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Mechanism of Oxyhaemoglobin Breakdown on Reaction with Acetylphenylhydrazine

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The reaction of oxyhaemoglobin and acetylphenylhydrazine, which results in haemoglobin denaturation and precipitation, was found to be influenced by H_2O_2 and superoxide ($O_2^{-\cdot}$) generated during the reaction. By analysing the different haemoglobin oxidation products, it was found that by influencing the rate at which oxyhaemoglobin was oxidized, H_2O_2 accelerated the overall haemoglobin breakdown, and $O_2^{-\cdot}$ inhibited it. By adding GSH (reduced glutathione) or ascorbate, it was possible to slow down the rates of both oxyhaemoglobin oxidation and $O_2^{-\cdot}$ production, and the overall rate of haemoglobin breakdown. These results are compatible with a mechanism involving production of the acetylphenylhydrazyl free radical, and with GSH, ascorbate and $O_2^{-\cdot}$ acting as radical scavengers and preventing its further reactions. The reaction produced choleglobin, as well as acetylphenyldiazine and methaemoglobin, which combined to form a haemichrome. The haemichrome was less stable and precipitated first. It was also less stable than the haemichrome formed by direct reaction of acetylphenyldiazine with methaemoglobin, and it is proposed that this is because the methaemoglobin produced from oxyhaemoglobin and acetylphenylhydrazine was modified by the free radicals and H_2O_2 produced in the reaction.

Phenylhydrazine and acetylphenylhydrazine are typical of a wide range of drugs that react with oxyhaemoglobin via a redox mechanism in which both the haemoglobin and the drug are oxidized by the haemoglobin-bound oxygen. In glucose 6-phosphate dehydrogenase deficiency and other related disorders of erythrocyte metabolism, this results in precipitation of the haemoglobin as Heinz bodies and premature destruction of the cells. Previous investigators have identified a number of products of the reaction. It appears that the oxyhaemoglobin is initially oxidized to methaemoglobin (Beaven & White, 1954) and then to a mixture of choleglobin, a rather ill-defined species considered to have a hydroxy group attached to one of the methene bridges of the porphyrin, and sometimes referred to as 'sulphaemoglobin', and haemichrome (Jandl *et al.*, 1960; Peisach *et al.*, 1975; Itano, 1970; Itano *et al.*, 1975, 1977). The haemichrome formed is considered by Itano and his co-workers to involve direct binding of the oxidized drug (phenyldiazine), but Peisach *et al.* (1975) do not consider the diazine to be involved in this way.

There has been some debate as to whether the physical presence of the Heinz bodies, or attack by other reaction products on vulnerable sites in the cell, is the major cause of erythrocyte destruction. Cohen & Hochstein (1964) showed that the reaction produced H_2O_2, and there is good evidence that, if this is not sufficiently removed, cellular damage ensues (Mills, 1957). More recently, superoxide ($O_2^{-\cdot}$) production has been detected, and considered as another contributor to cell breakdown (Goldberg & Stern, 1975, 1977; Goldberg *et al.*, 1976). Interest in the effects of H_2O_2 and $O_2^{-\cdot}$ has tended to obscure the proposals of Kosower & Kosower (1969) that drug free radicals could be important. Support for this has come from Misra & Fridovich (1976), who found evidence for the production of the phenylhydrazyl radical during the autoxidation of phenylhydrazine. Goldberg *et al.* (1976), although proposing a different mechanism, also implicate free-radical intermediates in the reaction of phenylhydrazine with haemoglobin. However, there is very little information on how much these reactive intermediates contribute either to the breakdown of haemoglobin or to destruction of the erythrocytes. In the first instance we report here on the oxidation and precipitation of purified oxyhaemoglobin on reaction with acetylphenylhydrazine, and the roles of H_2O_2, $O_2^{-\cdot}$ and drug free radicals in the processes. We have also considered how the reaction produces both choleglobin and haemichrome, the nature of the haemichrome, and how much each contributes

Abbreviation used: GSH, reduced glutathione.

to the finally denatured haemoglobin. A preliminary report of this work has been published (Winterbourn & French, 1977).

Methods

Oxyhaemoglobin was purified from normal erythrocyte haemolysates by column chromatography on DEAE-Sephadex (Huisman & Dozy, 1965). No superoxide dismutase or catalase could be detected in the purified haemoglobin. Methaemoglobin was prepared from purified oxyhaemoglobin by the addition of a slight excess of potassium ferricyanide, which was subsequently removed by passage through a column of Sephadex G-25. Acetylphenylhydrazine was obtained from BDH, Poole, Dorset, U.K. Phenylhydrazine hydrochloride, catalase, superoxide dismutase, xanthine oxidase, xanthine, GSH, adrenaline, sodium ascorbate and gallic acid were all obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. All spectral measurements were performed on Beckman spectrophotometers, either model 25 or Acta C III.

Reactions of acetylphenylhydrazine with haemoglobin were carried out in air-saturated phosphate buffer, pH 7.4, prepared from 0.067M-KH_2PO_4 and 0.067M-Na_2HPO_4. Unless otherwise stated, the temperature was 22°C, and the solutions contained 2mM-acetylphenylhydrazine, 25μM-haemoglobin, 100μM-EDTA, and where indicated 67μg of catalase/ml or 6.8μg of superoxide dismutase/ml. Concentrations of oxyhaemoglobin, methaemoglobin and haemichrome were calculated from the absorbance of the solution at 560, 577 and 630nm and the molar absorption coefficient of each species at these wavelengths (Winterbourn *et al.*, 1976).

Before this calculation was made, the absorbance corresponding to choleglobin was subtracted. Since it was not possible to prepare a solution of pure choleglobin, this had to be determined indirectly. Oxyhaemoglobin was made to react with acetylphenylhydrazine, the choleglobin concentration measured by the method of Lemberg *et al.* (1941), and the spectra of the original oxyhaemoglobin solution and the final mixture determined, after adding cyanide and ferricyanide to each. It was assumed that all the species except choleglobin were converted into cyanmethaemoglobin, and the difference in absorbance of the two solutions was due to choleglobin. This gave a nearly flat choleglobin spectrum over the range 560–700nm, with a millimolar absorption coefficient of approximately 4. Choleglobin concentrations were estimated from A_{700}, where oxyhaemoglobin, methaemoglobin and haemichrome absorb very little, and this value was subtracted from the absorbances at the other wavelengths. In spite of the approximations used in determining the spectral contribution of choleglobin, the concentrations of the haemoglobin species determined by this method appeared to be reliable. Close agreement was always found between the initial oxyhaemoglobin concentration and the sum of the components present at any time.

In mixtures containing oxyhaemoglobin and cyanmethaemoglobin, their concentrations were calculated from A_{540} and A_{577} with millimolar absorption coefficients of 47.5 and 30.9 (cyanmethaemoglobin) and 61.2 and 66 (oxyhaemoglobin). Haemoglobin precipitation was measured as the difference in A_{700} before and after centrifuging the solution for 10min at 10000rev./min ($r_{av.}$ 6cm) at 4°C.

Exogenous $O_2^{-\cdot}$ or H_2O_2 was generated by adding 6.7μg of xanthine oxidase/ml, 1.67mM-xanthine, and either 67μg of catalase/ml or 6.8μg of superoxide dismutase/ml to the solution. Production of $O_2^{-\cdot}$ was detected by its reaction with adrenaline to give adrenochrome, inhibited by adding superoxide dismutase (Misra & Fridovich, 1972).

Haemichromes were prepared from phenylhydrazine and methaemoglobin (Mannen & Itano, 1973). For this 4mol of ferricyanide/mol of haem group were added to 20μM-oxyhaemoglobin in a tonometer. After evacuation the solution was mixed with 1mol of phenylhydrazine (which had been held in the lid)/mol of haem group. Rates of precipitation of haemichromes formed with phenylhydrazine were measured at 37°C, by continuously recording A_{700}. The reactions were carried out in 0.067M-phosphate buffer, pH 7.4, containing 0.1mM-EDTA.

Results and Discussion

Effects of H_2O_2 and $O_2^{-\cdot}$ on the oxidation of haemoglobin by acetylphenylhydrazine

Fig. 1(*a*) shows a series of spectra recorded during the reaction of acetylphenylhydrazine with oxyhaemoglobin. Characteristic changes are the increase in the shoulder at 630nm, predominantly due to methaemoglobin, the shallowing of the trough at 560nm, primarily due to haemichrome, and an increase in baseline absorbance due to choleglobin. Continued incubation resulted in precipitation, causing a further increase in baseline absorbance. As shown in Figs. 1(*b*) and 1(*c*), these changes occurred more slowly in the presence of catalase, and more rapidly in the presence of superoxide dismutase.

To determine which steps in the proposed sequence

Oxyhaemoglobin → methaemoglobin → haemichrome / choleglobin

were influenced by H_2O_2 and $O_2^{-\cdot}$, the effects of catalase and superoxide dismutase on the concen-

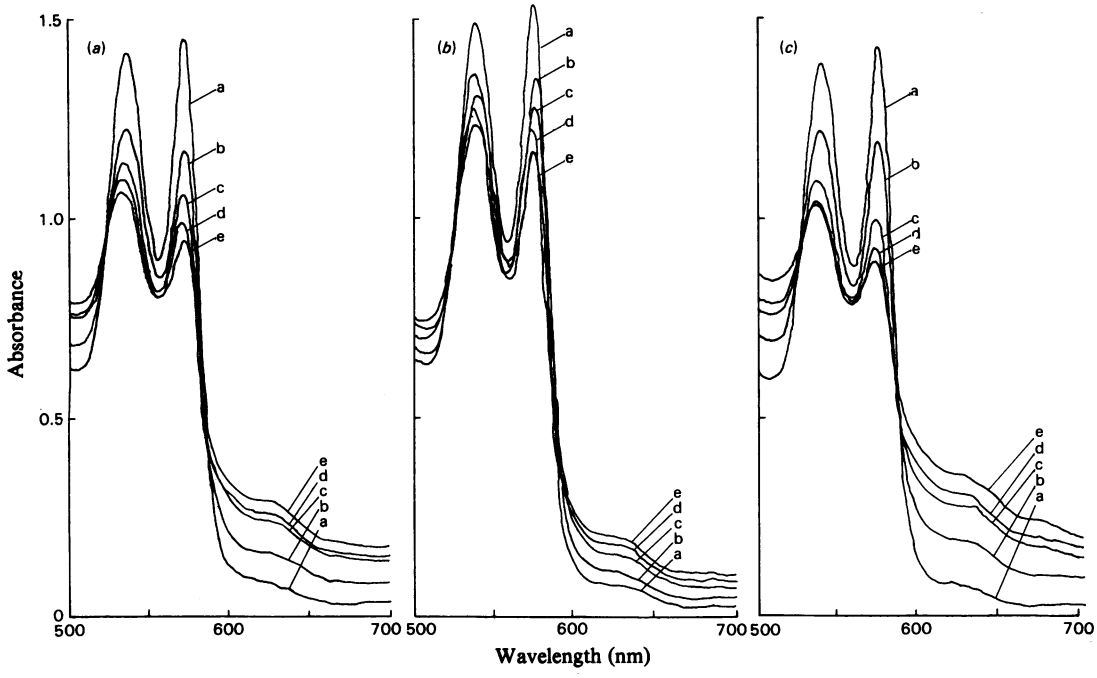

Fig. 1. *Spectra recorded during the reaction of oxyhaemoglobin with acetylphenylhydrazine, (a) with no additions, (b) plus catalase and (c) plus superoxide dismutase*

Details are given in the Methods section. Spectra were recorded at 6 min (a), and subsequently at 16 min intervals (b–e); total haemoglobin concentration 25 μM; path-length 1 cm.

tration changes of the haemoglobin species were considered. Addition of catalase to remove H_2O_2 generated during the reaction slowed down the rate of disappearance of oxyhaemoglobin to about a half, either in the presence of superoxide dismutase (Fig. 2*a*) or in its absence. Higher concentrations of H_2O_2 generated exogenously from xanthine and xanthine oxidase accelerated oxidation. Addition of catalase eliminated the xanthine oxidase effect. However, addition of superoxide dismutase to remove $O_2^{-\bullet}$ generated during the reaction increased the rate of oxyhaemoglobin oxidation about 1.5-fold, and exogenously generated $O_2^{-\bullet}$ slowed it down. The proportional increase in rate was approximately the same in the presence (Fig. 2*b*) or in the absence of catalase.

Superoxide dismutase also increased the rate of oxidation of oxyhaemoglobin when sufficient cyanide was present to convert the methaemoglobin formed into cyanmethaemoglobin, but not to inhibit the enzyme. Superoxide dismutase does not therefore act by preventing the reduction of methaemoglobin by $O_2^{-\bullet}$ (Winterbourn *et al.*, 1976). The effect of catalase is most likely to prevent further oxidation of oxyhaemoglobin by H_2O_2.

Removal of H_2O_2 decreased the rates of formation of haemichrome and choleglobin and the rate of precipitation (Table 1), and removal of $O_2^{-\bullet}$ increased these rates. The [choleglobin]/[haemichrome] ratio appeared to remain constant, between 0.63 and 0.80, during the course of the reaction, and to be independent of whether H_2O_2 or $O_2^{-\bullet}$ was removed or added exogenously. The haemichrome concentration was proportional to the total amount of methaemoglobin formed (i.e. the total concentration of methaemoglobin, haemichrome and choleglobin), regardless of whether $O_2^{-\bullet}$ or H_2O_2 was removed or added exogenously. This indicates that neither $O_2^{-\bullet}$ nor H_2O_2 participates in the conversion of methaemoglobin into haemichrome, and they influence the overall rate of haemoglobin breakdown by affecting only the first step of the above sequence.

Since catalase did not prevent choleglobin formation, free H_2O_2 must not be necessary for the hydroxylation of one of the methene bridges of the porphyrin in the acetylphenylhydrazine and oxyhaemoglobin reaction. We therefore propose that the initial oxidation product is a methaemoglobin–H_2O_2 complex formed by a one-electron donation from acetylphenylhydrazine to oxyhaemoglobin.

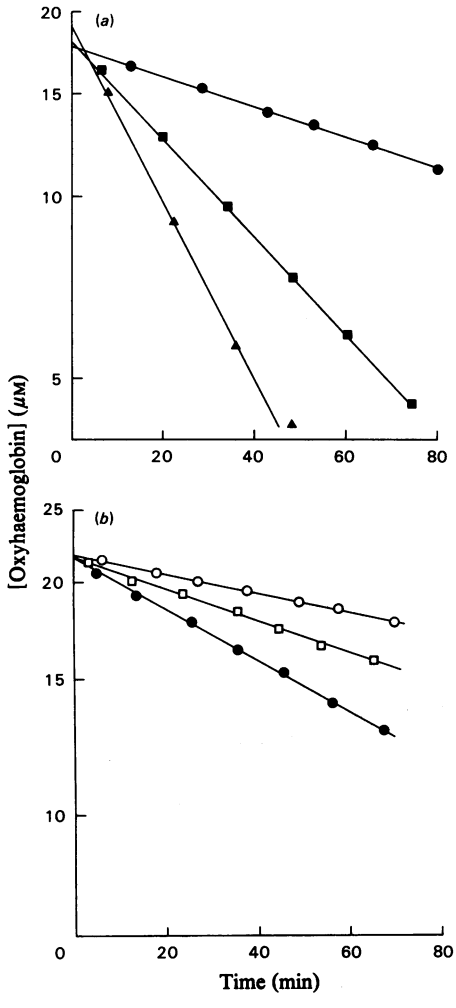

Fig. 2. *Semi-logarithmic plots of the rate of oxidation of oxyhaemoglobin by acetylphenylhydrazine*
Details are given in the Methods section. (*a*) Effect of varying the H_2O_2 concentration. All solutions contained superoxide dismutase; ■, no other additions; ●, plus catalase; ▲, plus xanthine and xanthine oxidase. (*b*) Effect of varying the $O_2^{-\cdot}$ concentration. All solutions contained catalase: □, no other additions; ●, plus superoxide dismutase; ○, plus xanthine and xanthine oxidase.

This would be similar to the complex that methaemoglobin forms with H_2O_2 when functioning as a peroxidase (Shiga & Imaizumi, 1975). Choleglobin formation can be considered as an internal peroxidation, with the haemoglobin-bound oxygen being sufficiently activated to attack a sterically favourable methene bridge. This is similar to the mechanism proposed by Brown (1976). Our results suggest that the complex can either break down to choleglobin, or dissociate to give methaemoglobin and H_2O_2. To account for the apparently constant proportions of choleglobin and haemichrome formed even when exogenous H_2O_2 is added, the dissociation must be reversible. Again, this is analogous to the peroxidase mechanism.

The superoxide produced from acetylphenylhydrazine and oxyhaemoglobin was detectable by its reaction with adrenaline. Increasing the pO_2, without significantly altering the extent of haemoglobin oxygenation, increased the rate of adrenaline oxidation, supporting the conclusions of Goldberg *et al.* (1976) and Misra & Fridovich (1976) that the source of $O_2^{-\cdot}$ is free rather than haem-bound O_2.

Effect of free-radical scavengers on oxyhaemoglobin oxidation and $O_2^{-\cdot}$ production

The $O_2^{-\cdot}$-dependent oxidation of adrenaline by oxyhaemoglobin was virtually eliminated by adding GSH. Ascorbate also inhibited $O_2^{-\cdot}$ production, but urate, xanthine and gallic acid all had no effect. The rate of oxidation of oxyhaemoglobin in the presence of acetylphenylhydrazine was markedly decreased by adding GSH (Fig. 3). Optimal concentrations of catalase and superoxide dismutase were present to eliminate any reactions of GSH with H_2O_2 or $O_2^{-\cdot}$. Under similar conditions, 0.3mM-ascorbate almost completely inhibited oxyhaemoglobin oxidation. Parallel experiments showed that reduction of the methaemoglobin formed during the reaction by GSH or ascorbate was too slow to account for the observed effects.

The results are compatible with GSH and ascorbate acting as scavengers for the acetylphenylhydrazyl radical, and preventing its reacting further with O_2 and oxyhaemoglobin. This is the most likely radical to be involved. Misra & Fridovich (1976) have proposed its formation during phenylhydrazine autoxidation, and by using e.s.r. and spin traps we have been able to detect the phenylhydrazyl rather than the phenyl radical during the reaction of phenylhydrazine with oxyhaemoglobin (C. C. Winterbourn & B. Gilbert, unpublished work). The alternative mechanism, with GSH acting to reduce acetylphenyldiazine (Kosower *et al.*, 1969) and preventing its reacting with O_2 to produce $O_2^{-\cdot}$ and phenyl radicals, as proposed by Goldberg *et al.* (1976), therefore seems less likely.

The ability of ascorbate to prevent oxidation of oxyhaemoglobin almost entirely cannot be explained solely by its acting as a radical scavenger. Reduction of methaemoglobin by ascorbate radicals may be a contributing factor. For a free-radical scavenger to be effective, the energetics of its reaction with a radical must be favoured over the reactions of its competitors. The reason why gallate and urate were

Table 1. *Oxyhaemoglobin and acetylphenylhydrazine: effects of catalase and superoxide dismutase on the formation of choleglobin, haemichrome and precipitate*

Oxyhaemoglobin (25 μM) was incubated with acetylphenylhydrazine (2 mM). Further details are given in the Methods section. Choleglobin and haemichrome concentrations were measured in experiments carried out at 22°C in which there was no precipitation. Precipitation was measured in separate experiments at 37°C. ΔA_{700} is the absorbance difference before and after centrifugation at 3000 rev./min for 5 min.

Enzymes added	[Choleglobin] (μM)		[Haemichrome] (μM)		Precipitation (ΔA_{700})
	32 min	65 min	32 min	65 min	65 min
None	1.7	2.9	2.2	3.7	0.54
Catalase	0.6	1.6	0.9	2.0	0.02
Superoxide dismutase	2.0	3.3	3.2	4.9	1.19
Catalase+superoxide dismutase	1.4	2.4	1.8	3.0	

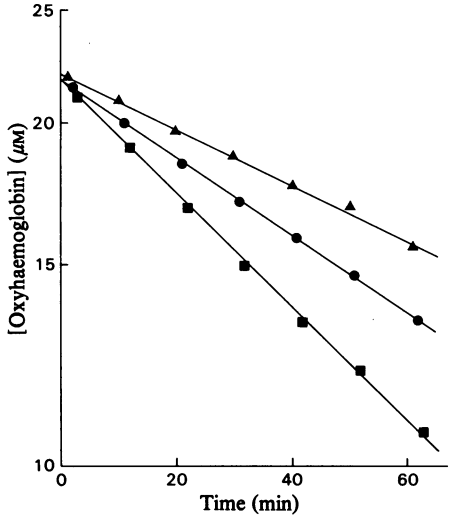

Fig. 3. *Semi-logarithmic plot of the rate of oxyhaemoglobin oxidation by acetylphenylhydrazine, and the effect of GSH*

Each solution contained oxyhaemoglobin (25 μM), acetylphenylhydrazine (2 mM), catalase and superoxide dismutase. Other details are given in the Methods section. ■, No GSH; ●, 33 μM-GSH; ▲, 100 μM-GSH.

ineffective may be that neither can compete with O_2 or oxyhaemoglobin for the acetylphenylhydrazyl radical. Alternatively they may react, but to give other radicals that also react with O_2 or oxyhaemoglobin. GSH and ascorbate may be particularly effective because both radicals show a strong preference for reacting with themselves (Bielski & Richter, 1975). It seems likely that $O_2^{-\cdot}$ also acts in the acetylphenylhydrazine–haemoglobin reaction as a free-radical scavenger. It is known to react with a number of radicals (Lee-Ruff, 1977), and such a role for $O_2^{-\cdot}$ would explain why its removal by superoxide dismutase accelerates haemoglobin oxidation.

Properties of haemichromes formed with phenyldiazine

Although phenyldiazine is very unstable in the presence of oxygen (Huang & Kosower, 1968*b*), we found that in the presence of methaemoglobin it reacted to form a haemichrome that underwent only very slight spectral changes over 1 h after introduction of air at 22°C. This result supports the conclusions of Itano (1970) that the diazine is stabilized by binding to methaemoglobin, rather than those of Peisach *et. al.* (1975), who have questioned the role of diazines in haemichrome formation in view of their rapid decomposition in the presence of O_2. It is most likely that the diazine binds to the sixth co-ordinate position of the haem, as proposed by Itano *et al.* (1975, 1977), but binding to another site on the globin (Huang & Kosower, 1968*a*), allosterically aiding haemichrome formation with the distal histidine residue, is also possible.

This part of the work was performed with phenylhydrazine rather than acetylphenylhydrazine, because only phenylhydrazine is rapidly oxidized to the diazine by ferricyanide (Mannen & Itano, 1973). The reaction of phenylhydrazine with oxyhaemoglobin gave similar products to those with acetylphenylhydrazine, but very much more rapidly. At 37°C, 20 μM-oxyhaemoglobin and 80 μM-phenylhydrazine, spectral changes were virtually completed within 5 min. Further incubation resulted in precipitation, during which the A_{700} of choleglobin remained constant, whereas the contribution of haemichrome to the spectrum declined. This suggests that the haemichrome and not choleglobin was being precipitated. Since no precipitation occurred until the oxidative reaction was complete, it was possible to study relative stabilities of haemichromes formed under different

Table 2. *Comparison of the stabilities of haemichromes formed from phenyldiazine and methaemoglobin, and from oxyhaemoglobin and phenylhydrazine*

Haemichromes were prepared by adding phenylhydrazine either to ferricyanide and methaemoglobin or to oxyhaemoglobin, as described in the Methods section. The phenylhydrazine/haemoglobin molar ratio was 4:1. The reactions were timed from the addition of phenylhydrazine. With oxyhaemoglobin, A_{700} increased initially as choleglobin was produced, and then formed a plateau, which was taken as the baseline. After the start of precipitation, the increase in A_{700} rapidly became linear with time. The change in A_{700}/10min is the slope of this line, and the time interval before precipitation is its intercept on the baseline.

	O_2	[Total haemoglobin] (μM)	[Haemichrome] (μM)	Time interval before precipitation (min)	ΔA_{700}/10min
Methaemoglobin+phenyldiazine	Absent	20	8	55	0.029
	Present	20	8	35	0.08
Oxyhaemoglobin+phenylhydrazine	Present	20	4.7	10	0.23

Table 3. *Effect of catalase, superoxide dismutase and GSH on the rate of precipitation of haemichromes formed from oxyhaemoglobin and phenylhydrazine*

Details are given in the Methods section and Table 2. The concentrations of choleglobin and haemichrome were those after the oxidation reaction was complete but before the onset of precipitation.

Additions	[Total haemoglobin] (μM)	[Choleglobin] (μM)	[Haemichrome] (μM)	Time interval before precipitation (min)	ΔA_{700}/10min
None	20	3.9	4.7	10	0.23
Catalase (13 μg/ml)	20	4.0	4.6	11.6	0.18
GSH (1 mM)	20	4.0	4.4	12.5	0.17
Catalase+GSH	20	4.0	4.2	14.1	0.14
Superoxide dismutase (2.7 μg/ml)	20			7.9	0.30
Catalase+superoxide dismutase	20			8.8	0.25

conditions. If the time for precipitation to start and the subsequent rate of precipitation are used as criteria, the haemichrome formed from oxyhaemoglobin and phenylhydrazine was far less stable than the haemichrome formed from methaemoglobin and the diazine (Table 2). The amount of haemichrome formed from oxyhaemoglobin and phenylhydrazine varied only slightly in the presence and absence of catalase, superoxide dismutase and GSH (Table 3). However, addition of catalase or GSH each gave a haemichrome with greater stability than the control, and the haemichrome formed in the presence of superoxide dismutase had a lower stability. The effects were additive, and were not present if catalase, GSH or superoxide dismutase were added before precipitation but after the reaction in solution was complete. It appears that the presence of catalase or the free-radical scavengers GSH and $O_2^{-\cdot}$ prevented reactions of H_2O_2 or free radicals with sites on the globin, producing modified methaemoglobins. It is suggested that although these combine with the diazine to form haemichromes, their protein conformation is altered, they are less stable and they are precipitated more readily than haemichromes formed from native haemoglobin.

Mechanism of the reaction of oxyhaemoglobin with acetylphenylhydrazine

The interpretation of the results given above is compatible with the following mechanism:

$$Hb^{3+}O_2^- + PhNHNHAc + H^+ \rightarrow Hb^{3+}H_2O_2 + PhN^{\cdot}NHAc \quad (1)$$

$$PhN^{\cdot}NHAc + Hb^{3+}O_2^- \rightarrow Hb^{3+}H_2O_2 + PhN{=}NAc \quad (2)$$

$$PhN^{\cdot}NHAc + O_2 \rightarrow O_2^{-\cdot} + PhN{=}NAc + H^+ \quad (3)$$

$$PhN^{\cdot}NHAc + O_2^{-\cdot} + H^+ \rightarrow O_2 + PhNHNHAc \quad (4)$$

$$Hb^{3+} + H_2O_2 \rightleftharpoons Hb^{3+}H_2O_2 \rightarrow \text{choleglobin} \quad (5)$$

$$Hb^{3+} + PhN{=}NAc \rightarrow Hb^{3+}PhN{=}NAc \quad (6)$$

$$Hb^{3+}O_2^- + \tfrac{1}{2}H_2O_2 \rightarrow Hb^{3+} + OH^- + O_2 \quad (7)$$

Oxyhaemoglobin has been represented as superoxoferrihaemoglobin ($Hb^{3+}O_2^-$). There is evidence supporting this representation (Collman *et al.*, 1976) in which the oxygen can be considered as activated and available as an oxidizing agent for acetylphenylhydrazine. It is proposed that the first step is the one-electron oxidation of acetylphenylhydrazine by the liganded $O_2^{-\cdot}$, to give a free-radical intermediate and

a methaemoglobin–H_2O_2 complex (reaction 1 above). The acetylphenylhydrazyl radical (PhN·NHAc) then participates in a number of competing reactions. Major ones appear to be with more oxyhaemoglobin to give the diazine (reaction 2), with O_2 to give $O_2^{-\cdot}$ (reaction 3), and with $O_2^{-\cdot}$ (reaction 4). Decreasing the contribution of any one of the reactions, e.g. by removing $O_2^{-\cdot}$ with superoxide dismutase, would increase the contribution of the others. Likewise the presence of other constituents that could compete for the radical would alter the overall course of the reaction. This occurs when GSH or ascorbate (H_2A) is present, and reactions (8) and (9) or (10) and (11) compete for the acetylphenylhydrazyl radical:

$$GSH + PhN{\cdot}NHAc \rightarrow GS{\cdot} + PhNHNHAc \quad (8)$$

$$2GS{\cdot} \rightarrow GSSG \quad (9)$$

$$H_2A + PhN{\cdot}NHAc \rightarrow HA{\cdot} + PhNHNHAc \quad (10)$$

$$2HA{\cdot} \rightarrow H_2A + A \quad (11)$$

The complex formed in reactions (1) and (2) is proposed to either dissociate into methaemoglobin and H_2O_2 or break down to choleglobin (5). It is possible that it could also function peroxidatively, and be responsible for some of the reactions attributed to H_2O_2. The methaemoglobin can then form haemichromes with the diazine (6). The H_2O_2 and free radicals also react with unidentified sites on any of the haemoglobin species, giving rise to less-stable haemichromes that are likely to produce the bulk of the Heinz-body precipitate. Choleglobin, although an irreversibly denatured haemoglobin, appears to be more stable than the haemichromes, and is probably only a minor component of the precipitate.

Conclusions

The reaction of other oxidizable drugs or metabolites with haemoglobin should be similar to the reaction of acetylphenylhydrazine. The haemoglobin is functioning as an oxidase, activating oxygen, by forming superoxoferrihaemoglobin ($Hb^{3+}O_2^-$), so that it may readily oxidize the drug (H_2X):

$$Hb^{3+}O_2^- + H_2X \rightarrow Hb^{3+} + H_2O_2 + X^{-\cdot} \quad (15)$$

The drug free radical, as well as H_2O_2, should therefore be a product common to this type of reaction. The $O_2^{-\cdot}$ bound to haemoglobin initiates haemoglobin breakdown, but the free $O_2^{-\cdot}$ radical itself does not contribute to the process. Indeed its main role is protective, apparently because of its ability to scavenge free radicals. Goldberg & Stern (1977) have come to a similar conclusion, namely that $O_2^{-\cdot}$ is not a major contributor to haemolysis in these reactions.

The protective role of GSH against acetylphenylhydrazine, and more generally drug-induced haemolysis, must be considered to be twofold. With glutathione peroxidase it is required to reduce H_2O_2 and lipid peroxides (Mills, 1957; Little *et al.*, 1970), but our evidence gives particular support to the role of GSH as a free-radical scavenger (Kosower & Kosower, 1969). It prevents haemoglobin denaturation by inhibiting haem oxidation and globin modification, and because it inhibits production of acetylphenyldiazine, by decreasing the amount of haemichrome formation. The significance of GSH inhibiting free-radical reactions with other erythrocyte constituents is yet to be established.

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