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The generation of hydroxyl and alkoxyl radicals from the interaction of ferrous bipyridyl with peroxides

Differential oxidation of typical hydroxyl-radical scavengers

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Reaction conditions by which the iron–chelate ferrous bipyridyl can be used as a Fenton reagent to generate specifically alkoxyl radical (•OR) from its corresponding alkyl hydroperoxide (ROOH) without producing hydroxyl radical (•OH) as a result of autoxidation are described. In this manner, the relative ability of common •OH-scavenging agents to react with •OH and various •OR species could be assessed. When •OH was generated from H_2O_2, 4-methylmercapto-2-oxobutyrate, ethanol and benzoate all were oxidized. When •OR (cumoxyl radical, t-butoxyl radical or ethoxyl radical) was generated specifically, each was found to oxidize 4-methylmercapto-2-oxobutyrate and ethanol. In contrast with •OH, however, none of the •OR radicals mediated the decarboxylation of benzoate. Cross-competition studies with the scavengers showed that, in contrast with the •OH-dependent reaction, the •OR-dependent oxidation of 4-methylmercapto-2-oxobutyrate and ethanol was not inhibited by benzoate. Rate constants for ferrous bipyridyl oxidation by ROOH and by H_2O_2 were found to be essentially the same, and therefore the differential oxidation of the various scavengers was not a reflection of iron–peroxide interaction, but rather an interaction between generated oxy radicals and the scavengers. In contrast with the H_2O_2 system, catalase did not inhibit the oxidation of 4-methylmercapto-2-oxobutyrate or ethanol by either the cumene hydroperoxide or the t-butyl hydroperoxide system, suggesting that the oxidizing species was not derived from H_2O_2. These results suggest that benzoate decarboxylation might serve as a more specific probe to detect the presence of •OH than either 4-methylmercapto-2-oxobutyrate or ethanol, which react readily with •OR.

The production of oxidative products from certain substrates such as methional, KTBA, ethanol and benzoate has been used to detect the generation of oxy radical intermediates in a variety of systems (Mathews & Sangster, 1965; Beauchamp & Fridovich, 1970; Cohen, 1978; Bors *et al.*, 1978, 1982; Sagone *et al.*, 1980; Cohen & Cederbaum, 1980; Halliwell, 1981). A problem with this methodology is that most scavengers are rather unspecific. For example, Pryor & Tang (1978) demonstrated that ethylene can be generated from methional by a variety of radicals. Hence an understanding of the specificity of various scavengers would be of value, especially in studies in complex biological systems.

The present paper concerns a comparison of the abilities of •OH and alkoxyl (•OR) radicals to catalyse the oxidation of KTBA, ethanol and benzoate to ethylene, acetaldehyde and CO_2 respectively. Alkoxyl radicals were selected since lipid radicals (•OL) are believed to be generated during the peroxidation of lipids (Aust & Svingen, 1982). The Fenton reaction (Walling, 1975) was used to generate •OH or •OR (eqns. 1 and 1a).

$$H_2O_2 + Fe^{2+} \rightarrow {}^{\bullet}OH + OH^- + Fe^{3+} \quad (1)$$

$$ROOH + Fe^{2+} \rightarrow {}^{\bullet}OR + OH^- + Fe^{3+} \quad (1a)$$

A problem to be overcome in such studies is the autoxidation of ferrous (whether chelated or un-

Abbreviation used: KTBA, 4-methylmercapto-2-oxobutyrate ('2-keto-4-thiolmethylbutyrate').

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chelated). For example, ferrous EDTA in phosphate buffer autoxidizes within less than 1 min (Cohen *et al.*, 1981). This autoxidation could result in the production of $^{\bullet}OH$ even in the absence of H_2O_2, according to reactions (1)–(3):

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\bullet -} \quad (2)$$

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

Hence a major consideration is the ability to generate $^{\bullet}OR$ without the production of $^{\bullet}OH$ due to autoxidation of ferrous. As described below, appropriate reaction conditions can be established with the iron chelate ferrous bipyridyl to generate $^{\bullet}OR$ specifically from its corresponding alkyl hydroperoxide, without the artifact production of $^{\bullet}OH$. In this way, the interaction of KTBA, ethanol and benzoate with $^{\bullet}OH$ and with $^{\bullet}OR$ could be assessed.

Experimental procedures

Preparation of ferrous bipyridyl

Stock solutions were prepared by dissolving 2 mg of $Fe(NH_4)_2(SO_4)_2,6H_2O$ (5 mM) per ml of 12 mM-2,2′-bipyridyl.

Oxidation of scavengers

Two different types of reaction conditions were utilized. Reactions were first carried out at 37°C in 25 ml Erlenmeyer flasks containing 100 mM-potassium phosphate buffer, pH 7.4, 3 mM-H_2O_2 or 3 mM-alkyl hydroperoxide, and 53 mM-ethanol or 30 mM-KTBA or 20 mM-[7-^{14}C]benzoate (specific radioactivity 200 d.p.m./nmol) as substrate. Some flasks did not contain any peroxide, and served as the autoxidation control. Reactions were initiated by the addition of 1 mM-ferrous bipyridyl, and the flasks were sealed with rubber septa and incubated. At specific time points, portions of the head space were removed and ethylene production from KTBA or acetaldehyde production from ethanol was determined by a gas-chromatography procedure (Winston & Cederbaum, 1983). In some experiments the reactions were terminated by the addition of dimethyl sulphoxide at a final concentration of 1.6 M. The large excess of dimethyl sulphoxide should compete effectively with the substrates and scavenge the generated radicals. This procedure was necessary for the benzoate studies, since, once the reactions were terminated, it was necessary to collect the $^{14}CO_2$ in hanging centre-well cups filled with Hyamine hydroxide (Winston & Cederbaum, 1982) for an additional 60 min. The centre wells were placed in vials containing 10 ml of Soluscint-O (National Diagnostics) plus 0.03 ml of acetic acid, and the radioactivity was determined by using an automatic quench-correction procedure. In some of the experiments with ethanol the reactions were also terminated with dimethyl sulphoxide, and the acetaldehyde was measured by a semicarbazone procedure (Cederbaum *et al.*, 1978). The results were in excellent agreement with the gas-chromatography procedure.

As described in the Results section, some autoxidation of the ferrous bipyridyl complex was found. Therefore a second type of reaction condition was utilized. The ferrous bipyridyl was allowed to incubate with the phosphate buffer for 30 min in open flasks. At this point the appropriate peroxide (H_2O_2, cumene hydroperoxide, t-butyl hydroperoxide or ethyl hydroperoxide) was added, and the flasks were sealed and incubated. This was defined as the zero time. Some flasks did not receive any peroxide after the 30 min preincubation period and served as autoxidation controls. Reactions were assayed as described above.

Oxidation of ferrous bipyridyl by peroxides

The rate of oxidation of the ferrous bipyridyl complex was followed by monitoring the decrease in absorbance maximum (520 nm) in the presence of H_2O_2 or the various alkyl hydroperoxides.

Results

Initial experiments were carried out with ligands with extended π-systems that can stabilize metal atoms in low formal oxidation states (Konig, 1968). The ferrous chelates of 1,10-*o*-phenanthroline, bathophenanthroline and 2,2′-bipyridyl in the presence of H_2O_2 catalysed the oxidation of KTBA to ethylene or of benzoate to CO_2. Interestingly, *o*-phenanthroline and bathophenanthroline themselves, in the absence of iron, catalysed a significant rate of oxidation of KTBA and benzoate in the presence of H_2O_2 (results not shown). The iron-independent interaction with H_2O_2 was not observed with 2,2′-bipyridyl, and therefore further experiments were carried out with this chelating agent. Neither bipyridyl itself, nor ferric bipyridyl, could mediate the oxidation of the three $^{\bullet}OH$ scavengers used in these studies (results not shown).

Ferrous bipyridyl-catalysed oxidation of ethanol

The ferrous bipyridyl complex was stable in water and did not autoxidize. However, in phosphate buffer some oxidation occurred. This can be observed further from the results shown in Fig. 1. On incubation of ferrous bipyridyl in phosphate buffer, pH 7.4, in the presence of ethanol (and in the absence of H_2O_2 or alkyl hydroperoxide) some acetaldehyde production occurred, which levelled off

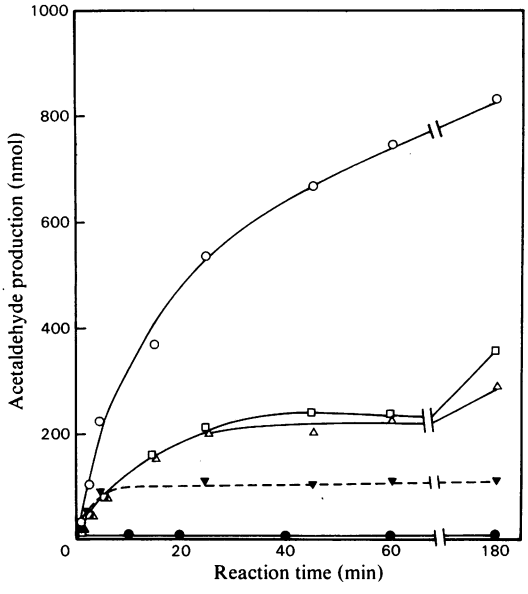

Fig. 1. *Time course for the production of acetaldehyde from ethanol by a ferrous bipyridyl-mediated Fenton reaction*

Reactions were carried out as described in the Experimental procedures section. Two different reaction conditions were studied: either reactions were initiated with the ferrous bipyridyl complex, or a 30min preincubation period with ferrous bipyridyl was performed before initiating the reaction with peroxide or water for the autoxidation control. Acetaldehyde was determined by head-space gas chromatography. Results are the means for three experiments. O, 3mM-H_2O_2, with or without the 30min preincubation; □, 3mM-cumene hydroperoxide, with or without the 30min preincubation; △, 3mM-t-butyl hydroperoxide, with or without the 30min preincubation; ▼, water system, without the 30min preincubation; ●, water system, with the 30min preincubation.

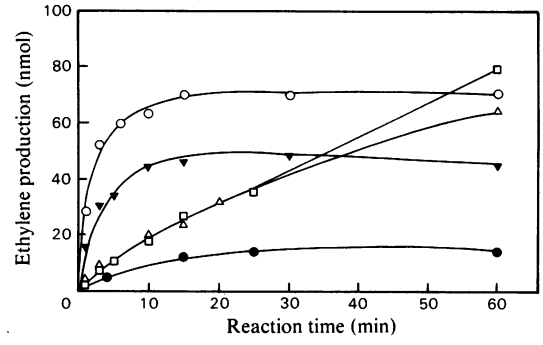

Fig. 2. *Time course for the production of ethylene from KTBA by a ferrous bipyridyl-mediated Fenton reaction*

Reactions were initiated by the addition of ferrous bipyridyl to flasks containing H_2O_2 or alkyl hydroperoxide or water (autoxidation control). Ethylene was determined by injecting 0.5ml portions of the head space directly into the gas chromatograph. Results are the means for three experiments. O, H_2O_2; □, cumene hydroperoxide; △, t-butyl hydroperoxide; ●, ethyl hydroperoxide; ▼, water system.

after approx. 10min (Fig. 1). Acetaldehyde production did not occur in the absence of phosphate buffer (results not shown), suggesting that the phosphate might be complexing to the iron in a manner that promotes autoxidation, e.g. lowering the redox potential. The fact that acetaldehyde was not produced in non-buffered water eliminated the possibility that a ligand anion radical (McWhinnie & Miller, 1969) was the oxidizing species responsible for the oxidation of ethanol.

When H_2O_2 was present, the rate and extent of acetaldehyde production were increased considerably over the rate and extent in the absence of the peroxide (Fig. 1). When cumene hydroperoxide or t-butyl hydroperoxide was present, the extent of acetaldehyde production was increased over that found for the autoxidation-dependent activity, i.e. at the point when the autoxidation system levelled off acetaldehyde was still being produced in the alkyl hydroperoxide systems (Fig. 1).

Because the autoxidation-dependent system levelled off after 10min, an experiment was performed in which ferrous bipyridyl was incubated with phosphate buffer for 30min. At this point, one of H_2O_2, cumene hydroperoxide or t-butyl hydroperoxide, or no peroxide, was added, the flasks were sealed, and the production of acetaldehyde was monitored. It was found that the peroxide-dependent activities remained essentially identical with what was observed in the absence of the 30min preincubation period (Fig. 1). However, acetaldehyde production essentially was negligible in the absence of peroxides after this preincubation (Fig. 1). This latter experiment established reaction conditions in which one could generate $^{\bullet}OH$ or $^{\bullet}OR$ without any significant contribution from an autoxidation-dependent reaction. Thus ethanol could be oxidized to acetaldehyde, not only by $^{\bullet}OH$, but also by two different alkoxyl radicals.

Ferrous bipyridyl-catalysed oxidation of KTBA

KTBA was oxidized to ethylene when the ferrous chelate was allowed to incubate in phosphate buffer without the addition of any peroxide (Fig. 2). The addition of H_2O_2 to the reaction system increased the rate and extent of ethylene production (Fig. 2). When any of the three different alkyl hydroperoxides replaced H_2O_2, the oxidation of KTBA to ethylene proceeded for at least 1h (Fig. 2). How-

ever, the rate of ethylene production was considerably lower than that which could be ascribed to autoxidation, suggesting that the ferrous bipyridyl was reacting with the hydroperoxides faster than it could autoxidize. In fact, the cumene hydroperoxide- or t-butyl hydroperoxide-dependent activity continued for over 1 h and surpassed the autoxidation-dependent rate of ethylene generation (Fig. 2).

When the ferrous bipyridyl complex was first incubated with phosphate buffer for 30 min, there was no production of ethylene in the absence of any peroxide after this preincubation period (Fig. 3). The kinetics of KTBA oxidation by •OH and by the three different •OR radicals was essentially the same after this preincubation period as before it. Thus KTBA can be oxidized to ethylene by •OH and by •OR.

Ferrous bipyridyl-catalysed oxidation of benzoate

The Fenton reaction between ferrous bipyridyl and H_2O_2 also promoted the decarboxylation of benzoate (Fig. 4). However, in contrast with what was observed with KTBA and ethanol, the three different •OR radicals were essentially inert in promoting decarboxylation of benzoate (Fig. 4). There was also no oxidation of benzoate by an autoxidation-dependent reaction (Fig. 4). Rates of $^{14}CO_2$ production in the absence of any peroxide, or in the presence of either cumene hydroperoxide, t-butyl hydroperoxide or ethyl hydroperoxide, were indistinguishable from background rates that occurred in the absence of ferrous bipyridyl. Thus •OH, but not •OR, catalyses the decarboxylation of benzoate.

There was no •OR-dependent decarboxylation of benzoate over a 10–20-fold range of concentrations of organic hydroperoxide or ferrous bipyridyl (results not shown). Therefore the lack of reactivity of benzoate with •OR was not due to a limiting hydroperoxide or iron concentration.

Cross-competiton experiments between •OH scavengers

The inability of •OR to promote the decarboxylation of benzoate does not necessarily mean that •OR cannot react with benzoate. It is possible that products other than CO_2 may result from the interaction of •OR with benzoate. To test this possibility, cross-competition experiments between •OH scavengers were conducted. The oxidation of KTBA, ethanol or benzoate by the ferrous bipyridyl •OH-generating system was first studied. Table 1 shows that there was cross-competition among these three substrates for oxidation.

It was reasoned that, if a scavenger reacted at all (or at an appreciable rate) with •OR, it should compete with either KTBA or ethanol for the

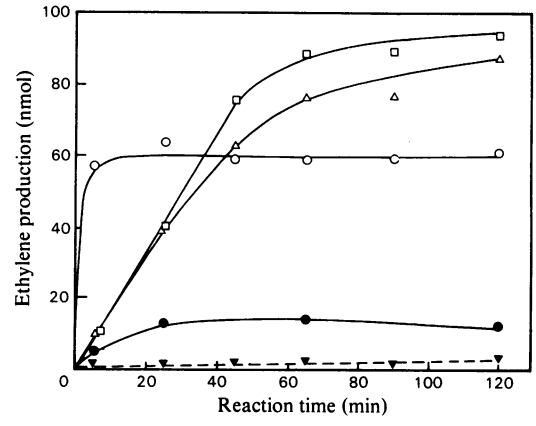

Fig. 3. *Time course for the producton of ethylene from KTBA by a ferrous bipyridyl-mediated Fenton reaction after a 30 min preincubation period with ferrous bipyridyl*

Reactions were initiated by the addition of H_2O_2, alkyl hydroperoxide or water, and ethylene was determined at various time points by head-space gas chromatography. Symbols are coded as described in the legend to Fig. 2. Results are the means for two experiments.

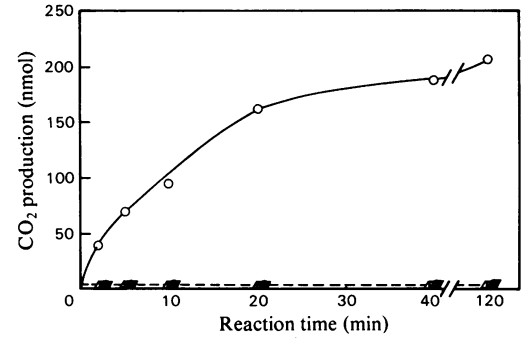

Fig. 4. *Time course for the production of CO_2 from benzoate by a ferrous bipyridyl-mediated Fenton reaction*

Reactions were carried out as described in the Experimental procedures section. ○, H_2O_2; □, cumene hydroperoxide; △, t-butyl hydroperoxide; ●, ethyl hydroperoxide; ▼, water system. Results are the means for four experiments.

generated •OR, just as it competed with these substrates for the generated •OH (Table 1). The oxidation of ethanol by the cumoxyl or the t-butoxyl radical was inhibited by KTBA (Table 1). In an analogous manner, the oxidation of KTBA by these two alkoxyl radicals was inhibited by ethanol (Table

Table 1. *Effects of competing 'OH-scavenging agents on the 'OH- and 'OR-dependent oxidation of substrates*
The production of ethylene from 30mM-KTBA or of acetaldehyde from 53mM-ethanol, or of CO_2 from 20mM-benzoate, was assayed as described in the Experimental procedures section in the presence of the indicated competing scavengers. Incubation times were 1h for KTBA and ethanol experiments, and 20min for benzoate experiments. Results are from three to five experiments. Control rates (nmol of product formed/60min for KTBA and ethanol, nmol of product formed/20min for benzoate) were: for the 'OH-dependent system, KTBA, 57.5 ± 4.8, ethanol, 763 ± 45, and benzoate, 122 ± 11; for the cumoxyl-dependent system, KTBA, 87 ± 8, and ethanol, 210 ± 13; for the t-butoxyl-dependent system, KTBA, 69 ± 6, and ethanol, 210 ± 24.

Substrate	Competing scavenger	Inhibition (%)		
		'OH-dependent	Cumoxyl-dependent	t-Butoxyl-dependent
KTBA	Ethanol (53mM)	52	25	32
	Mannitol (60mM)	60	0	7
	Benzoate (30mM)	43	2	6
Ethanol	KTBA (30mM)	85	52	45
	Mannitol (60mM)	41	4	7
	Benzoate (30mM)	60	1	5
Benzoate	KTBA (30mM)	98		
	Ethanol (53mM)	71		
	Mannitol (60mM)	76		

1). Cross-competition between KTBA and ethanol for 'OR was observed because both of these substrates can be oxidized by 'OR (Figs. 1–3). By contrast, neither benzoate nor mannitol inhibited the 'OR-dependent oxidation of KTBA or ethanol (Table 1) at concentrations that produced inhibition of the 'OH-dependent oxidation of KTBA or ethanol (Table 1). These results suggest that benzoate (and mannitol) appear to be considerably more reactive with 'OH than with 'OR. The results with mannitol are in agreement with the report that this scavenger inhibited the 'OH-dependent but not the 'OR-dependent bleaching of crocin (Bors *et al.*, 1981).

Formate, another commonly used scavenging agent, was found to inhibit the oxidation of KTBA by both 'OH and 'OR (cumene hydroperoxide and t-butyl hydroperoxide) to similar extents. For example, 30mM-formate inhibited ethylene production from 10mM-KTBA by 56, 56 and 31% for the H_2O_2, t-butyl hydroperoxide and cumene hydroperoxide systems respectively. Therefore formate appears to be relatively non-specific, similarly to KTBA and ethanol.

Oxidation of ferrous bipyridyl by H_2O_2 and alkyl hydroperoxides

The greater rate of oxidation of KTBA and ethanol by 'OH than by 'OR (Figs. 1–3) could reflect a difference in the rate of production of 'OH compared with 'OR, or a lower reactivity of 'OR with KTBA and ethanol. To evaluate the first possibility, the relative rates at which each of the peroxides could react with the ferrous bipyridyl complex (eqn. 1) was determined. A summary of the pseudo-first-order rate constants shows that the three alkyl hydroperoxides mediated the oxidation of the ferrous bipyridyl complex at similar rates to that found with H_2O_2 (Table 2). Differences in the rates of oxidation of substrates by 'OH and 'OR probably reflect the rates at which 'OH and 'OR react with the substrate (Pryor & Tang, 1978), and not the ability of ferrous bipyridyl to interact with the peroxide in the system.

Table 2. *Pseudo-first-order rate constants for the oxidation of ferrous bipyridyl by H_2O_2 and three alkyl hydroperoxides*
Rate constants were determined from pseudo-first-order rate plots. Final concentration of all peroxides was 1.0mM. Final concentration of ferrous bipyridyl was 133μM.

Peroxide	k (s^{-1})
H_2O_2	4.5×10^{-4}
Cumene hydroperoxide	4.2×10^{-4}
t-Butyl hydroperoxide	5.0×10^{-4}
Ethyl hydroperoxide	5.9×10^{-4}

Effect of catalase and superoxide dismutase

To verify further that 'OH was not being generated in the 'OR-generating systems, the effect of catalase was determined. As expected, catalase inhibited the oxidation of KTBA, ethanol and benzoate by the H_2O_2-dependent system (Table 3). This suggests that catalase reacts faster with H_2O_2 than ferrous bipyridyl does. At this elevated H_2O_2/catalase haem ratio, catalatic activity of catalase rather than peroxidatic activity with ethanol predominates (Oshino *et al.*, 1973). By contrast, catalase did not inhibit the oxidation of KTBA or ethanol by the cumene hydroperoxide or t-butyl

Table 3. *Effects of superoxide dismutase and catalase on the oxidation of 'OH-scavenging agents by 'OH and 'OR*
Values represent nmol of ethylene, acetaldehyde or CO_2 from KTBA, ethanol or benzoate respectively, and were assayed as described in the Experimental procedures section in the absence or in the presence of 100 μg of superoxide dismutase (SOD) or 100 μg of catalase. Concentrations of KTBA, ethanol and benzoate were 30, 53 and 20 mM respectively. Reaction times were 60 min for KTBA and ethanol and 20 min for benzoate. N.A., Not applicable, as CO_2 was not generated by the alkyl hydroperoxide system (Fig. 4).

		H_2O_2 system		Cumene hydroperoxide system		t-Butyl hydroperoxide system	
Scavenger substrate	Addition	Product formation (nmol)	Inhibition (%)	Product formation (nmol)	Inhibition (%)	Product formation (nmol)	Inhibition (%)
KTBA	Control	66	—	93	—	68	—
	SOD	62	−7	104	+2	70	+3
	Catalase	0	−100	93	0	73	+9
Ethanol	Control	773	—	220	—	212	—
	SOD	751	−3	217	−1	216	+2
	Catalase	184	−76	1230	+460	1240	+485
Benzoate	Control	107	—	N.A.		N.A.	
	SOD	109	+2				
	Catalase	0	−100				

hydroperoxide systems (Table 3). In fact, the pronounced stimulation of ethanol oxidation by catalase probably reflects the peroxidatic activity of catalase/alkyl hydroperoxide with ethanol (Rahimtula & O'Brien, 1977). The different responses of the H_2O_2 and the hydroperoxide systems to catalase further suggest that the oxidizing species generated when ferrous bipyridyl interacted with cumene hydroperoxide and t-butyl hydroperoxide was not derived from H_2O_2.

As would be expected from typical Fenton chemistry, superoxide dismutase had no effect on the rate of oxidation of KTBA, ethanol and benzoate (Table 3).

Discussion

Conditions under which an iron–chelate (ferrous bipyridyl) could be used in a typical Fenton-type reaction to generate 'OR, without generating 'OH, have been described and characterized. That 'OH does not appear to be produced in the 'OR-generating system can be seen from the following; (*a*) benzoate is not decarboxylated in the hydroperoxide-dependent systems, although it is in the H_2O_2-dependent system; (*b*) catalase does not inhibit the oxidation of KTBA by 'OR, but does inhibit the 'OH-mediated oxidation; (*c*) mannitol and benzoate are effective inhibitors of the oxidation of ethanol and KTBA by the 'OH-generating system but not of the oxidation mediated by the 'OR-generating system.

Previous investigations by others (Mathews & Sangster, 1965; Hoigne & Bader, 1975) eliminated the possible contribution of the superoxide radical or the perhydroxyl radical to the decarboxylation of benzoate by γ-irradiation of aqueous solutions. Benzoate was decarboxylated also by granulocytes during the phagocytosis of zymosan particles (Sagone *et al.*, 1980) and during NADPH-dependent microsomal electron transport (Winston & Cederbaum, 1982). The interaction of ferrous bipyridyl with H_2O_2 also results in the decarboxylation of benzoate (Fig. 4). However, 'OR is essentially inert in promoting this oxidation (Fig. 4). Since steric hindrance could have played a role in the failure to observe CO_2 production from benzoate by 'OR, experiments were performed with a small primary alkyl hydroperoxide, ethyl hydroperoxide. Although the ethoxyl radical oxidized KTBA, there was still no production of CO_2 from benzoate. These results suggest the possibility that decarboxylation of benzoate might serve as a more specific probe to detect the presence of 'OH than do KTBA or ethanol. Similarly, the inability of benzoate or mannitol to inhibit 'OR-dependent oxidation reactions at concentrations that inhibit 'OH-dependent reactions (Table 1) also might be useful to implicate a role for 'OR in a particular system, especially when such studies are carried out in conjunction with KTBA and ethanol as competing scavengers. At present, it is not known whether decarboxylation of benzoate may be catalysed by other radicals, and consequently the results described above are limited to 'OR.

Of interest are the apparent differences observed in the rates and/or amount of product formation due to autoxidation of the ferrous bipyridyl complex, as a function of the different substrate scavengers present in the reaction system. For example, with benzoate as the substrate, there is no CO_2 production in the

autoxidation control (water added instead of H_2O_2), whereas with KTBA as the substrate the rate of ethylene production by the autoxidation control is about one-half that of the H_2O_2-dependent rate (Fig. 2). This suggests the possibility that the various substrates may be involved in ligand interactions with the ferrous bipyridyl complex. At the ratio of iron to bipyridyl (1:2.2) used in these studies the ferrous bipyridyl complex would exist primarily as the bis complex. Because this complex is known to be a hexa-co-ordinate species (Konig, 1968), two available co-ordination sites exist for a ligand to associate with the complex. The phosphate buffer itself probably serves as a ligand to the complex, in view of the observation that, whereas the complex is stable in water, some autoxidation occurs in the presence of phosphate. Extending this ligand–complex interaction to the scavenger substrates, it is possible that the thioether moiety of KTBA may co-ordinate to the two available ligand sites of the ferrous bipyridyl complex, whereas benzoate does not react readily with the complex.

Previous experiments, based essentially on the stereospecific nature of several hydroxylation reactions as catalysed by the Fenton reaction, suggest the possibility that free $^{\bullet}OH$ may not be formed when the Fenton reaction is carried out at neutral pH (Walling, 1975; Groves & Swanson, 1975; Groves & Vanderpuy, 1976). Rather, some type of iron–$^{\bullet}OH$ intermediate may be formed, or the kinetic equivalent of $^{\bullet}OH$, the ferryl ion species, may be the actual oxidizing agent (Aust & Svingen, 1982). Preliminary studies have shown that when ferrous bipyridyl interacts with H_2O_2 there is liberation of oxygen (Winston *et al.*, 1983). When a different iron–chelate, ferrous diethylenetriaminepenta-acetic acid, reacts with H_2O_2, a net consumption of oxygen ensues (Cohen *et al.*, 1981). With the ferrous bipyridyl system, the interaction of the iron–chelate with alkyl hydroperoxide results in a consumption of oxygen, the opposite of which occurs with H_2O_2 (Winston *et al.*, 1983). Possibly the respective peroxides may form different oxo intermediates with ferrous bipyridyl, or different intermediates are formed when H_2O_2 reacts with different iron–chelates.

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