PERMEABILITY OF NORMAL AND GLUCOSE-6-PHOSPHATE DEHYDROGENASE DEFICIENT ERYTHROCYTES TO GLUTATHIONE*

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Glutathione (GSH) appears to be synthesized in mammalian erythrocytes (1, 2), and isotopic-labeling studies reveal that its turnover is rapid. However, the fate of erythrocyte GSH is unknown. We know of no erythrocyte enzyme that could split the γ-glutamyl linkage of GSH. The formation of mixed disulfides cannot account for the large amount of GSH that must be disposed of by the erythrocyte if a steady state is to be maintained. It is also noteworthy that there are no conditions that, in vivo, result in the accumulation of oxidized glutathione (GSSG) in the erythrocyte. Even under circumstances in which reduction of GSSG must be inadequate, as in glucose-6-phosphate dehydrogenase (G-6-PD) deficiency and in glutathione reductase deficiency, there is no accumulation of the disulfide.

Güntherberg and Rost (3), using N-ethylmaleimide (NEM) to complex GSH and thus prevent its oxidation to GSSG, were able to demonstrate that the level of GSSG in mammalian erythrocytes

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is much lower than previous studies had indicated. Using an improved method for the estimation of GSSG in red cells, we have found that the concentration of GSSG in normal and G-6-PD deficient eryathrocytes is exceedingly low, and that the red cell membrane is permeable to GSSG. This finding elucidates some of the previously poorly understood aspects of red cell GSH metabolism.

Methods and Materials

GSH was estimated by the 5,5′ dithiobis-(2-nitrobenzoic acid) (DTNB) method (4). GSSG levels were determined by the following technique: NEM was added to the sample in a final concentration of 0.01 M, and proteins were precipitated by trichloroacetic acid (TCA). The sample was centrifuged and TCA and NEM were removed from the supernatant by extraction with ether. Aliquots were taken for the estimation of GSSG using glutathione reductase ($NAD(P)H_2$: glutathione oxidoreductase E.C. 1.6.4.2) and the oxidation of NADPH at 340 mμ was followed in a Gilford Spectrophotometer, light path 1 cm. The assay system as constituted finally contained NADPH (Sigma Chemical Company), 0.12 mM, potassium phosphate buffer pH 6.8, 125 mM, and glutathione reductase 0.9 enzyme units (Calbiochem). Excellent recoveries of added GSSG were achieved with this procedure.

Fresh blood from normal and G-6-PD deficient subjects was drawn into heparin and centrifuged at 900 g for 30 minutes at 4^{o}. The buffy coat was removed and the cells were suspended in buffered saline, pH 7.4 (1 part 0.15 M potassium phosphate buffer to 9 parts of 0.15 M NaCl). Following recentrifugation and second removal of the remaining buffy coat, 1.0 ml of cells was suspended in 3.0 ml buffered saline, with or without

0.014 M glucose, in Warburg flasks with hydrogen peroxide (H_2O_2) in the center well (5). The flasks were stoppered and incubated in a Dubnoff Shaker at 37° for 4 hours with 100 oscillations per minute. At the end of incubations, the contents of the flask were centrifuged at 900 g for 30 minutes, and GSH and GSSG estimated in the supernatant medium and erythrocytes.

Results and Discussion

The results (Table 1) indicate that the level of GSSG in the normal and G-6-PD deficient erythrocytes was below the limit of detection (.005 μmoles/ml RBC). It was confirmed in separate experiments that this was true even when unwashed fresh red cells were studied. After treatment with H_2O_2, the level of GSH was unchanged in normal cells incubated with glucose, decreased in normal cells without glucose, and decreased in G-6-PD deficient cells with or without glucose. No appreciable amounts of GSH were observed in any of the media after incubation GSH lost after treatment with H_2O_2 diffusion was recovered, almost in its entirety, as GSSG. Oxidized glutathione was found not only within the erythrocytes, but also in the incubating medium. The distribution of GSH and GSSG in the cells and the medium is presented in Table 1 and 2. Estimates of hemolysis of erythrocytes during the course of incubation showed that less than 0.5% of the erythrocytes were lysed, and that therefore the GSSG in the medium could not have been derived from the GSH of hemolysed cells.

There is good reason to believe that the red cell membrane is impermeable to GSH. The GSH level of erythrocytes is not diminished after as many as 7 washings in 10 volumes of isotonic

Table 1

Permeability of GSSG in Normal and Glucose-6-Phosphate Dehydrogenase Deficient Erythrocytes Treated with Hydrogen Peroxide.

Subject	Glucose	Pre-Incubation GSH	Pre-Incubation GSSG	Post-Incubation GSH RBC	Post-Incubation GSH Med	Post-Incubation GSSG RBC	Post-Incubation GSSG Med	Recovery
Normal								
N1	+	212	<0.5	214	<6	<0.5	<0.5	101
N2	+	237	<0.5	207	<6	<0.5	<0.5	87
N3	+	171	<0.5	175	<6	<0.5	<0.5	102
N4	+	219	<0.5	236	<6	<0.5	<0.5	108
N1	0	212	<0.5	35	<6	72.0	14.5	97
N2	0	237	<0.5	59	<6	64.3	14.5	91
N3	0	171	<0.5	44	<6	51.4	13.0	101
N4	0	219	<0.5	48	<6	65.8	16.1	97
G6PD Def.								
D1	+	162	<0.5	70	<6	23.2	21.2	98
D2	+	180	<0.5	127	<6	9.0	15.4	98
D3	+	171	<0.5	140	<6	2.5	23.2	112
D1	0	158	<0.5	17	<6	50.1	19.3	99
D2	0	180	<0.5	31	<6	48.2	15.4	88
D3	0	171	<0.5	79	<6	32.8	14.5	101

All GSH and GSSG values are given as 1 x 10^{-2} µmoles/ml of red blood cells (RBC) or per 3.0 ml of medium (Med).

Table 2

Average Ratios of GSSG Content in Erythrocytes to Medium in Normal and G-6-PD Deficient Blood Treated with Hydrogen Peroxide

	# of Subjects	Glucose	GSSG RBC	GSSG Med	Ratio RBC/Med
Normal	4	0	63.4	4.8	13.2
G6PD Def.	3	+	11.6	6.6	1.7
	3	0	43.7	5.5	7.9

GSSG values are given as 1 x 10^{-2} µmoles/ml of red blood cells (RBC) or per ml of medium (Med).

saline solution (6). We have observed that the incubation of normal red cells in glucose-containing media failed to result in any diminution of red cell GSH levels or appearance of GSH in the surrounding medium. The permeability of red cells to GSSG has previously received only scant attention. It was suggested on the basis of indirect evidence that such leakage of GSSG might occur in blood after prolonged storage (7), but to our knowledge no direct measurements of the permeability of red cell membrane to GSSG have been reported. Furthermore, most earlier estimations of GSSG in the erythrocyte, including our own (8), have represented almost entirely the disulfide produced by the oxidation of GSH during the protein precipitation.

The technique described in this paper demonstrates that the concentration of GSSG in red cells is extremely low and that GSSG, which is formed in the erythrocyte, can readily pass from the red cell into the surrounding medium. Thus it is now possible to explain the fate of GSSG under circumstances in which GSSG reduction is impaired, and to account for the disposition of GSH that is synthesized by the red cell.

The GSSG gradient between the red cells and the surrounding media is summarized in Table 2. These data raise the question of whether G-6-PD deficient erythrocytes are more permeable to GSSG than normal erythrocytes. It is possible, however, that glucose may affect the loss of GSSG from erythrocytes; the gradient seems much greater in the absence of glucose than in its presence. Because of the limited number of observations, any conclusions regarding the mode of transport of GSSG into the surrounding medium must be deferred.

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