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INTERDEPENDENCE OF HEMOGLOBIN, CATALASE AND THE HEXOSE MONOPHOSPHATE SHUNT IN RED BLOOD CELLS EXPOSED TO OXIDATIVE AGENTS

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Abstract—Hydrogen peroxide, 1, 4-naphthoquinone-2-sulfonic acid and 6-hydroxydopamine were used as biochemical probes to study the interdependence of hemoglobin, catalase and the hexose monophosphate shunt in protection of red blood cell (red cell) function against superoxide, hydrogen peroxide and organic free radicals. It was shown that catalase may remove hydrogen peroxide both catalatically and peroxidatically in the red cell and that glucose metabolism supplies electron donors for the peroxidatic function of catalase. The hexose monophosphate shunt is known to participate in removal of hydrogen peroxide by supplying electrons for the action of glutathione reductase and glutathione peroxidase. Experiments in which red cell catalase was irreversibly inhibited by interaction with hydrogen peroxide and 3-amino-1, 2, 4-triazole showed that both catalase and the hexose monophosphate shunt share in the removal of hydrogen peroxide from red cells. The effect of the hemoglobin oxidation state on the interaction of red cells and oxidative agents was studied using red cell preparations containing hemoglobin, carbonmonoxyhemoglobin or methemoglobin. Oxyhemoglobin was able to accelerate the production of superoxide and hydrogen peroxide with agents like 1, 4-naphthoquinone-2-sulfonic acid, whereas oxyhemoglobin had little effect with 6-hydroxydopamine. In experiments with red cells containing oxyhemoglobin, the accumulation of catalase in the form of Compound II, with resulting loss of available catalatic activity, was directly proportional to formation of methemoglobin. The close relationship between loss of catalatic activity and formation of methemoglobin may indicate that catalase has a protective effect on hemoglobin. Experiments with red cells containing methemoglobin indicated that once methemoglobin is formed it protects the red cell from further loss of the catalatic activity of catalase. In some circumstances oxyhemoglobin was formed from methemoglobin as a by-product of the protective effect of methemoglobin on red cell function. Formation of oxyhemoglobin by this mechanism was many times faster than oxyhemoglobin formation by the methemoglobin reductase system.

The red blood cell (red cell) has a variety of mechanisms to protect hemoglobin and membrane function from superoxide and H_2O_2. While superoxide dismutase and catalase enzymatically remove superoxide and H_2O_2, respectively, red cell reducing equivalents, mainly glutathione, NADH and NADPH, may react directly with superoxide and H_2O_2 [1–3]. The activity of the hexose monophosphate shunt is important since it produces NADPH, the necessary cofactor for glutathione production by glutathione reductase. The reduction of H_2O_2 to water is catalyzed by glutathione peroxidase and possibly by the peroxidatic activity of hemoglobin [4].

We have recently studied the chemistry of red cell catalase using 1, 4-naphthoquinone-2-sulfonic acid [5], a vitamin K analog which reacts with oxyhemoglobin generating superoxide and H_2O_2 [6, 7]. It was demonstrated that catalase accumulates as Compound II* in red cells incubated with 1, 4-naphthoquinone-2-sulfonic acid when glucose is unavailable [5]. When glucose is added, the accumulated Compound II is reduced to ferricatalase by products of glucose metabolism. Experiments with red cell lysates [9] or purified components [10] have shown that NADPH and NADH can both donate electrons to Compound II and are probable intracellular electron donors. These results indicate that catalase may function both peroxidatically and catalatically in the red cell. It was also observed that methemoglobin formation was directly proportional to accumulation of Compound II. While the addition of glucose restored ferricatalase content within a 90-min period, the methemoglobin reductase system required between 24 and 48 hr to restore oxyhemoglobin [5].

In this paper we have explored the interdependence of hemoglobin, catalase and glucose metabolism in red cells exposed to H_2O_2, 1, 4-naphthoquinone-2-sulfonic acid and 6-hydroxydopamine. Cells were exposed to H_2O_2 mainly as a control to help in interpreting the more complex results

* The terminology used in this paper is based on the chemistry of catalase which is known to function either catalatically or peroxidatically *in vitro* [8]. Ferricatalase reacts with H_2O_2 to form the two-electron oxidation product, Compound I. Compound I may undergo either a concerted two-electron reduction by H_2O_2 (catalatic activity) or substrates such as ethanol (peroxidatic activity) with the formation of ferricatalase or two successive one-electron reductions by appropriate electron donors with the sequential formation of Compound II and ferricatalase (peroxidatic activity).

obtained with the organic agents. 1, 4-Naphthoquinone-2-sulfonic acid is an oxidizing agent which reacts with reducing agents to yield its semiquinone and quinol derivatives [7]. The semiquinone derivative may act as an oxidizing or reducing agent in the red cell. 1, 4-Naphthoquinone-2-sulfonic acid reacts with oxyhemoglobin to generate superoxide, H_2O_2 and methemoglobin [6, 7]. 6-Hydroxydopamine, a quinol, is a reducing agent which undergoes autoxidation to its quinone derivative [11]. The semiquinone of 6-hydroxydopamine may be formed as an intermediate in autoxidation [12]. Superoxide, H_2O_2, hydroxyl radical and singlet oxygen are formed during autoxidation of 6-hydroxydopamine [13, 14]. The quinone of 6-hydroxydopamine may be reduced to the quinol by products of glucose metabolism, giving rise to cycles of autoxidation and, therefore, superoxide and H_2O_2 production [15–17].

MATERIALS AND METHODS

Red cell preparations. Adult human blood was drawn daily into 3.8% sodium citrate solution. After centrifugation, plasma and white cells were removed, and red cells were washed three times with phosphate-buffered saline (9 parts 0.9% NaCl; 1 part 0.1 M KH_2PO_4/K_2HPO_4, pH 7.4). A 25% (v/v) suspension of red cells was prepared in Krebs–Ringer phosphate buffer (100 parts 0.154 M NaCl; 4 parts 0.154 M KCl; 1.5 parts 0.11 M $CaCl_2$; 1 part 0.154 M $MgSO_4$; 21 parts 0.1 M NaH_2PO_4/Na_2HPO_4, pH 7.4).

Experiments were carried out with three types of red cell preparations, characterized by the hemoglobin: oxyhemoglobin, carbonmonoxyhemoglobin and methemoglobin. For experiments with normal red cells, that is, containing oxyhemoglobin, red cells were collected from a 25% suspension in Krebs–Ringer phosphate and resuspended at 25% in fresh Krebs–Ringer phosphate with or without 5 mM D-glucose immediately prior to use. Red cells containing carbonmonoxyhemoglobin were prepared by blowing CO over the red cell suspension until the visible spectra of red cell lysates reached a maximum at 569 nm. The red cells were then washed once with oxygenated phosphate-buffered saline and resuspended at 25% in Krebs–Ringer phosphate with or without 5 mM D-glucose. Red cells containing methemoglobin were prepared by incubating a volume of red cells with an equal volume of 1% $NaNO_2$ in phosphate-buffered saline for 10 min at room temperature. After incubation, red cells were washed five times with phosphate-buffered saline to remove nitrite and resuspended at 25% in Krebs–Ringer phosphate with or without 5 mM D-glucose. Visible spectra of red cell lysates were obtained using a Cary 14 spectrophotometer. Ten microliters of a 25% red cell suspension in Krebs–Ringer phosphate were lysed in 1 ml H_2O, and absorbance was read against an H_2O reference. Red cell preparations contained essentially 100 per cent oxyhemoglobin, carbonmonoxyhemoglobin or methemoglobin at the beginning of experiments. After 90 min of incubation at 37°, red cells containing oxyhemoglobin or methemoglobin showed essentially no change in their hemoglobin. After 90 min at 37°, cells containing carbonmonoxyhemoglobin contained about 20 per cent oxyhemoglobin.

Agents producing superoxide and H_2O_2. The interactions of the red cells with three agents were studied: H_2O_2, 1, 4-naphthoquinone-2-sulfonic acid (Eastman Kodak Co., Rochester, NY) and 6-hydroxydopamine (Aldrich Chemical Co., Milwaukee, WI). In experiments with H_2O_2, wells containing 0.3 ml of 30% H_2O_2 were included in 25-ml stoppered Erlenmeyer flasks containing 1 or 2 ml of red cell suspension. This procedure allowed for the gaseous diffusion of H_2O_2 from the center well to the red cells at a steady rate [18]. Stoppered flasks with wells containing 0.3 ml of H_2O were used as controls. In other experiments, 1, 4-naphthoquinone-2-sulfonic acid was dissolved in Krebs–Ringer phosphate and a small volume was added to red cell suspensions such that there was little effect on incubation volume. Equal volumes of Krebs–Ringer phosphate without drug were added to red cell suspensions as controls. 6-Hydroxydopamine was dissolved in 140 mM NaCl, 1 mM KCl, pH 2.0. The low pH is necessary to prevent autoxidation of the 6-hydroxydopamine before addition to red cell suspensions. A small volume of 6-hydroxydopamine was added to red cell suspensions such that there was no significant change in pH (pH 7.4) or volume of the red cell suspensions. Equal volumes of 140 mM NaCl, 1 mM KCl, pH 2.0, without drug were added to red cell suspensions as controls.

Assay for catalatic activity of red cell catalase. Catalase activity was measured in red cell lysates by observing the decrease in absorbance at 240 nm of a hydrogen peroxide solution, as described by Beers and Sizer [19]. A 0.03 ml portion of a 25% red cell suspension was lysed in 1 ml H_2O and 3 ml 10^{-4} M EDTA, 0.1 M NaH_2PO_4, pH 7.4, was added to give 4.03 ml of red cell lysate. A 0.5 ml portion of this lysate was added to 4 ml of 0.05 M NaH_2PO_4, pH 6.8, and 2 ml of this mixture was added to a sample cuvette and 2 ml to a reference cuvette in a Cary 14 spectrophotometer. The contents of the sample cuvette were constantly mixed by a small magnetic stirring bar. A 0.04 ml portion of 0.78 M H_2O_2, 0.05 M NaH_2PO_4, pH 6.8, was injected into the sample cuvette, and the decrease in absorbance at 240 nm was measured. The decrease in absorbance was linear with time and proportional to catalase activity up to 20 sec after injection. Since this assay measures only ferricatalase and Compound I, any accumulation of Compound II or other forms [8] will appear as a loss of catalase activity.

Measurement of the flux of glucose metabolism. The flux of glucose through the hexose monophosphate shunt and glycolysis was measured by a modification of the method of Pescarmona *et al.** Two milliliters of a 25 per cent red cell suspension in Krebs–Ringer phosphate with 5 mM D-glucose was added to a 25-ml Erlenmeyer flask. A blank was run using a red cell suspension that had been boiled for 10 min. When appropriate, 1, 4-naphthoquinone-2-sulfonic acid, 6-hydroxydopamine, or wells containing H_2O_2 were added at this time. A 0.3 ml portion

* G. P. Pescarmona, A. Bosia, P. Arese and M. L. Sartori (University of Torino, Italy and University of Sassari, Italy), personal communication.

of D- [1-^{14}C]glucose (Amersham) in Krebs–Ringer phosphate with 5 mM D-glucose (0.5 μCi/0.3 ml) was added to each flask. Flasks were stoppered with wells containing 0.2 ml of 2 M KOH and incubated in a shaking water bath at 37° for 1 hr. The reaction was stopped by addition of 0.7 ml of 35% perchloric acid and incubated for 30 min to ensure that released $^{14}CO_2$ was trapped in the KOH. Contents of the well (KOH with trapped $^{14}CO_2$) were transferred into 10 ml Oxosol (National Diagnostic, NJ) and counted in a liquid scintillation counter. The flux through the hexose monophoshate shunt was calculated after subtracting blank values and expressed as μmoles CO_2 produced per hr per ml of red cells. After addition of and incubation with perchloric acid, the contents of the incubation flasks were transferred to centrifuge tubes, and the flasks were washed with 2 ml H_2O, bringing the total volume in the centrifuge tube to 5 ml. After centrifugation at 8000 *g* for 10 min, 1 ml of supernatant fluid was added to 1 ml of 0.5 M Tris, 0.78 M KOH and centrifuged to remove perchlorate as the potassium salt. One-tenth ml of this supernatant fluid was added to a 25-ml Erlenmeyer flask with 0.9 ml of 3.3 mM ATP, 1mM NADP, 6.5 mM $MgCl_2$, 1.4 I.U. glucose-6-phosphate dehydrogenase, 0.1 M Tris, pH 7.6, and 0.05 ml containing 2.8 I.U. hexokinase and 0.24 I.U. 6-phosphogluconate dehydrogenase. Flasks were stoppered with wells containing 0.2 ml of 2 M KOH and incubated in a shaking water bath at 37° for 30 min.The purpose of this procedure is to decarboxylate the remaining glucose, leaving intermediates of glycolysis as the only radioactive species in the flask. The reaction was terminated by the addition of 0.2 ml of 7% perchloric acid, and the flasks were further incubated for 30 min. The flask contents were centrifuged and 0.25 ml of the supernatant fluid was transferred to 10 ml Oxosol and counted in a liquid scintillation counter. The flux through glycolysis was calculated, after subtracting blank values and correcting for dilution, and expressed as μmoles glycolytic intermediates produced per hr per ml red cells.

RESULTS

Peroxidatic activity of catalase. Table 1 shows that, in the absence of glucose, both 4×10^{-5} M 1, 4-naphthoquinone-2-sulfonic acid and 6×10^{-4} M 6-hydroxydopamine (administered in three equal doses at 0, 30 and 60 min) caused accumulation of catalase Compound II. The catalase assay we used measures only ferricatalase and Compound I so that loss of catalase activity using this assay implies accumulation of Compound II. When glucose was added at 90 min to red cell suspensions incubated with 1, 4-naphthoquinone-2-sulfonic acid or 6-hydroxydopamine, intracellular electron donors formed by glucose metabolism restored ferricatalase, as shown at 150 and 240 min. Three equal doses of 6-hydroxydopamine were more effective than one larger dose in causing significant accumulation of Compound II, probably because autoxidation of 6-hydroxydopamine at pH 7.4 is complete in about 2 min and the resulting quinone is not reduced in the absence of glucose metabolism [15–17]. Hydrogen peroxide alone did not cause accumulation of Compound II (Table 1) even if added dropwise in high concentration (data not shown).

Removal of H_2O_2 by catalase and the hexose monophosphate shunt. Both catalase and the hexose monophosphate shunt (by supplying electrons for the action of glutathione reductase and glutathione peroxidase) can contribute to removal of H_2O_2 in the red cell. This can be emphasized by measuring the flux of glucose metabolism through the hexose monophosphate shunt in conditions where catalase is inactivated. 3-Amino-1, 2, 4-triazole irreversibly inhibits catalase in the presence of H_2O_2 by forming a complex between Compound I and 3-amino-1, 2, 4-triazole [20, 21]. By incubating red cells with 0.05 M 3-amino-1, 2, 4-triazole and using 4×10^{-3} M 1, 4-naphthoquinone-2-sulfonic acid as a source of H_2O_2, red cell catalase was irreversibly inactivated and did not recover with the addition of glucose (Table 2). Measurement of the flux of glucose through the hexose monophosphate shunt showed that $4 \times$

Table 1. Formation of Compound II and restoration of ferricatalase by glucose metabolism*

Agent	Catalase activity (mmoles $H_2O_2 \cdot sec^{-1} \cdot ml^{-1}$ red cells)			
	0 min	90 min	150 min	240 min
H_2O_2	1.4	1.4 †		
1, 4-Naphthoquinone-2-sulfonic acid	1.4	0.81 ‡	1.2	1.3
6-Hydroxydopamine	1.4	0.52 ‡	1.0	1.1

* One milliliter aliquots of a 25% red cell suspension in Krebs–Ringer phosphate without glucose were incubated at 37° for 240 min in a shaking water bath. One aliquot was incubated from zero time in a stoppered flask containing a well with 0.3 ml of 30% H_2O_2. A second aliquot contained 4×10^{-5} M 1, 4-naphthoquinone-2-sulfonic acid. A third aliquot received equal additions of 6-hydroxydopamine at 0, 30 and 60 min to give a total dose of 6×10^{-4} M 6-hydroxydopamine. Red cell catalase activity was measured at 0, 90, 150 and 240 min.

† Catalase activity remained at 1.4 up to 240 min in the absence of glucose.

‡ D-Glucose was added at 90 min to a concentration of 5 mM.

Table 2. Irreversible inactivation of catalase in the presence of 3-amino-1, 2, 4-triazole*

Additions	Catalase activity (mmoles $H_2O_2 \cdot sec^{-1} \cdot ml^{-1}$ red cells)		
	0 min	60 min†	120 min
None	1.2	1.3	1.0
1, 4-Naphthoquinone-2-sulfonic acid	1.2	0.44	0.67
3-Amino-1, 2, 4-triazole	1.2	1.1	0.96
1, 4-Naphthoquinone-2-sulfonic acid + 3-amino-1, 2, 4-triazole	1.2	0.07	0.07

* One milliliter aliquots of a 25% red cell suspension in Krebs–Ringer phosphate without glucose were incubated at 37° for 120 min in a shaking water bath. Additions made at zero time: 4×10^{-5} M 1, 4-naphthoquinone-2-sulfonic acid and 0.05 M 3-amino-1, 2, 4-triazole. Red cell catalase activity was measured at 0, 60 and 120 min.

† D-Glucose was added at 60 min to a concentration of 5 mM.

10^{-5} M 1, 4-naphthoquinone-2-sulfonic acid caused an 8-fold increase in flux (Table 3). With the addition of 0.05 M 3-amino-1, 2, 4-triazole and resulting inactivation of catalase, a 12-fold increase in flux was observed.

Catalase and hemoglobin. To study the relationship between catalase chemistry and red cell hemoglobin, we prepared red cells containing carbonmonoxyhemoglobin or methemoglobin before addition of superoxide and H_2O_2 producing agents. Cells containing carbonmonoxyhemoglobin approximate conditions where the heme group is unavailable for reaction; however, by the end of the 90-min incubation period, about 20 per cent of the hemoglobin reverted to oxyhemoglobin, so that some effect of oxyhemoglobin can be expected in these experiments. Cells containing methemoglobin were used to exaggerate the effect of the small amount of methemoglobin present or produced in conditions where superoxide and H_2O_2 are present.

Table 4 shows the accumulation of Compound II with different agents and red cell preparations. Incubation with H_2O_2 caused little or no accumulation of Compound II with any of the red cell preparations. 1, 4-Naphthoquinone-2-sulfonic acid in the presence of oxyhemoglobin caused almost half of the red cell catalase to accumulate as Compound II in 90 min. The removal of most of the oxyhemoglobin, as in preparations containing carbonmonoxyhemoglobin, resulted in only 14 per cent accumulation of Compound II. There was no accumulation of Compound II when red cells containing methemoglobin were incubated with 1, 4-naphthoquinone-2-sulfonic acid. 6-Hydroxydopamine caused equal Compound II accumulation in red cells containg oxyhemoglobin or carbonmonoxyhemoglobin. When red cells containing methemoglobin were incubated with 6-hydroxydopamine, however, little Compound II accumulated.

Besides causing no accumulation of Compound II, incubation with H_2O_2 also had no effect on the hemoglobin in red cell preparations containing oxyhemoglobin, carbonmonoxyhemoglobin or methemoglobin. Figure 1 shows the effects of 1, 4-naphthoquinone-2-sulfonic acid on hemoglobin in various red cell preparations. In the presence of oxyhemoglobin, 1, 4-naphthoquinone-2-sulfonic acid caused significant methemoglobin formation, as indicated by changes in the visible spectra at 500, 541, 577 and 631 nm (Fig. 1a). In the presence of carbonmonoxyhemoglobin, a much smaller amount of methemoglobin was formed, probably the result of interaction between 1, 4-naphthoquinone-2-sulfonic acid and the small amount of oxyhemoglobin present in these cells (Fig. 1b). Red cells containing methemoglobin showed no change in hemoglobin content after a 90-min incubation with 1, 4-naphthoquinone-2-sulfonic acid (Fig. 1c). The effects of 6-hydroxydopamine on hemoglobin in various red cell preparations are shown in Fig. 2. Significant met-

Table 3. Flux through the hexose monophosphate shunt in conditions causing catalase inactivation*

Additions	Flux through the hexose monophosphate shunt (μmoles glucose metabolized $\cdot hr^{-1} \cdot ml^{-1}$ red cells)
None	0.086
1, 4-Naphthoquinone-2-sulfonic acid	0.670
3-Amino-1, 2, 4-triazole	0.069
1, 4-Naphthoquinone-2-sulfonic acid + 3-amino-1, 2, 4-triazole	1.05

* Concentrations: 4×10^{-5} M 1, 4-naphthoquinone-2-sulfonic acid and 0.05 M 3-amino-1, 2, 4-triazole. The flux of glucose through the hexose monophosphate shunt was measured as described in Materials and Methods.

Table 4. Catalase activity in different red cell preparations exposed to various agents*

Agent	Red cell preparation	Per cent inhibition of catalase activity
H_2O_2	Oxyhemoglobin	7
H_2O_2	Carbonmonoxyhemoglobin	8
H_2O_2	Methemoglobin	0
1, 4-Naphthoquinone-2-sulfonic acid	Oxyhemoglobin	46
1, 4-Naphthoquinone-2-sulfonic acid	Carbonmonoxyhemoglobin	14
1, 4-Naphthoquinone-2-sulfonic acid	Methemoglobin	0
6-Hydroxydopamine	Oxyhemoglobin	47
6-Hydroxydopamine	Carbonmonoxyhemoglobin	51
6-Hydroxydopamine	Methemoglobin	9

* One milliliter aliquots of a 25% red cell suspension in Krebs–Ringer phosphate without glucose were incubated at 37° for 90 min in a shaking water bath. Three different red cell preparations were used as described in Materials and Methods. Preparation of red cell suspensions containing carbonmonoxyhemoglobin and methemoglobin did not result in loss of catalase activity. Doses of H_2O_2, 1, 4-naphthoquinone-2-sulfonic acid and 6-hydroxydopamine were as described in the footnote to Table 1. Red cell catalase activity was measured at 90 min and compared to the catalase activity of controls incubated without oxidative agents (see Materials and Methods).

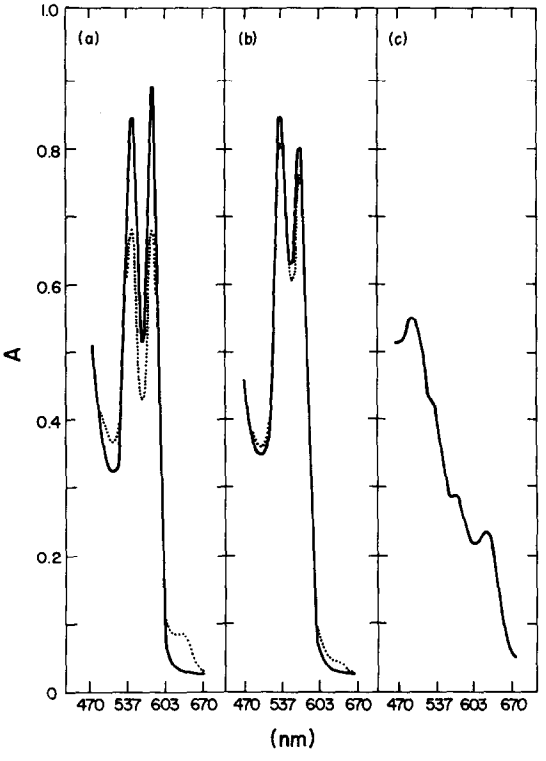

Fig. 1. Effects of 1, 4-naphthoquinone-2-sulfonic acid on red cell hemoglobin. One milliliter aliquots of a 25% red cell suspension in Krebs–Ringer phosphate without glucose were incubated at 37° for 90 min in a shaking water bath. Red cells containing oxyhemoglobin (a), carbonmonoxyhemoglobin (b) and methemoglobin (c) were prepared as described in Materials and Methods. Aliquots were incubated with or without 4×10^{-5} M 1, 4-naphthoquinone-2-sulfonic acid. At 90 min, red cell lysates were prepared and visible spectra were recorded as described in Materials and Methods. Key: (——) controls, (– – –) samples with 4×10^{-5} M 1, 4-naphthoquinone-2-sulfonic acid.

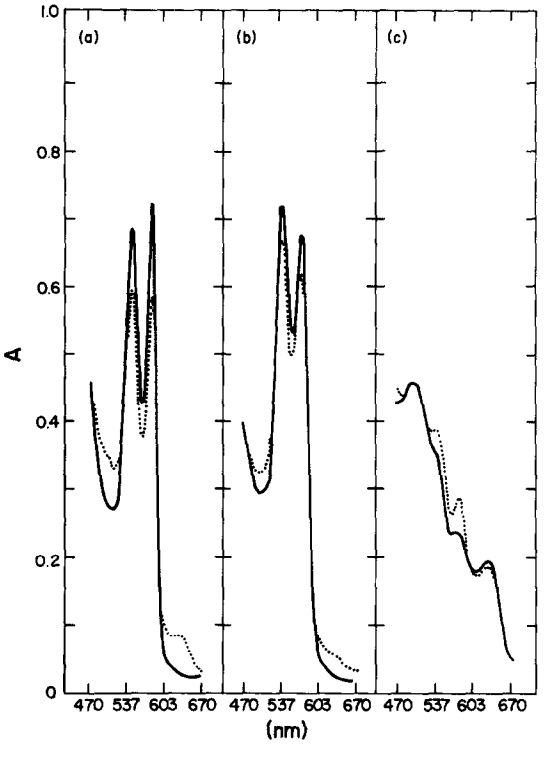

Fig. 2. Effects of 6-hydroxydopamine on red cell hemoglobin. Red cell suspensions were treated as described in Fig. 1 except that aliquots received equal additions of 6-hydroxydopamine at 0, 30 and 60 min to give a total dose of 6×10^{-4} M 6-hydroxydopamine. Preparation of 6-hydroxydopamine and treatment of controls are described in Materials and Methods. At 90 min, red cell lysates were prepared and visible spectra were recorded as described in Materials and Methods. Red cell preparations contained oxyhemoglobin (a), carbonmonoxyhemoglobin (b) or methemoglobin (c). Key: (——) controls, and (– – –) samples with 6-hydroxydopamine.

Table 5. Flux of glucose metabolism through the hexose monophosphate shunt and glycolysis in different red cell preparations*

Red cell preparation	Flux (μmoles glucose metabolized · hr^{-1} · ml^{-1} red cells)	
	Hexose monophosphate shunt	Glycolysis
Oxyhemoglobin	0.088	0.93
Carbonmonoxyhemoglobin	0.073	0.96
Methemoglobin	0.258	1.13

* Preparation of red cells and measurement of flux are described in Materials and Methods.

hemoglobin formation took place in red cells containing both oxyhemoglobin (Fig. 2a) and carbonmonoxyhemoglobin (Fig. 2b). In red cell preparations containing methemoglobin, incubation with 6-hydroxydopamine resulted in significant formation of oxyhemoglobin, as indicated by changes in the visible spectra at 470, 541, 577 and 631 nm (Fig. 2c).

The flux of glucose metablism. Table 5 shows the flux of glucose metabolism in red cell preparations containing oxyhemoglobin, carbonmonoxyhemoglobin and methemoglobin. In red cells containing oxyhemoglobin and carbonmonoxyhemoglobin, the flux through glycolysis was ten to thirteen times greater than through the hexose monophosphate shunt. In red cells containing methemoglobin, the flux through both pathways was increased but most striking was the 3-fold increase in the hexose monophosphate shunt.

The effects of H_2O_2, 1, 4-naphthoquinone-2-sulfonic acid and 6-hydroxydopamine on the flux of glucose metabolism in the three red cell preparations is shown in Table 6. The results are given as the increase or decrease in flux as compared to the controls in Table 5. The doses of H_2O_2 and 1, 4-naphthoquinone-2-sulfonic acid were the same as in previous experiments. It was found that only a single dose of 6-hydroxydopamine was required to observe major changes in the flux of glucose metabolism, unlike the results in experiments in the absence of glucose (Tables 1 and 4, Fig. 2).

Hydrogen peroxide, 1, 4-naphthoquinone-2-sulfonic acid and 6-hydroxydopamine all caused major increases in the flux of the hexose monophosphate shunt, with smaller decreases in the flux of glycolysis (Table 6). The decreases in glycolysis are approximately proportional to the increases in the hexose monophosphate shunt. Since our assay for glycolysis measures the flux of [1-^{14}C]glucose-6-phosphate into glycolysis, the entrance into glycolysis of unlabeled fructose-6-phosphate and glyceraldehyde-3-phosphate, formed from the pentoses produced by the hexose monophosphate shunt, will not be measured. Therefore, the actual total flux of glycolysis is little affected by the agents studied.

Hydrogen peroxide increased the flux through the hexose monophosphate shunt in all red cell preparations (Table 6), but the change in flux was twice as high in red cells containing methemoglobin as in red cells containing oxyhemoglobin or carbonmonoxyhemoglobin. 1, 4-Naphthoquinone-2-sulfonic acid caused the greatest increases in flux through the

Table 6. Flux of glucose metabolism in different red cell preparations exposed to various agents*

Agent	Red cell preparation	Change in flux (μmoles glucose metabolized · hr^{-1} · ml^{-1} red cells)	
		Hexose monophosphate shunt	Glycolysis
H_2O_2	Oxyhemoglobin	+0.185	−0.06
H_2O_2	Carbonmonoxyhemoglobin	+0.138	−0.06
H_2O_2	Methemoglobin	+0.352	−0.12
1, 4-Naphthoquinone-2-sulfonic acid	Oxyhemoglobin	+0.659	−0.23
1, 4-Naphthoquinone-2-sulfonic acid	Carbonmonoxyhemoglobin	+0.213	−0.06
1, 4-Naphthoquinone-2-sulfonic acid	Methemoglobin	+0.337	−0.20
6-Hydroxydopamine	Oxyhemoglobin	+0.570	−0.14
6-Hydroxydopamine	Carbonmonoxyhemoglobin	+0.333	−0.00
6-Hydroxydopamine	Methemoglobin	+0.540	−0.25

* Preparation of red cells and measurement of flux are described in Materials and Methods. Aliquots were incubated at zero time with wells containing 0.3 ml of 30% H_2O_2, with 4×10^{-5} M 1, 4-naphthoquinone-2-sulfonic acid, or with 2×10^{-4} M 6-hydroxydopamine. Control aliquots were incubated without oxidative agents (see Materials and Methods). The change in flux caused by oxidative agents is the increase (+) or decrease (−) in flux as compared to controls.

hexose monophosphate shunt in red cells containing oxyhemoglobin. The increase in red cells containing methemoglobin was half that of red cells containing oxyhemoglobin and least of all in red cells containing carbonmonoxyhemoglobin. 6-Hydroxydopamine caused about equal increases in flux through the hexose monophosphate shunt in red cells containing oxyhemoglobin and methemoglobin and slightly less of an increase in red cells containing carbonmonoxyhemoglobin.

DISCUSSION

One mechanism for a possible peroxidatic function of catalase in the red cell requires the availabilty of one-electron donors to convert Compound I to Compound II and, then, to convert Compound II to ferricatalase. The reverse of these reactions, one-electron oxidation of the ferric state to Compound II and one-electron oxidation of Compound II to Compound I, has been shown to occur with horseradish peroxidase [22] and may be possible with red cell catalase given appropriate one-electron acceptors. The accumulation of Compound II in the absence of glucose is evidence for the presence of one-electron donors or acceptors in the red cell. The conversion of accumulated Compound II to ferricatalase by glucose metabolism in the intact red cell constitutes evidence that catalase may be able to function intracellularly through a Compound II intermediate. We have previously used 1, 4-naphthoquinone-2-sulfonic acid as an intracellular source of H_2O_2 and observed accumulation of Compound II followed by restoration of ferricatalase after addition of glucose [5]. In this report, red cells were incubated with 1, 4-naphthoquinone-2-sulfonic acid and 6-hydroxydopamine as sources of H_2O_2 and one-electron donors or acceptors, while red cells were incubated with H_2O_2 alone as a control. In the absence of glucose, incubation with H_2O_2 did not cause accumulation of Compound II even if H_2O_2 was added dropwise in high concentration (not shown), whereas incubation with 1, 4-naphthoquinone-2-sulfonic acid or 6-hydroxydopamine caused significant accumulation of Compound II. Three equal doses of 6-hydroxydopamine added sequentially were more effective than one larger single dose in causing accumulation of Compound II, probably because autoxidation of 6-hydroxydopamine at pH 7.4 is complete in about 2 min and the resulting quinone is not reduced in the absence of glucose metabolism [15–17]. Both ferricatalase and Compound I are expected to be present in red cells incubated with 1, 4-naphthoquinone-2-sulfonic acid, 6-hydroxydopamine or H_2O_2 alone. Incubation with 1, 4-naphthoquinone-2-sulfonic acid or 6-hydroxydopamine but not with H_2O_2 alone makes one-electron donors or acceptors available and Compound II accumulates. Likely one-electron donors or acceptors are superoxide and the semiquinone derivatives of the organic agents. Conversion of accumulated Compound II to ferricatalase takes place when products of glucose metabolism become available. Experiments with red cell lysates [9] or purified components [10] have shown that NADPH and NADH can both convert Compound II to ferricatalase and are probable intracellular electron donors formed by glucose metabolism. The results indicate that one function of red cell catalase may be to remove free radicals through the Compound I to Compound II conversion and to utilize electrons from NADPH to NADH to restore ferricatalase.

A controversy exists over the relative importance of catalase and the hexose monophosphate shunt with associated glutathione reductase and glutathione peroxidase in the catabolism by the red cell of physiological concentrations of hydrogen peroxide. Results have focused on the importance of hexose monophosphate shunt activity [18, 23] and catalase activity [24], and on the shared role of both the hexose monophosphate shunt and catalase [25–27]. Eaton *et al.* [9] presented evidence which suggests that the formation of catalase Compound II and its reduction by NADPH activate the hexose monophosphate shunt by increasing intracellular $NADP^+$ levels. We have further demonstrated the participation of catalase in the removal of H_2O_2 by observing the flux of glucose metabolism through the hexose monophosphate shunt in experiments in which catalase was inhibited irreversibly. Using 1, 4-naphthoquinone-2-sulfonic acid as a source of intracellular H_2O_2, inactivation of catalase by 3-amino-1, 2, 4-triazole caused the flux through the hexose monophosphate shunt to increase by 50 per cent. This implies that H_2O_2 removed by catalase in control red cells is removed by the hexose monophosphate shunt with associated glutathione reductase and glutathione peroxidase in red cells with inactivated catalase.

Increases in the flux of glucose metabolism through the hexose monophosphate shunt and glycolysis can be used as an estimate of intracellular formation of superoxide, H_2O_2 and organic free radicals. Increases in the flux through the hexose monophosphate shunt are particularly informative since this pathway acts to maintain glutathione and is especially important in removal of H_2O_2 by glutathione peroxidase. Control studies on the flux of glucose metabolism in red cells containing oxyhemoglobin, carbonmonoxyhemoglobin or methemoglobin showed that there is a 3-fold increase in the hexose monophosphate shunt in red cells containing methemoglobin compared to red cells containing oxyhemoglobin. It has been suggested that the increase in flux in red cells containing methemoglobin results mainly from the multiple washing required to rid the red cells of nitrite used to convert oxyhemoglobin to methemoglobin [28]. In our laboratory, however, multiple washings of control red cell preparations did not increase flux through the hexose monophosphate shunt. The activity of the methemoglobin reductase system may account for part of these increases in flux in red cells containing methemoglobin.

Since both 1, 4-naphthoquinone-2-sulfonic acid and 6-hydroxydopamine are sources of not only H_2O_2 but also superoxide, quinols, semiquinones and quinones, controls were carried out with H_2O_2 alone. The removal of H_2O_2 by the red cell is very efficient even in the absence of glucose [29]. When the red cell is challenged with H_2O_2 alone, no accumulation of Compound II and no changes in hemoglobin content are observed in a 90-min incubation period. Accumulation of Compound II and changes in hemo-

globin seem to require the presence of reactive organic species and their products: quinols, semiquinones, quinones and superoxide. In particular, the conversion of Compound I to Compound II requires a one-electron donor. In the presence of glucose, H_2O_2 causes the expected increase in the flux through the hexose monophosphate shunt to maintain levels of reduced glutathione [29]. It has been observed previously that the hexose monophosphate shunt is capable of a much greater response to oxidative stress that glycolysis [27, 30]. The increase in flux through the hexose monophosphate shunt when red cells containing methemoglobin are incubated with H_2O_2 is double that of red cells containing oxyhemoglobin. The reason for this difference is not understood.

The effects of 1,4-naphthoquinone-2-sulfonic acid on the red cell can be understood by referring to its reaction with oxyhemoglobin. 1,4-Naphthoquinone-2-sulfonic acid reacts with oxyhemoglobin yielding methemoglobin, superoxide, H_2O_2, and the semiquinone and quinol derivatives [7]. In agreement with this reaction, when red cells containing oxyhemoglobin were incubated with 1, 4-naphthoquinone-2-sulfonic acid, methemoglobin formation was observed and the flux through the hexose monophosphate shunt increased. Intracellular H_2O_2 formation and the availability of one-electron donors resulted in the accumulation of the catalase intermediate, Compound II. Preparations of red cells containing carbonmonoxyhemoglobin had only small amounts of oxyhemoglobin (up to 20 per cent after a 90-min incubation) available for reaction with 1, 4-naphthoquinone-2-sulfonic acid, and therefore had much lower methemoglobin formation, accumulation of Compound II, and increase in flux through the hexose monophosphate shunt. 1, 4-Naphthoquinone-2-sulfonic acid can also react with sulfhydryl groups on methemoglobin yielding the semiquinone which can react further with methemoglobin to restore oxyhemoglobin [7]. In red cell preparations containing methemoglobin we did not observe oxyhemoglobin formation by reaction of 1, 4-naphthoquinone-2-sulfonic acid with methemoglobin or other cellular electron donors during the 90-min incubation period. However, the reaction of 1, 4-naphthoquinone-2-sulfonic acid with methemoglobin or other cellular donors produced enough free radicals or H_2O_2 to increase the flux through the hexose monophosphate shunt to 50 per cent of the increase observed in red cells containing oxyhemoglobin. More interesting was the observation that, in spite of this significant increase in flux through the hexose monophosphate shunt, no accumulation of Compound II was observed in red cells containing methemoglobin and incubated with 1, 4-naphthoquinone-2-sulfonic acid. This raises the possibility that methemoglobin has a protective effect on catalase activity.

6-Hydroxydopamine autoxidizes without requirement for red cell components. 6-Hydroxydopamine autoxidation yields superoxide, H_2O_2, hydroxyl radical, singlet oxygen and the quinone derivative with the semiquinone as a possible intermediate [11–14]. In the presence of glucose, the quinone resulting from 6-hydroxydopamine autoxidation may be reduced to the quinol by the products of glucose metabolism causing multiple cycles of autoxidation and H_2O_2 production [15–17]. For this reason, experiments where red cells were incubated with glucose required a smaller dose of 6-hydroxydopamine as a source of H_2O_2 and free radicals. Since oxyhemoglobin is not required for the autoxidation of 6-hydroxydopamine, incubation of red cells containing either oxyhemoglobin or carbonmonoxyhemoglobin with 6-hydroxydopamine resulted in equivalent Compound II accumulation and increases in flux through the hexose monophosphate shunt. More methemoglobin formation was observed in red cells containing oxyhemoglobin than in those containing carbonmonoxyhemoglobin because of the greater availability of heme groups on oxyhemoglobin for reaction with products of 6-hydroxydopamine autoxidation. With red cells containing methemoglobin, 6-hydroxydopamine caused the expected increase in flux through the hexose monophosphate shunt, but no Compound II accumulation was observed. The presence of methemoglobin had a protective effect on catalase activity possibly by scavenging one-electron donors which would otherwise cause the conversion of Compound I to Compound II. It was also observed that in the presence of 6-hydroxydopamine significant quantities of methemoglobin were restored to oxyhemoglobin at a rate much faster than the maximum rate of the methemoglobin reductase system. Methemoglobin is known to have peroxidatic activity *in vitro* [4] which presumably proceeds through one-electron step intermediates related to the peroxidatic intermediates, Compound I and Compound II. Methemoglobin may scavenge intracellular one-electron donors by this mechanism and thereby protect catalase activity. The restoration of oxyhemoglobin as a by-product of this reaction is equivalent to the formation of the peroxidatic intermediate, Compound III [31].

With substances like 1, 4-naphthoquinone-2-sulfonic acid and adriamycin [32], intracellular oxyhemoglobin can accelerate the formation of toxic substances with resultant methemoglobin formation. Our results show that methemoglobin, once formed, may scavenge toxic intermediates by functioning peroxidatically, restoring the oxyhemoglobin content of the red cell as a by-product of peroxidatic activity. It has been shown previously that catalase may prevent methemoglobin formation in the red cell [24–27, 29]. Our results with 1, 4-naphthoquinone-2-sulfonic acid and 6-hydroxydopamine indicate that the presence of methemoglobin may prevent accumulation of catalase Compound II and loss of catalatic activity.

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