form. Measurement of GSSG as the small difference in the amounts of GSH assayable before and after reduction has thus led to standard deviations larger than the calculated GSSG concentrations (Martin & McIlwain, 1959; Jocelyn, 1960). It is also clear that oxidation of a small proportion of the GSH to GSSG would cause large errors in the measurement of the latter. This

could account for higher GSSG concentrations being reported in blood than in other tissues (Bhattacharya *et al.* 1955).

These two difficulties had been overcome, for measurement of erythrocyte GSSG, by Güntherberg & Rost (1966). They prevented oxidation of GSH by making it react with NEM,* and measured GSSG directly, using glutathione reductase, after removal of excess of NEM (which is a potent inhibitor of the enzyme). The removal of NEM had to be effected by repeatedly washing the intact erythrocytes. Güntherberg & Rost (1966) were therefore unable to apply this method to the measurement of GSSG in other tissues.

The present paper reports the development of an assay for GSSG in tissue extracts, and its application to the measurement of GSH and GSSG concentrations in rat hearts perfused under a variety of conditions.

EXPERIMENTAL

Materials

Rats. Adult male albino rats (Wistar or Sprague-Dawley as indicated) were used. They were allowed free access to water and to stock laboratory diet 41 B (Oxoid Division, Oxo Ltd., London S.E.1, U.K.). Alloxan-diabetes was induced 46 h before the animals were used by intravenous administration (60 mg/kg) under ether anaesthesia. The animals had blood glucose concentrations in excess of 2 mg/ml; this was ascertained with Dextrostix (Ames Co. Ltd., Stoke Poges, Slough, Bucks., U.K.) before perfusion of hearts.

Chemicals. GSH, GSSG, yeast glutathione reductase (1 mg/ml, 70 units/mg) and NADPH were from Boehringer Corp. (London) Ltd., London W.5, U.K. GSSG solutions

* Abbreviations: NEM, *N*-ethylmaleimide; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid).

were standardized from the fall in E_{340} due to the oxidation of NADPH on addition of glutathione reductase. The GSSG supplied was found on this basis to be 85% pure by weight. The nature of the impurity (which is not removed by drying at 105°C overnight) is not known to the suppliers. Standard GSSG solutions were used to calibrate the total glutathione assay.

Alloxan was from Eastman Kodak Ltd., Rochester, N.Y., U.S.A.; NEM and DTNB were from the Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. DTNB (0.1 M) was dissolved in 0.1 M-potassium phosphate-2 mM-EDTA buffer, pH 7.0, to which NaHCO_3 (1.5 mg/ml) had been added after the pH adjustment, and this stock solution was stored in the deep-freeze. Heparin (from Evans Medical Ltd., Speke, Liverpool, U.K.) was dissolved in 0.9% NaCl to 2500 units/ml and diluted with an equal volume of veterinary Nembutal solution (Abbott Laboratories Ltd., Queenborough, Kent, U.K.) (60 mg/ml). Crystalline ox insulin (given by Boots Pure Drug Co. Ltd., Nottingham, U.K., or Burroughs Wellcome, Beckenham, Kent, U.K.) was dissolved in 3.3 M-HCl to yield a stock solution of 20 units/ml. Dowex 50 (AG 50 W-X2; 200-400 mesh; H^+ form), obtained from Bio-Rad Laboratories, Richmond, Calif., U.S.A., was washed with water until the eluate was colourless.

Media. Hearts were perfused with a bicarbonate-buffered medium (Krebs & Henseleit, 1932) gassed with either $\text{O}_2 + \text{CO}_2$ (95:5) (aerobic) or with $\text{N}_2 + \text{CO}_2$ (95:5) (anaerobic).

Methods and procedure

Heart perfusions. Rats (150-250 g) were injected intraperitoneally with Nembutal (15 mg) and heparin (625 units) about 5 min before hearts were removed and perfused by drip-through as described by Morgan, Henderson, Regen & Park (1961). Perfusions were for 8 min.

Preparation of heart extracts. Hearts were frozen at -70°C with the tissue clamp of Wollenberger, Ristau & Schoffa (1960), and were powdered in a percussion mortar kept at -70°C . For measurements on perfused hearts the powder from two hearts was pooled. For recovery experiments the powder from all hearts was pooled and mixed to homogeneity. Heart powder was extracted, by using a ground glass pestle and mortar, in the media described below.

Total water. This was determined by weighing a sample of the heart powder before and after freeze-drying.

Assay of total glutathione. Total glutathione was assayed by the method of Owens & Belcher (1965), in which glutathione cycles continuously between its oxidized and reduced forms, reduction of GSSG being effected by NADPH and glutathione reductase, and non-enzymic oxidation of GSH being effected by DTNB (Scheme 1). The ion of 5-carboxy-4-nitrothiophenol thus produced absorbs at 412 nm ($\epsilon 13.6 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$; Ellman, 1959). The reaction rate becomes linear (as a dynamic equilibrium between the two forms of glutathione is established) within 1 min and remains linear for several minutes. Within a range of glutathione concentrations (70-350 nM-GSSG under the experimental conditions described below) the rate of increase of extinction at 412 nm is linearly related to the concentration of glutathione present.

The procedure was as follows. About 0.2-0.3 g of heart powder was extracted with 2 ml of 6% (w/v) HClO_4 and centrifuged at 2000g for 10 min. A 10 μl portion of the supernatant in a cuvette volume of 2.5 ml was usually a suitable quantity for assay. The reaction was followed at 412 nm by using a 1 cm light-path cell in a Hilger-Gilford recording spectrophotometer. The assay medium (at pH 7.0) contained glutathione reductase (0.1 unit/ml), 100 μM -DTNB, 0.1 M-potassium phosphate buffer, 2 mM-EDTA and 50 μM -NADPH (added to start the reaction). Since the rate depends on the concentration of glutathione reductase, this must be diluted into solution (in 0.1 M-potassium phosphate-2 mM-EDTA buffer, pH 7.0) from the suspension in which it is supplied, and a constant amount used throughout a series of assays. Each series of assays was calibrated with standard GSSG, and the amount of extract used was adjusted so that the rate lay within the region in which rate was linearly related to GSSG concentration.

Assay of GSSG. About 1-2 g of heart powder was stirred into 4.0 ml of 0.05 M-NEM-0.1 M-tris hydrochloride-2 mM-EDTA buffer, pH 7.5. Then, 2-2½ min after the powder had been first immersed, 0.4 ml of 70% (w/v) HClO_4 was added, and the suspension centrifuged at 2000g for 10 min. A 3.8 ml portion of the supernatant was applied to a column (8 cm $\times$ 0.6 cm) of Dowex 50 (H^+ form) and allowed to run in under gravity. The column was washed with ten 2.5 ml portions of water under pressure to remove the excess of NEM. GSSG was then eluted with 0.2 M-NaCl-0.1 M-potassium phosphate-2 mM-EDTA buffer, pH 7.5. The position of elution of GSSG was known from prior calibration experiments in which the elution of larger amounts of GSSG had been followed by collecting 1 ml fractions. The pH of the eluate was adjusted to 7.0-7.5 with saturated KHCO_3 , by using a pH-meter. CO_2 bubbles were removed under reduced pressure. GSSG in the neutralized extracts was assayed by observing the fall in E_{340} when glutathione reductase was added in the presence of NADPH. Assays were performed in a final volume of 3.1 ml in a 4 cm-light-path cell read at 340 nm in a Unicam SP.500 spectrophotometer. The cell contained 2.0 ml of neutralized eluate, 1.1 ml of 0.1 M-potassium phosphate-2 mM-EDTA buffer, pH 7.5, and 30 nmol of NADPH; 20 μl (1.4 units) of glutathione reductase was added to start the reaction. Readings were made at intervals of 30 or of 40 s for about 10 min, until

a steady rate of decrease of E_{340} (about 0.002/min) had been attained after the initial rapid fall (about 0.03). This final background rate was extrapolated back to the time of addition of glutathione reductase to calculate the fall in E_{340} due to GSSG in the extract.

All spectrophotometric assays of GSSG and of total glutathione were performed at least in duplicate, and were repeated if satisfactory agreement was not obtained.

Recovery of total glutathione and of GSSG: effect of added GSH on GSSG measurement. The normal extraction and assay procedure was followed, except that the added GSH or GSSG was present in the mortar before addition of heart powder. When the effect of added GSH on GSSG measurement was being determined, GSH (33 mM) was first incubated at room temperature for $\frac{1}{2}$ h with NADPH (750 μ M) and glutathione reductase (0.3 unit/ml) in 0.1 M-tris hydrochloride-2 mM-EDTA buffer, pH 7.5, to ensure complete reduction of the GSH (preliminary measurement of the GSSG in the GSH had shown the above concentration of NADPH to be sufficient).

RESULTS

Recoveries of total glutathione and GSSG: effect of added GSH on GSSG measurement. The amount of glutathione added in recovery experiments was approximately equal to that endogenously present in the quantity of tissue extracted. In all experiments triplicate samples of heart powder, both with and without added glutathione, were extracted and

assayed. Results are expressed as means $\pm$ S.E.M. for three samples.

Recovery of GSSG in two separate experiments was $88 \pm 4\%$ and $109 \pm 2\%$.

When GSH (3 μ mol) (fully reduced) was added and the extract assayed for GSSG, the non-endogenous GSSG was measured as 3 ± 2.2 nmol. The endogenous glutathione measured in the amount of heart powder normally extracted for GSSG assay was: GSSG, 20 nmol; GSH, 0.3 μ mol.

Recovery of total glutathione (added as a GSH solution that would have contained a small percentage of GSSG) was $110 \pm 2\%$.

Concentrations of GSSG and GSH in the perfused rat heart. In the rat heart perfused under a variety of conditions (Table 1) the tissue content of glutathione was: GSH, 7.5–10.5 μ mol/g dry wt.; GSSG, 60–120 nmol/g dry wt. The percentage of glutathione present as GSSG was 1.5–2.7%. The dry weight of aerobically perfused hearts was 13.1% of the wet weight. Assuming aerobically perfused hearts to contain 33 ml of intracellular water/100 g wet wt. (P. J. Randle, D. J. Watts & P. J. England, unpublished work), the corresponding intracellular concentrations (if GSH and GSSG were distributed evenly throughout the intracellular water) would be: GSSG, 30–50 μ M; GSH, 3–4 mM.

The standard errors of measurements on hearts

Table 1. *Effects of glucose + insulin, 3-hydroxybutyrate, alloxan-diabetes and anaerobiosis on the GSSG and GSH content of perfused rat heart*

The concentrations of additions to the perfusion medium, where present, were: glucose, 1 mg/ml; insulin, 0.05 unit/ml; sodium DL-3-hydroxybutyrate, 0.7 mg/ml. Media were gassed with $O_2 + CO_2$ (95:5) (aerobic), except that to obtain anaerobic conditions N_2 was substituted for O_2 . Each measurement was made on two pooled hearts. Results are expressed as means $\pm$ S.E.M. The percentage of glutathione present as GSSG was calculated independently for each pair of hearts. Concentrations are expressed in nmol/g dry wt. Total glutathione is expressed as GSH. All differences from the appropriate controls were insignificant ($P > 0.05$). The breed of rat is indicated by W (Wistar) or S (Sprague-Dawley).

Expt. no.	Breed of rat	State of rat	Additions to perfusion medium	No. of heart pairs	Concn. of glutathione		% of total glutathione as GSSG
					GSSG	Total (as GSH)	
1	W	Normal	None	3	109 $\pm$ 8	8400 $\pm$ 120	2.60 $\pm$ 0.16
			Glucose+insulin	3	93 $\pm$ 6	8290 $\pm$ 110	2.25 $\pm$ 0.13
2	W	Normal	None	3	108 $\pm$ 12	9140 $\pm$ 260	2.35 $\pm$ 0.26
			Glucose+insulin	3	92 $\pm$ 18	8780 $\pm$ 470	2.07 $\pm$ 0.30
3	W	Normal	None	3	108 $\pm$ 13	8220 $\pm$ 210	2.64 $\pm$ 0.29
			3-Hydroxybutyrate	3	105 $\pm$ 11	8180 $\pm$ 370	2.45 $\pm$ 0.22
4	S	Normal	Glucose+insulin	3	118 $\pm$ 18	10300 $\pm$ 170	2.30 $\pm$ 0.36
			Diabetic	3	124 $\pm$ 11	10000 $\pm$ 520	2.52 $\pm$ 0.33
5	S	Normal	Glucose+insulin	2	84 $\pm$ 23	9300 $\pm$ 1150	1.77 $\pm$ 0.29
			Diabetic	3	85 $\pm$ 8	9680 $\pm$ 790	1.75 $\pm$ 0.05
6	S	Normal	Glucose+insulin	3 (aerobic)	103 $\pm$ 10	9610 $\pm$ 180	2.14 $\pm$ 0.19
				3 (anaerobic)	84 $\pm$ 11	9330 $\pm$ 190	1.80 $\pm$ 0.21
7	S	Normal	Glucose+insulin	3 (aerobic)	60 $\pm$ 5	7780 $\pm$ 460	1.54 $\pm$ 0.14
				3 (anaerobic)	71 $\pm$ 4	7920 $\pm$ 170	1.80 $\pm$ 0.05

perfused under the same conditions in a given experiment are small, but the differences between measurements on hearts perfused under the same conditions in different experiments are larger (e.g. aerobic values in Expts. 6 and 7). Within a given experiment, rats were of similar age and weight, and hearts from both control and experimental groups were perfused at the same time. This comparability does not hold between different experiments. Comparisons may therefore be made only between control and experimental groups in the same experiment, not between measurements obtained in different experiments.

The effect was investigated of including in the perfusion medium glucose (1 mg/ml) + insulin (0.05 unit/ml) (Expts. 1 and 2) or 3-hydroxybutyrate (0.7 mg/ml) (Expt. 3). In neither case was there a significant change in the concentration of GSH or of GSSG, or of the percentage of glutathione present as GSSG, in the hearts.

In the remaining experiments all hearts were perfused with media containing glucose + insulin. Hearts from normal rats were compared with hearts from alloxan-diabetic rats (Expts. 4 and 5). By using hearts from normal rats, the effect of anaerobiosis was investigated by gassing the perfusion medium with $N_2 + CO_2$ (95:5) instead of $O_2 + CO_2$ (95:5) (Expts. 6 and 7). Neither alloxan-diabetes nor anaerobiosis caused any change in the concentrations of GSH and GSSG, or in the percentage of glutathione present as GSSG, in the perfused hearts.

DISCUSSION

Measurements of tissue GSSG. All published measurements of tissue GSSG claiming reasonable accuracy have employed NEM to prevent autooxidation of GSH to GSSG. Various methods have been employed to remove excess of NEM, and to measure GSSG. Güntherberg & Rost (1966) treated intact erythrocytes with NEM, and were thus able to remove the excess of NEM by washing the cells before lysis. The concentration of GSSG, determined by direct spectrophotometric assay with glutathione reductase, was $45 \mu\text{mol/l}$ of packed cells. In the present experiments a cation-exchange resin was used to remove the excess of NEM before measurement of GSSG by the direct assay. The intracellular concentration of GSSG in rat heart was calculated to be $30\text{--}50 \mu\text{M}$. Güntherberg & Rapoport (1968) separated GSSG in rat liver extracts from excess of NEM by using columns of Sephadex G-10, and measured GSSG by the recycling assay of Owens & Belcher (1965). They report $30\text{--}40 \mu\text{mol}$ of GSSG/kg of liver, corresponding to an intracellular concentration about $60 \mu\text{M}$.

These values agree as closely as can be expected for measurements made on different tissues. However, Srivastava & Beutler (1967), who removed excess of NEM from lysed erythrocytes by extraction with ether, before measuring GSSG by the direct assay, reported that the concentration of GSSG was below $5 \mu\text{mol/l}$ of erythrocytes. As they do not give any details of the procedure or results of their recovery experiments (beyond stating that the recoveries of added GSSG were 'excellent'), it does not seem certain that ether extraction is a sufficiently effective method of removing excess of NEM.

The method of GSSG measurement, by using the direct assay, reported here is less sensitive than that of Güntherberg & Rapoport (1968), since they used the recycling assay of Owens & Belcher (1965). Güntherberg & Rapoport (1968) found, however, that their prepared extracts were inhibitory in the recycling assay. They attempted to overcome this difficulty by constructing standard curves in the presence of a fixed amount of extract. Their calculation entails the assumption of a linearity of response of their assay system that they were unable to test experimentally. The possibility therefore cannot be excluded that measurements made by this method are subject to systematic error.

Equilibrium and rate of glutathione reductase in perfused rat heart. It is known that $[\text{GSH}]^2[\text{NADP}^+]/[\text{GSSG}][\text{NADPH}]$ is about 10^3 M at pH 7.0 (Rost & Rapoport, 1964). Typical values for intracellular concentrations of glutathione in rat heart (assuming even distribution throughout the intracellular water) are: GSH, 3.5 mM ; GSSG, $40 \mu\text{M}$. If the glutathione reductase reaction were near equilibrium in the cell, the $[\text{NADP}^+]/[\text{NADPH}]$ ratio would therefore have to be 3×10^3 . Since glutathione reductase is present mainly in the cytoplasm of the cell (Rall & Lehninger, 1952), it is the ratio of cytoplasmic nicotinamide nucleotide concentrations that is relevant.

It might be expected that changes in the $[\text{NADP}^+]/[\text{NADPH}]$ ratio in the whole tissue should be reflected in changes in the $[\text{NADP}^+]/[\text{NADPH}]$ ratio in the cytoplasm. If the glutathione reductase reaction were near equilibrium, corresponding changes in the $[\text{GSSG}]/[\text{GSH}]$ ratio would occur. Perfusion of hearts with 3-hydroxybutyrate (Watts, 1969), or under anaerobic conditions (Williamson & Kreisberg, 1965), are both known to produce significant decreases in the whole-tissue $[\text{NADP}^+]/[\text{NADPH}]$ ratio. The present experiments have shown, however, that in neither case is there an alteration in the $[\text{GSSG}]/[\text{GSH}]$ ratio.

In the cytoplasm of rat liver the $[\text{NADP}^+]/[\text{NADPH}]$ ratio is less than 0.02 under a range of dietary conditions (Veech, Eggleston & Krebs, 1969). This ratio is unlikely to be very different in

rat heart. If the glutathione reductase reaction were at equilibrium, the ratio would have to be 100 000-fold higher. It may therefore be concluded that glutathione reductase is far removed from equilibrium in rat heart.

An enzymic reaction lies near equilibrium in the cell when the catalytic capacity of the enzyme greatly exceeds the net rate of the reaction. In this case significant changes in the net rate of the reaction can occur without an appreciable change in the concentration of the substrate. For a reaction that does not lie near equilibrium, however, a change in rate will be accompanied by a change in substrate concentration, whether or not the change in substrate concentration is the primary cause of the change in rate.

The GSSG concentration in rat heart has remained constant in the experiments reported here, under a range of perfusion conditions known to produce marked changes in the pattern of energy-producing metabolism (Garland & Randle, 1964; Newsholme & Randle, 1964). Since the glutathione reductase reaction does not lie near equilibrium, it follows that the rate of this reaction has also remained constant under the various perfusion conditions tested.

I thank Professor P. J. Randle for his advice and encouragement, and the Medical Research Council for a studentship.

REFERENCES

- Bhattacharya, S. K., Robson, J. S. & Stewart, C. P. (1955). *Biochem. J.* **60**, 696.
- Ellman, G. L. (1959). *Archs Biochem. Biophys.* **82**, 70.
- Garland, P. B. & Randle, P. J. (1964). *Biochem. J.* **93**, 678.
- Güntherberg, H. & Rapoport, S. (1968). *Acta biol. med. germ.* **20**, 559.
- Güntherberg, H. & Rost, J. (1966). *Analyt. Biochem.* **15**, 205.
- Jocelyn, P. C. (1960). *Biochem. J.* **77**, 363.
- Krebs, H. A. & Henseleit, K. (1932). *Hoppe-Seyler's Z. physiol. Chem.* **210**, 33.
- Martin, H. & McIlwain, H. (1959). *Biochem. J.* **71**, 275.
- Morgan, H. E., Henderson, M. J., Regen, D. M. & Park, C. R. (1961). *J. biol. Chem.* **236**, 253.
- Newsholme, E. A. & Randle, P. J. (1964). *Biochem. J.* **93**, 641.
- Owens, C. W. I. & Belcher, R. V. (1965). *Biochem. J.* **94**, 705.
- Rall, T. W. & Lehninger, A. L. (1952). *J. biol. Chem.* **194**, 119.
- Rost, J. & Rapoport, S. (1964). *Nature, Lond.*, **201**, 185.
- Srivastava, S. K. & Beutler, E. (1967). *Biochem. biophys. Res. Commun.* **28**, 659.
- Veech, R. L., Eggleston, L. V. & Krebs, H. A. (1969). *Biochem. J.* **115**, 609.
- Watts, D. J. (1969). Ph.D. Thesis: University of Bristol.
- Williamson, J. R. & Kreisberg, R. A. (1965). *Biochim. biophys. Acta*, **97**, 347.
- Wollenberger, A., Ristau, O. & Schoffa, G. (1960). *Pflügers Arch. ges. Physiol.* **270**, 399.