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IRON OXIDATION IN MOPS BUFFER. EFFECT OF PHOSPHORUS CONTAINING COMPOUNDS

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Fe^{2+} autoxidation in Mops buffer both in absence and presence of substoichiometric concentrations of EDTA, H_2O_2 and of Fe^{3+} is greatly affected by phosphorus containing compounds. They increase the lag phase, characteristic of Fe^{2+} oxidation in this buffer,[1] and decrease the rate of the reaction. This effect is due to the phosphates of the molecule. The ability of the different compounds tested to affect Fe^{2+} oxidation, however, appears to be influenced also by the rest of the molecule. The concentration of the different phosphorus containing compounds that inhibits 50% of Fe^{2+} oxidation is rather different. The effect exerted appears to be the result of an equilibrium between an inhibitory effect on the pathway of Fe^{2+} oxidation that occurs in Mops buffer and the onset of a different oxidation pathway of Fe^{2+} similar to that occurring in Na phosphate buffer.[1] A hypothesis is proposed that the phosphorus containing compounds inhibit Fe^{2+} oxidation by binding Fe^{3+} and decreasing its ability to accelerate Fe^{2+} autoxidation.[2] It is suggested that the presence *in vitro* and *in vivo* of phosphorus containing compounds may modify Fe^{2+} autoxidation and thus the production of oxygen active species.

KEY WORDS: Iron, buffer, antioxidant, ATP, CDP-choline, glycerophosphoinositol.

INTRODUCTION

Oxygen free radicals, which are produced during the four electron reduction of molecular oxygen to water, have been implicated as etiological agents in several pathological conditions[3]. As the chemistry of oxygen dictates a preference for accepting electrons "one at a time", transition metal ions are involved in the formation and reactivity of the oxygen radicals.[4] Under physiological conditions, iron is only slightly soluble. However, a variety of chelating agents can greatly increase its solubility and reactivity.[5,6] Very little ferrous iron is present, *in vivo*, in its free ionic form.[7,8] In animal tissues it is largely bound to proteins such as transferrin and ferritin. However, such sequestration is unlikely to be complete and iron ions may also become attached to other molecules including inorganic phosphate and phosphate esters such as nucleotides and nucleic acids. Also the phosphate groups of membrane lipids may bind iron salts.

Despite the fact that the iron autoxidation is known to be greatly affected by the presence of different anions and chelators, not much attention has been given to the details of the effect that these physiological chelators may have.

We have undertaken a study aimed at evaluating whether and to what extent phosphorus containing compounds affect Fe^{2+} autoxidation in Mops buffer. This buffer has low affinity for iron[9] and thus the effect of possible chelators on iron autoxidation may be studied without interference due to the interaction of iron with the buffer. The results are described in the present paper.

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MATERIALS AND METHODS

Mops, ATP, ADP, AMP, GTP, GDP, Glycerophosphoinositol, CDP-choline, EDTA were obtained from Sigma Chemical Co. (St. Louis, Mo, U.S.A.), 1,10-phenathroline 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 by the o-phenanthroline method according to Mahler and Elowe.[10] 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 was immediately read at 515 nm. Fe^{3+} was measured as thiocyanate complex as previously described.[1] The development of yellow colour during Fe^{2+} autoxidation in Mops buffer was followed by measuring the absorbance at 400 nm. The competition of EDTA and ATP for Fe^{3+} were studied by incubating various amounts of Fe^{3+} in the absence or presence of 10 μM ATP for 10 min in 5 mM Mops buffer, pH 7.3. To the samples were added 180 μM EDTA and after 20 min the absorbance spectrum of these solutions was read versus control samples without EDTA.

RESULTS

Effect of phosphorus containing compounds on Fe^{2+} autoxidation in Mops buffer

Figure 1 illustrates quite clearly the effect that ATP, ADP and AMP have on Fe^{2+} autoxidation in Mops buffer, pH 7.2. These nucleotides at 10 μM concentration decrease the rate and increase the lag time characteristic of iron autoxidation in this buffer. They delay also the development of the yellow colour that parallels iron autoxidation and the formation of formazan that occurs when iron autoxidation is conducted in the presence of NBT in this buffer[1] (results not shown). The inhibition of iron autoxidation is already exerted at very low concentration of nucleotide. The dependence of the inhibition from the nucleotide concentration, however, is related to the type of nucleotide (Figure 2). The pattern of ADP inhibition was further explored by studying the rate of Fe^{2+} autoxidation in the presence of different concentrations of the nucleotide (Figure 3). A direct correlation exists between ADP concentration and the protection from autoxidation exerted at long incubation times. On the contrary a biphasic effect is observed when Fe^{2+} autoxidation is measured after a short incubation: the increment of the lag phase induced by low concentrations of the nucleotide becomes less evident and eventually is decreased increasing the nucleotide concentrations.

Other compounds have been tested. As shown in Table I, GTP and GDP exert a inhibitory effect on Fe^{2+} autoxidation at concentrations lower that those of the corresponding adenine nucleotides. CDP-choline is able to interfere with the Fe^{2+} autoxidation but high concentrations of this compound are required. cAMP, cGMP, adenine, adenosine and glycerophosphoinositol do not affect Fe^{2+} autoxidation.

Also Na phosphate protects Fe^{2+} from autoxidation at rather low concentrations whereas higher concentrations of this anion are less efficient (Figure 4). A study of the rate of Fe^{2+} autoxidation in the presence of 200 μM shows that the high phosphate concentration changes the kinetic of this reaction (Figure 5a). In this condition the

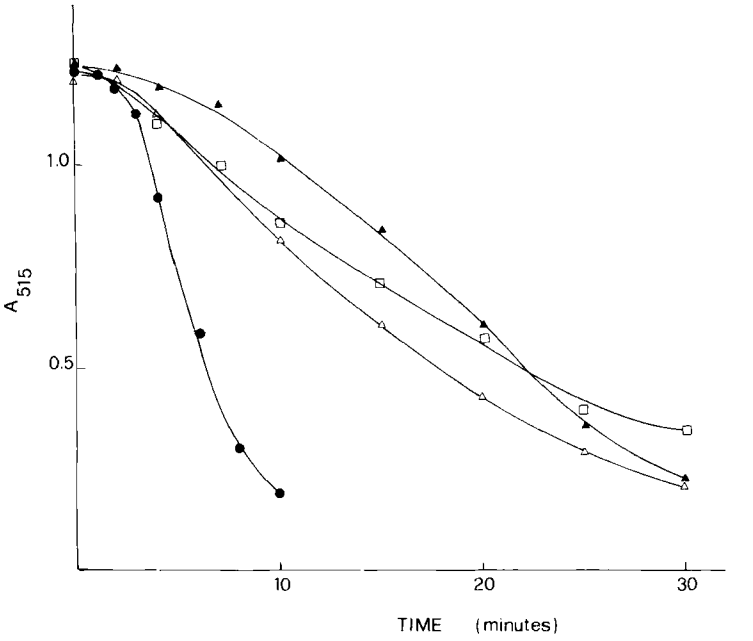


FIGURE 1 Time course of Fe^{2+} autoxidation in 5 mM Mops pH 7.2 in the presence of ATP, ADP and AMP. The disappearance of 150 μM Fe^{2+} from the standard solution (●) and in the presence of 10 μM ATP (△); 10 μM ADP (□); 10 μM AMP (▲) was determined by the o-phenanthroline method as described in the materials and methods section.

TABLE I
Effect of nucleotides on Fe^{2+} autoxidation

Fe^{2+} autoxidation was measured by the o-phenanthroline method in 5 mM Mops buffer pH 7.3. The sample was incubated for 10 min in the absence of 50 μM $FeCl_3$ and for 5 min in its presence. All concentrations shown are final reaction concentrations.

	Fe^{2+} oxidation			
	Mops Buffer		Mops buffer + 50 μM $FeCl_3$	
	ΔA_{515}	Inhibition	ΔA_{515}	Inhibition
Buffer (5 mM) + Fe^{2+} (0.15 mM)	1.233	–	1.250	–
+ ATP (10 μM)	0.395	68	0.297	73
+ ADP (10 μM)	0.209	83	0.275	78
+ AMP (10 μM)	0.259	79	0.010	99
+ cAMP (50 μM)	1.159	6	–	–
+ GTP (1 μM)	0.456	63	–	–
+ GDP (5 μM)	0.370	70	–	–
+ cGMP (50 μM)	1.196	3	–	–
+ Adenosine (100 μM)	1.156	6	1.212	3
+ Adenine (100 μM)	1.122	9	1.252	0
+ CDP-choline (50 μM)	1.110	10	–	–
+ CDP-choline (400 μM)	0.777	37	0.587	63
+ Glycerophosphoinositol (50 μM)	1.230	0	–	–
+ Na phosphate (10 μM)	0.567	54	–	–
+ Na pyrophosphate (10 μM)	1.250	0	–	–

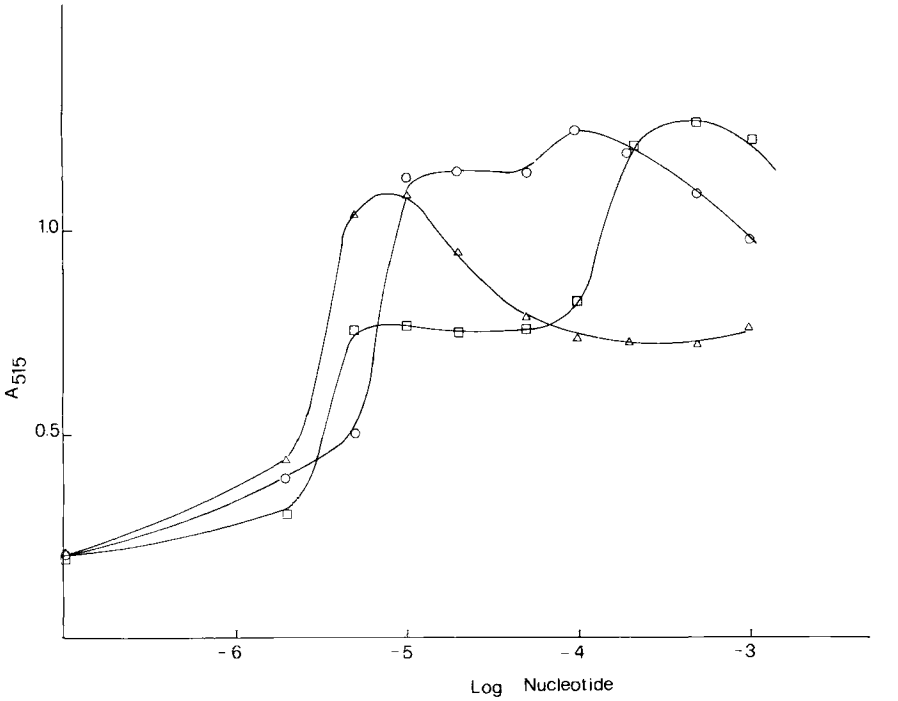


FIGURE 2 Effect of nucleotide concentration on Fe^{2+} autoxidation in 5 mM Mops buffer pH 7.2. The disappearance of 150 mM Fe^{2+} from the standard solution in the presence of increasing concentrations of ATP (□); ADP (△) and AMP (○) was determined after 10 min incubation by the o-phenanthroline method as described in the materials and methods section. The A_{515} of the sample at zero time was 1.130.

solution does not develop a yellow colour, parallel to Fe^{2+} autoxidation, as it does when the oxidation is conducted in the presence of 15 μM phosphate (Figure 5b). The absorbance spectra of these solutions are different (Figure 5c). The iron product of Fe^{2+} oxidation in the presence of 200 μM phosphate shows an absorbance spectrum similar to that obtained when the oxidation is conducted in phosphate buffer and similar to the spectrum of Fe^{3+} in this buffer.[1] The absorption spectrum of the iron oxidized in the presence of 15 μM phosphate, on the contrary, resembles with some differences that obtained in Mops buffer alone. The differences observed suggest that, also in the presence of 15 μM phosphate, some Fe^{2+} is oxidized through the pathway occurring in phosphate buffer. Na pyrophosphate has a stimulatory effect on Fe^{2+} autoxidation in Mops buffer (Figure 4) at all concentrations tested (1 μM–1 mM).

Effect of phosphorus containing compounds on Fe^{2+} autoxidation induced by EDTA, H_2O_2 and Fe^{3+} in Mops buffer

Fe^{2+} autoxidation in Mops buffer is greatly affected by substoichiometric concentrations of EDTA.[2] This chelator not only causes the oxidation of a stoichiometric amount of Fe^{2+} but also accelerates the oxidation of the free Fe^{2+}. When the reaction is conducted in the presence of the nucleotides AMP, ADP and ATP the oxidation of the unchelated Fe^{2+} is inhibited. The protection is exerted at very low concentra-

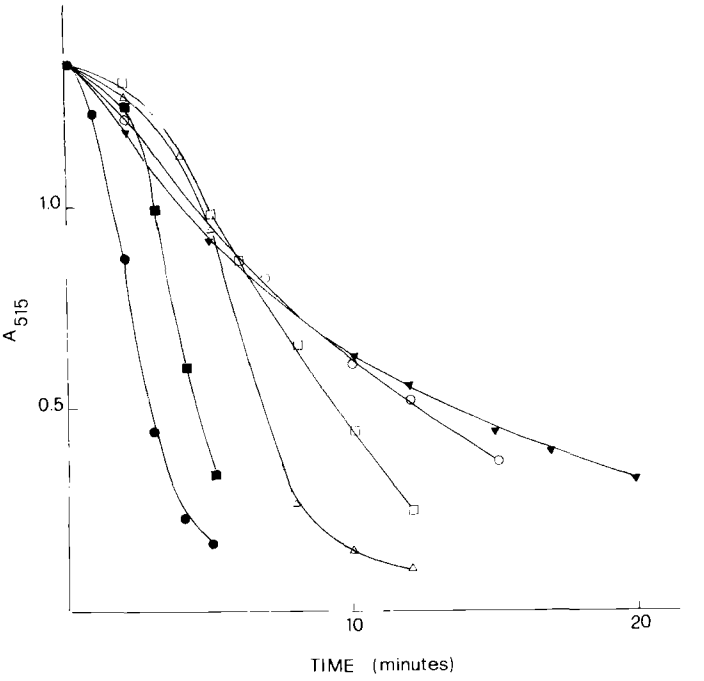


FIGURE 3 Time course of Fe^{2+} autoxidation in 5 mM Mops buffer pH 7.2 in the presence of different concentrations of ADP. The disappearance of 150 μM Fe^{2+} from the standard solution in the absence (●) and presence of 2 μM, (■), 5 μM (△), 10 μM, (□), 20 μM (○), 50 μM (▼) ADP was determined by the o-phenantholine method as described in the materials and methods section.

tions of the nucleotide (5–10 μM). The nucleotides however do not inhibit the oxidation of a stoichiometric amount of Fe^{2+} by EDTA that occurs in either water or Mops pH 6 (result not shown). Also Na phosphate inhibits the oxidation of free Fe^{2+} in Mops but at higher concentrations (20–50 μM) (Figure 6).

Fe^{2+} autoxidation in Mops buffer is also accelerated by addition of substoichiometric concentrations of H_2O_2[2]. ADP added to the reaction mixture does not appear to interfere with the direct oxidation of a stoichiometric amount of Fe^{2+} by H_2O_2. In water or Mops, pH 6 it certainly does not inhibit this direct oxidation (results not shown). However this nucleotide, in Mops buffer pH 7.3, inhibits the oxidation of Fe^{2+} exceeding H_2O_2 concentration. It exerts this effect by increasing the lag phase and decreasing the rate of Fe^{2+} autoxidation (Figure 7). The extent of the inhibition depends on the concentration of the nucleotide in the reaction mixture (Figure 7). Similar effects are observed in the presence of ATP (result not shown). ADP inhibits also the Fe^{3+} catalyzed oxidation of Fe^{2+} in Mops pH 7.3 (Figure 8). As observed for the ADP protection of Fe^{2+} from autoxidation in Mops buffer (Figure 3), a direct correlation exists between ADP concentration and the protection exerted at long incubation times whereas a biphasic effect is observed at short incubation times (Figure 8a). The amount of ADP required to inhibit Fe^{2+} autoxidation depends on the amount of Fe^{3+} added (Figure 8b). The other adenylic nucleotides ATP and AMP inhibit the Fe^{3+} catalyzed oxidation of Fe^{2+} whereas adenosine and adenine are

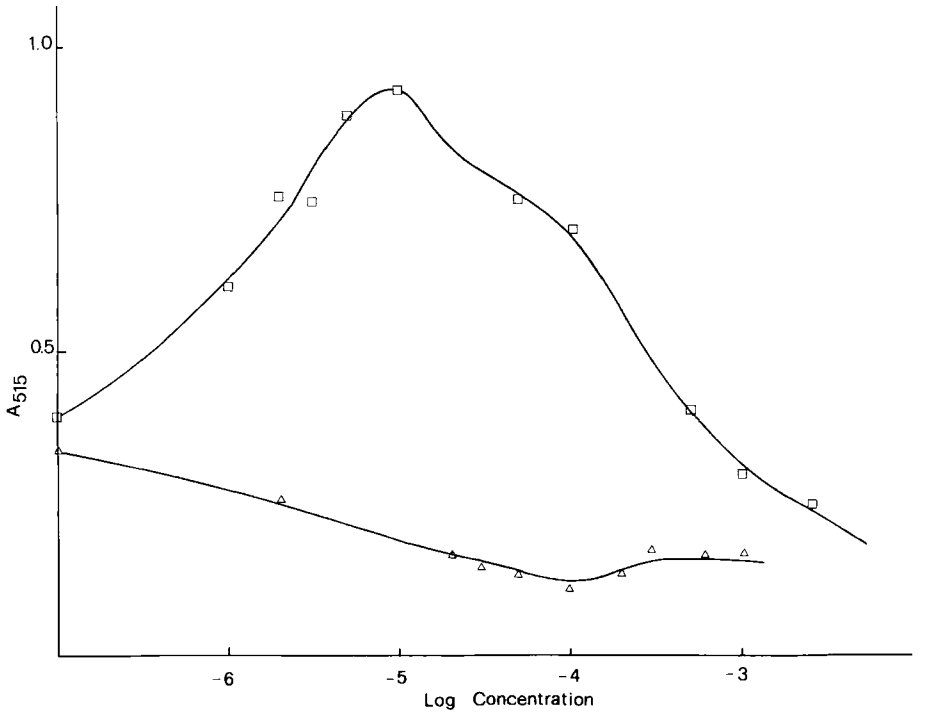
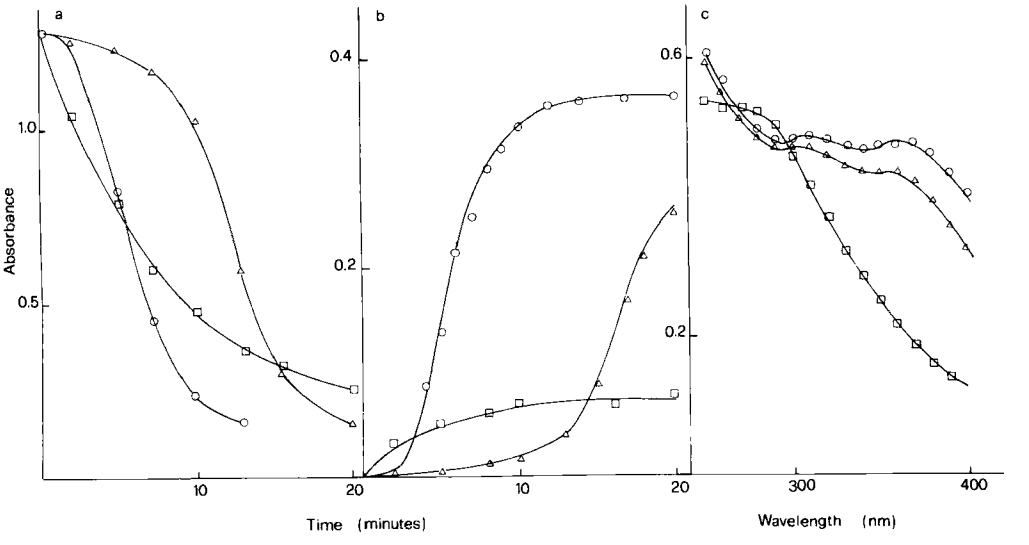


FIGURE 4 Effect of Na phosphate and Na pyrophosphate concentration on Fe^{2+} autoxidation in 5 mM Mops buffer pH 7.3. The disappearance of 150 μM Fe^{2+} from the standard solution in the presence of increasing concentrations of Na phosphate (□) and Na pyrophosphate (△) was determined after 5 min incubation by the o-phenanthroline method as described in the materials and methods section. The A_{515} of the sample at zero time was 1.240.



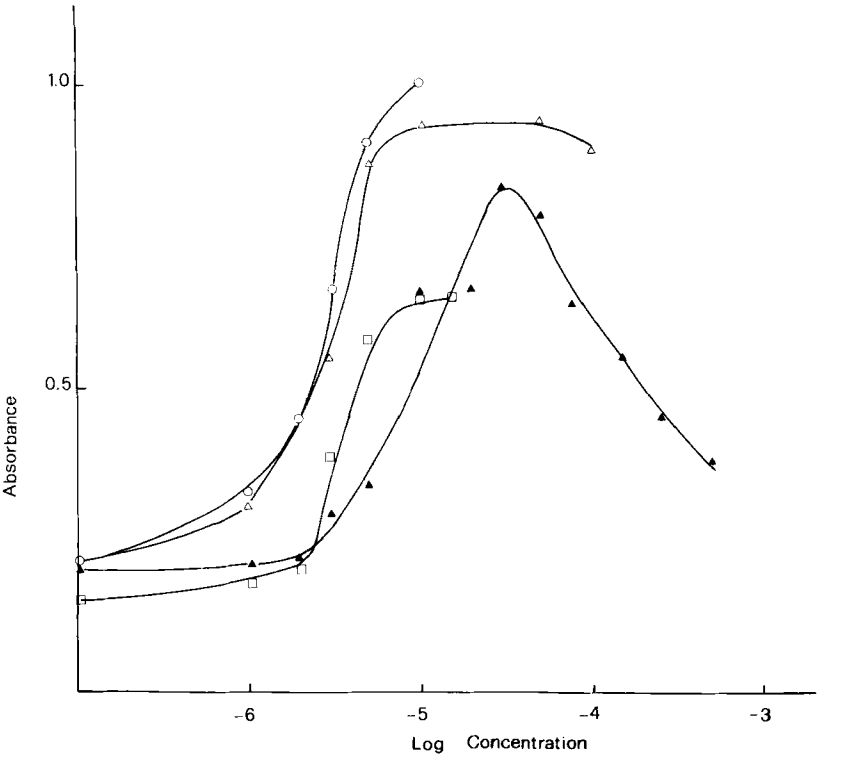


FIGURE 6 Effect of nucleotide and Na phosphate concentration on Fe^{2+} autoxidation accelerated by EDTA in 5 mM Mops buffer, pH 7.2. The disappearance of 150 μM Fe^{2+} from the standard solution, accelerated by 20 μM EDTA, in the presence of increasing concentrations of ATP (□), ADP (△), AMP (○) and Na phosphate (▲) was determined after 4 min incubation by the o-phenanthroline method as described in the materials and methods section. The A_{515} of the sample at zero time in the absence of EDTA was 1.360. The A_{515} of the sample containing 20 μM EDTA in water at the end of the incubation was 1.180.

uneffective. CDP-choline inhibits at a concentration much higher than the other nucleotides tested (Table I).

Competition between ATP and EDTA for Fe^{3+}

In Mops buffer pH 7.2 the Fe^{3+} EDTA complex has an absorbance spectrum with a maximum at 250–260 nm.[2] When the EDTA concentration is in excess, the absorbance at 260 nm is directly proportional to the concentration of Fe^{3+} in solution (Figure 9). In Figure 9 is also reported the absorbance spectrum of a sample where

FIGURE 5 Effect of Na phosphate on Fe^{2+} autoxidation in 5 mM Mops buffer pH 7.2. a) The time course of the disappearance of 150 μM Fe^{2+} in the absence (○) and presence of 15 μM (△) and 200 μM (□) Na phosphate was determined by the o-phenanthroline method. b) The time course of the development of yellow colour during the autoxidation of 150 μM Fe^{2+} in the absence (○) and presence of 15 μM (△) and 200 μM (□) Na phosphate was followed by measuring the absorbance at 400 nm. c) Absorbance spectra of the final product of Fe^{2+} autoxidation in the absence (○) and presence of 15 μM (△) and 200 μM (□) Na phosphate were determined after 40 min incubation.

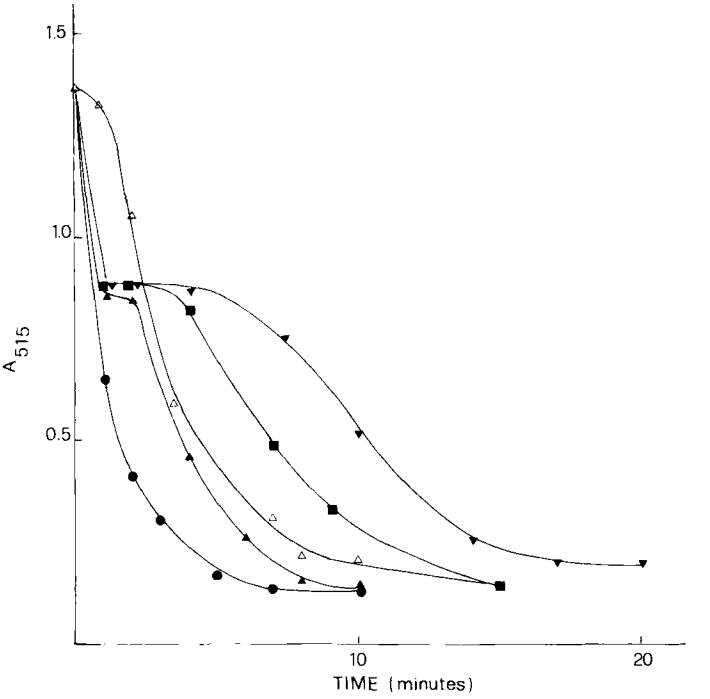


FIGURE 7 Effect of ADP on Fe^{2+} autoxidation accelerated by H_2O_2 in 5 mM Mops buffer pH 7.3. The disappearance of 150 μM Fe^{2+} from the standard solution (△) accelerated by 20 μM H_2O_2 in the absence (●) and presence of 5 μM (▲), 10 μM (■) and 15 μM (▼) ADP was determined by the o-phenanthroline method as described in the materials and methods section.

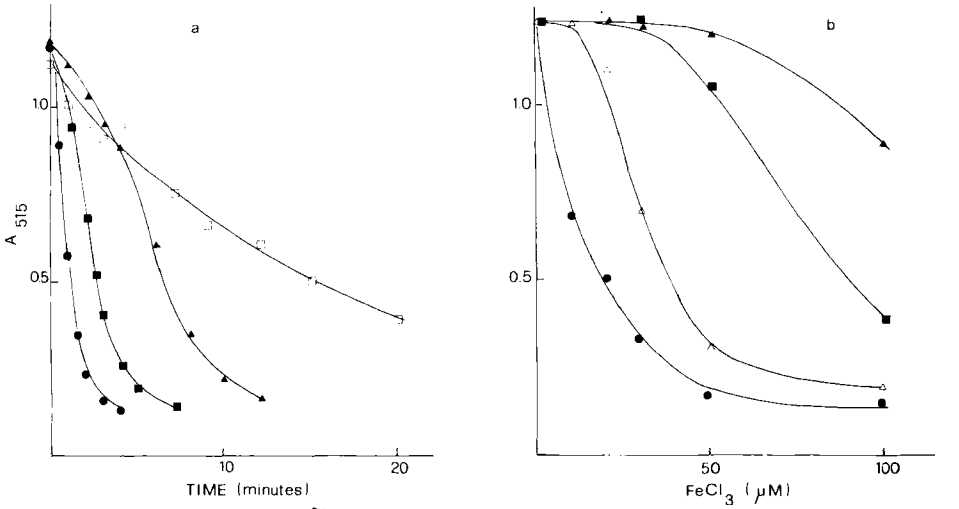


FIGURE 8 Effect of ADP on Fe^{2+} autoxidation accelerated by $FeCl_3$ in 5 mM Mops buffer pH 7.2. a) The time course of the disappearance of 150 μM Fe^{2+} from the standard solution accelerated by 50 μM Fe^{3+} in the absence (●) and presence of 2 μM (■), 5 μM (▲) and 15 μM (□) ADP was determined by the o-phenanthroline method. b) The disappearance of 150 μM Fe^{2+} from the standard solution accelerated by increasing concentrations of Fe^{3+} was determined after 4 min incubation in the absence (●) and presence of 2 μM (△), 5 μM (■) and 10 μM (▲) ADP by the o-phenanthroline method as described in the materials and methods section.

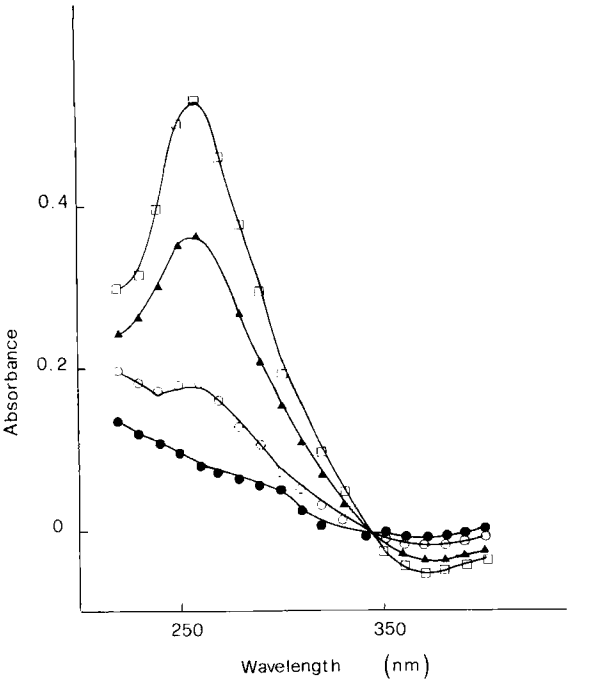


FIGURE 9 Effect of ATP on the absorbance spectrum of Fe^{3+} EDTA complex. Solutions containing 50 μM (○), 100 μM (▲), 150 μM (□) Fe^{3+} and 150 μM Fe^{3+}, 10 μM DP (●) were incubated for 10 min in 5 mM Mops buffer, pH 7.3. The samples were added with, 180 μM EDTA and after 20 min the absorbance spectrum of these solutions was read versus control samples without EDTA.

180 μM EDTA were added to 150 mM Fe^{3+} preincubated with 10 μM ATP. It is evident that in this experimental condition only a small amount of Fe^{3+} can react with EDTA to form Fe^{3+} EDTA complex.

DISCUSSION

Our results clearly demonstrate that phosphorus containing compounds greatly affect Fe^{2+} oxidation in Mops buffer. This is in agreement with the demonstration by Tien *et al.*[11] of only a slow oxygen consumption from ADP-Fe^{2+} complexes. Also the capacity of ferrous ion, in the presence of ADP, of generating $OH^{\cdot}$ from H_2O_2 for a considerable length of time agrees with an increased life time of Fe^{2+} in the presence of the nucleotide.[12] Also the di and triphosphate nucleotides were effective but the monophosphates were not. This discrepancy with out results may be due to the different buffering conditions. We have previously shown that Fe^{2+} autoxidation differs depending on the buffering conditions and that in particular Fe^{2+} autoxidation in Mops occurs through a pathway different from that in phosphate buffer. In the study reported, bicarbonate buffer was used and Fe^{2+} autoxidation in the presence of such anion is first order with respect to Fe^{2+} concentration[13] as in the presence of phosphate.[14]

The decreased Fe^{2+} oxidation observed in Mops buffer in the presence of nucleo-

tides does not appear to be due to the scavenging of $OH^{\cdot}$ by the purine[15] as adenine and adenosine are unaffective. Substitution of the adenine with the guanine nucleotide results in a better protection of Fe^{2+} from oxidation. Also the number of phosphates bound to the nucleoside influences the pattern of Fe^{2+} protection exerted by increasing concentration of the nucleotide. The complexity of the curves however cannot be interpreted by a simple explanation. The presence in the compound of a phosphodiester bound greatly reduces its capacity to inhibit Fe^{2+} oxidation: both cyclic nucleotides and glycerophosphoinositol do not protect Fe^{2+} from oxidation. The substituted nucleotide CDP-choline decreases Fe^{2+} autoxidation. The effect of the phosphorus containing compounds on Fe^{2+} autoxidation depends on their concentration: at low concentration they increase the lag time but at high concentration this phenomenon is less evident and some other process occurs. This is particularly evident when Na phosphate is studied. In the presence of high concentration of this anion, Fe^{2+} oxidation occurs through a different pathway[1] whose initial rate is faster compared to the situation in the presence of Mops buffer alone. Na pyrophosphate, on the other hand has no protective effect and accelerates Fe^{2+} oxidation also at low concentrations.

The influence exerted by phosphorus containing compounds on Fe^{2+} oxidation can be observed also when Fe^{2+} oxidation is conducted in the presence either of substoichiometric concentrations of EDTA, H_2O_2 or of $FeCl_3$ in Mops buffer. This result further demonstrates that the increased oxidation of the ferrous iron that occurs in the presence of these compounds in this buffer does not follow a different pathway but is an acceleration of the normal rate.[2]

The dependence of the amount of ADP required to inhibit Fe^{2+} oxidation on the concentration of Fe^{3+} added as catalyst suggests a possible molecular mechanism for the action of the nucleotide. The nucleotide may act as a ligand for Fe^{3+} and the ferric ion in this complex may lose its ability to accelerate Fe^{2+} oxidation. There is evidence that Fe^{3+} nucleotide complexes do exist also *in vivo*.[16-20]

Ferric ion forms a very stable complex with ATP and other similar nucleotide phosphates and the formation constants of Fe^{3+} ATP ($3.9 \times 10^6 M^{-1}$) and Fe^{3+} ADP ($4.6 \times 10^6 M^{-1}$) were determined.[21] ATP and ADP are able to inhibit Fe^{2+} oxidation accelerated by substoichimetric concentrations of EDTA. According to our hypothesis the nucleotide to accomplish its effect should be able either to substitute EDTA in the complex with Fe^{3+} or to form a ternary complex with the metal and the chelator. The formation constants of the Fe^{3+}-nucleotide complexes are much lower than that of Fe^{3+} EDTA ($\log Ka = 25$).[22] The difference of many orders of magnitude between these formation constants is against the hypothesis that the nucleotides may displace Fe^{3+} from EDTA. The formation constants of Fe^{3+} ATP and Fe^{3+} ADP, were however determined at pH 2 and in that assay condition the population of the nucleotides exists in solution as a species of one less negative charge (pKa of the secondary phosphates of ATP and ADP are 6.5 and 6.3 respectively).[23] Besides, EDTA and a metal in solution form different types of complexes [ML, MHL, MOHL, $M(OH)_2L$, $(MOHL)_2$] depending on the pH and this affects the formation of the ML complex (M metal, L ligand).[22] Whether and to what extent the formation of Fe^{3+} EDTA is affected in our experimental conditions we do not know. We have tried to evaluate the ability of ATP to interfere in the formation of the Fe^{3+} EDTA complex. When Fe^{3+} is incubated in the presence of ATP only a small amount of the metal is able to form the Fe^{3+} EDTA complex. The result obtained confirms that the nucleotide interacts with Fe^{3+} in the presence of EDTA, but it does not allow to distinguish

between the formation of Fe^{3+} ATP complex or of a ternary complex between ATP, Fe^{3+} and EDTA with spectrophotometric characteristics differing from that of the Fe^{3+} EDTA complex.

The results in their complex show that the rate of Fe^{2+} oxidation and thus the formation of active oxygen species may greatly vary depending on the presence of compounds normally present *in vivo*. In our opinion it is noteworthy that phospholipids, containing phosphorus in their polar heads, may have an influence on the oxidation of Fe^{2+}. It is well known that different results are obtained when lipid peroxidation is studied utilizing either fatty acids or phospholipids.[24,25] Our results might provide a new interpretation of those findings. Furthermore the different ability of AMP and CDP-choline and glycerophosphoinositol, that somehow resemble the polar head group of phosphatidic acid (PA), phosphatidylcholine (PC) and phosphatidylinositol (PI), to inhibit Fe^{2+} autoxidation suggests that the phospholipid composition of the membranes and liposomes under test may affect the results when lipoperoxidation is studied. We have in fact shown that the lipoperoxidation of PC, PA:PC (1:1 molar ratio) and PI:PC (1:1 molar ratio) vesicles differed in their dependence on Fe^{2+} concentration: PA containing vesicles required higher concentration of the metal.[26] This result could not be explained by also invoking the occurrence of a site specific reaction as it is more likely the binding of Fe^{2+} and thus the production of $OH^{\cdot}$ in the proximity of PA:PC and PI:PC instead of PC vesicles. In the light of the data presented the production of oxygen active species and the oxidative damage appears to be related not only to the ability of liposome to bind Fe^{2+} but also to its ability to interfere with Fe^{2+} oxidation.

Acknowledgements

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