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The Determination of Blood Glutathione

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The nitroprusside method for estimating glutathione is non-specific and suffers from the defect of an unstable colour, although recent improvements (Thompson & Watson, 1952; Grunert & Phillips, 1951) appear to make the method more satisfactory. Binkley, Fufii & Kimmell (1950) used a method based on the Sullivan & Hess (1936) reaction to estimate glutathione, γ -glutamylcysteine, cysteinylglycine and cysteine, but in our hands it failed to give reproducible results. We therefore had to seek another method and investigated the observation by Patterson, Lazarow & Levey (1949) that alloxan and glutathione interact to form a compound characterized by an absorption band at 305 m μ . Other sulphhydryl compounds, e.g. cysteine, react with alloxan but produce dialuric acid by reduction. This last-named substance has an absorption maximum at 275 m μ and does not interfere with measurements at 305 m μ . Some proteins react with alloxan but these can be removed by precipitation, as in the proposed method.

It appeared possible therefore that the reaction with alloxan could be used to estimate glutathione in blood, thus avoiding the interference of the peptides cysteinylglycine and γ -glutamylcysteine, which, present in blood, react in other methods as glutathione. A very sensitive method, using this reaction, has been developed which readily detects 15 μ g. of glutathione and can be used to estimate it in as little as 0.2 ml. of blood. We have not attempted to extend the method to the estimation of glutathione in tissues. The method was used to investigate the blood-glutathione concentration in some cases of therapeutic deep insulin coma.

METHOD

Principle. Whole lysed blood is deproteinized with trichloroacetic acid, neutralized with buffered NaOH and

alloxan is added to develop the compound with glutathione (GSH), which has a characteristic absorption at 305 m μ .

Reagents. 10% (w/v) Trichloroacetic acid.

0.24 M-Phosphate buffer, pH 7.6, prepared by mixing 0.24 M-Na₂HPO₄ with 0.24 M-KH₂PO₄ (88:12, v/v).

Neutralizing NaOH: this contains in 1.5 ml. of solution both 0.24 M-phosphate buffer and enough N-NaOH to neutralize 0.5 ml. of supernatant from protein precipitation (219:21, v/v).

Alloxan reagent: 1 mg. of alloxan monohydrate/ml. in dilute HCl (1 drop of conc. HCl/100 ml. of water). Prepared freshly and used within 5 min.

Standard GSH: 10 mg./100 ml. of water, freshly prepared.

0.1 M-Glycine solution.

Procedure. A specimen (0.2 ml.) of whole blood from a finger prick is added to 0.6 ml. of water. After it has been allowed to stand for 10 min. to haemolyse, 0.8 ml. of trichloroacetic acid is added, the liquids are mixed immediately with a glass rod and centrifuged for 5 min.

For each sample of blood two tubes are prepared: test (tube 1) and control (tube 2); each contains 0.5 ml. of glycine solution, and 2.9 and 3.9 ml. of phosphate buffer are added to (1) and (2) respectively. To each tube 0.4 ml. of supernatant from the deproteinized blood and then 1.2 ml. of neutralizing NaOH are added, with mixing.

An alloxan blank (tube 3: 0.5 ml. of glycine solution and 4.5 ml. of phosphate buffer), a phosphate blank (tube 4: 6 ml. of phosphate buffer) and a standard tube (tube 5: 0.5 ml. of glycine solution, 4.3 ml. of phosphate buffer and 0.2 ml. of standard glutathione) are also prepared.

Fresh alloxan solution (1 ml.) is added to tubes 1 (test), 3 (alloxan blank) and 5 (standard) and the contents of each tube are mixed. The final volume in each tube is 6 ml. This procedure is summarized in Table 1.

Readings. All extinctions are read in 2 cm. silica cells in a spectrophotometer set at 305 m μ . (A Uvispek spectrophotometer was used with hydrogen-arc lamp and slit width 19 = 0.38 mm., waveband width 5 m μ .) The control tube 2 is read against the phosphate blank immediately, as its absorption at 305 m μ increases slightly on standing. For completion of the reaction 20 min. is allowed and the test tube 1 and standard tube 5 are then read against the alloxan blank (tube 3).

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Table 1. *Preparation of tubes for estimation of glutathione*

Material	Vol. of material added (ml.)				
	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5
	Test	Control	Alloxan blank	Phosphate blank	Standard
Glycine	0.5	0.5	0.5	—	0.5
Phosphate buffer	2.9	3.9	4.5	6.0	4.3
Deproteinized blood	0.4	0.4	—	—	—
Neutralizing NaOH	1.2	1.2	—	—	—
Standard GSH	—	—	—	—	0.2
Alloxan	1.0	—	1.0	—	1.0

Fig. 1. 'Colour' development curves of reaction between glutathione and 1 mg. of alloxan in the presence of 5 μ g. of cysteine and 0.5 ml. of 0.1 M-glycine, in a reaction mixture of 6 ml. Readings of alloxan blank (a) and glutathione standard (b) were made against phosphate buffer. Standard less blank readings are indicated by (c).

The extinction of the alloxan blank rapidly decreases, whereas that of the standard increases, during the first 20 min. The maximum difference occurs between 20 and 40 min., during which time results are constant (Fig. 1).

Calculation. Let control reading against phosphate blank = E_A , test reading against alloxan blank = E_B , standard reading against alloxan blank = E_S . Then

$$40 (E_B - E_A)/E_S = \text{mg. of GSH/100 ml. of blood.}$$

RESULTS

The results of estimating GSH in blood from 14 normal adults (seven male, seven female) are given in Table 2. These values represent only GSH since oxidized glutathione does not react with alloxan (Patterson *et al.* 1949). The range of values is 24.4–37.3 mg./100 ml. and the mean 30.0, S.D. ± 4.0 . The mean value for males was 31.6 mg./100 ml., S.D. ± 4.4 , and for females 28.4 mg./100 ml., S.D. ± 2.9 . The difference between these means is not statistically significant ($P < 0.2 > 0.1$). A sex difference does not seem probable, but it would be

Table 2. *Concentration of glutathione in normal adult blood*

Concn. (mg./100 ml.)	
Males	Females
24.4	25.4
26.5	25.8
31.2	26.1
31.6	27.6
34.6	29.7
35.4	30.3
37.3	34.0
Mean 31.6	28.4
S.D. ± 4.4	± 2.9

All 14: mean 30.0, S.D. ± 4.0 .

unsafe to draw a firm conclusion from such a small series. When corrected by a recovery factor of 86 % (see below), the range of values becomes 28.4–43.4 mg./100 ml. and the mean 34.8 mg./100 ml.

Recovery experiments. In a series of recovery experiments in which known amounts of GSH were added to blood after lysis, 78–93 % (mean 86 %, S.D. ± 4) of the added GSH was accounted for. The result of one experiment is given in Table 3.

The reasons for the low variable recovery were not explored. It may be due to oxidation of GSH added to blood or to interfering substances in the supernatant after protein precipitation. Until the reasons for the low recovery are settled, the question of the advantage of using added GSH as an internal standard in the method is left open. If the low recovery is due to the oxidation of GSH added to blood, it seems likely that the proportion recovered represents the proportion of the total glutathione in blood present as GSH.

Effect of pH. The effect of various phosphate buffer mixtures is shown in Fig. 2, from which it is evident that the buffer mixture chosen gives maximum formation of the GSH-alloxan compound as measured by extinction.

Since the reaction is sensitive to pH changes, great care is necessary in the preparation and measurement of the neutralizing solution. Incomplete neutralization in particular gives rise to

Table 3. *Recovery of glutathione added to blood after lysis*

All values are given as mg. of glutathione/100 ml.

Blood GSH	GSH added	Theoretical value	GSH found	GSH recovered
26.9	11.66	38.56	37.8	10.9
26.9	23.32	50.22	46.9	20.0
26.9	34.98	61.88	56.9	30.0
	69.96			60.9
				(= 87% of GSH added)

Fig. 2. Effect of various phosphate buffer mixtures on the GSH-alloxan reaction.

Fig. 3. Calibration curves for glutathione with 1 mg. of alloxan, showing the influence of the volume of the solution. (a) Reaction volume of 3 ml., to which 3 ml. of phosphate buffer was afterwards added; (b) reaction volume of 6 ml.

Fig. 4. The presence of 0.2 ml. of 0.1M-glycine (a) improved the reaction between glutathione and 1 mg. of alloxan in 6 ml. of reaction mixture; (b) reaction without glycine.

errors, e.g. a deficiency of 0.05–0.1 ml. of the neutralizing solution may give errors of the order of –3 to –8%.

Effect of alloxan concentration. Calibration curves obtained from reaction mixtures of 3 ml. volume containing 1 mg. of alloxan show good proportionality between glutathione and extinction (Fig. 3a), but the same mixtures in 6 ml. with 1 mg. of alloxan gave a graph which did not intersect the origin (Fig. 3b). As would be expected, 6 ml. mixtures containing 2 mg. of alloxan produced a straight-line graph passing through the origin.

Effect of glycine. When 0.2 ml. of 0.1M-glycine was added, 1 mg. of alloxan in a 6 ml. mixture gave a good calibration curve (Fig. 4a), comparable to that given when 2 mg. of alloxan was used without glycine in 6 ml. Fig. 4b was obtained simultaneously without glycine. Thus the addition of glycine has an effect similar to that given by doubling the concentration of alloxan, although it does not give the same extinction values. It has, however, the great advantage of giving a much lower extinction in the blank.

Activation and inhibition. It was recognized that certain factors in the solutions (possibly heavy-metal ions) might be responsible for the anomalous calibration curves obtained in the absence of glycine. A number of compounds known to be 'sequestering agents' for metal ions were therefore included in the reaction mixture. Sodium cyanide and sodium dithionite reacted with alloxan in a way that produced a large extinction even in the

Table 4. *Effect of 'sequestering agents' on the reaction between glutathione and alloxan*

GSH (20 μ g.) was treated with 1 mg. of alloxan in 6 ml. of solution containing the sequestering agent (0.2 ml.; 0.1 M). Readings were made at 305 m μ against the appropriate blank.

Sequestering agent	<i>E</i> due to GSH
Nil	0.230
Na ₂ S ₂ O ₃	0.231
Sodium citrate	0.237
Sodium tartrate	0.238
Glycine	0.280
NaCN	} No reading possible because of high blank
Na ₂ S ₂ O ₄	

Table 5. *Effect of various reagents on the reaction between glutathione and alloxan*

GSH (19.02 μ g.) was treated with alloxan (2 mg.) in 6 ml. of solution containing the test reagent (0.3 ml.; 0.1 M). Readings were made against the appropriate blank at 305 m μ . The extinction readings of the blanks were all low, except for methionine (0.069 against phosphate blank).

Reagent	<i>E</i> due to GSH
Nil	0.285
Sodium citrate	0.300
Glycine	0.320
α -Alanine	0.322
Threonine	0.353
Aspartic acid	0.353
Serine	0.357
β -Alanine	0.271
Methionine	0.135

Table 6. *Inhibition by methionine and cysteine of the glutathione-alloxan reaction*

The reaction mixtures (6 ml.) contained alloxan (2 mg.) and, when present, GSH (19.02 μ g.), cysteine (2 μ g.) and methionine (2 μ g.).

Reaction mixture	<i>E</i> _{305 mμ}
A GSH	0.293
GSH + cysteine	0.274
Cysteine	0.004
B GSH	0.289
GSH + methionine	0.262
Methionine	0.009

absence of GSH. Of the other reagents chosen (Table 4), glycine had the greatest effect, but from Table 5 it can be seen that other amino acids had a similar or greater effect. Glycine was selected for the method purely as a matter of convenience. The maximum effect of glycine was given when 0.5 ml. of 0.1 M-glycine was included in 6 ml. of reaction mixture. Glycine also improved the recovery values of added GSH in the method with blood.

β -Alanine and methionine (Table 5) and cysteine (Table 6 and Fig. 5) have inhibitory effects on the GSH-alloxan reaction. The mechanism of this was not investigated, but there seems to be a distinguishing feature in that, in the presence of glycine, methionine inhibition is not shown, whereas cysteine inhibition is unchanged (Fig. 5). In the absence of glycine, 2 μ g. of either methionine or cysteine showed an appreciable inhibitory effect

Fig. 5. Comparison of the effect of glycine (0.5 ml.; 0.1 M) on the inhibitive effects of methionine (●) and cysteine (+) on the interaction between 19.02 μ g. of glutathione and 2 mg. of alloxan in 6 ml. of solution.

Fig. 6. Ultraviolet-absorption curves of the GSH-alloxan compound (G), the substance in blood reacting with alloxan (B) and of the alloxan compound formed in blood with added GSH (BG).

(Table 6), which could be important in some other applications of this method.

Specificity. The ultraviolet-absorption spectra of the compounds of alloxan with GSH (curve *G*), deproteinized blood (curve *B*) and deproteinized blood with added GSH (curve *BG*) are given in Fig. 6. The curves, especially *G*, are very similar to those given by Patterson *et al.* (1949). The molar extinction at 305 m μ , calculated from curve *G* on the basis of the substance in lowest concentration (GSH), is 17 130. This value is very close to the maximum value of 17 400 found by Patterson *et al.* In their case, alloxan and not GSH was the substance present in the lowest concentration. It is apparent that under the conditions of their and our experiments quantitatively similar formation of the alloxan-GSH compound occurs.

When blood supernatant was present (curves *B* and *BG*) the extinction due to the alloxan compound could not be measured at wavelengths below 270 m μ because of the high absorption by the neutralized supernatant. Although the absorption maxima of the alloxan compound with the reacting substance in blood supernatant (curves *B* and *BG*) occur at about 302 m μ and not at 305 m μ as with GSH, the curves *B* and *BG* are so nearly like that given by GSH (*G*) that it is reasonable to conclude that the substance from blood that reacts with alloxan is GSH.

The peptides cysteinylglycine and γ -glutamylcysteine in blood present the problem of possible interference by producing substances which may have absorption bands at or near 305 m μ . Because these compounds are not readily available, it has not been possible to prove the complete specificity of the method. Cysteinylglycine, however, is produced from GSH by controlled hydrolysis with hot 2M-phosphoric acid for 1 hr. (Binkley *et al.* 1950). After neutralization it was found that the alloxan-reacting material had been destroyed by the hydrolysis.

GSH (35 μ g.) in 2M-phosphoric acid, neutralized and allowed to react with alloxan, gave an extinction at 305 m μ of 0.393. GSH (35 μ g.) hydrolysed with hot 2M-phosphoric acid, neutralized and allowed to react with alloxan, gave an extinction at 305 m μ of 0.018. The fall in extinction, 0.375, due

to hydrolysis of the alloxan-reacting material, corresponds to the extinction of 0.377 given by 35 μ g. of GSH in the alloxan reaction. Thus if cysteinylglycine is formed by hydrolysis of GSH, it does not react with alloxan to give any absorption at 305 m μ and does not interfere with the estimation of GSH. Ergothioneine (29 μ g.) and alloxan did not cause any absorption at 305 m μ .

Conclusion. The effect of various substances on the interaction between GSH and alloxan indicates that any method for estimating GSH using absorption at 305 m μ must exclude interference by substances occurring naturally in blood. The influence of glycine on the reaction appears similar to its effect on the interference by amino acids in the Nakamura & Binkley method (1948) for cysteine, recorded by Hird & Springell (1954), in which glycine eliminated the depression of colour production caused by certain other amino acids.

Blood glutathione in therapeutic deep insulin coma

A short investigation was made of the blood GSH concentration in therapeutic deep insulin coma. By the courtesy of Dr A. S. Thorley, in 1953 four patients were studied. They were adult males who had had eight to 14 comas previously. Blood was collected after onset of coma until recovery. The results are given in Table 7. Blood GSH concentration was not affected in these patients by the state of hypoglycaemic coma or by the amount of insulin given to induce coma. Patient 4 had a slightly sub-normal blood GSH both during coma and after recovery when the blood glucose was in the normal range.

In prolonged hypoglycaemic coma, when a patient undergoing therapeutic deep insulin coma fails to recover after being given glucose, abnormal values of blood GSH may occur. In one patient values of 77–90 mg./100 ml. were found, the blood glucose being normal or above normal. In another, a value of 65 mg./100 ml. was obtained but in two others normal values (29.8, 31.2 and 33.9 mg./100 ml.) were found. There did not seem to be any simple relationship between the blood GSH and the prolonged coma.

Table 7. *Blood concentration of glutathione in therapeutic deep insulin coma*

Patient no.	Units of insulin given	No. of coma	Time (min.) after onset of coma				After recovery
			7	30-40	60-70	100	
			Blood GSH (mg./100 ml.)				
1	320	12	34.8	34.4	35.7	34.4	—
2	280	9	—	36.2	37.1	36.8	34.3
3	220	15	—	30.6	31.5	30.0	—
4	120	10	—	23.8	23.4	23.1	22.3

SUMMARY

1. The application of the reaction between glutathione and alloxan, giving a substance characterized by an absorption band at 305 m μ , to the estimation of glutathione in blood is described.

2. Certain substances influence the reaction: β -alanine, methionine and cysteine are inhibitors and citrate, glycine and other α -amino acids are activators.

3. Some observations of blood glutathione concentration in normal and prolonged therapeutic deep insulin coma are recorded.

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