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CONTRIBUTIONS OF SUPEROXIDE, HYDROGEN PEROXIDE, AND TRANSITION METAL IONS TO AUTO-OXIDATION OF THE FAVISM-INDUCING PYRIMIDINE AGLYCONE, DIVICINE, AND ITS REACTIONS WITH HAEMOGLOBIN

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Abstract—The influence of O_2^-, H_2O_2 and metal ions on the auto-oxidation of divicine, a pyrimidine aglycone, was studied. In air at pH 7.4, the hydroquinonic form oxidized within a few minutes. Superoxide dismutase (SOD) markedly decreased the initial rate, giving a lag phase followed by rapid oxidation. Although catalase or diethylenetriamine-penta-acetic acid (DTPA) alone had little effect, each in the presence of SOD further slowed the initial rate and increased the lag. H_2O_2 decreased the lag time, as did Cu^{2+}, Fe^{2+} or haemoglobin. GSH substantially increased the lag phase, but it eventually reacted with the divicine to form a 305 nm-absorbing adduct. These results indicate that an O_2^--dependent mechanism of divicine auto-oxidation normally predominates. Auto-oxidation can also occur by a mechanism involving H_2O_2 and transition metal ions or haemoglobin, and if both these reactions are prevented by SOD and DTPA or catalase, a third mechanism, requiring build-up of an autocatalytic intermediate, becomes operative. Oxyhaemoglobin did not react directly with divicine, but reacted with the H_2O_2 produced by divicine auto-oxidation to give mainly an oxidized derivative presumed to be ferrylhaemoglobin. Divicine was shown to reduce ferylhaemoglobin to methaemoglobin, and this reaction was probably responsible for the acceleratory effect of haemoglobin on divicine oxidation. These results indicate that O_2 rather than oxyhaemoglobin is likely to initiate divicine oxidation in the erythrocyte. Haemolytic crises, which are thought to result from this oxidation, occur only sporadically in glucose-6-phosphate dehydrogenase deficient individuals following ingestion of fava beans. A characteristic of the crises is acute depletion of erythrocyte GSH, and the vulnerability of these cells could relate to the ability of GSH, in combination with SOD, to protect against the autocatalytic mechanism of divicine auto-oxidation. Our demonstration of a variety of auto-oxidation pathways also suggests possible areas of individual variation.

Favism is an acute haemolytic disease associated with glucose-6-phosphate dehydrogenase (G6PD§) deficiency and ingestion of fava (broad) beans. Two pyrimidine aglycones, divicine (2,6-diamino-4,5-dihydroxypyrimidine) and isouramil (6-amino-2,4,5-trihydroxypyrimidine), can induce oxidative changes in erythrocytes, such as rapid depletion of GSH, and are generally thought to be the toxic constituents of the beans [1, 2]. They are present at high concentrations as their β-glucosides, vicine and convicine, but only become haemolytically active after hydrolysis, normally by a β-glucosidase following ingestion [1]. A feature of favism is that not all G6PD-deficient individuals are susceptible, and the condition may occur sporadically in the one individual. There is, therefore, an as yet unknown predisposing factor, which is not necessarily genetic.

Previous studies have shown that divicine and isouramil are very unstable in the presence of O_2, undergoing oxidation to their respective quinones and subsequently decomposing [3, 4]. They also readily oxidize pyridine nucleotides and GSH and can form addition products with GSH [4, 5]. Oxidation of divicine (or isouramil), with the accompanying production of H_2O_2 [4] and probably free radical intermediates [6], is a key step in its haemolytic action, and hence factors which affect auto-oxidation may also influence haemolysis. Most drugs which cause haemolysis in G6PD deficiency undergo an initial redox reaction with haemoglobin, producing free radical intermediates, H_2O_2 and oxidized haemoglobin derivatives [7, 8]. Whether this is the case with divicine and isouramil has not been established, since until now no studies of the reactions of these compounds with haemoglobin have been carried out. The present study was undertaken to establish how divicine auto-oxidation is influenced by extrinsic factors such as O_2^-, H_2O_2 and transition metal ions, and to determine the nature of any reactions between divicine and haemoglobin, in an attempt to understand the mechanism of haemolysis and to rationalize the reasons for individual susceptibility to haemolysis.

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§ Abbreviations: G6PD, glucose-6-phosphate dehydrogenase; DTPA, diethylenetriamine-penta-acetic acid; SOD, superoxide dismutase; GSH, reduced glutathione; GSSG, oxidized glutathione; and HPLC, high performance liquid chromatography.

MATERIALS AND METHODS

Materials: Divicine was prepared by hydrolysis of vicine (Serva, Heidelberg, F.R.G.) by heating 0.1 mmole at 100° for 15 min in 1 ml of 1 M HCl under N_2 in a sealed ampoule. It was stored at −20° under N_2 until immediately before use. Divicine concentrations were determined using $\varepsilon_{280} = 9800\ M^{-1}\ cm^{-1}$ [4]. The quinone species was prepared by neutralizing 0.1 ml of hydrolysed divicine solution with 0.095 ml of 1 M NaOH and allowing it to auto-oxidize by adding 1 ml of aerated 20 mM phosphate buffer, pH 7, containing 146 mM NaCl at 25°. The solution (100 μl) was immediately injected into a Waters model 510 HPLC with a Merck RP8 column (25 × 0.4 cm, i.d.) and eluted with air-saturated 0.1 mM diethylenetriamine-penta-acetic acid (DTPA) in water, neutralized to pH 7.0. The flow rate was 1 ml/min, and A_{240} of the eluate was monitored.

Oxyhaemoglobin was purified from erythrocyte lysates by DEAE-Sephadex column chromatography [9]. Methaemoglobin was prepared from oxyhaemoglobin by adding $K_3Fe(CN)_6$ and removing the excess on a column of Sephadex G25. Concentrations, expressed on a haem basis, were measured according to Beutler [10].

Catalase, superoxide dismutase (SOD), GSH and DTPA were obtained from the Sigma Chemical Co., St. Louis, MO, U.S.A., and Chelex-100 resin was from Bio-Rad, Richmond, CA.

Auto-oxidation of divicine. Reactions were carried out at 30° in 25 mM sodium phosphate buffer, pH 7.4. Divicine was always added to the mixture of other reagents immediately before monitoring the reaction. Sequential spectra or A_{280} changes were recorded with a Pye-Unicam PU 8800 spectrophotometer.

Haemoglobin oxidation. Either serial spectra between 700 and 500 nm were recorded, or the loss of oxyhaemoglobin was followed by continuously monitoring A_{577}. Solutions were in the same phosphate buffer as above.

RESULTS

Auto-oxidation of divicine. In accordance with Chevion *et al.* [4], divicine underwent rapid auto-oxidation in air at pH 7.4 (Fig. 1), with disappearance of the 280 nm peak corresponding to the hydroquinonic species and appearance of the 240 nm-absorbing quinonic species. This species was eventually converted to derivatives which absorb at shorter wavelengths. Disappearance of the hydroquinone, monitored by continuously recording A_{280}, occurred within a few minutes, with no perceptible lag (Fig. 2a). Addition of DTPA slowed the reaction slightly, without causing a lag (Fig. 2b) as did addition of EDTA or desferrioxamine (100 μM) or pretreatment of the buffer with Chelex resin.

In the presence or absence of DTPA, catalase alone did not affect significantly the rate of divicine auto-oxidation (not shown). SOD decreased the initial rate, giving a lag period when auto-oxidation was very slow, followed by rapid disappearance of the 280 nm peak. The lag phase was much longer in

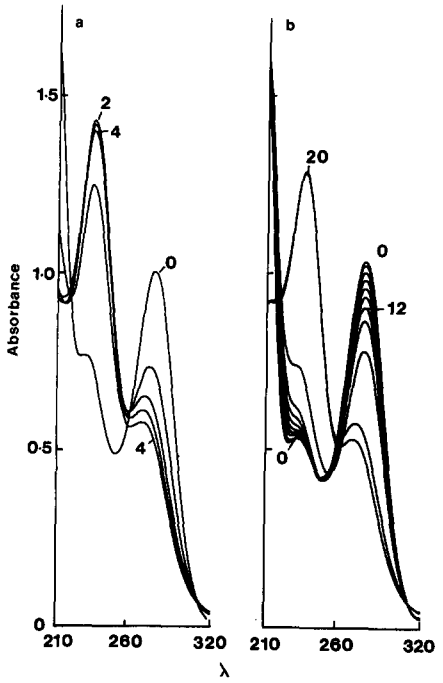

Fig. 1. Spectral changes on auto-oxidation of divicine (0.1 mM). (a) In pH 7.4 phosphate buffer at 30°; (b) in the same buffer containing DTPA (0.1 mM) and superoxide dismutase (10 μg/ml). Scans were recorded after addition of divicine at 1-min (a) or 2-min (b) intervals.

the presence (Fig. 2b) than in the absence (Fig. 2a) of DTPA. Lowering the SOD concentration progressively increased the initial rate and decreased the lag time; higher concentrations had no additional effect. Catalase substantially increased the lag phase seen with SOD alone, whereas it had little effect when DTPA and SOD were both present (Fig. 2). Thus, either catalase or DTPA in combination with SOD decreased the rate of divicine auto-oxidation to the same extent. Irrespective of the length of the lag phase, the rate of auto-oxidation eventually increased to a maximum that was approximately the same regardless of whether DTPA, catalase or SOD was present. Adding further SOD or catalase at this point made no difference. Formate (10 mM), which would be expected to scavenge hydroxyl radicals, had no effect on the course of the reaction under any of the above conditions.

These results suggest that normally divicine auto-oxidation is mediated primarily by O_2^-, but in the presence of SOD auto-oxidation is influenced by H_2O_2 and adventitiously present metal ions. These relationships were further investigated by adding H_2O_2 or metal ions directly (in the presence of SOD) and measuring their effects on the length of the lag period (defined as the time from the start until the point when the rate of absorbance change was maximal). This is also a measure of the initial reaction rate, which was inversely related to the lag period. In the absence of DTPA, H_2O_2, and Fe^{2+} or Cu^{2+} in the micromolar range, decreased the lag phase (Table 1). Cu^{2+} was more effective than Fe^{2+}. In the

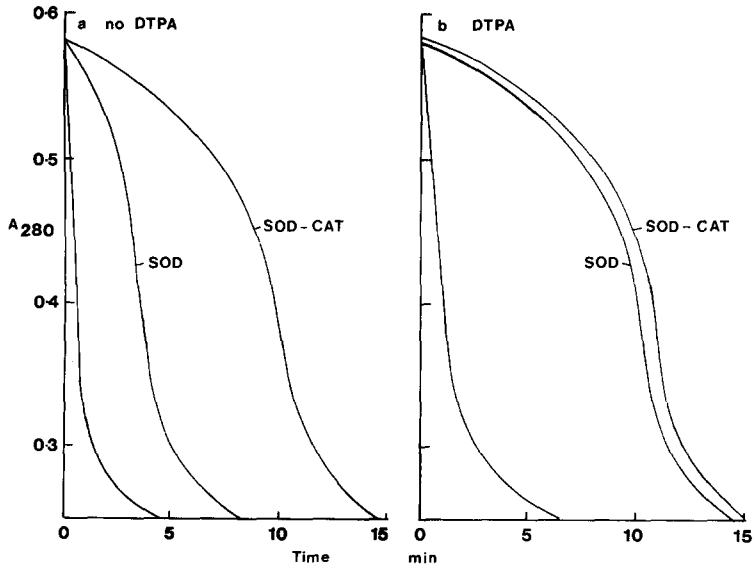

Fig. 2. Effects of superoxide dismutase and catalase on the rate of auto-oxidation of divicine (60 μM) in the (a) absence and (b) presence of DTPA (0.1 mM). Unmarked lines, no enzymes; SOD, plus superoxide dismutase (10 μg/ml); SOD-CAT, plus superoxide dismutase and catalase (20 μg/ml). Temperature, 30°.

presence of Fe^{2+} or Cu^{2+}, the lag was decreased further by H_2O_2 and increased by catalase. The effects of H_2O_2, Cu^{2+} and Fe^{2+} were abrogated by the presence of DTPA.

Haemoglobin (met- or oxy-) also decreased the lag phase of divicine oxidation in the presence of SOD and DTPA, although with 5 μM haem groups the effect was quite small (Table 1). H_2O_2 further decreased the lag, and catalase eliminated the effect of haemoglobin.

The findings with Fe^{2+} and H_2O_2 were corroborated by an experiment in which H_2O_2 (1 mM) was added to divicine (100 μM) under N_2. H_2O_2 had no effect in the presence of DTPA (100 μM), but in the presence of 0.5 mM $FeSO_4$, it caused a rapid decrease in A_{280}.

Table 1. Effects of metals and H_2O_2 on the rate of auto-oxidation of divicine in the presence of superoxide dismutase

	Lag time (min)	
Additive	No DTPA	+100 μM DTPA
None	4.0	8.7
Catalase	9.0	9.0
H_2O_2	1.7	8.3
Cu^{2+}, 0.25 μM	1.8	
Cu^{2+}, 0.5 μM	0.4	
Cu^{2+}, 1 μM	<0.4	8.7
Cu^{2+}, 1 μM, catalase	5.0	
Cu^{2+}, 0.25 μM, H_2O_2	1.2	
Fe^{2+}, 1 μM	2.2	
Fe^{2+}, 2 μM	1.3	8.7
Fe^{2+}, 2 μM, catalase	7.0	
Fe^{2+}, 1 μM, H_2O_2	0.8	
Methaemoglobin, 5 μM		6.3
Methaemoglobin, 5 μM, catalase		8.5
Methaemoglobin, 5 μM, H_2O_2		3.0
Oxyhaemoglobin, 5 μM		7.2
Oxyhaemoglobin, 5 μM, catalase		8.5
Oxyhaemoglobin, 5 μM, H_2O_2		2.7

Reactions were carried out at pH 7.4 and 30° with 100 μM divicine and 10 μg/ml superoxide dismutase. Concentrations, where added, were catalase (20 μg/ml) and H_2O_2 (200 μM). Lag times represent the time from addition of divicine to when the rate of change of A_{280} was maximum. Means of two to three assays which agreed ±5% are shown.

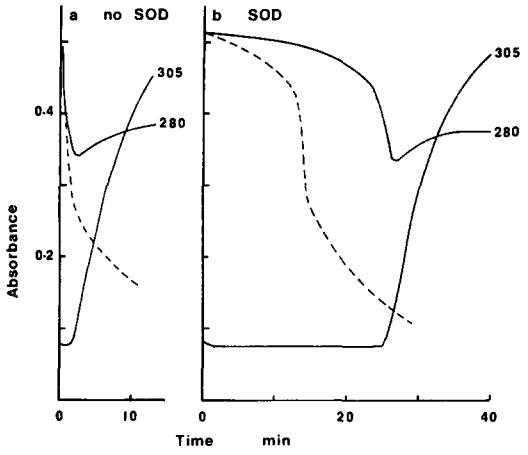

Fig. 3. Effect of GSH (200 μM) on the auto-oxidation of divicine (50 μM). (a) In pH 7.4 phosphate buffer containing 0.1 mM DPTA. (b) In the same buffer containing DTPA and superoxide dismutase (15 μg/ml). Absorbance changes at 280 and 305 nm were monitored continuously at 30°. (——) Changes in A_{280} under similar conditions but in the absence of GSH.

GSH is thought to be important in preventing the haemolytic effects of divicine. It had very little effect on the rate of divicine auto-oxidation (disappearance of the 280 nm-absorbing hydroquinone) in the absence of SOD but, in the presence of SOD, GSH decreased the initial rate and increased the lag time (Fig. 3). As the reaction proceeded, a product with A_{max} = 305 nm developed. Serial spectra showed that this species was responsible for the secondary rise in A_{280}. Divicine and GSH have been shown previously to form an adduct with this A_{max} [4, 5]. Our finding that formation of this adduct lagged behind the decrease in A_{280} shows that it is formed from a product of divicine auto-oxidation, possibly the quinone. The length of the lag phase in the presence of SOD was dependent on the GSH concentration. With no GSH, 200 μM and 400 μM, the lag was 14, 26 and 65 min respectively. Ascorbic acid (200 μM) in the presence of SOD approximately halved the lag phase before a rapid decrease in A_{280}. As ascorbate also absorbs at 280 nm, the absorbance change probably represents oxidation of both compounds.

Haemoglobin oxidation. The spectral changes that occurred on adding divicine to purified oxyhaemoglobin (Fig. 4a) are identical to those seen when H_2O_2 was added to oxyhaemoglobin, and represent the gradual formation of a haemoglobin–peroxide complex, the spectrum of which is shown in Fig. 6. By analogy with myoglobin, this is probably the ferryl species [11, 12] and will subsequently be referred to as such. Although its structure has not been fully elucidated, it is thought to have the form $[Fe^{IV}OH^-]^{3+}$. Very little methaemoglobin formation was apparent (only a slight shoulder at 630 nm in Fig. 4a), although methaemoglobin was more evident (with less overall oxidation) when the divicine:haemoglobin ratio was below 1. The divicine-induced changes were inhibited completely by catalase (Fig. 5), i.e. there was no direct reaction between oxyhaemoglobin and divicine. With methaemoglobin (Fig. 4b), divicine also caused spectral changes indicative of ferrylhaemoglobin formation that were fully inhibited by catalase. Again this is similar to what is observed when H_2O_2 is added to methaemoglobin. In this case, the ferryl species is thought to arise via an intermediate, short-lived ferryl radical [8, 11, 13].

Divicine was unable to cause oxidation of carbonmonoxyhaemoglobin. When oxyhaemoglobin was treated with CO and incubated with divicine in 80% air/20% CO, its spectrum remained unchanged for at least 1 hr, although divicine auto-oxidation occurred within a few minutes. The 240 nm-absorbing (quinone) species, purified from auto-oxidized divicine by HPLC and stabilized after elution under N_2, underwent no detectable reaction with oxyhaemoglobin. No haemoglobin spectral changes

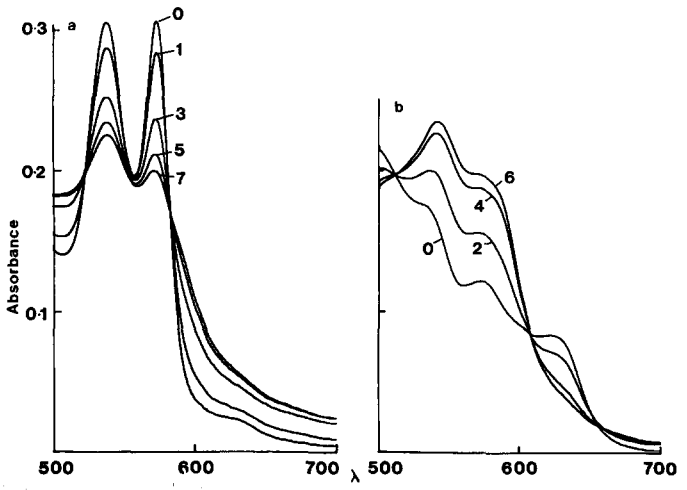

Fig. 4. Haemoglobin spectral changes on reaction with divicine. (a) Oxyhaemoglobin (20 μM), divicine (100 μM); (b) Methaemoglobin (20 μM), divicine (100 μM). The time (min) after addition of divicine is shown for each scan. The oxyhaemoglobin solution initially contained approximately 10% methaemoglobin; temperature, 30°.

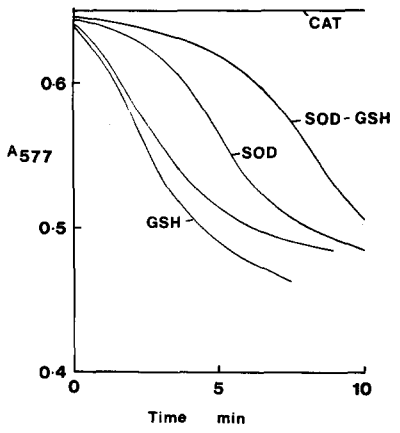

Fig. 5. Effects of catalase, superoxide dismutase and GSH on the rate of oxidation of oxyhaemoglobin (45 μM) by divicine (60 μM). Reactions were carried out at 30° in the presence of 0.1 mM DTPA. CAT, plus catalase (20 μg/ml); SOD, plus superoxide dismutase (10 μg/ml); GSH, plus GSH (200 μM).

were evident for at least 1 hr after mixing in air in the absence of catalase.

Addition of DTPA to divicine and oxyhaemoglobin did not alter the nature of the haemoglobin spectral changes but slowed the reaction slightly. Loss of oxyhaemoglobin absorbance at 577 nm (Fig. 5) lagged slightly behind divicine auto-oxidation under similar conditions (Fig. 2b). This lag undoubtedly represents the build-up of H_2O_2 and its reaction with oxyhaemoglobin (which, at these concentrations, takes place over several minutes). SOD increased the lag more markedly in the presence of DTPA (Fig. 5) than in its absence (not shown). GSH alone had little effect, but in the presence of SOD further increased the lag period (Fig. 5). The effects of these additives parallel their effects on divicine auto-oxidation, although the lag times in the presence of SOD were significantly shorter than for divicine auto-oxidation in the absence of haemoglobin

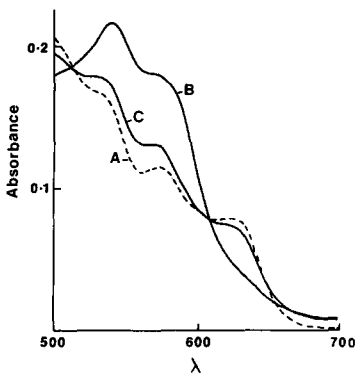

Fig. 6. Spectral changes of ferrylhaemoglobin on reaction with divicine. (A) (——) methaemoglobin (20 μM); (B) ferrylhaemoglobin, prepared by adding 200 μM H_2O_2 to A, then after 2 min adding catalase (20 μg/ml) and superoxide dismutase (10 μg/ml); and (C) product of the reaction of B with divicine (200 μM), scanned after 3 min.

(Figs. 2 and 3). This is probably due to the acceleratory effect of haemoglobin on divicine auto-oxidation, as was shown in Table 1.

The possibility that the acceleratory effect of haemoglobin on divicine oxidation was due to direct oxidation by ferrylhaemoglobin was investigated. Ferrylhaemoglobin was prepared from methaemoglobin and H_2O_2, and reacted with divicine, after first adding catalase to remove excess H_2O_2 and SOD to prolong divicine auto-oxidation. Ferrylhaemoglobin was reasonably stable under these conditions, undergoing little spectral change over 20 min, and auto-oxidation of divicine alone showed a lag phase of about 10 min. However, on addition of divicine to the ferrylhaemoglobin, its spectrum changed over 2–3 min, with no perceptible lag, to approach that of methaemoglobin (Fig. 6).

DISCUSSION

Our findings show that divicine auto-oxidation can occur by a variety of routes. The normally predominant mechanism is O_2^--dependent and effectively inhibited by SOD. This appears to be analogous to the mechanism of adrenaline auto-oxidation [14] implying the following reaction sequence (where RH_2, $RH^\cdot$ and R represent the hydroquinone, semiquinone and quinone forms of divicine respectively):

$$RH_2 + O_2 \rightarrow RH^\cdot + O_2^- + H^+ \text{ (slow)} \quad (1)$$

$$RH_2 + O_2^- + H^+ \rightarrow RH^\cdot + H_2O_2 \text{ (fast)} \quad (2)$$

$$RH^\cdot + O_2 \rightarrow R + O_2^- + H^+ \text{ (fast)} \quad (3)$$

After the initial step, reactions (2) and (3) would constitute a chain and be responsible for most of the reaction.

The second pathway, prevented by catalase or metal chelators, depends on transition metal ions and H_2O_2, which may be exogenous or produced from the O_2^- formed in reaction 1. Normally this pathway is much slower than reactions 2 and 3, and is of minor significance. However, it predominates if SOD is present, or if concentrations of transition metal ions, e.g. Cu^{2+} or Fe^{2+}, are micromolar or more. The absence of any effect of formate suggests that this reaction is not due to hydroxyl radicals. The reaction may involve direct reduction of the metal ions, which readily bind to the *o*-hydroquinone groups of divicine. Haemoglobin and H_2O_2 also accelerate divicine auto-oxidation, apparently via formation of peroxide complexes such as ferrylhaemoglobin with known peroxidative activity [11]. Although at an equivalent molar concentration, haemoglobin is less effective than Fe^{2+} or Cu^{2+}, in the erythrocyte the high haemoglobin concentration should outweigh this difference.

If both the above pathways are prevented by SOD and DTPA or catalase, divicine auto-oxidation still proceeds slowly, presumably via reaction 1, up to a point where another mechanism takes over and there is rapid auto-oxidation. The kinetics of this reaction suggest that there is gradual buildup of an autocatalytic intermediate, as yet unidentified.

GSH has little effect on the rate of auto-oxidation

of divicine in the absence of SOD but, with SOD present, it prolongs the lag phase. It is possible that sufficient GSH, or continual reduction of oxidized GSH, could prevent completely the rapid oxidation phase. It has been shown previously that divicine auto-oxidation causes rapid oxidation of GSH, to predominantly GSSG, but with some formation of adducts with 305 and 320 nm absorbance maxima [4, 5]. Our study suggests that, following oxidation, most of the divicine can be converted to the 305 nm-absorbing adduct. Ascorbate appears to enhance the oxidant effects of divicine and, in view of its high concentration in fava beans [2], may contribute to haemolysis in favism. Our finding that it accelerates divicine oxidation in the presence of SOD suggests a possible reason for this effect.

Although the sensitivity of divicine auto-oxidation towards metal ions has been recorded, the involvement of O_2^- and the inhibitory effect of SOD have not been described previously. Chevion *et al.* [4] saw no effect of SOD on auto-oxidation of isouramil, possibly because transition metal ions could have obscured the O_2^--dependent auto-oxidation mechanism.

Our studies have shown that, unlike most redox drugs that are active in G6PD deficiency, divicine does not react directly with oxyhaemoglobin. However, interactions between the two are not inconsequential. Auto-oxidizing divicine produces H_2O_2 which reacts with oxy- or methaemoglobin to form predominantly ferrylhaemoglobin. A little methaemoglobin is formed from oxyhaemoglobin, relatively more when the divicine:haemoglobin ratio is low, which is in keeping with the mechanism of myoglobin oxidation by H_2O_2 proposed by Whitburn [12]. Thus, the outcome of adding divicine to haemoglobin is production of the ferryl species, with the time course following that of auto-oxidation. The ferryl species $[Hb^{IV}OH^-]^{3+}$ can react directly with divicine, thus accelerating divicine oxidation. Methaemoglobin is produced, suggesting the following reaction:

$$[Hb^{IV}OH^-]^{3+} + RH_2 + H^+ \rightarrow Hb^{3+} + 2H_2O + RH$$

Ferrylhaemoglobin and the even more reactive intermediate formed during the reaction of H_2O_2 with methaemoglobin are also capable of oxidizing many other electron donors [8, 11]. These oxidized haemoglobin derivatives are likely to be major contributors to cell damage in the oxidant-stressed erythrocyte.

Divicine, particularly when stabilized against auto-oxidation, can reduce methaemoglobin to ferrous haemoglobin [15]. This reaction was not observed in the present study because (a) it is largely obscured by the reaction of haemoglobin with H_2O_2, and (b) even in the presence of catalase, with the divicine and haemoglobin concentrations being 50- to 100-fold lower than those used by Benatti *et al.*, reaction times were not long enough to observe a significant change.

With respect to the relevance of our results to the reactions of divicine (or isouramil) in normal and G6PD-deficient erythrocytes, it is apparent that oxidative changes are more likely to be initiated by auto-oxidation of divicine than by its reaction with oxyhaemoglobin. Thus, compounds such as SOD, GSH and ascorbate which affect the rate of auto-oxidation will, by this action, have secondary effects on the production of oxidized haemoglobin derivatives, and on oxidant damage in general. O_2^--dependent divicine auto-oxidation should not occur in the erythrocyte because of the high concentration of SOD, and the effects of other agents should be considered in the light of our results in the presence of SOD. Likewise, haemoglobin- or transition metal ion-dependent divicine oxidation should not occur when there is efficient removal of H_2O_2. However, in G6PD-deficient erythrocytes, the impaired function of the GSH-glutathione peroxidase pathway may allow this reaction to proceed. Haemoglobin would be expected to contribute more to the reaction than transition metal ions, unless their concentrations were excessively high, as may be the case in malaria-infected cells [16].

Even if both O_2^-- and H_2O_2-dependent pathways of divicine auto-oxidation are prevented, our results show that a third autocatalytic mechanism can operate. If this reaction occurs in the erythrocyte, deleterious effects of divicine might be expected. Its prevention may be a major factor in protection against haemolysis. Our finding that GSH may be able to do this by removing buildup of the autocatalytic intermediate suggests a possible reason why divicine preferentially affects G6PD-deficient cells. A major feature of the aetiology of favism is its variable expression among G6PD-deficient subjects. Our findings have highlighted the diversity of mechanisms for the divicine auto-oxidation that is thought to precipitate the crisis and, in this respect, suggest different areas where sources of this variability could be found.

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