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The Role of the Pentose Phosphate Pathway in the Reduction of Methaemoglobin in Human Erythrocytes

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The precise mechanism by which methaemoglobin is reduced to haemoglobin in intact erythrocytes is still unknown, though several hypotheses have been put forward. Reduced glutathione (Morrison & Williams, 1938), ascorbic acid and a few other naturally occurring compounds (Kiese, 1944) spontaneously reduce methaemoglobin *in vitro*, but the physiological role of these reductions is uncertain. The normal process of methaemoglobin reduction is associated with glucose metabolism (Wendel, 1930; Kiese, 1944), and seems to depend on the oxidation of either $NADPH_2$ or $NADH_2$ (Gutmann, Jandorf & Bodansky, 1947).

There are a number of indications that a $NADPH_2$-dependent methaemoglobin reductase is present in erythrocytes. Kiese (1944) partially purified an enzyme from horse erythrocytes which catalyses the reduction of methaemoglobin by $NADPH_2$, and to a lesser extent also by $NADH_2$. The addition of a cofactor, such as methylene blue, greatly increased the reduction rate. On further purification of the same enzyme from human erythrocytes, it became inactive unless a cofactor were added (Kiese, Schneider & Waller, 1957). Huennekens, Caffrey, Basford & Gabrio (1957) isolated from human erythrocytes a similar methaemoglobin reductase which also required a cofactor such as methylene blue or, under certain conditions, Fe^{3+} ions (Huennekens, Caffrey & Gabrio, 1958). It was suggested that a physiological cofactor might have been lost during purification.

Indirect evidence for a $NADH_2$-dependent methaemoglobin reductase in human erythrocytes was presented by Gibson (1948). Recently such an enzyme was partially purified; it was also found to be lacking in patients with hereditary methaemoglobinaemia (Scott & Griffith, 1959; Scott, 1960). Some authors believe that the $NADH_2$-dependent reduction of methaemoglobin accounts for the main part of the physiological reduction of this pigment (Gibson, 1948; Waller & Löhr, 1961; Jaffé, 1959).

Thus earlier observations indicate that the reduction of methaemoglobin may be associated both with the Embden–Meyerhof pathway (regeneration of $NADH_2$) and with the pentose phosphate pathway (regeneration of $NADPH_2$). However, the relative significance of these systems in the physiological reduction of methaemoglobin has not as yet been established. The experiments presented in this paper show that in human erythrocytes the utilization of glucose by the pentose phosphate pathway accounts for only a small part of the total methaemoglobin reduction. The main part, evidently, is reduced by the Embden–Meyerhof pathway. The former mechanism involves a direct reduction of methaemoglobin by reduced glutathione. The oxidized glutathione is in turn reduced by $NADPH_2$.

MATERIALS AND METHODS

Uniformly labelled [^{14}C]glucose (specific activity 3·77 mc/m-mole) was obtained from The Radiochemical Centre, Amersham, Bucks. Before use, it was diluted approximately 65-fold with inactive glucose. Scintillators and M-hyamine in methanol were purchased from Packard Instrument Co. Inc., La Grange, Ill., U.S.A. Glucose 6-phosphate was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. GSH, GSSG and NADP were obtained from Nutritional Biochemical Corp., Cleveland, Ohio, U.S.A.

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Heparinized blood from the hospital blood bank was centrifuged, and the plasma and the buffy coat were removed. The erythrocytes were washed twice with 10 vol. of 'phosphate-buffered saline' containing the following [final concentrations (m-equiv./l.)]: Na^+ ion, 160; K^+ ion, 2·2; Cl^- ion, 143; HPO_4^{2-} ion, 11. Erythrocytes containing methaemoglobin were prepared by treating the washed erythrocytes at room temperature for 10 min. with 3 vol. of phosphate-buffered saline containing 0·1% (v/v) of 'amyl nitrite'. The treated erythrocytes were subsequently washed four times to remove the amyl nitrite. This treatment oxidized about 80% of the haemoglobin. The packed erythrocytes were finally suspended in 5 vol. of a phosphate-buffered saline, pH 7·4, containing the following [final concentrations (m-equiv./l.)]: Na^+ ion, 163; K^+ ion, 6·7; Cl^- ion, 110; HPO_4^{2-} ion, 33.

Haemolysates were made by twice freezing and thawing the washed erythrocytes suspended in a phosphate-buffered saline, pH 7·4, containing the following [final concentrations (m-equiv./l.)]: Na^+ ion, 67; K^+ ion, 103; Cl^- ion, 103; HPO_4^{2-} ion, 33. To get rid of glutathione and of other small molecules, the haemolysates were dialysed against 100 vol. of the latter phosphate-buffered saline for 17 hr. at 4°. Before incubation the haemolysates were freed from stroma by centrifuging at 20 000*g* for 10 min.

The experiments were carried out at 37° in stoppered Warburg flasks containing 3 ml. of erythrocyte suspension. After temperature equilibration, the experiments were started by adding [^{14}C]glucose to give a final concentration of 2·3 mM. The CO_2 released during the incubation was trapped in the centre well in 0·2 ml. of 20% (w/v) KOH. The concentration of methaemoglobin was measured at the beginning and at the end of the incubation by the spectrophotometric method of Horecker & Brackett (1944), adapted for a Hilger Uvispek spectrophotometer. Before measurements, diluted samples were centrifuged at 20 000*g* for 10 min. to obtain optically clear solutions.

A few experiments were carried out in the conventional Warburg apparatus. Methaemoglobin reduction was followed continuously by measuring the additional oxygen uptake by the erythrocytes treated with amyl nitrite as compared with the untreated erythrocytes, since as haemoglobin is formed it becomes oxygenated. The total haemoprotein concentration was measured as cyanmethaemoglobin by the method of Ceilous (1960).

The activity of the pentose phosphate shunt was evaluated by measuring the radioactivity of the $^{14}CO_2$ produced from [^{14}C]glucose (Murphy, 1960). At the end of the incubations the KOH solution from the centre wells was transferred to a diffusion chamber with three compartments. To the second and third compartments were added 0·5 ml. of 50% (w/w) perchloric acid and 0·5 ml. of M-hyamine in methanol respectively. After the chamber had been evacuated, the KOH solution was acidified with the perchloric acid, and the CO_2 released was trapped in the hyamine solution. The radioactivity of this solution was measured in a Tri Carb Liquid Scintillation spectrometer (Packard Instrument Co. Inc.), after the addition of a dioxan–toluene solution of scintillators.

Anaerobic conditions were obtained by equilibrating the blood against pure nitrogen in a rotating tonometer at room temperature for ½ hr. The suspension was subsequently transferred under nitrogen to the incubation vessels.

The GSH concentration of the erythrocytes was determined both by the spectrophotometric method of Grunert & Phillips (1951), as modified by Beutler (1957), and by the amperometric method of Benesch, Lardy & Benesch (1955).

RESULTS AND DISCUSSION

Production of carbon dioxide in relation to the reduction of methaemoglobin in intact erythrocytes

Fig. 1 shows the reduction of methaemoglobin and the production of carbon dioxide in erythrocytes in the presence of [^{14}C]glucose. There is fairly good agreement between the manometric method (the curves) and the spectrophotometric method (columns *a* and *b*) for determination of methaemoglobin, although the latter is undoubtedly the more accurate. The reduction of methaemoglobin proceeded at a constant rate for more than 7 hr. This finding, as well as a comparison of columns *a* and *b* (initial methaemoglobin concentration 60 and 90% respectively), shows that, in accordance with the finding of Cox & Wendel (1942), the velocity of methaemoglobin reduction is independent of the methaemoglobin concentration within wide limits. Significant activity of the pentose phosphate shunt was found in all three series (Fig. 1) as judged from the amount of labelled carbon dioxide produced by the erythrocytes. There was only a slight increase, however, in the carbon dioxide released in the series containing methaemoglobin (columns a_1 and b_1) as compared with the one without methaemoglobin (column c_1). This is in striking contrast with the fact that we observed a 15-fold increase in production of carbon dioxide when, under comparable conditions, the pentose phosphate shunt was stressed by a forced regeneration of NADP.

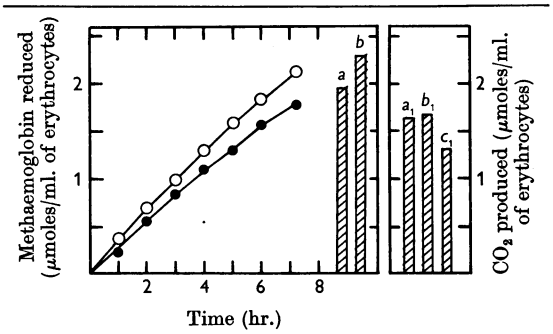


Fig. 1. Reduction of methaemoglobin and production of $^{14}CO_2$ by erythrocytes incubated with uniformly labelled [^{14}C]glucose (2·3 mM). Initial methaemoglobin concentrations: A, 60%; B, 90%; C, 0% [of the total haemoprotein concentration (5·45 g./100 ml. of erythrocyte suspension)]. ●, Reduction of methaemoglobin in A; ○, reduction of methaemoglobin in B (calculated from oxygen uptakes). Columns *a* and *b*: methaemoglobin reduced in A and B, determined spectrophotometrically at the end of the experiment. Columns a_1, b_1, c_1: CO_2 produced in A, B and C during the experiment.

This was obtained when the $NADPH_2$-dependent disulphide-reducing capacity of the erythrocytes was brought into action by adding cystamine to a final concentration of 1 mM (Eldjarn, Bremer & Börresen, 1962).

The slight increase in the production of carbon dioxide when methaemoglobin was present in the erythrocytes (Fig. 1) amounts to 0·47 and 0·53 μ-moles/hr./10 ml. of packed erythrocytes, whereas the simultaneous reduction of methaemoglobin amounts to 2·8 and 3·3 μmoles/hr./10 ml. of packed erythrocytes. If it is assumed that only this increase in the production of carbon dioxide is related to the reduction of methaemoglobin, then the amount of pigment which is reduced by the shunt oxidation of glucose is at most 17 % of the total reduction of methaemoglobin. These calculations are based on the fact that the release of 1 μmole of carbon dioxide accompanies the formation of 2 μmoles of $NADPH_2$, which in turn may reduce 1 μmole of methaemoglobin or 4 μmoles of haem. The calculated 17 % is a maximum value, however, since oxidation products other than methaemoglobin may have been formed during preincubation with amyl nitrite, and these products might also have regenerated NADP. On the other hand, the pronounced production of carbon dioxide in the incubation series containing no methaemoglobin (column c_1) introduces a considerable uncertainty in the assumption that only the increase over and above this blank value is related to the reduction of methaemoglobin. To reduce this blank value experiments similar to those described above were performed anaerobically, since it was expected that the production of carbon dioxide in erythrocytes containing no methaemoglobin would be mainly due to autoxidation of GSH. The GSSG thus formed would regenerate NADP, by way of the glutathione-reductase system, for the shunt oxidation of glucose.

Both with and without methaemoglobin in the erythrocytes the production of carbon dioxide was lower under anaerobic conditions than under aerobic conditions (Table 1). The larger reduction occurred in erythrocytes without methaemoglobin, bringing the blank value for the production of carbon dioxide down to a negligible amount. The difference between the values obtained with and without methaemoglobin is higher under anaerobic conditions (mean, 1·02 μmoles/hr./10 ml. of packed erythrocytes) than under aerobic conditions (mean, 0·43 μmole/hr./10 ml. of packed erythrocytes). If again it is assumed that only this difference is a measure of the part of methaemoglobin reduced by the pentose phosphate shunt, it follows that this pathway was responsible for 15 and 29 % of the total reduction (Table 1) under aerobic and anaerobic conditions respectively. However, the anaerobic experiments demonstrate that, even if the total shunt activity was related to the reduction of methaemoglobin, no more than 38 % of the total reduction could be ascribed to the shunt. It can therefore be safely concluded that, under optimum conditions for the reduction of methaemoglobin, not more than one-third of the reduction is derived from the pentose phosphate shunt. The greater part must depend on the Embden–Meyerhof pathway, as it is known that glucose utilization is necessary for methaemoglobin reduction.

If a specific $NADPH_2$-dependent methaemoglobin reductase were responsible for the reduction of methaemoglobin in erythrocytes, this reduction should be stoicheiometrically related to the labelled carbon dioxide produced through the activity of the pentose phosphate shunt. The minor role that is in fact played by the shunt throws some doubt on the existence of a specific reductase, the activity of which (see above) requires the presence of artificial cofactors. On the other hand, it is known that GSH reduces methaemoglobin directly, and that the GSSG thus formed can be reduced by $NADPH_2$ and glutathione reductase. The following experiments demonstrate that in intact erythrocytes the latter mechanism is sufficiently rapid to account for the part of the reduction of methaemoglobin that can be ascribed to the activity of the shunt.

Table 1. *Production of carbon dioxide in relation to the reduction of methaemoglobin during aerobic and anaerobic incubation*

Erythrocyte suspension B contained 5·59 g. of oxyhaemoglobin/100 ml. Suspension A contained the same concentration of total haemoprotein, but 74 % was in the form of methaemoglobin. Suspensions received uniformly labelled [^{14}C]glucose (2·3 mM) at zero time and were incubated at 37° for 5 hr. in air or in nitrogen. Results of duplicate experiments are expressed as μmoles/hr./10 ml. of packed erythrocytes.

	CO_2 formed in 5 hr.			Methaemoglobin reduced in 5 hr.
	In A	In B	A – B	In A
In air	2·80	2·46	0·34	2·96
	2·78	2·26	0·52	2·96
In nitrogen	1·40	0·36	1·04	3·64
	1·36	0·36	1·00	3·48

Reduction of methaemoglobin in haemolysates by reduced glutathione

When high concentrations of GSH are added to thoroughly dialysed haemolysates of methaemoglobin-containing erythrocytes a reduction to haemoglobin takes place (Fig. 2). This result is in agreement with those obtained by Morrison & Williams (1938). Under the specified conditions the initial reduction velocity was about 1·1 μmoles of methaemoglobin/hr./10 ml. of haemolysate.

To obtain conditions more comparable with those within intact erythrocytes a GSH-regenerating system, consisting of glucose 6-phosphate, NADP and GSSG, was added to the dialysed haemolysate. The glutathione concentration in these experiments was equal to that of intact erythrocytes, i.e. 1·5 mM as GSSG. The methaemoglobin concentration was 6 g./100 ml. of haemolysate. When comparing the results with those obtained with intact erythrocytes (Table 1) it should be remembered that in the latter case the methaemoglobin concentration inside the cells is about three times as high. During 5 hr. the haemolysate system reduced 0·30 μmole of methaemoglobin/hr./10 ml. of haemolysate. This value corresponds fairly well to the reduction in intact erythrocytes which was ascribed to the activity of the pentose phosphate shunt (Table 1, column 'A − B'). When GSSG was omitted, so that the system regenerated only $NADPH_2$, there was very little, if any, effect on the rate of reduction of methaemoglobin. This is in accordance with previous findings (Kiese, 1944). Thus GSH, in concentrations comparable with those in intact erythrocytes, may reduce methaemoglobin at a sufficient rate to account for the amount of methaemoglobin reduction that can be ascribed to the pentose phosphate shunt.

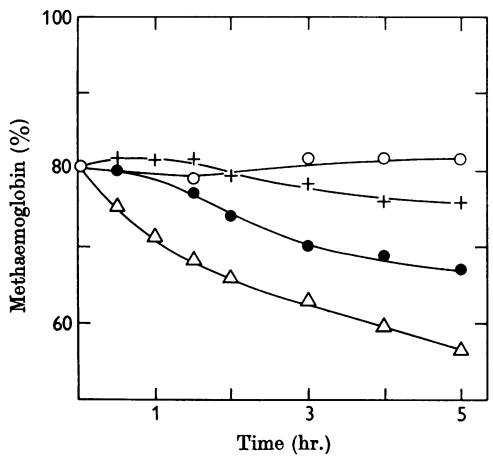


Fig. 2. The methaemoglobin-reducing effects of GSH and GSH-producing systems in dialysed haemolysates of erythrocytes containing methaemoglobin. The initial methaemoglobin concentration was 80% of the total haemoprotein concentration (7·45 g./100 ml. of haemolysate). The incubations were at 37° with the following additions: ○, nil; +, glucose 6-phosphate (6 mM) + NADP (0·2 mM); ●, glucose 6-phosphate (6 mM) + NADP (0·2 mM) + GSSG (1·5 mM); △, GSH (10 mM).

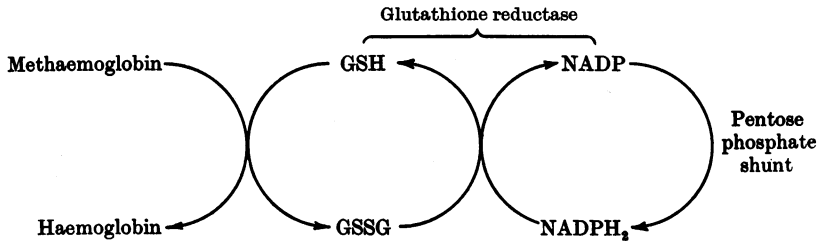
Reduction of methaemoglobin by reduced glutathione in intact erythrocytes

When erythrocytes containing no methaemoglobin were incubated at 37° in the presence of oxygen but without glucose, the GSH gradually disappeared. After 3 hr. the GSH concentration was about 75% of the initial value. In erythrocytes containing methaemoglobin the corresponding value was 50%. When these experiments were repeated under anaerobic conditions (Table 2) only a negligible disappearance of GSH was observed in the presence of haemoglobin, whereas with methaemoglobin the disappearance persisted. The initial GSH concentration was somewhat lower in the erythrocytes containing methaemoglobin than in those which did not, probably owing to some oxidation of GSH by amyl nitrite. When glucose was added to both, the GSH concentrations increased and reached the same level. When the erythrocytes containing methaemoglobin were incubated for 5 hr. without glucose the GSH content decreased by 2·72 μmoles/hr./10 ml. of packed erythrocytes,

Table 2. *Effect of methaemoglobin and glucose on the concentration of reduced glutathione in anaerobically incubated erythrocytes*

Suspension B contained 5·50 g. of oxyhaemoglobin/100 ml. Suspension A contained the same concentration of total haemoprotein, but 69% was in the form of methaemoglobin. Glucose (5·55 mM) was added at zero time and the suspensions were incubated in nitrogen for 5 hr. at 37°.

	Concn. of GSH (mM)		Change in GSH content (μmoles/hr./10 ml. of packed erythrocytes)	Methaemoglobin reduced (μmoles of haem/hr./10 ml. of packed erythrocytes)
	Zero time	5 hr.		
Suspension A + glucose	2·27	2·70	+0·86	18·1
Suspension A	2·22	0·86	−2·72	3·48
Suspension B + glucose	2·67	2·81	+0·28	—
Suspension B	2·70	2·55	−0·30	—



Scheme 1

a value of the same order as the amount of methaemoglobin simultaneously reduced (3·48 μmoles/hr./10 ml. of packed erythrocytes).

From the results in Table 2 it appears that the presence of glucose leads to a regeneration of GSH in incubated suspensions of erythrocytes containing methaemoglobin. The extent of this regeneration is at least 3·58 μmoles/hr./10 ml. of packed erythrocytes, i.e. the difference between the GSH content of suspension A with and without glucose (Table 2). Such an activity of glutathione reductase and of the shunt will give rise to 0·90 μmole of carbon dioxide/hr./10 ml. of packed erythrocytes. It should be stressed that these experiments are comparable with the anaerobic series of Table 1, where the mean rate of release of carbon dioxide was 1·02 μmoles/hr./10 ml. of packed erythrocytes. On the basis of these stoicheiometric agreements it seems reasonable to conclude that, in intact erythrocytes, part of the reduction of methaemoglobin which is coupled to the pentose phosphate shunt is initiated by a spontaneous reaction between GSH and methaemoglobin according to Scheme 1. Thus there seems to be no need to invoke the participation of a $NADPH_2$-dependent methaemoglobin reductase.

SUMMARY

1. The role of the pentose phosphate shunt in the reduction of methaemoglobin in human erythrocytes has been studied by measuring, under comparable conditions: (*a*) the activity of the pentose phosphate shunt, (*b*) the amount of methaemoglobin reduced, (*c*) the intracellular glutathione concentration.

2. The pentose phosphate shunt accounts for only a small part of the total methaemoglobin reduction.

3. The participation of the pentose phosphate shunt is fully explained by a direct non-enzymic reduction of methaemoglobin by reduced glutathione. The oxidized glutathione thus formed is in turn reduced by nicotinamide–adenine dinucleotide phosphate and glutathione reductase.

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