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IRON AUTOXIDATION IN MOPS AND HEPES BUFFERS

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Iron autoxidation in Mops and Hepes buffers is characterized by a lag phase that becomes shorter with increasing $FeCl_2$ concentration and pH. During iron oxidation in these buffers a yellow colour develops in the solution. When the reaction is conducted in the presence of nitro blue tetrazolium (NBT), blue formazan is formed. Of the many $OH^{\cdot}$ scavengers tested, mannitol and sorbitol are most effective in inhibiting Fe^{2+} oxidation, yellow colour development and NBT reduction. Some inhibition was also noted with catalase. The iron product of the oxidative reaction differs from Fe^{3+} in its absorption spectrum and its low reactivity with thiocyanate. Similar results are obtained when iron autoxidation is studied in unbuffered solutions brought to alkaline pH with NaOH. In phosphate buffer, no lag phase is evident and the absorption spectrum of the final solution is identical to that of Fe^{3+} in this buffer. The iron product reacts immediately with thiocyanate. When iron oxidation is conducted in the presence of NBT the formation of formazan is almost undetectable. Of the many compounds tested only catalase inhibits iron autoxidation in this buffer. The sequence of reactions leading to iron autoxidation in Good-type buffers[1] thus resembles that occurring in unbuffered solutions brought to alkaline pH with NaOH and greatly differs from that occurring in phosphate buffer. These results are in agreement with the observation that these buffers have very low affinity for iron.[1] The data presented define experimental conditions where Fe^{2+} is substantially stable for a considerable length of time in Mops buffer.

KEY WORDS: Iron, buffer, mannitol, catalase, superoxide dismutase.

INTRODUCTION

Transition metal such as iron or copper appear to have a major role in enhancing the reactivity of molecular oxygen. During the electron reduction of oxygen to water, "active oxygen" species are formed. Two of these species, O_2^- and $OH^{\cdot}$, are free radicals which are directly or indirectly, capable of damaging almost all known biomolecules.[2–4] This leads to cellular alteration and ultimately to tissue damage. An awareness of the fact that many of the damaging effects of oxygen could be attributed to the formation of oxygen centred radicals, prompted a re-examination of the basic biochemical mechanisms which are involved in such free radical-mediated injury to cells. A vast literature is available concerning the manner in which transition metals participate in the formation of oxygen free radicals and about their reactions with biomolecules such as DNA, proteins, phospholipids and carbohydrates.[5–7] These investigations have been conducted using either unbuffered solutions adjusted to the desired pH[8–10] or in a variety of different buffers.[11–13]

Buffers were shown to affect metal autoxidation.[14,15] The mechanism of autoxidation of transitional metal ions, such as Fe^{2+}, is in fact dependent on reaction con-

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ditions. The rate expression for Fe^{2+} autoxidation, varies in the presence of different anions. In the presence of phosphate[16] or bicarbonate[17] it is first order with respect to Fe^{2+} concentration. By contrast, a second order dependence on Fe^{2+} concentration is observed in the presence of sulfate[18] or nitrate[19]. The different rates of Fe^{2+} autoxidation may indicate that the reaction is proceeding by different pathways which in turn may influence the types, reactivities and lifetimes of the reactive species formed. In the investigation reported here, we have studied how Fe^{2+} autoxidizes in Mops, Hepes buffers in the neutral pH range and how compounds such as hydroxyl radical scavengers, catalase and superoxide dismutase affect Fe^{2+} autoxidation in these Good type buffers. These buffers are widely used in biochemical studies for the advantages that they provide compared to other buffers. The aim of this study is to provide a basic knowledge of Fe^{2+} autoxidation in these buffers. We have also compared the results with those obtained in two of the experimental conditions most used in free radical studies, namely phosphate buffer and unbuffered solutions adjusted to alkaline pH.

MATERIALS AND METHODS

Mops, Hepes and nitro blue tetrazolium (NBT) were obtained from Sigma Chemical Co (St. Louis, MO, U.S.A.), 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+} were prepared daily. Fe^{2+} determination was made by the o-phenanthroline method according to Mahler and Elowe.[20] 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 absorption was immediately read at 515 nm. Fe^{3+} was measured as thiocyanate complex. A 0.5 ml aliquot of the 1 ml reaction mixture was removed and added to 2.5 ml of glacial acetic acid. After 1 min 0.25 ml of 20% (w/v) KSCN was added. The absorption at 505 nm was measured after 2 min. Nitro blue tetrazolium reduction was studied by measuring the absorbance at 560 nm. The development of yellow colour during Fe^{2+} autoxidation in Mops and Hepes buffer was followed by measuring the absorption at 400 nm.

RESULTS

Effect of Buffer on Fe^{2+} Autoxidation

We have compared the autoxidation of Fe^{2+} in Mops and Hepes buffers with that in phosphate buffer. The only other ion present was the chloride associated with the source of iron, $FeCl_2$. In both Mops and Hepes buffers, Fe^{2+} autoxidation was greatly affected by the pH and the $FeCl_2$ concentration in the sample (Figure 1a). This unusual pattern was further explored by studying the rate of Fe^{2+} autoxidation: a lag phase was always present and its length was decreased by increasing either the pH (Figure 1b) or iron concentration (Figure 1c). Fe^{2+} autoxidation was not affected by buffer concentration at pH 7 whereas at pH 7.5 it was slightly accelerated as concentration of the buffering component was increased from 0.5 to 5 mM (results not shown). During Fe^{2+} autoxidation the solution develops a yellow colour whose

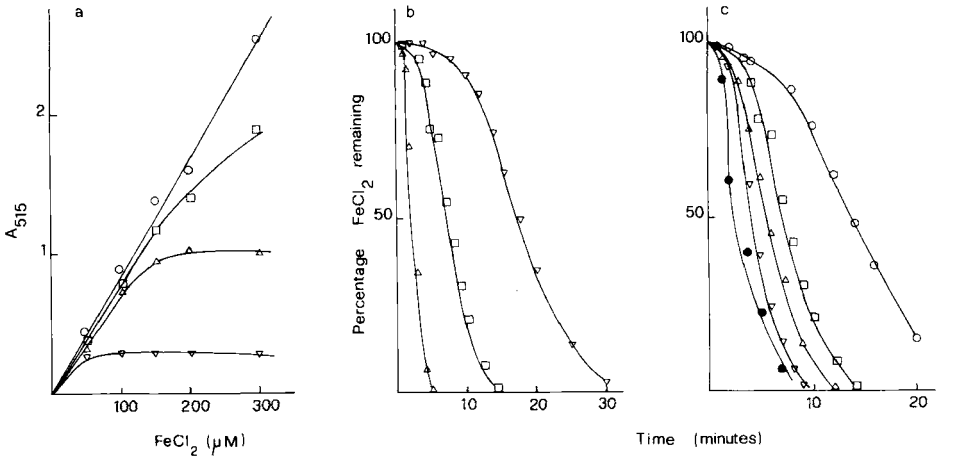

FIGURE 1 Effect of pH and initial concentration of Fe^{2+} on Fe^{2+} autoxidation in 5 mM Mops buffer. a) Increasing concentrations of Fe^{2+} were incubated for 3 min in ○ H_2O; □ Mops pH 7; △ Mops pH 7.3; ▽ Mops pH 7.5 and the remaining Fe^{2+} was determined as described in the materials and methods section. b) The autoxidation of 150 μM Fe^{2+} in 5 mM Mops pH ▽ 7; □ 7.3; △ 7.5 was determined at different times and expressed as percentage of Fe^{2+} remaining. c) Fe^{2+} autoxidation in 5 mM Mops pH 7.3 in the presence of ○ 50 μM; □ 100 μM; △ 150 μM; ▽ 200 μM and ● 300 μM Fe^{2+} was determined at different times and expressed as percentage of Fe^{2+} remaining.

intensity depends on iron concentration. The absorption spectrum of the solution greatly differs from that of an equal molar solution of $FeCl_3$ in the same buffer (Figure 2a). The absorbance of iron, autoxidized in Mops, is higher at all wavelengths tested and shows two peaks at 310 and 370 nm. Determination of the Fe^{3+} content of this

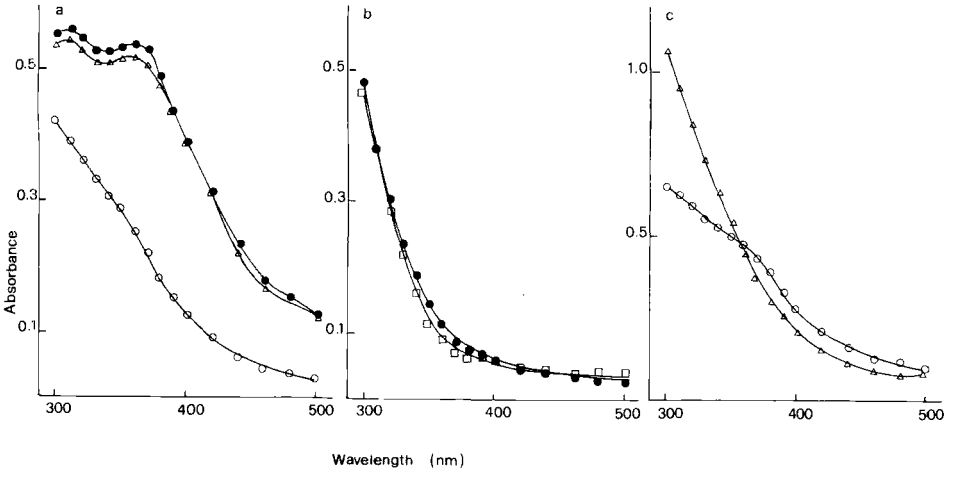

FIGURE 2 Absorbance spectra of the product of Fe^{2+} autoxidation in different buffering conditions. The absorbance spectra were determined after 20 min incubation of 150 μM Fe^{2+} in a) ● 5 mM Mops pH 7.3; △ 5 mM Hepes pH 7.3; b) ● 5 mM phosphate pH 7.3; c) ○ H_2O brought to pH 9 with NaOH. The absorbance spectra of 150 μM Fe^{3+} in a) ○ 5 mM Mops pH 7.3; b) □ 5 mM phosphate pH 7.3; c) △ H_2O brought to pH 9 with NaOH were also determined after 20 min incubation.

solution by the thiocyanate method shows that only a small amount of iron is present as this ion and therefore colour-reactive using the standard procedure (16–20% of the Fe^{3+} that might theoretically be present). When the reaction mixture is incubated in acid for longer than the standard 1 min, a proportionally higher amount of iron reacts with thiocyanate (results not shown). Comparison of the time course of Fe^{2+} autoxidation and yellow colour formation (A_{400}) indicates that ΔA_{400} slightly lags behind Fe^{2+} autoxidation. Besides this small difference the two phenomena are correlated (Figure 3). Iron autoxidation and yellow colour development do not occur when the experiments are conducted in the absence of oxygen (results not shown). In the presence of phosphate buffer, Fe^{2+} autoxidation does not result in the formation of a yellow colour. The absorption of Fe^{2+} autoxidized in phosphate buffer pH 7.3 does not significantly differ from that of an equal amount of Fe^{3+} in the same buffer (Figure 2b). The ferric products of the reaction is readily coloured by the thiocyanate method. In phosphate buffer, Fe^{2+} autoxidation depends on pH without any evident lag phase (Figure 4a,b).

When iron autoxidation is studied in unbuffered solutions the results depend on the pH obtained by NaOH addition. At pH 7–8.5 addition of the acidic iron solution almost instantaneously decreases the pH below 7 and iron oxidation does not occur. At pH 9.5 the iron added is almost instantaneously oxidized and this renders difficult the study of the effect of different compounds. In an unbuffered solution brought to pH 9 with NaOH, Fe^{2+} autoxidized with the formation of a yellow colour. The absorption spectrum of the solution differs from that of $FeCl_3$ added to the same solvent (Figure 2c) and is similar to that of Fe^{2+} autoxidized in Mops buffer (Figure 2a). The spectrum has a shoulder at about 360–370 nm. The final product appears therefore to be a mixture of $FeCl_3$ and of the product formed in Mops and Hepes buffer. As the first step of the chain reaction proposed for Fe^{2+} autoxidation forms O_2^- we added nitro blue tetrazolium to the reaction mixtures to detect it. NBT is

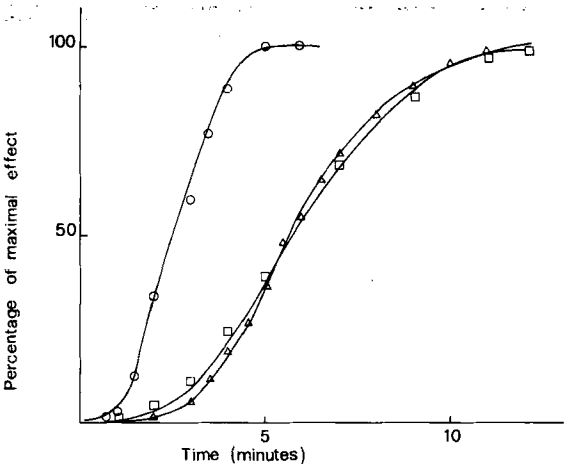

FIGURE 3 Time course of Fe^{2+} autoxidation in 5 mM Mops pH 7.3. The disappearance of 150 μM Fe^{2+} from the standard solution (□) was determined by the o-phenanthroline method as described in materials and methods section. The development of a yellow colour in this solution (△) was studied by monitoring the increase in A_{400}. NBT reduction (○) was measured in the presence of 50 μM NBT in the standard solution monitoring the formazan formation at 560 nm.

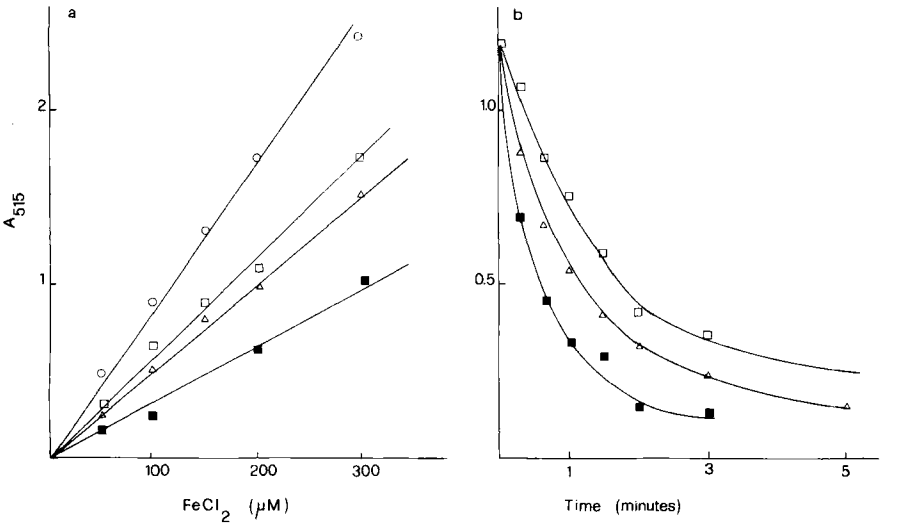

FIGURE 4 Effect of pH and initial concentration of Fe^{2+} on Fe^{2+} autoxidation in 5 mM phosphate buffer. a) increasing concentrations of Fe^{2+} were incubated for 1 min in ○ H_2O; □ phosphate pH 7; △ phosphate pH 7.2; ■ phosphate pH 7.5 and the remaining Fe^{2+} was determined as described in the materials and methods section. b) the autoxidation of 150 mMμM Fe^{2+} in 5 mM phosphate pH □ 7; △ 7.2 and ■ 7.5 was determined at different times.

reduced to formazan during Fe^{2+} oxidation in Mops and Hepes buffers whereas the formation of formazan is almost undetectable in the presence of phosphate buffer (Figure 5). In Mops buffer the rate of NBT reduction compared to Fe^{2+} autoxidation is similarly affected by pH (Figure 5), iron and buffer concentration (Figure 6a,b). NBT addition, however, shortens the lag phase and accelerates the rate of Fe^{2+} autoxidation, consequently the curves of NBT reduction are anticipated compared to Fe^{2+} autoxidation in its absence (Figure 3). Fe^{2+} autoxidation in the presence of NBT was studied following the formation of red ferroin from o-phenanthroline at 450 nm. At this wavelength the ΔA contribution due to formazan formation is minimal but still too relevant to allow a fine comparison of the time course of the two phenomena (results not shown).

In Mops buffer at pH 7.5, various $OH^{\cdot}$ radical scavengers were tested but only the polyols mannitol, sorbitol and to a lesser extent inositol inhibit Fe^{2+} autoxidation, NBT reduction (Table I) and ΔA (results not shown); formate, thiourea, ethanol, butan-1-ol have little or no effect. Also in Hepes buffer at pH 7.4, mannitol, added at different times after iron addition, blocks further formazan formation (Figure 7a). In this buffer, concentrations of mannitol as low as 0.5 mM are able to affect the formazan formation rate; increasing the concentration of this compound increases the lag phase and decreases the rate and the extent of NBT reduction (Figure 8). Sorbitol is active at similar concentrations whereas 50 mM inositol is slightly less efficient than 1 mM mannitol (results not shown). The extent of the effect exerted by these polyols depends on the pH: a higher concentration of the compound is required to produce the same effect on NBT reduction, Fe^{2+} autoxidation and ΔA_{400} at higher pH (results not shown). The pattern of inhibition of Fe^{2+} autoxidation in phosphate buffer at pH 7.5 differs markedly. Table I shows that all $OH^{\cdot}$ scavengers are low inhibitors.

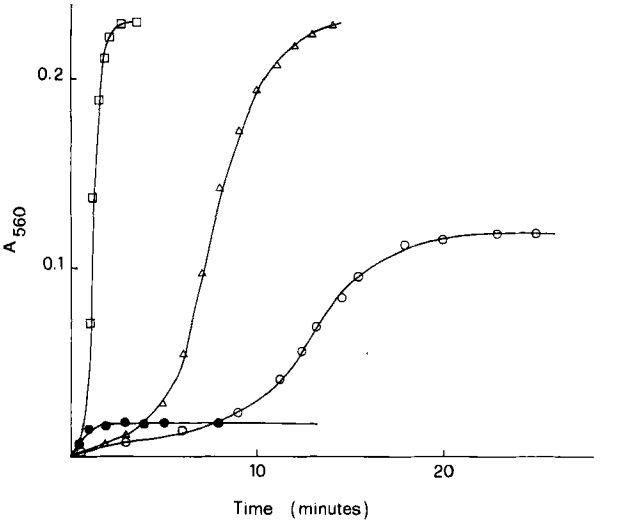

FIGURE 5 Effect of buffer on NBT reduction occurring during Fe^{2+} autoxidation. The reduction of 25 μM NBT was measured monitoring the formazan formation at 560 nm during the autoxidation of 50 μM Fe^{2+} in 5 mM □ Mops pH 7.5; △ Hepes pH 7.3; ○ Mops pH 7; ● phosphate pH 7.2.

Superoxide dismutase (0.2 mg) inhibits Fe^{2+} autoxidation and NBT reduction (Table I) and its inhibition depends on the pH of the Mops and Hepes buffers. At pH 7.2, in the presence of superoxide dismutase, NBT reduction lags 7–8 min behind iron addition and then starts at a reduced rate. At this pH, superoxide dismutase added 3 min after iron is able to greatly reduce the rate of formazan formation (results not

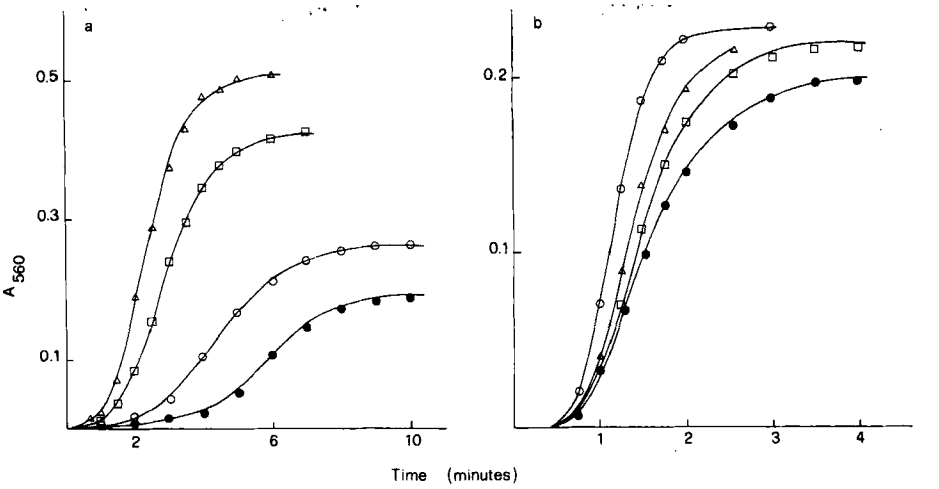

FIGURE 6 Effect of pH and Mops buffer concentration on NBT reduction occurring during Fe^{2+} autoxidation. The reduction of 25 μM NBT was measured monitoring the formazan formation at 560 nm during Fe^{2+} autoxidation in the presence of a) 5 mM Mops pH 7.3 containing ● 50 μM; ○ 100 μM; □ 150 μM and △ 200 μM Fe^{2+}; b) 50 μM Fe^{2+} in ● 0.5 mM; □ 1 mM; △ 1.5 mM and ○ 2.5 mM Mops pH 7.5.

TABLE I
Effect of $OH^{\cdot}$ scavengers on Fe^{2+} autoxidation

	NBT reduction				Fe^{2+} oxidation			
	Mops buffer		H_2O pH 9		Mops buffer		Phosphate buffer	
	ΔA_{560}	Inhibition	ΔA_{560}	Inhibition	ΔA_{515}	Inhibition	ΔA_{515}	Inhibition
Buffer (5 mM) + Fe^{2+} (0.15 mM)	0.811	–	0.240	–	1.343	–	1.078	–
+ Ethanol (50 mM)	–	–	–	–	1.347	0%	–	–
+ Butan-1-ol (50 mM)	0.707	13%	–	–	1.345	0%	1.062	1%
+ Formate (5 mM)	0.798	2%	0.227	5%	1.276	5%	1.069	1%
+ Mannitol (5 mM)	0.102	87%	0.032	87%	0.249	82%	0.954	11%
+ Sorbitol (5 mM)	0.050	94%	0.026	89%	0.209	84%	0.996	8%
+ Inositol (50 mM)	0.113	86%	–	–	0.519	61%	0.998	8%
+ Thiourea (50 mM)	0.804	1%	0.256	0%	1.226	9%	0.966	10%
+ SOD (0.2 mg/ml)	0.125	85%	0.030	87%	0.424	68%	0.975	10%
+ SOD (0.1 mg/ml)	0.356	56%	–	–	–	–	–	–
+ SOD (heat denatured (0.2 mg/ml)	0.131	84%	–	–	0.327	76%	0.984	9%
+ Catalase (0.1 mg/ml)	0.030	96%	0.010	96%	1.038	23%	0.705	35%
+ Catalase (heat denatured) (0.1 mg/ml)	0.440	46%	–	–	1.189	11%	1.079	0%
+ Albumin (0.2 mg/ml)	0.157	81%	0.036	85%	1.263	6%	1.078	0%

NBT reduction in Mops buffer, pH 7.5, was measured after 3 min; Fe^{2+} autoxidation was measured by the o-phenanthroline method after 3 min in phosphate buffer, pH 7.5 and after 5 min, in Mops buffer, pH 7.5. All concentrations shown are final reaction concentrations. Abbreviation: SOD superoxide dismutase.

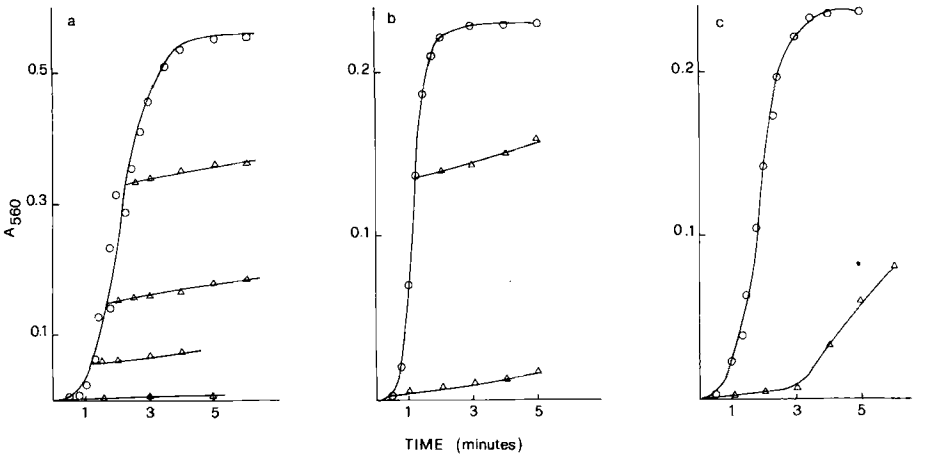

FIGURE 7 Effect of mannitol, catalase and superoxide dismutase on NBT reduction occurring during Fe^{2+} autoxidation in Good-type buffers. The reduction of 25 μM NBT was measured monitoring the formazan formation at 560 nm during Fe^{2+} autoxidation in the stated conditions. Addition of the compound under test was made either before Fe^{2+} addition or at different times during the incubations. The assay was conducted in the presence of a) 5 mM Hepes pH 7.4, 150 μM Fe^{2+} in the absence (○) or presence (△) of 10 mM mannitol; b) 5 mM Mops pH 7.5, 50 μM Fe^{2+} in the absence (○) or presence (△) of 0.1 mg/ml catalase; c) 5 mM Hepes pH 7.4, 50 μM Fe^{2+} in the absence (○) or presence (△) of 0.1 mg/ml superoxide dismutase.

shown). When superoxide dismutase is added to a Hepes buffer at pH 7.4 the complete inhibition of NBT reduction occurs only for 2–3 min and the successive rate is not so much affected as at pH 7.2 (Figure 7c). At pH 7.6, superoxide dismutase reduces only the rate and the final extent of formazan formation (results not shown). Heat dena-

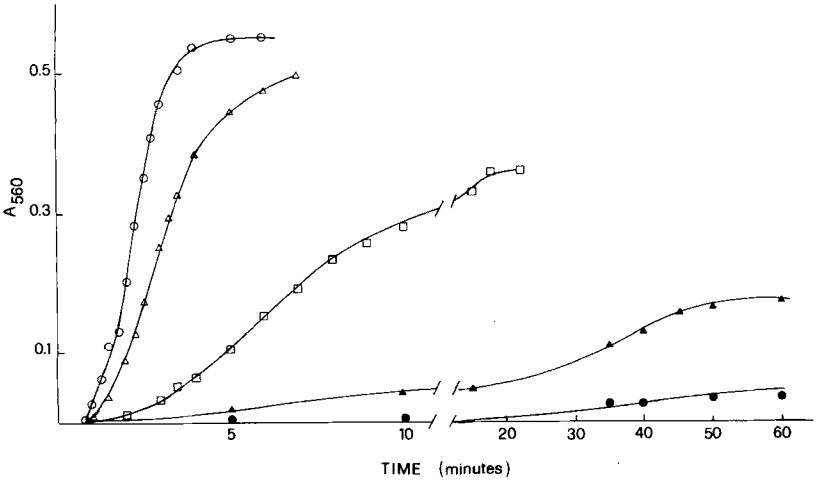

FIGURE 8 Effect of the concentration of mannitol on NBT reduction occurring during Fe^{2+} autoxidation in Hepes buffer. The reduction of 25 μM NBT was measured monitoring the formazan formation at 560 nm during the autoxidation of 150 μM Fe^{2+} in 5 mM Hepes pH 7.4 in the absence (○) or presence of △ 0.5 mM, □ 1 mM, ▲ 2.5 mM, ● 10 mM mannitol.

turation of superoxide dismutase did not, however, abolish its inhibitory activity (Table I). The prevention of Fe^{2+} autoxidation and NBT reduction caused by denaturated superoxide dismutase suggested that these effects might be ascribed to an unspecific protein effect rather than to the catalytic activity. Bovine serum albumin was very effective in decreasing NBT reduction and had a lower effect on Fe^{2+} autoxidation (Table I). In phosphate buffer at pH 7.5, 0.2 mg of both native and heat denatured superoxide dismutase only poorly protects Fe^{2+} from autoxidation (Table I).

NBT reduction in Mops and Hepes buffer was inhibited by catalase (Table I). Inhibition also occurs when the enzyme is added to the reaction mixture at different times after iron addition (Figure 7b). The heat denatured enzyme causes some inhibition of formazan formation although its effect is much lower than that of the native enzyme. Inhibition of Fe^{2+} autoxidation by both native and denatured enzyme is much less than the inhibition of NBT reduction (Table I). High inhibition of Fe^{2+} autoxidation by native catalase was, however, evident up to 2–3 min. In phosphate buffer at pH 7.5, of the many compounds tested, native catalase has the highest ability to interfere with Fe^{2+} autoxidation whereas the denatured enzyme is ineffective.

Iron added to unbuffered water brought to pH 9 with NaOH, in the presence of NBT, results in formazan formation. Mannitol, catalase, superoxide dismutase and albumin inhibit NBT reduction (Table I), ΔA_{400} and iron autoxidation (results not shown).

DISCUSSION

The results show that iron autoxidation is different in the presence of Good-type buffers and phosphate buffers. Iron autoxidation in either Mops or Hepes buffers is characterized by a lag phase as already reported by Lambeth *et al.*[15] This observation is consistent with a reaction mechanism in which an intermediate is slowly produced. This can then further participate as a catalyst in the autoxidation of the remaining Fe^{2+}. This slow process is clearly influenced by pH and Fe^{2+} concentration and suggests that both OH^- and Fe^{2+} iron are involved in the formation of this intermediate.

As early as 1907, Just[17] showed that the rate expression for oxidation of ferrous iron by dissolved oxygen in neutral solution showed a second order dependence on OH^- concentration. Different models proposed by Abel[21] and Goto *et al.*[22] however, did not clarify the mechanism. Fe^{2+} autoxidation in these zwitterion buffers introduced by Good *et al.*[1] is associated with the development of a yellow colour in solution. The absorption spectrum of this product differs from that of Fe^{3+} in the same buffers. This product, with its characteristic spectrum, is not formed by a direct reduction of the buffer by the ferrous iron, since neither the oxidation of iron nor the yellow colour are obtained when the reaction is conducted in the absence of oxygen. Moreover, it is unlikely that it is the product of the reaction of the buffering component with O_2^- generated by the Fe^{2+} autoxidation. In this case, increasing concentrations of buffering materials should compete with NBT for O_2^- thus lowering the formation of formazan. In fact, the opposite is observed. Furthermore, the similarities of the spectra, obtained in Mops and Hepes buffers, do not support the formation of a coloured iron buffer complex. Good-type buffers have no or very weak affinities for metals,[1] so the autoxidation of Fe^{2+} in these buffers more likely reflects the hydrolytic

and autoxidizing properties of iron in water. The results obtained in water brought to alkaline pH with NaOH support this hypothesis.

The product of the Fe^{2+} autoxidation in Good-type buffers seems to differ from Fe^{3+} in both its free ionic and its hydroxylated form not only in its absorption spectrum but also in respect of its low reactivity towards thiocyanate. As already mentioned, during Fe^{2+} autoxidation in either Mops or Hepes buffer, NBT is reduced to formazan. The O_2^- radical appears to be produced during the sequence of reactions leading to Fe^{2+} oxidation in these buffers. From the data available it is difficult to determine whether it is formed at an early or a late stage of the process. The increased rate of Fe^{2+} oxidation which is observed in the presence of a compound (NBT), that acts as a O_2^- scavenger suggests, however, that it is not an early intermediate in the sequence of reactions leading to Fe^{2+} oxidation and, furthermore, that this oxygen active species slows down Fe^{2+} oxidation, probably via a direct reaction with Fe^{3+} to form Fe^{2+}. Of the many compounds tested, mannitol and sorbitol are the most effective in preventing Fe^{2+} autoxidation. These compounds are known $OH^{\cdot}$ scavengers; however, they are effective at concentrations lower than those usually used to scavenge this radical (10 to 500 mM).[23,24] On the contrary thiourea, ethanol, formate and butan-1-ol exerted a small inhibition of Fe^{2+} autoxidation. Mops and Hepes were reported to be effective $OH^{\cdot}$ scavengers[25] and this may explain the results obtained with those scavengers. However the lack of inhibition of Fe^{2+} autoxidation by the buffers either in the absence or presence of $OH^{\cdot}$ scavengers suggests that free $OH^{\cdot}$ radical is not a likely intermediate in the sequence of reactions leading to Fe^{2+} autoxidation. Carbohydrates are known to bind metals avidly.[26] This may suggest that protection by mannitol and sorbitol is simply due to either their binding to the iron ions, or possibly to an intermediate iron complex with oxygen, O_2^- or some other reduced oxygen species. Mannitol is reported in the Merck Index as forming a stable complex with H_2O_2.

Superoxide dismutase is also an inhibitor of Fe^{2+} oxidation and NBT reduction and this suggests that O_2^- radicals are required. Heat denaturation of the superoxide dismutase did not however, abolish its inhibitory activity. It was previously shown that heat denatured superoxide dismutase did not totally lose its enzymic activity.[27,28] This might reflect either recombination of released copper and protein to restore superoxide dismutase activity as previously observed[27] or a non specific protein effect. Bovine serum albumin, included as a control, strongly inhibited the maximum extent of NBT reduction and decreased the rate of Fe^{2+} oxidation and the ΔA_{400}. It is unlikely that this effect is due to competition with NBT for the O_2^- radical as this oxygen active species is only poorly reactive in aqueous media and amino acids are not among the substances that can react with O_2^- with appreciable reaction rates.[29] On the other hand, albumin, acting as a chelating agent[30] could loosely bind the metal ions and affect their reactivity. Catalase interferes with Fe^{2+} autoxidation and its effect seems to be mostly related to its enzymic activity.

The rate of Fe^{2+} autoxidation in the presence of phosphate differs from that in Good-type buffers as no noticeable lag phase is evident. In the presence of this anion, the rate expression for Fe^{2+} autoxidation is first order, which is consistent with a discrete one-electron reduction pathway.[16] It was suggested that phosphate destabilizes the $Fe^{3+}O_2^-$ complex resulting in the formation of $HO_2^{\cdot}/O_2^-$ (a process presumably first order with respect to Fe^{2+}).[31] In our experimental system, however, the reduction of NBT by O_2^- was barely observable. Furthermore, superoxide dismutase did not interefere with Fe^{2+} autoxidation suggesting that this oxygen free

radical does not participate further in the autoxidation of the remaining Fe^{2+}. The participation of $OH^{\cdot}$ is also questionable since all scavengers of this oxygen radical which were tested did not prevent Fe^{2+} oxidation. Of the compounds tested, only native catalase affects Fe^{2+} autoxidation. In phosphate buffer, not only the pathway but also the iron product of Fe^{2+} autoxidation differs from that formed in Good-type buffers. In fact in this buffer the iron product did not significantly differ from Fe^{3+} in respect of both its spectrophotometric characteristics and its reactivity with thiocyanate.

The data presented, besides indicating a substantial difference in iron oxidation pathway in Mops and Hepes buffers compared to phosphate buffers, show some characteristics of iron oxidation in Good-type buffers which may have great advantage in the study of the physiology and pathology of iron. The existence of a lag phase in Fe^{2+} autoxidation, its dependence on pH and iron concentration, its inhibiton by mannitol at very low concentrations allows to define experimental conditions where Fe^{2+} is stable for a considerable length of time. This, together with the very low affinities of the buffering species[1] for the metal renders in our opinion these buffers particularly suitable for such studies. The ability of Hepes[25] and probably of Mops to scavenge $OH^{\cdot}$ may limit their use in some systems, for example, initiation of lipoperoxidation. However the rate constant of the reaction of Hepes with $OH^{\cdot}$ ($5.1 \times 10^9 M^{-1} s^{-1}$) is not much higher than that of other compounds that are frequently used in such studies. The rate constant of $OH^{\cdot}$ reaction with the chelator EDTA is in fact $2.76 \times 10^9 M^{-1} s^{-1}$[32] and that of the nucleotide component of another chelator ADP is $2.5 \times 10^9 M^{-1} s^{-1}$[33].

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