PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 148, 402–409 (1975)

Isolation and Some Characteristics of Glutathione Reductase from Rabbit Erythrocytes[1] (38548)

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Reduced glutathione (GSH) is essential to a number of cellular processes in erythrocytes. It is the major means of eliminating hydrogen peroxide from red blood cells (1), of protecting hemoglobin against oxidation (2) and of regenerating critical sulfhydryl groups in various enzymes (3). It is evident, therefore, that glutathione reductase (GR) is an important enzyme in erythrocyte metabolism because of its role in producing the GSH that is critical to various processes. Human erythrocyte GR has been isolated and characterized by previous investigators (4–7), and is reported to be a dimeric protein with a mol wt of 115,000–125,000 (5, 6). Although GR has also been isolated from rat liver (8), peas (9), baker's yeast (10) and *Penicillium chrysogenum* (11), there has apparently been little interest in similar enzymes from the erythrocytes of species other than human. During an investigation of metabolism in rabbit erythrocytes, we became interested in the characteristics of GR of rabbit red cells, and we report herein the isolation in homogeneous form of a monomeric rabbit erythrocyte GR with a mol wt of 60,000.

Materials and Methods. Enzyme isolation. New Zealand White rabbits, both male and female, weighing at least 2500 g were maintained on Wayne Rabbit Ration (Allied Mills, Inc., Chicago) and provided with water *ad libitum*. Blood was obtained by cardiac puncture using about 5 ml of anticoagulant (ACD, formula A, Brewer and Sing (12)) per 45 ml of blood. No more than 50 ml of blood were withdrawn at one time and a minimum recovery period of one week was allowed. The samples were immediately cooled in an ice bath and stored at 4° until used. Within 16 hr, they were centrifuged at 5,000*g* for 20–30 min; the plasma was removed and retained for assay. The buffy coat was aspirated and the red cells were resuspended for washing in buffer A (10 m*M* potassium phosphate, pH 7.2, 1 m*M* in EDTA, 20 μM in $NADP^+$, containing 14 m*M* 2-mercaptoethanol (2-ME)) which was rendered isotonic with NaCl. The washed red cells were again centrifuged and the washings and residual buffy coat were removed. The packed, washed red cells were hemolyzed in 9 volumes of buffer A, permitted to stand overnight at 4°, then centrifuged at 20,000*g* for 30 min and the stroma-free hemolysate (pH 7.4) was brought to 35% saturation in $(NH_4)_2SO_4$. The precipitate was discarded, the supernatant liquid (pH 7.4) was adjusted to 70% saturation in $(NH_4)_2SO_4$, and the resulting precipitate was collected, dissolved in buffer A and refractionated with $(NH_4)_2SO_4$ between 35 and 70% saturation. The active fraction, dissolved in buffer A containing 10 m*M* NaCl, was applied to a column of DEAE-Sephadex A-50 which had been equilibrated with 10 m*M* NaCl in buffer A (pH 7.2). GR was eluted at about 0.10–0.15 *M* NaCl by a linear gradient of NaCl in buffer A. The active fractions were combined, adjusted to pH 7.4 and concentrated by precipitation with $(NH_4)_2SO_4$ at 80% saturation. The precipitate was dissolved in buffer A (pH 6.3) containing 10 m*M* NaCl and was applied to a column of CM-Sephadex C-50 equilibrated with the same buffer. Under these conditions, the activity was retained on the column and could be eluted at about 0.1 *M* NaCl by a linear gradient of NaCl in buffer A at pH 6.3. The active fractions were combined, adjusted to pH 7.4, and concentrated by adding $(NH_4)_2SO_4$ to give 80% saturation. The precipitate was dissolved in buffer A

[1] Supported in part by Grant No. A-003 of the Robert A. Welch Foundation and in part by Contract F41609-72-C-0003 from the U. S. Air Force School of Aerospace Medicine.

and subjected to gel filtration through a column of Sephadex G-150 which had been equilibrated with 0.1 *M* NaCl in buffer A. The active fractions were combined and precipitated at 80% saturation of $(NH_4)_2SO_4$; the precipitated GR was dissolved in a small volume of buffer A and dialyzed against buffer A.

Enzyme assays. GR activity was determined at 25° in a 1 cm cuvette by following the oxidation of NADPH at 340 nm in a Zeiss PMQ II spectrophotometer equipped with a Zeiss TE converter and a Simpson recorder. Full scale expansions equivalent to 0.2 or 0.5 absorbance were employed. The routine reaction mixture consisted of 0.1 ml of 2 *M* NaCl, 0.4 ml of 0.5 *M* KH_2PO_4 (pH 6.8), 0.1 ml of a solution that was 5 m*M* in both EDTA and 2-ME, 0.1 ml of 10 m*M* oxidized glutathione (GSSG), 0.1 ml of 1.2 m*M* NADPH, enzyme solution (20 μl of hemolysate or 5 or 10 μl of purified GR solution) and deionized water to give a total volume of 1.0 ml. The reaction was initiated by addition of substrate and cofactor. GR activity was calculated from the recorded change in absorbance using a molar extinction coefficient of 6,200 $cm^{-1}M^{-1}$ for NADPH (13). One unit was defined as the amount of enzyme necessary to oxidize 1 μmole of NADPH per min under the above conditions. Protein concentrations were determined by the method of Lowry *et al.* (14) with bovine serum albumin as the standard. Specific activity was expressed as units of enzyme per mg of protein.

The effect of pH on GR activity was determined by adjusting stock 0.5 *M* phosphate solutions to approximately the final pH value desired and substituting them for the phosphate buffer in the assay mixture. GR activity was then measured as described above using 10 μl of the purified enzyme and the pH was again measured using a Radiometer TTT1c pH meter, a Radiometer PHA630T scale expander, and microelectrodes. This final value was taken as the pH of the reaction. The effect of ionic strength was studied by preparing a number of stock solutions of NaCl (0.5–3.0 *M*) for use in place of the 2 *M* NaCl used in the routine assay. For this study, the stock phosphate buffer was 0.25 *M*, pH 6.9; 10 μl of purified GR were used in each assay. Kinetics of the reaction as a function of concentration of both substrate and cofactor were studied by varying the concentration of one while holding that of the other constant. Concentrations of the stock NADPH solutions were determined spectrophotometrically at 259 nm using the extinction coefficient (15,200 $cm^{-1}M^{-1}$) reported by the vendor. Because the enzyme was active when NADH was substituted for NADPH, the kinetic parameters K_m and V_{max} were also estimated for NADH. The other conditions (pH, ionic strength and substrate concentration) were based on the optimal levels found using NADPH as cofactor. The effect of *p*-hydroxymercuribenzoate (pHMB) was studied under a number of conditions. Purified GR, with and without added NADPH, was treated with pHMB for 2–3 hr, then assayed. Other samples were incubated for 2 hr at 37° in the presence of 7.7 *M* urea, with and without added NADPH, then treated with pHMB for 2–3 hr and assayed. The final concentration of pHMB was 50 μ*M* and the samples were assayed using the routine procedure, except that 2-ME was omitted from the assay. At the end of 16–20 hr, all samples were again assayed, this time with 2-ME in the assay mixture.

Electrophoresis. Standard 7.5% polyacrylamide gels, similar to those described by Davis (15) were prepared using 0.15% ammonium persulfate and stock solutions of Tris–HCl (RDS-A) and acrylamide–bisacrylamide (RDS-C) purchased from Canalco, Inc. (Rockville, MD). After polymerization, the samples were mixed with an equal volume of 0.5 *M* Tris–HCl buffer (pH 6.3) containing 20% sucrose, placed on the gels, and overlayered with buffer prepared from Canalco stock buffer RDS-H. Electrophoresis was carried out at 3 mA per gel until the tracking dye added to the upper reservoir had traveled the length of the sample gels. Gels were fixed in 12.5% trichloroacetic acid (TCA), stained using 0.05% Coomassie Blue in 12.5% TCA and finally destained in 10% TCA. Identification of GR was made by preparing dupli-

cate gels, one of which was stained with Coomassie Blue and the other specifically for the enzyme For the latter procedure the gel was not fixed, but was immediately placed in a freshly prepared solution of 0.25 *M* Tris (pH 8.4) containing 3.4 m*M* GSSG, 0.36 m*M* NADPH, 0.052 m*M* dichlorophenolindophenol and 1.1 m*M* 3(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium (16). The site of glutathione reductase was marked by a blue precipitate as the tetrazolium was reduced to formazan. When the band was clearly discernible, the gels were removed from the stain and were stored in 7% acetic acid. Dodecyl sulfate (DS) electrophoresis was done in 10% gels containing 0.1% DS prepared by the method of Weber and Osborn (17). Samples were incubated at 37° with 1% DS, 0.14 *M* 2-ME and 8 *M* urea in 10 m*M* phosphate, pH 7.0, for at least 2 hr, then were applied to DS-polyacrylamide gels and electrophoresis was carried out at 8 mA per gel until the tracking dye had migrated nearly the length of the gel. The gels were fixed in 20% sulfosalicylic acid, then stained and destained as above.

Ultracentrifugation. The molecular weight was determined using the highspeed, meniscus depletion method (18) in a Beckman Spinco Model E analytical ultracentrifuge equipped with electronic speed control, using the An-D rotor at 19,963 rpm at 20°. Molecular weights were determined in two buffers; one was 75 m*M* in KCl and 11.5 m*M* in potassium phosphate, pH 7.0; the other was 50 m*M* in potassium phosphate, pH 7.2, and contained 1 m*M* EDTA, 20 m*M* $NADP^+$, and 0.14 *M* 2-ME. The Rayleigh interference patterns were photographed using Kodak Spectroscopic II-G photographic plates which were developed with Kodak D-19 developer. The fringe displacements were measured using a Nikon Model 6-C microcomparator and the natural logarithm of the fringe displacement was plotted against the square of the distance from the center of rotation.

Results. Hemolysates taken from weekly samplings of six to ten rabbits were combined, fractionated with $(NH_4)_2SO_4$, and the redissolved active fraction was stored frozen until enough material was accumulated to permit further purification. The results of an isolation experiment representing a total of 225 ml of packed, washed erythrocytes are summarized in Table I. The reason for the high recovery (129%) in the $(NH_4)_2SO_4$ fractionation steps is not apparent, but may have been due to removal of inhibitors. Chromatography of the combined $(NH_4)_2SO_4$ fractions on DEAE-Sephadex resulted in elution of the GR activity as a broad band starting between 0.1 and 0.15 *M* NaCl; however, only a modest increase in specific activity was achieved. Chromatography on CM-Sephadex proved highly beneficial as GR was retained and most of the contaminating protein passed through unretarded, resulting in a 42-fold increase in specific activity after elution of the enzyme

TABLE I. PURIFICATION OF GLUTATHIONE REDUCTASE FROM RABBIT ERYTHROCYTES.

Fraction	Total activity,[a] units	Specific activity[b]	Purification	Recovery, %
1. Soluble hemolysate[c]	89	0.0014	1	100
2. $(NH_4)_2SO_4$, 0.35–0.70[d]	115	0.088	61	129
3. $(NH_4)_2SO_4$, 0.35–0.70	115	0.17	118	129
4. DEAE-Sephadex, pH 7.2, and $(NH_4)_2SO_4$, 0.80	74	0.89	615	83
5. CM-Sephadex, pH 6.3, and $(NH_4)_2SO_4$, 0.80	142	37.5	25,840	160
6. Sephadex G-150, pH 7.2, and $(NH_4)_2SO_4$, 0.80	83	51.7	35,620	93

[a] Assays and units are described in the text.
[b] Units of enzyme/mg protein.
[c] Prepared as described in the text.
[d] All ammonium sulfate fractionations were performed at pH 7.4.

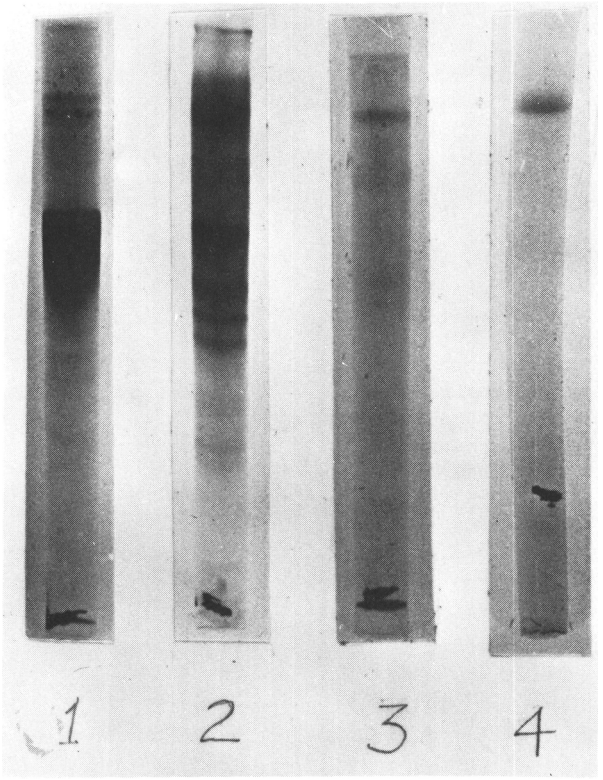

FIG. 1. Patterns obtained from polyacrylamide gel electrophoresis of GR at various stages of purification. Samples 1–3 were subjected to electrophoresis in standard 7.5% gels at 3 mA per gel, then stained with Coomassie blue. The position of the tracking dye is indicated by the dark reference mark near the bottom. The patterns show: (1) stroma-free hemolysate, (step 1, Table I), (2) concentrated DEAE-Sephadex eluate, (step 4, Table I), (3) purified glutathione reductase following Sephadex G-150 and concentration, (step 6, Table I). Gel 4 shows the pattern obtained by electrophoresis of purified glutathione reductase in the presence of DS. Enzyme from step 6 was treated with 1% DS and 0.14 *M* 2-ME in 8 *M* urea and 10 m*M* phosphate, pH 7.0, then electrophoresed at 8 mA per gel in 10% gels containing 0.1% DS.

as a relatively sharp peak at about 0.1 *M* NaCl. Subsequent gel filtration on Sephadex G-150 yielded a preparation with a specific activity of 51.7 units/mg of protein, representing a purification of 35, 620-fold; the total recovery was 93%. The specific activity of the final product was approximately one-third that reported for the enzyme isolated from human erythrocytes (4–6), and the overall purification was within the range obtained by previous workers from human red blood cells.

Samples from various stages of purification, when analyzed by gel electrophoresis, yielded the patterns shown in Fig. 1. The third gel represents purified GR obtained from step 6 and showed the preparation to be essentially homogeneous. Duplicate electrophoretic gels of the purified enzyme, when stained specifically for GR confirmed that the activity was associated with the protein band revealed by the standard protein staining technique. A high degree of homogeneity was also indicated by polyacrylamide gel electrophoresis in the presence of DS, as evidenced by the fourth pattern shown in Fig. 1. The single component was calculated by the procedure of Weber and Osborn (17) to have a mol wt of 60,000.

Determination of the molecular weight by ultracentrifugation was carried out under conditions which should not have caused dissociation and thus should have given the value for the native form. Two experiments in which different buffers were used gave mol wt of 57,000 and 62,800, calculated with an

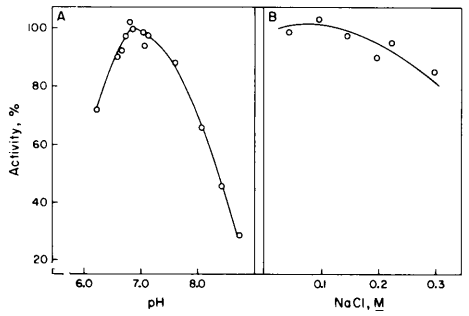

FIG. 2. The effect of the pH and ionic strength of the assay on GR activity. (A) The effect of pH was studied using 10 μl of enzyme solution in the routine assay (see text) at the pH values indicated; the final phosphate concentration was 0.2 *M* in each assay. (B) The effect of ionic strength was evaluated by assaying 10 μl samples of purified GR solution in the presence of 0.1 *M* phosphate, pH 6.9, at the indicated concentration of NaCl. Other details of the assay are given in the text.

assumed partial specific volume of 0.74 cc/g (10). The fact that these molecular weights are virtually identical with that obtained by DS gel electrophoresis indicates that rabbit erythrocyte GR is a single polypeptide chain of about 60,000 daltons, in contrast to the enzyme from human erythrocytes which is a dimer with a mol wt in the range of 115,000–125,000 (5, 6).

Effects of pH and ionic strength. The purified rabbit erythrocyte GR was maximally active at pH 6.8–7.0 (Fig. 2A), a value which agrees well with the range reported for the enzymes from human erythrocytes (4, 5) and *P. chrysogenum* (11). The ionic strength of the reaction mixture also influenced the observed activity, as is shown in Fig. 2B; these results are similar to those obtained with GR from other sources (5, 8, 11). On the basis of these results, the routine assay conditions described above were changed to yield final concentrations of NaCl and phosphate (pH 6.9) of 0.1 *M* each and the modified assay was used for the kinetic studies described below. This modification increased the measured activity about 10%, but served mainly to make conditions more nearly optimal and thus to minimize variations.

Effect of pHMB. Incubation of purified GR with 50 μM pHMB at 22° for 2–3 hr caused about a 55% loss of activity (Table II), and there was a similar decrease when

TABLE II. INHIBITION OF GLUTATHIONE REDUCTASE BY *p*-HYDROXYMERCURIBENZOATE.

Pretreatment[a]	pHMB, μM	Activity remaining, % Without 2-ME	With 2-ME
None	0	100	100
None	50	44	75
120 μM NADPH	50	1	43
7.7 *M* urea	50	45	14
7.7 *M* urea and 120 μM NADPH	50	0	8

[a] The purified enzyme was treated at 22° or 37° for 1.5–2 hr with the reagents listed, then the solution was rendered 50 μM with respect to pHMB. After 1.5–2 hr exposure to pHMB, the samples were assayed using the routine procedure (see text) except that 2-ME was omitted. The relative activities are listed under the column headed "Without 2-ME". After 16–20 hr the samples were again assayed using the routine assay (complete with 0.5 m*M* 2-ME); the relative activities are listed under the column headed "With 2-ME."

the enzyme was first treated with 7.7 *M* urea at 37° for 2 hr then with pHMB. After 16–20 hr, the activity of the sample treated with both urea and pHMB was only 14% of the original; thus, it appeared that an essential sulfhydryl group became slowly available in the presence of urea. When the enzyme was treated with 120 μM NADPH, with or without urea, and then with pHMB as above, there was complete loss of activity within 2 hr (Table II). The partial loss of activity observed with pHMB even in the absence of NADPH or urea might be explicable on the basis that some nonessential sulfhydryl groups are accessible in this enzyme and that the reaction of these with pHMB produces a comformational change that lowers activity. It appears from the data in Table II that 2-ME was capable of partially reversing the effect of pHMB alone, but in the presence of urea recovery of activity did not occur, presumably because of greater disruption of the conformation.

Kinetic characteristics. The effects of substrate and cofactor concentrations on initial velocity are shown in Figs. 3 and 4, from which the following kinetic parameters were

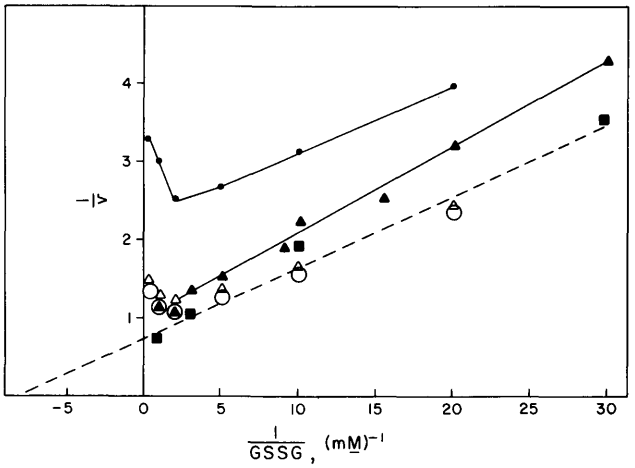

FIG. 3. Effect of GSSG concentration on the rate of reaction. Purified GR, 10 μl, was assayed in 0.1 *M* NaCl and 0.1 *M* phosphate, pH 6.9, containing EDTA and 2-ME as detailed in the text. The reciprocal of the velocity, in μmoles NADPH/min/ml, is shown as a function of the reciprocal of GSSG concentration (mM) at 12.2 (●), 56.5 (△), 89.8 (○) and 126 μM NADPH (▲). The dashed line includes points (■) representing an infinite concentration of NADPH as extrapolated from the data of Fig. 4.

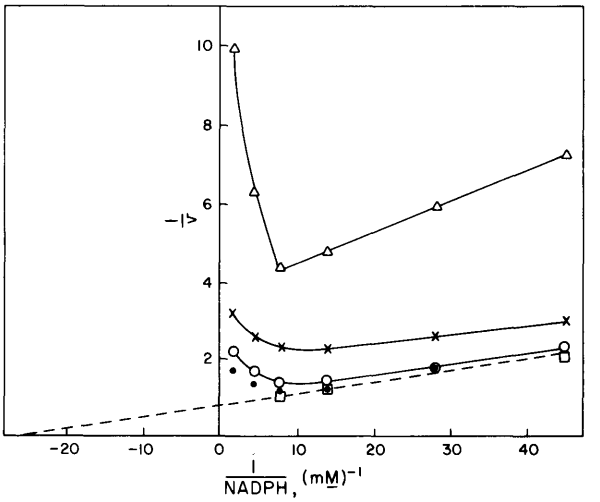

FIG. 4. Effect of NADPH concentration on the rate of reaction. The reciprocal of the velocity, in μmoles NADPH/min/ml, is shown as a function of the reciprocal of NADPH concentration (m*M*) at 33 (△), 100 (×), 333 (○) and 1,000 μ*M* GSSG (●). The dashed line includes points (□) representing an infinite concentration of GSSG as extrapolated from the data in Fig. 3.

calculated: for GSSG, K_m = 120 μM; for NADPH, K_m = 37 μM; and V_{max} = 23 μmole NADPH oxidized/min/mg protein. The K_m value for each compound was determined at saturating concentrations of the other. The value of V_{max} for rabbit erythrocyte GR was based on enzyme concentrations calculated using $E^{1\%}_{230}$ = 15.4 as reported by Mavis and Stellwagen (10) for GR from baker's yeast, as the limited amount of material precluded determination of this value for the rabbit erythrocyte GR. The enzyme was capable of utilizing NADH in place of NADPH, and in the presence of 1 m*M* GSSG, K_m for NADH was 420 μM (Fig. 5). V_{max} was 3 μmoles NADH oxidized/min/mg protein.

As shown in Figs. 3 and 4, substrate inhibi-

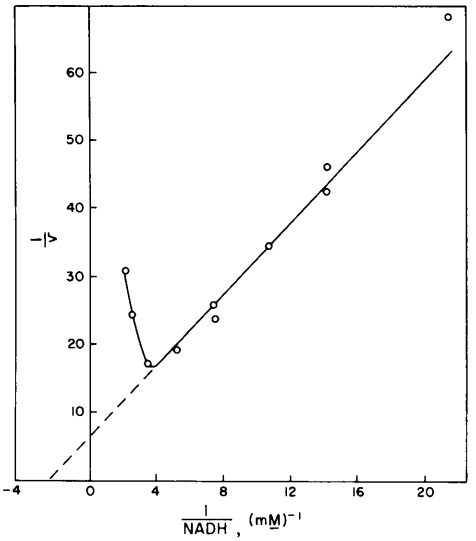

FIG. 5. Effect of NADH concentration on the rate of reaction. Purified GR solution was assayed in 0.1 *M* NaCl and 0.1 *M* phosphate, pH 6.9, containing EDTA and 2-ME as explained in the text. The reciprocal of the velocity, in μmoles NADH/min/ml, is shown as a function of NADH concentration (m*M*). The concentration of GSSG was 1.0 m*M*.

tion was caused both by substrate (above 0.5–1.0 m*M* GSSG) and cofactor (above 100–125 μ*M* NADPH). Inhibition due to either could be eliminated or reduced by increasing the concentration of the other; for example, inhibition was not caused by GSSG when the NADPH was 126 μ*M* (Fig. 3). When NADH was used as the cofactor, substrate inhibition occurred above 250 μ*M* NADH (Fig. 5). As shown in Fig. 5, GSSG was less effective in overcoming inhibition caused by high concentrations of NADH.

Discussion. Differences in the properties of GR from rabbit erythrocytes and catalytically similar enzymes from unrelated biological forms are certainly to be expected; in addition there are also substantial differences in the properties of the GR isolated from rabbit erythrocytes and those of the enzyme others have isolated from human red cells. In the first place, the isolation procedures used for human erythrocyte GR (4–6) gave low yields when applied to rabbit red cells, and had to be modified. Perhaps the most interesting difference between the two enzymes from erythrocytes, however, is that human GR is a dimer with a mol wt variously determined as 115,000–125,000, whereas the rabbit enzyme is a monomer of 60,000 mol wt. Catalytically similar enzymes from pea seedlings (9) and rat liver (19), also have been reported to be monomeric proteins of 60,000 and 44,000 mol wt, respectively.

Because of differences in the assay mixtures, it is difficult to compare the kinetic values obtained herein for rabbit red cell GR with those of similar enzymes from other sources, particularly since it has been shown (7) that the composition of the reaction mixture markedly influences the values obtained (*e.g.*, changing the assay mixture from 0.03 to 0.3 *M* phosphate changed K_m from 19 to 125 μ*M* (7)). Even with that limitation in mind it is interesting to compare some values of K_m and V_{max} for enzymes of different sources, as shown in Table III. From these data it is evident that the K_m values of the rabbit enzyme toward GSSG, NADPH and NADH are generally higher than those of GR from other sources, and that V_{max} values for the rabbit GR are lower than the others. It would appear that metabolism in the red blood cells of rabbits may be less dependent upon an efficient glutathione reductase system than that in human erythrocytes. The much lower V_{max} toward NADH may reflect the use of nonoptimal concentrations of GSSG in our experiments, as lack of enzyme prevented determination of $K_{m,\ GSSG}$ in the presence of NADH. With respect to substrate inhibition, comparison is also difficult because of the wide discrepancy in the literature. For example, Icén (5) has reported no substrate inhibition of human erythrocyte GR by either substrate or cofactor (up to 1.5 m*M* GSSG or 0.2 m*M* NADPH) whereas Staal and Veeger (7) found inhibition by both (at 1.5 m*M* GSSG). The enzyme from *P. chrysogenum*, on the other hand, was reported by Woodin and Segel (11) to exhibit substrate inhibition by NADPH (at 6 m*M*) but not by GSSG (at 100 m*M*). Only the latter investigators mention substrate inhibition due to NADH; they report inhibition above 0.8 m*M* NADH. The results with pHMB are in agreement with a proposed model containing free SH groups not involved in catalysis and a disulfide bridge which is reduced as the first step in catalysis (20). Reaction of the nonessential SH groups

TABLE III. COMPARISON OF SOME KINETIC VALUES OF GLUTATHIONE REDUCTASES FROM DIFFERENT SOURCES.[a]

Parameter	Erythrocytes				*Penicillium chrysogenum* (11)	Rat Liver (8)
	Rabbit	Human (5)[b]	Human (7)	Human (4)		
K_m , GSSG	120	67; 14[d]	19–125[c]	96; 16.7[d]	55	50
K_m , NADPH	37	9.2	9.5–13.3[c]	16	10	3
K_m , NADH	420	250	200–300[c]	193	300	—
V_{max}, NADPH	23	166	140–260[c]	182	450	1.6–9.7[c]
V_{max}, NADH	3	152	36–45[c]	115	110	—

[a] K_m values are expressed in μM; V_{max} values in μmoles/min/mg protein.
[b] The numbers in parentheses indicate the reference citation from which the values are taken.
[c] Values were affected by the conditions of assay.
[d] Determined with NADH.

appears to disrupt the structure to a limited extent whereas reduction of the essential disulfide bridge followed by pHMB gives a total inactivation.

Summary. Glutathione reductase from rabbit erythrocytes was purified to homogeneity and found to be a monomer with a mol wt of 60,000. Both NADPH and NADH were capable of acting as cofactors for the reduction of GSSG and the following kinetic values were obtained: $K_{m,\ GSSG}$ = 120 μM; $K_{m,\ NADPH}$ = 37 μM; V_{max} = 23 μmoles NADPH/min/mg protein, $K_{m,\ NADH}$ = 420 μM; V_{max} = 3 μmoles NADH/min/mg protein. Rabbit erythrocyte GR exhibited substrate inhibition, and was susceptible to inhibition by *p*-hydroxymercuribenzoate under certain conditions.

The invaluable assistance of Mrs. Mary E. Bayliss and Mrs. Stella H. Wilkes with some of the experiments is gratefully acknowledged.

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Received September 6, 1974, P.S.E.B.M. 1975, Vol. 148.