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THE APPLICATION OF 5,5′ DITHIOBIS-2-NITROBENZOIC ACID TO THE DETERMINATION OF THE ACID-SOLUBLE DISULPHIDE CONTENT OF BLOOD

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Abstract—The susceptibility of the thiol group of reduced 5,5′-dithiobis-2-nitrobenzoic acid (DTNB)* to selective oxidation by aliquots of sulphosalicylic acid-extracted blood is described, and the effect attributed to the low pK_a of the thiol group of the reduced reagent compared with that of glutathione. An analytical procedure is described which corrects for this tendency to reagent oxidation, and enables a true measure of the blood thiol content to be estimated.

THE content of acid-soluble sulphydryl compounds in biological tissues may be measured by potentiometric,[1, 2] enzymatic,[3] iodometric,[4] or colorimetric techniques.[5] 5,5′ dithiobis-2-nitrobenzoic acid (DTNB) is a recent addition to the assembly of colorimetric thiol reagents, and its application to the estimation of sulphydryl compounds in blood has been described by Beutler,[6] and by Stevenson.[7] The present communication describes the use of this reagent in the estimation of the acid-soluble disulphide content of blood, and underlines some precautionary measures necessary in the general application of the reagent.

MATERIALS AND METHODS

GSH and GSSG were purchased from Sigma Chemical Co., CNB and DTNB from Aldrich Chemical Co. A sample of the latter melted at 239·5/241·5°. (Found: C, 42·52%; H, 2·10%; N, 7·10%; S, 15·68%. Calc. for $C_{14}H_8N_2O_8S_2$: C, 42·40%; H, 2·04%; N, 7·06%; S, 16·2%.) This material was used without further purification. Other chemicals were obtained from Hopkin and Williams Ltd., or B.D.H. Ltd., AnalaR grades being used where available.

Blood was taken by cubital venepuncture from healthy human donors, heparinized, and saturated with carbon monoxide at 0°. Deproteinization was carried out by homogenizing one volume of whole blood with nine volumes of ice-cold 3% SSA in a Potter–Elvejhem homogenisor at 2000 rpm. After standing for 20 min, protein was removed by centrifugation at 400 *g* and 0°. Thereafter the deproteinized blood sample was stored at 0° until required for assay.

Soluble thiols were estimated potentiometrically,[8] or by the following colorimetric procedure:

* The following abbreviations will be used throughout this paper: DTNB: 5·5′dithiobis-2-nitrobenzoic acid; CNB: 5-chloro-2-nitrobenzoic acid; GSH: reduced glutathione; GSSG: oxidized glutathione; SSA: sulphosalicylic acid; SSAST: supernatant from SSA-extracted blood; EDTA: ethylenediamine tetraacetic acid.

To 4·0 ml DTNB, $0{\cdot}5 \times 10^{-3}$ M in 1·0 M phosphate buffer pH 7·0 (10^{-3} M with respect to EDTA), was added 1·0 ml SSAST. The optical density of the resulting yellow colour was read at 410 mμ in a 1·0 cm cell in the Unicam S.P. 500 spectrophotometer. In order to obtain reliable duplication it was found necessary to develop the colours in stoppered tubes which had been rinsed previously with DTNB reagent. This procedure yielded the "free thiol" content of the blood sample. Electrolytic reduction[8] of a further aliquot of deproteinized blood, followed by application of the colorimetric procedure outlined above gave the "total thiol" content.

The spectra of reduced DTNB samples were measured as follows: a 1 cm cuvette was sealed with a rubber cap carrying needles for the delivery and egress of argon. A steady stream of argon (freed from contaminating traces of oxygen by washing in an acidic solution of vanadyl sulphate over zinc amalgam[9] was passed through the cell and solutions were injected through the cap as follows: 0·2 ml DTNB ($0{\cdot}5 \times 10^{-3}$ M in 0·01 M pyrophosphate buffer pH 9·0) and 1·1 ml 0·01 M pyrophosphate buffer pH 9·0. Addition of 0·2 ml 0·05 M sodium borohydride in 0·01 M pyrophosphate buffer pH 9·0 followed 5 min later, and after a further 15 min, 1·0 ml of a 1·0 M solution of the chosen buffer was injected anaerobically. The spectrum was measured after a further 5-min gassing period in a Cary Model 11 recording spectrophotometer, following removal of the gassing tubes.

The pK_a of the thiol group of reduced DTNB was measured colorimetrically: 5 ml of a 10^{-3} M solution of DTNB in 0·003 M NaOH was added to 10·0 mg sodium borohydride in a stream of oxygen-free argon. After 10 min, 0·25 ml N/1 HCl (also oxygen free) was added to the reduced DTNB in order to destroy excess borohydride: 0·05 ml of the resultant solution was added to 2·7 ml 0·01 M oxygen-free formate or acetate buffer in the pH range 3·7–5·9. The optical density was measured at 20° in a Hilger–Gilford reaction kinetics spectrophotometer, and the pH measured at 20° with a Beckman expanded scale pH meter (model 76).

RESULTS

A comparison of blood disulphide levels determined by the potentiometric and DTNB procedures in three donors is shown in Table 1. The free thiol colours were

TABLE 1. COMPARISON OF POTENTIOMETRIC (Ag) AND COLORIMETRIC (DTNB) ASSAY PROCEDURES FOR TOTAL BLOOD THIOL AND DISULPHIDE LEVELS

		SH (μmoles/ml blood)			
		Potentiometric		Colorimetric*	
Subject	Sex	Total	%SS	Total	%SS
R.B.	M	1·03	Nil	1·05	76
M.W.	M	0·98	Nil	0·91	62
L.H.	M	1·30	Nil	1·32	71

"Free thiol" colours allowed to develop for up to 30 min before measuring E^1_{410}.

developed and allowed to stand while the electrolytic reduction and colour development of the total thiols was carried out: the optical densities of all solutions were then read together. It can be seen that considerable amounts of disulphide were

registered in the colorimetric procedure, while none were detected potentiometrically. In order to check whether this discrepancy could be attributed to fading of the colorimetric reagent, the time course of colour development in a number of blood samples was examined. The data for one such experiment (representative of several) are plotted in Fig. 1. The colours developed in DTNB solutions containing GSH or

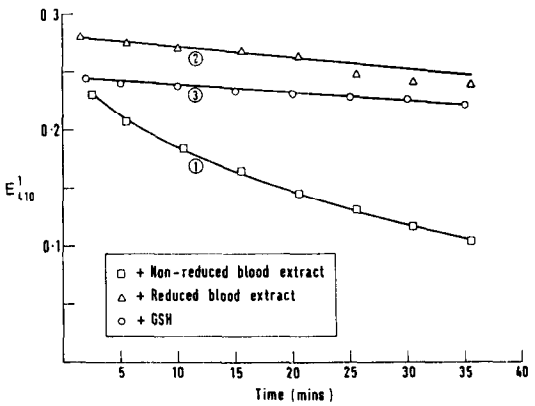

FIG. 1. Colour Loss in presence of acid extract of whole blood. 4 ml DTNB ($0{\cdot}5 \times 10^{-3}$ M in 1·0 M phosphate buffer pH 7·0, 10^{-3} M with respect ot EDTA) containing:
1. 0·9 ml SSAST (non-reduced) + 0·1 ml 3% SSA; 2. 0·9 ml SSAST (reduced) + 0·1 ml 3% SSA; and 3. 1·0 ml 10^{-4} M GSH in 3% SSA.

electrolytically reduced SSAST were relatively stable compared with that developed in reagent containing unreduced SSAST. In this latter case, progressive colour fading occurred, so that in 30 min, more than 60 per cent of the colour developed initially had been lost.

This colour fading could have been due to oxidation—either of the glutathione present in the deproteinized blood supernatant, or of the reduced reagent itself. That the latter is the correct explanation is confirmed by the results of the experiments described in Table 2 and Fig. 2. Table 2 compares the percentage loss of thiol when glutathione or aliquots of SSAST were incubated either in presence of buffered DTNB, or in buffer alone for 1 hr at 25°. Where incubations were carried out in the absence of DTNB, thiol estimations were performed potentiometrically.[8] In those solutions containing DTNB, GSH and reduced DTNB were components of the reversible equilibrium:

$$\text{GSH} + \text{RSSR} \rightleftharpoons \text{GSSR} + \text{RSH}$$
$$(\text{R} = \text{1-Carboxy-2-nitrophenyl-5-})$$

so that loss in E^1_{410} represented the combined oxidation of glutathione-SH and reduced DTNB -SH: in the absence of DTNB, however, the silver estimations recorded only the oxidation of glutathione -SH.

It is evident from Table 2 that GSH in phosphate buffer at pH 7·0 was resistant to oxidation as measured by silver assay, while some oxidation took place as recorded by DTNB assay. However, in presence of deproteinized blood supernatant, an

TABLE 2. PERCENTAGE LOSS OF THIOL AFTER 1 HOUR'S INCUBATION IN AIR AT 25°

Subject	In buffered DTNB		In buffer alone (Ag Assay)	
	+ SSAST	+ GSH	+ SSAST	+ GSH
M.T.	46·0	6·5	15·0	Nil
S.F.	47·0	9·1	10·0	Nil
D.M.	47·0	7·8	8·6	Nil

Incubations
In buffered DTNB: 1 ml SSAST or 1 ml GSH (2×10^{-4} M) + 4·0 ml $0{\cdot}5 \times 10^{-3}$ M DTNB in 1·0 M phosphate buffer pH 7·0 (10^{-3} M with respect to EDTA).
In buffer: 1 ml SSAST or 1 ml GSH (2×10^{-4} M) + 4·0 ml 1·0 M phosphate buffer pH 7·0 (10^{-3} M with respect to EDTA).

average of 11·2 per cent oxidation was detected by silver assay and 47 per cent by DTNB assay. It is apparent that the increased level of oxidation in the DTNB assay must be attributed to oxidation of reduced reagent.

Figure 2 illustrates the data from a similar experiment: aliquots of glutathione or glutathione and SSAST were incubated continuously either in buffered DTNB or in

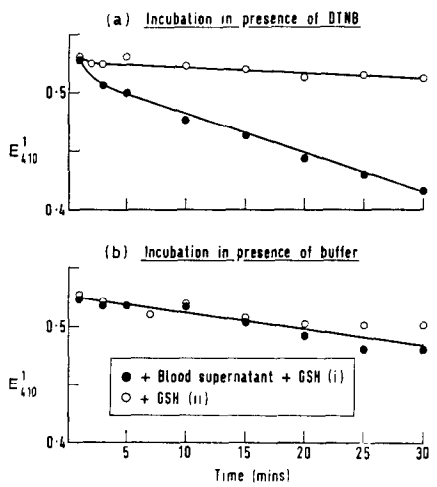

FIG. 2. Autoxidation of reduced DTNB and glutathione.

(a). (i) 40 ml DTNB ($0{\cdot}5 \times 10^{-3}$ M in 1·0 M phosphate buffer pH 7·0, 10^{-3} M with respect to EDTA) + 9 ml SSAST + 1 ml 10^{-3} M GSH.

(ii) 40 ml DTNB ($0{\cdot}5 \times 10^{-3}$ M in 1·0 M phosphate buffer pH 7·0, 10^{-3}M with respect to EDTA) + 8 ml 3% SSA + 2 ml 10^{-3} M GSH.

(b). (i) 40 ml 1·0 M phosphate buffer pH 7·0 (10^{-3} M with respect to EDTA) + 9 ml SSAST + 1 ml 10^{-3} M GSH.

(ii) 40 ml 1·0 M phosphate buffer pH 7·0 (10^{-3} M with respect to EDTA) + 8 ml 3% SSA + 2 ml 10^{-3} M GSH.

For thiol estimation added 4 ml to 0·05 ml 0·04 M DTNB in 1·0 M phosphate buffer (10^{-3} M with respect to EDTA) and measured E^1_{410}.

buffer alone at 25°. The thiol content of samples incubated in buffer alone was estimated by addition of an aliquot of the incubate to DTNB solution and recording the colour developed at 410 mμ within 2 min, thus measuring the rate of oxidation of glutathione throughout the incubation. In presence of buffered DTNB, monitoring the optical density at 410 mμ gave a combined measure of the rate of autoxidation of the reduced reagent and of reduced glutathione. It is apparent from Fig. 2 that the presence of acid-soluble blood supernatant accelerated considerably the rate of autoxidation of reduced DTNB, while having little effect on the autoxidation of glutathione.

Addition of solutions of ferric and cupric salts, in concentrations equivalent to those found in deproteinized blood supernatants, to solutions containing GSH and DTNB, did not give oxidation rates comparable to those obtained by the addition of an equivalent volume of SSAST: the rates in presence of added metal were approximately 1/10th of those found with SSAST. Addition of ashed supernatants gave similar results. The oxidation effect could not be eliminated by increasing the concentration of EDTA. Further, the effect was associated with the gross cellular fraction of the blood—no oxidation of reduced DTNB was obtained when aliquots of deproteinized serum were added to DTNB solutions containing GSH.

In order to compensate for the colour fading which occurred in DTNB solutions containing SSAST, the optical density at 410 mμ was followed for 10 min, and the curves extrapolated to zero time. A series of estimations according to this procedure gave the figures listed in Table 3 for the content of total SH and disulphide in whole blood.

TABLE 3. ESTIMATION OF SULPHYDRYL AND DISULPHIDE CONTENT OF WHOLE BLOOD (via extrapolation of DTNB autoxidation curves)

Subject	Sex	Total SH*	% SS
M.A.	F	1·08	6
B.H.	F	0·89	Nil
N.C.	M	1·35	Nil
J.D.	M	1·17	3
R.J.	M	1·34	Nil
R.B.	M	1·05	Nil

* μMoles/ml whole blood.

The measurement of the absorption spectrum of DTNB at various pH's revealed the presence of an isosbestic point at 359 mμ, confirming the existence of a dissociable equilibrium $RSH \rightleftharpoons RS^- + H^+$ (see Fig. 3).

A p*Ka* of 4·8 ± 0·1 was calculated for the sulphydryl group of reduced DTNB after substitution in the equation:

$$pKa = pH + \log \frac{E_S^- - E}{E - E_{SH}}$$

and correction for the activity of the buffer salts used,[10] where

E_{SH} = Optical density of a solution containing only the undissociated form of reduced DTNB;

E_{S^-} = Optical density of a solution containing only the dissociated form of reduced DTNB;

E = Optical density of a solution containing a mixture of the RSH and RS^- forms of reduced DTNB.

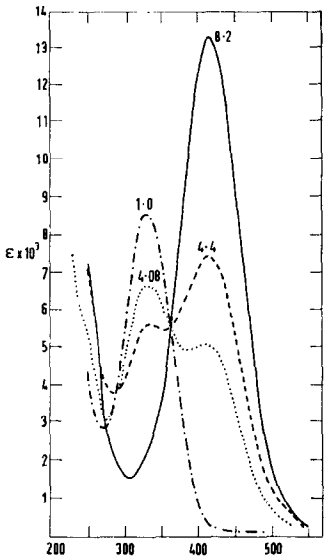

FIG. 3. Absorption spectra of reduced DTNB. pH's marked on curves. Experimental procedure described in 'Materials and Methods' section.

An examination of the absorption spectra of 10^{-4} M solutions of CNB in N/10 HCl and N/10 NaOH revealed the absence of absorption of 410 mμ in both cases (the nearest absorption band being at 282 mμ) and confirmed that ionisation of the carboxyl group of reduced DTNB was not associated with colour changes at 410 mμ.

DISCUSSION

Sulphosalicylic acid extracts of whole blood accelerated the rate of autoxidation of reduced DTNB solutions while not affecting the rate of autoxidation of glutathione under the same conditions of pH and temperature: this effect is attributed to the difference in p*Ka* of the sulphydryl groups of reduced DTNB and glutathione respectively. The rate of autoxidation of -SH compounds is proportional to the concentration of $-S^-$ form present: Calvin[11] has reported a p*Ka* of 8·7 for the SH group of glutathione, while the author of this paper has determined a value of 4·8 for the p*Ka* of the SH group of reduced DTNB. Hence at pH 7·0 (the pH chosen for colorimetric assay), in a mixture containing reduced glutathione and reduced DTNB, more than 99 per cent of the glutathione will be in the unionized form, while more than 99 per cent of the reduced DTNB will be dissociated.

Thus the pH chosen for the DTNB-GSH reaction is also the pH at which the reagent is almost completely dissociated, and therefore susceptible to oxidation. However it is possible to compensate for reagent oxidation by measuring the rate of decolourisation and extrapolating back to zero time. Such a procedure yields blood

disulphide figures comparable to those obtained previously by a potentiometric procedure.[8]

The identity of the component present in acid-extracted blood which is responsible for the oxidation effect is unknown. It is clear that the effect cannot be attributed to heavy metals: it may be that traces of iron porphyrin in the acid extract are responsible for the effects described.

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