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Generation of Hydrogen Peroxide, Superoxide and Hydroxyl Radicals during the Oxidation of Dihydroxyfumaric Acid by Peroxidase

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1. Dihydroxyfumarate slowly autoxidizes at pH 6. This reaction is inhibited by superoxide dismutase but not by EDTA. Mn^{2+} catalyses dihydroxyfumarate oxidation by reacting with $O_2^{-\bullet}$ to form Mn^{3+}, which seems to oxidize dihydroxyfumarate rapidly. Cu^{2+} also catalyses dihydroxyfumarate oxidation, but by a mechanism that does not involve $O_2^{-\bullet}$. 2. Peroxidase catalyses oxidation of dihydroxyfumarate at pH 6; addition of H_2O_2 does not increase the rate. Experiments with superoxide dismutase and catalase suggest that there are two types of oxidation taking place: an enzymic, H_2O_2-dependent oxidation of dihydroxyfumarate by peroxidase, and a non-enzymic reaction involving oxidation of dihydroxyfumarate by $O_2^{-\bullet}$. The latter accounts for most of the observed oxidation of dihydroxyfumarate. 3. During dihydroxyfumarate oxidation, most peroxidase is present as compound III, and the enzymic oxidation may be limited by the low rate of breakdown of this compound. 4. Addition of *p*-coumaric acid to the peroxidase/dihydroxyfumarate system increases the rate of dihydroxyfumarate oxidation, which is now stimulated by addition of H_2O_2, and is more sensitive to inhibition by catalase but less sensitive to superoxide dismutase. Compound III is decomposed in the presence of *p*-coumaric acid. *p*-Hydroxybenzoate has similar, but much smaller, effects on dihydroxyfumarate oxidation. However, salicylate affects neither the rate nor the mechanism of dihydroxyfumarate oxidation. 5. *p*-Hydroxybenzoate, salicylate and *p*-coumarate are hydroxylated by the peroxidase/dihydroxyfumarate system. Experiments using scavengers of hydroxyl radicals shown that $OH^{\bullet}$ is required. Ability to increase dihydroxyfumarate oxidation is not necessary for hydroxylation to occur.

The enzyme horseradish peroxidase (donor–H_2O_2 oxidoreductase, EC 1.11.1.7) readily catalyses the oxidation of dihydroxyfumaric acid under aerobic conditions, 1 mol of O_2 being taken up per mol of dihydroxyfumarate oxidized (Swedin & Theorell, 1940; Chance, 1952). Studies of this reaction by various workers (Yamazaki, 1957, 1974; Yamazaki & Piette, 1963) have led to the proposal that aerobic oxidation of dihydroxyfumarate by peroxidase is initially due to a H_2O_2-dependent oxidation of this compound by the enzyme: this interpretation was largely based on studies of inhibition by catalase preparations. However, later stages of the oxidation were suggested to involve the superoxide radical, $O_2^{-\bullet}$. Indeed, Nilsson *et al.* (1969) obtained e.s.r.* evidence for generation of $O_2^{-\bullet}$ by a mixture of peroxidase and dihydroxyfumarate at pH 10.8. Also, Yamazaki & Yamazaki (1973) showed that oxidation of 210 μM-dihydroxyfumarate by 0.42 μM-peroxidase in the presence of ethanol is transiently inhibited on addition of superoxide dismutase.

When oxidizing dihydroxyfumarate, peroxidase becomes largely converted into compound III, which appears to be an oxygenated ferroperoxidase (Wittenberg *et al.*, 1967) and may be formed by a direct reaction between $O_2^{-\bullet}$ and the ferri-enzyme (Sawada & Yamazaki, 1973). Compound III breaks down slowly to give ferri-enzyme, $O_2^{-\bullet}$ and H_2O_2 (Rotilio *et al.*, 1975).

Unfortunately, the results obtained in the early studies of inhibition of dihydroxyfumarate oxidation by catalase are ambiguous, as it is likely that the catalase samples used were heavily contaminated with superoxide dismutase (Halliwell, 1973; Trotta *et al.*, 1974). Studies of the oxidation of indol-3-ylacetic acid by peroxidase suggest that H_2O_2 is not required to initiate the reaction (e.g. Ricard & Job, 1974).

During the oxidation of dihydroxyfumarate by peroxidase, aromatic compounds added to the reaction mixture become hydroxylated (Buhler & Mason, 1961). The concentration of dihydroxyfumarate (30 mM) used in this system is far higher than that used in previous studies of the oxidation reaction (Chance, 1952; Yamazaki & Yamazaki, 1973). Hydroxylation of *p*-coumaric acid (4-hydroxycinnamic acid) to caffeic acid (3,4-dihydroxycinnamic

* Abbreviation: e.s.r., electron spin resonance.

acid) by the peroxidase/dihydroxyfumarate system is inhibited by superoxide dismutase and by free Cu^{2+}, free Mn^{2+} or Fe^{2+} chelated to EDTA at concentrations which scavenge $O_2^{-\bullet}$ (Halliwell, 1975; Halliwell & Ahluwalia, 1976). Since this hydroxylation is also inhibited by scavengers of the hydroxyl radical, $OH^{\bullet}$, Halliwell & Ahluwalia (1976) proposed that $O_2^{-\bullet}$ and H_2O_2, formed during the oxidation of dihydroxyfumarate, combine together by the reaction first postulated by Haber & Weiss (1934) and shown in eqn. (1):

$$H_2O_2 + O_2^{-\bullet} \rightarrow OH^{\bullet} + OH^- + O_2 \qquad (1)$$

Although the Haber–Weiss reaction has been invoked to explain many aspects of the interactions of $O_2^{-\bullet}$ with biochemical systems (Bors *et al.*, 1974; Fridovich, 1975), McClune & Fee (1976) and Halliwell (1976) have been unable to demonstrate this reaction.

In the present paper, I report studies on the mechanism of oxidation of dihydroxyfumarate by peroxidase, carried out with the concentrations of enzyme and substrate required for hydroxylation. The objects of this work were both to check the proposed role of H_2O_2 and $O_2^{-\bullet}$ in the oxidation, in view of the recent results with indolylacetic acid, and to gain more information about the chemical species that might be available for hydroxylation, especially $OH^{\bullet}$. The oxidation reaction has been followed in two ways: by the loss of dihydroxyfumarate, measured as ΔA_{300} (Goscin & Fridovich, 1972), and by uptake of O_2, measured with an O_2 electrode. Because of the limited solubility of O_2 in the reaction mixture, the latter technique only provides information about the first few minutes of the oxidation reaction, but it is extremely useful for following the initial stages.

Experimental

Materials

Dihydroxyfumaric acid was purchased from Koch–Light Laboratories, Colnbrook, Bucks., U.K. Catalase was obtained from Boehringer Corp., London W.5, U.K.: it was completely free from superoxide dismutase activity (Halliwell, 1973). Erythrocuprein was prepared as described by McCord & Fridovich (1969). The manganese-containing superoxide dismutase from *Bacillus stearothermophilus* (Bridgen *et al.*, 1975) was obtained from M.R.E., Porton, Salisbury, Wilts., U.K. Neither enzyme contained catalase activity. Where indicated, erythrocuprein was denatured by heating at 100°C for 30min and cooling before use. Potassium superoxide was obtained from ICN Pharmaceuticals, Plainview, NY, U.S.A.

p-Coumaric acid and horseradish peroxidase (type VI) were purchased from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. The peroxidase preparation was dissolved in potassium phosphate buffer (8.3mM, pH6) and its concentration calculated from A_{403} ($\varepsilon_{403} = 10^5 M^{-1} \cdot cm^{-1}$) (Sawada & Yamazaki, 1973). The A_{403}/A_{278} ratio was 2.90.

Methods

Enzyme assays. Superoxide dismutase ($O_2^{-\bullet}$–$O_2^{-\bullet}$ oxidoreductase, EC 1.15.1.1) was assayed by the cytochrome *c* method; 1 unit inhibits the reduction of cytochrome *c* by 50% under the assay conditions of McCord & Fridovich (1969). Catalase (H_2O_2–H_2O_2 oxidoreductase, EC 1.11.1.6) was assayed by the fall in A_{240} as H_2O_2 was destroyed; the assay conditions were those of Luck (1963), except that 8.3mM-KH_2PO_4, adjusted to pH6 with KOH, was the buffer used in the reaction mixture; 1 unit of catalase is that amount which catalyses the breakdown of 1 μmol of H_2O_2/min under these conditions.

Oxidation of dihydroxyfumarate. This was followed by the fall in A_{300}. Reaction mixtures contained, in a total volume of 3ml, the following reagents at the final concentrations stated: dihydroxyfumarate (30mM), KH_2PO_4 (8.3mM), peroxidase (26nM) and sufficient KOH to adjust the pH to 6. Where indicated, *p*-coumaric acid (2.5mM), or other phenol (2.5mM), was also added. Reaction mixtures were incubated, with shaking, at 25°C. At intervals, samples (20μl) were removed from each reaction mixture, added to 10ml of water and A_{300} was recorded at once (Halliwell & Ahluwalia, 1976).

O_2 uptake by the above reaction mixtures was followed in a final volume of 3 ml at 25°C by using a Rank O_2 electrode (Rank Bros., Cambridge, U.K.) connected to a mV recorder. The solubility of O_2 in the reaction mixtures was measured by the method of Chappell (1964).

Hydroxylation of aromatic compounds. Hydroxylation of these added to the above reaction mixtures was followed as described by Halliwell & Ahluwalia (1976).

Results

Where concentrations of reagents are specified, they refer to the final concentrations in the appropriate reaction mixtures, unless otherwise stated.

Autoxidation of dihydroxyfumarate

Solutions of dihydroxyfumarate were found to autoxidize slowly at pH6. Fig. 1 shows that the uptake of O_2 is almost completely inhibited by addition of 10 units of superoxide dismutase, but the heat-denatured enzyme has no effect. Addition of Cu^{2+} (0.1μmol) greatly increases the rate of oxidation (e.g. in one experiment 0.1μmol of $CuSO_4$ caused the breakdown of 9.2μmol of dihydroxyfumarate in

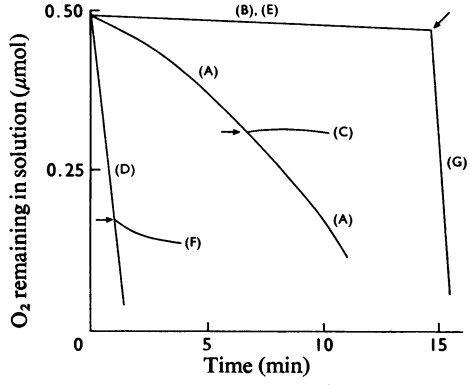

Fig. 1. *O_2 uptake by a solution of dihydroxyfumarate*
Reaction mixtures contained, in a total volume of 3ml, dihydroxyfumarate (90μmol), KH_2PO_4 (25μmol) and sufficient KOH to adjust the pH to 6. O_2 uptake was followed by using the O_2 electrode (see the Experimental section). (A), No addition; (B), +10 units of erythrocuprein; (C), as (A), but 10 units of erythrocuprein injected at the arrow; (D), +$MnCl_2$ (0.1μmol); (E) +$MnCl_2$ (0.1μmol) and 10 units of erythrocuprein; (F), as (D) but 10 units erythrocuprein added at arrow; (G), as (B), but $CuSO_4$ (0.1μmol) added at the arrow. Erythrocuprein had no effect on O_2 uptake in the presence of $CuSO_4$. Units of erythrocuprein are as defined by the cytochrome *c* assay (see the Experimental section). The results with Mn^{2+} and Cu^{2+} were confirmed by using the assay based on ΔA_{300} (see under 'Methods').

30min at 25°C, as followed by ΔA_{300}, but this increased rate is not affected by superoxide dismutase. Mn^{2+} also catalyses dihydroxyfumarate oxidation, but this is completely prevented by the presence of superoxidase dismutase (Fig. 1).

EDTA at a final concentration of 1mM completely prevents the stimulation of dihydroxyfumarate oxidation by $CuSO_4$ (0.1μmol) or $MnCl_2$ (0.1μmol). However, the autoxidation of dihydroxyfumarate in the absence of added Mn^{2+} or Cu^{2+} is not due to contamination by traces of these metal ions, as EDTA (1–2mM) does not decrease the uptake of O_2.

Incubation of dihydroxyfumarate (30mM) with H_2O_2 (10–180mM) at 25°C for 30min does not increase the ΔA_{300}, and so there will be no non-enzymic reaction between dihydroxyfumarate and any H_2O_2 formed during incubations.

Addition of *p*-coumaric acid or other phenol (2.5mM) does not increase the rate of autoxidation of dihydroxyfumarate.

Oxidation of dihydroxyfumarate by peroxidase

This reaction was followed at the pH and concentrations of enzyme and dihydroxyfumarate which give optimal rates of hydroxylation of added aromatic compounds (Buhler & Mason, 1961). Fig. 2(*a*) shows that oxidation, followed by ΔA_{300}, is almost completely stopped by addition of 10 units of super-

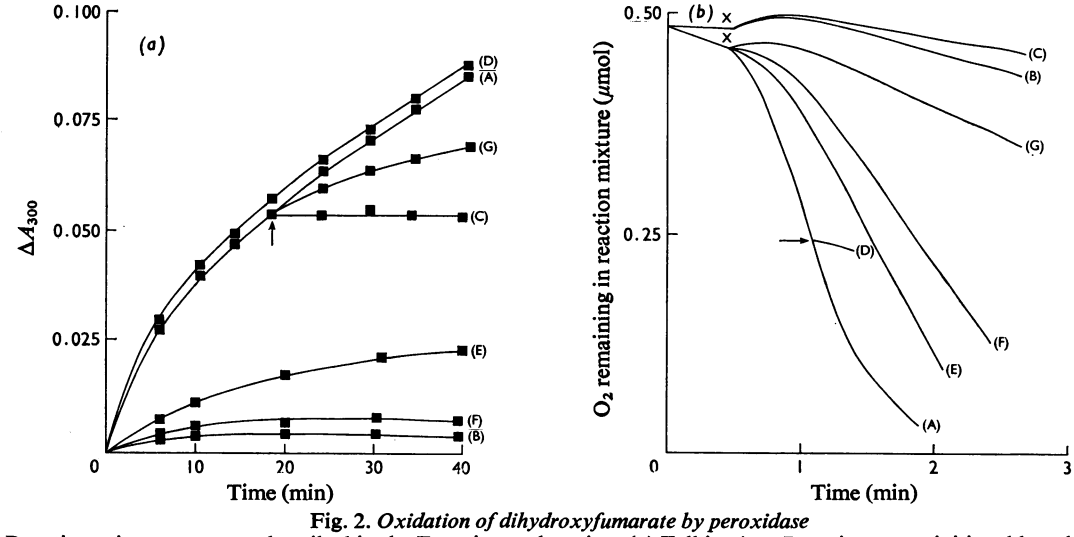

Fig. 2. *Oxidation of dihydroxyfumarate by peroxidase*
Reaction mixtures were as described in the Experimental section. (*a*) Fall in A_{300}. Reactions were initiated by adding peroxidase. The results presented have been corrected for the small non-enzymic breakdown of dihydroxyfumarate. (A), Complete; (B), +10 units of erythrocuprein; (C), as (A), but 10 units of erythrocuprein added at the arrow; (D), +4400 units of catalase; (E), +22000 units of catalase; (F), +44000 units of catalase; (G), as (A), 44000 units of catalase added at the arrow. (*b*) Uptake of O_2. The non-enzymic rate of oxidation was followed for 30s in each case and the reaction initiated by injecting peroxidase at the point X. (A), Complete; (B), +10 units of erythrocuprein; (C), +50 units of erythrocuprein; (D), as (A), but 50 units of erythrocuprein added at the arrow; (E), +4400 units of catalase; (F), +9000 units of catalase; (G), +44000 units of catalase.

oxide dismutase (erythrocuprein and the manganese-containing superoxide dismutase are equally effective), but heat-denatured enzyme has no effect. Inhibition also occurs if superoxide dismutase is added after the reaction has started. Similar results are obtained when the initial stages of dihydroxyfumarate oxidation are examined with the O_2 electrode (Fig. 2*b*).

Addition of 4400 units of catalase (completely free of superoxide dismutase; see Halliwell, 1973) decreases the O_2 uptake by the peroxidase/dihydroxyfumarate system (Fig. 2*b*) but does not inhibit oxidation as followed by ΔA_{300} (Fig. 2*a*). This suggests that H_2O_2 is formed in the reaction mixture and the catalase is breaking it down to release O_2, but that this H_2O_2 is not required for dihydroxyfumarate oxidation. However, when the amount of catalase added is increased to 44000 units, the rate of O_2 uptake is further diminished and inhibition of dihydroxyfumarate oxidation does occur (Fig. 2*a*). This large amount of catalase itself slightly increases the slow breakdown of dihydroxyfumarate in the absence of peroxidase: the results presented in Fig, 2 have been corrected for this. When 44000 units of catalase are added after the peroxidase has begun oxidation of dihydroxyfumarate, maximal inhibition only occurs after a period of 5–10 min (Fig. 2*a*).

Bovine serum albumin (2 mg) has no effect on the oxidation reaction, nor does addition of H_2O_2, tested up to a final concentration of 7.5 mM.

The effects of Cu^{2+} and Mn^{2+} on the peroxidase-catalysed oxidation of dihydroxyfumarate were also examined. The rate in the presence of $CuSO_4$ (0.1 μmol) is only slightly greater than the non-enzymic rate caused by Cu^{2+}, suggesting that Cu^{2+} inhibits the peroxidase-catalysed reaction. This is consistent with its ability to catalyse the dismutation of $O_2^{-\bullet}$ (Rabani *et al.*, 1973). Cu^{2+} (0.1 μmol) has no inhibitory effect on peroxidase as measured by the guaiacol assay (Chance & Maehly, 1955), since guaiacol oxidation does not involve $O_2^{-\bullet}$.

Swedin & Theorell (1940) and Chance (1952) suggest that Mn^{2+} stimulates dihydroxyfumarate oxidation by peroxidase. However, I found that when allowance is made for the non-enzymic breakdown induced by Mn^{2+} (0.1 or 0.2 μmol), no stimulation of the peroxidase-catalysed reaction is evident under the reaction conditions used in the present paper.

The effect of scavengers of $OH^{\bullet}$ on the oxidation by peroxidase was also examined: 0.1 M-ethanol, 50 mM-sodium formate, 10 mM-thiourea and 0.1 M-mannitol each inhibit dihydroxyfumarate oxidation by 11–18%, suggesting that $OH^{\bullet}$ may play a small role in the oxidation.

Effect of phenols on the peroxidase/dihydroxyfumarate system

Addition of *p*-coumarate (2.5 mM) greatly increases the rate of oxidation of dihydroxyfumarate by peroxidase (Fig. 3). More than 95% of the *p*-coumarate added to these reaction mixtures remains unchanged, but a small amount is hydroxylated to give caffeate (Halliwell & Ahluwalia, 1976). Caffeate also increased the rate of dihydroxyfumarate oxidation by peroxidase. However, addition of the amount of caffeate that would be formed during 30 min incubation of peroxidase and dihydroxyfumarate with *p*-coumarate only stimulated oxidation by 100%, whereas *p*-coumarate stimulates it to a much greater extent (Fig. 3). Studies using the O_2 electrode showed that the increased rate of oxidation in the presence of *p*-coumarate is visible within the first few seconds of the reaction, when no caffeate has been formed. Further, a concentration of superoxide dismutase which completely blocks formation of caffeate only slightly inhibits the increased rate of dihydroxyfumarate oxidation in the presence of *p*-coumarate (Halliwell & Ahluwalia, 1976). It is therefore concluded that the stimulation of oxidation is largely, if not completely, due to *p*-coumarate itself.

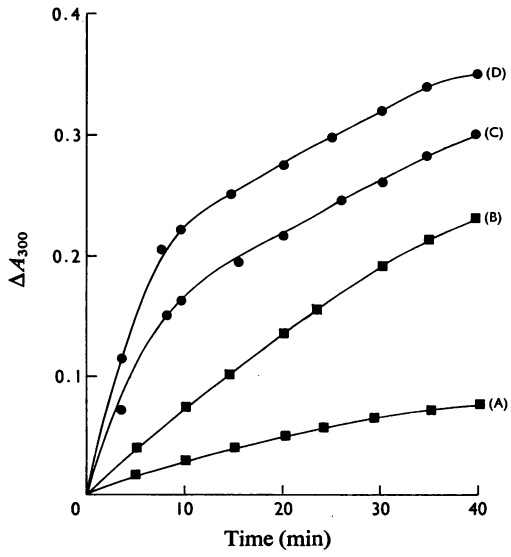

Fig. 3. *Effect of p-coumarate and H_2O_2 on oxidation of dihydroxyfumarate by peroxidase*

Reaction conditions were as described in the Experimental section. Reactions were initiated by adding peroxidase. (A), No phenol; (B), +*p*-coumarate (2.5 mM); (C) +*p*-coumarate (2.5 mM) and H_2O_2 (3.7 mM); (D), +*p*-coumarate (2.5 mM) and H_2O_2 (7.4 mM). H_2O_2 at either concentration did not increase the rate of oxidation of dihydroxyfumarate in the absence of *p*-coumarate.

Oxidation of dihydroxyfumarate by peroxidase in the presence of *p*-coumarate is much more sensitive

Table 1. *Effect of monophenols on the mechanism and rate of oxidation of dihydroxyfumarate by peroxidase*
The reaction conditions were as described in the Experimental section. Phenols were present at a final concentration of 2.5mM. Where indicated, erythrocuprein, catalase or $MnCl_2$ were added in the amounts stated. Preliminary experiments showed that the decreased inhibition by dismutase in the presence of *p*-coumarate is not due to inhibition of this enzyme by the phenol. The initial rate of oxidation of dihydroxyfumarate, designated as 1.0, was $\Delta A_{300} = 0.003$/min, and the time course was similar to that shown in Fig. 2(*a*).

Parameter of reaction	Phenol added: None	*p*-Coumarate	Salicylate	*p*-Hydroxybenzoate
Relative initial rate of oxidation of dihydroxyfumarate	1.0	2.4	1.0	1.1
Inhibition of oxidation by superoxide dismutase (%)				
10 units	90	18	90	30
50 units	95	53	98	—
100 units	99	62	—	—
Inhibition of oxidation by catalase (%)				
4400 units	0	72	0	30
9000 units	0	76	—	—
22000 units	54	100	—	—
Stimulation by $MnCl_2$* (0.2μmol)	No	Yes	No	No

* Rates corrected for the breakdown induced by Mn^{2+} in the absence of peroxidase (see the text).

to catalase and less sensitive to superoxide dismutase (equal activities of erythrocuprein and the manganese-containing enzyme give exactly the same degree of inhibition) than is oxidation in the absence of phenol (Table 1). However, higher concentrations of dismutase do inhibit the oxidation partially; heat-denatured enzyme does not.

The initial rate of dihydroxyfumarate oxidation in the presence of peroxidase and *p*-coumarate is stimulated by H_2O_2 (Fig. 3). Also, Mn^{2+} (0.2μmol) increases the rate of oxidation by 100% (after correction for non-enzymic breakdown). It is noteworthy that dihydroxyfumarate oxidation in the presence of Mn^{2+} (0.2μmol) and *p*-coumarate is now completely unaffected by superoxide dismutase (100 units) or Cu^{2+} (0.1μmol) but is still completely inhibited by 44000 units of catalase.

Table 1 shows the effect of two other monophenols, both substrates for hydroxylation by the peroxidase/dihydroxyfumarate system, on the rate of oxidation. Salicylate has no effect on the rate of oxidation, nor on its sensitivity to superoxide dismutase, catalase or Mn^{2+}. *p*-Hydroxybenzoate stimulates oxidation only slightly, but this increased rate is more sensitive to catalase and less sensitive to superoxide dismutase. However, Mn^{2+} (0.1–0.2μmol) does not stimulate further. The rate of hydroxylation of *p*-coumarate is about twice that of the other two phenols, when assayed as described by Halliwell & Ahluwalia (1976).

Formation of peroxidase compound III

In confirmation of previous results (Chance, 1952; Yamazaki, 1957), it was found that the absorption spectrum of peroxidase rapidly shifts to that of compound III when dihydroxyfumarate is added. The effect of phenols on formation of compound III was studied; it was, of course, necessary to use much higher concentrations of enzyme (1μM) than those used in studies of oxidation (26nM), in order to follow

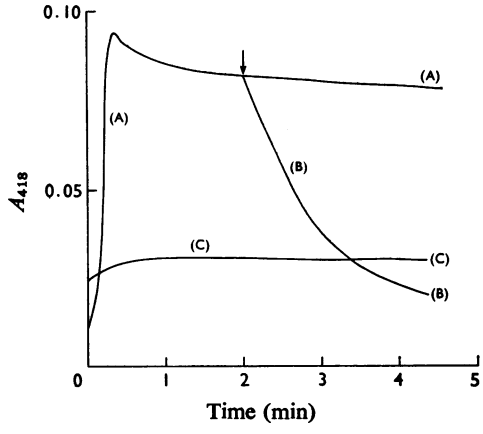

Fig. 4. *Effect of p-coumarate on peroxidase compound III*
Formation of compound III when peroxidase was added to a solution of dihydroxyfumarate was followed by ΔA_{418}, as described by Yokota & Yamazaki (1965). (A), Complete; (B), as (A), but 1μmol of *p*-coumarate added at the arrow; (C), *p*-coumarate (2.5mM) present in the reaction mixture before peroxidase was added.

Table 2. *Effect of hydroxyl scavengers on the rate of hydroxylation of p-coumarate by the peroxidase/dihydroxyfumarate system*
Hydroxylation and dihydroxyfumarate oxidation were assayed as described in the Experimental section. The reason why thiourea stimulates dihydroxyfumarate oxidation is unknown, but it shows that inhibition of hydroxylation by this compound is not due to an inhibition of peroxidase. (Thiourea did not induce any oxidation of dihydroxyfumarate in the absence of peroxidase.) The radical rate constants were taken from the compilation by Anbar & Neta (1967).

Compound tested	Concentration (mM)	Rate constant for reaction with $OH^{\bullet}$ ($M^{-1} \cdot s^{-1}$)	Inhibition of *p*-coumarate hydroxylation (%)	Inhibition of dihydroxyfumarate oxidation (%)
Mannitol	50	1.0×10^9	23	0
	100		40	0
Ethanol	50	1.2×10^9	30	0
	100		47	0
Formate	20	2.7×10^9	52	0
	50		92	24
Thiourea	10	4.7×10^9	80	—
	50		100	None (50% stimulation)
Urea	10	$<7.0 \times 10^5$	0	0
KCl	100	$<10^3$	0	0

the spectral changes. Fig. 4 shows that when *p*-coumarate (2.5mM) is present in the reaction mixture, the rise in A_{418}, which can be used to measure compound-III formation (Chance, 1952; Yokota & Yamazaki, 1965) is much less marked. Further, addition of *p*-coumarate to a reaction mixture containing dihydroxyfumarate and peroxidase results in an immediate loss of compound III. No such effects were observed with *p*-hydroxybenzoate or salicylate: Yokota & Yamazaki (1965) also observed that salicylate does not interact with compound III.

Mechanism of the hydroxylation reaction

Halliwell & Ahluwalia (1976) proposed that $OH^{\bullet}$ is required for hydroxylation by the peroxidase/dihydroxyfumarate system, since the $OH^{\bullet}$ scavengers mannitol, formate, ethanol and Tris inhibit hydroxylation at concentrations having little effect on dihydroxyfumarate oxidation. We have extended this work to include thiourea, a powerful scavenger on $OH^{\bullet}$ (Anbar & Neta, 1967; Heikkila *et al.*, 1976). Table 2 summarizes the results obtained using these scavengers. It may be seen that their ability to inhibit hydroxylation is correlated, at least qualitatively, with the rate constants for their reactions with $OH^{\bullet}$. Inhibition of hydroxylation cannot be attributed to an effect on dihydroxyfumarate oxidation. Urea (10mM) and KCl (100mM) which do not react with $OH^{\bullet}$, do not inhibit hydroxylation. Thiourea also inhibits hydroxylation of salicylate and *p*-hydroxybenzoate.

Discussion

The effect of superoxide dismutase on autoxidation of dihydroxyfumarate suggests that this compound reduces O_2 to $O_2^{-\bullet}$, which then reacts with more dihydroxyfumarate (eqns. 2–4)

$$\text{dihydroxyfumarate} + O_2 \rightarrow [X]^{\bullet} + O_2^{-\bullet} \quad (2)$$

$$2H^+ + O_2^{-\bullet} + \text{dihydroxyfumarate} \rightarrow [X]^{\bullet} + H_2O_2 \quad (3)$$

$$[X]^{\bullet} + O_2 \rightarrow \text{dioxosuccinate} + O_2^{-\bullet} \quad (4)$$

where $[X]^{\bullet}$ is a free radical formed by loss of one electron from dihydroxyfumarate. Evidence consistent with these reactions is provided by the observation that the e.s.r. spectrum of $[X]^{\bullet}$, generated under anaerobic conditions, is immediately lost when O_2 is admitted (Yamazaki & Piette, 1963). Hence a solution of dihydroxyfumarate at pH6 always contains some $O_2^{-\bullet}$ and H_2O_2. Cu^{2+} catalyses breakdown of dihydroxyfumarate by a different mechanism. However, the stimulation by Mn^{2+} is abolished by superoxide dismutase. Since Mn^{2+} reacts with $O_2^{-\bullet}$ to form the powerful oxidant Mn^{3+} (eqn. 5) (Halliwell & Ahluwalia, 1976; Kono *et al.*, 1976), the stimulation can be accounted for by reactions 5 and 6, where the rate of reaction 6 is greater than that of reaction 2:

$$O_2^{-\bullet} + Mn^{2+} + 2H^+ \rightarrow Mn^{3+} + H_2O_2 \quad (5)$$

$$Mn^{3+} + \text{dihydroxyfumarate} \rightarrow Mn^{2+} + [X]^{\bullet} \quad (6)$$

When peroxidase is added, the rate of dihydroxyfumarate oxidation increases. This increased rate is still largely dependent on $O_2^{-\bullet}$, as shown by the inhibition by superoxide dismutase and Cu^{2+}. However, high concentrations of catalase also inhibit the oxidation. Since catalase is known to be inefficient at breaking down low concentrations of H_2O_2 (Halliwell, 1974), the large amount of enzyme required to inhibit suggests that a small quantity of H_2O_2 in the reaction mixture is required for

oxidation to proceed, but, as Fig. 2 shows, more H_2O_2 than this is being generated, at least in the initial stages of the reaction. Hence there is no stimulation of oxidation by added H_2O_2 (Fig. 3). When catalase is added after the reaction has started, inhibition does not reach the maximum value for 5–10min, presumably until sufficient H_2O_2 has been destroyed (Fig. 2*a*). It could be argued that this inhibition by high concentrations of catalase is due to some property of the protein other than the ability to decompose H_2O_2. However, bovine serum albumin has no effect.

These results can be accommodated within the scheme of Yamazaki & Piette (1963), in which the peroxidase uses H_2O_2 to oxidize dihydroxyfumarate (eqn. 7):

$$\text{Dihydroxyfumarate} + H_2O_2 \xrightarrow{\text{Peroxidase}} [X]^\bullet + H_2O \quad (7)$$

$[X]^\bullet$ can then react with O_2 to give $O_2^{-\bullet}$ (eqn. 4), which will cause oxidation of more dihydroxyfumarate (eqn. 3). This non-enzymic phase of the oxidation makes a major contribution, as shown by the effects of superoxide dismutase.

Peroxidase in the presence of dihydroxyfumarate exists largely as compound III. Compound III does not react with dihydroxyfumarate (Yokota & Yamazaki, 1965) and so the rate of the enzymic stage (eqn. 7) may well be governed by the rate of breakdown of this ferro–enzyme complex to the active ferri–enzyme, which is low (Rotilio *et al.*, 1975).

Addition of *p*-coumarate greatly stimulates dihydroxyfumarate oxidation. This can be understood from the results in Fig. 4, which show that *p*-coumarate causes a breakdown of compound III. If these results are extrapolated to the much lower concentrations of enzyme used in the oxidation studies, one would expect more active enzyme to be available for oxidation, and the enzymic phase of the oxidation might be expected to play a much more significant role. That this is the case is shown by the increased sensitivity of dihydroxyfumarate oxidation in the presence of *p*-coumarate to inhibition by catalase, and the decreased effect of superoxide dismutase (Table 1). Indeed, Fig. 3 shows that the rate of oxidation is now being restrained by the supply of H_2O_2. Mn^{2+} further increases the rate of oxidation, but it does not do so by interaction with free $O_2^{-\bullet}$, since superoxide dismutase does not prevent the stimulation. Indeed, the presence of Mn^{2+} and *p*-coumarate together completely changes the mechanism of the reaction.

p-Coumarate is hydroxylated by the peroxidase/dihydroxyfumarate system. Ability to stimulate oxidation is not required for hydroxylation, since salicylate is also hydroxylated, but the increased rate of oxidation induced by *p*-coumarate seems to result in an increased rate of hydroxylation. *p*-Hydroxybenzoate stimulates oxidation slightly, and alters its sensitivity to catalase and superoxide dismutase, but not to Mn^{2+}. It is possible that *p*-hydroxybenzoate decomposes compound III at a rate too low to be detected in experiments of the type shown in Fig. 4, or perhaps converts it into another peroxidase compound with equal A_{418}, although there is no spectral evidence for this.

The studies with thiourea confirm the proposal of Halliwell & Ahluwalia (1976) that hydroxylation of aromatic compounds is due to formation of $OH^\bullet$ (Table 2). Circumstantial evidence for $OH^\bullet$ formation is also provided by the observation that $OH^\bullet$ scavengers slightly inhibit oxidation of dihydroxyfumarate by peroxidase. How does $OH^\bullet$ arise? The first proposal might be the Haber–Weiss reaction. However, attempts to demonstrate the Haber–Weiss reaction in this labotatory (Halliwell, 1976), by McClune & Fee (1976) and by earlier workers (reviewed by Fee, 1977) have all failed. Nevertheless, the results in the present paper, and others (Fridovich, 1975), strongly suggest that $OH^\bullet$ is generated in solutions containing $O_2^{-\bullet}$, by some mechanism. Elucidation of this requires further investigation.

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