Complex-formation and reduction of ferric iron by 2-oxo-4-thiomethylbutyric acid, and the production of hydroxyl radicals

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2-Oxo-4-thiomethylbutyric acid (OMBA) is a widely used oxygen-radical-scavenging agent and has been used for the detection of 'OH-like species in a variety of systems. This scavenger reacts with other radicals and is therefore not specific for 'OH. Since iron is required in most systems for the generation of 'OH-like species, studies were carried out to investigate the possible interaction of OMBA with iron. Fe^{3+} reacted with OMBA to produce complexes that gave rise to discrete spectra. Intense purple complexes, with broad absorbance maxima of 525–550 nm, were found at OMBA/ Fe^{3+} ratios of up to 1:1, whereas red complexes with a prominent shoulder between 440 and 480 nm were found at higher OMBA/ Fe^{3+} ratios. OMBA caused reduction of ferric iron to the ferrous state, as detected with 2,2'-bipyridyl as the indicator. This reduction occurs in the dark, can be photo-accelerated especially by light with wavelengths near the absorbance maximum of the respective complexes, and is increased as the OMBA/ Fe^{3+} ratio is elevated. The presence of phosphate buffer quenches the purple and red ferric-ion–OMBA complexes and lowers the rate of reduction of Fe^{3+} by OMBA about 10-fold. The resulting ferrous-ion–OMBA–phosphate complex is very stable against autoxidation. Both the ferrous-ion–OMBA and ferric-ion–OMBA complexes reacted with H_2O_2 , with the subsequent production of ethylene gas from OMBA. The interaction with H_2O_2 resulted in discrete spectral changes of both the ferrous-ion–OMBA and ferric-ion–OMBA complexes. The ferrous-ion–OMBA/ H_2O_2 or ferric-ion–OMBA/ H_2O_2 system appeared to produce 'OH free radicals via a Fenton-type of reaction since ethylene production was inhibited by competitive 'OH scavengers. Ferrous-ion–OMBA complex reacted with H_2O_2 not only to produce ethylene from the OMBA, but also to promote the oxidation of another scavenger, ethanol. The ability of OMBA to chelate iron, to promote reduction of ferric iron and to react with H_2O_2 to produce potent oxidizing radicals may play a role in the lack of specificity of OMBA as a scavenger of oxygen radicals.

INTRODUCTION

Beauchamp & Fridovich (1970) studied the production of ethylene from methional ($\text{CH}_3\text{--S--CH}_2\text{--CH}_2\text{--CHO}$) as an indicator for the production of hydroxyl radicals during the oxidation of xanthine by xanthine oxidase. OMBA (2-oxo-4-thiomethylbutyric acid), a keto acid analogue of methional ($\text{CH}_3\text{--S--CH}_2\text{--CH}_2\text{--CO--CO}_2\text{H}$), was shown to yield ethylene in peroxidase-catalysed reactions (Yang, 1969; Lieberman & Kunishi, 1971). The production of ethylene from either methional or OMBA has been utilized for the detection of 'OH-like species in a variety of chemical, enzymic and biological systems (Bors *et al.*, 1982; Cohen *et al.*, 1976; Heikkilä *et al.*, 1976; Tauber & Babior, 1977; Weiss *et al.*, 1977; Winterbourne, 1981, 1983; Komiyama *et al.*, 1982). Our laboratory has used OMBA along with several other 'OH scavengers to detect the production of 'OH-like species by rat liver microsomal preparations (Cohen & Cederbaum, 1980; Cederbaum & Dicker, 1983) and reconstituted systems containing purified cytochrome P-450 and NADPH-cytochrome P-450 reductase (Winston & Cederbaum, 1983a,b). Pryor & Tang (1978) pointed out that ethylene

can be generated from methional by a variety of radicals, and hence methional was not specific for 'OH. This lack of specificity was extended to OMBA, as ethylene was produced from the interaction of OMBA with 'OH as well as with several different alkoxyl radicals (Winston *et al.*, 1983).

In previous studies with microsomal preparations, it was observed that OMBA differed somewhat from other scavengers such as dimethyl sulphoxide or benzoate in response to certain additions to the reaction system. For example, the rate of OMBA oxidation was 3–10-fold greater than the rates of oxidation of dimethyl sulphoxide or benzoate (Winston *et al.*, 1984). The rate of oxidation of all the scavengers was increased by the addition of EDTA to the system (probably reflecting the chelation of iron ions to produce an iron-ion–EDTA chelate), but, however, the increase in OMBA oxidation (2–3-fold) was much less than the increase in the oxidation of dimethyl sulphoxide or benzoate (10-fold). Similar results were found with another chelating agent, DTPA (Winston *et al.*, 1984). Since iron ions are required for the microsomal production of the 'OH (or 'OH-like species) necessary for the subsequent oxidation of

Abbreviations used: OMBA, 2-oxo-4-thiomethylbutyric acid; DTPA, diethylenetriaminepenta-acetic acid.

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OMBA, dimethyl sulphoxide or benzoate (Cederbaum & Dicker, 1983), it was considered that some of the anomalous responses given by OMBA in comparison with dimethyl sulphoxide or benzoate could reflect interaction of OMBA with iron ions to produce an iron-ion-OMBA chelate, with the subsequent interaction of this chelate with H_2O_2 to yield ethylene. The goal of the current study was to investigate the possible interaction of OMBA with iron ions, and to determine whether 'OH-like species could be produced from the interaction of ferrous-ion-OMBA and ferric-ion-OMBA complexes with H_2O_2 .

METHODS

Spectra were recorded with either a Perkin-Elmer 557 dual-beam dual-wavelength spectrophotometer or a Cary model 14 spectrophotometer. Solutions were prepared freshly daily. Fe^{3+} was added either as FeCl_3 or $\text{FeNH}_4(\text{SO}_4)_2$. The ratios of OMBA to Fe^{3+} are indicated in the Figures or the legends to Figures. Experiments were carried out either in water (Figs. 1-4) or in phosphate buffer, pH 7.4 (Fig. 5). To monitor the reduction of Fe^{3+} in the presence of OMBA, various amounts of $\text{FeNH}_4(\text{SO}_4)_2$ plus OMBA were mixed together, and at various time points 0.1 ml portions were removed and immediately placed into 50 mM-2,2'-bipyridyl. The absorbance at 520 nm was then recorded.

The production of ethylene from OMBA or of acetaldehyde from ethanol was assayed by head-space gas-chromatography procedures, as previously described (Winston & Cederbaum, 1983a,b). Reactions were carried out in 25 ml Erlenmeyer flasks containing OMBA, $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ or $\text{FeNH}_4(\text{SO}_4)_2$ and other additions, and were initiated by the addition of H_2O_2 . The precise reaction conditions for the various experiments are shown in detail in the Figure legends. The flasks were sealed with rubber septa, and 1 ml portions of the head space were removed at various time points with a gas-tight syringe and injected into a Hewlett-Packard model 5840 gas chromatograph for the detection of ethylene and acetaldehyde. The water used to prepare all solutions and the phosphate buffer were passed through columns of Chelex-100 to remove contaminating iron. OMBA was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), 2,2'-bipyridyl was from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.); other chemicals were reagent grade from Fisher Scientific Co. (Springfield, NJ U.S.A.).

RESULTS

Spectral characteristics of iron-ion-OMBA complexes

The data presented as Fig. 1 represents the absolute spectra of aqueous complexes of ferric-ion-OMBA that are formed when the molar ratios of OMBA/ FeCl_3 were varied over the range of 0.2-30:1. At OMBA/ Fe^{3+} ratios ranging from 0.2:1 to 1:1 intensely purple-coloured complexes characterized by wide charge-transfer bands with maxima at about 536 nm that tailed gradually into the near-i.r. region are noted (spectra 1-4). At OMBA/ Fe^{3+} ratios of 2-4:1, a loss of absorbance over this range becomes evident (spectra 5 and 6). The solutions giving rise to spectrum 5 remained a deep purple, but, however, spectrum 6 had a characteristic reddish-brown tint. At even higher OMBA/ Fe^{3+} ratios (8-30:1) intensely red-coloured solutions were observed,

Fig. 1. Interaction of various concentrations of OMBA with FeCl_3 .

The concentration of Fe^{3+} was 5 mM in all experiments, and the concentration of OMBA was zero for spectrum 1, and increased to 1.0, 2.5, 5, 10, 20, 40, 75 and 150 mM for spectra 2-9 respectively. The isosbestic point at 572 nm is indicated on the Figure.

characterized by a prominent shoulder between 440 and 480 nm that increased in absorbance with increasing ligand/ Fe^{3+} ratios (spectra 7-9). Identical spectra were obtained when $\text{FeNH}_4(\text{SO}_4)_2$ replaced FeCl_3 in these reaction mixtures. OMBA in the absence of Fe^{3+} did not absorb over the range of wavelengths shown in Fig. 1.

Reduction of Fe^{3+} in the presence of OMBA

Because ferric iron reacted with OMBA to generate charge-transfer complexes, attempts were made to determine the nature of the co-ordinating ligands by using resonance Raman spectroscopy (Bradley *et al.*, 1981; Basu *et al.*, 1982). When the ferric-ion-OMBA complex samples were exposed to the argon laser beam of the Raman spectrophotometer (about 30 mW) the coloured complexes were found to be photo-bleached. Ferrous-ion-OMBA complex did not yield observable coloured complexes, which suggested the possibility that the bleaching phenomenon was reflecting the formation of a reduced species. Freshly prepared purple (OMBA/ Fe^{3+} ratio 1:1) and red (OMBA/ Fe^{3+} ratio 8:1) ferric-ion-OMBA complexes (prepared as in Fig. 1) were drawn into 100 μl capillary tubes and blown out into solutions containing 20 mM-2,2'-bipyridyl or 50 mM- $\text{K}_3\text{Fe}(\text{CN})_6$. No apparent visible colour change was observed. Similarly, another portion was drawn into the capillary tubes and this time placed in the laser light-path with the 514.5 nm argon line in tune, i.e. near the apparent absorbance maximum of the purple-complex's charge-transfer band. The tubes were removed from the light-path after 10 s. Visual examination of the tube revealed a distinct clear zone where the light-beam had passed through the capillary tube. The red complex did not have this clear zone. The solutions were blown into the bipyridyl and ferricyanide solutions. The purple complex gave rise to either a pink or blue colour in the presence of bipyridyl or ferricyanide respectively, indicating that ferrous iron was present. The red complex, even though not photo-bleached in the laser light-path, also resulted in the production of either a pink or a blue colour when added to the bipyridyl or ferricyanide indicators. When the red complex was placed in the 488.0 nm laser beam,

i.e. near the maximum of the shoulder of the red-complex's spectrum (Fig. 1), there was indeed a clear zone in the capillary tube, and the resulting solutions formed when the contents of these tubes were mixed with the bipyridyl and ferricyanide indicators showed even more intense visual pink or blue colour changes. Thus it would appear that OMBA promotes reduction of ferric iron, and that this reduction is facilitated by absorbing specific light energies. On the basis of these qualitative initial studies, further characterization of the reduction of ferric iron in the presence of OMBA was carried out.

When Fe^{2+} ions are added to a solution of excess 2,2'-bipyridyl, the red $(\text{Fe}^{2+})_3$ -bipyridyl complex is formed (Gillard, 1979). The ferric-ion-bipyridyl complex, which is yellow, does not absorb in this region. Further, the very high formation constant of the $(\text{Fe}^{2+})_3$ -bipyridyl complex (10^{29}) renders it sufficient to readily displace many other ligands. Results shown in Fig. 2 indicate that immediately upon mixing the ferric salt with OMBA (1:1 ratio) reduced iron is generated, as indicated by the formation of a reduced charge-transfer complex when the sample was placed in the bipyridyl (trace b). The longer the ferric salt incubated with OMBA, the greater is the intensity of the resulting ferrous-ion-bipyridyl complex charge-transfer band that forms (traces b-g in Fig. 2). Thus the reduction of Fe^{3+} in the presence of OMBA occurs slowly over time. A plot of the increase in

Fig. 2. Reduction of Fe^{3+} in the presence of OMBA

$\text{FeNH}_4(\text{SO}_4)_2$ (0.17 mM) and OMBA (0.17 mM) were mixed in a 1:1 ratio, and at various time points portions were removed and added to 50 mM-2,2'-bipyridyl, and each sample was scanned over the wavelength range 400–600 nm. Spectrum a was obtained in the absence of OMBA, and hence reflects the spectrum of ferric-ion-2,2'-bipyridyl complex. Spectrum b was obtained immediately after mixing Fe^{3+} with OMBA, and spectra c, d, e, f and g were obtained 5, 10, 20, 40 and 60 min respectively after mixing Fe^{3+} with OMBA.

absorbance of the charge-transfer band maximum (i.e. 520 nm) as a function of time is shown in Fig. 3(a) and thus represents the kinetic profile of the reduction process. A rapid initial reduction occurs, followed by a slower linear phase of reduction. The spectra shown in Fig. 2 are essentially identical with those obtained when ferric-ion-bipyridyl complex was reduced with $\text{Na}_2\text{S}_2\text{O}_4$ and when $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ was added to bipyridyl directly (results not shown). The spectrum of ferric-ion-bipyridyl complex is shown in the same Figure (trace a, absence of OMBA) to demonstrate that only reduced iron can give rise to the charge-transfer region of the visible-absorption spectra shown.

Effect of OMBA concentration on reduction of Fe^{3+}

Because different OMBA/ Fe^{3+} ratios produced different coloured complexes (Fig. 1) it was of interest to determine how the reduction of Fe^{3+} was affected by varying the concentration of OMBA. The data shown in Fig. 3(b) summarize the results obtained 5 min after $\text{FeNH}_4(\text{SO}_4)_2$ was mixed with solutions of increasing OMBA concentration. The rate of reduction of Fe^{2+} in the presence of OMBA was dependent upon the amount of OMBA in the solution, e.g. more than 3 times the amount of reduced iron was present when the OMBA/ Fe^{3+} ratio was 10:1 than when it was 1:1.

Photo-induced reduction of ferric-ion-OMBA complex

The attempted resonance Raman studies indicated that the reduction phenomenon was photo-sensitive, displaying some dependency upon the excitation wavelength used to excite the complexes resulting from the mixing of OMBA with Fe^{3+} . To investigate this further, an experiment was conducted with the use of the monochromator setting of a Perkin-Elmer 557 UV-Vis spectrophotometer to irradiate a purple ferric-ion-OMBA complex (1:1 molar ratio), to determine if there was a correlation between the light energy and the rate at which the complex was reduced. Fig. 4 shows that the greatest degree of reduction of ferric-ion-OMBA complex occurred at wavelengths corresponding to the broad absorbance maximum of the characteristic charge-transfer band of the purple complex (as described in Fig. 1). As the irradiating light-source was decreased in energy (i.e. shifted towards red wavelength) the reduction of Fe^{3+} was more refractory. To the blue side of the maximum there also was a decrease in Fe^{3+} reduction; however, no difference was observed between 360 and 430 nm light-irradiation. Reduction of Fe^{3+} by OMBA also occurred in the dark, but the rate was accelerated in the light (results not shown).

Effects of phosphate buffer

Many investigations involving the chemistry of oxygen activation in biological systems use phosphate-buffered reaction mixtures. Therefore it was of interest to determine whether or not the ligation of OMBA to Fe^{3+} and the resulting reduction of the Fe^{3+} was altered as a result of phosphate being present in the medium. When phosphate buffer (50 mM final concentration, pH 7.4) was added to a solution of the purple ferric-ion-OMBA complex prepared as the 1:1 chelate, an immediate change to a yellow solution was observed. The colour change was reflected in the loss of the charge-transfer band of the purple complex as seen in the spectra shown in Fig. 5. Similar observations were made when

Fig. 3. Time course and effect of OMBA concentration on reduction of Fe^{3+}

Results shown in (a) show the time course for reduction of $\text{FeNH}_4(\text{SO}_4)_2$ by a molar equivalent amount of OMBA. In the experiment shown in (b) the OMBA/ Fe^{3+} ratio was varied from 1:1 to 2:1, 5:1 and 10:1. The Fe^{3+} and OMBA were mixed for 5 min, portions were removed and added to the 2,2'-bipyridyl, and the absorbances at 520 nm were recorded.

Fig. 4. Photo-induced reduction of ferric-ion-OMBA complex

Ferric-ion-OMBA complex at a 1:1 molar ratio was placed in a spectrophotometer, with the monochromator set at either 360, 430, 520, 630 or 750 nm. The sample was placed in the light-path and irradiated for 10 min, after which a portion was removed and placed in 2,2'-bipyridyl, and the spectrum scanned from 400 to 600 nm. Results show the absorbance at 520 nm, the absorbance maximum of the ferrous-ion-2,2'-bipyridyl complex.

phosphate was added to solutions of ferric-ion-OMBA complexes (prepared over a range of 0.2–40:1 OMBA/ Fe^{3+} ratios) (results not shown). Portions of the yellow solution that resulted from phosphate addition to purple ferric-ion-OMBA complex solutions were added to bipyridyl as discussed above and analysed spectro-

Fig. 5. Effect of phosphate buffer on the ferric-ion-OMBA charge-transfer complex

A 1:1 molar ratio of OMBA (5 mM) with Fe^{3+} (5 mM) was mixed in the absence of phosphate (spectrum A) or in the presence of 50 mM-phosphate, pH 7.4 (spectrum B), and the spectrum was scanned.

photometrically. The spectra obtained showed the typical ferrous-ion-bipyridyl complex profile. The concentrations of ferrous iron remained essentially constant for several hours after addition of phosphate and showed little loss

even after 5 days at room temperature. Thus it would appear that phosphate has a marked stabilizing effect on the ferrous form of the iron in these solutions. In other experiments it was noted that the rate of reduction of Fe^{3+} in phosphate buffer by OMBA was about 10-fold lower than the rate in water (results not shown).

Reactivity with H_2O_2 and production of oxy radicals

The above results have shown that the interaction of Fe^{3+} with OMBA results in an apparent ferrous co-ordination complex. It was of interest to study the interaction of iron-ion-OMBA complexes with H_2O_2 and to assess its role in the production of oxy radicals. To this end ferrous-ion-OMBA and ferric-ion-OMBA complexes were prepared and allowed to react with H_2O_2 as described in the Methods section. As can be seen in Table 1, both ferrous-ion-OMBA and ferric-ion-OMBA complexes react with H_2O_2 to produce ethylene. Very small amounts of ethylene were produced in the absence of H_2O_2 . Under the experimental conditions of Table 1, the ferric-ion-OMBA complex resulted in approx. 30% of the ethylene produced by the ferrous-ion-OMBA complex. The production of ethylene by both ferrous-ion-OMBA and ferric-ion-OMBA complexes was inhibited by competing $\cdot\text{OH}$ -scavenging agents (Table 1), which suggests that the reaction system involved, at least in part, a classical Fenton-type reaction in which a ferrous-ion-OMBA complex reacted with H_2O_2 to produce $\cdot\text{OH}$.

Fig. 6 shows that the addition of Fe^{2+} to OMBA results in a bathochromic shift of the OMBA spectrum with an increase in absorbance over the 290–360 nm range (spectra a and b in Fig. 6). Upon the addition of H_2O_2 there is a red-shift in the spectrum of ferrous-ion-OMBA complex (spectra e and f in Fig. 6). It is apparent that the red-shift was the result of H_2O_2 interaction with ferrous-ion-OMBA complex and not OMBA alone, as the latter reaction results in a blue-shift with a concomitant loss of the shoulder in the spectrum of OMBA (spectra c and d in Fig. 6). Thus H_2O_2 can react with OMBA (in the absence of iron ions) to affect changes

in its structure, although the significance of this reaction to oxygen-radical chemistry remains unclear, as ethylene was not a product of this interaction (results not shown). The difference spectrum presented as Fig. 7 shows that spectral changes accompany H_2O_2 interaction with ferric-ion-OMBA complex, the most distinguishing features being the appearance of a spectral intermediate at 295 nm and an isosbestic point at 424 nm. The appearance of the spectral intermediate at 295 nm also is accompanied by the loss of a species with 250 nm absorbance in the sample cuvette. Thus the interaction of both ferrous-ion-OMBA and ferric-ion-OMBA complexes with H_2O_2 is associated with dramatic changes in the spectra of these complexes.

The production of ethylene was linear for up to 5 min and then began to level off slowly with essentially little change over the time span of 30–180 min. In the absence of H_2O_2 a very slow but definite increase in ethylene was seen. This may reflect reduction of Fe^{3+} by OMBA, followed by a very low rate at which the ferrous-ion-OMBA complex autoxidizes to make $\text{O}_2^{\cdot-}$ and thus H_2O_2 . No ethylene was seen after 3 h when OMBA was incubated without iron ions (results not shown).

The spectral data in Fig. 2 demonstrated that ferrous iron accumulates with time after the mixing of ferric salt and OMBA. Therefore the longer Fe^{3+} is incubated with OMBA before the addition of H_2O_2 the greater should be the amount of ethylene produced in the reaction. Fig. 8 shows that the production of ethylene increased as a function of the length of time in which Fe^{3+} was incubated with OMBA before the addition of H_2O_2 . Thus the ferrous iron that accumulates is readily able to participate in a Fenton reaction to generate $\cdot\text{OH}$, which in turn is manifest by the oxidation of OMBA to produce ethylene.

Oxidation of ethanol by ferrous-ion-OMBA complex

To determine whether the reduced complex that forms when OMBA reacts with $\text{FeNH}_4(\text{SO}_4)_2$ could affect the oxidation of another scavenger in the presence of H_2O_2 , a ferric-ion-OMBA complex was first prepared by

Table 1. Ethylene production from the interaction of iron-ion-OMBA complexes with H_2O_2 : effect of competing $\cdot\text{OH}$ -scavenging agent

OMBA (10 mM) was incubated with either $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ or $\text{FeNH}_4(\text{SO}_4)_2$ (0.14 mM) for 15 min, and reactions were initiated by the addition of H_2O_2 (final concentration 30 mM). Ethylene was measured after 15 min of reaction as described in the Methods section. Competitive inhibitor concentrations were: mannitol, 100 mM; ethanol, 53 mM; benzoate, 30 mM; dimethyl sulphoxide, 30 mM. Results are from three experiments with ethanol and mannitol and two experiments with benzoate and dimethyl sulphoxide. Urea (50 and 100 mM) and KCl (100 mM), which are poor scavengers of radicals such as $\cdot\text{OH}$, did not inhibit the production of ethylene by either the ferrous-ion-OMBA complex or the ferric-ion-OMBA complex systems. In fact, a slight increase (10–20%) was observed with the latter.

	Reaction system	Addition	Ethylene production (nmol)	Effect of addition (%)
(a)	Ferrous-ion-OMBA, no H_2O_2	—	1.1	—
	Ferrous-ion-OMBA, + H_2O_2	None	154	—
	Ferrous-ion-OMBA, + H_2O_2	Mannitol	84.7	–45
	Ferrous-ion-OMBA, + H_2O_2	Ethanol	58.5	–62
	Ferrous-ion-OMBA, + H_2O_2	Benzoate	26.2	–83
	Ferrous-ion-OMBA, + H_2O_2	Dimethyl sulphoxide	44.7	–71
(b)	Ferric-ion-OMBA, no H_2O_2	—	0.9	—
	Ferric-ion-OMBA, + H_2O_2	None	49.8	—
	Ferric-ion-OMBA, + H_2O_2	Mannitol	16.9	–58
	Ferric-ion-OMBA, + H_2O_2	Ethanol	10.0	–75

Fig. 6. Spectral interactions between OMBA and ferrous-ion-OMBA complex with H_2O_2

Spectrum a represents the spectrum of 10 mM-OMBA alone, and for spectrum b $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ (0.5 mM) was added to the OMBA. For spectra c and d H_2O_2 (3 mM) was added to the OMBA and the spectrum was recorded 5 min (spectrum c) or 10 min (spectrum d) later. For spectra e and f H_2O_2 (3 mM) was added to the ferrous-ion-OMBA complex and the spectrum was recorded either immediately (spectrum e) or after 5 min (spectrum f).

addition of Fe^{3+} to a solution containing a 60-fold excess of OMBA. Thus prepared, the resulting red complex was added to reaction vessels containing ethanol. The reaction was initiated 10 min after the preparation of the iron-ion-OMBA complex by the addition of H_2O_2 . Approx. 50 nmol of acetaldehyde and 85 nmol of ethylene were produced. Since the amount of acetaldehyde produced was undoubtedly reflecting an inhibited rate due to the presence of competing excess OMBA, an experiment was designed to determine the capacity of iron-ion-OMBA complex in the presence of H_2O_2 to oxidize ethanol at OMBA concentrations that would not effectively compete with the oxidation of ethanol. Fig. 9 summarizes these experiments. When the OMBA concentration was zero there was no acetaldehyde production in the presence of Fe^{3+} plus H_2O_2 (since no OMBA was present to reduce the Fe^{3+}). Over the range of OMBA concentrations from 0.05 to 0.25 mM there was an increasing rate of acetaldehyde production with a

Fig. 7. Spectral interactions between ferric-ion-OMBA complex and H_2O_2

OMBA (10 mM) and $\text{FeNH}_4(\text{SO}_4)_2$ (0.17 mM) were mixed, followed by the addition of H_2O_2 (15 mM), and the spectrum was recorded after 5, 10, 15, 20, 25 and 30 min (spectra 2–7 respectively). For spectrum 1 no H_2O_2 was added.

Fig. 8. Production of ethylene from OMBA as a function of the incubation time of Fe^{3+} (0.5 mM) with OMBA (10 mM) before the addition of H_2O_2 (3.0 mM)

Ethylene was measured 5 min after the addition of H_2O_2 .

barely detectable amount of ethylene production. Over the range 0.5–1.0 mM-OMBA a linear decrease in acetaldehyde production with a concomitant increase in ethylene production was observed. Over the range 2–20 mM-OMBA acetaldehyde production decreased

Fig. 9. Ability of the ferric-ion-OMBA complex to promote the oxidation of ethanol in the presence of H_2O_2

The concentration of OMBA was varied from 0 to 20 mM in the presence of 0.5 mM- $\text{FeNH}_4(\text{SO}_4)_2$, 3.0 mM- H_2O_2 and 50 mM-ethanol. Reactions were initiated by the addition of H_2O_2 , and after 60 min of incubation portions of the head space were removed for the assay of acetaldehyde (●) or ethylene (○).

markedly, along with a dramatic increase in ethylene production. Thus, when the OMBA concentration is low and cannot effectively compete with the ethanol, there was a pronounced oxidation of acetaldehyde resulting from the Fenton reaction between (reduced)-iron-ion-OMBA complex and H_2O_2 . When the OMBA concentration is high, the competition effect becomes more prominent and acetaldehyde production begins to give way to ethylene production. These results show that in the presence of Fe^{3+} and OMBA a substantial degree of reduction of the Fe^{3+} occurs such that the reaction system can actually affect the oxidation of another 'OH-scavenging agent in the mixture.

DISCUSSION

The widely used oxygen-radical-scavenging agent OMBA was found to react with Fe^{3+} to produce complexes that give rise to discrete spectra that are dependent on the ligand/ Fe^{3+} ratio. Intense purple complexes characterized by a broad absorbance maxima over the range 525–550 nm were found at equivalent molar ratios of OMBA and Fe^{3+} , and red complexes characterized by a prominent shoulder between 440 and 480 nm were found at elevated OMBA/ Fe^{3+} ratios. The latter situation is likely to predominate in most experimental conditions since OMBA is often employed in concentrations ranging from 1 to 10 mM, whereas the concentration of iron ions, endogenous or added, is usually much less. The visible-region absorption observed for various OMBA/ Fe^{3+} ratios arises as a result of chelation by the ligand. The exact nature of this chelation is as yet undetermined. Methionine has been reported (McAuliffe *et al.*, 1966) to give a red-brown complex with Fe^{3+} , with a 3:1 methionine/ Fe^{3+} ratio. The chelation in this case, however, was through the carboxy and α -amino groups. The sulphur atom was not involved. For OMBA, the possibility of chelation via the carboxy group and an enolate form of the α -carbonyl group must be considered. A blue colour is observed for Fe^{3+} complexes of enolates (Hughes, 1981). The bonding of the thioether sulphur atom, a 'soft' Lewis base, is favoured for 'soft' metal acids, such as Ag(I) or Pt(II), but not for 'hard' metal acids, such as Fe(III) (Angelici, 1975). Further work is

required to identify the OMBA- Fe^{3+} interaction. Of interest is the observation that both the purple and the red ferric-ion-OMBA complexes display reduction of ferric to the ferrous state. This reduction is accelerated when the complex absorbs specific light energy, e.g. 525 nm for the purple complex and 480 nm for the red complex, but also occurs in the dark. The kinetic profile shows that there is an initial rapid reduction, followed by a slow linear phase, and that the reduction is dependent on the OMBA/ Fe^{3+} ratio (Fig. 3). This may be of importance in various experimental conditions in which excess OMBA plus iron ions (added or present in the sample) are incubated together. The resulting ferrous-ion-OMBA complex can react with H_2O_2 to produce 'OH, and thus promote the production of ethylene from OMBA. Hence the ability of OMBA to complex and reduce Fe^{3+} can ultimately result in the production of 'OH or 'OH-like species even in the absence of an added reducing agent for the Fe^{3+} .

The presence of phosphate buffer quenches the purple and the red ferric-ion-OMBA complexes and lowers the rate of reduction of Fe^{3+} by OMBA about 10-fold. The resulting ferrous-ion-OMBA-phosphate complex is remarkably stable against autoxidation, even after several days. In this regard, stabilization of ferrous-ion-OMBA complex against autoxidation by phosphate is reminiscent of the ability of phosphate to stabilize ferrous-ion-DTPA complex against autoxidation (Cohen *et al.*, 1981), but in contrast with the acceleration by phosphate of the autoxidation of ferrous-ion-EDTA (Cohen & Sinet, 1983) or of ferrous-ion-2,2'-dipyridyl (Winston *et al.*, 1983). The presence of a stable ferrous complex may be significant in experiments in which OMBA, Fe^{3+} and phosphate buffer are incubated together before the addition of H_2O_2 or of biological systems that generate $\text{O}_2^{\cdot-}$ and H_2O_2 , e.g. xanthine oxidase, microsomal preparations and phagocytes. It is possible that a cycle in which Fe^{3+} is reduced by OMBA to ferrous iron, followed by a Fenton-type reaction in which ferrous-ion-OMBA complex reacts with H_2O_2 to regenerate ferric iron, which can again be reduced to ferrous iron by OMBA, may be set up and result in significant rates of 'OH production.

The ferrous-ion-OMBA and ferric-ion-OMBA com-

plexes react with H_2O_2 , as shown by the various spectral changes depicted in Figs. 6 and 7, and by the production of ethylene from OMBA and of acetaldehyde from ethanol. The interaction of ferrous-ion-OMBA complex with H_2O_2 appeared to reflect a Fenton-type reaction with the production of free $\cdot\text{OH}$, since ethylene production is inhibited by competitive $\cdot\text{OH}$ scavengers. The Fenton-type reaction appears to occur primarily between ferrous-ion-OMBA complex with H_2O_2 , and not uncomplexed Fe^{2+} with H_2O_2 . The ferric-ion-OMBA complex also reacts with H_2O_2 to produce $\cdot\text{OH}$, as indicated by the production of ethylene through a process sensitive to inhibition by competitive $\cdot\text{OH}$ scavengers such as mannitol or ethanol. The production of $\cdot\text{OH}$ by the ferric-ion-OMBA complex/ H_2O_2 system most probably reflects first the reduction of Fe^{3+} in the presence of OMBA to produce the ferrous-ion-OMBA complex, followed by a Fenton-type reaction between the ferrous-ion-OMBA complex and H_2O_2 . Results in Fig. 8 show that the longer the incubation time between Fe^{3+} and OMBA before the addition of H_2O_2 , the greater was the production of ethylene. This correlates with the increased production of ferrous iron from the reduction of Fe^{3+} by OMBA as described in Fig. 2. It has previously been shown that Fe^{3+} can be reduced when complexed to adriamycin in the absence of an external reducing agent (Sugioka & Nakano, 1982; Gutteridge, 1984).

Ferrous-ion-OMBA complex reacted with H_2O_2 not only to produce ethylene but also to promote the oxidation of another $\cdot\text{OH}$ scavenger, ethanol. Low concentrations of OMBA are most effective in promoting the oxidation of ethanol, since at high concentrations the OMBA competes with the ethanol for the generated $\cdot\text{OH}$ (Fig. 5). Surprisingly, the decrease in acetaldehyde as the concentration of OMBA was increased is greater than the increase in ethylene. For example, at 0.25 mM-OMBA about 380 nmol of acetaldehyde and about 8 nmol of ethylene are produced, whereas at 10 mM-OMBA about 50 nmol of acetaldehyde plus 80 nmol of ethylene are produced (Fig. 9); thus the 'apparent yield' of $\cdot\text{OH}$ is decreased from about 390 nmol of product formation to about 130 nmol. This could reflect the possibility that other products not being measured could result from the interaction of OMBA with $\cdot\text{OH}$, or that the interaction of alcohols with $\cdot\text{OH}$ proceeds via a chain mechanism in which the interaction with 1 molar equivalent of $\cdot\text{OH}$ results in the production of more than 1 more equivalent of acetaldehyde (Merz & Waters, 1948; Walling, 1975). In this regard we have previously noted that the yield of product from the oxidation of ethanol by $\cdot\text{OH}$ generated by model systems such as the xanthine oxidase or the iron-ion-EDTA complex + ascorbate reaction was greater than that found for other $\cdot\text{OH}$ scavengers such as OMBA and dimethyl sulphoxide (Cederbaum & Berl, 1982; Cederbaum *et al.*, 1983; Feerman *et al.*, 1985).

The ability of OMBA to complex and reduce Fe^{3+} may explain previous findings with microsomal systems in which several differences were noted between OMBA and other $\cdot\text{OH}$ scavengers such as benzoate and dimethyl sulphoxide (Feerman & Cederbaum, 1983; Winston *et al.*, 1984). Although the production of $\cdot\text{OH}$ by microsomal preparations requires iron ions, certain chelates, e.g. ferric-ion-EDTA and ferric-ion-DTPA complexes, are much more effective than unchelated Fe^{3+} or ferric ATP or ferric citrate in catalysing $\cdot\text{OH}$ production (Winston *et al.*, 1984). This greater effective-

ness appears to reflect the ability of ferric-ion-EDTA and ferric-ion-DTPA complexes to act as particularly good electron sinks (as compared with the other ferric chelates) for NADPH-cytochrome P-450 reductase (Morehouse *et al.*, 1984; Winston *et al.*, 1984). In the absence of added iron ion or chelating agent, the rate of OMBA oxidation to ethylene was much greater than the rates of oxidation of dimethyl sulphoxide or benzoate. This could reflect the ability of OMBA but not benzoate or dimethyl sulphoxide to chelate and reduce adventitious ferric iron, with the subsequent interaction of ferrous-ion-OMBA complex with H_2O_2 (generated by microsomal electron transfer) to produce $\cdot\text{OH}$, and subsequently ethylene. In addition, the ferrous-ion-OMBA complex in phosphate buffer is more stable against autoxidation than is unchelated Fe^{2+} in phosphate buffer, and it is the ferrous form of iron that reacts with H_2O_2 to produce $\cdot\text{OH}$. Thus in these microsomal studies iron ions complexed with either OMBA, EDTA or DTPA are more effective than unchelated ions (or ferric ATP and ferric citrate complexes) in catalysing the production of $\cdot\text{OH}$. It is possible that the ability of OMBA to complex with iron ions and thus affect the reduction of the iron ions coupled with the interaction of the complex with H_2O_2 (and perhaps other peroxides and oxidants) to produce ethylene plays a role in the lack of specificity of OMBA as a scavenger of oxygen radicals (Pryor & Tang, 1978; Winston *et al.*, 1983).

These studies were supported by U.S. Public Health Service Grant AA-03312 and Research Career Development Award 5-KO2 AA-0003 (A.I.C.) from the National Institute on Alcohol Abuse and Alcoholism, and by the Revson Foundation (G. W. W.).

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Received 19 July 1985/29 October 1985; accepted 16 December 1985