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AUTO-OXIDATION OF DIALURIC ACID, DIVICINE AND ISOURAMIL

SUPEROXIDE DEPENDENT AND INDEPENDENT MECHANISMS

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Abstract—The toxicity of dialuric acid to pancreatic β cells, and the haemolytic action of divicine and isouramil involve auto-oxidation and redox cycling reactions. Divicine and isouramil are produced on hydrolysis of the fava bean glycosides, vicine and convicine. The mechanism of auto-oxidation of the three compounds as well as the acid hydrolysis product of vicine (provisionally assigned the structure 2-amino-4,5,6-trihydroxypyrimidine) has been studied. All four pyrimidines auto-oxidized rapidly at neutral pH, generating H_2O_2 by an $O_2^{\cdot-}$-dependent chain mechanism. Superoxide dismutase inhibited the initial oxidation, but inhibition was transitory, and after a lag period rapid oxidation occurred. The lag period varied with pH, temperature and pyrimidine concentration, and was much shorter for isouramil and divicine than for dialuric acid and acid-hydrolysed vicine. The initial rate of dialuric acid oxidation was greater and the acceleration less pronounced than with the other pyrimidines. A mechanism common to all four pyrimidines has been shown by kinetic analysis to account for nearly all the observations in the presence and absence of superoxide dismutase. Autocatalysis in the latter case is attributed mainly to the reactions

reduced pyrimidine + oxidized pyrimidine $\rightleftharpoons$ 2 pyrimidine radical
pyrimidine radical + $O_2 \rightarrow$ oxidized pyrimidine + $O_2^{\cdot-}$

Rate constants for these and other reactions are reported. At pH 7.4 and 37° the lag period before 100 μM acid-hydrolysed vicine underwent rapid oxidation was approx. 15 min. Isouramil and divicine at an equivalent concentration gave lags of <1 min, which became less at higher concentrations. Thus intracellular superoxide dismutase should provide only transitory protection against the oxidation products of dialuric acid, divicine or isouramil. Prolonged protection should only be achieved if accumulation of oxidized pyrimidine is also prevented.

Dialuric acid [1–4], divicine and isouramil [5, 6] are cytotoxic pyrimidines that are thought to act via redox cycling mechanisms. Dialuric acid, the reduced form of alloxan, destroys pancreatic β cells and is used for the experimental induction of diabetes [7]. It can also lyse red blood cells [8, 9]. Divicine and isouramil are present as their corresponding glycosides, vicine and convicine, in fava beans [6, 10] and are the likely cause of haemolysis of glucose-6-phosphate dehydrogenase (G6PD)‖ deficient red cells in favism [5, 6].

All three pyrimidines readily auto-oxidize, producing H_2O_2 and $O_2^{\cdot-}$ [1, 11–15]. Transition metal ion-catalysed hydroxyl radical production has also been measured [11, 12]. Hydroxyl radicals, or other oxidants produced in metal catalysed reactions, have been implicated in the cytotoxicity of alloxan [2–4, 16, 17] and divicine or isouramil [18, 19]. Although these compounds are structurally similar (Fig. 1), previous studies have suggested differences in their auto-oxidation mechanisms. In particular, the role of $O_2^{\cdot-}$ requires clarification. Superoxide dismutase (SOD) has been reported to markedly decrease the initial rate of divicine oxidation, giving a lag phase followed by a rapid reaction [14]. SOD also inhibits auto-oxidation of dialuric acid, but no lag phase has been described [12]. With isouramil, however, no effect of SOD on auto-oxidation rate was found [13]. In other respects, the compounds are similar. Oxidation of each is accelerated by iron or copper ions, and slowed by chelators [12–14]. Catalase decreases the rate of metal catalysed oxidation of divicine or dialuric acid, but has little effect in the presence of chelators [12, 14]. No effect of catalase on isouramil auto-oxidation was found [13].

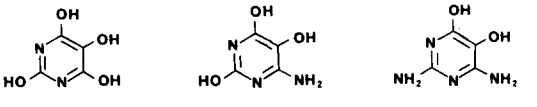

Fig. 1. Structural formulae of dialuric acid, divicine and isouramil.

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‖ Abbreviations: GSH, reduced glutathione; G6PD, glucose-6-phosphate dehydrogenase; SOD, superoxide dismutase; DH_2, reduced pyrimidine; $DH^{\cdot}$, pyrimidine radical; D, oxidized pyrimidine.

A complicating factor may be the nature of the product of vicine hydrolysis used in previous studies. This has been assumed to be divicine, whether it was prepared using β-glucosidase [6, 18–20] or acid hydrolysis [14, 15]. However, a recent ESR study [21] has shown that the two hydrolysis products differ and a structure of 2-amino-4,5,6-trihydroxypyrimidine (i.e. an isouramil isomer) has been proposed for acid-hydrolysed vicine.

The present study was undertaken to elucidate the auto-oxidation mechanisms of dialuric acid and the fava bean pyrimidines, comparing synthetic divicine with the products of vicine hydrolysis by β-glucosidase and by HCl. We confirm that the two hydrolysis products are different and show that all the pyrimidines auto-oxidize by a major route that is a chain reaction with $O_2^{\cdot-}$ as an intermediate. When this is blocked by SOD, oxidation occurs following a lag period, and involves a reaction between the oxidized and reduced forms of each compound.

MATERIALS AND METHODS

Materials. Hydrolysis of vicine (Serva, Heidelberg, F.R.G.) was performed either by heating 0.1 mmole at 100° for 15 min in 1 ml of 1 M HCl, under N_2 in a sealed ampoule, or by incubating 3 mg with 0.3 mg β-glucosidase (Sigma) for 4 hr at 37° under N_2 in pH 5.0 acetate buffer. Dialuric acid was synthesized according to Biltz and Damm [22], and kindly supplied by Dr. R. Munday (Ruakura Animal Research Centre, Hamilton, N.Z.). Isouramil was synthesized by the method of Bien *et al.* [23]. Divicine hydrochloride, synthesized according to Zav'Yalov and Pokvisneva [24], was purified by recrystallization from water and gave C, H and N analyses within 0.1% of expected values. All were stored at −20° under N_2. Stock solutions of 5 or 10 mM in 20 mM HCl were prepared daily, and kept N_2-bubbled during use. Concentrations were determined using ε_{280} 14,100 $M^{-1}cm^{-1}$ for isouramil [13] and ε_{273} 16,000 $M^{-1}cm^{-1}$ for dialuric acid [25]. Chevion *et al.* [13] give ε_{280} 9800 for divicine but do not give the source of their material. We confirmed this value for synthetic divicine and β-glucosidase-hydrolysed vicine, using reduction of Fe^{3+}(*o*-phenanthroline) at low pH to calibrate the pyrimidine concentration. Using this method acid-hydrolysed vicine also gave ε_{250} of approx. 9800 $M^{-1}cm^{-1}$. Concentration changes on oxidation were determined assuming that the oxidized products had zero absorbance at the measured wavelengths. As a consequence of overlap of the peaks, concentration changes would have been underestimated by up to 20% with divicine, somewhat less with acid-hydrolysed vicine. Alloxan solutions were standardized by measuring A_{273} following reduction to dialuric acid with a 50-fold excess of cysteine. SOD, catalase, GSH and alloxan were from Sigma (St. Louis, MO).

Auto-oxidation studies. Loss of reduced pyrimidine was followed by continuously monitoring A_{280} (divicine and isouramil) or A_{273} (dialuric acid), using a Pye-Unicam PU 8000 spectrophotometer. Kinetic studies were carried out at 23° in 50 mM phosphate buffer containing 50 μM diethylenetriamine-pentaacetic acid (DTPA). Reactions were initiated by adding up to 50 μl of pyrimidine solution in 20 mM HCl to 1 ml of reaction buffer. Absorbance

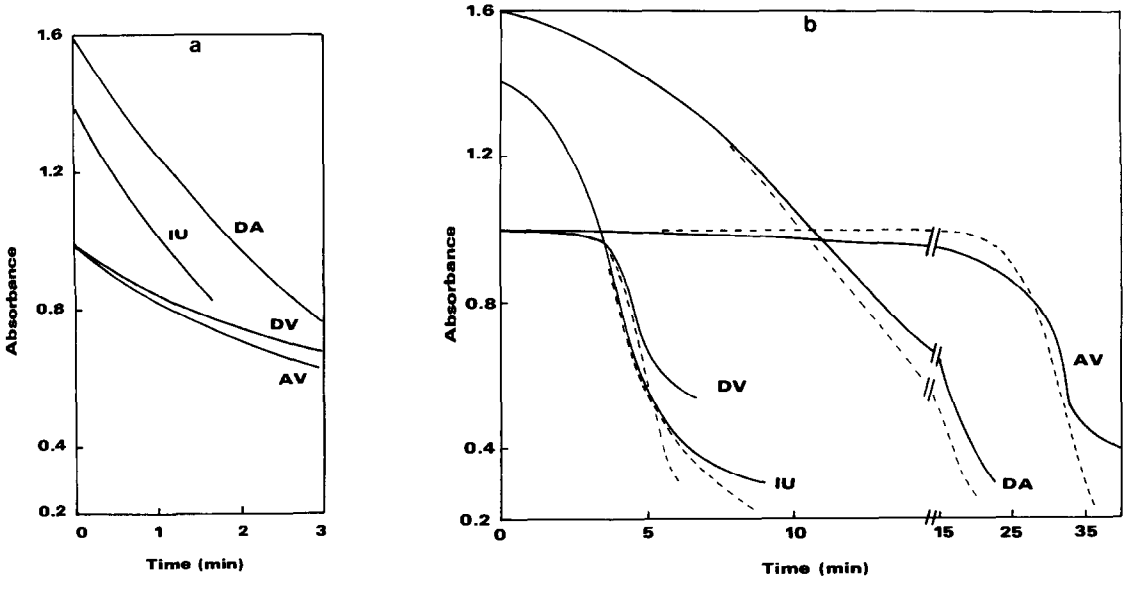

Fig. 2. Time course for oxidation in the absence (a) and presence (b) of SOD. Dialuric acid (DA), divicine (DV), isouramil (IU) and acid-hydrolysed vicine (AV) were each at 100 μM in pH 7.4 phosphate buffer containing DTPA, and incubated at 23°. SOD concentrations were 50 μg/ml for divicine, and 10 μg/ml in all other cases. Absorbances at 273 nm (dialuric acid) or 280 nm (others) were continuously monitored. Solid lines represent observed data. Broken lines represent curves predicted from the rate constants given in Table 1, except where the two curves are superimposable, where only the observed curves are shown. This is the case in Fig. 2a except for acid-hydrolysed vicine for which a simulation was not possible (see text).

measurements were commenced approx. 10 sec after mixing, and initial rates were determined from that time. Other studies were performed at 37° with 50 mM phosphate pH 7.4 containing 50 μM DTPA. O_2 uptake was measured under the same conditions using 3 ml of solution and a Clarke-type O_2 electrode (Model 25, Yellowsprings Instruments, Yellowsprings, OH).

RESULTS

Superoxide-dependent oxidation

All the pyrimidines, at 100 μM in aerated pH 7.0 phosphate buffer containing DTPA, underwent rapid auto-oxidation at 23°, measured as a decrease in absorbance (Fig. 2a) or uptake of O_2 occurring over the same time scale (not shown). Spectral analysis (not shown) indicated conversion to species with absorption maxima at lower wavelengths, as observed previously [13, 14, 25]. Synthetic divicine and the products of vicine hydrolysis all had a 280 nm absorption maximum and gave a peak at 240 nm on oxidation. However, this peak was of lower intensity with acid-hydrolysed vicine than with the other preparations. At concentrations <100 μM, divicine and acid-hydrolysed vicine oxidized at comparable rates, but at higher concentrations, divicine oxidation was substantially slower. Initial oxidation rates increased with increasing concentration of pyrimidine (Fig. 3) and were approximately doubled by saturating the solutions with O_2 instead of air (Fig. 3). No lags were apparent.

Oxidation in the presence of superoxide dismutase

Addition of SOD initially produced a marked inhibition of oxidation (paralleled by inhibition of O_2 uptake), but in each case this was transitory, with a lag period followed by rapid oxidation (Fig. 2b). The long lag for acid-hydrolysed vicine has been reported previously [14]. The lag was much shorter for synthetic divicine, which behaved like vicine hydrolysed with β-glucosidase, and for isouramil. With dialuric acid, although the rate of oxidation increased with time, the initial rate was higher than with the other pyrimidines, so the lag phase was less apparent. SOD concentrations higher than those used in Fig. 2b did not alter lag times or maximum rates. Neither did addition of further SOD just before the end of the

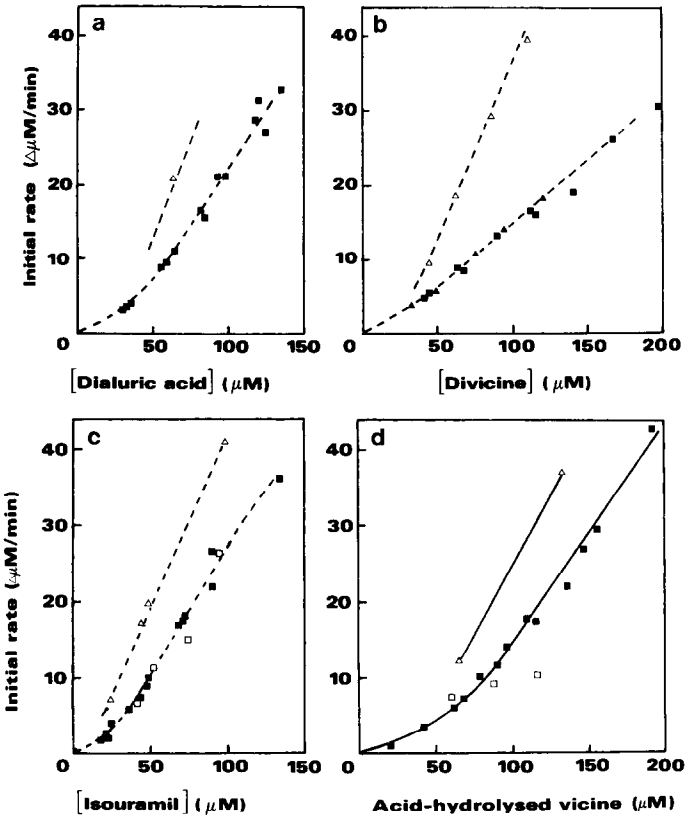

Fig. 3. Plots of initial rate of oxidation versus concentration of (a) dialuric acid, (b) divicine, (c) isouramil and (d) acid-hydrolysed vicine. Reactions were followed in the absence of SOD as in Fig. 2a, with various concentrations of pyrimidine: ■, in air; △, in O_2; □, with 40 μM oxidized acid-hydrolysed vicine or 70 μM oxidized isouramil present; ▲, (Fig. 3b) vicine hydrolysed with β-glucosidase. The points represent experimental observations, the broken lines are computer simulations based on the rate constants given in Table 2. Calculations were not made for acid-hydrolysed vicine, and a solid line has been drawn through the experimental points. Computer calculations were also made for isouramil in the presence of 70 μM oxidized isouramil. The curve (not shown) was almost the same as that with no oxidized isouramil present.

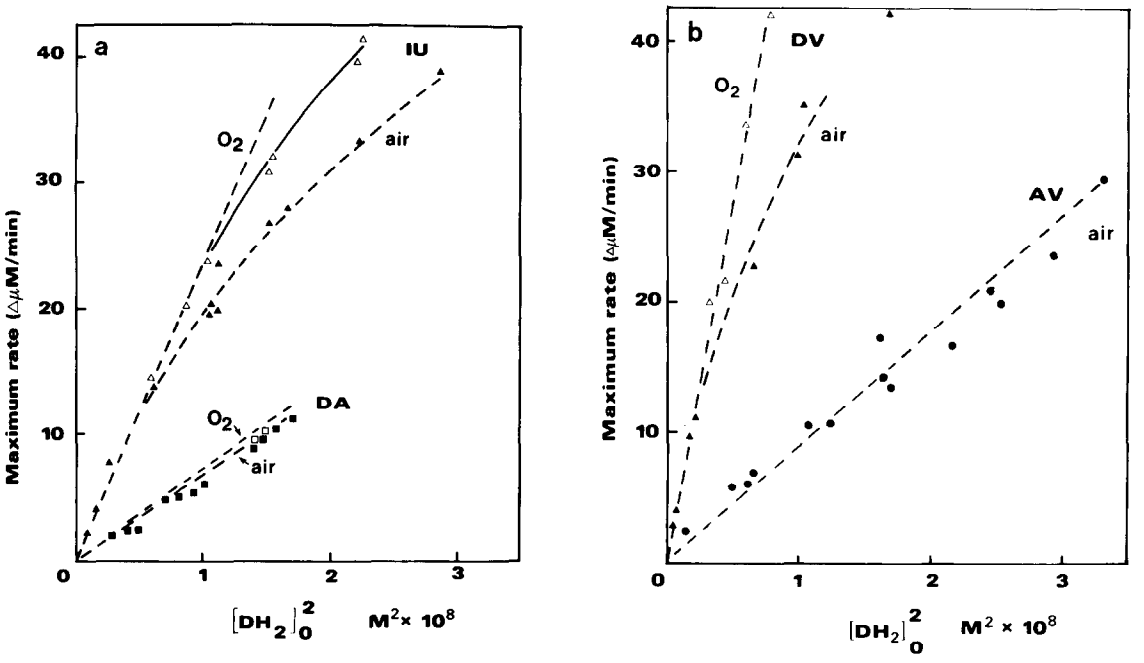

Fig. 4. Concentration dependence of the maximum rate of oxidation in the presence of SOD (50 μg/ml for divicine, 10 μg/ml for the other pyrimidines). (a) Dialuric acid (air) ■, (O_2) □; isouramil (air) ▲, (O_2) △. (b) Divicine (air) ▲; (O_2) △; acid-hydrolysed vicine (air) ●. Conditions as in Fig. 2b, with varying concentrations of pyrimidine. Experimental points are plotted. Broken lines are calculated curves using the rate constants in Table 1, the solid lines representing best fit through the experimental points.

lag phase. Divicine required at least 5 times more SOD for maximum effect than did the other pyrimidines.

Rates of oxidation of the pyrimidines were maximal when their concentrations had dropped to half the initial concentration (e.g. Fig. 2b) and as shown in Fig. 4, maximum rates were proportional to $[DH_2]_0^2$ (where $[DH_2]_0$ is the initial pyrimidine concentration). Bubbling with O_2 increased the maximum rate of oxidation at the higher but not the lower concentrations of isouramil and divicine, thereby extending the range of linearity (Fig. 4). O_2 had little effect with acid-hydrolysed vicine (not shown) or dialuric acid (Fig. 4). O_2 increased the initial rate of dialuric acid oxidation 1.5–2-fold but did not affect initial oxidation rates of the other pyrimidines.

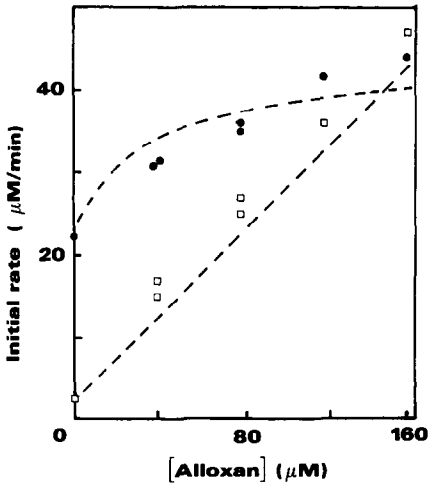

Fig. 5. Effect of alloxan on the initial rate of oxidation of dialuric acid (100 μM). Points represent rates measured in the absence (●) and presence (□) of SOD (10 μg/ml). Broken lines are calculated curves using the rate constants in Table 1.

There was an inverse relationship between the lag period (measured as the time taken to reach the maximum rate of oxidation) and the initial concentration of each pyrimidine. Thus, decreasing the concentration from 100 to 25 μM increased the lag from 12 to 50 min for dialuric acid, from 4 to 16 min for isouramil, from 4 to 7 min for divicine and from 30 to 120 min for acid-hydrolysed vicine. Lag periods tended to decrease with the length of time that stock solutions had been prepared. This is presumably because introduction of O_2 allowed small amounts of oxidized pyrimidine to accumulate. Dialuric acid solutions were particularly sensitive, and often after several samplings they gave maximum oxidation rates almost immediately after adding to the pH 7 buffer. This may be why acceleration of dialuric acid oxidation was not apparent previously [12].

Adding alloxan (the oxidation product of dialuric acid) to dialuric acid eliminated the lag period in the presence of SOD and gave initial rates of dialuric acid oxidation that increased with increasing alloxan concentration (Fig. 5). In the absence of SOD, adding alloxan had much less effect. A similar exper-

iment was not possible with divicine or isouramil, because stable oxidation products cannot be isolated. However, when either was allowed to auto-oxidize (approx. 80%), then further divicine or isouramil was added, rapid oxidation occurred without a lag. As examples, the initial rate of oxidation of 40 μM isouramil increased from 0.5 to 21 μM/min when 90 μM oxidized isouramil was added and with 50 μM acid-hydrolysed vicine, adding 40 μM oxidation product increased initial oxidation from <0.5 to 11 μM/min. No increase in rate was apparent in the absence of SOD (see Fig. 3).

Omission of DTPA increased rates of auto-oxidation approx. 2-fold. Catalase (10 μg/ml), in the presence of DTPA, altered initial rates in the absence of SOD, or lag times and maximum rates in the presence of SOD, by no more than 5%. In accordance with there being little involvement of H_2O_2 under these conditions, adding catalase after the completion of each reaction led to recovery of 45–47% of the O_2 consumed. This indicates that >90% of the O_2 was converted to H_2O_2 as a stable end product. These observations agree with previous reports showing that oxidation can be accelerated by a mechanism involving H_2O_2, but only in the presence of added or adventitious transition metal ions [12–14]. This reaction has been excluded from the present study by the inclusion of DTPA in buffers.

Reaction mechanism

The following mechanism is proposed to account for the observations:

$$DH_2 + O_2 \rightarrow O_2^{\cdot-} + DH^{\cdot} + H^+ \quad (1)$$

$$H^+ + DH_2 + O_2^{\cdot-} \rightarrow DH^{\cdot} + H_2O_2 \quad (2)$$

$$DH^{\cdot} + O_2 \rightarrow D + O_2^{\cdot-} + H^+ \quad (3)$$

$$H^+ + D + O_2^{\cdot-} \rightarrow DH^{\cdot} + O_2 \quad (4)$$

$$DH_2 + D \rightarrow 2DH^{\cdot} \quad (5)$$

$$2DH^{\cdot} \rightarrow DH_2 + D \quad (6)$$

$$2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (7)$$

In the presence of excess SOD, reaction (7) suppresses reactions (2) and (4). If reaction (3) is quantitative for the DH$^{\cdot}$ produced in (1) and (5), the net reaction is (8) and the rate is given by (9), in which $k_5 \gg k_1$.

$$DH_2 + O_2 \rightarrow H_2O_2 + D \quad (8)$$

$$-d[DH_2]/dt = k_1[DH_2][O_2] + k_5[DH_2][D] \quad (9)$$

Oxidation begins slowly, mainly because of the small concentration of D initially present, but as D accumulates the reaction accelerates and reaches maximum rate when [D] = [DH_2] and

$$(-d[DH_2]/dt)_{max} = 0.25\, k_5[DH_2]_0^2 \quad (10)$$

Figure 4 shows that this relation is obeyed in all cases with O_2-saturated solutions at $[DH_2]_0 < 100$ μM. The values of k_5 so obtained are listed in Table 1. As k_6 [DH$^{\cdot}$]/$k_3[O_2]$ increases, reaction (6) competes with (3), thus decreasing oxidation rates below those calculated from (9) by k_6 [DH$^{\cdot}$]^2. Since [DH$^{\cdot}$] is largely determined by the balance between reactions (5) and (3), it is approx. equal to 2 k_5 [DH_2][D]/$k_3[O_2]$, and the calculated rate decrease is then proportional to $k_6(k_5/k_3)^2[DH_2]^2[D]^2/[O_2]^2$. This enables values of k_6/k_3 to be obtained from the data in Fig. 4 (Table 1).

The extremely small initial oxidation rates and long lag periods and their insensitivity to varying [O_2] for divicine and acid-hydrolysed vicine show that reaction (1) is practically negligible for these pyrimidines, and also imply that the initial [D] $<10^{-5}$ $[DH_2]_0$. The initial rates (Fig. 2b) indicate higher values of k_1 for dialuric acid and isouramil. Estimates of k_1 obtained from computer aided calculations of the time course of oxidation and its variation with [O_2], assuming $[D]_0 = 0.01\ [DH_2]_0$ are given in Table 1.

Computer simulations using the rate constants in Table 1 have been made to compare the experimental data with those predicted from the mechanism. Agreement is satisfactory for the rate curves (Fig.

Table 1. Measured and assumed rate constants ($M^{-1}\,sec^{-1}$)

	Isouramil	Divicine	Acid-hydrolysed vicine	Dialuric acid	Dialuric acid at pH 5.7 [27]
k_1*	0.2	$<3 \times 10^{-3}$	$<1 \times 10^{-4}$	0.5	210
k_2†	2×10^4	2.5×10^5	ND	1.5×10^4	$<1 \times 10^3$
k_3‡	1.2×10^5	1×10^5	$>3 \times 10^5$	$>3 \times 10^5$	3×10^7
k_4†	2×10^5	1×10^7	ND	1×10^5	5×10^5
k_5*	170	370	60	45	ignored
k_6§	1×10^8	1×10^8	1×10^8	1×10^8	8×10^7
k_7‖	6×10^5	6×10^5	6×10^5	6×10^5	negligible
k_{12}§	ignored	2×10^8	ND	ignored	2.5×10^8
k_6/k_3^2 (Msec)*	7×10^{-3}	1×10^{-2}	$<1 \times 10^{-3}$	$<1 \times 10^{-3}$	

* Determined from data in presence of SOD.
† Adopted to account for data in absence of SOD.
‡ Obtained from k_6/k_3^2, assuming $k_6 = 1 \times 10^8$.
§ Assumed.
‖ Observed rate constant for disproportionation of the equilibrium mixture of $O_2^{\cdot-}$ and HO_2 at pH 7 [26].
ND, not determined (see text).

2b), except at high percentage oxidations for divicine and acid-hydrolysed vicine, probably because absorption by the oxidized products at 280 nm has been neglected. There is good agreement for pyrimidine concentration dependence in air and O_2 (Fig. 4) and reasonable agreement with observed dependence of dialuric acid oxidation rate on alloxan concentration (Fig. 5). The mechanism also accounts for the observed inverse relationship between lag period and $[DH_2]_0$, since it predicts that when reactions (1) and (6) are negligible, the time to 50% oxidation is $(2.3 \log[DH_2]_0/[D]_0)/k_5[DH_2]_0$.

In the absence of SOD, $DH^\cdot$ and $O_2^{\cdot-}$ produced in (1) and (5) react by either of two pathways. The first leads to pyrimidine oxidation by the chain sequence (2) and (3). The second consumes radicals in (6) and (7), or products and radicals in (4) and (6). The oxidation rate depends on a competition between these pathways. The observed rates (Fig. 3) exceed the rate of radical production calculated from equation (9) for dialuric acid, isouramil and acid-hydrolysed vicine, although not divicine, even after appreciable accumulation of D (>10% oxidation). If the rate-determining chain propagation step is (2) and the major initiating reaction is (1) (which is approximated in the initial stages of dialuric acid oxidation) then the mechanism leads to

$$-d[DH_2]/dt = k_2(k_1/k_7)^{1/2}[DH_2]^{3/2}[O_2]^{1/2} \qquad (11)$$

The data for dialuric acid nearly satisfy this relation but this must be regarded as fortuitous, since a similar dependence on $[DH_2]^{3/2}[O_2]^{1/2}$ was approximated for isouramil and acid-hydrolysed vicine where the data in the presence of SOD have shown the dominant initiating reaction to be (5). Further, equilibrium between reactions (3) and (4) is not maintained, because this would require a greater increase of oxidation rate with increasing [D] than was observed. For this reason, computer-aided calculations of oxidation rates were used to test the mechanism. Of the seven rate constants required for this purpose k_7 is known from the literature, and k_1, k_5, and k_6/k_3^2 have been determined from the data in the presence of SOD. Two adjustable parameters (k_2 and k_4) are available to fit the data in its absence. The values listed in Table 1 have been adopted in calculating the progress of oxidation (Fig. 2A), and variation of oxidation rates with $[DH_2]$, $[O_2]$ and [D] (Figs 3 and 5), with results (broken lines) in reasonable agreement with the data for three of the four pyrimidines studied. Ten-fold lower assumed values of k_6 do not affect this agreement or the values required for k_2 and k_4, provided k_5^2 is reduced by the same factor. The data for acid-hydrolysed vicine follow a similar pattern but have been excluded from kinetic analysis because the parameters required in the presence of SOD predict a lag of 60 sec or more in its absence, before maximum oxidation rate is attained. This was not observed. With this exception, the mechanism accounts satisfactorily for the data. In particular, it explains why oxidation rates occurring by a non-chain mechanism in the presence of SOD can exceed those in its absence, as was most strikingly observed for divicine. The high values of k_5 and k_4 for this pyrimidine lead to high $[DH^\cdot]$ as D accumulates during oxidation. This favours radical consumption in (6) (proportional to $[DH^\cdot]^2$) over chain propagation (proportional to $[DH^\cdot]$). In contrast, radical concentrations are extremely low in the initial stages of oxidation in the absence of SOD, when $[D] \ll [DH_2]$. This favours high chain yields, and eliminates the lag seen in the presence of SOD.

Houee-Levin *et al.* [27] have studied the auto-oxidation of dialuric acid using pulse radiolysis and propose a mechanism broadly similar to ours, but omitting reaction (5). This omission partially accounts for the discrepancies in rate constants reported by these authors and ourselves, noted in Table 1. Differences of pH may also contribute. Our evidence above indicates that reaction (5) must have played a major role in their experiments since they used alloxan concentrations up to 5×10^{-3} M, whilst their value of k_1 is not consistent with the oxidation rates we observed in the presence of SOD, and their very small dependence on $[O_2]$. Their mechanism includes the reaction

$$H^+ + O_2^{\cdot-} + DH^\cdot \rightarrow D + H_2O_2 \qquad (12)$$

This reaction may occur under our conditions but its rate constant cannot be determined from the data. We have ignored it for dialuric acid and isouramil because the data can be explained without it. If it is included with $k_{12} = 2 \times 10^8\ M^{-1}\ sec^{-1}$ (as reported for dialuric acid [27]) the data can be approximately but less satisfactorily fitted provided k_2 is increased seven-fold, and k_4 two-fold for DA and 10-fold for isouramil. For divicine, however, the exceptionally high SOD concentration needed to suppress reaction (2) shows that $k_2 > 2 \times 10^5\ M^{-1}\ sec^{-1}$, and this requirement can be met only if reaction (12) is included. The rate constants quoted fit the data for an assumed value of k_{12} of $2 \times 10^8\ M^{-1}\ sec^{-1}$.

In summary, we conclude that the mechanism satisfactorily accounts for the data, but there are considerable uncertainties in the values estimated for k_2 and k_4 arising from lack of knowledge of k_{12}.

Auto-oxidation at pH 7.4 and 37°

Kinetic studies were carried out at pH 7.0 and 23° where reactions were slow enough to follow by conventional spectrophotometry. Reactions were faster and measurements were less accurate at physiological temperature and pH, but similar concentration relationships were observed under both sets of conditions. At pH 7.4 and 37°, initial rates in the absence of SOD were 2.5 times higher than those in Fig. 3 for dialuric acid and acid-hydrolysed vicine, 3 times higher for divicine and 9 times higher for isouramil. Maximum oxidation rates in the presence of SOD, determined by k_5, were also increased (Table 2). The greatest increase was again seen with isouramil, so that at this pH it oxidized faster than divicine. As a result of reaction (5) being faster, the effect of SOD was more transitory, as reflected by the lag times in Table 2. Presumably these conditions are similar to those where Chevion *et al.* [13] saw no inhibition of isouramil oxidation by SOD.

DISCUSSION

This study has shown that dialuric acid and the fava

Table 2. Rate constants for reaction (5) and lag times for auto-oxidation in the presence of SOD at pH 7.4 and 37°

Compound	k_5 ($M^{-1}\,sec^{-1}$)	Lag (min)*
Dialuric acid	140	1.5
Isouramil	3000	0.11
Divicine	1280	0.8
Acid-hydrolysed vicine	300	7.0

Values of k_5 were determined from the slopes of lines similar to those in Fig. 4, on the basis of the relationship: max. rate = $k_5/4[DH_2]_0^2$.

* Time until maximum rate was attained, measured with 100 μM pyrimidine.

bean-derived pyrimidines auto-oxidize by a common mechanism, involving an $O_2^{\cdot}$-dependent chain, and an autocatalytic reaction between the oxidized and reduced pyrimidine that predominates when the chain is suppressed by SOD. The latter reaction explains why inhibition by SOD is transitory and becomes less at higher pyrimidine concentrations. The relatively faster reaction of O_2 with dialuric acid compared with the other pyrimidines explains why initial inhibition of dialuric acid oxidation by SOD is less effective and there is a less defined lag period.

Our results confirm the observation of Pedersen *et al.* [21] that hydrolysis of vicine by acid and β-glucosidase gives different products. We show that the two compounds are spectrally similar but auto-oxidize at different rates, with the β-glucosidase product behaving in the same way as synthetic divicine. The acid hydrolysis product auto-oxidized slightly faster than divicine in the absence of SOD, but considerably more slowly with SOD present. These differences must be taken into account in interpreting previous studies such as [14, 15] in which acid hydrolysis has been assumed to produce divicine. The present study suggests that mechanistic analogies can be drawn between the two compounds, but relative reaction rates differ. Pedersen *et al.* [21] report greater stability of the "true" divicine radical. This is consistent with our observation that more SOD is required to inhibit $O_2^{\cdot}$-dependent chain oxidation with divicine, due to higher values of k_2 and k_4, than for the other pyrimidines. With the lag phase being so much shorter for divicine and isouramil than for acid-hydrolysed vicine, it is not surprising that SOD inhibition of auto-oxidation was not apparent in previous studies [13].

The cellular toxicity of dialuric acid (or alloxan) and the favic compounds divicine and isouramil is thought to involve their redox reactions [1–6]. Suppression of oxidation, therefore, should protect against toxicity. The results of this study imply that SOD alone should give only transitory suppression of divicine or isouramil oxidation but it should be more effective with dialuric acid, particularly with low concentrations. However, if accumulation of oxidized pyrimidine as well as $O_2^{\cdot}$ could be prevented, chain oxidation and the autocatalytic reaction would be suppressed, and this should result in prolonged and effective inhibition of auto-oxidation. It appears that the combination of SOD and GSH can perform this function. In the absence of SOD, GSH can decrease the net rate of pyrimidine oxidation, but it sets up a redox cycle with continued GSH consumption and conversion of O_2 to H_2O_2 [13, 28, 29]. However, in the presence of SOD, GSH extends the lag period and decreases the maximum rate of oxidation of all three pyrimidines [14, 28, 29], so that with a 10-fold GSH excess, oxidation is almost completely suppressed. GSH appears to act by competing with reaction (3) for $DH^{\cdot}$, thereby preventing the buildup of oxidized pyrimidine. Not only does the pyrimidine remain reduced but there is little GSH oxidation or H_2O_2 accumulation. Cells well endowed with SOD and GSH, therefore, should be able to inhibit redox cycling. This may explain the protective effect of thiols against alloxan diabetes [7, 30], and why normal red cells are not particularly vulnerable to favic haemolysis. Furthermore, the sensitivity of G6DP deficient cells may not only be due to their impaired ability to remove H_2O_2 [5, 6, 13], but may also stem from their GSH levels being too low to prevent divicine and isouramil from redox cycling.

This study has described a mechanism for the auto-oxidation of divicine, isouramil and dialuric acid that should provide a basis for understanding how these compounds exert their cytotoxicity. A further oxidation pathway involving reactions of H_2O_2 and either transition metal ions or haemoglobin [12, 14] is suppressed if there is efficient H_2O_2 removal and metal sequestration, but could become significant if H_2O_2 is not adequately dealt with. This could be the case in red cells with their high haemoglobin content. Furthermore, all three pyrimidines are capable of releasing iron from ferritin (Monteiro and Winterbourn, unpublished), and this might serve to catalyse oxidation. Thus factors that regulate metal catalysed auto-oxidation could influence the rate at which the pyrimidines auto-oxidize and also need to be considered in relation to haemolysis and β cell toxicity.

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