Transport Accounts for Glutathione Turnover in Human Erythrocytes

By G. Lunn, G. L. Dale, and E. Beutler

Human erythrocytes were incubated with $^{3}$H-glycine to label the glutathione pool. These cells were then used to determine the rate of oxidized glutathione (GSSG) transport out of erythrocytes. For 6 normal individuals, the mean transport rate was 6.7 nmole GSSG/hr/ml red cells. This transport rate would suggest a half-life of 4.7 days for the erythrocytic glutathione, which is in close agreement with the observed in vivo half-life of 4 days. These data demonstrate that GSSG transport can account for the observed turnover of erythrocytic glutathione.

GLUTATHIONE (GSH) is one of the major constituents of erythrocytes and is a vital factor in protecting the cell from oxidative challenges. In 1955, Dimant et al. demonstrated that glutathione in human erythrocytes had a half-life of 4 days; however, the metabolism of erythrocytic glutathione has not been fully elucidated. The synthesis of GSH has been shown to occur in two ATP-requiring steps, but the mechanism of GSH degradation has remained uncertain. Jackson, Azzopardi and Jayle and Palekar et al. suggested that -y-glutamyl transpeptidase was present in red cells, and the latter authors proposed that the -y-glutamyl cycle might, therefore, account for the observed turnover rate for glutathione. However, more recently it has been shown that -y-glutamyl transpeptidase is not present in red cell stroma; its putative presence was apparently due to contamination of the red cell preparations with leukocytes.

Srivastava and Beutler reported that erythrocytes exposed to oxidative stress will actively extrude oxidized glutathione (GSSG); this transport requires ATP and is inhibited by fluoride. Several additional studies have demonstrated that resealed red cell membranes, lens, and liver cells, in addition to erythrocytes, will transport GSSG when the intracellular level of GSSG is high. These observations led to the hypothesis that GSSG transport might explain the turnover of glutathione in the red cell. However, the analytic methods employed were not sufficiently sensitive to detect the minute quantities of GSSG that would be expelled by red cells with normal GSSG levels. Using a radiochemical labeling of glutathione, we report that the GSSG transport rate for unstressed erythrocytes is sufficient to account for the observed in vivo turnover rate.

MATERIALS AND METHODS

Labeling of Red Cell Glutathione

Whole blood from a normal donor was collected in heparin, and leukocytes and platelets were removed by passage through microcrystalline cellulose-α-cellulose as previously described. The cells were then washed 3 times and incubated at 20% hematocrit for 4 hr at 37°C in 1% bovine serum.
albumin, 8 mM glucose, 62 mM NaCl, 40 mM NaH₂PO₄/Na₂HPO₄, 35 mM Na-TES (N-Tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid), pH 7.4 (Buffer A) with 15 μCi ³H-glycine (23 μCi/nmol)/ml RBC. After preincubation, the erythrocytes were washed twice in saline at room temperature, allowed to stand 3 min at 37°C in saline, and then washed twice again in saline at room temperature to remove most of the residual ³H-glycine.

Specific Activity of Glutathione

After preincubation and washing, 5.0 ml of the cell suspension were lysed and incubated for 15 min at 37°C with an excess of t-butylhydroperoxide to oxidize GSH to GSSG. After protein precipitation with 5 ml of 4% perchloric acid, neutralization of the extract with 1 M K₂CO₃, removal of potassium perchlorate, and fourfold dilution with water, the sample was applied to 10 x 95 mm Dowex-1 column (formate form). The column was washed with water and eluted with a 100-ml linear gradient of 0–1 M ammonium formate; 3.5-ml fractions were collected (Fig. 1). The GSSG concentration of the fractions was assayed as previously described.

Transport Experiments

Erythrocytes containing labeled glutathione were incubated in Buffer A at a hematocrit of 20% at 37°C in a shaking water bath. The pH of the suspension was monitored on a Radiometer blood pH meter and was maintained at 7.38 ± 0.04. During the incubation, aliquots were taken, centrifuged, and the supernatants saved for subsequent GSSG analysis. To correct the extracellular radioactivity for the contribution from small amounts of lysed cells, samples of supernatant were also assayed for hemoglobin by measuring absorbance at 410 nm.

Two milliliters of supernatant from the transport experiment were diluted with 14 ml of 0.5 mM glycine, 0.5 mM GSSG, pH 7, and each mixture was applied to a 10 x 95 mm column of Dowex-1 and chromatographed as described in the above section.

An alternative experimental procedure was also used to measure the supernatant ³H-glutathione in each study. One-milliliter samples of supernatant were diluted with 8 ml of 0.5 mM glycine, 0.5 mM GSSG, pH 7, and applied to 5 x 60 mm columns of Dowex-1 (formate form). The columns were then washed 4 times with 1-ml portions of water and eluted with 2 ml of 4 N formic acid followed by 1 ml

![Fig. 1. Chromatography of ³H-glutathione. Erythrocytes were incubated with ³H-glycine to label the glutathione pool as outlined in Materials and Methods. A portion of the cells were lysed, incubated with t-butylhydroperoxide to oxidize all of the GSH to GSSG, and then chromatographed on Dowex-1 (formate form). The column was eluted with a 0–1 M ammonium formate gradient. Radioactivity is represented by the open circles; the closed circles are GSSG (nmole/ml). The radioactivity that appeared before fraction 1 is from residual ³H-glycine.](from)
water. This mini-column procedure for the isolation of $^3$H-GSSG was developed to allow more rapid analysis of samples in these transport studies.

**Materials.** Bovine serum albumin, oxidized glutathione (GSSG), and N-Tris (hydroxymethyl) methyl-2-aminomethane sulfonic acid (TES) were obtained from Sigma Chemical Co., St. Louis, Mo. Amersham (Arlington Heights, Ill.) supplied the $2^3$H-glycine (23 Ci/m mole), and Mallinkrodt Chemical Co. (St. Louis, Mo.) provided the Handifluor for liquid scintillation counting. Bio-Rad Laboratories (Richmond, Calif.) supplied the Dowex-1 ion exchange resin (200–400 mesh), and it was converted to the formate form.

**RESULTS**

Human erythrocytes were incubated with $^3$H-glycine to label the glutathione pool. Previous studies with glutathione synthetase indicate that labeling occurs under these circumstances from both net synthesis of GSH and from glycine...
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exchange with preexisting glutathione. The specific activity of the labeled glutathione was determined after chromatographic isolation of GSSG (Fig. 1); for experiment 1, the specific activity was 1930 cpm/nmole GSSG.

The labeled erythrocytes were then incubated in a buffered medium, and the transport of 3H-GSSG out of the cells was monitored by chromatography of supernatant fractions collected at various time intervals (Fig. 2). A peak of radioactivity progressively appeared on the chromatograms between fractions 12 and 16; spectrophotometric assay of carrier GSSG (t = 120 min supernatant) demonstrated that the radioactivity coeluted with GSSG.

Besides the 3H-glycine, which was collected in the first 6 fractions of each graph in Fig. 2, 2 of the time points (t = 90 and 150 min) also show an additional intermediate peak of radioactivity between the glycine and GSSG. This intermediate peak was shown to be reduced glutathione by two criteria; first, after aerobic precipitation of the supernatant by perchloric acid, this intermediate peak disappeared, and the size of the GSSG peak increased. Secondly, the size of the intermediate peak correlated with the degree of hemolysis at any given time point, indicating it represented glutathione released by cell lysis.

The same supernatants depicted in Fig. 2 were also analyzed by the mini-columns of Dowex-1 as discussed in the Materials and Methods section. These raw data, as well as the associated hemolysis corrections, are shown in Table 1. The hemolysis corrections are important in that they demonstrate that the increase in extracellular 3H-GSSG with time is not the result of cell lysis. While the hemolysis corrections are quite significant, they represent no more than 25% of the total supernatant radioactivity after the 30-min time point.

Figure 3B depicts the appearance of extracellular GSSG (corrected for hemolysis) as a function of time in experiment 1 as analyzed by the mini-column method; Fig. 3A shows the same data calculated from the gradient-columns (Fig. 2). The mini-columns gave the more reproducible and linear results of the two techniques; presumably, this discrepancy is due to the cumulative errors involved with summing the radioactivity of individual fractions (Fig. 2) compared to the determination of a single point in the batch-wise elution from the mini-Dowex-1 columns.

The slope of the graph in Fig. 3B indicates that the GSSG transported by the red cells was 2400 cpm/ml supernatant/hr or 9600 cpm GSSG/hr/ml RBC. Based on the 3H-GSSG specific activity determined in Fig. 1, this represents a transport rate

<table>
<thead>
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<th>Incubation Time (min)</th>
<th>Total Extracellular GSSG (cpm/ml supernatant)</th>
<th>Percent Hemolysis</th>
<th>Hemolysis Correction (cpm/ml supernatant)</th>
<th>Corrected Extracellular GSSG (cpm/ml supernatant)</th>
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<tbody>
<tr>
<td>5</td>
<td>545</td>
<td>0.06</td>
<td>310</td>
<td>235</td>
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<tr>
<td>30</td>
<td>1,680</td>
<td>0.08</td>
<td>415</td>
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<td>62</td>
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<td>730</td>
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<td>7,120</td>
<td>0.22</td>
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<td>5,680</td>
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</table>

These data are from the mini-columns for experiment 1 (gradient columns for these time points are shown in Fig. 2).

The final data are plotted in Fig. 3B.
Fig. 3. Transport of 3H-glutathione by erythrocytes. For experiment 1, the 3H-GSSG transported by erythrocytes is shown as a function of time. (A) The total radioactivity associated with each GSSG peak in Fig. 2 was determined and corrected for hemolysis. (B) The 3H-GSSG transported, as measured by the mini-column of Dowex-1 (Table 1), is plotted versus incubation time.
Table 2.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Transport Rate (nmole GSSG/hr/ml RBC)</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>5.0</td>
</tr>
<tr>
<td>B</td>
<td>7.2</td>
</tr>
<tr>
<td>C</td>
<td>6.1</td>
</tr>
<tr>
<td>D</td>
<td>6.5</td>
</tr>
<tr>
<td>E</td>
<td>6.9</td>
</tr>
<tr>
<td>F</td>
<td>8.5</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>6.7 ± 1.2</td>
</tr>
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</table>

of 5.0 nmole GSSG/hr/ml RBC. Table 2 gives the observed transport rate for the red cells of 6 normal individuals (analyzed by the mini-column method). The mean transport rate is 6.7 nmole GSSG/hr/ml RBC. Based on a total glutathione pool of 2.2 μmole/ml RBC, this transport rate would predict a half-life for erythrocyte glutathione of 4.7 days, which is in excellent agreement with the observed half-life of 4 days.

**DISCUSSION**

When the hexose monophosphate shunt is not operative, as in G-6-PD-deficient or chromate-treated erythrocytes, erythrocyte GSSG content can be greatly elevated by treatment with oxidizing agents such as hydrogen peroxide, azoester, or t-butylhydroperoxide. Under such circumstances, GSSG rapidly leaves the red cell.4 This egress of GSSG is an active process requiring ATP that can be inhibited by fluoride.7,8 Outward transport of GSSG in various animal species can be demonstrated when the GSSG level is artificially increased; the rate of GSSG transport in various animals closely parallels the rate of in vivo GSH turnover.15

All previous studies of GSSG transport from erythrocytes have been carried out on erythrocytes or on resealed membranes containing very high levels of GSSG. The techniques previously employed were not sufficiently sensitive to detect transport of the very small amounts of GSSG that would, in reality, be sufficient to account for the normal turnover of human red cell GSH. We have now succeeded in making such measurements by labeling intracellular GSH with radioactive glycine and measuring the extruded GSSG after chromatographic purification.

These experiments indicate for the first time that erythrocytes that have not been exposed to an oxidative stress do actively transport glutathione in an outward direction and that this rate is rapid enough to account for the observed degradation of the erythrocytic glutathione. Presumably, this transport represents the basal level of the rapid transport which occurs when GSSG levels are markedly elevated. This latter system has been proposed as a defense mechanism for the erythrocyte against extreme oxidative challenges.14

**REFERENCES**

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5. Palekar AG, Tate SS, Meister A: Formation of 5-oxoproline from glutathione in erythrocytes by the gamma glutamyltranspeptidase-cyclo-transferase pathway. Proc Natl Acad Sci USA 71:293, 1974


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