30% in C-1, and 10% in C-3+C-4, of the glucose molecule.

5. Possible metabolic routes which might explain this unexpected metabolic similarity of the two isomers are discussed.

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The Reduction of Oxidized Glutathione in Erythrocyte Haemolysates in Pernicious Anaemia

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The erythrocytes and cells from certain tissues of vitamin B_{12}-deficient rats (Ling & Chow, 1953, 1954; Register, 1954; Kasbekar, Lavate, Rege & Sreenivasan, 1959) and chicks (Hsu, Chow & Okuda, 1959) but not mice (Jaffé, 1958) have unusually low concentrations of reduced glutathione, which rise to within the normal ranges soon after adequate administration of the vitamin. The reason for these low concentrations, however, has not been determined. Stekol (1954) found, using labelled glycine or cystine, that the synthesis of glutathione, *in vivo*, from its three amino acid components was not significantly different from the normal in the liver of vitamin B_{12}-deficient rats, Kasbekar & Sreenivasan (1959); who found a small decrease in the rate of this synthesis in erythrocytes taken from vitamin B_{12}-deficient rats, could not rule out that this might have been due to an observed slight haemolysis of the incubated cells.

Vitamin B_{12} has been implicated in the reduction of disulphides in rat-liver homogenates (Dubnoff, 1950), and it is therefore possible that the reduction of oxidized glutathione (a disulphide) is impaired

as a result of a deficiency of the vitamin. Such an impairment would probably entail low concentrations of reduced glutathione together with correspondingly high concentrations of oxidized glutathione. In addition, a deficiency in one of the components of the system reducing oxidized glutathione might be observed. This system, which involves glutathione reductase and triphosphopyridine nucleotide together with the corresponding dehydrogenases, catalyses the transfer of hydrogen from oxidizable substrates to oxidized glutathione (Rall & Lehninger, 1952; Conn & ennesland, 1951; Francoeur & Denstedt, 1954).

To test this theory, the concentrations of reduced and oxidized glutathione, triphosphopyridine nucleotide and oxidizable substrates have been measured in haemolysates of erythrocytes from patients with pernicious anaemia, before and after treatment with vitamin B_{12}, and compared with the corresponding values from normal erythrocytes. The outcome of this investigation is reported in this paper, a brief account of which has already been given (Jocelyn, 1959).

EXPERIMENTAL

Materials. Solutions of oxidized triphosphopyridine nucleotide (TPN), oxidized glutathione (GSSG) (both obtained from Boehringer und Sohne, Mannheim) and glucose 6-phosphate (British Drug Houses Ltd., Poole) (dried *in vacuo* before use) were made up immediately before the experiments. Disodium ethylenediaminetetra-acetate (EDTA) and 2-amino-2-hydroxymethylpropane-1:3-diol (tris) were also from British Drug Houses Ltd. and reduced glutathione (GSH) was a gift from The Distillers' Co., Speke, Liverpool.

Blood from hospital patients with pernicious anaemia was kindly made available by Dr R. H. Girdwood, Department of Medicine, University of Edinburgh. Diagnosis had been confirmed by sternal puncture and blood-film examination. Figures for the red-cell counts were obtained from the Haematology Laboratory, Royal Infirmary, Edinburgh.

Preparation of the haemolysates. Packed human erythrocytes were obtained by centrifuging fresh oxalated blood for 10 min. at 1000 *g* and removing the plasma and buffy coat. They were washed once by suspending in an equal volume of 0·9% sodium chloride solution and recentrifuging. The separated erythrocytes were haemolysed with 9 vol. of oxygen-free water and the haemolysates used immediately.

Estimation of reduced glutathione. To the haemolysates (1–3 ml.), after incubation with the required additions, was added 5% metaphosphoric acid (0·5 ml.) to precipitate the proteins. After centrifuging, the clear, colourless, supernatant solution (3 ml.) was used for estimating GSH by the nitroprusside method (Grunerts & Phillips, 1951). All estimations were carried out in duplicate. The standard deviation of the readings from the mean for 20 estimations was $\pm 6{\cdot}5\,\mu$moles/100 ml. When haemolysates (3 ml.) containing added GSH (up to 0·8 μmole), EDTA (2 μmoles) and water to 4·5 ml. were incubated for up to 2 hr. at 37°, recoveries of the GSH averaged 95%. EDTA was included in all the experiments since its omission gave erratic results. There was no difference between the GSH content of haemolysates estimated at once or after incubation at 37° for 2 hr. The addition of GSSG (0·5 μmole) before this incubation did not affect the values obtained (see Pirie, 1959). GSH formed in haemolysates during incubation with the additions described was determined by subtracting from the GSH content of these mixtures that of the unincubated haemolysates alone.

Estimation of glutathione-reductase activity. The method of Collier & McRae (1955) was modified as follows: to the 1:10 haemolysate (1 ml.) were added GSSG (0·5 μmole), glucose 6-phosphate (0·5 μmole), TPN (0·1 μmole), EDTA

Table 1. *Recovery of oxidized glutathione added to haemolysates*

Mixtures of 1:10 haemolysate (3 ml.), glucose 6-phosphate (0·5 μmole), TPN (0·1 μmole), EDTA (2 μmoles), 0·1 M-tris–HCl solution, pH 7·4 (0·5 ml.) and GSSG as indicated in a final volume of 4·5 ml. were incubated at 37°. After 30 min. the proteins were precipitated and the GSH formed during the incubation estimated. The amount formed when no GSSG had been added to the mixture (0·02 μmole) was subtracted and the figures then obtained halved to give values for GSSG. Each recovery figure given represents the mean from duplicate incubations with the same haemolysate.

GSSG (μmole)	
Added	Found
0·05	0·09
0·10	0·11
0·15	0·15
0·20	0·20
0·25	0·24
0·30	0·30
0·35	0·35
0·40	0·38
0·45	0·43
0·50	0·50

Table 2. *Recovery of glucose 6-phosphate added to haemolysates*

Mixtures of 1:10 haemolysate (1 ml.), GSSG (0·5 μmole), TPN (0·1 μmole), EDTA (2 μmoles), 0·12 M-tris–HCl solution, pH 7·4 (0·5 ml.) and glucose 6-phosphate as indicated, to a final volume of 4·5 ml. were incubated at 37°. After 30 min. the proteins were precipitated and the GSH formed was estimated. GSH formed without added glucose 6-phosphate (0·04 μmole) was subtracted. The resultant figures were halved to give the stated recoveries which were the mean from duplicate incubations with the same haemolysate.

Glucose 6-phosphate (μmoles)	
Added	Found
0·05	0·04
0·10	0·10
0·15	0·145
0·20	0·18
0·25	0·235

(2 μmoles) and 0·12 M-tris–HCl solution, pH 7·4 (0·5 ml.) to a final volume of 4·5 ml. The mixture was incubated at 37° for 6 min., the proteins were precipitated and the GSH formed was estimated. This amount was used as the measure of glutathione-reductase activity since it varied linearly with changes in the volume of haemolysate used.

Estimation of oxidized glutathione. When amounts of GSSG up to 0·5 μmole were added to haemolysates together with glucose 6-phosphate and TPN, a quantitative conversion into GSH occurred within 30 min. (Table 1). Consequently, any GSH formed when GSSG was omitted represented the original concentration of GSSG in the haemolysates, thus providing a simple method for measuring the concentration in erythrocytes.

Estimation of triphosphopyridine nucleotide-reducing substrates. If TPN and excess of GSSG were both added to haemolysates, the amount of reduction which could occur was then limited by the concentration of glucose 6-phosphate also added. As shown in Table 2, the GSH formed after 30 min., with varying additions of glucose 6-phosphate, was 90–100% of the amount expected theoretically if 1 mole was required to reduce 1 mole of GSSG. Any GSH formed when no glucose 6-phosphate was added therefore represented the concentration of this substance or of other TPN-reducing substrates originally present in the haemolysate.

Estimation of total triphosphopyridine nucleotide. When GSSG and glucose 6-phosphate, but no TPN, were added to haemolysates, a slow reduction of the GSSG still occurred owing to the total (oxidized and reduced) TPN content of the haemolysate. To determine its concentration, the amount of GSH formed after 15 min. was compared with the amount formed in the same time when very small quantities of TPN (1–7 μm-moles) were added. Under these conditions the additional GSH formed was proportional to the concentration of added TPN up to 5 μm-moles (Fig. 1). The addition of diphosphopyridine nucleotide (up to 0·1 μmole) had no effect on the figures obtained. The TPN content of the original haemolysate was therefore found by extrapolation as shown for three different haemolysates in Fig. 1.

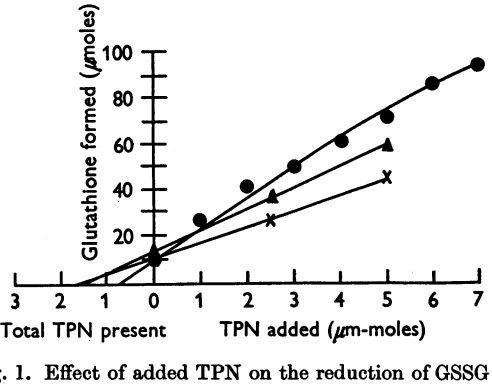

Fig. 1. Effect of added TPN on the reduction of GSSG in erythrocyte haemolysates. To 1 ml. of three different haemolysates (×, ▲, ●) was added GSSG (0·5 μmole), glucose 6-phosphate (0·5 μmole), EDTA (2 μmoles), TPN as indicated and 0·12 M-tris–HCl solution, pH 7·4 (0·5 ml.), to a final volume of 4·5 ml. The mixture was incubated for 15 min. at 37°, the proteins were precipitated and the GSH formed was estimated. The total TPN originally present in the haemolysates was found by extrapolation as shown.

An assay method, with glutathione reductase to estimate total TPN, has been described (Anderson, 1953), but not previously applied to erythrocytes.

RESULTS

With the methods described, glutathione-reductase activity and the concentration of GSH, GSSG, total TPN and TPN-reducing substrates have all been estimated on the same haemolysates. A range of the values found in haemolysates from the erythrocytes of ten normal human subjects is given in Table 3. Apart from the values for GSSG, these ranges are in good agreement with those found by other workers (Collier & McRae, 1955; Levitas, Robinson, Rosen, Huff & Perlzweig, 1947; Schlenk, 1942; see Jocelyn, 1958). The same estimations have also been performed on the haemolysates of erythrocytes from seven patients with pernicious anaemia both before and after treatment with vitamin B_{12}. The results are tabulated in Table 4 together with the red-cell count before treatment.

There are no significant departures ($> 2 \times$ S.D.) from the normal range of the values found in all seven untreated cases for GSH, glutathione-reductase activity and (in six of the cases) for total TPN. Also the activity of the enzyme is not affected by the addition of vitamin B_{12} to the haemolysate.

On the other hand, in three of the cases before treatment with vitamin B_{12}, the values obtained for GSSG are significantly above the normal range ($P = 0{\cdot}05$; $< 0{\cdot}01$; $< 0{\cdot}01$) and in the same three untreated cases an abnormally high level ($P < 0{\cdot}01$) of TPN-reducing substrates is also evident. Soon after administration of the vitamin (3–7 days) the two cases which could be followed show a large drop in the concentrations of TPN-reducing substrates and GSSG so that both are within the normal range. The losses in GSSG are accompanied

Table 3. *Range of values found in haemolysates of erythrocytes from ten normal subjects*

Glutathione-reductase activity is expressed as glutathione formed in 6 min. from 1 ml. of 1:10 haemolysate under the conditions given in Fig. 1. The other values are for 100 ml. of packed cells.

Substance	Concn. (μmoles) Range	Mean ± S.D.
GSH	160–300	220 ± 41
GSSG	0–15	5 ± 6·3
TPN-reducing substrates	14–40	25 ± 8·5
Total TPN	0·5–1·7	1·1 ± 0·4
Glutathione reductase	0·29–0·50	0·37 ± 0·08

Table 4. *Range of values found in haemolysates of erythrocytes from subjects with pernicious anaemia before and after treatment with vitamin B_{12}*

The dose of vitamin B_{12} injected in each case was 1 mg. Red-cell counts given are values before treatment. Other values are given as μmoles/100 ml. of packed cells except for glutathione-reductase activity which is expressed as GSH (μmoles) formed in 6 min. from 1 ml. of 1:10 haemolysate; values in brackets are the corresponding activities found when vitamin B_{12} was added to the haemolysate before estimation at a concentration of 0·1 μg./ml. Other values were estimated in duplicate and, except for TPN, both results are given. These are based on the mean of the two readings shown for GSH.

Subject	Time of venepuncture (before or after dose of vitamin B_{12})	Red-cell count (millions)	GSH	GSSG	Glutathione-reductase activity	Total TPN	TPN-reducing substrates
1	Before	1·8	223, 237	53·5, 56·5	27 (28)	0·7	65, 70
	3 days after		323, 343	0·5, −0·5	28, 29	0·6	10, 15
2	Before	2·0	255, 267	28·5, 30·5	42 (38)	2·4	74, 72
	7 days after		301, 317	0·5, 7	—	—	18, 18
3	Before	1·39	186, 190	15, 7	—	—	—
	10 days after		200, 207	−1·5, 1	—	—	—
4	Before	0·9	270, 264	15, 18	34 (29)	1·2	55, 56
5	Before	—	262, 250	0, 8	35 (32)	1·1	10, 15
6	Before	2·5	150, 160	10, 2	38 (34)	1·25	30, 35
7	Before	1·76	238, 238	−0·5, 2·5	50 (51)	1·2	15, 20

by a gain in the concentration of GSH of approximately the amount expected if the lost GSSG had been reduced to GSH *in vivo*. In both cases the new concentration of GSH is significantly above the normal range.

DISCUSSION

The reduction of GSSG in haemolysates, under the conditions used, involves both glutathione reductase and glucose 6-phosphate dehydrogenase but since addition of the purified dehydrogenase does not increase the rate of the reduction, this is actually limited by the activity of glutathione reductase, of which it is therefore a convenient measure (Collier & McRae, 1955). The use of this glutathione-reductase system to estimate the erythrocyte concentration of one of its components, GSSG, glucose 6-phosphate (or TPN-reducing substrates) or total TPN merely involves omitting this component from the additions made, when its endogenous concentration limits the amount of GSH which can be formed or, for TPN, the rate of its formation.

Glutathione reductase has occasionally been used (e.g. Martin & McIlwain, 1959) to convert GSSG into GSH but the enzyme has usually been supplied from an exogenous source and used to reduce the GSSG present in a protein-free filtrate. In estimating GSSG in erythrocytes by using the reductase which they already contain, the reduction is carried out before precipitating the proteins. This offers an important advantage over other procedures used.

Thus it is uncertain whether (or to what extent) GSSG is formed during the precipitation of proteins from a solution containing GSH. Consequently, subsequent values found may overestimate the concentration of GSSG in the original solutions. However, if GSSG is reduced to GSH before precipitation of the proteins and then estimated from the difference between the GSH content of the protein-free filtrates from reduced and unreduced solutions, any oxidation occurring during the precipitation will be common to both solutions and cancel out. Thus though the GSH values found will be smaller by the amounts oxidized, those for GSSG will represent the true values present before precipitation. Applying this method to haemolysates of erythrocytes, the values obtained for GSSG are in fact considerably lower than those found in human blood or erythrocytes by other workers. Thus the mean value for GSSG (from ten normal subjects) expressed as GSH is 0–6 % of the total glutathione (GSH + GSSG) found whereas Bhattacharya, Robson & Stewart (1955) found values for blood GSSG of 10–25 % of total glutathione.

The GSH formed when glucose 6-phosphate is omitted from the glutathione reductase system probably represents mainly that arising through the presence of endogenous glucose 6-phosphate. The other chief substrate in erythrocytes involving a TPN-reducing dehydrogenase is 6-phosphogluconic acid. This substance is capable of reducing GSSG in haemolysates (Carson, Flanagan, Ickes & Alving, 1956) but does not seem to do so under the conditions used in the present work since, though

6-phosphogluconate arises from the dehydrogenation of glucose 6-phosphate, only 1 mole of GSSG is in fact reduced by the latter substance [cf. Conn & Vennesland (1951) who found a 13–25% further reduction from glucose 6-phosphate added to wheat-germ extract].

From the results for these estimations performed on haemolysates of erythrocytes both from normal subjects and from patients with pernicious anaemia, some points may be noted:

(1) The finding that, in untreated pernicious anaemia, the concentrations of GSH are within the normal range contrasts with the low concentrations found in erythrocytes from vitamin B_{12}-deficient animals (see introduction) and conflicts with the finding of Ling & Chow (1953) of a low concentration in the one case of pernicious anaemia they examined.

(2) These authors observed a rise in GSH, after treatment of their one case with vitamin B_{12}, similar to the rise observed in vitamin B_{12}-deficient animals after administering the vitamin. Thus the elevation of GSH noted in the present work in two of the cases after treatment with vitamin B_{12} would seem to be in accordance with these findings. This additional GSH seems to originate from a reduction of the abnormal amount of GSSG found in the untreated cases because, after treatment, this GSSG falls approximately to the extent that the GSH rises. Thus it is possible that the low concentration of GSH found in the vitamin B_{12}-deficient animals is also due to conversion into GSSG and that this process is reversed when the vitamin is given.

(3) In the three untreated cases where high concentrations of GSSG were found, the concentration of TPN-reducing substrates was also high. Since in the two cases examined after treatment both of these values had reverted to within the normal range, it seems likely that their elevation was due to a single deficiency correctable by vitamin B_{12}. A deficiency in glucose 6-phosphate-dehydrogenase activity, which would inhibit both the utilization of glucose 6-phosphate through the pentose phosphate pathway and also the reduction of GSSG, has been shown (Carson *et al.* 1956; Beutler, 1959) to account for the low concentrations of GSH found in erythrocytes of subjects with certain drug-sensitive haemolytic anaemias. Such a deficiency however, necessarily affects the activity of glutathione reductase when measured in haemolysates by coupling the two enzyme reactions, and in the present work glutathione-reductase activities were found to be normal and uninfluenced by vitamin B_{12}.

A block in the glycolytic pathway from glucose to lactate in erythrocytes would also be likely to lead to an accumulation of glucose 6-phosphate and this explanation would be in accord with the impaired tolerance to glucose found in vitamin B_{12}-deficient rats (Ling & Chow, 1954). Though such a block could not plausibly account for an accumulation of GSSG, there is the converse possibility that GSSG, present in excessive amount, might partly reversibly inactivate thiol-dependent enzymes of this pathway (e.g. glyceraldehyde 3-phosphate dehydrogenase) in the manner demonstrated by Hopkins, Morgan & Lutwak-Mann (1938). The high concentrations of TPN-reducing substrates found in the cases of untreated pernicious anaemia would then be a consequence of the high concentrations of GSSG.

The concentrations of GSSG probably reflect an intracellular equilibrium between its loss by reduction and its formation from GSH by oxidation. This would explain why some GSSG coexists with some TPN-reducing substrates even in normal subjects (Table 3). Since, despite the high concentrations found, the reduction of GSSG is unimpaired in untreated pernicious anaemia, the possibility arises that the rate of oxidation of GSH is increased above the normal in this disease and that this is the primary defect correctable by administration of vitamin B_{12}.

SUMMARY

1. The reduction of oxidized glutathione to the reduced form in erythrocyte haemolysates containing glucose 6-phosphate and oxidized triphosphopyridine nucleotide has been used as the basis for estimating glutathione-reductase activity and the concentrations of oxidized glutathione, triphosphopyridine nucleotide-reducible substrates and total triphosphopyridine nucleotide.

2. These estimations, together with that of reduced glutathione, have been performed on haemolysates from erythrocytes from ten normal human subjects and seven with pernicious anaemia.

3. In untreated pernicious anaemia, the concentrations of reduced glutathione, glutathione reductase and triphosphopyridine nucleotide were within the normal range whereas in three cases those of oxidized glutathione and triphosphopyridine nucleotide-reducing substrates were above it.

4. After treatment with vitamin B_{12}, the oxidized glutathione appeared to be converted into reduced glutathione, and the concentration of triphosphopyridine nucleotide-reducing substrates fell to within the normal range.

5. The significance of these findings is discussed and the suggestion made that vitamin B_{12} plays a part in inhibiting the oxidation of reduced glutathione.

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The Effect of some Protein Solutions on the Oxidation of Glutathione in Oxygenated Erythrocytes

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Though much is known about the oxidation *in vitro* of reduced glutathione (Barron, 1951; Isherwood, 1959), the corresponding intracellular reaction has been little studied. Meldrum (1932) aerated glucose-depleted ox erythrocytes and observed a slow fall in the concentration of glutathione which, since it was prevented by the addition of glucose, he attributed to its intracellular oxidation. Klebanoff (1957) using this system found that besides glucose, adenosine, inosine and 33% plasma (but not bovine-serum albumin) prevented the loss of glutathione by oxidation when added to the saline in which the cells were suspended. The effect of plasma was said to be due to its supplying 'utilizable metabolites to the cell'.

Enzymes have also been described which convert glutathione into its oxidized form with or without oxygen itself. Among these are glutathione peroxidase (Mills, 1957), cytochrome *c*–cytochrome *c* oxidase (Boeri, Baltscheffsky, Bonnichsen & Gustav-Paul, 1953), glutathione–homocystine transhydrogenase (Racker, 1955*b*) and dehydroascorbic acid reductase (Hopkins & Morgan, 1936). Apart from the last these have all been found in animal tissues but their quantitative importance as routes for the intracellular oxidation of glutathione is not known. However, in view of the speed of the metal-catalysed oxidation *in vitro* (see Isherwood, 1959) and the presence of such catalytic metals (e.g. iron and copper) in the tissues, it seems likely that the major proportion of glutathione oxidized intracellularly is oxidized by direct reaction with oxygen itself.

In the presence of glucose, net intracellular oxidation of glutathione cannot normally be observed (Meldrum, 1932; Klebanoff, 1957). This is probably due to the activity of the widely distributed enzyme, glutathione reductase which mediates the reduction of the oxidized glutathione by means of reduced triphosphopyridine nucleotide (Rall & Lehninger, 1952). Regeneration of the latter from its oxidized form requires the metabolism of glucose (see Horecker, 1951). To investigate systematically the intracellular oxidation of glutathione, it is therefore necessary to use cells depleted of glucose, thus enabling the oxidation to be followed, uncomplicated by any subsequent reduction of the oxidized glutathione formed.