

Predominant Role of Catalase in the Disposal of Hydrogen Peroxide Within Human Erythrocytes

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Purified enzymes were mixed to form a cell-free system that simulated the conditions for removal of hydrogen peroxide within human erythrocytes. Human glutathione peroxidase disposed of hydrogen peroxide (H_2O_2) at a rate that was only 17% of the rate at which human catalase simultaneously removed hydrogen peroxide. The relative rates observed were in agreement with the relative rates predicted from the kinetic constants of the two enzymes. These results confirm two earlier studies on intact erythrocytes, which refuted the notion that glutathione peroxidase is the primary enzyme for removal of hydrogen peroxide within erythrocytes.

HYDROGEN PEROXIDE (H_2O_2), and certain other derivatives of oxygen, are being increasingly recognized as toxic intermediates in a wide variety of human disorders. Although many different cells and tissues are affected, erythrocytes have historically served as the cells providing understanding of the ways in which H_2O_2 is detoxified. In a previous article in this journal, we pointed out the importance of catalase in the removal of H_2O_2 within human erythrocytes, and we reached the conclusion that catalase handles approximately half of the H_2O_2 , while the glutathione peroxidase/reductase system accounts for the other half.¹ An unexpected finding was that the increased dependence on the glutathione peroxidase/reductase mechanism did not occur until more than 98% of the catalase had been inactivated.² This suggested that catalase and the glutathione peroxidase function intracellularly in a very different manner from that previously ascribed to them. For the present report, we developed a cell-free system, containing catalase and glutathione peroxidase, as a means of reproducing the intracellular mechanisms of H_2O_2 detoxification. The two enzymes were obtained through exclusion chromatography from human hemolysate and were exposed to low and constant flows of H_2O_2 , mimicking intracellular conditions with respect to concentrations of reduced glutathione (GSH) and nicotinamide adenine dinucleotide phosphate (NADPH). Catalase and glutathione peroxidase were used in the same proportion of activity as were found to be present in hemolysates. Relatively large amounts of glutathione reductase and glucose-6-phosphate dehydrogenase (G6PD) were added. In this cell-free system, the rate of generation of 6-phosphogluconate was a measure of the rate at which H_2O_2 was being removed by mechanisms, such as that of glutathione peroxidase, leading to oxidation of GSH or NADPH.

MATERIALS AND METHODS

Glucose oxidase, glucose-6-phosphate dehydrogenase, 6-phosphogluconic dehydrogenase (6PGD), glutathione reductase and horseradish peroxidase were from Boehringer (Mannheim, Germany). Commercial catalase and glutathione peroxidase, both from human erythrocytes, were from Sigma (St Louis, MO). Blood from nine normal volunteers was collected in tubes containing EDTA as the anticoagulant. Leukocytes and platelets were removed by the method of Beutler et al.³ After the blood was centrifuged, plasma was removed, and the erythrocytes were washed twice by being suspended in 5 vol 0.15 mol/L NaCl, followed by centrifuging. An hemolysate was prepared by the addition of 1 mL of packed erythrocytes to 9 mL of water. After 10 minutes, the preparation

The present findings differ from the results with intact cells, however, in showing that glutathione peroxidase accounts for even less than 50% of the removal of hydrogen peroxide. A means is proposed for calculating the relative contribution of glutathione peroxidase and catalase in other cells and species. The present results raise the possibility that the major function of glutathione peroxidase may be the disposal of organic peroxides rather than the removal of hydrogen peroxide.

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was centrifuged for 15 minutes at 16,000g at 4°C, and the supernatant fluid was collected and concentrated with a CF-25 ultrafiltration cone (Amicon), as described earlier.⁴ The hemolysate was concentrated to a volume of 1 mL, to which were added 4 mL of Krebs-Ringer solution/20 mmol/L Tes (N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid) buffer (pH 7.4) (KRT). The composition of the Krebs-Ringer solution is given elsewhere.⁵ An aliquot (100 μ L) of the hemolysate was set aside for enzyme determination. The remainder was applied to a Sephacryl S-200 column of 1.5 \times 95 cm, that had been equilibrated with KRT buffer. One bed volume of KRT buffer was passed through the column. Fractions of 3.2 mL were collected at a flow rate of 6.4 mL/hour, activities of catalase and glutathione peroxidase were measured, and the hemoglobin concentration was determined (Fig 1). Fractions containing catalase, glutathione peroxidase and hemoglobin, and (in some runs) NADPH diaphorase, were collected, pooled, and concentrated on CF-25 ultrafiltration cones to a final volume of 0.5 mL.⁴ Assays for enzymatic activities and protein concentrations were repeated after ultrafiltration. No cross-contamination was observed between the two or three concentrated fractions. Some 6PGD activity, however, was present in the glutathione peroxidase preparation. This 6PGD was then removed by passing the glutathione peroxidase preparation through a 0.4 \times 2.5 cm column containing 2,5'-adenosine diphosphate (ADP) Sepharose gel that had been suspended in 0.01 mol/L phosphate buffer, pH 6.0. At this point, all preparations were free of 6PGD activity and ready for the in vitro metabolic studies (specific activities: catalase 45 to 54 k/mg, glutathione peroxidase 2.5 to 3.2 U/mg).

Activity of erythrocytic catalase was expressed as, k, the first-

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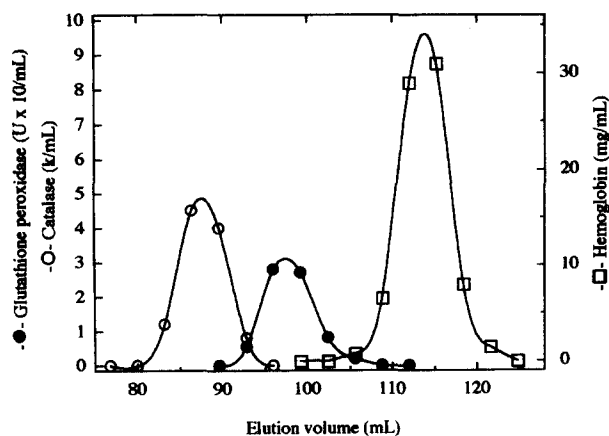


Fig 1. Separation, after exclusion chromatography on a Sephacryl S-200 column, of a normal hemolysate (see Material and Methods).

order kinetic constant (sec^{-1}) for disappearance of H_2O_2 , as determined at a wavelength of 240 nm with a recording spectrophotometer.⁶ When multiplied by the concentration of H_2O_2 , k gives the rate at which H_2O_2 is used at that concentration. Catalase activity per unit volume (k/mL) of a solution was based on the first-order kinetic constant observed when an aliquot of a solution was added to an assay mixture. The kinetic constant was then multiplied by the dilution factor (assay volume/aliquot volume). A unit (U) of activity of glutathione reductase and glutathione peroxidase represents an enzymatic rate of one μmole of NADPH oxidized per minute. Assays of glutathione peroxidase were performed at 37°C by the method of Beutler,⁷ as follows, except that the reaction was performed at pH 7.4 in KRT buffer. The reaction mixture had a final volume of 1 mL and contained, at the following final concentrations: GSH 2 mmol/L, NADPH 200 $\mu\text{mol/L}$, *t*-butyl hydroperoxide 70 $\mu\text{mol/L}$, and yeast glutathione reductase (2 U/mL of reaction mixture). The reaction was followed from the rate of disappearance of NADPH, as determined at 340 nm with a recording spectrophotometer. The presence of a relative excess of glutathione reductase served to keep the GSH at a constant concentration of 2 mmol/L. Because catalase has almost no affinity for *t*-butyl hydroperoxide, this substrate provides a reliable measurement of glutathione peroxidase in the presence of erythrocytic catalase. After collection, partial purification and concentration of the separate fractions (catalase, glutathione peroxidase, and hemoglobin), the three preparations were mixed in a reaction mixture in the same proportions as were present in the crude hemolysates. The final concentrations of catalase purified from human erythrocytes varied between 0.16 k and 0.60 k/mL, in which k represents the first-order rate constant (sec^{-1}) of catalase. Final concentrations for human purified glutathione peroxidase were between 0.016 and 0.06 U/mL. The final concentration of human hemoglobin, when present, was between 10 and 20 $\mu\text{mol/L}$ according to the concentration of the two antioxidant enzymes present in the reaction mixtures. The reaction was then exposed to H_2O_2 , which was generated at a constant rate by glucose and glucose oxidase.² The reaction mixture had a final volume of 1.0 mL and contained: GSH 2 μmoles , NADP⁺ 2 nmoles, glucose-6-phosphate 1.0 μmole , glutathione reductase (yeast) 2 U, glucose-6-phosphate dehydrogenase (yeast) 10 μg , glucose 5.0 μmoles , and glucose oxidase (5 picomoles).

Some experiments were performed with commercially available, highly purified catalase and glutathione peroxidase from human erythrocytes. Before use, the enzymes were thoroughly washed on ultrafiltration cones to remove all preservatives, then resuspended in KRT. Activities of these enzymes were measured as for hemolysates

just before they were added to incubation mixtures. Incubations were in Pyrex tubes at 37°C for 20 to 30 minutes in a metabolic shaker. The reaction was stopped at the end of the incubation by heating of the tubes at 100°C for 2 minutes. Supernatant fluid was removed after the tubes were centrifuged at 2000g for 2 minutes, then kept on ice before determination of the amount of 6-phosphogluconate (6PG) that had been generated during the incubation. Concentrations of 6PG were measured with a spectrophotometer at 340 nm in a 0.5 mL reaction mixture containing 0.1 mol/L Tris-HCl, pH 8.0, 0.05 mol/L MgCl_2 , 1 mmol/L NADP⁺, 5 μg yeast 6PGD, and 50 μL of the incubation mixture. The net increase in NADPH corresponded to the amount of 6PG generated during the exposure of catalase and glutathione peroxidase to the H_2O_2 flow. All incubations and determinations were in duplicate.

RESULTS

At a GSH concentration of 2 mmol/L, the Michaelis constant (K_m) for H_2O_2 of purified, human erythrocytic glutathione peroxidase was found to be 28 $\mu\text{mol/L}$ and the enzyme used H_2O_2 at a rate that was 2.5 times the rate at which it used *t*-butyl hydroperoxide. The erythrocytes of nine normal Caucasian subjects contained 28.7 ± 6.4 (mean \pm standard deviation [SD]) U of glutathione peroxidase per gram of hemoglobin, and the catalase activity was 312 ± 44 k per gram of hemoglobin. Whether human glutathione peroxidase and catalase were purchased or partially purified, cost and practical limitations prohibited incubations of the two enzymes at intracellular concentrations, especially given the need for a relative excess of reagent glutathione reductase. For the *in vitro* metabolic studies, these two enzymes were kept at concentrations proportional to the erythrocytic values, but at levels well below the erythrocytic concentrations. At the intracellular ratio of catalase to glutathione peroxidase and at a constant rate of generation of H_2O_2 , the rate of generation of 6PG remained almost the same over a range of concentrations of the two enzymes (Table 1).

The rate of formation of 6PG was 1.33 ± 0.33 nmoles $\text{mL}^{-1} \text{min}^{-1}$ at a concentration of 0.6 k/mL of catalase and 0.06 U/mL of glutathione peroxidase in the presence of an excess of G6PD and glutathione reductase and at a constant flow of H_2O_2 of 12 nmoles $\text{mL}^{-1} \text{min}^{-1}$ (Fig 2). In the presence of glutathione peroxidase only, the generation of 6PG was 9.73 ± 1.47 nmoles $\text{mL}^{-1} \text{min}^{-1}$, in agreement with the rate expected from the fact that one mole of 6PG is formed for each mole of H_2O_2 reduced. Exposure of catalase to the same flow of H_2O_2 , in the absence of glutathione peroxidase, led to the generation of 6PG at a rate of 0.21 ± 0.03 nmoles $\text{mL}^{-1} \text{min}^{-1}$ (Fig 2). In the presence of both enzymes in the

Table 1. Rate of 6PG Generation During Exposure of Catalase and Glutathione Peroxidase, at Different Concentrations, to H_2O_2 at the Same Rate of H_2O_2 Flow ($5.3 \text{ nmol mL}^{-1} \text{min}^{-1}$)

Catalase (k/mL)	Glutathione Peroxidase (U/mL)	6PG (nmol $\text{mL}^{-1} \text{min}^{-1}$)
0.15	0.015	1.62
0.30	0.030	1.34
0.60	0.060	1.36
1.00	0.100	1.33

A system of regeneration of GSH and NADPH was present (see Materials and Methods).

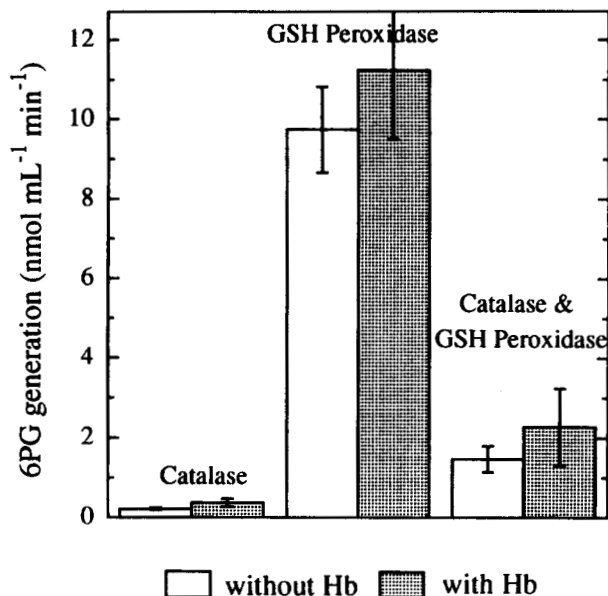


Fig 2. Generation of 6PG over 30-minute during exposure of catalase, glutathione peroxidase, and both enzymes, to a H₂O₂ flow of 12.0 nmol mL⁻¹ min⁻¹ in the presence of constant concentration of GSH (2 mmol/L) and NADPH (2 μmol/L). Values represent the mean ± SD of seven experiments.

erythrocytic proportion, the glutathione peroxidase, therefore, handled only 15% of the generated H₂O₂ (Fig 2). Exposure of the same reaction mixture to different rates of H₂O₂ generation elicited proportional amount of 6PG for all three enzyme combinations (Fig 3). The higher values of 6PG observed at zero flow of H₂O₂ may be related to autooxidation of reduced glutathione that is dependent on temperature

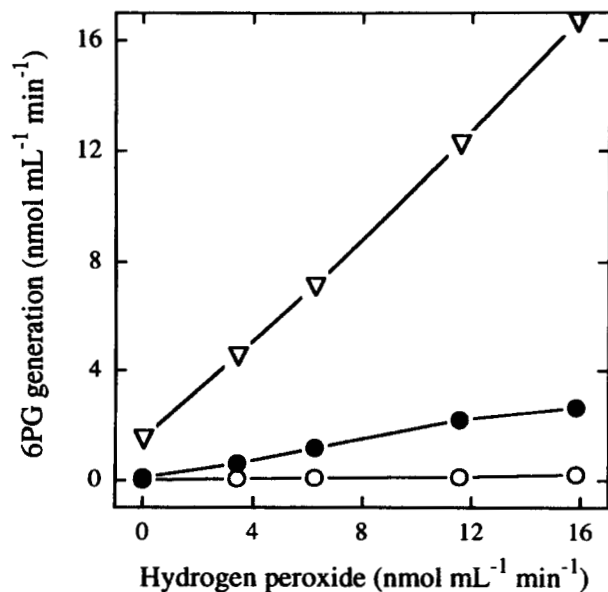


Fig 3. Formation of 6PG at different flows of H₂O₂ under the conditions specified in Fig 1. (▽) glutathione peroxidase; (○) catalase; (●) catalase plus glutathione peroxidase.

and pH.⁸ During the incubation, which never exceeded 30 minutes, the rate of generation of 6PG remained constant. Under the conditions specified above, the rate of formation of 6PG was not greatly altered by the addition to the catalase/ glutathione peroxidase system of spin-trapping agents or such other enzymes as NADPH diaphorase, superoxide dismutase (Fig 2 and Table 2) or glutathione-S-transferase (not shown). A small, but consistent, stimulation of the rate of 6PG production was seen when purified human hemoglobin was present (Table 2). Several incubation mixtures, containing decreasing amount of catalase in the presence of a constant activity of glutathione peroxidase, were prepared and were exposed to the same flow of H₂O₂ for 30 minutes. At the catalase/glutathione peroxidase ratio observed in normal erythrocytes, the formation of 6PG was about one-fifth to one-tenth of that expected if the glutathione peroxidase were fully active (far right of Fig 4).

Expected rates of 6PG formation were assumed to be the rate at which glutathione peroxidase was functioning in a hypothetical reaction mixture containing glutathione peroxidase, catalase, and GSH (2 mmol/L) at known concentrations and H₂O₂ at a known and constant rate of generation. The maximal velocity of the glutathione peroxidase was taken as the activity with H₂O₂ (100 μmol/L) as the substrate. The Km of the glutathione peroxidase was considered to be 28 μmol/L. An assumption that the reaction had reached a steady state (constant H₂O₂ concentration) allowed the concentration of H₂O₂ to be calculated. This value allowed calculation, in turn, of the rate at which the glutathione peroxidase was functioning. The observed rate of 6PG formation was similar to the expected rate, and all rates followed a relatively smooth curve upward at progressively lower concentrations of catalase (Fig 4). With the addition of purified hemoglobin, the curve was shifted upward slightly, but remained concave. Curves of similar shape were observed when commercially prepared enzymes were used (Fig 5).

DISCUSSION

Until mid-century, catalase was regarded as the enzyme responsible for disposing of H₂O₂. In 1957, Mills⁹ discovered glutathione peroxidase and demonstrated that it used GSH

Table 2. Effect of Different Agents on the Rate of Production of 6PG During Exposure of Catalase and Glutathione Peroxidase to H₂O₂ Flows (7.2 nmol mL⁻¹ min⁻¹) in the Presence of a System for Regeneration of NADPH and GSH

Addition (final concentration)	6PG Generation (% of baseline values)
None	[100]
NADPH diaphorase (5 mU/mL)	125
Superoxide dismutase (5 μg/mL)	105
Hemoglobin (10 nmoles/mL)	116
Hemoglobin plus superoxide dismutase	106
Methemoglobin (10 nmoles/mL)	114
N-tert-butyl-α-phenylnitron (1 mmol/L)	100
Hemoglobin plus N-tert-butyl-α-phenylnitron (1 mmol/L)	126

Values are expressed as a percentage of the rate of 6PG generation without an added agent.

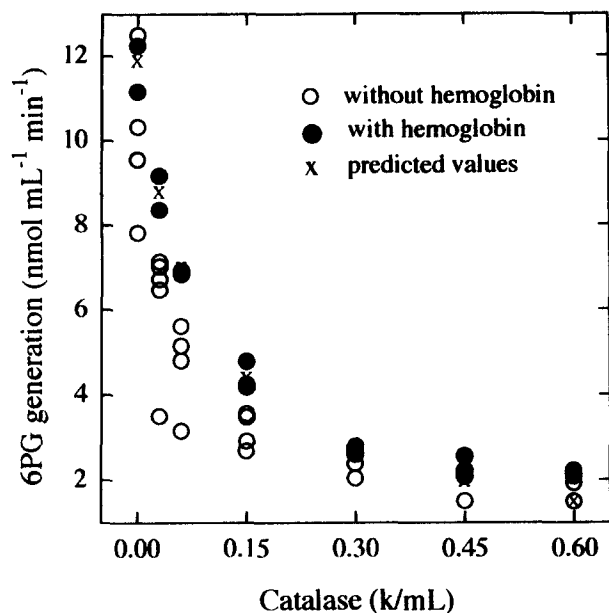


Fig 4. Rates of formation of 6PG in the presence of increasing amounts of catalase and at fixed concentrations of glutathione peroxidase (0.06 U/mL), NADPH (2 μ mol/L), and GSH (2 mmol/L). The rate of generation of H_2O_2 was constant (12.0 nmol mL⁻¹ min⁻¹). The concentration of hemoglobin was 10 μ mol/L.

to reduce H_2O_2 to water. The resulting oxidized glutathione was known to be brought back to the reduced state by glutathione reductase, using NADPH. A prevailing view that emerged was that glutathione peroxidase is the main player in H_2O_2 detoxification. This view derived from studies on erythrocytes with a reduced capability to generate adequate amounts of NADPH, of which G6PD deficiency is the paradigmatic example.¹⁰ Catalase was regarded as a fossil enzyme.¹¹ That catalase, itself, requires NADPH was suggested, however, by the early studies of Eaton et al¹² and was proved by the more recent discovery that mammalian catalase has tightly bound NADPH⁴ and uses NADPH to remain active in the constant presence of H_2O_2 .¹³ In two previous studies reported in this journal, we used intact human erythrocytes to estimate the relative contributions of catalase and glutathione peroxidase in the removal of H_2O_2 . Normal cells were compared with those with a genetic deficiency of catalase¹ or with varying degrees of aminotriazol-induced inactivation of catalase.² We concluded that catalase accounts for the disposal of about half of the H_2O_2 that might be generated at physiologically and pathologically realistic rates. Glutathione peroxidase was presumed to account for the other half. Anomalies in the kinetics of H_2O_2 removal in the second study, however, made it difficult to attribute half of the H_2O_2 disposal entirely to glutathione peroxidase. Moreover, glutathione peroxidase was assumed in those two studies to be the only mechanism by which the removal of H_2O_2 was accompanied by the oxidation of NADPH or GSH.

The present cell-free system consisted of a mixture of the enzymes involved in the disposal of H_2O_2 . This system allowed the concentration of individual components to be changed so as to provide better insight into the H_2O_2 -removing role of each

component than could be gained with studies of intact cells. Both the observed and predicted contributions by glutathione peroxidase were much less than those of catalase. Calculations giving the expected rate of glutathione peroxidase gave also an estimate of the steady-state concentration of H_2O_2 . Under the conditions of each of the tables and figures in this report, the concentrations of H_2O_2 were well below 28 μ mol/L, the K_m for H_2O_2 of glutathione peroxidase at a GSH concentration of 2 mmol/L. At such relatively low concentrations of H_2O_2 , the activities of catalase and glutathione peroxidase would each be proportional to the H_2O_2 concentration, and glutathione peroxidase would remove H_2O_2 at a rate that is V_{max}/kK_m times the rate at which H_2O_2 would be removed by catalase. V_{max} and K_m are the constants of glutathione peroxidase at a specified concentration of GSH, and k is the first-order rate constant of catalase. For any given ratio of glutathione peroxidase to catalase activity, therefore, the fraction of total H_2O_2 that is removed by glutathione peroxidase should remain the same over a range of concentrations of the two enzymes or of rates of generation of H_2O_2 . That expectation is confirmed by the observations of Table 1 and Fig 3. This approach provides an estimate of the maximum contribution of glutathione peroxidase, relative to that of catalase, even though the concentration of H_2O_2 may be unknown. Activity of catalase is simply proportional to the H_2O_2 concentration, whereas activity of glutathione peroxidase follows Michaelis-Menten relationships and begins to level off as the concentration of H_2O_2 gets above the K_m of glutathione peroxidase. We consider the generation rates and concentrations of H_2O_2 in this and our earlier studies^{1,2} to be

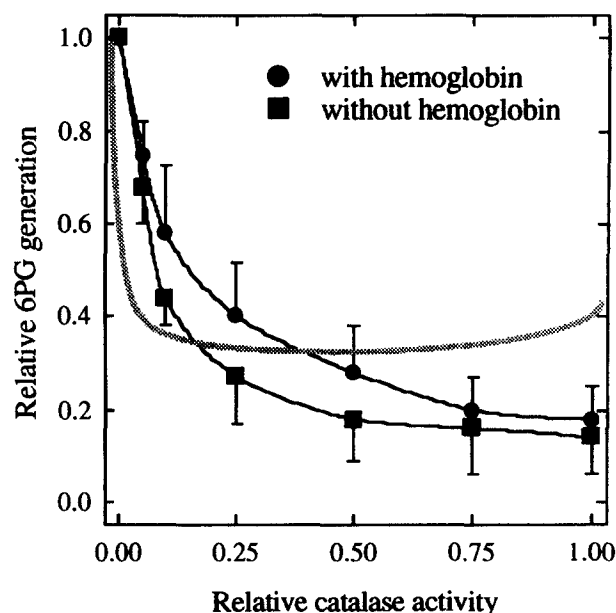


Fig 5. Relative rates of production of 6PG during incubation of human glutathione peroxidase and catalase (from commercial enzymes) to H_2O_2 generated at a constant rate (12 nmol mL⁻¹ min⁻¹) by the glucose plus glucose oxidase system in the presence of NADPH (2 μ mol/L), G6P (1 mmol/L), GSH (2 mmol/L), yeast G6PD (10 μ g/mL), and yeast glutathione reductase (2 U/mL). Dotted line represents the relative hexosemonophosphate shunt activity of previous experiments in intact erythrocytes.² Bars represent the SD of five experiments.

toxicologically realistic. Those who believe that the rates and concentration are even higher must contend with the fact that the activity of glutathione peroxidase, relative to that of catalase, would then be even lower.

Estimates of the expected rate of glutathione peroxidase at low concentrations of H_2O_2 would be sensitive to errors in the determination of the K_m . The possibility of such an error deserves consideration because the K_m was determined in the micromolar range of H_2O_2 , whereas the concentration of H_2O_2 in these studies was largely in the nanomolar range. The agreement between expected and observed results (Fig 4), however, refutes that possibility. Earlier attention to the K_m of glutathione peroxidase and to the activities of the two enzymes in erythrocytes would have allowed anticipation of the growing number of studies^{1,2,14-18} discounting the notion that glutathione peroxidase is the major means by which H_2O_2 is removed in erythrocytes. Aebi and Suter¹⁹ mentioned the possible use of this approach in 1974. Because the assignment of a maximum rate for glutathione peroxidase, relative to that of catalase, does not depend on knowledge of the H_2O_2 concentration, this approach could be applicable to other tissues and species. The ping-pong kinetics of glutathione peroxidase would require, however, that the concentration of GSH be known before the K_m of glutathione peroxidase for H_2O_2 could be calculated.²⁰ Pending the outcome of those studies, we suggest that the function of glutathione peroxidase may be the disposal of organic peroxides rather than the removal of H_2O_2 .

Our earlier studies indicated that catalase accounts for about half of the H_2O_2 removed in human erythrocytes.^{1,2} Glutathione peroxidase was presumed to account for the other half. The present study indicates, however, that glutathione peroxidase could account for only a small portion of the remaining half, as measured by the rate of oxidation of NADPH and GSH. This relationship is reflected in Fig 5, where the rate of 6PG generation can be seen to decrease less with intact cells than with cell-free solutions, as progressively higher catalase activities are considered. A question requiring addressing is the nature of this unknown portion of H_2O_2 -removing ability. The well-recognized pseudo-peroxidase activity of hemoglobin²¹ may account for at least some of the missing portion. At the relatively low concentrations of components used in this study, some increase in rate of 6PG production is consistently seen when hemoglobin is present (Table 2, Figs 2, 4, and 5). Catalase oxidizes NADPH in the process of protecting itself from being inactivated by its own toxic substrate (H_2O_2).¹³ It is unlikely, however, that this accounts for much of the missing portion, as catalase oxidizes NADPH at less than 7% of the rate at which it converts H_2O_2 to oxygen and water.¹³

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