Glutathione Peroxidase: The Primary Agent for the Elimination of Hydrogen Peroxide in Erythrocytes*

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Experiments were designed to evaluate the competition between erythrocyte catalase and glutathione peroxidase for their common substrate, H_2O_2. When extracellular H_2O_2 concentrations were maintained at an upper limit of 10^{-6} M, the oxidation of glutathione accounted for the major fraction of H_2O_2 added to the cells. At higher concentrations, the usual decomposition of H_2O_2 by catalase became increasingly prominent. When normal (catalase-rich) erythrocytes were exposed to low-level, steady-state H_2O_2 concentrations for up to 24 hours, progressive oxidation of hemoglobin was followed by progressive osmotic fragility increases leading to eventual lysis in isotonic medium. These changes were preceded by the disappearance of intracellular reduced glutathione. The loss in reduced glutathione could be prevented by the addition of glucose to provide a substrate for reduction of the oxidized form of glutathione. Glutathione peroxidase activity sustained by the generation of reduced nicotinamide adenine dinucleotide phosphate (required by glutathione reductase) protected cells against methemoglobin formation and osmotic fragility changes. Catalase-deficient erythrocytes, viz., duck cells and azide-treated human cells, were similarly protected against the toxic effects of low H_2O_2 concentrations by sustained glutathione peroxidase activity. It is concluded that under physiologic conditions glutathione peroxidase linked to hexose shunt activity represents the major pathway of H_2O_2 metabolism in intact erythrocytes.

Hydrogen peroxide may be generated in living cells through the action of certain enzyme systems, e.g., xanthine oxidase, or from the autoxidation of hydrogen-donor molecules, e.g., dihydric phenols, or as a consequence of exposure to ionizing radiation. Catalase has long been considered to be the primary scavenger of intracellular H_2O_2. In this paper we present evidence that in erythrocytes the enzyme glutathione peroxidase predominates in this important role, rather than catalase.

GSH[1] peroxidase was first reported by Mills (1957) and was subsequently shown to be present in a wide variety of tissues (Mills, 1960). We have confirmed its presence in normal rat and mouse tissues and in certain tumor cells (Hochstein and Cohen, 1962). The enzyme has been isolated from bovine erythrocytes and shown to be distinct from both catalase and hemoglobin (Mills, 1959).

In intact cells, the activity of GSH peroxidase is sustained by the continuous reduction of oxidized glutathione by glutathione reductase (Fig. 1). Glutathione reductase is $NADPH_2$-dependent, and in erythrocytes the main source of $NADPH_2$ is from hexose shunt activity, that is, from the dehydrogenation of glucose 6-phosphate and 6-phosphogluconate. An alternate source in other tissues is from isocitric dehydrogenase activity and malic dehydrogenase activity, but not in erythrocytes where an intact Kreb's cycle is missing.

GSH peroxidase was first detected in erythrocyte lysates as a consequence of its ability to protect hemoglobin from oxidative breakdown (Mills, 1957). In a subsequent study with intact erythrocytes Mills and Randall (1958) employed ascorbic acid as a stressor agent; oxidation of hemoglobin was attributed to H_2O_2 generated from a reaction between ascorbic acid and oxyhemoglobin. In cells incubated with sodium azide to inhibit catalase, marked formation of methemoglobin and choleglobin was noted. It was demonstrated that the addition of glucose to cells was protective, and evidence was presented that a portion of the protective mechanism involved the functioning of GSH peroxidase. However, in cells with intact uninhibited catalase only minimal oxidation of hemoglobin occurred, and so the presence of GSH peroxidase was obscured by the strong protective action of catalase. Thus these studies established the presence of GSH peroxidase and its potential for protecting hemoglobin against oxidative breakdown; but since GSH peroxidase activity was observed in intact cells only after preliminary inhibition of catalase, it appeared that catalase was still the dominant enzyme concerned with the detoxication of H_2O_2.

In this current manuscript we present the results of studies in which the effects of H_2O_2 on intact erythrocytes were studied directly; commercial H_2O_2 was employed and its rate of addition to cells was maintained at low levels by means of a gaseous diffusion technique. With this technique we have been able to demonstrate the primary role played by GSH peroxidase in protecting both catalase-rich and catalase-deficient erythrocytes against oxidative damage by H_2O_2. In our experiments, catalase was incapable of protecting cells against oxidation of hemoglobin or increases in osmotic fragility. In addition, we have been able to confirm by quantitative measure that oxidation of GSH is the major pathway of H_2O_2 metabolism in intact erythrocytes.

Experimental Details

Penicillin G was obtained from Bristol Laboratories. Glucose-6-P and NADP were obtained from California Corporation for Biochemical Research. All other chemicals were reagent grade.

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[1] The following abbreviations have been employed: GSSG and GSH, for the oxidized and reduced forms of glutathione, respectively; NADP and $NADPH_2$ for the oxidized and reduced forms of nicotinamide adenine dinucleotide phosphate (formerly triphosphopyridine nucleotide), respectively; glucose 6-P for glucose-6-phosphate; and EDTA for ethylene diamine tetraacetic acid.

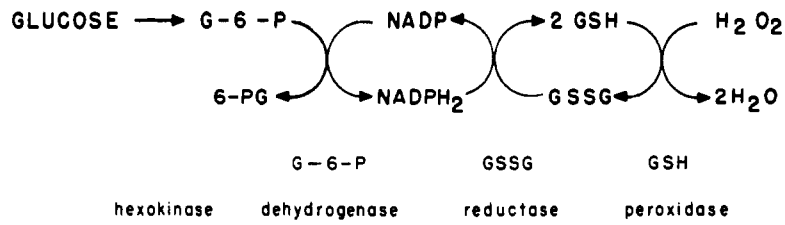

FIG. 1.—The detoxication of hydrogen peroxide via the glucose-linked glutathione peroxidase pathway. The abbreviations G-6-P and 6-PG have been employed for glucose 6-phosphate and 6-phosphogluconate, respectively.

Isotonic saline buffered at *p*H 7.4 according to the method of Dacie (1954) was used throughout this investigation as the incubation medium for erythrocytes.

Heparinized human whole blood, or blood obtained from the common Long Island duck, was utilized immediately or was stored at 5° for up to 24 hours. The cells were washed three times in 4–6 volumes isotonic saline, the buffy coat was removed, and the cells were resuspended in buffer at the desired hematocrit. The absence of significant catalase activity in duck cells and in azide-treated human cells was confirmed by visual observation of failure to form oxygen bubbles with concomitant oxidation of hemoglobin upon the addition of several drops of 3% H_2O_2 to test samples. In addition, inhibition of catalase by azide at the concentrations employed in these experiments was confirmed by quantitative assay; no residual catalase activity could be detected, i.e., inhibition was greater than 99.95%. Catalase was measured by titration of residual H_2O_2 with $KMnO_4$ after incubation of lysate with H_2O_2 at 0° and at *p*H 7.0.

Reduced glutathione in erythrocytes was measured with the technique of Grunert and Phillips (1951) as modified by Beutler (1957), and with further minor volume adjustments. Color development was performed at room temperature. Readings were taken within 15 seconds after color development; on longer standing, the color tended to fade.

Methemoglobin was determined by the technique of Evelyn and Malloy (1938).

Osmotic fragilities were determined on measured

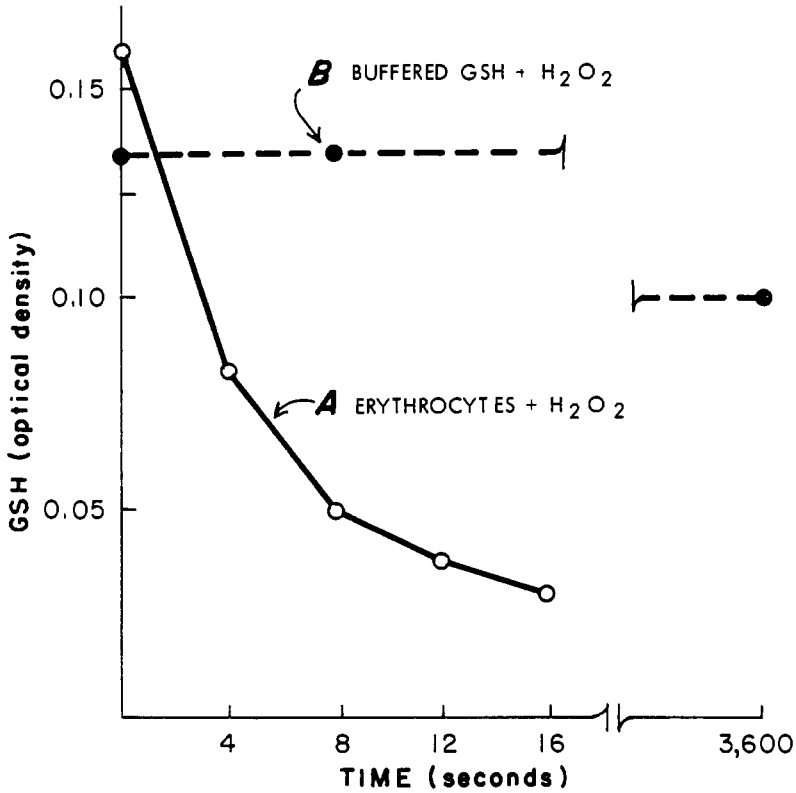

FIG. 2.—Catalysis of the reaction between GSH and H_2O_2 by erythrocyte glutathione peroxidase. The reaction was run at room temperature. Sodium azide (400 μg/ml) was added to inhibit erythrocyte catalase. EDTA (25 μg/ml) was added to inhibit the autoxidation of GSH in buffer. The reaction of curve A was initiated by the addition of 1.6 ml of a 17% (v/v) suspension of washed erythrocytes in buffer, to test tubes containing 0.4 ml H_2O_2 (0.8 μequiv) in buffer. The reaction was stopped at the times indicated by the rapid addition of a suspension of 2 ml isobutanol in 6 ml 2.5% (w/v) HPO_4. The isobutanol served both to cause rapid hemolysis of the cells and to extract unreacted H_2O_2. Samples were centrifuged and the isobutanol layer was removed by aspiration prior to analyses for GSH. Reaction rates: curve A (intracellular GSH) = 1.9×10^{-2} OD units/second, for the first 4 seconds; curve B (GSH in buffer) = 1.25×10^{-5} OD units/second. The rate for curve B has been corrected for a small autoxidation rate of GSH in buffer, viz., 0.31×10^{-5} OD units/second. No loss in GSH was observed in control samples for which the H_2O_2 and the isobutanol-HPO_4 reagent were premixed and then added simultaneously to erythrocyte samples.

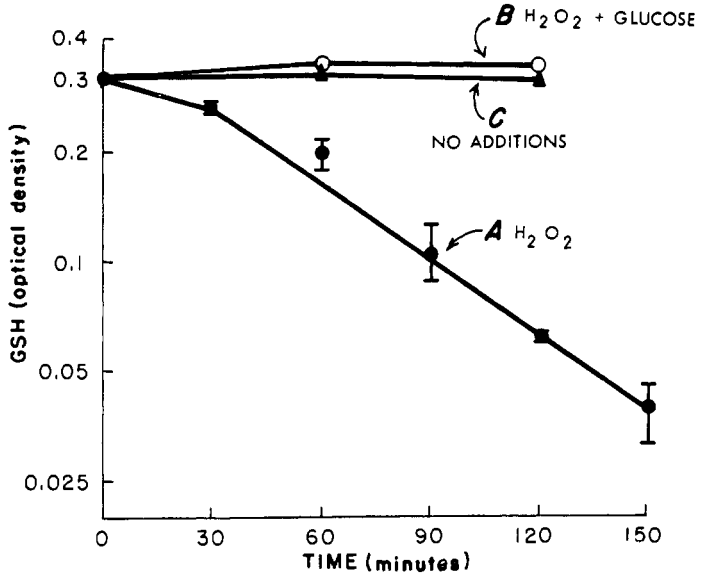

FIG. 3.—Erythrocyte glutathione peroxidase activity in the presence of uninhibited catalase. Washed erythrocytes were suspended in buffer at a hematocrit of 25%. Three-ml aliquots were incubated at 37° with 0.3 ml 30% H_2O_2 in the center well of the Warburg flasks. For curve B, glucose was added to a concentration of 200 mg/100 ml. For curve C (control), the samples were incubated with water in the center well rather than the 30% H_2O_2. The points for curve A represent the average from duplicate vessels; the bars indicate the range. Initial intracellular GSH = 62.5 mg/100 ml. Total GSH per vessel = 1.52 μequiv. First-order reaction rate constant, $k = \frac{2.3}{t} \times \log \frac{\text{GSH @ 30 min}}{\text{GSH @ 150 min}}$

$= \frac{2.3}{120 \text{ min}} \times \log \frac{0.278}{0.041} = 0.0159 \text{ min}^{-1}$.

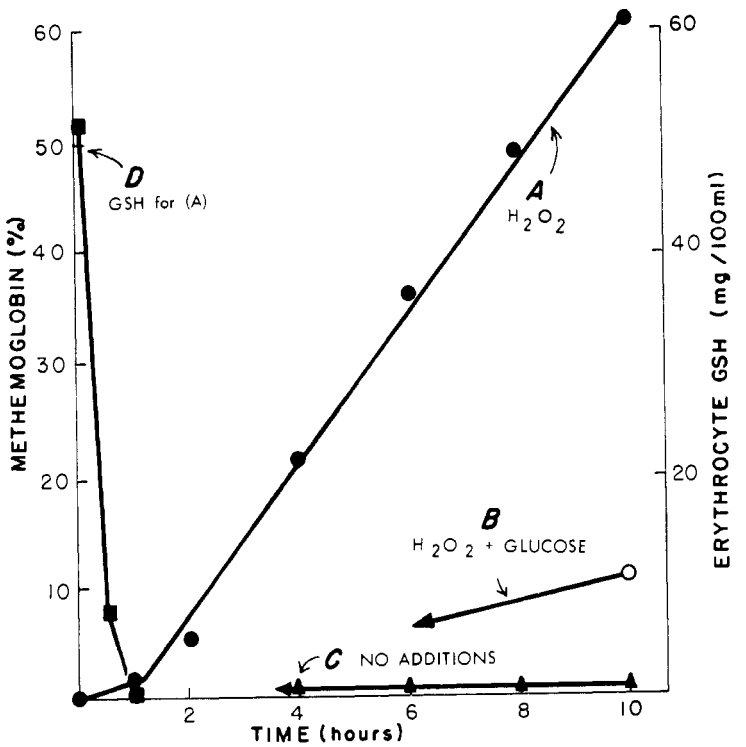

FIG. 4.—Methemoglobin formation induced by hydrogen peroxide. Washed human erythrocytes were suspended in buffer at a hematocrit of 5%. Four-ml aliquots were incubated at 37° in Warburg flasks with either 0.3 ml 30% H_2O_2 (curves A and B) or 0.3 ml distilled water in the center well (curve C). For curve B glucose was added to the erythrocytes to a concentration of 200 mg/100 ml. GSH data have been plotted for curve A.

aliquots of cell suspensions with the method described by Dacie (1954). In order to avoid difficulties arising from the presence of both oxyhemoglobin and methemoglobin, the samples were converted to cyanomethemoglobin and analyzed at 540 mμ. The cyanomethemoglobin reagent was incorporated directly into the buffered saline of various tonicities by 10-fold dilution of a stock solution containing 500 mg $K_3Fe(CN)_6$, 125 mg KCN, and 1 g $NaHCO_3$ per liter distilled water.

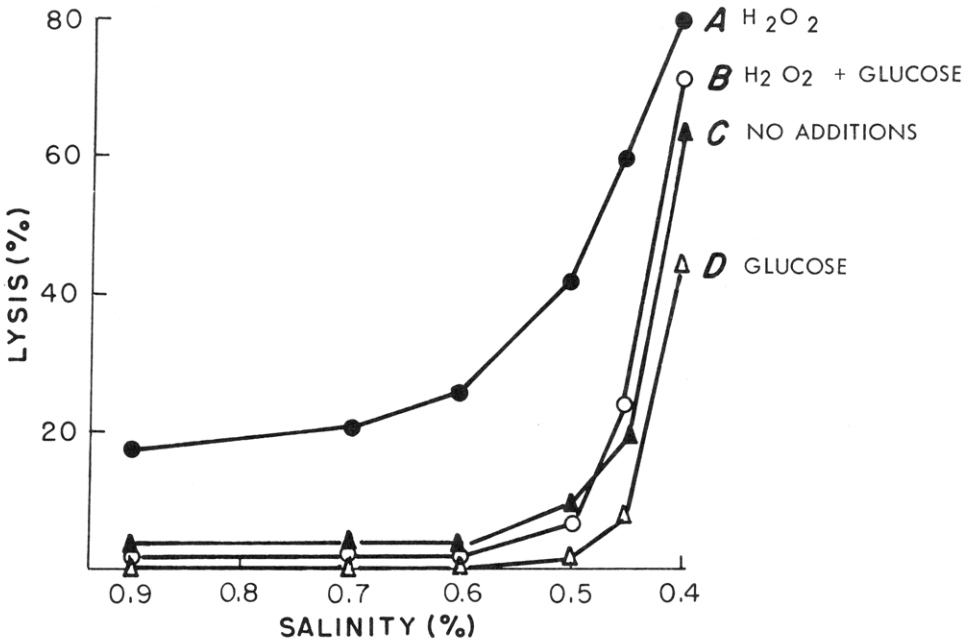

FIG. 5.—Erythrocyte fragility changes induced by hydrogen peroxide. Washed human erythrocytes were suspended at a hematocrit of 10% in buffer containing 25 mg Penicillin G per 100 ml. Glucose was added where indicated to a concentration of 200 mg/100 ml. Samples were incubated at 37° in Warburg flasks containing either 0.3 ml 30% H_2O_2 (curves A and B) or 0.3 ml distilled water (curves C and D) in the center well. Osmotic fragilities were determined after 21 hours.

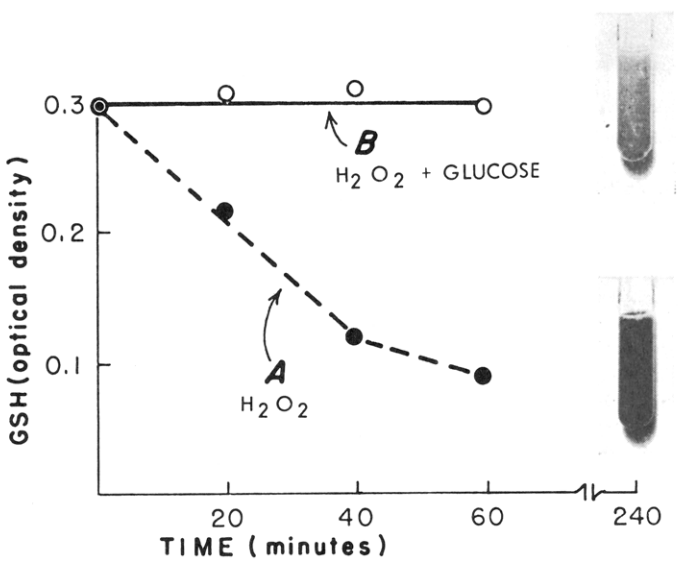

FIG. 6.—Prevention of GSH loss and methemoglobin formation in erythrocytes with inhibited catalase. Washed human erythrocytes were suspended at a hematocrit of 22% in buffer. Sodium azide was added to a final concentration of 7.5 mg/100 ml in order to inhibit catalase. Aliquots (3.5 ml) of erythrocyte suspension were incubated at 37°, with 0.25 ml of 30% H_2O_2 in the center well of the Warburg vessels. In curve B, glucose was added to the erythrocyte suspension to a final concentration of 200 mg/100 ml. In the photographic inset, erythrocyte samples at 4 hours were transferred to test tubes and photographed with 3,000 speed (ASA rating) Polaroid film. A Corning No. 2418 filter was employed in order best to observe the methemoglobin absorption band. No GSH loss or methemoglobin formation was observed in control vessels incubated in the absence of H_2O_2. The inhibition of catalase by azide was confirmed by visual observation of methemoglobin formation and failure to form oxygen bubbles upon the addition of several drops 3% H_2O_2 to an aliquot of erythrocyte suspension.

H_2O_2 Diffusion.—Studies were carried out as described previously (Cohen and Hochstein, 1961) in standard Warburg manometer flasks at 37°. The H_2O_2 passed by gaseous diffusion from the center well to the erythrocytes in the main chamber. Control samples were incubated with water instead of H_2O_2 in the center well.

Estimates of diffusion rates were obtained by collecting the H_2O_2 in 0.25 N H_2SO_4 and titrating with standardized 0.01 N $KMnO_4$. Titrations performed after 0.5–3.0 hours of diffusion indicated that 40–60% of the H_2O_2 transferred to the main compartment was localized in the vapor droplets on the side walls. In studies with catalase-deficient cells it was essential to avoid contact with these vapor droplets, since there was sufficient H_2O_2 present to cause complete oxidation

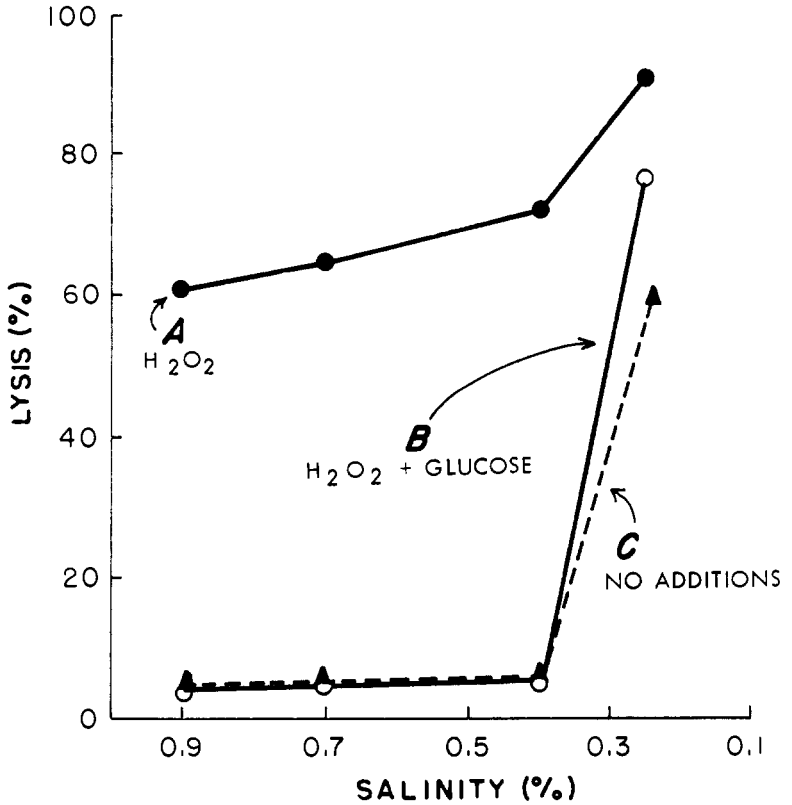

FIG. 7.—Protection of catalase-deficient duck erythrocytes against H_2O_2-induced osmotic fragility changes. Washed duck erythrocytes were suspended at a hematocrit of 10% in buffer containing 16 mg Penicillin G/100 ml. Seven-ml aliquots of erythrocyte suspension, in open beakers, were exposed to H_2O_2 vapor with the modified diffusion technique described in the Experimental section; 5% H_2O_2 was employed. For curve B, glucose was added to the erythrocytes to a concentration of 300 mg/100 ml. For curve C (control), the sample was exposed to water vapor rather than H_2O_2 vapor. The absence of significant endogenous catalase activity in duck cells was confirmed by visual observation of methemoglobin formation and failure to form oxygen bubbles upon the addition of several drops of 3% H_2O_2 to an aliquot of erythrocyte suspension.

of GSH and visible oxidation of hemoglobin; samples were therefore removed rapidly by medicine dropper after preliminary gentle swirling to obtain homogeneity. In studies with catalase-rich cells, this precaution was not necessary since the relatively concentrated H_2O_2 in the vapor droplets was decomposed upon contact with erythrocyte catalase. Estimates of the amount of H_2O_2 reaching the erythrocytes during incubation were 6.0 μequivalents/hour (Fig. 3) or 4.5 μequivalents/hour (Figs. 4–6), depending upon the geometry of the two separate sets of vessels employed.[2]

In osmotic fragility studies with catalase-deficient cells (see Fig. 7) Warburg vessels could not be employed because the transfer of concentrated H_2O_2, washed down by streaming of fluid from the side walls, could not be controlled during the long incubation period. Therefore the following modification was used: A 20-ml beaker containing 7 ml H_2O_2 was placed in the center of a cubic plastic container; similar beakers containing erythrocyte suspensions were placed in the corners. In order to avoid cross-contamination by contact, rubber padding was employed to hold the beakers rigidly in place, equidistant from the H_2O_2 source, and separated both from the side walls of the container and from one another. A piece of absorbent tissue, held in place across the opening of the container by the plastic cover employed to seal the system, absorbed vapor droplets which ordinarily would have collected there and dropped into the open beakers below.

Glutathione Peroxidase Efficiency Studies.—Washed erythrocytes were suspended in 4–6 volumes of buffer and permitted to stand at room temperature or at 37° for 1 hour in order to deplete the cells of trace amounts of glucose or other metabolizable substrates. The cells were then isolated by centrifugation and resuspended in buffer at the hematocrits listed in Tables I and II. Three-ml aliquots were placed in 25-ml Erlenmeyer flasks containing Teflon-encased magnetic stirrers.

A stock dilution of 0.3% H_2O_2 in distilled water was prepared. Further dilutions of 50–500-fold were prepared as required in buffer. The exact concentration of H_2O_2 in the stock dilution was determined by titration with 0.01 N $KMnO_4$. The H_2O_2 was added to the erythrocyte samples dropwise from a siliconized pipet with a finely ground tapered tip. The rate of flow was controlled at 6–10 drops per minute by an air leak from a needle valve; the pipet delivered approximately 40 drops per ml. Magnetic stirring of the erythrocyte sample was commenced while the H_2O_2 drop was forming at the tip of the pipet, and was continued for several seconds after delivery. The erythrocyte specimen was then replaced with the next sample, and so on. This process was repeated for each time period, i.e., every 30 seconds for the addition of 40 drops in 20 minutes. Three minutes were permitted

[2] In an earlier report (Cohen and Hochstein, 1961) we were unaware of the relatively large amounts of H_2O_2 contained in the vapor droplets and, since a rinsing technique was employed, our estimates of the rate of addition of H_2O_2 to the erythrocytes was too high by a factor of two.

TABLE I

EFFECT OF THE MANNER OF ADDITION OF H_2O_2 ON GSH PEROXIDASE EFFICIENCY[a]

Experiment	H_2O_2 Dilution	H_2O_2 Addition (drops/min)	GSH Peroxidase Efficiency: Untreated	GSH Peroxidase Efficiency: Plus Azide
(1) Decreasing drop rate	1/10,800	40/1	12%	73%
	same	40/20	31%	
(2) Decreasing concentration	1/5,000	5/2½	29%	67%
	1/20,000	20/10	47%	
(3) Decreasing amount	1/35,000	44/40	41%	57%
	same	22/20	50%	
(4) Duck cells	1/10,000	40/3	78%	
(5) Duck cells	1/6,000	40 @ 0′	67%	

[a] Human erythrocytes were suspended at a hematocrit of 33%; duck erythrocytes were suspended at a hematocrit of 25%. The initial intracellular GSH levels were in the range 57–71 mg/100 ml for the human cells, and 106–134 mg/100 ml for the duck cells. Sodium azide (300 μg), in isotonic buffer (0.1 ml) was added where indicated to 3.0-ml aliquots of erythrocyte suspension. The exact amount of H_2O_2 added in each experiment was determined by titration with $KMnO_4$. The efficiency of GSH peroxidase is defined as:
(μequiv GSH oxidized/μequiv H_2O_2 added) × 100%

to elapse after the last drop of H_2O_2 had been added before specimens were prepared for GSH analyses. Control specimens were handled in the same manner, with the exception that buffer without H_2O_2 was added. For all GSH peroxidase efficiency studies duplicate specimens were carried through the addition procedures.

RESULTS AND DISCUSSION

A. Demonstration of GSH Peroxidase Activity in Intact Cells.—Catalysis of the reaction between endogenous GSH and H_2O_2 by a factor present in intact erythrocytes is demonstrated in Figure 2. The intracellular reaction proceeded very rapidly and was essentially complete in 15 seconds; in contrast, virtually no reaction was observed in the same time period in the absence of cells. The catalysis of the reaction was attributed to the enzyme GSH peroxidase reported and isolated by Mills (1957; 1959). In the example shown in Figure 2, the initial rate of the enzyme-catalyzed reaction was 1400 times faster than that of the uncatalyzed reaction.

In order best to observe the peroxidase activity illustrated in Figure 2, sodium azide was added to the cells to inhibit catalase. In the absence of azide, very little loss in GSH was observed due to the rapid decomposition of the H_2O_2 by catalase. However, GSH peroxidase activity can be observed in the absence of catalase inhibitors if the rate of H_2O_2 addition is carefully controlled. For example, in Figure 3 a slow rate of addition of H_2O_2 was maintained during the course of the observed reaction by means of a gaseous diffusion technique in closed Warburg flasks. The reaction exhibited first-order kinetics with regard to GSH, i.e., at a presumed steady-state H_2O_2 concentration the rate of oxidation of GSH was proportional to the GSH concentration. The decline in intracellular GSH (curve A) was completely prevented by the addition of glucose to the suspending medium (curve B). The absence of a GSH decline in the presence of glucose could have been due either to a glucose-dependent peroxidase activity or to the regeneration of GSH from GSSG via the reaction scheme depicted in Figure 1. That the latter explanation is the correct one is substantiated by the observation that glucose-6-P dehydrogenase–deficient erythrocytes subjected to diffusing H_2O_2 in the presence of added glucose exhibited marked GSH loss (Cohen and Hochstein, 1961); the peroxide sensitivity of these enzyme-deficient cells is therefore linked specifically to their inability to generate the $NADPH_2$ required by GSSG reductase. In curve A the lag period observed during the first half hour is attributed to some reduction of GSSG due to the presence of trace amounts of metabolizable substrates, and also to the time required for saturation of the atmosphere with H_2O_2 vapor.

B. Evaluation of the Competition Between Catalase and GSH Peroxidase.—It has been shown by Keilin and Hartree (1945; 1955), by Chance (1950), and others that catalase can function as a peroxidase in coupled oxidations with hydrogen-donor molecules. The catalatic activity of catalase, that is, the decomposition of H_2O_2 into oxygen and water, appears to be a special case of peroxidatic activity in which the donor molecule is a second molecule of H_2O_2. Catalatic activity is favored at high H_2O_2 concentrations, while peroxidatic activity is favored at low H_2O_2 concentrations. It has been suggested (Chance, 1951) that catalase may serve a physiologic role as a regulator of the concentration of intracellular H_2O_2, acting peroxidatically at very low H_2O_2 concentrations (10^{-9} M) and acting catalatically with increasing effectiveness at higher H_2O_2 concentrations. However, the endogenous hydrogen acceptor required to sustain peroxidatic activity has not been established. On the other hand, the relatively high intracellular GSH level in erythrocytes (2.5×10^{-3} M) provides a suitable hydrogen donor and suggests that the presumed role of catalase might be usurped by GSH peroxidase.

TABLE II

GSH PEROXIDASE EFFICIENCY AT SLOW H_2O_2 ADDITION RATES[a]

Expt	Hematocrit	H_2O_2 Added (drops/min)	H_2O_2 Added (A) (μequiv)	GSH Oxidized (B) (μequiv)	GSH Oxidized (% initial amount)	Efficiency [(B)/(A) × 100%]
1	33%	40/20	0.53	0.39	16%	74%
2	29%	40/20	0.68	0.41	14%	61%
3	33%	40/20	0.70	0.43	22%	61%
4	17%	60/20	1.01	0.55	33%	54%
5	33%	80/40	1.38	0.72	27%	52%

[a] The initial intracellular GSH levels in these experiments were in the range 60–90 mg/100 ml. Dilutions of stock 30% H_2O_2 were made in isotonic buffer as follows: For experiment 1, 1 part H_2O_2 in 40,000 parts buffer; for experiments 2–5, 1 part H_2O_2 in 30,000 parts buffer. The GSH losses in these experiments amounted to 0.060–0.075 OD unit (GSH-nitroprusside complex). The average deviations from the mean for each set of duplicate specimens taken through the entire procedure was roughly ± 0.0025 OD unit (i.e., duplicates were reliable to within 0.005 OD unit).

TABLE III
REVERSAL OF ERYTHROCYTE GSH LOSS[a]

Experiment	Additions	GSH (Optical Density) No Further Treatment	After Glucose	After Glucose-6-P plus NADP
(1) Human cells	Buffer	0.280	0.280	0.280
	H_2O_2	0.240	0.280	0.280
(2) Human cells	Buffer	0.345	0.340	0.350
	H_2O_2	0.120	0.345	0.345
(3) Duck cells	Buffer	0.260	0.260	
	H_2O_2	0.150	0.255	

[a] The experiments illustrate reversal of both small GSH losses (expt 1) and large GSH losses (expt 2) for normal human erythrocytes, and also for acatalasic duck erythrocytes (expt 3). After the GSH had been oxidized by the addition of buffered H_2O_2, solid glucose was added to the intact cells to a final concentration of 200 mg/100 ml, or the cells were lysed in 2 parts water, and glucose-6-P (6 μmoles) plus NADP (0.4 μmole) were added to 4.5 ml of lysate (Jocelyn, 1960). Samples were then incubated at 37° for 30 minutes, after which time they were reanalyzed for GSH.

We therefore attempted to evaluate the competition between catalase and GSH peroxidase of intact cells for their common substrate, H_2O_2. Our initial approach was via diffusion studies of the type illustrated in Figure 3. The rate of oxidation of intracellular GSH was compared with the rate of addition of H_2O_2, and the results were expressed as an efficiency of the GSH peroxidase reaction. For instance, in Figure 3, the first-order reaction rate constant was $k = 0.0159$ min^{-1} (see footnote to figure), the total GSH per vessel was 1.52 μmoles, and the rate of addition of H_2O_2 to the erythrocytes was 6.0 μequivalents/hour. Therefore, efficiency at 0 time $= [(0.0159\ min^{-1} \times 1.52\ \mu equiv\ GSH)/(0.10\ \mu equiv\ H_2O_2\ min^{-1})] \times 100\% = 24\%$.

However, the diffusion technique is not particularly well suited for such stoichiometric studies because of the occasional transfer of droplets of concentrated H_2O_2 during the shaking process. Therefore, in order to maintain better control over the upper limit of H_2O_2 concentrations in contact with the cells, we abandoned the diffusion technique and resorted to a dropwise addition of dilute H_2O_2. In Table I are presented data illustrating the effects of changing some of the variables in the dropwise addition technique on the efficiency of the GSH peroxidase reaction. The efficiency was greater for slower drop rates of addition of H_2O_2 (expt 1), and for lower concentrations of H_2O_2 per drop (expt 2). Apparently, when the concentration of H_2O_2 in contact with erythrocytes exceeded a certain critical level, catalatic decomposition of the H_2O_2 resulted in smaller losses of GSH. In expt 3 the efficiency was greater when smaller total amounts of H_2O_2 were added. This latter effect was undoubtedly related to the first-order kinetics observed for the GSH peroxidase reaction at constant H_2O_2 concentration (Fig. 3). With duck erythrocytes, the efficiency of GSH peroxidase was high at all H_2O_2 addition rates (expts 4 and 5). Duck erythrocytes are deficient in catalase (Kaziro *et al.*, 1952) and therefore the oxidation of GSH by H_2O_2 was not in competition with catalatic activity. However, even with human erythrocytes with high endogenous catalase levels, the predominant role of GSH peroxidase at low H_2O_2 concentrations was apparent. As illustrated in Table II, when the concentration of H_2O_2 in contact with the cells was decreased below a critical level the loss in GSH accounted for the major fraction of the oxidizing equivalents of added H_2O_2. The critical concentration of H_2O_2 in these experiments was in the range of 3×10^{-6} M. For example, in expt 3 of Table II, 0.0088 μmole of H_2O_2 (i.e., 1 drop = 1/40 ml × 0.70 μequiv/ml × 1 μmole/2 μequiv) was added to an average extracellular volume of 2.5 ml of medium (3.5 ml average total volume − 1.0 ml suspended erythrocytes) for an average H_2O_2 concentration of 3.5×10^{-6} M.

It is unlikely that the nitroprusside-positive material destroyed by H_2O_2 could be any substance other than GSH, for several reasons. First, in erythrocytes interference with the measurement of GSH with the nitroprusside method is limited to only several per cent (Woodward, 1935; Register, 1954; Koj, 1962), while good efficiency data were obtained for losses of as much as 30% of the apparent GSH (see experiments 4 and 5, Table II). Second, in Figure 3, there was no indication for a faster-reacting component present in small amount, and the first-order kinetics were consistent with but a single component comprising the bulk of the nitroprusside-positive material. Last, as illustrated in Table III, the substance which disappears on reaction with H_2O_2 can be completely regenerated by the addition of glucose to intact cells in accord with the reaction scheme depicted in Figure 1, and also more specifically by the addition of glucose-6-P and NADP to lysates.

C. An Important Protective Role for Glutathione Peroxidase.—With the gaseous diffusion technique it was possible to maintain an automatic continuous addition of small amounts of H_2O_2 and thereby subject erythrocytes to low-level steady-state H_2O_2 concentrations for prolonged periods of time. In such experiments, once the GSH level had fallen in glucose-deprived cells, progressive oxidation of hemoglobin ensued (Fig. 4). The oxidation of hemoglobin was observed visually as a darkening of the erythrocytes and also by spectrophotometric determination of methemoglobin. The initial lag period in the oxidation of hemoglobin (curve A) is attributed to the protective action of GSH peroxidase. The addition of glucose to the medium in order to maintain GSH peroxidase activity (via the continuous regeneration of GSH from GSSG) resulted in protection against methemoglobin formation (curve B). That the protective role of glucose is derived from hexose shunt activity as depicted in Figure 1, is evidenced by the fact that glucose-6-P dehydrogenase–deficient erythrocytes, in the presence of glucose, exhibited methemoglobin formation on prolonged exposure to diffusing peroxide.[3]

In no instance with glucose-deprived normal erythrocytes, or with glucose-deprived catalase-deficient erythrocytes (as described in section *D*), or with glucose-6-P dehydrogenase–deficient cells in the presence of glucose,[3] was methemoglobin formation observed unless the GSH level had first fallen. Additional evidence for the predominant protective role played by GSH peroxidase is available from experiments in which erythrocytes, in the presence of glucose, were subjected to diffusing H_2O_2 for long periods of time. In some instances oxidation of hemoglobin was noted despite the continuing presence of glucose.[4] This phenomenon

[3] To be published.

[4] Two such examples are as follows: Experiment 1, after 21 hours, GSH 8 mg/100 ml, methemoglobin 70%; experiment 2, after 21 hours, GSH 28 mg/100 ml, methemoglobin 14%. The GSH levels at zero time were in the range 60–90 mg/100 ml. At 21 hours the presence of glucose in concentrations in excess of 100 mg/100 ml was confirmed by testing with Glucostat reagent (Worthington).

was always associated with a marked decline in the intracellular GSH concentration; the decline was most probably due to H_2O_2-produced inactivation of the enzymes of the repair mechanism illustrated in Figure 1. In any event, the hemoglobin oxidation was clearly associated with decreased intracellular GSH, and hence with decreased GSH peroxidase activity.

Further oxidative breakdown of hemoglobin beyond the methemoglobin stage was noted after prolonged exposure to H_2O_2. This was recorded as a decrease in the total hemoglobin measured as cyanomethemoglobin, and by the appearance of substances which absorbed light in the range of 610–640 mμ but which did not react with cyanide. No attempt was made to characterize further or to identify the hemoglobin breakdown products.

On prolonged exposure of glucose-deprived erythrocytes to H_2O_2, further oxidative damage was apparent in the form of progressive osmotic fragility increases leading to eventual lysis. For instance, in Figure 5, after 21 hours, the unprotected cells were strongly methemoglobinemic and exhibited decreased resistance to osmotic stress (curve A); in addition, some 20% of the cells had already undergone lysis in the isotonic incubation medium. In contrast, the cells in which the GSH level had been maintained by the presence of glucose were protected against major fragility changes (curve B). Fragility changes probably represent direct oxidative damage to cell membranes after the primary and secondary protective agents (GSH and hemoglobin, respectively) have been titrated out by the H_2O_2.

These data indicate a fundamental role for GSH peroxidase in protecting erythrocytes against the toxic effects of H_2O_2. The ability of H_2O_2 to mimic the hematological effects of certain drugs on glucose-6-P dehydrogenase–deficient individuals (Beutler, 1960; Tarlov *et al.*, 1962) lends credence to suggestions (Mills and Randall, 1958; Cohen and Hochstein, 1961) that H_2O_2 toxicity is an important factor in drug-induced hemolysis.

D. What is the Role of Catalase?—In order to assess the relative importance of catalase in protecting against H_2O_2 damage, we investigated the sensitivity of catalase-deficient cells. We measured the three major criteria of cell damage in erythrocytes: loss of GSH, methemoglobin formation, and increased osmotic fragility. For instance, in Figure 6 the catalase of human erythrocytes was inhibited with azide, and the cells were then subjected to H_2O_2 with the closed diffusion system. In the absence of glucose the usual fall in GSH occurred. However, the loss in GSH was completely prevented by the presence of glucose. In this regard, catalase-deficient cells reacted no differently than catalase-rich cells; they were completely dependent upon a supply of glucose for protection. Once the level of GSH had fallen in the glucose-deprived cells methemoglobin formation became evident (photographic inset), whereas cells in which the GSH level was maintained by the presence of glucose were fully protected. These results were again directly comparable to those obtained with catalase-rich erythrocytes.

In Figure 7 results of osmotic fragility studies are illustrated for duck erythrocytes which have very low endogenous catalase levels (Kaziro *et al.*, 1952); a modified H_2O_2 diffusion chamber was employed in these experiments (see Experimental section). Note in Figure 7 that with lysis and osmotic fragility changes as criteria of cell damage, only those cells which were deprived of glucose were sensitive. Thus sustained GSH peroxidase activity fully protected catalase-deficient cells against major cellular damage. In contrast, erythrocytes with diminished GSH peroxidase activity (i.e., glucose-deprived normal erythrocytes) were not protected by the presence of large amounts of endogenous catalase. The failure of catalase to protect normal cells cannot be attributed to subcellular compartmentalization of enzymes, since catalase was also present in the suspending medium due to lysis of cells, and still the H_2O_2-induced damage remained progressive.

The question of which enzyme, catalase or GSH peroxidase, predominates in detoxifying H_2O_2 under physiologic conditions revolves about the problem of estimating endogenous intracellular H_2O_2 levels. At the levels at which we have worked, or lower, GSH peroxidase would have to be assigned a role of singular importance. At higher levels, catalatic decomposition of H_2O_2 would become increasingly important. However, had the endogenous H_2O_2 level been higher than or even equal to our working range, then we would have observed declining GSH levels in the glucose-deprived cells incubated without added H_2O_2 (see Fig. 3, curve C). Likewise, with glucose-6-P dehydrogenase–deficient cells incubated with glucose, but without H_2O_2, we would have observed a declining GSH level—but again the GSH level was stable (Cohen and Hochstein, 1961). Therefore the amounts of H_2O_2 added by diffusion must have greatly exceeded the endogenous levels.

It is concluded that catalase does not play an important role in protecting erythrocytes against endogenous H_2O_2. These experiments confirm the suggestion by Keilin and Hartree (1945) that catalatic decomposition of H_2O_2 is *not* an important metabolic pathway in erythrocytes. On the other hand, GSH peroxidase appears to be the major metabolic pathway for H_2O_2 as determined by both stoichiometric and pharmacologic criteria. The widespread distribution of GSH peroxidase in tissues (Mills, 1960; Hochstein and Cohen, 1962) portends a potential replication of this role wherever H_2O_2 is generated endogenously in low concentration.

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Comparison of Polarographic and Spectrophotometric Assays for Cytochrome c Oxidase Activity*

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The rate of oxygen uptake measured polarographically in the system: ascorbate → cytochrome c → cytochrome c oxidase → oxygen was compared with one-fourth the initial rate of oxidation of ferrocytochrome c measured spectrophotometrically in the system: ferrocytochrome c → cytochrome c oxidase → oxygen, using identical mixtures except for the presence of ascorbate in the polarographic measurements. In contrast with the conclusions of other investigators, our data show that the two methods can be brought into agreement when certain conditions are fulfilled. Thus, it is possible to use the polarographic (or manometric) method for assaying cytochrome c oxidase activity without measurable interference from ascorbate.

Cytochrome c oxidase activity is usually assayed (a) by measuring the rate of oxidation of soluble ferrocytochrome c (spectrophotometric method) or (b) by measuring the rate of oxygen uptake when the cytochrome c is continuously reduced by an appropriate reducing agent such as ascorbic acid[1] (polarographic or manometric method). The following equations summarize the passage of electrons in the two systems:

(a) ferrocytochrome c → cytochrome c oxidase → O_2
(b) reducing agent → cytochrome c → cytochrome c oxidase → O_2

Reaction (a) is first order with respect to ferrocytochrome c, but the rate constant varies with the total concentration of cytochrome c in the reaction mixture (Smith and Conrad, 1956).

If the reduction of cytochrome c by ascorbate in system (b) is rapid compared to the rate of oxidation of ferrocytochrome c by the oxidase, the cytochrome c should be nearly completely reduced during the reaction, and the rate of oxygen uptake in system (b) should be equal to one-fourth the initial rate of oxidation of ferrocytochrome c in system (a) when the total concentration of cytochrome c is the same.

Several studies indicate that the above condition does not hold in the reaction mixtures usually employed with system (b) (see Minnaert, 1961; Yonetani, 1962). Thus we have tested the variables concerned to ascertain whether conditions could be found where the rate of oxygen uptake in system (b) is the same as the calculated corresponding rate in system (a). The data show that the two rates are the same when the concentrations of ascorbic acid, cytochrome c, and enzyme are adjusted so that the cytochrome c is more than 85% reduced during the reaction and provided that there has been no aggregation of the particulate oxidase preparations.

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[1] The advantages of ascorbic acid have been discussed by Minnaert (1961) and Yonetani (1962).

Methods and Reagents

Enzyme Preparations.—As a source of cytochrome c oxidase, the insoluble membrane fragments bearing the respiratory chain system were prepared from beef heart according to the method of Keilin and Hartree (1947), modified by disintegrating the mince in a Waring Blendor (Chance, 1952). Some preparations of particles were made deficient in cytochrome c by extracting the mince with buffer as described by Tsou (1952). Beef heart mitochondria were prepared by the method of Crane *et al.* (1956).

Cytochrome c was either prepared from beef heart by the Keilin-Hartree (1945) and Margoliash (1954) procedures, or was purchased from the Sigma Chemical Co. (type III). The cytochrome c was reduced with hydrogen and palladium, as previously described (Smith, 1954). The total concentration of cytochrome c was assessed from the optical density at 550 mμ of the compound reduced with $Na_2S_2O_4$, using 27.6 as the mM extinction coefficient (Margoliash, 1954).

Ascorbate.—A solution of ascorbic acid (Fisher reagent grade) was neutralized to *p*H 7.0 with NaOH and made 0.001 M with EDTA and 0.5 M with respect to ascorbate. This mixture was either used immediately or stored in the frozen state; in either case the same results were obtained.

Spectrophotometric Assay of Cytochrome c Oxidase.—The assay was performed at 25° in 0.05 M buffer made from a mixture of NA_2HPO_4 and KH_2PO_4 as described by Smith (1954), the first-order rate constant being calculated in each case. The initial rates were then obtained by multiplying the rate constant by the concentration of cytochrome c.

Polarographic Measurement of Cytochrome c Oxidase Activity.—The rate of oxygen uptake was measured with the Clark oxygen electrode (Clark, 1956) calibrated for each experiment with buffer saturated with air at 25°. The reagents were incubated in a bath at 25°;