Biochem. J. (1964), **93**, 232

The Estimation of Glutathione in Rat Tissues

A COMPARISON OF A NEW SPECTROPHOTOMETRIC METHOD WITH THE GLYOXALASE METHOD

By B. E. DAVIDSON and F. J. R. HIRD

Russell Grimwade School of Biochemistry and School of Agriculture, University of Melbourne, Parkville, N. 2, Victoria, Australia

(*Received* 16 *December* 1963)

The method for the estimation of oxidized and reduced glutathione (GSSG and GSH) based on the glyoxalase reaction (Woodward, 1935; Martin & McIlwain, 1959) is the only method available with a high degree of specificity. The present paper suggests another specific method based on thiol–disulphide exchange reactions.

Pihl, Eldjarn & Bremer (1957) have shown that exchange reactions between GSH and certain disulphides result in the formation of GSSG, which then reacts with $NADPH_2$ in the presence of glutathione reductase:

$$GSH + RSSR \rightleftharpoons GSSR + RSH$$

$$GSH + GSSR \rightleftharpoons GSSG + RSH$$

$$GSSG + NADPH_2 \xrightarrow[\text{reductase}]{\text{Glutathione}} 2GSH + NADP$$

Investigations on a similar system (Hird, 1962) showed that after a short delay the rate of change of extinction at 340 mμ was linear and was roughly proportional to the GSH concentration. Since specificity is conferred by glutathione reductase (Conn & Vennesland, 1951) we have re-examined the conditions under which the reactions can be used for the quantitative estimation of GSH.

To test the new method we have compared it with the established glyoxalase method, which for greater convenience has been adapted for use in the recording pH-stat.

MATERIALS AND METHODS

Reagents. $NADPH_2$ type II (87–98%), glutathione reductase types I and III (1·0 μmole unit/mg. and 1 μmole unit/2 μg. respectively) and GSSG were purchased from Sigma Chemical Co.; pyruvic aldehyde (40% in water), cystamine hydrochloride and DL-homocysteine hydrochloride were from L. Light and Co. Ltd.; GSH and cysteine hydrochloride were from S.A.F. Hoffmann–La Roche and Co. Ltd. Glass-distilled water was used for preparing solutions.

Preparation of tissue extracts. Rats were stunned and bled before removal of the tissue, but for those estimations on brain tissue the stunning was omitted. With liver, brain and kidney, the tissue was removed, weighed and dispersed in 5 parts (v/w) of cold 0·1 M-potassium phosphate buffer, pH 8·2, containing EDTA (10 mM), in a Potter–Elvehjem homogenizer. The dispersion was placed in a boiling-water bath for 2 min. in Pyrex test tubes (6 in. × 0·5 in.) and then cooled in an ice bath before being centrifuged at 17 000*g* for 25 min. at 3°. The clear pale-amber supernatant liquid contained no glutathione-reductase or glyoxalase activity.

In the preparation of muscle extracts a modified technique had to be used. The tissue was minced with scissors and then dispersed in approx. 3 parts (v/w) of buffer. The dispersion was heated for 3 min. with stirring at 1½ min. After centrifuging it was necessary to filter the supernatant liquid (Whatman no. 40 paper) to remove floating lipid material. The pH of these extracts was 7·2–7·5 and was adjusted to 8·2 with N-NaOH.

When tissues were extracted with 3% (w/v) sulphosalicylic acid the same ratios of homogenizing medium to tissue were used and the dispersion was allowed to stand for 30 min. before centrifuging as above.

Spectrophotometric estimation of reduced and oxidized glutathione. The experimental procedure used was similar to that described by Hird (1962). Solutions of $NADPH_2$, glutathione reductase and GSH in phosphate–EDTA buffer were prepared fresh each day. These solutions were stable for at least 9 hr. at 3°.

The reaction system used after the appropriate conditions had been established consisted of: 0·25 μmole unit of glutathione reductase added in 0·1 ml.; 0·2–0·7 mg. of $NADPH_2$ in 0·1 ml.; 0·16–1·6 μmoles of GSH in 0·1–1·2 ml.; 0·1 M-potassium phosphate buffer, pH 8·2, made 10 mM with respect to EDTA, to make a final volume of 1·6 ml. after the addition of cystamine.

These solutions were mixed in a silica cuvette (10 mm. light-path) and placed in the housing of the spectrophotometer (Unicam SP. 500 or Beckman DK2). A spacer was used below the cuvette to allow volumes down to 1·4 ml. to be read. The cell housing was maintained at 38 ± 0·1°. After 5 min. the extinction at 340 mμ was measured against a suitable blank and followed for a further 14 min. This period of time allows any GSSG in the sample to be reduced. At 15 min. 0·04–0·10 ml. of cystamine solution was added with a Perspex spatula to ensure rapid and complete mixing without removal of the cuvette from the housing. The extinction was then measured at suitable intervals until it fell to zero, or for 10–12 min., depending on the amount of GSH in the unknown solution.

The GSSG was estimated by the fall in extinction when the sample was added to $NADPH_2$ and glutathione reductase (see Fig. 1). In the system used 0·1 μmole of GSSG gives a fall in extinction of 0·33.

Glyoxalase estimation of reduced and oxidized glutathione. The system used was based on the manometric method of Martin & McIlwain (1959), but the production of protons was followed at pH 6·0 in a pH-stat (Radiometer, Copenhagen). An apoglyoxalase preparation was made as described by Martin & McIlwain (1959). These workers had found that, if a sample of GSSG was incubated with the apoglyoxalase before the addition of the pyruvic aldehyde, the GSSG was reduced and could be estimated as GSH. However, such activity was not present in our preparation, and accordingly GSSG was reduced with glutathione reductase and $NADPH_2$ before the addition of the apoglyoxalase and the pyruvic aldehyde.

The full procedure used was as follows: water, the unknown sample of GSH and EDTA (to 1 mM), collectively giving a volume of 1·3 ml., were placed in the reaction vessel (at 30±0·02°), and if necessary the pH was adjusted to 7·0–8·5 with N-NaOH so as to be within the optimum pH range of the glutathione reductase. Then $NADPH_2$ (0·3 mg. in 0·1 ml.) and glutathione reductase (0·2 μmole unit in 0·1 ml.) were added and the mixture was incubated for 5 min. This is long enough to give complete reduction of at least 0·2 μmole of GSSG. After this time 2 ml. of the apoglyoxalase suspension (15%) and 0·1 ml. of the pyruvic aldehyde (10%) were added, the pH was adjusted to approx. 6, the KCl open junction to the reference electrode was renewed, and the protons produced were titrated with 30 mM-NaOH at pH 6·0. After about 2 min. the rate was linear and the reaction was followed for a further 5 min.

Polarographic investigations. GSH, GSSG and $GSSO_3H$ all give well-defined current–voltage waves with half-wave potentials at −0·5, −0·5 and −1·35 v respectively at pH 9·0 with Ag/AgCl as the reference electrode. The GSH wave is anodic and the GSSG and $GSSO_3H$ waves are cathodic.

The polarograph used was a Polarecord type E261 and the reaction vessel contained KCl (0·7 g.) as carrier electrolyte, urea (5·7 g.), tris buffer, pH 9·0 (0·1 M), gelatin (0·003%), the sample being analysed, and water in a final volume of 12 ml. Oxygen was removed in the usual way by purging with nitrogen. When sulphite was used 0·5 g. of anhydrous Na_2SO_3 was added as a solid.

RESULTS AND DISCUSSION

Glyoxalase method. The system used responded linearly to increments of GSH up to 0·4 μmole, which was the upper limit chosen for convenience. At pH 7·0 in the absence of GSH there was a rapid background production of protons by the pyruvic aldehyde and the glyoxalase preparation. This was markedly decreased at the pH finally chosen (6·0) and was equivalent to only 0·04 μmole of GSH.

Glutathione-reductase method. Fig. 1 shows that when GSH was added to the system containing glutathione reductase and $NADPH_2$ the extinction fell, owing to a small amount (approx. 2%) of GSSG in the sample. After this reduction there remained a slow steady fall in extinction, i.e. before the addition of cystamine to the system. This was due to a slow oxidation of GSH which was not lessened by increasing the concentration of EDTA. This rate of change of extinction is referred to as the 'pre-cystamine rate'. After the addition of cystamine the rate of change of extinction is greatly increased, and this 'post-cystamine rate' of change is the basis of the method of estimation. It might be expected that subtraction of the pre-cystamine rate from the post-cystamine rate should give a closer relationship to the GSH concentration, but in some circumstances, e.g. in the presence of some other thiols, this leads to errors and such a subtraction has therefore not been made.

The post-cystamine rate became linear with time at pH 8·2 but departed from linearity above this value. Fig. 2 shows the range of concentrations over which there is a linear response to concentration of GSH. At pH values less than 8·2 this relationship is not quite linear and for these reasons pH 8·2 has been used.

Effect of the cystamine:glutathione ratio. Fig. 3 shows the results obtained when the GSH concentration was held constant as the cystamine concentration was altered. Similar results were found when the cystamine concentration was kept constant and that of GSH varied. It is therefore apparent that for a linear reaction rate the cystamine and GSH concentrations must be of similar magnitude. A more detailed examination of the rates (Fig. 4) shows that the optimum ratio is about 0·6. We have used a ratio of 1, so that at low con-

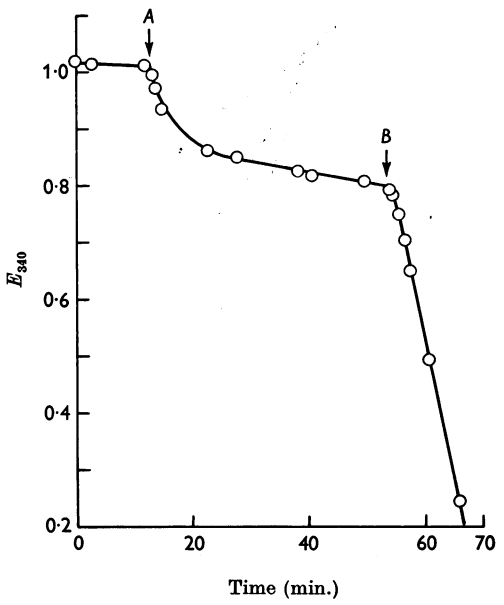

Fig. 1. Reduction of cystamine by GSH and $NADPH_2$ with glutathione reductase. Initially present were 0·3 mg. of $NADPH_2$ and 0·25 mg. of glutathione reductase in 1·4 ml. The first addition (*A*) was GSH (1·0 μmole in 0·1 ml.), and the second addition (*B*) was cystamine (1·0 μmole in 0·1 ml.). Other conditions are given in the text.

centrations there would be sufficient cystamine present to give a suitable change in extinction (0·1 μmole of disulphide had an extinction at 340 mμ of 0·33).

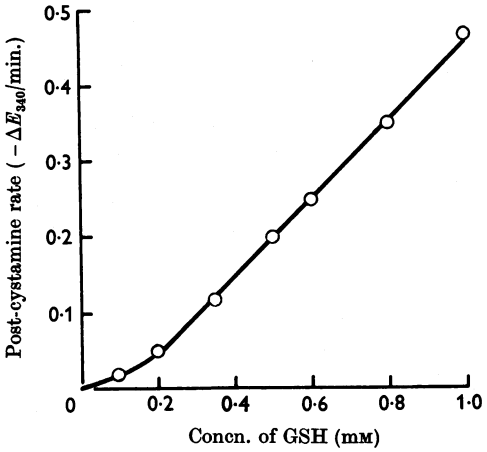

Fig. 2. Standard curve. The cystamine:glutathione ratio was 1. Other conditions are given in the text.

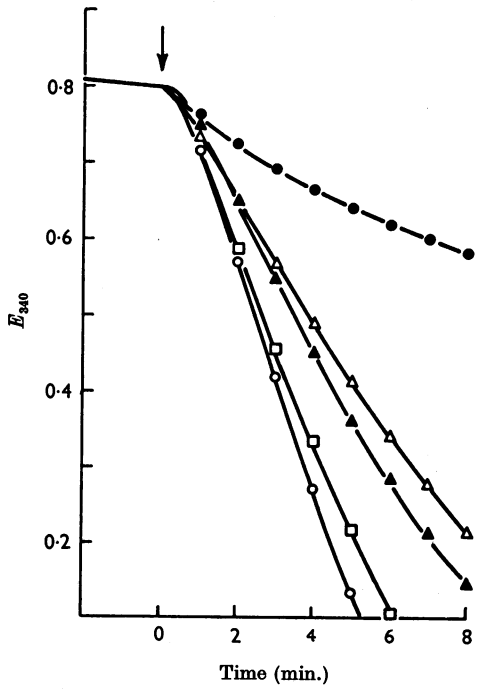

Fig. 3. Effect of cystamine:glutathione ratio. The GSH concentration was 0·5 mM; the cystamine concentration was: 0·1 mM (▲); 0·25 mM (○); 0·5 mM (□); 1·0 mM (△); 10 mM (●). Cystamine was added at the time indicated by the arrow. Other conditions are given in the text.

Effect of thiols. Under the aerobic conditions used, GSH was slowly oxidized (see Fig. 1) and so also were other thiols. As cysteine and homocysteine are the most likely thiols to be present in tissue extracts their effect on the system has been investigated. Table 1 shows that the presence of these compounds increased the pre-cystamine rate, since when they are oxidized to disulphides they act in the same way as cystamine. At higher concentrations of thiol the post-cystamine rate was also affected, presumably by competition with GSH for the thiol–disulphide exchange reactions involved. From these results it appears that when the post-cystamine rates are used homocysteine and cysteine will interfere if they are present at concentrations greater than 25 % of the GSH concentration.

There is no information on the amounts of cysteine and homocysteine in rat tissues used, but the values given for appropriate tissues of the cat by

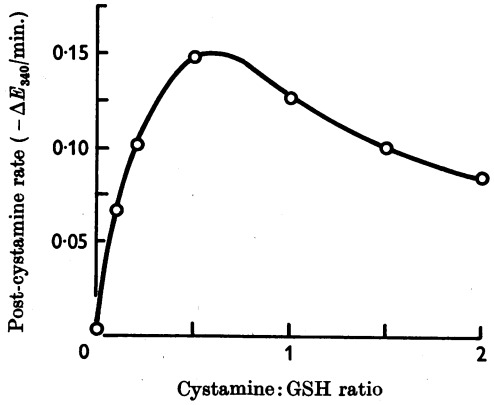

Fig. 4. Effect of cystamine:glutathione ratio on the post-cystamine rate. The GSH concentration was 0·5 mM. For those ratios with no linear component the maximum rate was used.

Table 1. *Effect of cysteine and homocysteine on reaction rates*

The GSH and cystamine concentrations were 0·4 mM. Other conditions are given in the text.

Addition	Final concn. (mM)	Pre-cystamine rate ($-\Delta E_{340}$/min.)	Post-cystamine rate ($-\Delta E_{340}$/min.)
None	—	0·005	0·146
Homocysteine	0·01	0·004	0·145
Homocysteine	0·10	0·007	0·137
Homocysteine	0·50	0·012	0·111
Homocysteine	1·00	0·015	0·088
None	—	0·003	0·131
Cysteine	0·01	0·004	0·131
Cysteine	0·02	0·007	0·132
Cysteine	0·05	0·020	0·128
Cysteine	0·10	0·049	0·124

Tallan, Moore & Stein (1954) showed cyst(e)ine in quantities less than would interfere; homocyst(e)ine was not reported. The presence of thiols could always be indicated by a higher pre-cystamine rate than usual.

Preparation of extracts for analysis. Heating of extracts (Thompson & Martin, 1959) was found to be the simplest method of preparing them free of protein precipitants that inhibited glutathione reductase. In solution this procedure caused less than 3 % loss of GSH or GSSG on heating for up to 5 min., and there was no decrease in GSH estimated in tissue extracts between 2 and 5 min. heating. In addition endogenous glutathione reductase and glyoxalase were destroyed by heating.

The recoveries of GSH added to liver and brain dispersions before and after heating were 99–102 %.

Comparison of glyoxalase and glutathione-reductase methods. Extracts were prepared in phosphate buffer as outlined above and GSH was estimated in duplicate by each method on tissues from a number of different animals. The results (Table 2) show that there is good agreement between the methods, with the glutathione-reductase method giving results 4–10 % higher. The results on kidney (see below) indicated losses with this tissue during the heating procedure. It therefore seemed desirable, even though there was full recovery of GSH with liver and brain, to compare the heating procedure with extraction by sulphosalicylic acid. The glutathione-reductase method does not work satisfactorily in the presence of sulphosalicylate, and so the comparison was made with the glyoxalase method. The results (Table 3) show that both methods of extraction give the same GSH content except with muscle, where the phosphate extraction consistently gave 10 % more GSH.

Glutathione in kidney. It was found by both methods of estimation that only very small amounts of GSH were present in heated extracts of kidney, and only about 20 % of GSH added to the homogenizing medium could be recovered. However, if sulphosalicylic acid were used for extraction the kidney content was found to be 4·2 μmoles/g. fresh wt. It therefore appeared that GSH was lost if the kidney were homogenized and heated in phosphate buffer. The cause of this loss was investigated.

The protein precipitate that resulted from the heat treatment was washed a number of times with 3 % sulphosalicylic acid to remove free GSH, dispersed in 8 M-urea and examined polarographically. The addition of sulphite did not result in the appearance of current–voltage waves characteristic of either GSH or $GSSO_3H$. This indicated that the GSH was not being lost by mixed disulphide formation with thiols (oxidation) or disulphides (interchange reactions) of the tissue protein.

Examination of the original kidney extract itself in the polarograph did not reveal any current–voltage wave due to diffusible thiol or to disulphide, thus eliminating the possibility of loss of GSH by γ-glutamyltransferase activity. The addition of sulphite to the original extract produced a well-defined thiol wave, indicating the possible presence of a disulphide complex undergoing sulphitolysis.

GSH added to the extract gave the normal thiol wave. However, GSSG added to the extract could not be detected as the thiol or disulphide wave, but on the addition of sulphite the expected thiol wave appeared.

It appears therefore that in kidney tissue much of the GSH is oxidized during the extraction procedure and then the GSSG forms complexes with an unknown substance in the extract. Such complex-formation has also been observed (under anaerobic conditions only) with NAD, $NADH_2$, NADP, $NADPH_2$ and water extracts of flour. In these cases also if GSSG is added it is not detected in the polarograph and it is not reduced by glutathione reductase. As with the kidney extracts the GSSG still reacts with sulphite to give a thiol wave. The presence therefore of this unknown substance in kidney extracts prepared by heating invalidates this method of extraction for this tissue.

General comments on the methods. With the glutathione-reductase method the fall in extinction after the addition of the sample to the glutathione

Table 2. *Comparison of estimation of glutathione by different methods*

The GSH content of each tissue was estimated in duplicate by each method. Experimental details are given in the text. The average values are reported with the ranges in parentheses.

Tissue	No. of animals used	GSH content (μmoles/g. fresh wt.) Glyoxalase method	Glutathione-reductase method
Liver	6	7·39 (5·5–8·5)	8·33 (5·6–9·9)
Brain	3	2·01 (1·77–2·31)	2·03 (1·83–2·28)
Muscle	3	1·15 (1·06–1·25)	1·19 (1·06–1·28)

Table 3. *Comparison of different methods of tissue extraction*

The GSH content of each tissue was estimated in duplicate by each method. Experimental details are given in the text. The average values are reported with the ranges in parentheses.

Tissue	No. of animals used	GSH content (μmoles/g. fresh wt.) 3 % Sulphosalicylic acid extraction	Phosphate buffer–heat extraction
Liver	2	6·22 (5·89, 6·55)	6·30 (5·87, 6·72)
Brain	3	1·74 (1·59–1·82)	1·77 (1·53–1·90)
Muscle	5	1·03 (0·74–1·35)	1·13 (0·82–1·36)
Kidney	3	4·2 (3·78–4·55)	0·56 (0·1–1·6)

reductase and $NADPH_2$ can be used to determine GSSG. The rate after the addition of cystamine gives total glutathione. However, GSH undergoes some oxidation during preparation of the extract and only total glutathione has been estimated in this study. The lower limit of the method as described is approx. 0·1 μmole, and greater sensitivity can be achieved by using more glutathione reductase, anaerobic conditions and a more sensitive spectrophotometer. Since the cystamine concentration must approximately equal the GSH concentration a trial run is sometimes necessary.

The method described therefore is comparable in sensitivity and ease of manipulation with the glyoxalase method. The simple extraction procedure of heating, which works well for liver, muscle and brain, is, however, inapplicable for kidney, and with this tissue another method of extraction of GSH must be sought.

SUMMARY

1. The glyoxalase method for the estimation of glutathione has been adapted for use in the pH-stat.

2. A new method for the estimation of glutathione based on thiol–disulphide exchange reactions with cystamine and glutathione reductase has been developed. The reactions are followed spectrophotometrically at 340 mμ.

3. The method as described can estimate down to 0·1 μmole of glutathione.

4. It has been compared with the glyoxalase method and found to give similar results with rat liver, brain and muscle. The averages of these were 7·32, 1·90 and 1·1 μmoles/g. fresh wt. respectively.

5. Extracts of kidney tissue at pH 8·2, extracts of flour, and nicotinamide coenzymes form complexes with oxidized glutathione in such a way that it is unavailable for reduction by glutathione reductase.

We acknowledge some financial support from the Wheat Industry Research Council.

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Metabolism of 11-Oxygenated Steroids

3. SOME 1-DEHYDRO AND 9α-FLUORO STEROIDS*

BY I. E. BUSH† AND V. B. MAHESH‡
Department of the Regius Professor of Medicine, The Radcliffe Infirmary, Oxford

(*Received* 28 *January* 1964)

In the two previous papers of this series (Bush & Mahesh, 1959*a*, *b*) it was shown that the reduction of orally administered 11-oxo steroids to the related 11β-hydroxy steroids in man was greatly affected by the stereochemistry of the α-surface of the steroid molecule. In particular it was found that angulation of the A/B ring junction as in the 5β-(H) steroids completely prevented this reduction, that a 2α-methyl group greatly inhibited it, and that the partial angulation of the A/B ring junction caused by the introduction of a 1,2-double bond into a Δ^4-3-oxo steroid inhibited it appreciably (Bush & Mahesh, 1958*a*). These findings were taken to suggest that the 11-oxo steroid associated with the responsible enzyme ('steroid 11β-ol dehydrogenase'; Hurlock & Talalay, 1959) over the 'upper' part of the α-surface of the steroid, i.e. with the closest apposition to the enzyme surface occurring with the α-oriented hydrogen atoms attached to C-1, C-2, C-12 and C-21 when present (Fig. 8 in Bush & Mahesh, 1959*b*). It was also possible to give a reasonable explanation of the increase in biological activity achieved by introducing a 2α-methyl group into cortisol (11β,17α,21-trihydroxypregn-4-ene-3,20-dione) and the paradoxical decrease achieved by a similar substitution

* Part 2: Bush & Mahesh (1959*b*).

† Present address: Worcester Foundation for Experimental Biology, Shrewsbury, Mass., U.S.A.

‡ Present address: Department of Endocrinology, Eugene Talmadge Memorial Hospital, Augusta, Georgia, U.S.A.