THE NITROPRUSSIDE METHOD FOR THE DETERMINATION OF BLOOD GLUTATHIONE

BY

R. H. S. THOMPSON AND D. WATSON

From the Department of Chemical Pathology, Guy's Hospital Medical School, London

(RECEIVED FOR PUBLICATION JUNE 18, 1951)

Recent studies on alloxan diabetes (Lazarow, 1949), together with observations on the effect of adrenocorticotrophic hormone on available sulphhydryl compounds (Conn, Louis, and Johnston, 1949), have focused the attention of a number of laboratories on the level of blood glutathione in diabetes and other conditions.

In the course of work in this laboratory on the effects produced in the body by various heavy metals the need arose for the estimation of blood glutathione levels by some method other than an iodometric one. Brückmann and Wertheimer's (1947) modification of the nitroprusside method described by Fujita and Numata (1939) was therefore adopted, but was not found to give consistent results in our hands. A number of methods for the determination of glutathione (GSH) in blood and tissues by means of the nitroprusside reaction have been described in the literature, but most of these have suffered from a rapid fading of the final colour and, in some instances, from the instability of one or more of the reagents. For these reasons the various steps in the procedure of Brückmann and Wertheimer (1947) have been examined in detail. A modified method has been worked out which is simple and has the double advantage of giving greater sensitivity and also greater stability of the final colour.

Experimental

Reagents.—The following were used.

- (1) 10% (w/v) trichloroacetic acid (AR).
- (2) Ammonium sulphate, solid (AR).
- (3) Ammonium sulphate, saturated aqueous solution.
- (4) M-sodium nitroprusside (AR), 0.05 in saturated $(\text{NH}_4)_2\text{SO}_4$.
- (5) 8 N Ammonium hydroxide (AR).
- (6) Standard glutathione (B.D.H.) solution, 1 mg./ml. This solution must be freshly prepared for each estimation.

All solutions must be prepared with glass-distilled water, and all glassware must be scrupulously clean.

Method.—A quantity, 0.5 ml., of freshly drawn blood (oxalated or heparinized) is added to 1.5 ml. of glass-distilled water in a 15 ml. centrifuge tube, and allowed to stand for five minutes to lake. Then 2 ml. of 10% trichloroacetic acid are added, and the mixture well stirred with a glass rod and allowed to stand for five minutes, after which it is centrifuged for five minutes at 3,000 r.p.m. Then 2 ml. of the clear, protein-free supernatant are pipetted into a stoppered 10 ml. graduated cylinder. Solid $(\text{NH}_4)_2\text{SO}_4$ is added to saturate the solution (by tipping in 1.4 g. contained in a marked tube); after shaking the volume is made up to 3 ml. with saturated $(\text{NH}_4)_2\text{SO}_4$.

For the production of the colour 0.5 ml. of 0.05 M-nitroprusside reagent is added, followed by 0.7 ml. of 8N NH_4OH .

After transferring into cells of 1 cm. optical depth the colour intensity is read in a photoelectric colorimeter 30 seconds after the addition of the NH_4OH , using an Ilford spectrum green (604) filter. A reagent blank determination is carried out using 1 ml. water and 1 ml. 10% trichloroacetic acid, with all subsequent additions as for the blood filtrate. All tubes are compared against water.

Standards.—The standard glutathione solution is diluted tenfold with H_2O to give a solution containing 100 μg . GSH/ml. To prepare a standard curve 0.2, 0.4 . . . 1.0 ml. samples (i.e. 20, 40, . . . 100 μg . GSH) are each diluted with H_2O to 1.0 ml. Then 1.0 ml. of 10% trichloroacetic acid is added to each, and the estimation carried out as for the blood filtrates. One standard tube (containing 60 or 80 μg . GSH) is included in each series of blood estimations.

Results

We have avoided the use of saponin and of saturation with NaCl before precipitation of proteins as in the method of Brückmann and Wertheimer (1947), because of the difficulty which we experienced in obtaining clear, protein-free supernatants without recourse to filtering. By initially diluting the blood with 1.5 ml. of water an adequate degree of laking is obtained; centrifugation after precipitating the proteins with trichloroacetic acid then results in a well-packed precipitate with a clear supernatant. The use of ammonium sulphate rather than NaCl to saturate this supernatant results also in greater colour intensities for a given concentration of GSH.

Fujita and Numata (1939) and Brückmann and Wertheimer (1947) used 1% sodium nitroprusside in 0.4% $(\text{NH}_4)_2\text{SO}_4$. This reagent is extremely unstable, whereas 0.05 M (1.5%) sodium nitroprusside in saturated $(\text{NH}_4)_2\text{SO}_4$ is stable for at least a week. It was also found to give greater intensities than the 1% solution. We have also used 8N NH_4OH instead of the 25% Na_2CO_3 recommended by Brückmann and Wertheimer (1947) on account of the greater optical density produced (E for 60 μg . GSH=0.130 with 25% Na_2CO_3 , 0.170 with 35% K_2CO_3 , and 0.295 with 8N NH_4OH).

The results which we have obtained with the method described above using pure solutions of GSH are shown in Table I, mean values and standard deviations being given for the extinctions obtained in 10 different experiments.

It will be seen that the colour intensities follow Beer's law for amounts up to 100 μg . Higher quantities have not been studied, since blood levels do not normally exceed this range.

TABLE I
OPTICAL DENSITIES OF COLOURS DEVELOPED FROM AQUEOUS SOLUTIONS OF GSH *

	GSH (μg .)				
	20	40	60	80	100
Mean optical densities ..	0.093	0.187	0.281	0.377	0.467
S.E.	± 0.005	± 0.005	± 0.008	± 0.009	± 0.006

* Readings at 1 cm. cell depth using an Ilford green filter 604. Ten observations were made at each concentration.

DETERMINATION OF BLOOD GLUTATHIONE

27

The variability from day to day in the results obtained with these standard solutions is not large, and is almost certainly due chiefly to changes in room temperature. We have confirmed the findings of Bierich and Rosenbohm (1933) and Grunert and Phillips (1951) that the colour intensity decreases with increasing temperature; it is therefore advisable to carry out the estimations at a standard temperature, although, if, as is recommended, a standard solution of GSH is included in each series of blood estimations, the final error due to temperature changes is not great.

Stability of Colour.—Under the final conditions chosen the rate of fading of the colour up to 50–60 seconds is sufficiently low to permit reasonably accurate reading at 30 seconds after the addition of the NH_4OH . The optical densities (uncorrected for the blank) obtained at varying intervals up to 90 seconds are shown in Table II.

TABLE II
OPTICAL DENSITY OF SOLUTIONS AT INTERVALS AFTER ADDITION OF NH_4OH *

GSH ($\mu\text{g.}$)	Uncorrected Optical Densities at					
	20	30	40	50	60	90 sec.
0	0.071	0.071	0.073	0.077	0.080	0.083
25	0.186	0.184	0.184	0.185	0.187	0.177
50	0.316	0.311	0.308	0.305	0.302	0.290
75	0.442	0.439	0.432	0.424	0.418	0.401
75	0.445	0.440	0.435	0.428	0.420	0.390
100	0.532	0.525	0.516	0.508	0.500	0.461
100	0.530	0.520	0.513	0.510	0.505	0.475

* Readings at 1 cm. cell depth using an Ilford green filter 604. Ten observations were made at each concentration.

Recovery of Added GSH.—To assess the accuracy of the method when applied to blood, recovery experiments have been carried out with known amounts of GSH added to samples of blood immediately after drawing and before precipitation of proteins (Table III).

TABLE III
RECOVERY OF GSH ADDED TO BLOOD SAMPLES IMMEDIATELY AFTER DRAWING

GSH ($\mu\text{g.}$) in Original Blood Sample	GSH ($\mu\text{g.}$) Added	GSH ($\mu\text{g.}$) Found	GSH ($\mu\text{g.}$) Recovered	Recovery (%)
70.0	10	79.6	9.6	96
75.5	15	89.4	13.9	93
80.2	15	95.0	14.8	99
77.6	20	96.6	19.0	95
60.5	20	80.0	19.5	98

Estimation of GSH in Blood.—The GSH contents of samples of blood from 21 healthy adults (15 male and six female) have been determined by this method. No significant difference was found between the values for males and females. In each case the packed cell volume was determined and found to be within the normal range. The mean value for the GSH level was 30.7 mg./100 ml. (S.E. = ± 2.05).

In a study of the GSH content of various tissues Leaf and Neuberger (1947) found that the values obtained by an iodometric method were, in the case of liver and kidney, often considerably higher than those obtained by the nitroprusside method of Fujita and Numata (1939); a similar discrepancy between these two methods had been described earlier by Bierich and Rosenbohm (1933). We have therefore compared, on samples from 13 different healthy subjects, the results obtained by our method with those found by the method of Woodward and Fry (1932). In each case the nitroprusside method gave a lower value than that obtained iodometrically, the difference varying from 0.7 to 16.8 mg./100 ml.

TABLE IV
COMPARISON OF BLOOD GSH LEVELS IN 13 DIFFERENT SUBJECTS BY NITROPRUSSIDE
AND IODOMETRIC METHODS

Nitroprusside (mg. GSH/100 ml.)	Iodometric (mg. GSH/100 ml.)	Difference
32.1	34.6	-2.5
30.0	30.7	-0.7
35.4	37.4	-2.0
29.3	41.8	-12.5
31.6	35.1	-3.5
30.1	36.8	-6.7
27.9	31.6	-3.7
29.4	32.0	-2.6
31.6	35.2	-3.6
31.7	48.5	-16.8
30.8	34.6	-3.8
33.4	34.3	-0.9
32.5	40.2	-7.7

Discussion

One of the chief disadvantages in the use of the nitroprusside reaction for the quantitative determination of thiols has been the instability of the colour. Although the observed rate of fading in the method described here is small a few experiments were done in the hope of minimizing this. Abderhalden and Wertheimer (1923) had reported that the addition of cyanide decreased the rate of fading, and in view of the reactivity of thiols with traces of metals we therefore investigated the effect of the addition of KCN and of various metal-binding agents before developing the colour. Neither KCN, 8-hydroxyquinoline, sodium diethyldithiocarbamate, nor α -benzoin oxime ("cuppron") had any observed effect in the concentrations studied. This finding agrees in general with the results of Herrmann and Moses (1945), who also studied the effect of various metal-binding agents, although these workers did report some stabilization with hydroxyquinoline.

The trichloroacetic acid extracts can safely be left for at least one hour at room temperature before carrying out the nitroprusside reaction without subsequent loss of colour intensity. In agreement with Leaf and Neuberger (1947) we have found that if the NH_4OH is not added immediately after the addition of the nitroprusside reagent the colour intensity produced is low.

Unless the reaction is carried out at a carefully controlled temperature, it is inadvisable to attempt to work from any precalibrated standard curve; a standard GSH solution should, instead, be included in each series of estimations.

Since some workers are now interested in blood GSH levels in diabetes, we felt it desirable to know whether any interference was to be expected, under the conditions of our method, from the slowly developing nitroprusside reaction given by aceto-acetic acid. The level of total ketone bodies in the blood in health is not usually much in excess of 1 mg./100 ml. expressed as β -hydroxybutyric acid (Weichselbaum and Somogyi, 1941; Greenberg and Lester, 1944; Billing, 1951). Amounts of aceto-acetic acid roughly equivalent to blood levels of 4 mg./100 ml. gave no measurable colour at 30 seconds, although by 90 seconds a significant degree of colour had already developed; amounts of aceto-acetic acid much above this level would of course interfere even at 30 seconds.

Summary

The nitroprusside method for the estimation of blood glutathione has been studied, and modifications made in order to increase the sensitivity and decrease the rate of fading of the final colour.

Our thanks are due to the volunteers who provided blood samples in the course of this work, and to Mr. D. H. Burchett for technical assistance.

REFERENCES

- Abderhalden, E., and Wertheimer, E. (1923). *Pflüg. Arch. ges. Physiol.*, **198**, 122.
Bierich, R., and Rosenbohm, A. (1933). *Hoppe-Seyl. Z.*, **215**, 151.
Billing, B. H. (1951). Unpublished results.
Brückmann, G., and Wertheimer, E. (1947). *J. biol. Chem.*, **168**, 241.
Conn, J. W., Louis, L. H., and Johnston, M. W. (1949). *J. Lab. clin. Med.*, **34**, 255.
Fujita, A., and Numata, I. (1939). *Biochem. Z.*, **300**, 246.
Greenberg, L. A., and Lester, D. (1944). *J. biol. Chem.*, **154**, 177.
Grunert, R. R., and Phillips, P. H. (1951). *Arch. Biochem.*, **30**, 217.
Herrmann, H., and Moses, S. G. (1945). *J. biol. Chem.*, **158**, 33.
Lazarow, A. (1949). *Physiol. Rev.*, **29**, 48.
Leaf, G., and Neuberger, A. (1947). *Biochem. J.*, **41**, 280.
Weichselbaum, T. E., and Somogyi, M. (1941). *J. biol. Chem.*, **140**, 5.
Woodward, G. E., and Fry, E. G. (1932). *Ibid.*, **97**, 465.