Erythrocyte Glutathione Reductase

By ERNEST BEUTLER AND MARY K. Y. YEH

THE EXACT ROLE of reduced glutathione (GSH) in the economy of the red blood cell remains to be defined. Drug-induced hemolytic anemia due to glucose-6-phosphate dehydrogenase deficiency is associated with low and with unstable GSH. Yet, a condition has been described in which red cell glutathione is virtually absent with only mild hemolysis being observed, and destruction of red cell glutathione by N-ethyl-maleimide does not appear to seriously impair red blood survival. The enzyme which is required to maintain glutathione in the reduced state is glutathione reductase. This enzyme normally transfers reducing power from the coenzyme TPNH to oxidized glutathione (GSSG) and thus restores this compound to the reduced form, GSH. Recently, it has been found that deficiency of glutathione reductase may be associated with sensitivity to drug-induced hemolysis and with nonspherocytic congenital hemolytic anemia.

The enzyme glutathione reductase was originally characterized in animal tissues by Rall and Lehninger, who assayed it in various rat tissues and found it to be TPN specific. More recently, Langdon succeeded in preparing highly purified GSSG reductase from rat liver, and confirmed the absolute specificity of this enzyme for TPN. Racker, on the other hand, found that glutathione reductase from yeast and beef liver could catalyze hydrogen transfer from both DPNH and TPNH, but more rapidly with the latter. Francoeur and Denstedt assayed crude human red cell preparations for GSSG-reductase activity and found that both TPN and DPN were effective as coenzymes. This observation has been confirmed by Carson et al.

The present studies have been carried out to determine (1) whether the DPN- and TPN-active glutathione reductase activity of human red cells is due to a single enzyme or whether two enzymes, each with a different coenzyme specificity, could be separated; (2) whether the DPN-active enzyme of red cells could be harnessed to reduce oxidized glutathione (GSSG) in the intact erythrocyte. At the same time, it has been possible to characterize the enzyme glutathione reductase in relatively purified preparations.

METHODS

Glutathione reductase was assayed spectrophotometrically by a modification of the method of Racker. Reduced triphosphopyridine nucleotide (TPNH) and diphosphopyridine nucleotide (DPNH) were obtained from Sigma Chemical Co. A solution containing approximately 1 mg./ml. was prepared in .01 M potassium phosphate buffer, pH 8.0. The solution was stored at approximately -20 C. After it was thawed, 0.1 ml. was added to 2.9 ml. of 0.33 M potassium phosphate buffer at pH 8.4 and the optical density of 340 μ was de-

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termmed. Using an extinction coefficient of $6.22 \times 10^6$ cm$^{-1}$ M$^{-1}$, the volume of the stock solution which contained 1 μM of TPNH or DPNH was calculated.

Oxidized glutathione was obtained from Sigma Chemical Co. A weighed portion was dissolved in water and the pH was adjusted to approximately 7 by the addition of 1 N NaOH. The volume of the solution was adjusted by the addition of water to give a final concentration of 2.0 per cent. No correction was made in routine assays for water of hydration or small amounts of contaminants. However, for the calculation of Michaelis-Menten constants, such corrections have been made. The solution was stored at −20 C. and prepared fresh at approximately monthly intervals. 1 per cent bovine albumin solution was prepared in 0.1 M phosphate buffer, pH 7.0, by dilution from Armour 30 per cent bovine albumin.

The assay mixture was prepared in a 3.0 ml. quartz cuvette with a 1.00 cm. light path:
- TPNH (or DPNH) solution—volume to contain 0.3 μM.
- Bovine albumin solution—0.1 ml.
- 1 M phosphate buffer, pH 7.0—0.15 ml.
- Enzyme solution—to contain 3–50 units.
- Distilled water—sufficient to make total volume of 2.70 ml.

The cuvette was covered with parafilm and the contents were mixed by inverting several times. The optical density of the solution was measured at 1 minute intervals for 3–6 minutes. Then, 0.3 ml. of neutralized GSSG solution was added and additional readings were made at ½ to 1 minute intervals for an additional 5 to 10 minutes. Enzyme activity was expressed in units. One unit of enzyme was that amount which gave rise to a change in optical density of .001 O.D. units/min. after GSSG had been added to the system. If the optical density was decreasing before GSSG was added to the assay system, indicating the presence of TPNH oxidase activity, the rate of this change was subtracted in calculating the glutathione reductase activity. Rarely, when enzyme activity was very great, the rate of decrease of optical density diminished near the conclusion of the period of observation. Under these circumstances availability of TPNH apparently had become a limiting factor, and the initial rate was used in computing the enzyme activity.

Chromatographic purification of the enzyme was carried out on CG-50, type II resin, supplied by Rohm and Haas Co. The resin was washed and cycled according to the method of Hirs et al. It was then equilibrated with 0.01 M potassium phosphate buffer at pH 7.0. All chromatographic separations were carried out at room temperature. To achieve reproducible results it was found necessary to control carefully the rate of flow of the eluting buffer over the column. Maintaining a constant head of pressure did not suffice: the flow rate tended to decrease steadily. A constant flow rate was assured by using a Harvard infusion pump* to perfuse the column with fluid.

Protein determinations were carried out by measuring optical density at 280 and 260 mμ or by a modification of the method of Lowry.

RESULTS

A. Purification of the Enzyme

Packed human red cells were hemolyzed in four volumes of distilled water. The stroma was removed by centrifugation at approximately 16,000 x g at 4 C. for 1 hour. Sufficient water was added to de-stromatized hemolysate to bring it to a volume of 20 times the original volume of erythrocytes. This fraction has been labeled “de-stromatized 1:20 hemolysate.” Sufficient solid ammonium sulfate was added to bring the de-stromatized 1:20 hemolysate to 55 per cent saturation. The preparation was allowed to stand at 4 C. for 24–84 hours, after which the precipitate was collected by centrifugation at 20,000

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Table 1

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Unita/mg. Protein</th>
<th>Per Cent Recovery</th>
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<tbody>
<tr>
<td></td>
<td>TPN</td>
<td>DPN</td>
</tr>
<tr>
<td>1:20 Destromatized hemolysate</td>
<td>3.97</td>
<td>0.76</td>
</tr>
<tr>
<td>A (55% (NH₄)₂SO₄ ppt. dissolved in water)</td>
<td>83.33</td>
<td>14.65</td>
</tr>
<tr>
<td>F (heated 62 C, 6 min.)</td>
<td>147.6</td>
<td>29.5</td>
</tr>
<tr>
<td>Entire column</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Tube #13 (most active tube)</td>
<td>340</td>
<td>—</td>
</tr>
</tbody>
</table>

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The precipitate was mixed with water and brought to approximately the original volume of red cells, and was labeled "A." It was then heated in a 70 C water bath for 10 minutes. The heavy coagulum which formed was removed by centrifugation and the supernatant solution was dialyzed overnight against distilled water at a temperature of 4 C. The precipitate which formed was virtually free of enzyme activity and was removed by centrifugation and discarded. The supernatant was labeled "F." The yield of enzyme and its purification is presented in table 1.

B. Chromatographic Characterization

Several chromatographic systems have been investigated with respect to their capacity to purify the enzyme further. The most satisfactory system has been found to consist of a convex gradient of potassium chloride in 0.01 M potassium phosphate buffer, pH 7.3, beginning with a concentration of 0 molar KCl and approaching a concentration of 0.4 M KCl, at room temperature. The enzyme preparation was washed into the top of the resin column with a small quantity of 0.01 M potassium phosphate buffer, pH 7.3. It was allowed to equilibrate with the resin overnight at 4 C. The gradient was achieved by pumping with a syringe-type perfusion pump 0.4 M KCl in 0.01 M phosphate buffer at a rate of 0.191 ml./min. Samples were collected each 30 minutes. The pH of the eluate was relatively stable, but tended to fall gradually to a pH of 6.60. Three peaks of enzyme activity were eluted from such a column when preparations had been heated in a 70 C water bath. The first peak was found in the first few tubes and has been labeled I. The second peak appeared at between 41 and 77 ml. and has been labeled II. The third peak, III, appeared after 77-134 ml. of fluid had passed over the column (fig. 1). On rechromatography of these peaks, using the same system, peak I yields a small peak in the position of I and additional peaks in the positions of II and III (fig. 2). Peak II when rechromatographed appears at the same location where it originally appeared (fig. 3). When peak III is rechromatographed, very little of it can be recovered, but small amounts of enzyme activity appear throughout the chromatogram. As shown in figure 4, peak III appears together with the red pigment present in the preparation "F." The absorption spectrum
Fig. 1.—Chromatography of "preparation F." The conditions of chromatography and enzyme assay are described in the text. The enzyme preparation was allowed to equilibrate overnight with the resin. A convex gradient from 0 M KCl in 0.01 M potassium phosphate buffer, pH 7.3, was used. Each tube represents the flow of approximately 5.7 ml. of buffer.

of this pigment resembles a mixture of oxyhemoglobin and hemoglobin. It probably consists largely of hemoglobin and catalase derivatives, as these proteins were among those identified on starch gel electrophoresis of the enzyme solution.\textsuperscript{17} It seemed possible that peak III merely represented glutathione reductase which had in some way become bound to hemoglobin and was carried with hemoglobin in the chromatogram. However, mixing peak II glutathione reductase with crystalline hemoglobin prepared by the method of Drabkin,\textsuperscript{18} or with the hemoglobin remaining in the supernatant solution after 55 per cent ammonium sulfate precipitation of the 1:20 de-stromatized hemolysate failed to change its chromatographic mobility. Further investigation revealed that peak III appears only when chromatography of enzyme heated to 62 C. or more is carried out. It has not been possible to chromatograph unheated, dialyzed 55 per cent ammonium sulfate fraction because of caking of the resin column. However, if this material is heated to a temperature of 55 C. for 6 minutes, only peaks I and II appear, but the recovery of the enzyme under these conditions is very poor.
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Fig. 2.—The results of rechromatography of peak I (fig. 1) in the same system, as described for figure 1.

Fig. 3.—Rechromatography of peak II (fig. 1) in the same system, as described for figure 1.

C. Relative TPNH/DPNH Activity of Red Cell Glutathione Reductase

As indicated in table 1, there was no significant change in the relative TPN and DPN activity of enzyme through 27-fold purification by salting out and heat. Similarly, in the chromatographic separation or purification of the enzyme, the ratio of TPNH activity to DPNH activity appeared unaltered in peaks I and II. However, a consistent decrease in activity with DPNH as substrate was found in peak III. Dialysis of this fraction, which contains a very substantial concentration of potassium chloride, results in restoration of the ratio of activity with DPNH to that with TPNH to the original level.

D. Effect of Salt Concentration on DPN and TPN-linked Activity

Because of the finding that the third chromatographic peak had an altered activity ratio with TPNH and DPNH which could be restored to normal by dialysis, the effect of various salts on relative TPNH and DPNH activity have been investigated. Typical results, which have been confirmed several times in our laboratory, are presented in figures 5 and 6. Potassium chloride and sodium chloride were found to increase markedly the activity of glutathione reductase when TPNH served as hydrogen donor. However, when DPNH
served as hydrogen donor the same salts caused a substantial decrease in enzyme activity (fig. 5). Pre-incubation of enzyme with salt for several days had no additional effect, and the salt effect was reversed completely by dialyzing added salt from the enzyme solution. Like potassium chloride and sodium chloride, increasing ionic strength by varying the quantity of potassium phosphate buffer used caused a striking increase in enzymatic activity when TPNH was the substrate. However, unlike sodium and potassium chloride, the increasing concentration of phosphate salt produced little change in DPNH activity of the enzyme (fig. 6). These changes could not have been due to small changes in pH because of the relatively flat pH optimum curves of the enzyme over the limited range of 6.3 to 8.0, as presented in figure 7.

E. Stability of the Enzyme

Red cell glutathione reductase, when purified as we have described to the "preparation F" stage, is remarkably stable. No loss of enzyme activity is noted during column chromatography over many hours at room temperature or on storage for many days at 4 C.

F. Michaelis Constants

Michaelis-Menten constants for the enzyme have been determined on the "preparation F" prepared with the temperature of the enzyme solution controlled at 62 C. during heating and on unheated preparation.
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G. Attempts to Achieve Glutathione Reduction through a DPN-linked System In Vivo

Red blood cells contain lactic dehydrogenase and are able to reduce DPN when supplied with lactate as a substrate. An attempt was therefore made to determine whether intracellular GSSG could be reduced in erythrocytes suspended in sodium lactate. Intracellular GSH was oxidized by suspending normal human erythrocytes in a saline-phosphate buffer and oxygenating a suspension at frequent intervals (fig. 8). When the glutathione content of the red cells had fallen to one-half of its original value, the suspension was divided into two aliquots. One was incubated for 1/2 hour with 1 per cent sodium nitrite, the other with saline. Both aliquots were washed five times in buffered saline. Nitrite-treated and control cells were then suspended either in glucose, .01 M final concentration, lactate, .02 M final concentration, or in buffered saline. They were then incubated for 3 hours, and methemoglobin determinations were carried out at intervals by the method of Evelyn and Malloy.10 As expected, both glucose and lactate served as effective substrates for methemoglobin reduction. The rapid reduction of methemoglobin in the presence of lactate confirms that the red cell is able to utilize lactate for reductive power under the conditions of the experiment. In contrast, while glucose served as an effective substrate for reduction of glutathione, as evidenced by the rapid rise in the concentration of reduced glutathione, lactate had no effect on the reduced glutathione content. In the suspension incubated with lactate, the GSH continued to disappear at the same rate as had been the case with the buffered saline control.

Discussion

We have confirmed the findings of Francoeur and Denstedt10 and of Carson et al.11 that human red cell GSH reductase is active both with TPNH and DPNH as a hydrogen donor. Purification of this enzyme has failed to separate a DPN-active enzyme from a TPN-active enzyme. Similarly, Racker9 was unable to separate DPNH and TPNH activity on purification of yeast or beef liver enzyme. It seems unlikely, therefore, that we are dealing with two separate enzymes, although it is entirely possible that separate molecular species might not differ sufficiently to permit us to distinguish them by the technics employed.

For those assaying this enzyme, it is important to recognize that the concentration of various electrolytes markedly influence its enzymatic activity. Thus, increasing amounts of chloride ion increase the activity of glutathione reductase when TPNH serves as the substrate, but decreased enzyme activity when DPNH was the substrate. Phosphate ion increased enzymatic activity when TPNH was the substrate but had little or no effect on DPNH activity. Racker, studying the yeast enzyme, had also found marked salt effects. He reported that the DPN-linked enzyme was markedly stimulated by phosphates and was inhibited by potassium or sodium chloride. In the case of the yeast enzyme, in contrast to the human red cell enzyme, salt concentrations did not appreciably affect TPN activity.9

It was of interest that although DPNH serves as a substrate for GSSG re-
Fig. 5.—The effect of KCl and NaCl added to the assay system on glutathione reductase activity using TPNH and DPNH as a substrate. Each cuvette contained 0.15 ml. 1 M potassium phosphate buffer, pH 7.0; 0.1 cc. bovine albumin; 0.3 cc. TPNH or DPNH solution; 0.3 cc. neutralized GSSG; 0.5 cc. of enzyme “F” diluted 1:10; 0 cc., 0.2 cc., 0.4 cc., or 0.8 cc. of 1 M NaCl or 1 M KCl; and sufficient water to bring the total volume to 3.0 ml. Enzyme activity is expressed as units per ml. of the original enzyme solution. Increasing the salt strength caused approximate doubling of enzyme activity with TPNH as a substrate but caused marked inhibition of enzyme activity when DPNH served as a substrate.

duction in vitro, the lactic dehydrogenase system could not achieve reduction of GSSG in the intact red cell, even under conditions where methemoglobin was reduced readily. This might be explained on the basis of an intracellular organization which does not permit lactic dehydrogenase-linked DPN reduction to link with GSSG reductase. However, it seems more likely that the explanation may be found in certain thermodynamic considerations.\textsuperscript{20} The redox potential of the lactate-pyruvate system ($E'_0 = -0.19V$) is higher than that of the glucose-6-phosphate-6 phosphogluconic deltalaconate system ($E'_0 = -0.32$). The latter may, therefore, be thermodynamically better situated to
reduce GSSG, the redox potential of which has not been well established. The fact that the lactate-pyruvate system is capable of reducing methemoglobin is compatible with a much higher redox potential ($E'_o = +0.17$) of the hemoglobin-methemoglobin system. Thus, the DPNH-DPN ratio achieved in the erythrocyte suspended in the lactate substrate may be sufficiently high to mediate methemoglobin reduction, but not sufficiently high to achieve the reduction of appreciable amounts of GSSG. In vivo, however, it is the triose phosphate dehydrogenase system rather than the lactic dehydrogenase system which reduces DPN to DPNH. The redox potential of this system ($E'_o = -0.29\text{V}$) is nearly as low as that of the glucose-6-phosphate-6 phosphogluconic deltalaactone system, and is considerably lower than that of the lactic dehydrogenase system. It is possible, therefore, that with a glucose substrate, the red cell may be able to utilize the DPN pathway more effectively.
Fig. 7.—The effect of pH on the activity of glutathione reductase. One M potassium phosphate buffer solutions ranging in pH from 5.80 to 8.00 were prepared. Each cuvette contained 0.3 ml. buffer; 0.1 ml. bovine albumin; 0.37 ml. TPNH or 0.27 ml. DPNH (see Methods); 0.3 cc. neutralized GSSG; and 0.25 ml. 1:10 dilution of enzyme preparation "F" in the case of the TPNH assays, and 0.5 ml. in the case of the DPNH assays. Sufficient water was added to give a total volume of 3.00 ml. In each case the enzyme activity is expressed as units/ml. of the original enzyme solution. The pH is expressed as the pH of the buffer solution when diluted 1:10. Over the relatively narrow pH range studied, there was little change in enzyme activity.

Fig. 8.—The failure of lactate to act as an adequate substrate for GSSG reduction in intact erythrocytes. Under experimental conditions in which lactate could serve as a substrate for methemoglobin reduction, lactate, unlike glucose, failed to result in the reduction of GSSG. The conditions of the experiment are described in the text.
Table 2.—Michaelis-Menten Constants for the Enzyme Glutathione Reductase

<table>
<thead>
<tr>
<th></th>
<th>GSSG</th>
<th>TPNH</th>
<th>DPNH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heated enzyme</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPNH activity</td>
<td>$2.499 \times 10^{-5}$ M</td>
<td>$6.55 \times 10^{-8}$ M</td>
<td></td>
</tr>
<tr>
<td>DPNH activity</td>
<td>$1.771 \times 10^{-5}$ M</td>
<td>$1.45 \times 10^{-6}$ M</td>
<td></td>
</tr>
<tr>
<td>Unheated enzyme</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPNH activity</td>
<td>$2.539 \times 10^{-5}$ M</td>
<td>$5.774 \times 10^{-8}$ M</td>
<td></td>
</tr>
<tr>
<td>DPNH activity</td>
<td>$1.477 \times 10^{-5}$ M</td>
<td>$1.013 \times 10^{-6}$ M</td>
<td></td>
</tr>
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</table>

for GSSG reduction than when lactate serves as substrate. It is possible that the glutathione reductase system may act as a link between DPN and TPN systems in the erythrocyte. To our knowledge, other transhydrogenating mechanisms have not been fully described in the erythrocyte, although it has recently been suggested that the LDH system may act in this way.22

SUMMARY

1. The physiologic roles of glutathione in the red cell and of glutathione reductase are reviewed briefly.
2. The partial purification of glutathione reductase by a combination of salting out, heating and chromatographic procedures is described.
3. Throughout purification, no appreciable change in the ratio of activity of the enzyme with DPN and TPN as a coenzyme has been found.
4. Potassium chloride and sodium chloride increased markedly the enzymatic activity when TPNH served as hydrogen donor. However, when DPNH was the hydrogen donor these salts caused a decrease in enzyme activity. Potassium phosphate buffer increased enzymatic activity with TPNH but produced little change with DPNH.
5. Michaelis-Menten constants were computed for the purified enzyme preparations.
6. The DPN-linked system did not result in GSSG reduction when lactate served as a substrate in intact erythrocytes.

SUMMARIO IN INTERLINGUA

1. Es presentate un breve revista del rolos physiologic de glutathiona in le erythrocyto e etiam de reductase de glutathiona.
2. Es describite le purification partial de reductase de glutathiona per un combination de methodos, incluse calefaction e chromatographia.
3. In le curso del purification, nulle appreciabile alteration esseva trovate in le proportion de activitate del enzyma con DPN e TPN como coenzyma.
4. Chloruro de kalium e chloruro de natrium augmentava marcatemente le activitate enzymatic quando TPNH serviva como donator de hydrogeno. Tamen, quando DPNH eseva le donator de hydrogeno, iste sales causava un reduction in le activitate enzymatic. Phosphato de kalium como tampon augmentava le activitate enzymatic con TPNH sed produceva pauc alteration con DPNH.
5. Constantes de Michaelis-Menten esseva computate pro le purificate preparatos enzymatic.
6. Le sistema in ligation a DPN non resultava in le reduction de glutathiona oxydate quando lactato serviva como substrato in erythrocytos intacte.
REFERENCES


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ERYTHROCYTE GLUTATHIONE REDUCTASE

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