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# EFFECT OF THE REDOX STATE OF THE RED BLOOD CELL COMPONENTS ON THE INACTIVATION OF GLUTATHIONE PEROXIDASE BY DIVICINE

MARIA ROSA CIRIOLO†, IRENE MAVELLI†† and GIUSEPPE ROTILIO†

†Department of Biology, 2nd University of Rome and CNR Center of Molecular Biology, University of Rome "La Sapienza", Rome (Italy)

††Institute of Applied Biochemistry, University of Rome "La Sapienza", Rome (Italy)

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The redox state of red blood cell components was found to have profound effects on the specific inactivation of erythrocyte glutathione (GSH) peroxidase by divicine, a hydroquinone imine molecule of fava beans likely to be responsible, through redox cycling, of the oxidative damage of red blood cells ultimately resulting in the hemolysis of favism. Oxidation of hemoglobin is a necessary step for the inactivation to take place, apparently as a $H_2O_2$-MetHb adduct. On the other hand, the presence of either reduced NADP or glutathione enhances the inactivating effect although NADPH inhibits the oxidation of hemoglobin, and this suggests a catalytic role for MetHb in the inactivation process.

Key words: Red Blood Cells, Methemoglobin, Glutathione Peroxidase, Divicine, Oxidative Damage

## INTRODUCTION

The cell employs several lines of defence against the toxic products of oxygen reduction such as $O_2^-$ and $H_2O_2$. The most important one appears to be a proper level of activity of the enzymes, that are competent to lower the steady state concentration of these species, and are located at or near the site where they are generated. Human pathology deserves a special consideration in this respect. Favism is a hemolytic disease affecting some glucose-6-phosphate dehydrogenase-deficient individuals, when they eat fava beans. An apparently acute decrease of GSH-peroxidase activity has been shown to occur during the hemolytic crisis[1]. In the search for a toxic factor of the beans, which may selectively discriminate glucose-6-phosphate dehydrogenase-deficient red blood cells, it has been suggested that the $\beta$-glucosides vicine and convicine[2] could serve as the source of damage. Vicine and convicine are $\beta$-glucosides of two pyrimidine aglycones, divicine and isouramil respectively, which are very reactive and unstable upon air exposure. In particular they undergo autooxidation of the native hydroquinonic species, producing $H_2O_2$ and the quinonic form of the aglycone. The latter species can be converted back to the hydroquinone by reduction. Even



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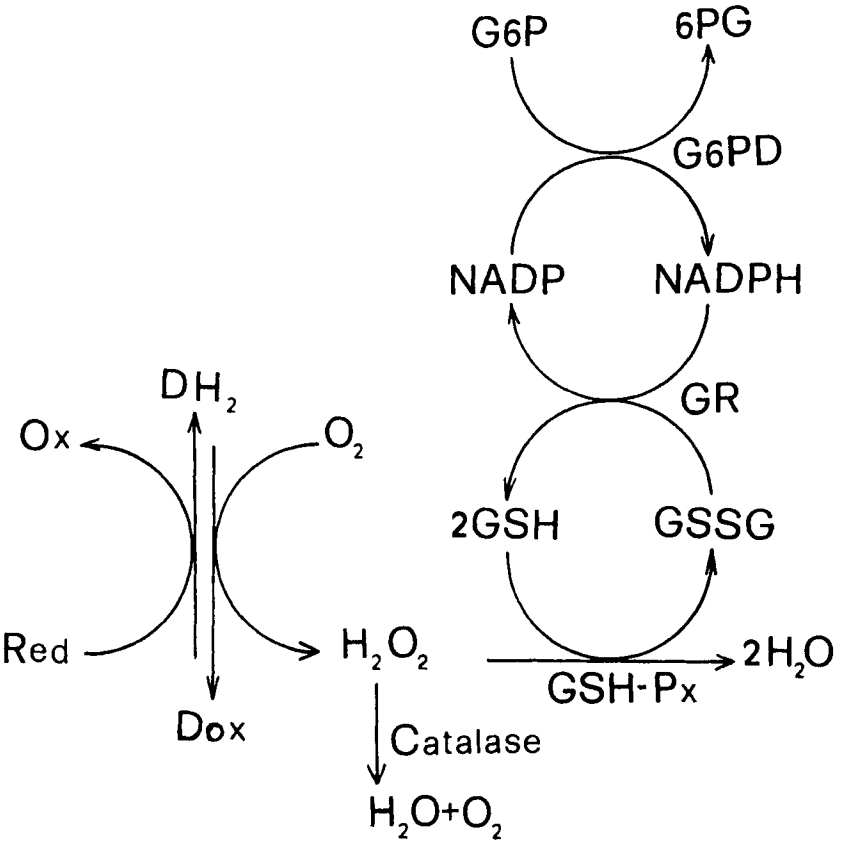


FIGURE 1 Mechanism of action of the fava pyrimidines in the red blood cell.

$DH_2$ = reduced divicine or isouramil
$D_{ox}$ = oxidized divicine or isouramil
G6PD = glucose-6-phosphate dehydrogenase
GR = glutathione reductase
GSH-Px = glutathione peroxidase.

catalytic amounts of the aglycone may become an indefinite source of peroxide if a large excess of reducing agents, such as ascorbate, is present to keep the redox cycle going for a long time[3].

Protection against the toxic effects of peroxides depends on the efficiency of the enzymes, such as GSH-peroxidase and catalase, which remove the damaging agents (Figure 1). The reactions of peroxides are potentially noxious to dehydrogenase-deficient red blood cells, which fail to increase their hexose monophosphate shunt activity because of glucose-6-phosphate dehydrogenase deficiency. On the other hand, GSH-peroxidase activity decrease occurred only in the favic individuals during crisis[1] and seems therefore to be specifically linked to the hemolytic event. Moreover the treatment of normal red blood cells with divicine plus L-ascorbate resulted in a significant inactivation of GSH-peroxidase and hemoglobin oxidation[4]. The purpose of the present investigation is to study the role of the oxidation state of hemoglobin on

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the response of the $H_2O_2$-removing enzymes. In addition the effect of reducing agents naturally present in the red blood cell, such as GSH and NADPH, was considered in relation to the possibility that they may interact with either the drug or the enzymes.

## MATERIALS AND METHODS

GSH and NADPH were products of Boehringer Mannheim. Vicine was from Serva, and L-ascorbic acid was from Fluka AG, Buchs, Switzerland. Catalase and β-glucosidase were obtained from, Sigma chemical Co., St. Louis, MO. Superoxide dismutase was prepared from bovine erythrocytes[5].

Heparinized blood was centrifuged at 2500 rpm for 10 min. at 5°C and the plasma was carefully separated. The erythrocytes pellet was washed three times with cold phosphate-buffered saline, care being taken in removing the buffy coat at every washing. Washed red blood cells were incubated in phosphate-buffered saline containing 5 mM glucose (5% v/v), at 37°C in a shaking water bath. Hemolysates were obtained by sonication of 5% v/v suspensions of red blood cells.

Preparation of MetHb-containing red blood cells involved their incubation for 20 min. with 0.2 M $KNO_2$, in phosphate-buffered saline containing 5 mM glucose, followed by several repeated washings with phosphate-buffered saline to remove $NO_2^-$ and $NO_3^-$. Conversion of oxyhemoglobin into CO-hemoglobin was obtained by repeated deareation-equilibration cycles of packed red blood cells with CO in a Thumberg tube followed by dilution of the cells with aerated buffer. Superoxide dismutase was measured by a polarographic method[6], with an Amel (Milan, Italy) polarographic unit, model 465. Catalase activity was assayed by an UV method[7]. GSH-peroxidase activity was determined spectrophotometrically with t-butyl hydroperoxide[8] or $H_2O_2$[9] as substrates. Units of these two enzymes were expressed as U = moles/l substrate transformed. $min^{-1}$. mg $Hb^{-1}$. Both these enzyme activities were assayed in a Perkin-Elmer model Lambda 3 spectrophotometer. Hemoglobin concentration was determined as the cyano-met derivation[10].

## RESULTS

GSH-peroxidase was considerably inactivated upon addition of divicine plus L-ascorbate to intact normal erythrocytes and, concomitantly oxyhemoglobin was oxidized[4]. Figure 2 shows that the rate of oxyhemoglobin oxidation follows first-order kinetics. The kinetics of inactivation of GSH-peroxidase were first-order only in the final part, while in the first hour of incubation enzyme activity was affected only slightly.

The correlation between the increase of MetHb content and the inactivation of GSH-peroxidase activity was demonstrated by

i) prevention of both effects when hemoglobin was converted into CO-hemoglobin before treatment of intact red cells with divicine and L-ascorbate,

ii) a consistently enhanced extent of GSH-peroxidase inactivation in nitrite-pretreated red blood cells (Table I).

Under the conditions used divicine had a significant effect only in the presence of equimolar amounts of L-ascorbate. In fact other reducing agents, like NADPH and

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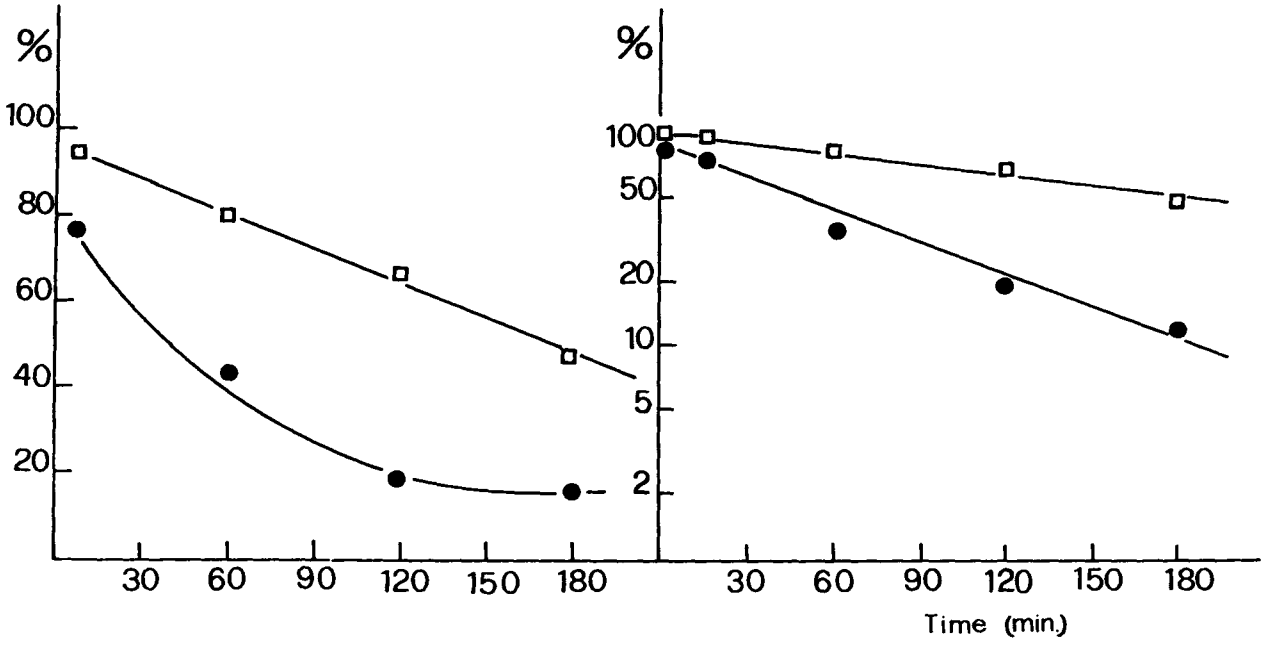


RE 2 Time course of the oxidation of oxyhemoglobin (•) and inactivation of GSH-peroxidase (□) upon incubation of red blood cells with divicine
rbate (for conditions see test).
el B shows the first-order plots of data of panel A.

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TABLE 1

rrelation between the effect of divicine and ascorbate on the GSH-peroxidase activity of human red blood cells and the ligation state of hemoglobi

| TING Hb TION STATE | $HbO_2$ | | MetHb | | COHb | |
|---|---|---|---|---|---|---|
| | MetHb % total Hb | GSH-PEROXIDASE % activity | MetHb % total Hb | GSH-PEROXIDASE % activity | MetHb % total Hb | GSH-PEROXIDA % activity |
| dition | 5 ∓ 3 | 100 | 70 ∓ 8 | 100 | 6 ∓ 1 | 100 |
| ne added | 4 ∓ 2 | 93 ∓ 1 | 66 ∓ 7 | 90 ∓ 3 | 6 ∓ 1 | 100 |
| ne + orbate added | 44 ∓ 20 | 72 ∓ 2 | 82 ∓ 17 | 47 ∓ 5 | 9 ∓ 2 | 100 |

l cell suspensions (5% v/v) were incubated under gentle stirring for 3 h at 37°C. Divicine = 2 mM vicine + 11 U/ml β-glucosidase. scorbic acid was 2 mM. Values are expressed as the means ∓ SD (n = 5).

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TABLE II
Effect of reducing agents on GSH-peroxidase inactivation and hemoglobin oxidation by divicine in red blood cells (A) and hemolysates[a] (B).

| ADDITIONS | A | | B | |
|---|---|---|---|---|
| | MetHb % total Hb | GSH-PEROXIDASE % activity | MetHb % total Hb | GSH-PEROXIDASE % activity |
| none | 5 ∓ 3 | 100 | 4 ∓ 3 | 100 |
| divicine (D) | 4 ∓ 2 | 93 ∓ 1 | 28 ∓ 16 | 58 ∓ 2 |
| D + L-ascorbate (A) | 44 ∓ 20 | 72 ∓ 2 | 75 ∓ 28 | 39 ∓ 9 |
| D + GSH | 18 ∓ 7 | 87 ∓ 2 | 58 ∓ 21 | 48 ∓ 2 |
| D + NADPH | 10 ∓ 5 | 94 ∓ 3 | 21 ∓ 5 | 44 ∓ 1 |
| D + A + GSH | 94 ∓ 20 | 61 ∓ 7 | 95 ∓ 22 | 96 ∓ 14 |
| D + A + NADPH | 68 ∓ 21 | 64 ∓ 5 | 52 ∓ 24 | 58 ∓ 13 |

GSH, NADPH, L-ascorbic acid concentrations were 2 mM. Red blood cells were incubated as in Table I, while [a]hemolyzed red blood cells were incubated for 2 h at 37°C.

GSH scarcely enhanced the effects of divicine (Table IIA). Apparently the reduction of oxidized divicine was essential to both inactivation of GSH-peroxidase and MetHb formation, and was significant only when an appropriate reducing agent, such as L-ascorbate, was capable of indefinitely maintaining a redox cycle with divicine.

In contrast, in hemolysates, oxidation of the hemoglobin and inactivation of GSH-peroxidase took place with divicine alone and these effects were augmented by addition of either L-ascorbate or GSH (Table IIB). However when both reducing agents were added full protection of GSH-peroxidase was obtained in spite of a high extent of hemoglobin oxidation. In the presence of NADPH the MetHb was much less although GSH-peroxidase was inactivated to approximately the same extent as with GSH and L-ascorbate.

The hemolysate incubations caused a significant decrease of catalase activity as well, which was specifically prevented by addition of NADPH (Table III).

Both GSH-peroxidase inactivation and MetHb formation were significantly prevented by externally added catalase, while superoxide dismutase was ineffective, indicating an important role for $H_2O_2$ in the process.

TABLE III
Effect of reducing agents on catalase inactivation by divicine in hemolyzed red blood cells.

| ADDITIONS | CATALASE % activity |
|---|---|
| none | 100 |
| divicine (D) | 55 |
| D + L-ascorbate (A) | 60 |
| D + GSH | 53 |
| D + NADPH | 105 |
| D + A + GSH | 52 |
| D + A + NADPH | 100 |

The values reported are the results of one experiment and the extent of the changes observed was confirmed by five separated experiments. Conditions as in Table II.

Concentrations of reagents were the same as in Table II.

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In all experiments reported the red blood cell superoxide dismutase activity was never affected.

## DISCUSSION

It has previously been reported[4] that in intact red blood cells divicine plus L-ascorbate induced hemoglobin oxidation and GSH-peroxidase inactivation, while neither superoxide dismutase nor catalase activity were substantially modified under the same conditions. The involvment of $H_2O_2$ in determining the effects observed is indicated by the prevention of GSH-peroxidase inactivation and MetHb formation by externally added catolase. When $H_2O_2$ is formed in the reaction of divicine with oxygen (Figure 1), the pyrimidine moiety of the molecule loses two hydrogens and forms a very unstable intermediate that decomposes in the absence of reducing agents, while in the presence of reducing agents the intermediate is reduced back to the hydrogenated pyrimidine and can now react with oxygen once again[3]. The results of the present work emphasize the role of an appropriate reducing agent to establish a redox cycle between the two states of the pyrimidine moiety of divicine. In fact L-ascorbate in its oxidized form (dehydroascorbate) can rapidly enter the cell, where it takes up reducing equivalents which appear to come from NADH[11]. Once reduced, it leaves the cell as ascorbate at a constant rate[12], then becoming available for another redox cycle. This is apparently not the case for externally added GSH and NADPH (Table IIA), while intracellular GSH and NADPH seem to be enough to sustain this cycle to a certain extent (Table IIB).

The inactivation of GSH-peroxidase appears to be clearly related to the formation of MetHb. In fact substantial protection against GSH-peroxidase inactivation and MetHb formation was obtained in CO-saturated erythrocytes, while prior transformation of hemoglobin into MetHb increased GSH-peroxidase inactivation (Table I). The kinetics of oxidation of oxyhemoglobin and inactivation of GSH-peroxidase also suggest that the primary effect of divicine plus L-ascorbate is MetHb formation. The delay of enzyme inactivation with respect to hemoglobin oxidation indicate that a critical amount of MetHb has to be present to initiate the inactivation process. This clearly reflects the damage threshold posed by the natural enzyme defenses present in the red blood cells. A peroxidizing adduct between $H_2O_2$ and MetHb or MetHb derivative may be a likely candidate for the damaging species[13,14]. Such systems are capable of altering several amino acid residues including tyrosine, tryptophan and cysteine in vitro[15]. On the other hand a proper coordination of hemin with a susceptible target protein, in the presence of reducing agents and peroxides can selectively damage the protein by site-directed $OH^{\cdot}$ production[16].

In the experiments with hemolysates also catalase was inactivated and this inactivation was prevented by NADPH. This confirms the protective effect of NADPH on catalase exposed to $H_2O_2$ or $H_2O_2$ sources[17,18,19].

When both GSH and L-ascorbate were present in the experiments with hemolysates, their effects on MetHb formation apparently added to each other, while GSH-peroxidase was protected. It seems that only when another reducing agent is preferentially engaged in the redox cycle of divicine, GSH may interact with the enzyme in a way that will enhance its activity against $H_2O_2$. When however GSH is limiting, some activity-linked amino acid residues of the enzymes, perhaps the active site selenocysteine, are more prone to oxidation[20]. In the experiments with hemolysates

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NADPH alone potentiated only the action of divicine on GSH-peroxidase inactivation but not that on hemoglobin oxidation. The most likely explanation is that NADPH readily reduced MetHb, via MetHb reductase, and this reaction can be responsible for the low content of MetHb measured in our experiments. This experiment indicates that catalytic amounts of MetHb are required, the inactivation extent being comparable to that observed with equivalent concentrations of other reducing agent leading to much higher MetHb formation (see Table II).

In conclusion, the exposure of red blood cells to divicine clearly imposes a challenge on their redox balance, which may be particularly detrimental to cells deficient of enzymes of the NADPH-regenerating cycles, such as glucose-6-phosphate dehydrogenase. Under such conditions inactivation of GSH-peroxidase has been shown to be a likely event and this will impair detoxification of $H_2O_2$ and/or lipid hydroperoxides. An unusual degree of accessibility of deficient red blood cells to the fava bean aglycones in some glucose-6-phosphate dehydrogenase-deficient individuals may therefore be an important factor in determining the susceptibility to hemolysis of the deficient red blood cells of favic patients.

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