Glutathione

Helmut Klotzsch and Hans-Ulrich Bergmeyer

Blood contains 28 to 52 mg.% glutathione, which is exclusively located in the erythrocytes[1]. The values for other organs differ greatly. The glutathione content of cat organs has been determined by *H. H. Tallan et al.*[2]. The non-enzymatic methods used at present, for example, the colour reaction with sodium nitroprusside[3] or phosphotungstic acid[4], give results which are not reproducible when applied to biological material. Other methods, for example, iodometric determination, are less specific. The enzymatic methods are preferable for biological material because of their specificity (for a review, see[5]).

Principle

According to[6,7] glutathione (GSH) reacts quantitatively with methylglyoxal in the presence of glyoxalase I (Gl-I) to give *S*-lactyl-GSH:

(1) $$GSH + \text{methylglyoxal} \longrightarrow S\text{-lactyl-GSH}$$

According to[8] oxidized glutathione (GSSG) is quantitatively reduced to GSH by reduced triphosphopyridine nucleotide (TPNH) and glutathione reductase (GR):

(2) $$GSSG + TPNH + H^+ \longrightarrow 2\,GSH + TPN^+$$

Lactyl-GSH is measured directly at 240 mμ. The oxidation of TPNH is measured by the decrease of optical density at 340 or 366 mμ.

The equilibria of both reactions lie far to the right. Under the conditions stated here the reactions proceed stoichiometrically.

The two forms of glutathione are determined in the same assay mixture. The GSH is determined first (by measurements of the optical density at 240 mμ) and then GSSG is estimated in the same cuvette (by measurements of the optical density at 340 or 366 mμ).

Reagents

1. Potassium dihydrogen phosphate, KH_2PO_4, A. R.
2. Dipotassium hydrogen phosphate, K_2HPO_4, A. R., anhydrous
3. Methylglyoxal
   freshly distilled (*e.g.* 30% aqueous solution of Fluka & Co.): steam-distill a commercial methylglyoxal solution (*ca.* 30%) (*e.g.* in the *Parnass-Wagner* micro-distillation apparatus).
4. Albumin (egg)
5. Perchloric acid, A. R., sp. gr. 1.67; *ca.* 70% (w/w)
6. Sodium hydrogen carbonate, $NaHCO_3$, A. R., anhydrous
7. Potassium carbonate, K_2CO_3, A. R., anhydrous

---

1) *Hoppe-Seyler-Thierfelder:* Handbuch der physiologisch- und pathologisch-chemischen Analyse. 10th ed., Springer-Verlag, Berlin, Göttingen, Heidelberg 1953, Vol. V, p. 44.
2) *H. H. Tallan, S. Moore* and *W. H. Stein*, J. biol. Chemistry *211*, 927 [1954].
3) *A. Fujita* and *I. Numata*, Biochem. Z. *300*, 246, 257 [1939].
4) *K. Shinohara*, J. biol. Chemistry *109*, 665 [1935]; *110*, 263 [1935].
5) *J. W. Patterson* and *A. Lazarow* in: Glutathione, a Symposium. Academic Press, New York 1954, p. 63.
6) *E. Racker*, J. biol. Chemistry *190*, 685 [1951].
7) *Th. Wieland, K. Dose* and *G. Pfleiderer*, Biochem. Z. *326*, 442 [1955].
8) *T. W. Rall* and *A. L. Lehninger*, J. biol. Chemistry *194*, 119 [1952].

8. Reduced triphosphopyridine nucleotide, TPNH
   sodium salt, TPNH-Na_4. Commercial preparation, see p. 1030.
9. Glyoxalase I, Gl-I
   from yeast, solution in 30% glycerol; specific activity at least 300 units*)/mg. Commercial preparation, see p. 981.
10. Glutathione reductase, GR
    from yeast, suspension in 2.8 M ammonium sulphate solution; specific activity at least 60 units*)/mg. Commercial preparation, see p. 979.

Purity of the enzyme preparations

Relative to their respective specific activities, glutathione reductase and glyoxalase I must not contain more than 0.1% G6P-DH, 6-PGDH and TPNH oxidase. Glyoxalase I must be absolutely free from glyoxalase II.

Preparation of Solutions

To prevent the growth of micro-organisms sterilize the containers.

I. Phosphate buffer (0.25 M; pH 6.8):
   a) Dissolve 3.4 g. KH_2PO_4 in doubly distilled water and make up to 100 ml.
   b) Dissolve 4.35 g. K_2HPO_4 in doubly distilled water and make up to 100 ml.
   Mix 50 ml. of solution a) with 61 ml. of solution b). Check the pH (glass electrode).

II. Methylglyoxal (*ca.* 0.1 M):
   Dilute the aqueous solution (distillate) *ca.* five-fold with doubly distilled water. Check the concentration (enzymatic determination, see p. 283).

III. Albumin (*ca.* 1% w/v):
   Dissolve 100 mg. albumin (egg) in doubly distilled water, make up to 10 ml. and centrifuge or filter off any insoluble material.

IV. Sodium hydrogen carbonate (5% w/v):
   Dissolve 5 g. $NaHCO_3$ in doubly distilled water and make up to 100 ml.

V. Reduced triphosphopyridine nucleotide (*ca.* 0.012 M β-TPNH):
   Dissolve 10 mg. TPNH-Na_4 in 1 ml. $NaHCO_3$ solution (IV).

VI. Glyoxalase I, Gl-I (1 mg. protein/ml.):
   Dilute the stock solution with 30% (v/v) glycerol.

VII. Glutathione reductase, GR (1 mg. protein/ml.):
   Dilute the stock suspension with 3.0 M ammonium sulphate solution.

VIII. Perchloric acid (*ca.* 6% w/v):
   Dilute 5.2 ml. 70% $HClO_4$ to 100 ml. with doubly distilled water.

IX. Potassium carbonate (1.0 M):
   Dissolve 13.8 g. anhydrous K_2CO_3 in doubly distilled water and make up to 100 ml.

Stability of the solutions

Store all the solutions and suspensions, stoppered, in a refrigerator at 0 to 4° C. In this state they keep for several weeks. Prepare the dilute methylglyoxal and TPNH solutions freshly each week.

---

* A unit is the amount of enzyme which converts 1 μmole of substrate in 1 min. at 25° C.

Procedure

Deproteinization

To avoid the oxidation of the glutathione blood must be deproteinized directly after collection.

Pipette successively into a centrifuge tube:

5 ml. ice-cold perchloric acid solution (VIII)
5 ml. blood.

Mix thoroughly with a thin glass rod and centrifuge for 10 min. at 3000 g. Use

0.5 ml.

of the supernatant for the assay.

Spectrophotometric measurements

Wavelength: 240 mμ for GSH; 340 or 366 mμ for GSSG; light path: 1 cm.; final volume: 2.93 ml. for GSH and 3.04 ml. for GSSG; room temperature. Measure against the control.

For the GSH determination pipette successively into the cuvettes:

Control:	2.55 ml. phosphate buffer (solution I)
	0.50 ml. sample (deproteinized and neutralized)
	0.15 ml. albumin solution (III)
Experimental:	2.25 ml. phosphate buffer (solution I)
	0.15 ml. albumin solution (III)
	0.50 ml. sample (deproteinized)
	0.01 ml. GI-I solution (VI).

Mix thoroughly with a small glass or plastic rod flattened at one end and read the optical density E_1. Mix into the experimental cuvette

0.02 ml. methylglyoxal solution (II).

Read the optical density after 8, 10 and 12 min. By extrapolation to the time of addition of the methylglyoxal obtain E_2. Once again mix in

0.02 ml. methylglyoxal solution (II),

read the optical density after 2, 4 and 6 min., and extrapolate to the time of the first methylglyoxal addition: E_3.

$E_3 - E_2 = \Delta E_{methylglyoxal}$; $E_2 - E_1 - \Delta E_{methylglyoxal} = \Delta E_{GSH}$.
ΔE_{GSH} is used for the calculations.

For the GSSG determination mix into the same cuvettes

0.08 ml. TPNH solution (V)

and read the optical density E_4 at 366 mμ (or 340 mμ). Mix into the experimental cuvette

0.01 ml. GR suspension (VII)

and follow the decrease in optical density. Read the optical density after 8, 10 and 12 min. By extrapolation to the time of the GR addition obtain the optical density E_5. $E_4 - E_5 = \Delta E_{GSSG}$ is used for the calculations.

Calculations

a) GSH: According to *E. Racker*[6] the extinction coefficient of *S*-lactyl-GSH is $\varepsilon_{240} = 3.37$ cm.²/μmole. Therefore with a final volume in the cuvette of 2.93 ml.

$$\frac{\Delta E_{GSH} \times 2.93}{3.37} = \mu\text{moles GSH/assay mixture}$$

$$\mu\text{moles GSH} \times 307 = \mu\text{g. GSH}$$

b) GSSG: The extinction coefficient of TPNH is $\varepsilon_{340} = 6.22$ cm.²/μmole or $\varepsilon_{366} = 3.3$ cm.²/μmole. Therefore with a final volume in the cuvette of 3.04 ml.

$$\text{at } 340\ \text{m}\mu\text{:}\ \frac{\Delta E_{GSSG} \times 3.04}{6.22} = \mu\text{moles GSSG/assay mixture}$$

$$\text{at } 366\ \text{m}\mu\text{:}\ \frac{\Delta E_{GSSG} \times 3.04}{3.30} = \mu\text{moles GSSG/assay mixture}$$

$$\mu\text{moles GSSG} \times 612 = \mu\text{g. GSSG.}$$

To obtain the glutathione content per ml. of sample, the dilution occurring on deproteinization must be taken into account.

Blood contains *ca.* 80% of its weight of water, 1 ml. blood weighs 1.06 g. On deproteinization 5 ml. blood = 5.30 g. gives $5.3 \times 0.8 + 5$ ml. = 9.24 ml. extract. This corresponds to 9.24 ml. filtrate for each 5 ml. blood taken.

0.5 ml. of the supernatant (corresponding to 0.2706 ml. blood) is taken for the assay. To convert to glutathione/ml. blood multiply by 3.70.
Therefore for

GSH (measurements at 240 mμ)

$\Delta E_{GSH} \times 988$ = μg. GSH/ml. blood
$\Delta E_{GSH} \times 3.22$ = μmoles GSH/ml. blood

GSSG (measurements at 340 mμ)

$\Delta E_{GSSG} \times 1108$ = μg. GSSG/ml. blood
$\Delta E_{GSSG} \times 1.81$ = μmoles GSSG/ml. blood

GSSG (measurements at 366 mμ)

$\Delta E_{GSSG} \times 2085$ = μg. GSSG/ml. blood
$\Delta E_{GSSG} \times 3.41$ = μmoles GSSG/ml. blood

Specificity and Sources of Error

Insufficient purity of the reagents, especially the enzymes, gives erroneous results. For example, if the glyoxalase I preparation still contains glyoxalase II, then too little GSH is found. If the glutathione reductase contains TPNH oxidase, then the GSSG values will be too high.

The GSH contained in blood is oxidized extremely rapidly to GSSG. For example, if the blood is allowed to stand for *ca.* 2 hours before deproteinization, then all the glutathione is found as GSSG. For reasons which are at present unknown, glutathione added to tissue homogenates is not completely recovered.

In the presence of large amounts of aspartathione and isoglutathione high values for glutathione are found.