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OXYGEN TOXICITY. THE INFLUENCE OF ADENINE-NUCLEOTIDES AND PHOSPHATE ON Fe^{2+} AUTOXIDATION

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$FeCl_2$ in Na phosphate buffer autoxidizes forming active oxygen species which damage deoxyribose. Di- and triphosphate adenine-nucleotides inhibit both Fe^{2+} autoxidation and deoxyribose damage in Na phosphate buffer pH 7.4. The inhibition is related to the number of charges of the adenine-nucleotide molecule: ATP at pH 7.4 is a better inhibitor than ADP; at a pH (6.5) close to the pK's of the third and fourth charge of ADP and ATP, ADP inhibition is greatly decreased whereas ATP inhibition is slightly affected. The extent of ATP inhibition of Fe^{2+} autoxidation depends both on ATP/Mg^{2+} and ATP/Fe^{2+} ratios in the reaction mixture. Formation of a Fe^{2+}-nucleotide complex appears to be the mechanism through which ATP and ADP inhibit autoxidation and thus the generation of active oxygen species. These findings are discussed in relation to physiological and pathological fluctuations of nucleotide concentrations.

KEY WORDS: Oxygen toxicity, iron, adenine-nucleotides, Na phosphate

The damaging effects of O_2 are attributed to the formation of intermediates of oxygen reduction i.e., superoxide ($\dot{O}_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^\cdot$).[1] $\dot{O}_2^-$ and H_2O_2 are produced in many enzymic reactions[2] and, in the presence of iron, yield $OH^\cdot$ [3] the highly reactive species that can attack and destroy almost all known biomolecules[4–6]

$$\dot{O}_2^- + Fe^{3+} \longrightarrow O_2 + Fe^{2+} \quad (1)$$

$$H_2O_2 + Fe^{2+} \longrightarrow OH^\cdot + OH^- + Fe^{3+} \quad (2)$$

Reduced iron, besides reacting with H_2O_2, can directly reduce molecular oxygen producing $\dot{O}_2^-$, H_2O_2, $OH^\cdot$ and finally water. Iron appears thus to play a major role in the generation of the damaging oxygen radical. Hydrogen peroxide, however, has a steady-state concentration in the cell of $1\ 10^{-8}M$[7] whereas the concentration of O_2 in most normal cells at pH 7 is about 10^4-fold higher than that of H_2O_2.[8,9] For this reason Fe^{2+} autoxidation in the cell might be a major source of damaging oxygen radicals and thus should be tightly controlled. Very little ferrous ion is present *in vivo* in the free ionic form[10,11] mostly being ligated to proteins or to various low molecular weight compounds.[3] Nucleotides and inorganic phosphate are regarded as likely iron chelators,[3] and are known to affect the redox chemistry of iron. In the presence of phosphate anion Fe^{2+} readily autoxidizes[12] producing $OH^\cdot$.[13,14] This important intra- and extracellular buffer would thus act as a prooxidant. On the contrary we have

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recently shown, in the presence of a buffering species (Mops) with low affinity for iron, that mono-, di- and triphosphate adenine-nicleotides inhibit Fe^{2+} autoxidation.[15] Floyd[16] reported that di- and triphosphate nucleotides can decrease Fe^{2+} autoxidation in bicarbonate buffer. Both results indicate nucleotides as antioxidants. It is conceivable that Fe^{2+} autoxidation and thus free radical generation, may be affected by metabolic fluctuations of the concentration of these ligands. Intracellular phosphate ion concentration is variable depending on cellular type and intracellular compartment: its concentration, however, is in the millimolar range. Adenine-nucleotide concentration, on the contrary, greatly varies in different physiological conditions.

To evaluate the role that Na phosphate and adenine-nucleotides have in the control of Fe^{2+} autoxidation I have studied whether adenine nucleotides exert an antioxidant effect on Fe^{2+} autoxidation in the presence of Na phosphate; the molecular mechanism of their action; the influence of $MgCl_2$ and of pH on nucleotide effect.

MATERIALS AND METHODS

Mops, ATP, ADP, AMP, 2-deoxy-D-ribose were obtained from Sigma Chemical Co. (St. Louis Mo. USA). Na_2HPO_4, NaH_2PO_4, 1,10-phenanthroline were obtained from Merck (Darmstadt, Germany). All other chemicals were the highest purity available.

Fe^{2+} determination

A solution of $FeCl_2$ (10 mM) was prepared in Chelex resin-treated distilled water. Fe^{2+} determination was made by the o-phenanthroline method according to Mahler and Elowe.[17] All incubations were carried out in 5 mM phosphate (Na_2HPO_4/NaH_2PO_4) buffer. The pH values of buffer were adjusted at room temperature. The 1 ml samples to be analyzed were prepared by the addition of adenine-nucleotide, followed eventually by $MgCl_2$, to the buffer. The reactions were started by addition of 150 μM $FeCl_2$ and incubated at room temperature. At the time stated, the reactions were stopped by addition of 0.2 ml of 25 mM 1,10-phenanthroline, and A_{515} was immediately read.

Damage to deoxyribose

Damage to deoxyribose was determined by the thiobarbituric acid-reactive material according to Tien *et al.*[18] The 1 ml samples to be analyzed were prepared by the successive addition to the buffer of the adenine-nucleotide and of 2.8 mM 2-deoxy-D-ribose. The reaction was started by addition of 150 μM $FeCl_2$. After 4 min incubation at room temperature, the reactions were stopped by addition of 10 μl of 1% (w/v) butylated hydroxytoluene (alcoholic solution). Thiobarbituric acid-reactivity was developed by heating for 15 min at 100°C after addition of 1.5 ml 20% (v/v) glacial acetic acid pH 3.5 and of 1.5 ml 0.8% (w/v) thiobarbituric acid in water. Resulting chromogens were measured at 532 nm against appropriate blanks.

RESULTS

Effect of nucleotides on Fe^{2+} autoxidation and deoxyribose degradation

The effect of increasing concentrations of adenine-nucleotides on Fe^{2+} autoxidation was studied in the presence of 5 mM Na phosphate buffer pH 7.4 (Figure 1). Whereas

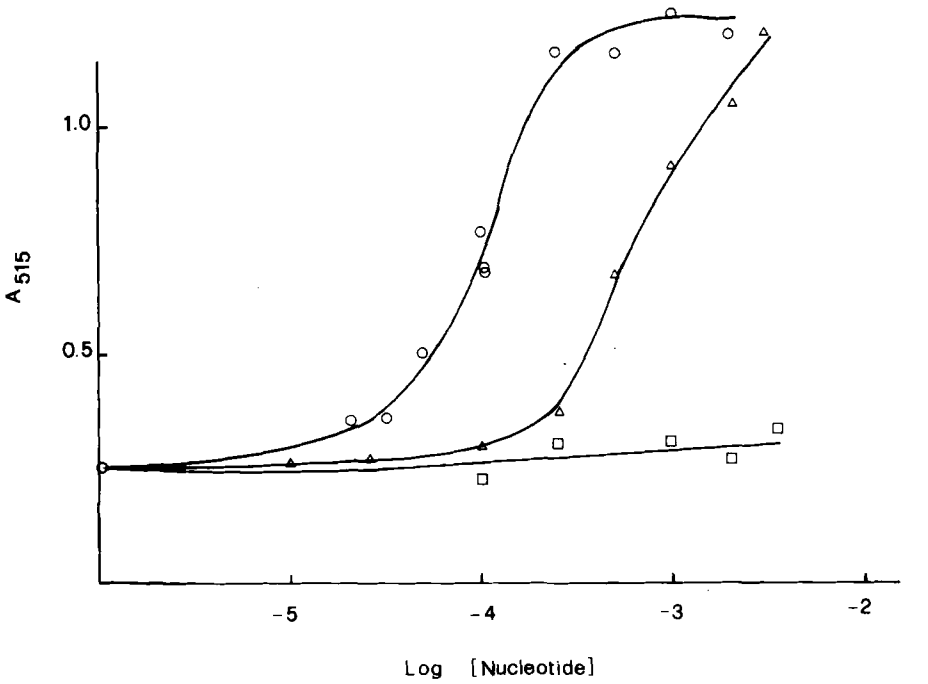

FIGURE 1 Effect of nucleotide concentration on Fe^{2+} autoxidation in Na phosphate buffer. The effect of AMP (□), ADP (△) and ATP (○) concentration on the disappearance of 150 μM $FeCl_2$ from the solution was measured in the presence of 5 mM Na phosphate buffer, pH 7.4 after 4 min incubation.

AMP, at all concentrations tested, does not affect Fe^{2+} autoxidation, ADP and ATP exert an inhibitory effect. The dependence of the inhibition on the nucleotide concentration is related to the type of nucleotide. The concentration of nucleotide causing half-maximal effect ($C_{0.5}$) is 0.1 mM and 0.65 mM for ATP and ADP respectively. These nucleotides are also able to inhibit the degradation of deoxyribose caused by OH° generated by Fe^{2+} autoxidation in Na phosphate buffer[14] (Figure 2). The pattern of inhibition of deoxyribose damage is similar to that of Fe^{2+} autoxidation: ATP being a better inhibitor ($C_{0.5}$ 0.2 mM) than ADP ($C_{0.5}$ 1 mM). AMP does not affect deoxyribose degradation.

Effect of $FeCl_3$ and $FeCl_2$ concentration on ATP inhibition of Fe^{2+} autoxidation

The inhibition of Fe^{2+} autoxidation in 5 mM Na phosphate buffer, pH 7.4 exerted by 150 μM ATP in not significantly decreased by $FeCl_3$ up to 150 μM (results not shown). The effect of ATP concentration on the rate of autoxidation of different concentrations of Fe^{2+} was then studied. From the results presented in Figure 3, it is evident that a direct correlation exists between the amount of Fe^{2+} present in the assay and the amount of ATP required to inhibit its autoxidation.

Effect of pH and $MgCl_2$ concentration on ATP and ADP inhibition of Fe^{2+} autoxidation

We have studied the inhibition of Fe^{2+} autoxidation by ATP and ADP in Na phosphate buffer, pH 6.5. As shown in Table 1, the lower pH only slightly affect ATP

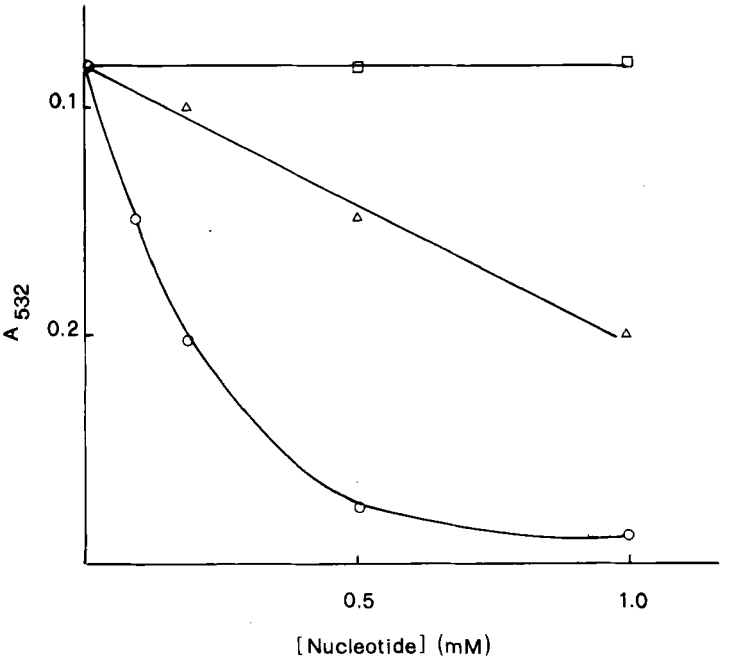

FIGURE 2 Effect of nucleotide concentration on deoxyribose damage caused by Fe^{2+} autoxidation in Na phosphate buffer. The effect of AMP (□), ADP (△) and ATP (○) concentration on the damage to deoxyribose, caused by the autoxidation of 150 μM $FeCl_2$ in 5 mM Na phosphate buffer, pH 7.4, was measured after 4 min incubation.

inhibition of Fe^{2+} autoxidation whereas ADP inhibition is decreased 50%. $MgCl_2$ decreases the inhibition of Fe^{2+} autoxidation exerted by ATP. This effect is most evident at the highest $MgCl_2$ concentration tested (5 mM) and at low ATP concentrations (Table 1).

TABLE I
Effect of pH and Mg^{2+} concentration on ADP and ATP inhibition of Fe^{2+} oxidation in 5 mM Na phosphate

	pH 6.5	pH 7.4		
			+ 2.5 mM Mg^{2+}	+ 5 mM Mg^{2+}
	ΔA_{515}	ΔA_{515}	ΔA_{515}	ΔA_{515}
Control	0.96	0.96	0.76	0.71
+ ADP 1 mM	0.64	0.31	–	–
+ ATP 0.1 mM	0.74	0.66	0.76	0.71
+ ATP 0.2 mM	–	0.21	0.63	0.66
+ ATP 0.5 mM	–	0.16	0.41	0.54
+ ATP 1 mM	0.16	0	0.04	0.24

The reaction was started by the addition of 150 μM Fe^{2+}. The change in Fe^{2+} concentrations was measured after 2 min incubation.

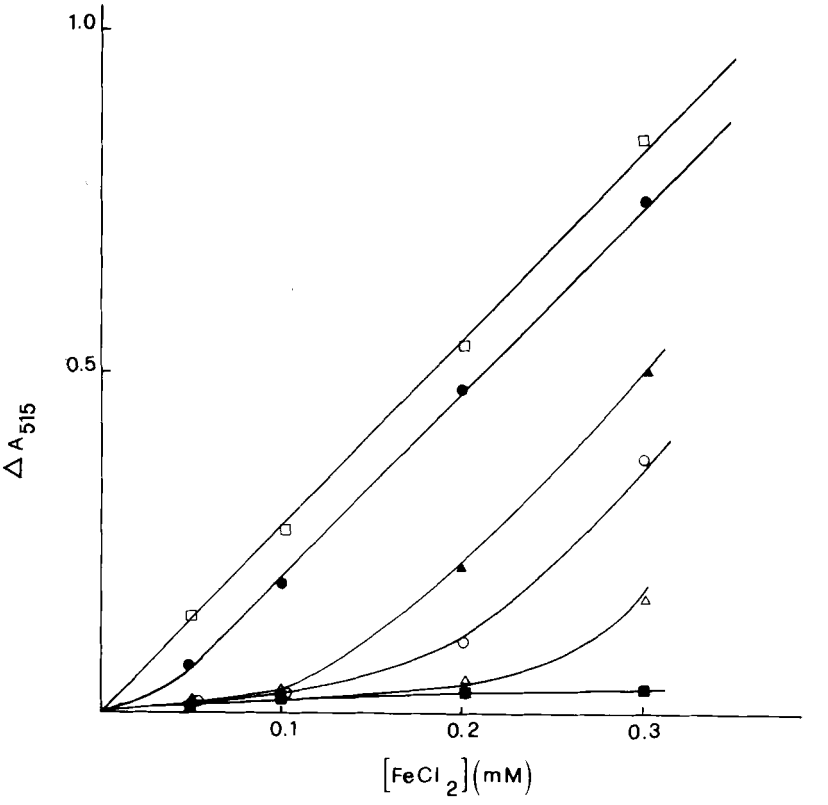

FIGURE 3 Effect of Fe^{2+} concentration on ATP inhibition of Fe^{2+} autoxidation in Na phosphate buffer. The autoxidation of increasing concentrations of Fe^{2+} in 5 mM Na phosphate buffer, pH 7.4 was measured in the absence (□) or presence of 10 μM (●), 50 μM (▲), 0.1 mM (○), 0.15 mM (△) and 0.5 mM (■) ATP. The change of Fe^{2+} concentration observed after 1 min incubation is reported (ΔA_{515}).

DISCUSSION

Fe^{2+} autoxidation in Na phosphate buffer results in the generation of $OH^{\cdot}$ and in the degradation of the detector molecule deoxyribose as already reported.[14] The adenine-nucleotides di- and triphosphate inhibit deoxyribose degradation. This effect cannot be ascribed to the $OH^{\cdot}$ scavenging ability of the purine moiety[19] as AMP is uneffective. The correlation between nucleotide inhibition of the generation of thiobarbituric acid-reactive material from deoxyribose and of Fe^{2+} autoxidation suggests the latter as the cause of the former phenomenon. In the presence both of a buffering species with low affinity for metals[15] and of a unbuffered solution pH 7,[20] adenine-nucleotides were shown to inhibit Fe^{2+} autoxidation. In those experimental conditions Fe^{2+} autoxidation was accelerated by Fe^{3+} [21] which acts as a catalyst. Nucleotides in the micromolar range were able to inhibit Fe^{3+} stimulated Fe^{2+} autoxidation and the active nucleotide concentration was directly proportional to the Fe^{3+} concentration in the assay.[15] We proposed that nucleotides may bind either the Fe^{3+} catalyst or a

Fe^{3+} related active species decreasing its reactivity and thus Fe^{2+} autoxidation. Fe^{2+} oxidation in phosphate buffer is known not to be affected by Fe^{3+} [12,21] and it is thus unlikely that nucleotides inhibit Fe^{2+} oxidation by interacting with an inactive substance. In fact nucleotide inhibition of Fe^{2+} autoxidation in phosphate buffer is not affected by Fe^{3+}. However, di- and triphosphate nucleotides may compete with Na phosphate for Fe^{2+} and the redox potential of the iron-nucleotide complex may be lower than that of the iron-phosphate complex. The results presented show a direct correlation between the amount of Fe^{2+} present in the assay and the amount of ATP required to inhibit its oxidation suggesting the formation of the complex Fe^{2+}-ATP as essential for the inhibition to occur.

The ability of di- and triphosphate adenine-nucleotides, compared to the monophosphate form, to interact with Fe^{2+} is apparently related to the number of charges of these ligands. The third and fourth charge of ADP and ATP have pK's of about 6.3 and 6.5 respectively.[22] To evaluate the importance of pH we have studied adenine-nucleotide inhibition of Fe^{2+} autoxidation in Na phosphate buffer, pH 6.5. The decrease of the negative charges decreases ADP but not ATP effectiveness, indicating that three charges are essential to ligate Fe^{2+} in an effective complex. Nucleotides are known to form complexes with bivalent metal ions, in particular Mg^{2+}.[23] This cation would thus be expected to compete with Fe^{2+} for ATP and to decrease inhibition of Fe^{2+} oxidation. This in fact occurs.

The results presented indicate that in the presence of phosphate at pH 7.4, an important buffer *in vivo*, and of physiological Mg^{2+} concentrations, ADP and ATP inhibit Fe^{2+} oxidation by molecular oxygen and thus oxygen free radical generation. These nucleotides may thus be part of the cellular mechanism of defence from oxygen toxicity preventing the generation of oxygen active species by Fe^{2+} autoxidation. Under physiological conditions nucleotide concentration would be sufficient to complex uncorpartimentalized iron whose concentration is extimated to be rather low.[24] However, a dramatic drop both in ATP and ADP concentrations, may increase free iron concentration. In fact, iron may be released from nucleotides upon hydrolysis and the new equilibrium among Fe^{2+}, Mg^{2+} and nucleotides would favour the formation of the nucleotide-Mg^{2+} complex. Intracellular acidosis may further increase free iron concentration. These events may occur during severe forced physical exercise and anoxia. Gutteride *et al.*[25] observed that a high concentration of bleomycin detectable iron is often present in the sweat of intensively-exercised athletes. It is known also that athletes can become anemic through prolonged and severe physical exercises[26,27] and that a significant decrease in iron concentration is observed in the heart from patients with "sudden" heart death.[28]

In the above conditions, oxidation of delocalized iron by phosphate anion may be accentuated and $OH^{\cdot}$ produced. This may cause the formation of organic radicals that lead to cellular damage.

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References

1. Slater, T.F., *Biochem.*, **222**, 1–15, (1984)
2. Bors, W., Saran, M. and Czapski, G. in "*Biological and clinical aspects of superoxide and SOD*" (Bannister W.H. & Bannister, J.V. eds.) pp. 1–31 Elselvier, Amsterdam (1980)
3. Halliwell, B. and Gutteridge, J.M.C. *Biochem. J.*, **219**, 1–14, (1984).
4. Fridovich, I. *Ann. Rev. Biochem.*, **44**, 147–159, (1975)
5. Fridovich, I. *Science*, **201**, 875–880, (1978).
6. Halliwell, B. in "*Age Pigments*" (Sohah R.S. ed.) pp. 1–62, Elselvier, Amsterdam (1981).
7. Boveris, A. and Change, B. *Biochem. J.*, **134**, 707–716, (1973).
8. Boveris, A. *Adv. Exp. Med. J. Biol.*, **78**, 67–70, (1977).
9. Boveris, A. and Cadenas, E. in "*Superoxide and superoxide dismutase*" Volume II, pp. 15–30 CRC (1982).
10. Gutteridge, J.M.C., Rowley, D.A. and Halliwell, B. *Biochem. J.* **199**, 263–265, (1981).
11. Gutteridge, J.M.C., Rowley, D.A. and Halliwell, B. *Biochem. J.*, **206**, 605–609, (1982).
12. Cher, M. and Davidson, N. *J. Am. Chem. Soc.*, **77**, 793–798, (1954).
13. Winston, G.W., Harvey, W., Berl, L. and Cederbaum, A.I. *Biochem. J.*, **216**, 415–421, (1983).
14. Gutteridge, J.M.C. *Biochem. J.*, **224**, 761–767, (1984).
15. Tadolini, B. and Sechi, A.M. *Free Radical Res. Commun.*, **4**, 161–172, (1987).
16. Floyd, R.A. *Arch. Biochem. Biophys.*, **225**, 263–270, (1983).
17. Mahler, H.R. and Elowe, D.G. *J. Biol. Chem.*, **210**, 165–179, (1954).
18. Tien, M., Svingen, B.A. and Aust, S.D. *Arch. Biochem. Biophys.*, **216**, 142–151, (1982).
19. Scholes, G., Ward, J.F. and Weiss, J. *J. Mol. Biol.*, **2**, 379–391, (1960).
20. Tien, M., Morehouse, L.A., Bucher, J.R. and Aust, S.D. *Arch. Biochem. Biophys.*, **218**, 450–458, (1982).
21. Tadolini, B. *Free Radical Res. Commun.*, **4**, 173–182, (1987).
22. Nakai, C. and Glinsmann, W. *Biochemistry*, **16**, 5636–5641, (1977).
23. Burton, K. *Biochem J...*, **71**, 388–395, (1959).
24. Young, S.P., Roberts, S. and Bomford, A. *Biochem. J.*, **232**, 819–823, (1985).
25. Gutteridge, J.M.C., Rowley, D.A., Halliwell, B., Cooper, D.F. and Heeley, D.M. *Clin. Chim. Acta*, **145**, 267–273, (1975).
26. Frederickson, L.A., Puhl, J.Z. and Runyan, W.S. *Med. Sci. Sports Exercise*, **15**, 271–276, (1983).
27. Paulev, P.E., Jordal, R. and Pedersen, N.S. *Clin. Chim. Acta*, **127**, 19–27, (1973).
28. Chipperfield, B. and Chipperfield, J.R. *Am. Heart J.*, **95**, 732–737, (1979).

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