Regeneration of Reduced Glutathione in Erythrocytes: Stoichiometric and Temporal Relationship to Hexose Monophosphate Shunt Activity

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The stoichiometric and temporal relationships between glutathione reduction and hexose monophosphate shunt (HMPS) activity in normal red blood cells were investigated using azoester to oxidize reduced glutathione (GSH) and an ionization chamber-electrometer apparatus to measure continuously the $^{14}$CO$_2$ derived from $^{14}$C-glucose. Under air, azoester produced rapid oxidation of GSH followed by rapid regeneration. The HMPS response was delayed and occurred after the period of maximal GSH regeneration. Due to the generation of hydrogen peroxide by azoester, cumulative shunt activity was far in excess of that theoretically required to regenerate GSH oxidized directly by azoester. Under carbon monoxide, no hydrogen peroxide was generated by azoester, and a stoichiometric relationship existed between GSH regeneration and HMPS activity. Again, however, the response of the HMPS was temporally dissociated from GSH regeneration. These findings demonstrate that under carefully controlled conditions there is a stoichiometric relationship between the regeneration of GSH and CO$_2$ production by the HMPS but that this stoichiometry is not the result of a "direct linkage" of the two reactions.

Activity of the hexose monophosphate shunt (HMPS) pathway in red blood cells is related primarily to maintenance of glutathione in the reduced state (GSH). The latter compound and the enzyme glutathione peroxidase constitute the major source of protection of the red cell against oxidant injury by hydrogen peroxide. Study of the reactions involved in this pathway has been facilitated by the use of methyl phenylazoformate (azoester) which produces rapid but reversible direct oxidation of red cell GSH and by the development of a technique for continuous monitoring of carbon dioxide generated by the HMPS in intact RBC. The ionization chamber-vibrating reed electrometer apparatus is sensitive enough to provide a continuous recording of radioactive carbon dioxide derived from intact RBC incubated with $^{14}$C-labeled glucose. It is thus possible to examine the linkage between glutathione reductase and the HMPS by determining the temporal relationship between GSH regeneration and CO$_2$ production from HMPS. These studies indicate that there is a stoichiometric relationship between GSH regeneration and CO$_2$ production in normal human red blood cells, but that CO$_2$ production lags con-
siderably behind GSH regeneration. This suggests that the first two steps in the HMPS pathway are not directly linked to GSH regeneration but rather that the red cell incurs an "NADPH debt" during the rapid regeneration of GSH and that this debt is then gradually restored by accelerated activity of the HMPS.

MATERIALS AND METHODS

HMPS Activity

Venous blood was collected in heparinized tubes from laboratory personnel with normal values for hematocrit and hemoglobin and no history of anemia. Samples were centrifuged at 1000 g for 10 min. The plasma and buffy coat were removed, and the red cells were washed once in 5 volumes of saline and resuspended in a pH 7.4 buffer containing 145 mM Na+, 5 mM K+, 20 mM glycyglycine, 5 mM glucose, and 145 mM Cl−. Three to four milliliters of RBC were suspended in a final volume of 10 ml in a 50-ml triple-headed distilling flask to which was then added 5 μCi of radioactive glucose labeled in the first or second position. A few experiments were done using glucose labeled in the third position (14C-1 and 14C-2-glucose was obtained from Amersham-Searle Corp., Arlington Heights, Ill. 14C-3-glucose was obtained from New England Nuclear, Boston, Mass.).

The inlet of the flask was connected to a gas cylinder containing either compressed air or carbon monoxide. The outlet arm of the flask was connected to a 275-ml Cary-Tolbert ionization chamber and a Cary Model 401 vibrating reed electrometer (Cary Instruments, Monrovia, Calif.).

The third arm of the flask was covered with a rubber stopper through which reagents could be added or samples withdrawn through a spinal needle. The use of the ionization chamber-electrometer apparatus for continuous monitoring of CO2 produced by cell suspensions, and our modifications of this apparatus, have been described in detail elsewhere.8 A duplicate system was used so that 14CO2 derived from 14C-1-glucose and 14C-2-glucose could be measured simultaneously. The incubation flasks were maintained at 37°C throughout these experiments and were stirred continuously. CO2 production was calculated from the millivolt reading recorded at various times during the course of the experiment.

The response of the HMPS to stimulation with azoester (Calbiochem, Los Angeles, Calif.) was calculated from the area beneath the curves of continuously recorded millivolt readings less the base-line activity. Values for CO2 derived from 14C-1-glucose and 14C-2-glucose were added to obtain total HMPS activity. As the rate of activity of the HMPS increases, an increasing amount of pentose is recycled through the shunt via transketolase. Thus, the carbon atom which was originally in the second position is now in the first. The degree to which recycling occurs can be estimated from the amount of 14CO2 derived from 14C-2-glucose. Glucose labeled on the third carbon atom yields trivial amounts of 14CO2 unless glucose utilization via the HMPS is greatly increased.

GSH Regeneration

Regeneration of red cell GSH after exposure to azoester was carried out in an incubation system identical to that used in the metabolic studies except that radioactive glucose was omitted. Paired flasks were incubated with and without supplemental glucose (5 mM). Samples were withdrawn from each flask for determination of base-line GSH concentration immediately before the addition of 2.6 μM of azoester per ml of RBC. Additional samples were then removed at 2, 5, 15, 30, and 60 min after the addition of azoester. Regeneration of GSH at a given time was calculated from the difference between the concentration of GSH in flasks with and without glucose. GSH values were determined using 5,5'-dithiobis-(2 nitrobenzoic acid) according to the method of Beutler, Duron, and Kelly.10

Hydrogen Peroxide Generation

The generation of hydrogen peroxide during incubation of RBC with azoester was estimated using the catalase inhibition technique of Cohen and Hochstein.11 RBC were incubated as in the metabolic experiments except that radioactive glucose was omitted and aminotriazol (0.05 M) was added to the flasks. In the presence of aminotriazol, hydrogen peroxide and catalase form an
REDUCED GLUTATHIONE IN ERYTHROCYTES

![Graph](image)

**Fig. 1.** Regeneration of red blood cell GSH under air. Azoester (2.6 μmoles per ml RBC) was added at time zero. Results are expressed as μmoles of GSH per ml RBC ± SE.

irreversible complex. Hydrogen peroxide generation can be detected by serial determinations of catalase. Catalase determinations, before and after the addition of azoester, were performed using stroma-free hemolysates equal to a 1:1500 dilution of packed RBC. Duplicate 1.0-ml aliquots of the hemolysate were incubated with sodium perborate substrate for 3 min, and residual perborate was titrated with 1.0 N potassium permanganate according to the method of Tarlov and Kellermeyer.2 This system provides a qualitative estimate of peroxide generation, and results are expressed as per cent fall in catalase activity during a 60-min incubation with azoester.

**RESULTS**

**Regeneration of GSH**

Figure 1 illustrates the regeneration of GSH when azoester was added to RBC under air. In samples without glucose, the fall in GSH was prompt and sustained. The fall was nearly maximal by 5 min and at 15 min was 1.75 μM/ml below the control value. At that time the corresponding value in samples with glucose was 1.3 μM/ml higher, indicating regeneration of at least that much GSH during the first 15 min. Values for GSH then remained relatively constant through the remainder of the experiment.

A similar set of experiments was carried out with RBC preincubated and maintained under 100% carbon monoxide (Fig. 2). In these samples the fall in GSH after addition of azoester was less marked, but regeneration was more complete, and the difference between samples with and without glucose was nearly identical to that observed in the previous experiments under air, i.e., the apparent regeneration of GSH was 1.3 μM/ml of RBC during the first 15 min.

**Response of the HMPS to Azoester**

The response of the HMPS to azoester (2.6 μM/ml RBC) is illustrated in Fig. 3. Maximim activity of the HMPS occurred approximately 20 min after
the addition of azoester, after the regeneration of GSH was nearly complete. Even if one assumes that maximum shunt activity was achieved instantaneously, this would account for only 50% of the observed regeneration of GSH during the first 5 min. On the other hand, total shunt activity over a 2-hr period produced the equivalent of 1.5 μM of CO₂/ml RBC, based on the yield of ¹⁴CO₂ from ¹⁴C-1 and ¹⁴C-2-glucose. A small amount of ¹⁴CO₂ was detected using ¹⁴C-3-glucose, bringing the total to 1.63 μM of CO₂, or the equivalent of about five times that amount of shunt activity required to regenerate 1.3 μM of GSH per ml RBC. Further, shunt activity was still accelerated more than four times its base-line rate 2 hr after stimulation with azoester.

Stimulation of the HMPS with azoester produced quite different results when the incubations were done under carbon monoxide. This is illustrated in Fig. 4. Again, CO₂ production from the shunt lagged behind the observed regeneration of GSH, but in these experiments activity of the HMPS had returned close to
the base-line rate at 60 min. Cumulative shunt activity calculated from the $^{14}\text{C}-1$ and $^{14}\text{C}-2$-glucose was 0.35 $\mu M$/ml RBC. Under these circumstances a small amount of additional $^{14}\text{CO}_2$ was detected from $^{14}\text{C}-3$-glucose, totaling approximately 0.05 $\mu M$/ml RBC. Thus, total HMPS activity response to azoester under CO was 0.40 $\mu M$. This is the equivalent of 1.6 $\mu M$ of GSH per ml RBC, a value remarkably similar to the amount actually measured in the regeneration experiments.

**Generation of Hydrogen Peroxide by Azoester**

The results of peroxide generation experiments offer a possible explanation for the discrepancy in HMPS response to azoester under air and under carbon monoxide. These results are presented in Fig. 5. In the absence of supplemental glucose there was a 70% reduction in catalase activity in RBC incubated 60 min with azoester in the presence of aminotriazole. The presence of glucose had a protective effect and resulted in a greater than 50% reduction in the apparent amount of peroxide generated. In contrast, peroxide production by azoester was virtually nil under carbon monoxide, with or without supplemental glucose.
DISCUSSION

These studies confirm the observation made by several other investigators that azoester is a useful compound for studying the regeneration of GSH in red cells. This compound produces very rapid oxidation of GSH which is reversible in the presence of glucose and a functioning HMPS.

The oxidation of GSH by azoester is a direct reaction not dependent on the presence of oxygen. However, Kosower, Song, and Kosower have shown that in the presence of oxygen a number of free radicals are generated, and, as demonstrated in the present study, this may lead to accumulation of hydrogen peroxide in the red cell. The latter phenomenon is probably responsible for the exaggerated HMPS response which is far in excess of that required to regenerate the GSH oxidized directly by the azoester. On the other hand, when the oxidation-regeneration studies are carried out under carbon monoxide, no peroxide is generated, and a stoichiometric relationship exists between HMPS activity and the measured GSH regeneration.

Use of the ionization chamber electrometer apparatus provides an additional dimension to the study of these reactions. Since the rate of CO₂ production is recorded continuously, changes in the activity of the HMPS which result from changes in experimental conditions or addition of reagents can usually be detected within 3 min. Although the studies under carbon monoxide confirmed the predicted stoichiometric relationship between GSH regeneration and HMPS activity, the temporal relationship between these two events indicates that the series of reactions involved in this pathway are not linked directly. In other words, the glutathione reductase reaction, in which NADPH is oxidized in the reduction of oxidized glutathione (GSSG), occurs rapidly and independent of CO₂ production from the HMPS. The most simple explanation for this phenomenon is that the shunt in intact RBC responds primarily to an increase in the concentration of NADP⁺ which results from the rapid reduction of GSSG. The concentration of GSSG would therefore play a secondary role in controlling HMPS activity. However, the precise mechanism by which the red cell is able to regenerate GSH in advance of HMPS activity is not entirely clear.

The available pool of NADPH, based on measurements by several investigators, may be quite variable but is probably insufficient to account completely for the rapid rate of GSH regeneration we have observed, and other reactions as yet unidentified may be involved. Although they do not relate directly to this study, experiments by Paniker, Srivastava, and Beutler with glutathione reductase-deficient RBC indicate clearly that the glutathione reductase reaction cannot be the limiting step in glutathione regeneration or in HMPS activity. The observation that hemolysates from both normal and glucose-6-phosphate dehydrogenase-deficient RBC can utilize NADH to regenerate GSH is even more provocative evidence that glutathione reduction and HMPS activity are not necessarily concurrent events.

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