The catalase within normal, intact human erythrocytes was completely inactivated with amino triazole. The rate of CO₂ evolution, when the cells were subsequently incubated with ¹³C-labeled glucose, provided a measure of the rate at which H₂O₂ was being oxidized by the glutathione peroxidase/reductase system for the disposal of H₂O₂. This rate was determined in control cells and in catalase-inactivated cells while the cells were exposed to H₂O₂, which was generated at various constant and predetermined rates by glucose oxidase. The results indicated that catalase handles approximately half of the generated H₂O₂. The glutathione peroxidase/reductase mechanism accounted for the other half. These results are in agreement with our earlier findings on erythrocytes of a subject with a genetic deficiency of catalase. However, an unexpected result with the present approach was the finding that the increased dependence on the glutathione peroxidase/reductase mechanism did not occur until greater than 98% of the catalase had been inactivated. The latter observation indicates that catalase and the glutathione peroxidase/reductase system function intracellularly in a manner very different from that previously ascribed to them. An explanation of the findings requires that the two methods of H₂O₂ disposal function in a coordinated way, such as a sequential action in which the glutathione peroxidase/reductase system is the rate-limiting step.

**MATERIALS AND METHODS**

Glucose oxidase and horseradish peroxidase were from Boehringer Mannheim (Mannheim, Germany). Blood from seven normal volunteers was collected in tubes containing sodium heparin as an anticoagulant. The rate of the hexose monophosphate shunt (HMS) in erythrocytes was determined by methods similar to those used earlier. Leukocytes and platelets were removed by the method of Beutler et al. Plasma was removed after centrifuging, and the erythrocytes were washed twice by suspension in 5 vol of 0.15 mol/L NaCl, followed by centrifuging. The cells were mixed with Krebs-Ringer solution/20 mmol/L Tes (N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid) buffer (pH 7.4)/5 mmol/L glucose (KRTG). The composition of Krebs-Ringer solution is given elsewhere. The packed cell volume was 35% to 40%. The incubation mixtures consisted of 1.0 mL of this erythrocyte suspension, 1.0 mL of KRTG solution (with or without glucose oxidase), and 0.3 mL (0.5 μCi) of [1-¹³C] glucose, [2-¹³C] glucose (specific activity, 3 mCi/mmol), or [1-¹³C] glucose (specific activity, 4 mCi/mmol). Incubations were performed at 37°C in a metabolic shaker at 90 cycles per minute in 25-mL vials to which were attached rubber caps containing disposable center wells. All incubations were performed in duplicate. Throughout the incubation period (60 minutes), the center wells contained 0.2 mL 1N NaOH. At the end of the incubations, a variety of studies provides evidence that one mechanism or the other is functioning to remove H₂O₂ in erythrocytes. In a previous study, reported here, we compared normal erythrocytes with those of a single subject with a genetic deficiency of catalase. The results suggested that catalase disposes of half the H₂O₂ generated in erythrocytes. In the present study, we compared normal erythrocytes with erythrocytes that had been artificially depleted of catalase activity. Because genetic deficiencies of catalase are rare, the latter approach made it possible to determine the extent to which the previous observations were true with numerous samples, and with varying degrees of catalase inactivation.

**ALTHOUGH ESSENTIAL** for most forms of life on this planet, oxygen is the source of certain toxic substances, such as hydrogen peroxide and the superoxide radical. Investigations over the past few decades have begun to show the extent to which organisms have had to evolve methods for disposing of these toxic derivatives, as well as the extent to which injury to cells occurs in those diseases and toxic reactions in which these defenses are breached. For the first half of this century, catalase (Fig 1A) was regarded as the means for disposal of H₂O₂. Subsequent studies of mammalian erythrocytes, and especially human erythrocytes, have played a historical role in providing understanding as to how H₂O₂ is disposed of in vivo. Such studies led to the discovery of the glutathione peroxidase/reductase mechanism (Fig 1B) for disposal of H₂O₂, as well as to the notion that this mechanism, rather than catalase, was responsible for removal of H₂O₂. That notion arose from the unusual susceptibility to peroxidative damage of human erythrocytes that are genetically deficient in glucose-6-phosphate dehydrogenase (G6PD) (Fig 1C) and, therefore, limited in ability to generate nicotinamide adenine dinucleotide phosphate (NADPH), which is required by the glutathione peroxidase reductase mechanism (Fig 1B). However, Kirkman and Gaetani demonstrated that mammalian catalase has bound NADPH, which becomes oxidized to NADP⁺, and displaced by unbound NADPH in the course of both preventing and reversing the inactivation of catalase by its own substrate, H₂O₂. Thus, both means of disposal of H₂O₂ have been shown to be dependent on the availability of NADPH.

A variety of studies provides evidence that one mechanism or the other is functioning to remove H₂O₂ in erythrocytes. In a previous study, reported here, we compared normal erythrocytes with those of a single subject with a genetic deficiency of catalase. The results suggested that catalase disposes of half the H₂O₂ generated in erythrocytes. In the present study, we compared normal erythrocytes with erythrocytes that had been artificially depleted of catalase activity. Because genetic deficiencies of catalase are rare, the latter approach made it possible to determine the extent to which the previous observations were true with numerous samples, and with varying degrees of catalase inactivation.
erythrocytes. Variable amounts of glucose oxidase were added to
KRTG solution to a final enzyme concentration of 4 to 9
mmol/L. Under these conditions, the formation of methemoglobin was less than 2.3%. Control erythrocytes were
exposed to the same concentration of glucose oxidase but with omission of the AT. In some experiments, glutathione-depleted cells were prepared by resuspending aliquots of the preincubated, packed erythrocytes in KRTG solution and exposing the cells for 15 minutes to either N-ethylmaleimide (NEM; 3 μmol/mL of packed erythrocytes) or 1-chloro-2,4-dinitrobenzene (CDNB; 3 μmol/mL).

Studies of purified catalase were with catalase purified from hu-
man erythrocytes by the method reported elsewhere, and with
the enzyme incubated in KRTG solution. Determinations of catalase-bound NADPH concentrations were by a method of alkaline extraction and enzymatic cycling after the catalase solution had been incubated for 60 minutes at 37°C in the presence of glucose oxidase at a final concentration of 2.2 mmol/L, with or without 20 mmol/L AT. Comparable amounts of free NADPH were also exposed under these conditions, and the concentration of NADPH was determined. Catalase activity of erythrocytes was measured on aliquots obtained at intervals throughout the experiments and assayed as hemolysates. The activity was expressed as the first-order kinetic constant of the rate of disappearance of H₂O₂, at an initial concentration of 10 mmol/L, as measured by absorbance at 240 nm. The extent of AT-inactivation of catalase, as determined by this assay, was confirmed in certain samples by measurements of activity of the catalase at much lower concentrations of H₂O₂, as follows. For estimates of activity at 10 to 100 nmol/L H₂O₂, aliquots of the hemolysate-H₂O₂ mixture were removed at intervals and added to a cuvette containing homovanillic acid and horseradish peroxidase, and fluorescence was measured. Measurements at 10 to 50 μmol/L H₂O₂ were performed in the same manner, except 4-aminoantipyrine replaced homovanillic acid, and absorbance was measured. Enough horseradish peroxi-
dase was added in both methods to give almost instantaneous con-
sumption of the remaining H₂O₂.

RESULTS

Table 1 lists the HMS rates of erythrocytes incubated without glucose oxidase. The erythrocytes were of three types: controls, cells in which the catalase had been completely inactivated, and glutathione-depleted cells. Under these resting conditions, the catalase-inactivated cells had an HMS rate that was 2.5 times that of the control cells. The difference was highly significant (P < .0002). Three experiments with glutathione-depleted cells showed that such cells have a decreased HMS rate in the resting condition (Table 1). Table 2 lists the HMS rates of the same cells when exposed to H₂O₂ flows resulting from glucose oxidase at

Table 1. HMS Rates in Resting Erythrocytes

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Controls</th>
<th>Catalase-Inactivated</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>51</td>
<td>92</td>
</tr>
<tr>
<td>2</td>
<td>45</td>
<td>168</td>
</tr>
<tr>
<td>3</td>
<td>42</td>
<td>127</td>
</tr>
<tr>
<td>4</td>
<td>59</td>
<td>116</td>
</tr>
<tr>
<td>5</td>
<td>41</td>
<td>105</td>
</tr>
<tr>
<td>6</td>
<td>47</td>
<td>111</td>
</tr>
<tr>
<td>7</td>
<td>51</td>
<td>135</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>47.9 ± 6.0</td>
<td>122.0 ± 24.7</td>
</tr>
</tbody>
</table>

Erythrocytes without exposure to H₂O₂ from glucose oxidase at the time of estimation of the HMS rate. The rates are expressed per millimeter of erythrocytes. Rates for glutathione-depleted cells were 21.5 in experiment 1, 13.1 in 2, and 17.8 in 4.
Table 2. HMS Rates of Erythrocytes in the Presence of Various Amounts of Glucose Oxidase

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Glucose Oxidase (nmol/L)</th>
<th>HMS Rates (nmol &quot;CO2/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls</td>
<td>Catalase-Inactivated</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>311</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>396</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>438</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>454</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>697</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>814</td>
</tr>
<tr>
<td>7</td>
<td>13</td>
<td>1,152</td>
</tr>
</tbody>
</table>

Rates are expressed per millimeter of erythrocytes. Rates for glutathione-depleted cells were 53.1 in experiment 1, 45.0 in 2, and 38.9 in 4.

different concentrations. Catalase-inactivated cells had an HMS rate 60% to 100% higher than that of control cells (P < .0014). These results were obtained only from cells with fully inactivated catalase. Inactivation of the catalase was measured at concentrations of H2O2 ranging from nanomolar to millimolar levels (see Materials and Methods), and in no case could residual catalase activity be detected. In the three experiments with complete depletion of glutathione, the HMS rate of the glutathione-depleted cells was much less than that of controls, both without (Table 1) and with (Table 2) exposure to H2O2 generated by glucose oxidase. No differences were observed between the HMS activities of glutathione-depleted erythrocytes obtained by use of either thiol-inhibitors (NEM or CDNB).

The data shown in Fig 2 represent the observed HMS rate, minus the resting HMS of the corresponding cells, plotted against the HMS rate that would be expected if the glutathione peroxidase/reductase mechanism disposed of all H2O2. The expected rate was obtained by dividing the rate of generation of H2O2 by 2 (corresponding to the two molecules of NADPH generated by each molecule of G6P passing through the HMS). The slope for the control cells (± SEE) was 0.62 ± 0.04, whereas the slope for the catalase-inhibited cells was 1.05 ± 0.03. Figure 3 illustrates this relationship in an experiment in which cells from one subject were simultaneously studied at different rates of H2O2 generation. Figure 4 shows the percentage inactivation of catalase of normal erythrocytes, that have been exposed to various flows of H2O2 over a period of 30 minutes in the presence of 20 mmol/L AT. Inactivation reached a maximum at H2O2 flows of 200 to 400 nmol/h/mL, and was faster in glutathione-depleted cells at lower H2O2 flows than in cells without glutathione depletion, whereas the inactivation of the latter followed a sigmoid curve (Fig 4). Figure 5 depicts the HMS rate in erythrocytes having normal glutathione levels and different degrees of catalase inactivation. When the cells were subsequently washed, then exposed to a H2O2 flow of 250 to 750 nmol/h, little, if any, change in the HMS rate occurred until inactivation of the catalase was nearly complete. The HMS rate then increased sharply (Fig 5). A similar plot was obtained with erythrocytes that were not exposed to exogenous H2O2 after the initial, partial inactivation of catalase (Fig 5, inset). Studies of catalase-bound NADPH of purified human catalase, with and without AT, during exposure to glucose oxidase-generated H2O2 flows, demonstrated that inactivation of the enzyme is accompanied by resistance to NADPH oxidation (Fig 6).
Fig 4. Catalase inhibition resulting from exposure to different flows of H₂O₂ in the presence of AT (20 mmol/L). The erythrocytes had been exposed to the different flows at 37°C for 15 minutes. Normal erythrocytes (○); erythrocytes depleted of glutathione by previous exposure to CDNB for 30 minutes at 37°C (●).

DISCUSSION

The lack of an understanding of the role of NADPH in mammalian catalase function led to the notion that H₂O₂ was handled largely or entirely by the glutathione peroxidase/reductase mechanism. The glutathione peroxidase/reductase mechanism was known to require NADPH20,21, and people who had erythrocytes with a genetic deficiency of G6PD22,23 and, therefore, the ability to generate NADPH, were susceptible to peroxidative damage to their erythrocytes. The catalase of higher animals came to be regarded as a fossil enzyme.24 However, Tarlov and Kellermeyer25 observed reduced activity of catalase in G6PD-deficient erythrocytes exposed to peroxidative stress in vivo. Eaton et al26 and Sullivan et al27 found a correlation between the catalase activity and the rise and fall of NADPH concentrations. Eaton et al26 observed recovery of catalase activity when NADPH was added, and hypothesized, as was later proved with purified catalase by others,28,29 that NADPH reversed the conversion of catalase to an inactive form (compound II) by H₂O₂. Further evidence for the role of catalase in mammalian cells came from the work of Scott et al30 with resealed erythrocytes and glutathione-depleted cells.24

Jacob et al31 and Aebi and Suter32 observed that the HMS activity was greater in the erythrocytes of people with a genetic deficiency of catalase than in the erythrocytes of normal people. As with the studies of Gaetani et al33, the rate of ¹⁴CO₂ evolution from ¹⁴C-labeled glucose was considered, in the present study, to be a measure of the HMS rate and, therefore, the rate at which H₂O₂ was being removed by the glutathione peroxidase/reductase mechanism (Fig 1). This assumption is made valid by several features of metabolism in human erythrocytes and of H₂O₂ disposal. The HMS is the principal source of CO₂ and NADPH in human erythrocytes.34 The CO₂ comes from the first carbon and, to a lesser extent, the second carbon of glucose.35 One molecule of NADPH is oxidized in the process of removing each molecule of H₂O₂ via the glutathione peroxidase/reductase mechanism (Fig 1B). Although mammalian catalase uses NADPH to prevent and reverse the inactivation of catalase by H₂O₂, only one molecule of NADPH is oxidized for every four to 14 molecules of H₂O₂ removed by catalase.4 Glutathione remains largely in the reduced form,36 and concentrations of 6-phosphogluconate remain negligible4 when human erythrocytes with normal G6PD activity are exposed to H₂O₂ flows, and NADPH oxidation in the range used in this study. Some earlier investigators exposed erythrocytes to H₂O₂ by the direct addition of H₂O₂ or by diffusion of gaseous H₂O₂. However, both of those methods cause the concentration of H₂O₂ at the surface, or at the site of H₂O₂ addition, to be much higher than elsewhere in the suspension of erythrocytes. The generation of H₂O₂ by glucose oxidase, as in the 1950 experiments of Chance,37 is uniform throughout the suspension and relatively constant. We used glucose oxidase in the present study at concentrations resulting in 6- to 20-fold stimulation of the HMS in cells with normal activities of G6PD and catalase. Based on H₂O₂ flows that are minimally adequate to produce decreases in glutathione concentrations in G6PD-deficient erythrocytes,5 this range seemed to be

Fig 5. Relative rates of the HMS of normal erythrocytes from three subjects having different residual activities of catalase and exposed to H₂O₂ generated at rates from 250 to 750 nmol/h. The relative rate is the ratio of the HMS activity of the erythrocytes with different degrees of catalase inactivation to the HMS activity of the same erythrocytes without catalase inactivation but exposed to the same exogenous H₂O₂ flow. (Inset) Relative rate of HMS activity versus percent catalase inactivation of erythrocytes from two normal subjects*, but without exposure to exogenous H₂O₂ after inactivation of catalase.
ACTIVE ROLE OF CATALASE IN HUMAN ERYTHROCYTES

realistic. Intracellular catalase can be completely inactivated by the AT-H$_2$O$_2$ mechanism if the erythrocytes are incubated without glucose, but the exclusion of glucose results in the depletion of glutathione, the formation of methemoglobin, and the development of abnormal osmotic fragilities. Such derangements of the erythrocyte were avoided in the present study by the use of a method of AT-H$_2$O$_2$ inactivation in which glucose was present during the incubation.

As judged by a doubling of the HMS rate when catalase was completely inactivated, catalase accounted for approximately half of the disposal of H$_2$O$_2$, even when the HMS rate was varied from the resting rate to 20 times the resting rate by the action of various amounts of added glucose oxidase (Tables 1 and 2). Similar observations were made with the erythrocytes of a man with a genetic deficiency of catalase. The similarity of results between the two approaches decreases the possibility that observations with AT-treated cells were the result of some effect of AT, other than inactivation of catalase. On the other hand, the use of AT-treated normal cells allowed observations to be made on cells with varying degrees of catalase inactivation.

At the nanomolar concentrations$^{10}$ of H$_2$O$_2$ present under our incubation conditions, the H$_2$O$_2$ concentration is well below the Michaelis constant for glutathione peroxidase. In addition, catalase has first-order kinetics over a wide range of H$_2$O$_2$ concentrations. Both enzymes, therefore, should have had activities proportional to the intracellular concentration of H$_2$O$_2$, and worked intracellularly to have given a diagonal straight line in Fig 5. What is unexpected is that doubling of the HMS rate did not occur until the catalase was greater than 98% inactivated (Fig 5). This represents a belated correlation with the 1971 observations of Aebi$^{28}$ who found that fewer symptoms were present in those catalase-deficient people who had at least a small percentage of the normal amount of catalase activity in their erythrocytes and fibroblasts. A simple excess of catalase activity (over glutathione peroxidase reductase activity) would have resulted in a plot similar to that of Fig 5, but would not have given the 50:50 stoichiometry indicated in Table 2 and Figs 2 and 3 or the properties observed with glutathione-depleted cells.

The commonly used assay for catalase activity$^{14}$ is performed at millimolar concentrations of H$_2$O$_2$, whereas the intracellular concentration was in the nanomolar range. Therefore, we found it necessary to determine the amount of catalase inactivation at much lower H$_2$O$_2$ concentrations, in order to exclude the possibility that such revised estimates of catalase inactivation would cause the plot of Fig 5 to be a diagonal line. Determinations of activity of the catalase at nanomolar and micromolar concentrations of H$_2$O$_2$ (see Materials and Methods) confirmed that the percentage inactivation of the catalase was the same at each range of H$_2$O$_2$ concentration. Because the same concavity is observed with endogenously generated H$_2$O$_2$ (Fig 5, inset), the concavity cannot be attributed to the fact that the H$_2$O$_2$ was often generated extracellularly. Of the mechanisms that can be hypothesized to account for Fig 5, all seem to require that the intracellular properties of catalase and mechanisms of H$_2$O$_2$ disposal are very different from those that have been recognized from studies of the diluted or purified enzymes. For example, a mechanism in which H$_2$O$_2$ is handled sequentially by catalase and glutathione peroxidase, with catalase being present in excess, would account for the observations in the tables and figures, including the 50:50 stoichiometry and the observations on glutathione-depleted cells. The possibility that intracellular function can differ greatly from extracellular function is made conceivable by the finding and explanation of such a severe discrepancy in activity of erythrocytic G6PD$^{39}$ and by the recent recognition of the phenomenon of macromolecular crowding.$^{30}$ Evolving techniques in the field of physical biochemistry may allow an evaluation of why the intracellular mechanisms of action of catalase and glutathione peroxidase differ greatly from presently recognized mechanisms. If so, the development will be yet another example of how concepts with broad biological implications have been gained through studies of human erythrocytes.

REFERENCES

Importance of catalase in the disposal of hydrogen peroxide within human erythrocytes

GF Gaetani, HN Kirkman, R Mangerini and AM Ferraris