Biochemical Pharmacology, 1966, Vol. 15, pp. 809–816. Pergamon Press Ltd., Printed in Great Britain.

ESTIMATION AND IDENTIFICATION OF THIOLS IN RAT SPLEEN AFTER CYSTEINE OR GLUTATHIONE TREATMENT: RELEVANCE TO PROTECTION AGAINST NITROGEN MUSTARDS

C. R. BALL

Chester Beatty Research Institute, Institute of Cancer Research:
Royal Cancer Hospital, London, S.W.3.

(*Received* 9 *December* 1965; *accepted* 10 *February* 1966)

Abstract—A chemical method is described for the specific estimation of glutathione in the protein-free fraction of tissues. Following administration of cysteine to rats there is a rise in protein-free thiol in all tissues, which is related to the degree of protection afforded against the toxicity of alkylating agents. It has now been shown that cysteine itself accounts for at least 90 per cent of the accumulating thiol after cysteine injection. Following administration of glutathione there is also a rise in total thiol concentration, but less than half of this is due to glutathione, the remainder being due to an unidentified β-amino-thiol. The relevance of the increased thiol concentration to mechanisms of protection against the toxicity of alkylating agents is discussed.

INTRODUCTION

THE PROTECTION of rats against the toxicity of certain nitrogen mustards by cysteine and other thiols has recently been reviewed by Connors.[1]

Protection by cysteine is related to the amount by which it increases the non-protein thiol (NPSH) content of tissues,[2] on the time interval between cysteine and mustard administration,[2, 3] and on the chemical reactivity of the mustard.[2] It has been proposed that protection can be ascribed to an intracellular reaction between cysteine and the mustard.[1, 2, 4, 5] However, the thiol which accumulates in all tissues following the administration of cysteine has not been identified as cysteine. Bacq and Alexander have, in fact, proposed that thiols which protect against alkylating agents and radiation do so indirectly.[6, 7] They believe that the protective thiols bind initially to proteins, causing the release of another thiol which is the protective agent. Recently, Revesz and Modig,[8] in a new theory of radiation protection, showed that the administration of cysteamine to Ehrlich ascites cells *in vitro* resulted in an increase of the intracellular glutathione concentration, and they propose that cysteamine *per se* is not protective, but that protection is a consequence of the increased glutathione concentration.

In this paper a chemical method is described for the specific estimation of glutathione in the protein-free fraction of tissues. Combined with chromatography, this method has been used to identify the thiols that accumulate in tissues following the administration of cysteine and glutathione.

MATERIALS AND METHODS

The specific estimation of glutathione

In preliminary experiments two specific methods for the estimation of cysteine gave inconsistent results.[9, 10] A report by Gadal[10] that the reaction of glyoxylic acid with cysteine could be used to distinguish it from glutathione has been the basis of our method for the identification of thiols in tissues. β-amino thiols such as cysteine form thiazolidines, with glyoxylic acid, which give no colour with the thiol reagent DTNB, 5,5'-dithiobis-(2-nitro) benzoic acid, Ellman's reagent.[12] In principle it is possible to estimate glutathione colorimetrically in a mixture of glutathione and cysteine after removal of the cysteine by reaction with glyoxylic acid. A measurement of the total thiol content of the mixture enables the concentration of cysteine to be estimated by difference.

In phosphate buffer (pH 6·8) at 60° the reaction between cysteine and glyoxylic acid is complete in 5 min (Fig. 1). At lower temperatures (e.g. 22°, Fig. 1) the reaction proceeds more slowly. At 60°, however significant oxidation of glutathione occurs in 5 min (Fig. 2). This oxidation of glutathione could be prevented by the use of more

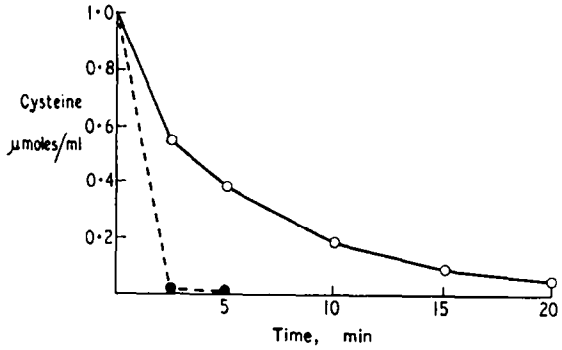

FIG. 1. Effect of temperature on the reaction between L-cysteine hydrochloride and glyoxylic acid monohydrate in 0·5 M phosphate buffer.
—○—, 22°; - - ● - -, 60°; pH, 6·8; 0·5 mg/ml glycoxylic acid.

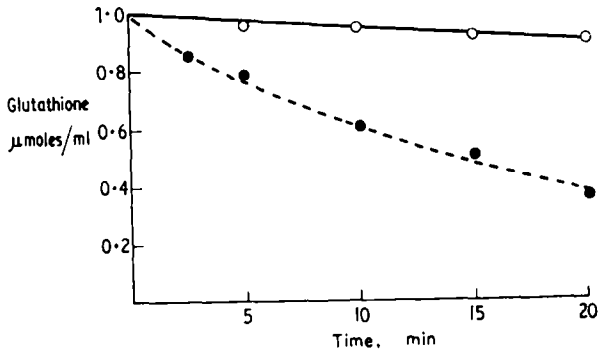

FIG. 2. Effect of the presence of ascorbic acid on the rate of oxidation of glutathione in 0·5 M phosphate buffer at 60°.
—○—, pH 6·8, 1mg/ml ascorbic acid; - - ● - -, pH 6·8.

acid conditions, but was not feasible since there was a corresponding reduction in the rate of reaction of cysteine with glyoxylic acid. Continuous gassing with oxygen-free argon also prevented oxidation of the glutathione, but this was not practical for large numbers of estimations. In the presence of ascorbic acid only slight oxidation of glutathione occurred in 5 min at 60° (Fig. 2), and under these conditions cysteine reacted completely with glyoxylic acid (Fig. 3).

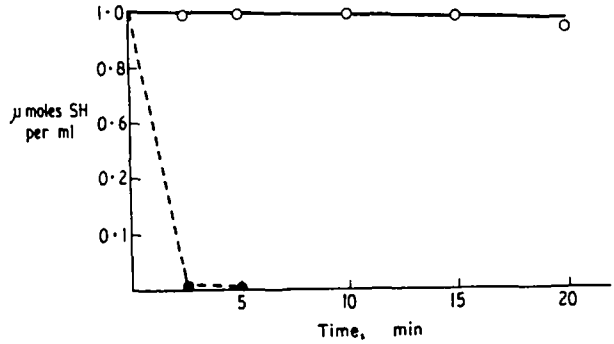

FIG. 3. Rate of reaction of L-cysteine hydrochloride and glutathione under the conditions used for the specific estimation of the two thiols.
—○—, glutathione; - - ● - -, cysteine, pH 6·8; 1 mg/ml ascorbic acid, 0·5 mg/ml glyoxylic acid.

The results obtained in estimating the concentrations of cysteine and glutathione in standard mixtures of the two thiols are shown in Table 1. The specificity of the Ellman reagent and the glyoxylic acid reaction is shown in Table 2.

Application of the glyoxylic reaction to tissue extracts

Female Chester Beatty rats, 8 weeks old, weighing 200–250 g were used, and maintained on rat cake and water *ad libitum*. L-cysteine hydrochloride and glutathione were

TABLE 1. ESTIMATION OF VARIOUS STANDARD SOLUTIONS OF L-CYSTEINE HYDROCHLORIDE AND GLUTATHIONE

Thiol content of standard solutions		Estimated thiol content (range in three independent estimations)	
Glutathione (μmoles/ml)	Cysteine (μmoles/ml)	Glutathione (μmoles/ml)	Cysteine (μmoles/ml)
0·50	0·25	0·46–0·49	0·25–0·26
0·25	0·25	0·23–0·25	0·25–0·265
0·125	0·25	0·12–0·13	0·24–0·26
0·25	0·50	0·25–0·29	0·45–0·52
0·25	0·25	0·24–0·27	0·24–0·27
0·25	0·125	0·24–0·26	0·12–0·15

administered intraperitoneally at 1 g/kg and 2 g/kg respectively, in aqueous solution adjusted to pH 7 with sodium hydroxide. At intervals after administration, groups of animals were killed by cervical dislocation, the spleens removed and blotted with

tissue paper to remove peritoneal fluid. Each spleen was weighed and homogenised in 4 ml ice-cold 5% sulphosalicylic acid (SSA) solution with an Ultra-Turrax homogeniser. After centrifugation the total thiol content of each supernatant was estimated by the addition of 5 ml of 0·5 M phosphate buffer (pH 6·8) containing ascorbic acid (1 g/l.) to a one ml aliquot of the supernatant, followed immediately by one ml of the Ellman reagent (150 mg DTNB in 100 ml phosphate buffer). The resulting colour was read at 412 mμ against a reagent blank.

The glutathione content of the supernatant was estimated by incubating a one ml aliquot with 5 ml of 0·5 M phosphate buffer (pH 6·8) containing ascorbic acid (1 g/l.) and glyoxylic acid (0·5 g/l.) for 5 min at 60°. The solution was then quickly cooled to room temperature, 1 ml of Ellman reagent added and read at 412 mμ. Glutathione standards in 5% SSA were routinely taken through both procedures to give calibration curves. Results were expressed as μmoles SH/g wet weight tissue. In all cases standard deviations were 5-10 per cent mean value.

TABLE 2. THE SPECIFICITY OF THE DTNB AND GLYOXYLIC ACID REACTIONS FOR VARIOUS SULPHUR COMPOUNDS

Compound (0·5 mM in 5% SSA)	Optical density with DTNB alone	Optical Density with DTNB after glyoxylic acid incubation
L-cysteine hydrochlorine	1·03	0
Glutathione	1·03	0·94
Cysteamine hydrochloride	1·00	0
DL-homocysteine	1·10	0
DL-penicillamine	1·08	0
AET*	1·05	0·98
2,3-dimercaptopropanol (BAL)	1·72	0·13
L-methionine	0	
Ergothioneine	0	—
Taurine	0	—
Cysteic acid	0	—
Cysteine thiosulphonate	0	—

* AET (S-2-aminoethylisothiourea dihydrobromide) was left 15 min in 5% SSA before use.

Chromatography

For chromatography more concentrated SSA extracts of spleen were required. Treated or control spleens were homogenised in 5% SSA at 500 mg wet wt./ml and 100 μl spots run in tertiary butanol: formic acid: water (70:15:15) by ascending chromatography. Standard solutions of cysteine, homocysteine, glutathione and cysteamine (50 μ molar) were diluted ten-fold in treated spleen SSA supernatant and these solutions run as markers.

DTNB (25 mg) was dissolved in ethanol (10 ml) and an equal volume of petroleum ether: pyridine (4:1) added. The dried chromatogram was dipped in this solution and dried. A yellow spot, which fades slowly, is produced when more than about $2{\cdot}5 \times 10^{-9}$ moles SH are present.

RESULTS

Intraperitoneal administration of L-cysteine hydrochloride (1 g/kg) gave a uniform increase in SSA soluble SH in all tissues 30 min after treatment (Table 3). The thiols contributing to this rise is non-protein SH have been identified in rat spleen by a combination of the glyoxylic acid reaction and chromatography as described above. Spleen has been chosen since it is known to be very sensitive to the action of the alkylating agents.[13] Figure 4 shows the rise in SSA soluble SH in rat spleen following the i.p. administration of cysteine. At 5 min the treated animals had a non-protein SH

TABLE 3. TOTAL NON-PROTEIN THIOL (NPSH) LEVELS IN RAT TISSUES 30 MIN AFTER L-CYSTEINE HYDROCHLORIDE (1 g/kg)

Tissue	Control NPSH μmoles SH/g wet wt.	Treated NPSH μmoles SH/g wet wt.	Rise in NPSH μmoles SH/g wet wt.
Liver	6·5	11·0	4·5
Spleen	3·4	8·7	5·3
Gut	5·1	10·4	5·2
Lung	2·4	7·1	4·7
Muscle	0·8	4·4	3·6
Bone marrow	3·7	7·9	4·2

level some 4·0 μmoles/g wet wt. higher than control rats. This level remained high for about 1 hr and returned to normal in about 2·5 hr. The glutathione level in the treated rats showed no significant change from control levels and the entire rise in SH was due to a thiol which reacted with glyoxylic acid (Fig. 4, Table 4). Chromatography of SSA

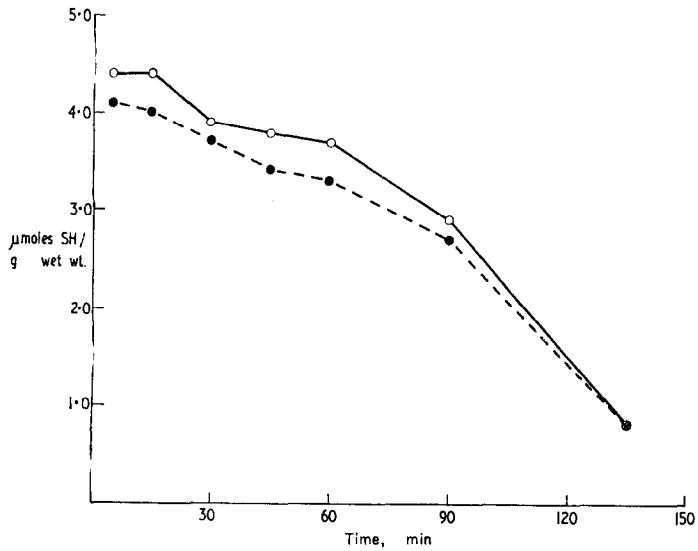

FIG. 4. Increase above control of total NPSH and cysteine in rat spleen following administration of cysteine (1 g/kg). Five animals per point. Control values: NPSH, 3·2 ± 0·2 μmole/g wet wt. Cysteine: none.
—○—rise in NPSH; - - ● - - rise in cysteine.

extracts of spleen taken from animals 30 min after injection of cysteine showed two distinct spots, one which was identical with the single spot seen in control spleens and proved to be glutathione, and a second faster moving spot which was shown to be cysteine (Table 5). No evidence was obtained for the presence of any other thiol following the administration of cysteine.

A different pattern was seen in rat spleen following intraperitoneal administration of 2 g/kg of glutathione. The level of non-protein SH only reached its maximum 30 min after injection (Fig. 5, Table 6). Furthermore, the rise in SH was not entirely accounted for by the administered thiol, as was the case for cyteine, since not only did the glutathione concentration increase but there also appeared a thiol, or thiols, which reacted with glyoxylic acid. The maximum in the concentration of glutathione occurred at 30 min, when the level in treated animals was about 1·5 μmoles per g wet wt. higher

TABLE 4. THIOL LEVELS IN RAT SPLEEN AFTER ADMINISTRATION OF L-CYSTEINE HYDROCHLORIDE (1 g/kg)

Time (min)	Total NPSH μmoles/g wet wt.	Glutathione μmoles/g wet wt.	Cysteine μmoles/g wet wt.
Control	3·2	3·2	—
5	7·6	3·5	4·1
15	7·6	3·6	4·0
30	7·1	3·4	3·7
45	7·0	3·6	3·4
60	7·0	3·8	3·2
90	6·1	3·5	2·7
135	4·0	3·3	0·8

TABLE 5. CHROMATOGRAPHY OF THE PROTEIN-FREE FRACTION OF RAT SPLEEN 30 MIN AFTER L-CYSTEINE HYDROCHLORIDE (1 g/kg)

Sample	R_f values (tertiary butanol: formic acid: water 70:15:15)		
Treated	0·62	0·70	—
Control	0·62	—	—
Treated + cysteine	0·62	0·70	—
Treated + glutathione	0·62	0·70	—
Treated + cystamine	0·62	0·71	0·81
Treated + homocysteine	0·62	0·70	0·78

than in control animals (Fig. 5, Table 6). The thiol reacting with glyoxylic acid did not attain its maximum concentration in spleen until 45 min after the injection of glutathione, when the level was about 2·6 μmoles/g wet wt. Chromatography of SSA spleen extracts of animals taken 45 min after the injection of glutathione showed one spot only, corresponding to glutathione. It is probable that the glyoxylic acid reacting thiol arising after glutathione treatment is cysteinylglycine (γ-glutamyl cysteine, the other possible hydrolysis product from glutathione is not a β-amino thiol). As cysteinylglycine was not available, this could not be confirmed.

DISCUSSION

Following injection of cysteine there is a rise in total NPSH which, at 30 min, is of the same order in all tissues (Table 3). The mean rise, 4·6 μmole SH per g wet wt. is equivalent to dose of 750 mg/kg, 75 per cent of the administered dose. It would seem that the tissues can deal with a dose of 250 mg/kg, probably by oxidation to cystine, and it has previously been noted[2] that doses below this level do not produce a rise in NPSH.

In rat spleen the accumulating NPSH is accounted for predominantly by cysteine (the sensitivity of the chromatographic method is such that a small rise of a β-amino thiol other than cysteine would not be detected). This finding adds further support to the postulate that cysteine protection against the toxicity of nitrogen mustards

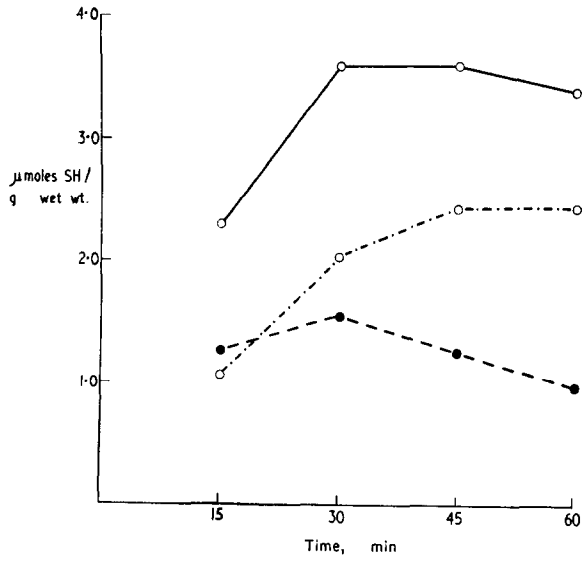

FIG. 5. Increase above controls of total NPSH, glutathione and β-aminothiol following administration of glutathione (2 g/kg). Control values: NPSH and glutathione 3·2 ± 0·2 μmoles/g wet wt. Cysteine: none.
—○— rise in total NPSH;
---●-- rise in glutathione; ·—○—·, rise in β-amino thiol.

TABLE 6. THIOL LEVELS IN RAT SPLEEN AFTER ADMINISTRATION OF GLUTATHIONE (2 g/kg)

Time (min)	Total NPSH μmoles/g wet wt.	Glutathione μmoles/g wet wt.	β-amino-thiol μmoles/g wet wt.
Control	3·1	3·1	—
15	5·4	4·3	1·1
30	6·7	4·6	2·0
45	6·7	4·3	2·4
60	6·5	4·0	2·4

results from an intracellular reaction between the nitrogen mustard and cysteine. Protection has only been obtained where the high level of cysteine observed (Fig. 4, Table 4) corresponds in time to the reaction of the mustard with intracellular sites.[1, 2]

The results obtained with glutathione (Fig. 5, Table 6) show that there is resistance to cellular penetration of glutathione. Although the maximum rise in NPSH represents 70 per cent of the administered dose, it is only reached after 30 min. The actual rise in glutathione is only small (equivalent to 25 per cent dose) as it appears to be transformed to a β-amino thiol, possibly by reversal of glutathione synthesis to give cysteinylglycine.

Glutathione gives little protection against the toxicity of nitrogen mustards,[14, 15] although the level of NPSH at the time of administration of the mustard is similar to that after cysteine pretreatment. This is consistent with the proposal that protection by cysteine is a result of its high competition factor for mustards relative to glutathione and other thiol-containing peptides.[1]

Acknowledgements—The author wishes to thank Dr. T. A. Connors for advice and Miss Gillian Howard for technical assistance. This work has been supported by a Medical Research Council Scholarship and by grants to the Chester Beatty Research Institute (Institute of Cancer Research: Royal Cancer Hospital) from the Medical Research Counci land the British Empire Cancer Campaign for Research, and by the Public Health Service Research Grant No. CA-03188-08 from the National Cancer Institute, U.S. Public Health Service, and will be submitted as part of a Ph.D. thesis in the University of London.

REFERENCES

1. T. A. CONNORS, *Europ. J. Cancer* (in press) (1966).
2. G. CALCUTT, T. A. CONNORS, L. A. ELSON and W. C. J. ROSS, *Biochem. Pharmac.* **12**, 833 (1963).
3. T. A. CONNORS, A. JENEY and M. JONES, *Biochem. Pharmac.* **13**, 1545 (1964).
4. P. SALERNO and H. L. FRIEDELL, Western Reserve Univ., Atomic Energy Medical Research Project, Cleveland, Ohio. Contract W 31-109-Eng. 78.
5. E. GOLDENTHAL, M. NADKARNI and P. SMITH, *Radiat. Res.* **10**, 571 (1959).
6. Z. M. BACQ and P. ALEXANDER, *Nature, Lond.* **203**, 162 (1964).
7. Z. M. BACQ, *Chemical protection against ionising radiation.* Thomas, Springfield (1965).
8. L. REVESZ and H. MODIG, *Nature, Lond.* **207**, 430 (1965).
9. S. ROSTON, *Analyt. Biochem.* **6**, 486 (1963).
10. F. J. R. HIRD and P. H. SPRINGELL, *Biochem. J.*, **56**, 417 (1954).
11. P. GADAL and J. SERIS, *Acad. Sci., Paris. c.r. hebd. Séanc* **254**, 1341 (1962).
12. G. L. ELLMAN, *Archs Biochem. Biophys.* **82**, 70 (1959).
13. A. J. THERKELSON, *Acta Radiol.* **49**, 49 (1958).
14. E. L. BRANDT and A. C. GRIFFIN, *Cancer*, **4**, 1030 (1951).
15. A. WEISBERGER, R. HEINLE and B. LEVINE, *J. Clin. Invest.* **31**, 217 (1952).