A Colorimetric Micro-Method for the Determination of Glutathione

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1. A rapid colorimetric and apparently specific micromethod for the determination of total glutathione in small amounts of tissue is described. Generally, less than 30mg. of tissue is sufficient and this is homogenized in ice-cold 3% metaphosphoric acid. The product is filtered through sintered glass and neutralized or diluted before being added to a cuvette containing phosphate buffer, pH 7.1, 5,5'-dithiobis-(2-nitrobenzoic acid), EDTA and glutathione reductase. Addition of NADPH₂ to the system initiates a progressive reduction of 5,5'-dithiobis-(2-nitrobenzoic acid) by catalytic amounts of GSH, and this causes a colour increase at 412m μ . The rate of this change, calculated over 5min., is proportional to the total amount of glutathione present, and consequently unknown concentrations may be determined by reference to standards. 2. A preparation (based on that of Racker, 1955) of a suitable sample of glutathione reductase from yeast is described. 3. A less specific and less sensitive determination of extracted thiol groups with 5,5'-dithiobis-(2-nitrobenzoic acid) at pH 8.0, based on observations of Ellman (1959) and Jocelyn (1962), is also described. 4. Although the precise nature of the reaction is not known, evidence is put forward to support a process of cyclo-reduction. GSSG is reduced enzymically to GSH, which reacts with 5,5'-dithiobis-(2-nitrobenzoic acid) to produce a coloured ion:

(E_{max} 412m μ) and a mixed disulphide. This disulphide reacts with further quantities of GSH to liberate another ion and GSSG, which then re-enters the cycle.

After the isolation of glutathione (Hopkins, 1921) many attempts were made to devise a satisfactory method for the determination of small amounts of this tripeptide in tissues. Recently, two new reagents have been introduced, namely bis-(*p*-nitrophenyl) disulphide (Stevenson, McDonald & Roston, 1960) and the more water-soluble DTNB* (Ellman, 1959; Beutler, Duvon & Kelly, 1963; Jocelyn, 1962) both of which have been used for thiol estimation. The procedure described below utilizes DTNB, but in contrast with other methods it measures the increase in extinction at 412m μ when either GSSG or GSH, glutathione reductase, NADPH₂ and DTNB are brought together at the appropriate pH. Under suitable conditions the rate of change in extinction is proportional to the total amount of glutathione present. The mechanism of the reaction is not yet clear, but it appears

to be specific and can be used for approx. 0.2 μ g. of glutathione in a 1cm. spectrophotometric cell. By using highly active glutathione reductase and micro-cells it should be possible to increase the sensitivity five- to ten-fold.

MATERIALS AND METHODS

DTNB was obtained from the Aldrich Chemical Co. Inc., Milwaukee, Wis., U.S.A. DTNB (39.6mg.) and NaHCO₃ (15mg.) were dissolved in 5ml. of 0.1M-phosphate buffer, pH 7.0, and the volume was adjusted to 10ml. with buffer. The reagent is stable at -15° in the dark.

NADPH₂ was obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. A solution of 4mg./ml. of 0.02N-NaOH, stable at -15°, was kept for a maximum of 2 days.

NADH₂ was obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A., and 4mg./ml. was dissolved in 0.02N-NaOH.

EDTA was obtained from L. Light and Co. Ltd., Colnbrook, Bucks. A solution of 73.1mg. was adjusted to pH 7.0

* Abbreviation: DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid), i.e. di-(5-carboxy-4-nitrophenyl) disulphide.

with 0.1N-NaOH and made up to 250 ml. with water. This reagent was added to eliminate any variations in response that could be caused by extraneous heavy-metal ions.

Trisodium phosphate (British Drug Houses Ltd., Poole, Dorset) was used as an aqueous solution containing 150 g. of $\text{Na}_2\text{PO}_4 \cdot 12\text{H}_2\text{O}$ /l.

Metaphosphoric acid solution (3.0%, w/v), made by dissolving sticks in deionized or glass-distilled water at room temperature, was prepared daily. Latterly this reagent was stirred with 2 g. of decolorizing charcoal/100 ml. for 20 min. at room temperature to remove oily impurities that frequently appeared.

GSH was supplied by British Drug Houses Ltd. A standard solution of 2 mg. in 20 ml. of 3.0% (w/v) HPO_3 was prepared daily and kept at 0° to limit hydrolysis and prevent oxidation.

GSSG was supplied by C. F. Boehringer und Soehne G.m.b.H., and standard solutions were made up in water.

Glutathione reductase was either purchased from the Sigma Chemical Co. (glutathione reductase type III) or C. F. Boehringer und Soehne G.m.b.H., or prepared from yeast as described below. Fresh solutions of the Sigma product were made daily by diluting approx. 1:80, and similarly the Boehringer sample was diluted 1:20. Repeated freezing and thawing caused considerable loss of activity, and ox-plasma albumin was not added as a stabilizer since it interfered with the assay reaction.

The enzyme was prepared from yeast by a method based on Racker (1955) but included a number of modifications. A sample (1 lb.) of baker's yeast (The Distillers Co. Ltd., Speke, Liverpool) is crumbled into a mortar and 20 ml. toluene added. A paste is made that is allowed to stand for 4 hr.; then 200 ml. of 0.66M- Na_2HPO_4 is added and the mixture stirred continuously at room temperature for 24 hr. The suspension is centrifuged at 1600g long enough for a clear supernatant to form; it is then decanted and kept. The sediment is resuspended in another 200 ml. of 0.66M- Na_2HPO_4 and stirred at room temperature for a further 24–36 hr. The centrifugation is repeated, and the two supernatants are combined, heated at 53–58° in a water bath for 15 min. while being stirred and then centrifuged at 14000g for 5 min. in an angled centrifuge. The supernatant, which should be clear, is removed and its volume measured after cooling to approx. 0°. One-half its volume of acetone at –12° is added slowly with stirring and the mixture is allowed to stand for 1 hr. at 4° with occasional stirring. After this, Racker's (1955) technique is followed up to the addition of protamine sulphate, when, in the present case, 2.5 ml. of 2% (w/v) protamine sulphate is added slowly at 4°. After a few minutes the suspension is centrifuged for 5 min. at –3° and 14000g and the supernatant, in a plastic centrifuge tube, is adjusted to pH 5.5 with about 2.5 ml. of 1N-acetic acid and cooled to near freezing in acetone–solid CO_2 mixture, and 6–8 ml. of 95% (w/v) ethanol at –12° is added with stirring. After standing for 5 min. the precipitate is collected by centrifugation at 14000g for 5 min. and kept partly dissolved at –15° in 20 ml. of water. This suspension may or may not be diluted further to give suitable activity, but it should be divided into 1 ml. portions and frozen at –15° to avoid daily thawing and re-freezing, which results in loss of activity. Under these conditions the enzyme is stable for at least 9 months. When dilution is necessary it is best done on each 1 ml. sample.

Ox-plasma albumin was purchased from Armour Pharma-

ceutical Co., Eastbourne, Sussex and was used directly as described.

Sulphur-containing compounds (described under specificity and interfering substances) were very kindly given by Dr H. Heath, Department of Chemical Pathology, University College Hospital Medical School, London.

Extinction measurements were made on either an Optica recording spectrophotometer or a manually operated instrument with glass cells of 1 cm. light-path.

RECOMMENDED METHOD AND RESULTS

The addition of certain reducing agents, including GSH, to DTNB results in a change of spectrum and intensification of yellow colour (Ellman, 1959); the peak of absorption is at 412m μ . In the procedure described below small quantities of GSSG or GSH bring about this change but to a quantitatively greater degree, suggesting the operation of a cyclic process (see the Discussion section).

Calibration of enzyme. The method depends on the rate of an enzymically controlled colour change being directly proportional to the amount of glutathione present. It is therefore necessary to find the range over which this is true for a given enzyme preparation.

Initially the reductase is diluted so that it gives a change in extinction at 412m μ of about 0.02/min. with 0.5 μ g. of GSH in the cuvette, and to ensure that the enzyme is never limiting it is calibrated with either acidic or neutral samples of standard glutathione, depending on whether high or low concentrations are to be measured. If the tissue being investigated contains, e.g., more than 40 mg. of glutathione/100 g. wet wt., then dilution of the extract with water, before estimation, is permissible. Under these circumstances standards are prepared so as to contain between 1 and 20 μ g./ml. after 1:1 or 1:2 dilution. With less than 40 mg./100 g. wet wt. of tissue or with an enzyme of low activity the metaphosphoric acid must be removed by neutralization, and hence standards in 3% metaphosphoric acid are treated with 0.3 ml. of trisodium phosphate/ml. of metaphosphoric acid solution and finally contain between 1 and 20 μ g. of glutathione/ml.

A 0.2 ml. sample is added to a cuvette containing 2.5 ml. of 0.05M-phosphate buffer, pH 7.1, 0.8 ml. of 1mM-EDTA (sodium salt) and 0.03 ml. of DTNB. The cuvette is shaken and 2 min. later 0.1 ml. of suitably diluted enzyme and 0.1 ml. of NADPH_2 are added. The cuvette is shaken again, the time recorded and the uniform change in extinction at 412m μ /min. noted from the first to the fifth minute. The experiment is repeated with various amounts of glutathione and with a blank, since often a background reaction occurs, presumably owing to glutathione contamination of the enzyme. The amounts at

which glutathione is limiting are thus determined and the tissue extract, described below, is treated accordingly so that its glutathione content falls within these values.

A stock mixture of buffer and EDTA (25:8, v/v) may be made. The reference blank contains 3.7 ml. of this solution with 0.03 ml. of DTNB.

Preparation of tissue extract. The tissue is excised from the animal and, after being weighed quickly on a torsion balance (maximum load 50 mg.), is dropped into 10 ml. of ice-cold 3% metaphosphoric acid and homogenized thoroughly. Suitable quantities of some organs are as follows: rat liver, adrenal, retina, kidney, mouse adrenal, 20 mg.; blood, 0.1 ml. in 9.9 ml. of acid. With blood, to prevent oxidation of GSH during denaturation, carbon monoxide is passed through the sample for a few minutes to displace oxygen from oxyhaemoglobin (Numata, 1940). The acidic tissue extract is removed by Pasteur pipette from the homogenizer and placed in a glass tube (approx. 15 cm. $\times$ 1 cm.) that has at one end a sintered-glass filter, porosity grade 4 (average pore size 5–10 μ). The space above the liquid is flushed with nitrogen and pressure applied at 15–20 lb./in.² long enough for 2–3 ml. to be collected in an ice-cooled test tube. By using a manifold, any number of such ultrafiltrations can be made at the same time.

Experiments have shown that the extract must be kept ice-cold at all times. The glutathione recovered was not affected by the quantities of

either denatured protein or metaphosphoric acid encountered in the recovery experiments and the efficiency of recovery varied from 90 to 105% (Table 1).

Determination of total thiol. A 0.5 ml. sample of the filtered extract containing about 5 μ g. of GSH is added to a mixture of 1.5 ml. of 0.5 M-potassium-sodium phosphate buffer, pH 8.0, and 0.03 ml. of DTNB. The blank contains 0.5 ml. of 3% metaphosphoric acid instead of the tissue extract and the reaction is carried out in very carefully cleaned test tubes. The stable colour is read at 412 m μ after 3 min. in a 2 cm. light-path micro-cell and it is compared with that from duplicate standards containing 5 μ g. of GSH/0.5 ml. of 3% metaphosphoric acid. The extinction at 412 m μ is linearly related to thiol present; thus the quantity of thiol in the extract may be calculated.

All glassware concerned with this estimation must be free from reducing substances. A convenient way of ensuring this is to treat the apparatus with a very little ethanol followed by concentrated nitric acid. After the violent reaction has subsided the glass is washed with deionized water and then acetone, and dried at 100°. Occasionally, with fresh buffer, prepared by adding 0.5 N-potassium hydroxide to 0.5 M-potassium dihydrogen phosphate until pH 8.0 is reached, a high blank colour is formed. This may be lessened by boiling the buffer for about 15–20 min.

Determination of total glutathione. If the tissue

Table 1. *Glutathione recovery from various tissue extracts*

Experimental details are given in the text.

Tissue in 3% (w/v) HPO ₃	Recovery of thiol from an extract sample			Recovery of total glutathione from an extract sample		
	Amount added (μ g.)	Amount found (μ g.)	Recovery (%)	Amount added (μ g.)	Amount found (μ g.)	Recovery (%)
Mouse liver (28.3 mg./10 ml.)	—	—	—	0	12.0	—
				9.0	21.5	105
				18.0	30.4	102
Rat liver (25.0 mg./10 ml.)	—	—	—	0	19.4	—
				10	29	96
				20	39	98
				30	51	105
				40	58.5	98
Rat blood (0.1 ml./10 ml.)	0	10.6	—	0	10.7	—
	10	19.6	90	10	20.5	97
	20	30.3	98.5	20	31.2	102
	30	40.6	100	30	40	98
Rat liver (150 mg./10 ml.)	0	172	—	—	—	—
	18	191	106			
	0	140	—			
	18	159	106			
	0	130	—			
	18	147	95			

glutathione content is low or the enzyme inactive then the excess of metaphosphoric acid, which depends on the amount of protein precipitated, must be neutralized before the enzymic process is initiated. Trisodium phosphate is a most effective base, but simple titration is, however, undesirable since according to the initial excess of metaphosphoric acid the final Na^+ concentration at neutrality will vary, and this in turn greatly effects the enzymic activity. Experience has shown that the addition of 0.3 ml. of trisodium phosphate solution/ml. of extract or standard provides a suitable solution for assay, although dilution when possible does increase the sensitivity. In favourable circumstances, as mentioned above, the extracts and standards may be diluted directly approx. 1:1 with water. In either case a 0.2 ml. sample is taken and assayed (as described under Calibration of enzyme) and the glutathione calculated by reference to standard solutions.

Extracts and standards must be treated identically, the latter being prepared at time of homogenizing and kept at 0° until neutralized or diluted or both along with the filtered extracts.

Notes on the method. (a) Total thiol. Ellman (1959) specified that the thiol estimation should be carried out at pH 8.0. Since the glutathione is extracted into acid, the effect of final pH on colour development was studied by using 3.0 ml. of 0.5 M-phosphate buffer, 0.03 ml. of DTNB and 5 μg . of GSH in 0.5 ml. of 1% (w/v) metaphosphoric acid. The result is summarized in Table 2.

The molarity of buffer did not effect the final colour formed, and the optimum amount of DTNB needed was 0.03 ml. A blank was always necessary, since occasionally fresh buffer at pH 8.0 gave a considerable colour, but it did not appear if the buffer had stood for a few days at room temperature. Neither hydrogen peroxide treatment nor quick boiling prevented this phenomenon, but saturation with carbon dioxide decreased it.

(b) Total glutathione. After the discovery of a reaction between glutathione, DTNB, glutathione reductase and NADPH_2 , a sample of the enzyme was prepared as described above and used to

estimate 30 μg . of GSSG under the conditions of Rall & Lehninger (1952). The observed rate of change in extinction at 340 m μ was not noticeably altered by the addition of 0.01 ml. of DTNB, but 0.1 ml. of DTNB completely stopped the reaction. Since the estimation was not inhibited with low quantities of DTNB, measurements were possible at 412 m μ when very small quantities of glutathione were used and when the optimum quantity of DTNB to be added was 0.03 ml.

The reaction exhibited a sharp optimum at pH 7.1 in the presence of 0.011% ox-plasma albumin and 0.05 M-phosphate buffer, but since the protein exerted an inhibitory action (Fig. 1) its use was finally dropped. Either glutathione or NADPH_2 may be added to the cuvette last, but since there is a 2 min. period in which GSH reacts with DTNB it is better that NADPH_2 is added after this reaction is completed, thus giving a uniform change in extinction from the beginning. The rate of colour change is proportional to the total glutathione irrespective of the percentage of each form present (Fig. 2), but the colour before the addition of NADPH_2 varies directly with the GSH present and also depends on the amount of protein in solution.

Experiments showed that 3% (w/v) sulphosalicylic acid was unsuitable for tissue extraction, since it produced a 66% inhibition in the cuvette that could not be overcome by increased ox-plasma albumin or DTNB. Metaphosphoric acid, neutralized carefully with trisodium phosphate, did not interfere markedly with the reaction.

Fig. 1. Inhibitory action of ox-plasma albumin on the system used for total glutathione determination. The cuvette contained: 2.5 ml. of 0.5 M-sodium phosphate buffer, pH 7.1; 0.8 ml. of 1 mM-EDTA (sodium salt); 0.03 ml. of DTNB; 0.1 ml. of enzyme; 5 μg . of GSSG; 0.1 ml. of NADPH_2 ; ox-plasma albumin as indicated. The protein was added in aqueous solution. One activity unit is defined as $10^{-4} \times \Delta E_{420\text{m}\mu}/\text{min}$.

Table 2. Effect of pH on colour development in the reaction between reduced glutathione and DTNB

The system contained 3.0 ml. of 0.5 M-phosphate buffer, 0.03 ml. of DTNB and 5 μg . of GSH in 0.5 ml. of 1.0% (w/v) HPO_3 .

Buffer pH	Max. $E_{412\text{m}\mu}$	Time for colour development (min.)
6.0	0.092	5
7.0	0.168	2
8.0	0.174	0.5

Fig. 2. Relationship between rate of colour development, [GSH]+[GSSG] and [GSH]/[GSSG] ratio. The cuvettes contained: 2.5 ml. of 0.5 M-sodium phosphate buffer, pH 7.1; 0.8 ml. of 1 mM-EDTA (sodium salt); 0.03 ml. of DTNB; 0.1 ml. of enzyme; 0.1 ml. of NADPH₂; glutathione. A, [GSH]+[GSSG], 7 μg./cuvette; [GSH]/[GSSG] ratio 6:1; B, [GSH]+[GSSG] 7 μg./cuvette; [GSH]/[GSSG] ratio 1:6.

Specificity and interfering substances. No detectable enzymic reaction occurred with any of the following compounds in solution: 10 μg. of taurine, cysteic acid, cystathione, methionine sulphone, methionine, thiolhistidine or djenkolic acid; 5 μg. of homocysteine or lanthionine; or 1 mg. of ascorbic acid. The compounds listed in Table 3 gave no enzymic reaction but did give a yellow colour with DTNB, and therefore must be considered to interfere with the thiol estimation.

It appeared that cysteine was the only naturally occurring compound that caused any serious interference, but further tests showed that it in no way affected the rate of colour formation. Since non-glutathione thiol appeared not to interfere with the change in extinction at 412 mμ it was hoped that thiol groups could be measured from the extinction at 412 mμ at *t*₀ (i.e. just before the addition of NADPH₂) and total glutathione from the rate of colour change in the first few minutes after the addition of NADPH₂. Experiments revealed difficulties. A calibration curve showed that, the more thiol groups there were present, the longer it took for a stable colour to be formed, and, moreover, the amount of enzyme in the cuvette

Table 3. Substances found to interfere with the determination of thiol groups with DTNB at pH 8.0 in 0.5 M-phosphate buffer

The system contained 3.0 ml. of 0.5 M-phosphate buffer, 0.03 ml. of DTNB and the sulphur compound in 0.5 ml. of 1% (w/v) HPO₃.

Substance	E _{412mμ}	Time for colour development (min.)
Ergothioneine (10 μg.)	0.02	5
Methionine (10 μg.)	0.008	3
ββ-Diaminoethyl disulphide (10 μg.)	0.02	5
2,3-Dimercaptopropanol (BAL) (10 μg.)	0.73	2
Mercaptoethylamine (10 μg.)	0.62	3
Cysteine hydrochloride (3 μg.)	0.05	3

Table 4. Glutathione content of various tissues

Experimental details are given in the text. The values in parentheses are taken from the literature.

Tissue	Wt. of sample (mg.)	Glutathione content (mg./100 g. wet. wt.)	
		GSSG + GSH	GSH
Nerve	40	17.2	—
Adrenal (mouse)	10	114 ± 3	110
Retina (rat)	18.5	43.1	30
		42	28
Blood plasma (human)	1.0 (ml.)	0.2 ± 0.05	—
Erythrocytes	0.3 (ml.)	62 ± 12 (60*)	—
Pituitary gland (rat)	8	81	60
Liver (rat)	20	245 ± 5 (243†)	235 ± 10 (241†)

* Danielli (1954).
† Bhattacharya, Robson & Stewart (1956).

affected the extinction at t_0 . Finally, the volume of extract needed to give a satisfactory reading before the addition of NADPH_2 gave too great a change in extinction at $412\text{m}\mu$ afterwards. Clearly two separate estimations were necessary.

Results. Some tissues have been briefly examined from laboratory animals and the glutathione contents found are shown in Table 4. In general, they agree with values stated already in the literature.

DISCUSSION

Precisely how the enzymic reaction proceeds is not known, but DTNB has been observed to produce a spectrally identical colour with various reducing agents such as sodium metabisulphite, 2,3-dimercaptopropanol, GSH, ferrous ammonium sulphate (Ellman, 1959), cysteine, sodium sulphide, 50% (w/w) hydrazine hydrate and, to a smaller extent, 'quinol' and 'metol' in sodium hydrogen carbonate solution. With alkali a slightly different colour is produced, and with ascorbic acid, glucose, formaldehyde, 2-methyl-1,4-naphthaquinone or sodium chloride there is no reaction. It therefore seems that there is a progressive reduction of DTNB, triggered off by catalytic amounts of glutathione in the presence of the reductase and NADPH_2 . Supporting this is the fact that 0.02 ml. of DTNB, at pH 8.0, treated with 0.05 ml. of aq. 1% (w/v) sodium sulphide solution or with the enzyme (with 30 μg . of GSSG, to facilitate rapid reaction) yields colours of identical spectra and intensity (Fig. 3). Similar results are obtained with 2% (w/v) GSH,

but 0.2 ml. of 2% (w/w) sodium metabisulphite produces only 50% of the colour intensity.

The necessity for the complete system of buffer, glutathione, DTNB, NADPH_2 and enzyme suggests a cyclic reduction, ceasing only when the enzyme, DTNB or NADPH_2 is spent. NADH_2 is only 50% as efficient as NADPH_2 , an observation of interest in view of the lack of specificity of the reductase for NADPH_2 (Racker, 1955). Both NAD and NADP are ineffective. The role of NADPH_2 is critical, since when it is spent the reaction stops abruptly only, to re-start on the addition of more nucleotide. If the enzyme is not in excess it appears to be expendable or unstable, since after a few minutes the reaction stops. Possibly DTNB reacts with the enzyme to yield an inactive form (Mapson & Isherwood, 1963) or directly with the thiol groups of the active centres. Further, it is relevant that excess of DTNB will inhibit the assay even with substantial amounts of enzyme present. The reaction is completely inhibited by 0.1 mM-*p*-chloromercuribenzoate, is effected by heat (Fig. 4) and is completely abolished by 0.71 M-sodium chloride in the presence of neutralized 0.28 M-meta-phosphoric acid.

Since the yellow colour produced is always in excess of that expected from the stoichiometric reduction of DTNB by GSH, the operation of a cyclic process seems virtually proven. Briefly, the postulated mechanism may be summarized in the following series of equations where DTNB is written as ArSSAr :

ArSH (as the ion) is measured at $412\text{m}\mu$.

Fig. 3. Non-enzymic (—) and enzymic (....) reduction of DTNB. Both systems contained: 2.5 ml. of 0.05 M-sodium phosphate buffer, pH 7.1; 0.8 ml. of 1 mM-EDTA (sodium salt); 0.02 ml. of DTNB; 0.1 ml. of enzyme; 0.1 ml. of NADPH_2 ; either 30 μg . of GSSG or 0.05 ml. of sodium sulphide (approx. 1%, w/v).

Fig. 4. Effect of temperature. Each cuvette contained: 2.5 ml. of 0.05 M-sodium phosphate buffer, pH 7.1; 0.8 ml. of 1 mM-EDTA (sodium salt); 0.03 ml. of DTNB; 0.1 ml. of enzyme; 0.1 ml. of NADPH_2 ; 2 μg . of GSSG. The cuvettes were maintained at: A, 20°; B, 38°; C, 45–50°; D, 60°; E was obtained with boiled enzyme. At 60° neither cooling nor the addition of NADPH_2 had any stimulatory effect.

The product GSSAr is cleaved either non-enzymically with GSH or enzymically to yield ArSH and GSSG or ArSH and GSH according to one of the following equations:

It is probable that GSH completes the reduction of ArSSG (eqn. 3) and relatively unlikely that ArSSG acts as a substrate for enzymic reduction (eqn. 4). Thus the GSH is regenerated and, with excess of NADPH₂ and constant amount of enzyme, the rate of colour production is proportional to the total glutathione. Never has the colour produced exceeded the theoretical maximum where all the DTNB has reacted. It does not appear necessary therefore to postulate the formation of a new and highly coloured complex.

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