DECREASED GLUTATHIONE CONTENT OF HUMAN ERYTHROCYTES PRODUCED BY METHYL PHENYLAZOFORMATE[1]

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We report here that methyl phenylazoformate (I) produces a rapid decrease in the reduced glutathione (GSH) content of human erythrocytes. One may also infer from our results a probable mechanism by which phenylhydrazine (II) and acetylphenylhydrazine (III) cause a diminution in the GSH content of red blood cells.

$C_6H_5N{=}NCOOCH_3$ (I) $C_6H_5NHNH_2$ (II) $C_6H_5NHNHCOCH_3$ (III)

The oxygen requirement for the action of phenylhydrazine and acetylphenylhydrazine suggested that an oxidized form of II (or III) was the effective reagent. We had found that the ester I was an excellent substrate for chymotrypsin (1), demonstrating its biological potential. Further, the acid derived from the ester by hydrolysis is extremely unstable, with an estimated half-life of 0.5 seconds at pH 7.4. (2) An intermediate, phenyldiimide ($C_6H_5N{=}NH$) is readily derived from all three sources (I, II, and III) and provides a common pathway for their action as shown in Eqs. 1-3.

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(1) $C_6H_5NHNH_2 + O_2 \rightarrow C_6H_5N{=}NH + H_2O_2$

(2) $C_6H_5NHNHCOCH_3 + O_2 \rightarrow H_2O_2 + C_6H_5N{=}NCOCH_3 \xrightarrow{H_2O} C_6H_5N{=}NH + CH_3COOH$

(3) $C_6H_5N{=}NCOOCH_3 \xrightarrow{H_2O} CH_3OH + C_6H_5N{=}NCOOH \rightarrow CO_2 + C_6H_5N{=}NH$

Phenyldiimide may be further oxidized by oxygen to yield benzenediazonium ion. (Eq. 4) (2)

(4) $C_6H_5N{=}NH + O_2 + H^+ \rightarrow H_2O_2 + C_6H_5N{\equiv}N^+$

The addition of the azoester I to a suspension of red blood cells leads to the changes in erythrocyte GSH content shown in Fig. 1. The

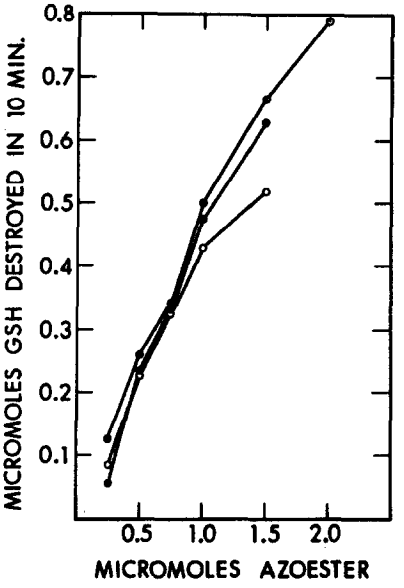

Fig. 1

A plot of micromoles of GSH destroyed against micromoles of azoester I used for blood from three different individuals. Methyl phenylazoformate was dissolved in dimethylsulfoxide (DMSO) and diluted with 0.1 M sodium chloride-0.1 M glycylglycine (GG) buffer (pH 7.4) to yield an azoester solution in 6% DMSO-GG. (This solution should be used as soon as possible.)

Suspensions of washed normal R.B.C. made with sodium chloride-GG were mixed with an equal volume of azoester solution to give a final concentration of 35% R.B.C. in 3% DMSO. Reaction was allowed to proceed at 4° C. and GSH was determined according to the method of Beutler (3).

data in the figure demonstrate that 1 micromole of azoester I causes the loss of approximately 0.5 micromole of GSH. The effect of the azoester on the GSH content of the cells is rapid and about 80-90% of the GSH loss occurs within ten minutes at 4° C. Preliminary experiments indicate that less GSH disappears when the experiment is carried out under nitrogen. Methemoglobin formation is shown by the appearance of a cyanide-sensitive absorption band at 6300 A. Accurate measurement of the degree of methemoglobin formation is precluded by the formation of other products which absorb in this region of the spectrum. The formation of oxidized glutathione, GSSG, is demonstrated by the regeneration of GSH in azoester treated red blood cells after incubation with glucose at 37° C. (Table I) Other aspects of GSH regeneration will be treated in a separate communication. (4)

Table 1

Regeneration of GSH in Normal Erythrocytes[a]

Experiment No.	Azoester	GSH (mg./100 ml. RBC) before incubation	after incubation +glucose[b]	after incubation -glucose[c]
1	+	8	54	9
	-	64	61	52
2	+	2	46	4
	-	51	53	42
3	+	1	76	
	-	88	88	
4	+	0	56	0
5	+	1	48	5
6	+	0	54	1

[a] Suspensions of washed normal red blood cells were treated with azoester I (1.8 - 2.2 μmole/ml. incubation mixture) as described under Figure 1, then incubated for 2 hours at 37°.

[b] Incubated with glucose, 11 μmoles/ml. incubation mixture

[c] Incubated without glucose

Tetravalent iron has been proposed as an intermediate in the divalent iron-hydrogen peroxide reaction (Fe^{++}-H_2O_2) by Cahill and Taube (5), but Sutin and his coworkers failed to detect the trivalent iron dimer, $(FeOH)_2^{++++}$, found in other reactions in which Fe^{++++} is a probable intermediate. (6) We have, for this reason, chosen a divalent iron-peroxide complex in the reaction scheme designed to explain our results, rather than the tetravalent iron (Fe^{++++}) which should easily be derived from such a complex.

To rationalize the effect of the azoester I on the GSH content of red cells, a mechanism, outlined in Eqs. 5-10, is proposed. This mechanism includes enzymatic hydrolysis of the azoester (slow decomposition of the ester in pure buffer, generation of the active species within the red cell), sensitivity of phenyldiimide to oxidizing agents (i.e., phenyldiimide is a strong reducing agent), stoichiometric relationship of azoester added to GSH loss, and the formation of methemoglobin and GSSG.

(5) $C_6H_5N{=}NCOOCH_3 \xrightarrow[\text{esterase}]{H_2O} CH_3OH + C_6H_5N{=}NCOOH \xrightarrow{\text{rapid}} C_6H_5N{=}NH$

(6) $Fe^{++}O_2 + C_6H_5N{=}NH \longrightarrow Fe^{++}OOH^- + C_6H_5N{\equiv}N^+$
oxyhemoglobin — peroxyhemoglobin

(7) $Fe^{++}OOH^- + C_6H_5N{=}NH \rightarrow Fe^{+++} + C_6H_5\cdot + N_2 + 2\ OH^-$
methemoglobin; phenyl free radical

(8) $Fe^{++}OOH^- + GSH \longrightarrow Fe^{+++} + GS\cdot + 2\ OH^-$
glutathione free radical

(9) $C_6H_5\cdot + GSH \rightarrow C_6H_6 + GS\cdot$

(10) $2\ GS\cdot \longrightarrow GSSG$

It should be noted specifically that the reactive phenyl radical may abstract a hydrogen atom from some other species in the red blood cell, e.g., reduced pyridine nucleotides. Reduced glutathione would eventually provide a hydrogen atom for these secondary radicals, since

the GS· radical is one of the most stable that can form from the components of the cell.

Clearly, our mechanism poses a central role for the peroxyhemoglobin complex in the loss of GSH. Peroxyhemoglobin probably corresponds to the intermediate suspected by Beutler who established the importance of oxyhemoglobin in the loss of GSH caused by acetylphenylhydrazine. (7,8) The formation and decomposition of peroxyhemoglobin may be formulated in a general way to cover those cases in which a drug is, or readily forms by metabolic degradation, a powerful reducing agent, as shown in Eqs. (11)-(13).

(11) Drug $\longrightarrow$ RH (or NO_2^-, etc.)

two-electron donor (reducing agent)

(12) $RH + Fe^{++}O_2 \longrightarrow R^+ + Fe^{++}OOH^-$

oxyhemoglobin peroxyhemoglobin

(13) $Fe^{++}OOH^- + XH$ (which may be RH) $\rightarrow Fe^{+++} + 2\ OH^- + X.$

one-electron donor methemoglobin free radical

The role of free hydrogen peroxide (Eqs. 1,2; ref. 9) is less certain since it causes a smaller and slower loss of GSH from red cells than the azoester I. (10) Under certain conditions, hydrogen peroxide may react with hemoglobin to form peroxyhemoglobin. However, this reaction must compete with the reaction of hydrogen peroxide with catalase.

The stability of the Fe^{+++} species in vivo in the presence of various one-electron reducing agents is uncertain, but reduction to Fe^{++} will surely occur in some cases, as in the finding by Jandl and coworkers (11) that phenylhydrazine reduces methemoglobin.

Conclusions: The azoester I should be a useful reagent for studies on the relationship of GSH to various aspects of red cell metabolism (e.g.,12) and should also be considered for studies on the relationship of GSH to malaria in view of the recent finding (13) that phenylhydrazine (III) prolongs the survival of plasmodium-infected mice.

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