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IRON OXIDATION IN MOPS BUFFER. EFFECT OF EDTA, HYDROGEN PEROXIDE AND $FeCl_3$

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The effect of EDTA and H_2O_2 on iron autoxidation in Mops buffer depends on the pH of the solution. At acidic pH, EDTA caused the oxidation of a stoichiometric amount of iron. At neutral and alkaline pH, EDTA and H_2O_2 not only oxidizes a stoichiometric amount of iron but also causes the oxidation of the Fe^{2+} exceeding the concentration of these compounds. In the presence of EDTA, oxidation of Fe^{2+} in exceeding the concentration of these compounds has a shorter lag phase and an increased rate compared with that in the absence. The solution developes a yellow colour whose intensity is proportional to the amount of Fe^{2+} exceeding the concentration of these compounds in solution. When the reaction is conducted in the presence of NBT, formazan formation is greatly reduced compared to the control without EDTA and H_2O_2. The Fe^{3+}-EDTA complex and Fe^{3+} affected iron oxidation, development of the yellow colour and NBT reduction in a similar fashion. In all these experimental conditions, iron oxidation is greatly reduced in the presence of mannitol, sorbitol and catalase. In phosphate buffer, EDTA oxidized a stoichiometric amount of iron without affecting free Fe^{2+} oxidation. Fe^{3+} has no effect on iron oxidation in this buffer.

KEY WORDS: Iron, buffer, EDTA, hydrogen peroxide, iron(III).

INTRODUCTION

Molecular oxygen is the major biological oxidizing agent, however a spin restriction makes it unreactive. Oxygen must be activated in order to react with organic compounds. Ions of transition metals, such as iron or copper, have variable valencies and by transfering single electrons can activate dioxygen.[1] Ferrous salts in aerobic aqueous solution readily autoxidize, forming active species such as superoxide (O_2^-) hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH\cdot$).

The iron-oxygen chemistry is complex and its dependence upon reaction conditions is known. The rate expression for Fe^{2+} autoxidation depends on the presence of anions.[2–4] Some of these anions are used as buffering species.[2,3] Phosphate is known to bind iron and to affect its rate of autoxidation.[3] To minimize iron interaction with the buffering species unbuffered solutions are frequently used in the study of the damage to biological molecules caused by free radicals produced in the presence of iron in different experimental conditions.[5–7] In these unbuffered conditions very easily unrecognized variations in the pH of the solution may occur and this may affect the rate of Fe^{2+} autoxidation that is known to be influenced by pH.[8] Other anionic buffering species such as Mops and Hepes have low affinity for Fe^{2+} and do not interfere with its rate of autoxidation.[9,10] We have shown that iron oxidation in these

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Good-type buffers is similar to iron oxidation in unbuffered solutions adjusted to alkaline pH with NaOH.[11] These buffers thus appear to be suitable to substitute unbuffered solutions in the study of iron catalyzed production of free oxygen radicals and of iron participation in their reactions with biomolecules.

A profitable use of these buffers in complex systems requires a basic knowledge of the effect on iron oxidation in these buffers of compounds frequently utilized in free radical studies. In the investigation reported here we have studied iron autoxidation in Mops buffer in the presence of EDTA (a chelator commonly used), of H_2O_2 (that with Fe^{2+} participates to the Fenton's reaction: $Fe^{2+} + H_2O_2 \rightarrow OH\cdot + OH^- + Fe^{3+}$) and of $FeCl_3$ (that was suggested to participate as initiator of lipid peroxidation).[12] We have also studied how compounds such as $OH\cdot$ scavengers, catalase and superoxide dismutase affect Fe^{2+} autoxidation under the different experimental conditions. For comparison the effect of these compounds on iron autoxidation in phosphate buffer was studied.

MATERIALS AND METHODS

Mops, nitro blue tetrazolium and EDTA were obtained from Sigma Chemical Co (St. Louis, MO. USA), 1,10-phenanthroline and potassium thiocyanate were obtained from Merck (Darmstadt, Germany). Solutions were prepared in Chelex resin-treated water. The pH values of buffer were adjusted at room temperature. Stock solutions of Fe^{2+} and Fe^{3+} were prepared daily. Fe^{2+} determination was made using the o-phenanthroline method according to Mahler and Elowe.[13] The 1 ml samples to be analyzed, after the addition of Fe^{2+}, were incubated at room temperature for the time stated and then mixed with 0.2 ml of 25 mM 1,10 phenanthroline. The absorbance at 515 nm was read immediately. Fe^{3+} determination was made using the thiocyanate method. A 0.5 ml aliquot was removed from the 1 ml reaction mixture and mixed with 2.5 ml of glacial acetic acid. After 1 minute 0.25 ml of 20% (w/v) KSCN was added. The absorbance at 505 nm was measured after 2 min. Nitroblue tetrazolium reduction was followed by measuring the absorbance at 560 nm. The development of yellow colour during Fe^{2+} autoxidation in Mops buffer was followed by measuring the absorbance at 400 nm.

RESULTS

Effect of EDTA on Fe^{2+} autoxidation in different buffers

EDTA causes a very rapid oxidation of an equivalent amount of Fe^{2+} in distilled water at pH 6 without affecting the remaining "free" Fe^{2+} which seems rather stable at this pH (Fig. 1a). In Mops buffer, pH 7.2, by contrast, substoichiometric concentrations of EDTA accelerate the rate of autoxidation of the "unchelated" Fe^{2+} compared to the rate in the absence of EDTA (Figure 1a). At EDTA concentrations less than that of Fe^{2+}, the development of yellow colour accompanying Fe^{2+} autoxidation in this buffer occurs much more rapidly (Figure 3a). The absorption spectrum of the product, however, does not differ from that observed during Fe^{2+} autoxidation in this buffer (Figure 2a). The rate of the ΔA_{400} is increased, whereas, the intensity of the colour is decreased with increasing EDTA concentration (Figure 3a). Addition, at the end of the incubation time, of an EDTA concentration exceeding that of iron does not

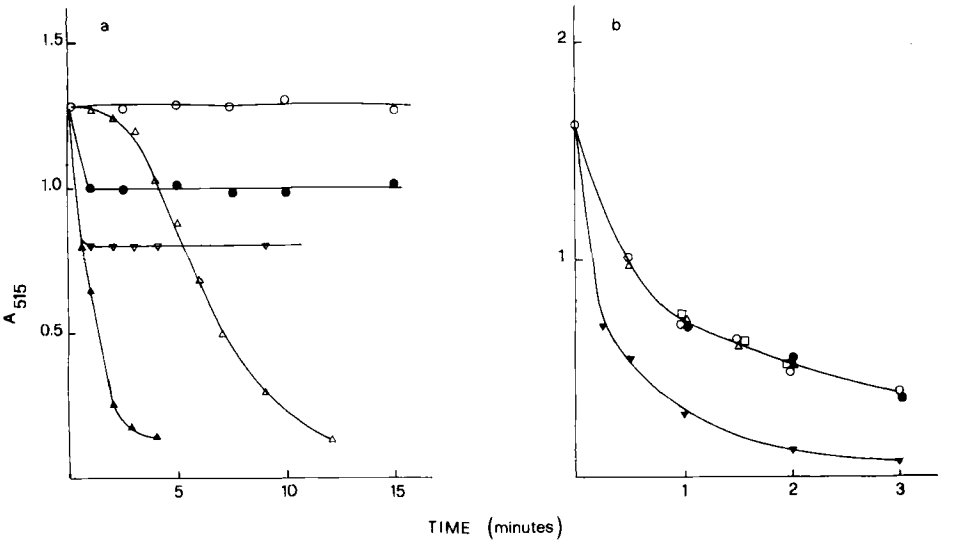

FIGURE 1 Effect of substoichiometric concentration of EDTA on Fe^{2+} autoxidation in different buffering conditions. The autoxidation of 150 μM Fe^{2+} was measured by the o-phenanthroline method as described in the materials and methods section. The reaction mixture contained: a) H_2O pH 6 in the absence (○) or presence of (●) 30 μM EDTA; 5 mM Mops pH 7.2 in the absence (△) or presence of either (▲) 50 μM EDTA or (▽) 50 μM EDTA and 10 mM mannitol; b) 5 mM phosphate, pH 7.2 in the absence (○) or presence of (▼) 50 μM EDTA, (△) 50 μM Fe^{3+}, (□) 100 μM Fe^{3+}, (●) 150 μM Fe^{3+}.

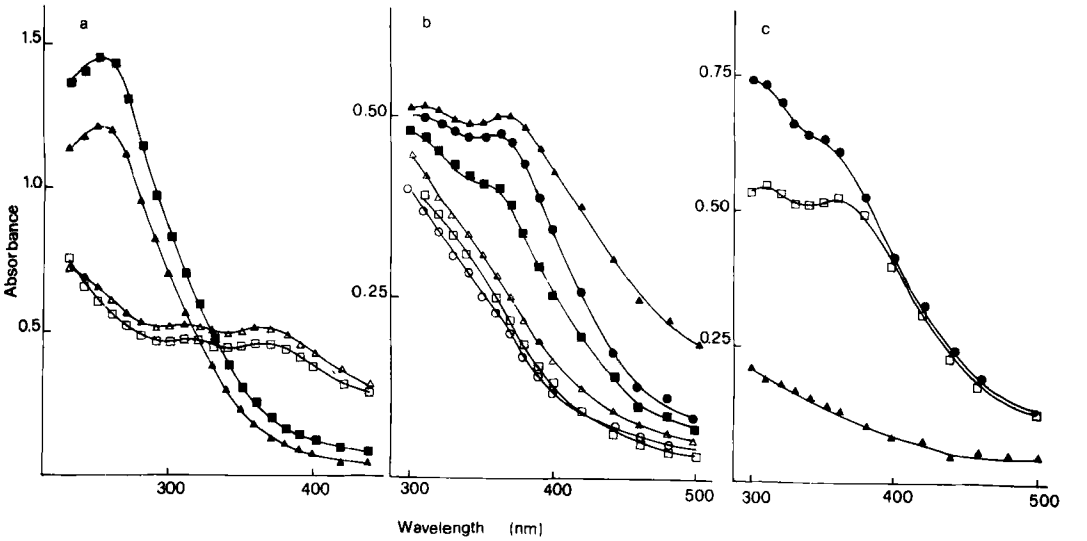

FIGURE 2 Absorbance spectra of the product of Fe^{2+} autoxidation in the presence of EDTA, H_2O_2 and Fe^{3+} in 5 mM Mops pH 7.2. The absorbance spectra were determined after 20 min incubation of the reaction mixture containing; a) 150 μM Fe^{2+}, 20 μM EDTA (△); 150 μM Fe^{2+}, 180 μM EDTA (■); 150 μM Fe^{3+}, 180 μM EDTA (▲); b) 150 μM Fe^{2+} in the absence (▲) or presence of 30 μM (●); 50 μM (■); 150 μM (△) H_2O_2; 150 μM Fe^{3+} in the absence (□) or presence of 150 μM H_2O_2 (○); c) 150 μM Fe^{2+} in the absence (□) or presence of 50 μM Fe^{3+} (●); 50 μM Fe^{3+} (▲). After a 20 min incubation the reaction mixture in a) containing 150 μM Fe^{2+}, 20 μM EDTA (△) was added with 180 μM EDTA (□).

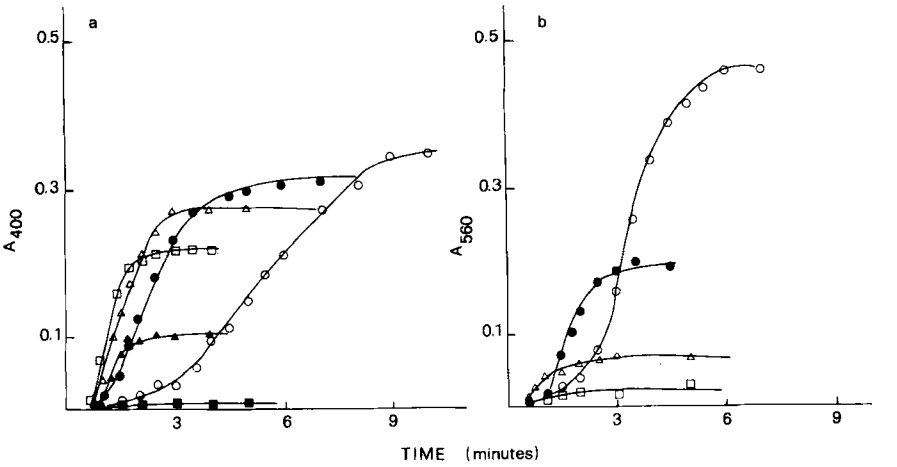

FIGURE 3 Effect of EDTA concentration on the development of yellow colour and on NBT reduction during Fe^{2+} autoxidation in 5 mM Mops pH 7.2. a) The development of yellow colour during the autoxidation of 150 μM Fe^{2+} was followed measuring the absorbance at 400 nm in the absence (○) or presence of 10 μM (●), 20 μM (△), 50 μM (□), 100 μM (▲) and 150 μM (■) EDTA. b) NBT reduction was measured following formazan formation at 560 nm in solutions containing 150 μM Fe^{2+}, 25 μM NBT in the absence (○) or presence of 10 μM (●), 20 μM (△) and 50 μM (□) EDTA.

significantly modify the adsorption spectrum (Figure 2a). When the concentration of EDTA is higher than Fe^{2+} (1.2:1), the reaction mixture does not turn yellow and it has an absorption spectrum similar to that of the EDTA complex with Fe^{3+} (1.2:1) (Figure 2a). When the EDTA:Fe^{2+} complex (1:3) was prepared in distilled water and an aliquot corresponding to 0.15 mM iron was added after various intervals of time

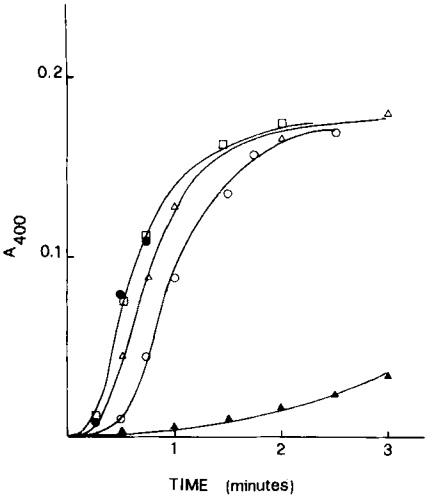

FIGURE 4 Effect of preincubation of the complex EDTA:Fe^{2+} on the development of yellow colour occurring during Fe^{2+} autoxidation in 5 mM Mops, pH 7.2. The development of yellow colour during Fe^{2+} autoxidation was followed measuring the absorbance at 400 nm. The reaction was started by addition either of 150 μM Fe^{2+} in the absence (▲) or presence of 50 μM EDTA (○) or the complex 150 μM Fe^{2+}:50 μM EDTA preincubated for 5 (△), 15 (□) or 60 (●) min.

TABLE I.

Effect of OH· scavengers on Fe^{2+} autoxidation.

Fe^{2+} autoxidation was measured by the o-phenanthroline method in Mops buffer, pH 7.2, in the presence of 20 μM EDTA or 30 μM $FeCl_3$ after 3 min incubation. Absorbance at 400 nm was measured after 3 min incubation. All concentrations shown are final reaction concentrations. Abbreviation: SOD superoxide dismutase

	Absorbance at 400 nm		Fe^{2+} oxidation			
	Mops buffer + EDTA		Mops buffer + EDTA		Mops buffer + Fe^{3+}	
	A_{400}	Inhibition	ΔA_{515}	Inhibition	ΔA_{515}	Inhibition
Buffer (5 mM) + Fe^{2+} (0.15 mM)	0.220		1.184		1.002	
+ Butan-l-ol (50 mM)	0.210	5%	1.066	10%	0.732	23%
+ Formate (5 mM)	0.200	9%	1.065	10%	0.832	17%
+ Mannitol (5 mM)	0.033	85%	0.213	82%	0.171	83%
+ Sorbitol (5 mM)	0.042	81%	0.308	74%	0.201	80%
+ Inositol (5 mM)	0.030	86%	0.592	50%	–	–
+ Thiourea (50 mM)	0.165	85%	1.007	15%	0.802	20%
+ SOD (0.2 mg/ml)	0.042	81%	0.391	67%	–	–
+ Catalase (0.2 mg/ml)	0.010	95%	0.002	100%	–	–

(5 min., 15 min and 1 h) to Mops buffer pH 7.2, the ΔA_{400} of the reaction mixtures was increased and its intensity was similar to that obtained when 0.15 mM Fe^{2+} was added directly to the buffer in the presence of 50 μM EDTA (Figure 4). However, the rate of the yellow colour formation was increased by increasing the preincubation time of the complex (Figure 4). Mannitol and sorbitol did not affect Fe^{2+} oxidation which was caused by EDTA in distilled water at pH 6 (result not shown). In Mops buffer pH 7.2,

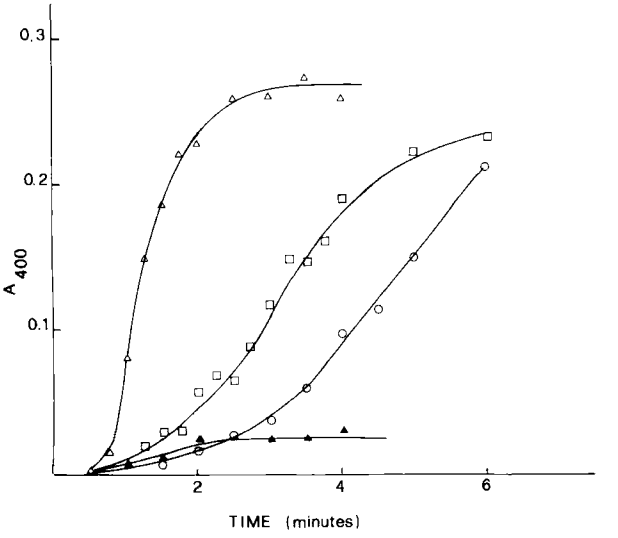

FIGURE 5 Effect of mannitol and catalase on the development of yellow colour occurring during Fe^{2+} autoxidation in the presence of EDTA. The development of yellow colour during the autoxidation of 150 μM Fe^{2+} was followed measuring the absorbance at 400 nm. The reaction mixtures in 5 mM Mops pH 7.2 contained besides Fe^{2+} (○), 30 μM EDTA ((△) added with either 0.1 mg/ml catalase (□) or 10 mM mannitol (▲).

mannitol inhibits only the oxidation of that amount of Fe^{2+} which exceeds the EDTA concentration (Figure 1a). Mannitol and sorbitol, in the presence of substoichiometric concentrations of EDTA, inhibit not only Fe^{2+} autoxidation but also the development of the yellow colour; thiourea and formate have some effect at high concentrations whereas butan-1-ol is ineffective (Table 1). Catalase and superoxide dismutase, under these experimental conditions, increase the lag time of yellow colour development and of Fe^{2+} autoxidation (Figure 5 and Table 1). Addition of substoichiometric concentrations of EDTA greatly inhibits NBT reduction (Figure 3b).

In phosphate buffer pH 7.2, addition of substoichiometric concentrations of EDTA causes the almost instantaneous oxidation of an equivalent amount of Fe^{2+} without any apparent effect on the oxidation of the "unchelated" iron. The rates of oxidation in the absence and presence of EDTA are in fact parallel, but the curve is shifted by the amount of iron oxidized by EDTA (Figure 1b).

Effect of hydrogen peroxide on Fe^{2+} autoxidation in Mops buffer

H_2O_2 causes the oxidation of a stoichiometric amount of Fe^{2+} in distilled water at pH 6, without affecting that amount of the Fe^{2+} which exceeds the H_2O_2 concentration. The amount of Fe^{3+} formed is easily determined by the thiocyanate method (results not shown). In Mops pH 7.2, a concentration of H_2O_2 lower than that of iron in the reaction mixture causes the oxidation of all of the Fe^{2+}. However, determination of the Fe^{3+} content of this solution by the thiocyanate method shows that besides the Fe^{3+} produced by the direct reaction of $FeCl_2$ with H_2O_2 a small amount of the remaining iron becomes coloured (results not shown). As for the case of EDTA, during the oxidation of excess iron, a yellow colour is developed. Its rate of formation increases, whilst its intensity decreases with increasing H_2O_2 concentration (Figure 6).

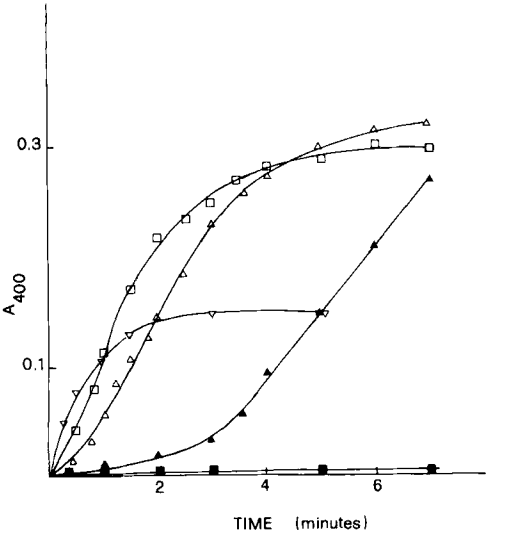

FIGURE 6 Effect of H_2O_2 on the development of yellow colour occurring during Fe^{2+} autoxidation in 5 mM Mops pH 7.2. The development of yellow colour during the autoxidation of 150 μM Fe^{2+} was followed measuring the absorbance at 400 nm. The reaction mixture contained besides Fe^{2+} (▲), 7.5 μM H_2O_2 (△), 15 μM H_2O_2 in the absence (□) or presence of 10 mM mannitol (■), 75 μM EDTA (▽).

The absorption spectra of the reaction mixtures containing substoichiometric amounts of H_2O_2 are given in Figure 2b. When a H_2O_2 amount stoichiometric with Fe^{2+} is added to the reaction a light yellow colour is formed and its absorption spectrum is similar to that of the $FeCl_3$ formed during the reaction (Figure 2b). Mannitol inhibits the yellow colour formation (Figure 6) and the oxidation of only that amount of the iron which exceeds the H_2O_2 concentration. In fact, it has no effect on the oxidation of iron which is mixed in stoichiometric proportions with H_2O_2 in distilled water at pH 6 (results not shown). Addition of H_2O_2 inhibits formazan formation when the reaction is conducted in the presence of NBT (results not shown).

Effect of $FeCl_3$ on Fe^{2+} autoxidation in different buffers

The possibilty that the disappearance of the lag time and the increased rate of yellow colour formation which was observed in Mops buffer in the presence of low concentrations of EDTA and H_2O_2 was due to the formation of Fe^{3+} was considered. Addition of 20 μM $FeCl_3$ to the standard reaction mixture greatly advances the onset of Fe^{2+} oxidation and of the formation of the yellow colour (Figure 7), whose intensity is not changed. The absorption spectrum of the solution can be seen to be more or less the addition of the spectrum of the added Fe^{3+} and the spectrum which is obtained from Fe^{2+} in this buffer in the absence of Fe^{3+} at much longer times (Figure 2c). $FeCl_3$ addition also affects NBT reduction. Reduction begins much sooner but the extent is greatly lowered (results not shown). Similar results are obtained when an equal amount of Fe^{3+} as the Fe^{3+}:EDTA (1.1:1) complex, is added (Figure 7). Again mannitol inhibits the observed phenomena. The amount of mannitol required to inhibit Fe^{2+} autoxidation depends on the amount of Fe^{3+} added. If

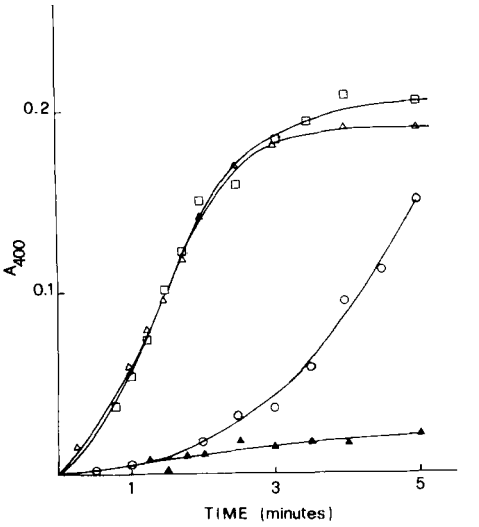

FIGURE 7 Effect of Fe^{3+} on the development of yellow colour occurring during Fe^{2+} autoxidation in 5 mM Mops, pH 7.2. The development of yellow colour during the autoxidation of 150 μM Fe^{2+} was followed measuring the absorbance at 400 nm. The reaction mixture contained besides Fe^{2+} (○), 20 μM Fe^{3+} (△), 20 μM Fe^{3+} as Fe^{3+}:EDTA (1.1.:1) complex (□), 20 μM Fe^{3+} in the presence of 10 mM mannitol (▲).

we plot the protection exerted by the polyol against its concentration, it is evident that the amount of mannitol required to cause 50% inhibition of Fe^{3+} stimulated Fe^{2+} oxidation depends on the Fe^{3+} concentration in the sample (Figure 8b). $FeCl_3$ exerts its effects on iron oxidation only when the pH of the Mops or Hepes buffer is in the neutral to alkaline range. In fact, it does not stimulate Fe^{2+} autoxidation in Mops pH 6.7. Addition of 50 up to 150 μM $FeCl_3$ to $FeCl_2$ in phosphate buffer does not increase Fe^{2+} autoxidation to any significant extent (Figure 1b).

DISCUSSION

Our results clearly demonstrate that the effect of EDTA on iron autoxidation depends on the buffering conditions. In distilled water at pH 6, EDTA causes a rapid oxidation of a stoichiometric amount of Fe^{2+} without affecting the unchelated Fe^{2+}. In the presence of Mops buffers, iron autoxidation depends on the pH. At acidic pH, the effect of EDTA is similar to that obtained in distilled water at pH 6; at neutral and slightly alkaline pH, EDTA not only oxidized a stoichiometric amount of iron but also causes the oxidation of the "free" Fe^{2+}. Disappearance of Fe^{2+} from the solution occurs much more quickly than in the absence of the chelating agent. During iron oxidation the solution develops a yellow colour whose intensity depends on the amount of free Fe^{2+} originally present in the solution. EDTA was observed to affect lipid peroxidation in an analogous manner.[6] Increasing molar ratios of the iron

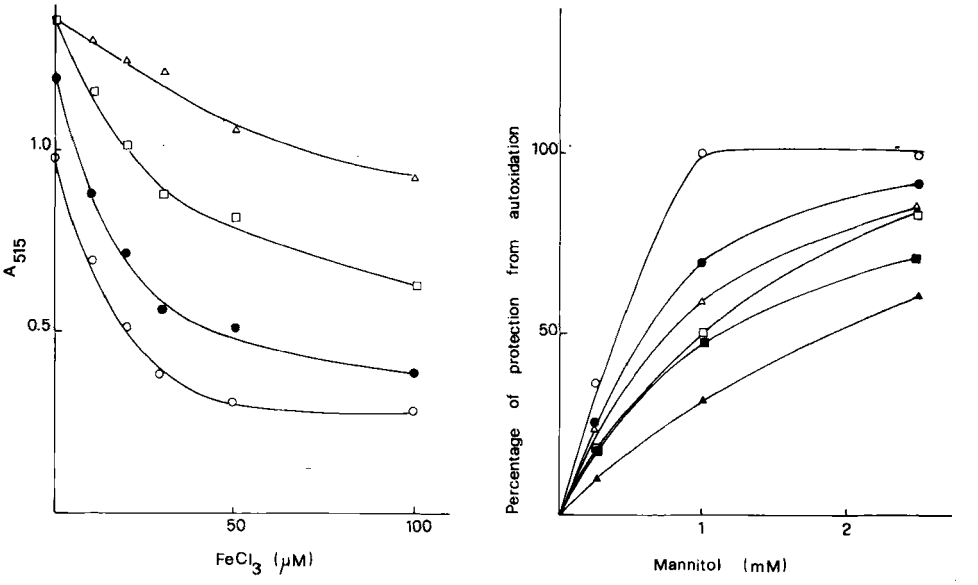

FIGURE 8 Effect of mannitol on the Fe^{2+} autoxidation in 5 mM Mops pH 7.2 in the presence of increasing concentrations of Fe^{3+}. Fe^{2+} autoxidation was measured by the o-phenanthroline method after 5 min incubation in 5 mM Mops pH 7.2 as described in the materials and methods section: a) the reactions were conducted in the presence of increasing concentrations of Fe^{3+} and in the absence (○) or presence of 0.25 mM (●), 1 mM (□) and 2.5 mM (△) mannitol; b) the experimental data from a) were plotted as percentage of protection exerted by different concentration of mannitol taking as 100% the ΔA ($t_0 - t_5$) for each Fe^{3+} concentration. ○ no Fe^{3+}; ● 10 μM; △ 20 μM; □ 30 μM; ■ 50 μM and ▲ 100 μM Fe^{3+}.

chelate, EDTA-Fe^{2+}, when added to linoleate caused increased rates of diene conjugation. However, the extent of conjugated diene formation was decreased as the EDTA to Fe^{2+} molar ratios was increased. The addition of either catalase or mannitol to the reaction mixture inhibited the rates of diene conjugation. Mannitol and catalase in our experimental system decrease the rate of iron oxidation. The above results were obtained in unbuffered water brought to neutral pH with NaOH. We have previously shown that in Good-type buffers, which are known not to bind metals, the pathway of iron autoxidation is similar to that occurring in unbuffered water brought to alkaline pH.[11] Furthermore, Gutteridge *et al.*[14] reported that intermediate molar ratios of EDTA and Fe^{2+} promote maximal extents of lipid peroxidation. It was suggested that the addition of EDTA (at concentrations less than that of Fe^{2+}) to a ferrous iron solution creates two pools of iron: EDTA-Fe^{2+} (rapidly oxidizing) and unchelated Fe^{2+} (slowly autoxidizing).[6] During the rapid autoxidation of iron, H_2O_2 appeared to be formed. This could be cleaved by unchelated Fe^{2+} to form OH· and Fe^{3+}. We have demonstrated that H_2O_2 addition in fact mimicks the effect of EDTA. However the direct reaction of H_2O_2 with Fe^{2+} would be expected to produce either a stoichiometric amount of Fe^{3+} or twice the stoichiometric amount of Fe^{3+} if the OH· produced reacts with Fe^{2+}. On the contrary, 15 μM H_2O_2 was observed to fully oxidize 150 μM $FeCl_2$. Furthermore, the Fe^{3+} ion greatly accelerates Fe^{2+} oxidation which points to Fe^{3+} (the product of the rapid oxidation reaction) as the component which catalyzes the oxidation of unchelated Fe^{2+}.

This hypothesis gains support from the fact that addition of Fe^{3+} not only decreases the lag time and accelerates the rate of iron oxidation, but also inhibits NBT reduction as was observed for the EDTA-Fe^{2+} complex. OH· scavengers affect Fe^{3+}-catalyzed iron autoxidation in a similar manner to their effect on the EDTA-catalyzed reaction. In both these experimental systems, mannitol and sorbitol are more efficient inhibitors than other OH· scavengers. A similar result was obtained when iron autoxidation was studied in Mops and Hepes buffers.[11] It was suggested that these carbohydrates exert their effect, not by scavenging OH· radicals, but more likely by binding a catalytic iron intermediate. At neutral and alkaline pH, the active form of Fe^{3+} may be a complex with hydroxyl ions (OH^-). Carbohydrates are known to bind metals avidly with their hydroxyl groups displacing OH^-[15]. The Fe^{3+} as the Fe^{3+} carbohydrate complex may thus loose its ability to catalyze the oxidation of Fe^{2+}.

The data obtained support the idea that EDTA, H_2O_2 and Fe^{3+} each accelerate the pathway of iron oxidation occurring in Good-type buffers[9] and give some hint as to a possible mechanism. In these buffers, iron autoxidation is characterized by a lag phase, during which an intermediate is slowly produced.[11] Our observations suggest that during this period Fe^{3+} is formed. The lag phase can be shortened by increasing the pH of the solution. It would appear that Fe^{3+} can act catalytically when complexed with OH^- and this may explain the dependence on the hydroxyl ion concentration. This hypothesis does not of course exclude a direct effect of OH^- on the production of Fe^{3+} as suggested by Goto *et al.*[8] The iron product which is formed when the oxidation reaction is accelerated by either EDTA, H_2O_2 or Fe^{3+} has the same absorption spectrum and the same reduced reactivity with thiocyanate as the product which is formed during iron autoxidation in Mops and Hepes buffer. Thus it appears to differ from Fe^{3+}. The absorption spectrum of the iron compound, produced during all these reactions, is not affected by an EDTA concentration exceeding the iron concentration whilst EDTA greatly affects the spectrum of Fe^{3+}. These data further support the hypothesis that Fe^{3+}, in its ionic and or hydroxylated

form, is not the final product of iron oxidation under these experimental conditions. They are also not consistent with the possibility that a Fe^{3+} buffer complex is the final product as its association constant would have to be at least comparable with that of the Fe^{3+} EDTA complex (log Ka = 25).[16] When Fe^{2+} autoxidation is conducted in the presence of NBT, formazan is formed.[11] Addition of EDTA, H_2O_2 and Fe^{3+} to the reaction mixture decreases NBT reduction by O_2^-. This effect is probably due to a direct competition of Fe^{3+} with NBT for O_2^-.

In phosphate buffer, EDTA directly oxidized a stoichiometric amount of Fe^{2+} without affecting the oxidation of the free Fe^{2+}. This suggests that Fe^{3+} does not have any catalytic effect in this buffer as previously shown.[3] Addition of Fe^{3+} does not in fact increase Fe^{2+} autoxidation. A possible explanation for these results is that the phosphate ion strongly binds Fe^{3+} and prevents it from exerting any effect on iron oxidation.

In conclusion, the observations reported here clearly demonstrate that results obtained using an EDTA-iron complex in one type of buffer can not necessarily be reproduced in other buffering conditions. The data may also help to clarify the sequence of reactions, that leads to iron autoxidation, in buffers which have no, or weak, affinities for metals and to understand the effect of EDTA in some experimental systems.

Acknowledgements

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