

# Polyamine inhibition of lipoperoxidation

## The influence of polyamines on iron oxidation in the presence of compounds mimicking phospholipid polar heads

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Polyamines appear to inhibit peroxidation of vesicles containing acidic phospholipids. A correlation exists between polyamine binding to phospholipid vesicles and its protective effect. However, phosphatidylinositol-containing vesicles which bind spermine are not protected by the polyamine [Tadolini, Cabrini, Landi, Varani & Pasquali (1985) *Biogenic Amines* 3, 97–106]. In the present paper I tested the hypothesis that polyamines, in particular spermine, by forming a ternary complex with iron and the phospholipid polar head may change the susceptibility of  $\text{Fe}^{2+}$  to autoxidation and thus its ability to generate free oxygen radicals. Different compounds mimicking phospholipid polar heads were studied, namely AMP, mimicking phosphatidic acid, CDP-choline, mimicking phosphatidylcholine, and glycerophosphoinositol, mimicking phosphatidylinositol. The results support the proposed hypothesis. In the presence of CDP-choline or of glycerophosphoinositol, spermine poorly affects  $\text{Fe}^{2+}$  autoxidation, whereas a considerable inhibition is observed in the presence of AMP. The ability of other phosphorus-containing compounds (ATP, ADP, cyclic AMP, sodium phosphate) to affect  $\text{Fe}^{2+}$  autoxidation in the presence of polyamines was also evaluated to understand the molecular mechanism of this phenomenon. It is proposed that polyamines may be part of the passive cellular defence mechanism against the oxidative damage caused by  $\text{Fe}^{2+}$ .

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### INTRODUCTION

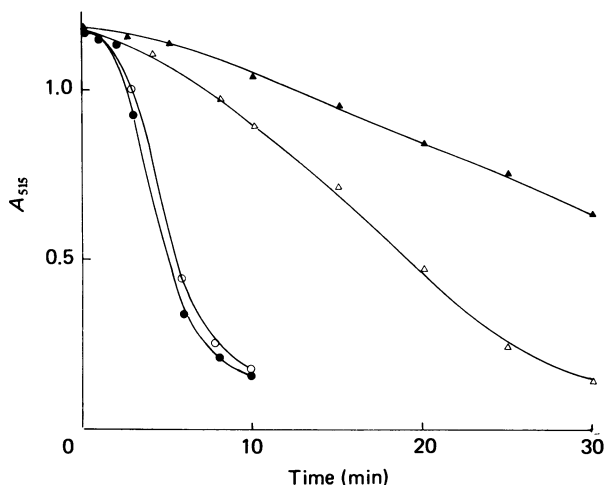
The aliphatic polyamines spermine and spermidine and their diamine precursor, putrescine, are non-protein nitrogenous bases widely distributed in prokaryotic and eukaryotic cells (Bachrach, 1973). They are known to interact with anionic cell constituents, among which are acidic phospholipids in biomembranes (Tabor, 1960a; Tadolini, 1980; Schuber *et al.*, 1983; Tadolini *et al.*, 1984, 1985a). The phospholipid–polyamine complex appears to exert a stabilizing effect on the membranes (Tabor, 1960a; Jellinck & Perry, 1967), possibly through the inhibition of lipid peroxidation (Tabor, 1960b; Kitada *et al.*, 1979, 1981; Tadolini *et al.*, 1984, 1985b). We have demonstrated that protection from lipid peroxidation occurs only when spermine is bound to phospholipid vesicles (Tadolini *et al.*, 1984, 1985b). In fact zwitterionic phospholipid vesicles, such as phosphatidylcholine vesicles, which do not bind spermine, are not protected by the polyamine. The effects of spermine on the peroxidation of vesicles containing different types and densities of acidic phospholipids are generally in agreement with the binding of spermine to the same vesicles (Tadolini *et al.*, 1985b). However, when vesicles containing phosphatidylinositol instead of phosphatidic acid, phosphatidylserine and cardiolipin are used, these are not protected from peroxidation, although spermine binds the vesicles.

We have investigated the possible molecular mechanism by which spermine may inhibit lipid peroxidation. Spermine may compete with iron and mobilize it from the anionic sites of phospholipids. Metal interaction with target molecules appears to be essential for oxidative damage to arise (Hodgson & Fridovich, 1975; Van Hemmen & Meuling, 1977; Samuni *et al.*, 1981). The experimental data, however, were only partially consistent with this hypothesis (Tadolini *et al.*, 1985b).

The hypothesis that spermine bound to phospholipid vesicles may be able to interact with iron and lower its reactivity was also proposed (Tadolini *et al.*, 1984). To verify the possible effect of polyamines bound to phospholipids on iron reactivity, we have studied whether and to what extent these amines affect  $\text{Fe}^{2+}$  autoxidation in Mops buffer in the presence of compounds mimicking phospholipid polar heads. The compounds studied were AMP, CDP-choline and glycerophosphoinositol, which mimic respectively phosphatidic acid, phosphatidylcholine and phosphatidylinositol. Other phosphorus-containing compounds were studied to understand better the molecular mechanism of the phenomenon.

### MATERIALS AND METHODS

Mops, ATP, ADP, AMP, cyclic AMP, CDP-choline, glycerophosphoinositol, EDTA, spermine tetrahydrochloride, spermidine trihydrochloride and putrescine dihydrochloride were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 1,10-Phenanthroline was obtained from Merck (Darmstadt, Germany). All procedures were carried out in plastic or acid-washed glassware, and solutions were prepared in Chelex-resin-treated water. The pH values of buffer were adjusted at room temperature. Stock solutions of  $\text{Fe}^{2+}$  were prepared daily.  $\text{Fe}^{2+}$  autoxidation was evaluated by determining the  $\text{Fe}^{2+}$  content by the *o*-phenanthroline method (Mahler & Elowe, 1954). All incubations were carried out in 5 mM-Mops buffer, pH 7.2, at room temperature. The 1 ml samples to be analysed were prepared by the successive addition to the buffer of the phosphorus-containing compound and of the polyamine. When the accelerated  $\text{Fe}^{2+}$  autoxidation was studied, 30  $\mu\text{M}$ - $\text{H}_2\text{O}_2$ , 30  $\mu\text{M}$ - $\text{FeCl}_3$  and 15  $\mu\text{M}$ -EDTA were first added to the



**Fig. 1.** Effect of spermine on the time course of  $\text{Fe}^{2+}$  autoxidation in 5 mM-Mops, pH 7.2, in the presence or absence of AMP

The disappearance of  $150 \mu\text{M-Fe}^{2+}$  from the standard solution (●) and in the presence of  $50 \mu\text{M-spermine}$  (○),  $10 \mu\text{M-AMP}$  (△) or  $50 \mu\text{M-spermine} + 10 \mu\text{M-AMP}$  (■) was measured.

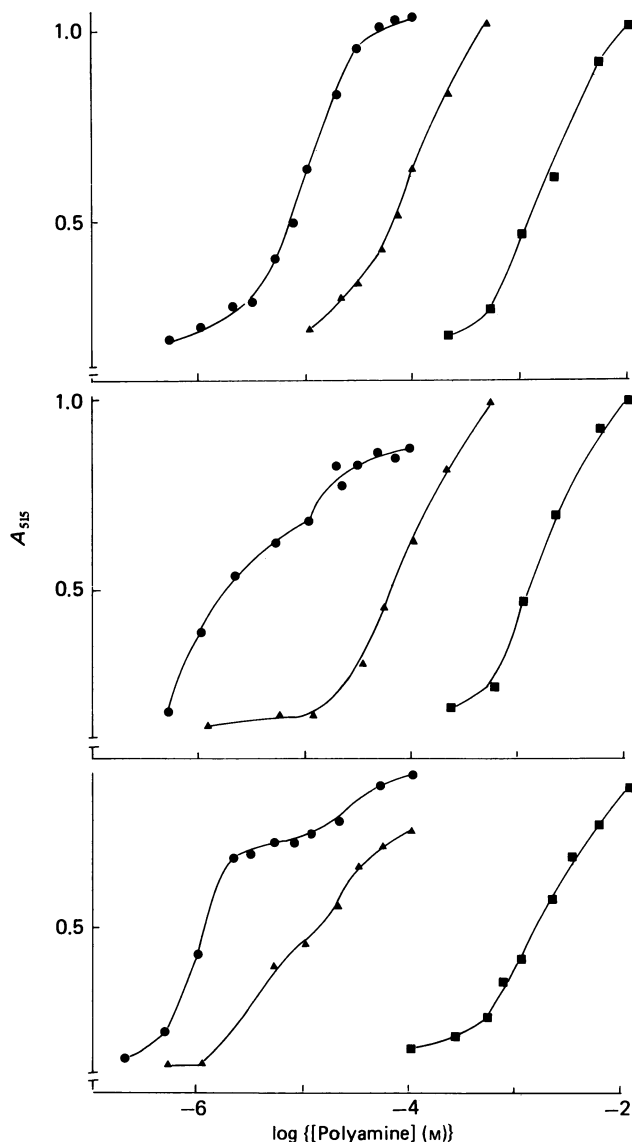
buffer. The reactions were started by addition of  $150 \mu\text{M-FeCl}_2$ . At the time stated, the reactions were stopped by addition of 0.2 ml of 25 mM-1,10-phenanthroline, and  $A_{515}$  was immediately read.

## RESULTS

### Effect of polyamines on $\text{Fe}^{2+}$ autoxidation in the presence of AMP, CDP-choline and glycerophosphoinositol

When the rate of  $\text{Fe}^{2+}$  oxidation in Mops buffer is measured in the presence of  $50 \mu\text{M-spermine}$ , this polycation only slightly affects the reaction. By contrast, spermine greatly decreases the rate of  $\text{Fe}^{2+}$  oxidation when the reaction is conducted in the presence of  $10 \mu\text{M-AMP}$  (Fig. 1). I studied the effect of the concentrations of spermine, spermidine and putrescine on  $\text{Fe}^{2+}$  oxidation in the presence of an AMP concentration ( $10 \mu\text{M}$ ) that is itself effective, but at an incubation time when its inhibition is over (25 min) (Fig. 2a). These amines are able to inhibit  $\text{Fe}^{2+}$  autoxidation almost completely, and the concentration causing half-maximal effect ( $C_{0.5}$ ) is  $9 \mu\text{M}$ ,  $90 \mu\text{M}$  and  $1.6 \text{ mM}$  for spermine, spermidine, and putrescine respectively. Also, CDP-choline was shown to affect  $\text{Fe}^{2+}$  oxidation in Mops buffer, although higher concentrations were required to obtain a smaller effect (Tadolini & Sechi, 1987). I studied the effect of spermine in the presence of this compound. The inhibition exerted by  $0.4 \text{ mM-CDP-choline}$  is over in 25 min, whereas the small inhibition exerted by  $40 \mu\text{M}$  of this nucleotide derivative lasts only 15 min. In both these conditions the spermine effect, also at the highest concentration tested ( $0.1 \text{ mM}$ ), is limited (Fig. 3).

Also, in the presence of glycerophosphoinositol ( $50 \mu\text{M}$ ), which does not itself significantly affect  $\text{Fe}^{2+}$  oxidation, the spermine effect is limited and high concentrations of this amine are required ( $C_{0.5} > 0.1 \text{ mM}$ ) (Fig. 3).



**Fig. 2.** Effect of polyamine concentration on  $\text{Fe}^{2+}$  autoxidation in 5 mM-Mops, pH 7.2, in the presence of AMP (a), ADP (b) or ATP (c)

The disappearance of  $150 \mu\text{M-Fe}^{2+}$  from the standard solution containing  $10 \mu\text{M}$  nucleotide and increasing concentrations of spermine (●), spermidine (▲) and putrescine (■) was measured after 25 min incubation.

### Effect of polyamines on $\text{Fe}^{2+}$ autoxidation in the presence of other adenine nucleotides and sodium phosphate

In order to clarify the molecular mechanism of the synergic effect of polyamines and AMP on the inhibition of  $\text{Fe}^{2+}$  oxidation, I studied the polyamine effect in the presence of other adenine nucleotides, namely ADP, ATP and cyclic AMP. Both ADP and ATP inhibit  $\text{Fe}^{2+}$  oxidation, whereas cyclic AMP does not significantly affect it (Tadolini & Sechi, 1987). As for AMP, the polyamine effect was studied at an incubation time when the eventual nucleotide effect was over (25 min for ADP and ATP, 15 min for cyclic AMP). The  $C_{0.5}$  values of spermine, spermidine and putrescine were respectively

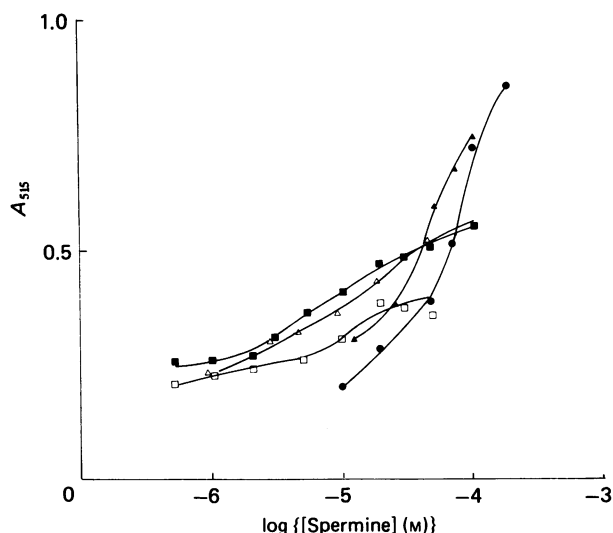


Fig. 3. Effect of spermine concentration on Fe<sup>2+</sup> autoxidation in 5 mM-Mops, pH 7.2, in the presence of CDP-choline, glycerophosphoinositol, cyclic AMP and sodium phosphate

The disappearance of 150  $\mu\text{M}$ -Fe<sup>2+</sup> from the solutions was measured. The samples containing the different compounds under test were incubated for different times: CDP-choline, 0.4 mM (■), 25 min; CDP-choline, 40  $\mu\text{M}$  (□), 15 min; glycerophosphoinositol, 50  $\mu\text{M}$  (△), 15 min; cyclic AMP, 50  $\mu\text{M}$  (●), 15 min; sodium phosphate, 20  $\mu\text{M}$  (▲), 25 min.

2  $\mu\text{M}$ , 90  $\mu\text{M}$  and 1.4 mM in the presence of 10  $\mu\text{M}$ -ADP (Fig. 2b) and 1.3  $\mu\text{M}$ , 6.5  $\mu\text{M}$  and 1.1 mM in the presence of 10  $\mu\text{M}$ -ATP (Fig. 2c). When 50  $\mu\text{M}$ -cyclic AMP was used, inhibition of Fe<sup>2+</sup> oxidation was observed in the presence of spermine, although high concentrations of this amine were required ( $C_{0.5}$  90  $\mu\text{M}$ ) (Fig. 3). High concentrations of spermine ( $C_{0.5}$  60  $\mu\text{M}$ ) were also able to decrease Fe<sup>2+</sup> oxidation in the presence of 20  $\mu\text{M}$ -sodium phosphate (Fig. 3), a compound itself able to inhibit Fe<sup>2+</sup> oxidation at low concentrations (Tadolini & Sechi, 1987).

#### Effect of spermine on Fe<sup>2+</sup> oxidation accelerated by H<sub>2</sub>O<sub>2</sub>, FeCl<sub>3</sub> and EDTA in the presence of ATP

Spermine (10  $\mu\text{M}$ ) (Table 1) is able to increase the inhibition exerted by 15  $\mu\text{M}$ -ATP on Fe<sup>2+</sup> oxidation accelerated by either 30  $\mu\text{M}$ -H<sub>2</sub>O<sub>2</sub> or 30  $\mu\text{M}$ -FeCl<sub>3</sub>. When Fe<sup>2+</sup> oxidation is accelerated by a sub-stoichiometric concentration of EDTA (15  $\mu\text{M}$ ), both ATP (10  $\mu\text{M}$ ) and ATP plus spermine (10  $\mu\text{M}$ ) decrease the oxidation, although to a lesser extent.

## DISCUSSION

Direct reaction of molecular oxygen with most organic compounds is spin-forbidden. In order for oxidation of these compounds to occur, dioxygen must be activated. The most important mechanism of dioxygen activation involves its complexation and/or reduction by a transition metal. In the process of transition-metal autoxidation, partially reduced forms of oxygen are generated, which are capable of oxidizing biomolecules leading to cellular alterations and ultimately to tissue damage (for a review, see Slater, 1984). Transition metals

Table 1. Effect of spermine in Fe<sup>2+</sup> oxidation accelerated by H<sub>2</sub>O<sub>2</sub>, FeCl<sub>3</sub> and EDTA in the presence of ATP

The disappearance of 150  $\mu\text{M}$ -Fe<sup>2+</sup> from the solution was measured after 5 min incubation by the *o*-phenanthroline method.

Addition	$\Delta A_{515}$
Control	0.750
+ H <sub>2</sub> O <sub>2</sub> (30 $\mu\text{M}$ )	1.247
+ H <sub>2</sub> O <sub>2</sub> (30 $\mu\text{M}$ ) + ATP (15 $\mu\text{M}$ )	0.580
+ H <sub>2</sub> O <sub>2</sub> (30 $\mu\text{M}$ ) + ATP (15 $\mu\text{M}$ ) + spermine (10 $\mu\text{M}$ )	0.380
+ FeCl <sub>3</sub> (30 $\mu\text{M}$ )	1.250
+ FeCl <sub>3</sub> (30 $\mu\text{M}$ ) + ATP (15 $\mu\text{M}$ )	0.252
+ FeCl <sub>3</sub> (30 $\mu\text{M}$ ) + ATP (15 $\mu\text{M}$ ) + spermine (10 $\mu\text{M}$ )	0.105
+ EDTA (15 $\mu\text{M}$ )	1.250
+ EDTA (15 $\mu\text{M}$ ) + ATP (10 $\mu\text{M}$ )	0.762
+ EDTA (15 $\mu\text{M}$ ) + ATP (10 $\mu\text{M}$ ) + spermine (10 $\mu\text{M}$ )	0.431

thus appear to have an integral role in the generation of these reactive 'oxy radicals', and the chemistry of the reaction of dioxygen with transition metals, principally iron, has been investigated for many years. The mechanism by which autoxidation of Fe<sup>2+</sup> and generation of oxy radicals occurs, however, is still not certain (for reviews, see Halliwell & Gutteridge, 1984; Aust *et al.*, 1985). The mechanism of autoxidation of transition-metal ions is in fact dependent on the presence of anions and chelators. The rate of Fe<sup>2+</sup> autoxidation may be modified by molecules present in the cellular environment which might be able to influence the generation of the highly reactive oxygen species that initiate oxidative damage.

From the data presented here, it is evident that spermine is capable of affecting Fe<sup>2+</sup> autoxidation, but only in the presence of phosphorus-containing compounds. The  $C_{0.5}$  of spermine in the presence of different phosphorus-containing compounds, however, differs greatly. In fact, the  $C_{0.5}$  in the presence of 10  $\mu\text{M}$ -AMP is 9  $\mu\text{M}$ , compared with > 0.1 mM when 0.4 mM-CDP-choline or 50  $\mu\text{M}$ -glycerophosphoinositol is present. These compounds were chosen to mimic phosphatidic acid, phosphatidylcholine and phosphatidylinositol polar heads respectively. The results obtained show that there is a strong correlation between spermine inhibition of Fe<sup>2+</sup> oxidation in the presence of the compound mimicking the phospholipid polar head and spermine inhibition of peroxidation of vesicles containing phospholipid with such a polar head (Tadolini *et al.*, 1985b). These results suggest that spermine, in the presence of phospholipid polar heads, may affect Fe<sup>2+</sup> oxidation and thus generation of oxy radicals. The studies conducted with other adenine nucleotides, to clarify the mechanism of the polyamine effect, show that the  $C_{0.5}$  depends on the charges and the structural features of nucleotides and also on the charges of polyamine molecules. Nakai & Glinsmann (1977) showed that polyamines bind ATP, ADP and AMP and that the interaction depends on the same parameters. Thus the inhibition by polyamine of Fe<sup>2+</sup> oxidation appears to be due to the formation of a complex between the polyamine and the phosphorus-containing compound. We have previously proposed that the effect of phosphorus-containing compounds on Fe<sup>2+</sup> oxidation in

Mops buffer may be due to the formation of a complex with iron (Tadolini & Sechi, 1987). Spermine interaction with the nucleotide, however, apparently does not displace iron from the phosphorus-containing compound. In this case iron would become free, the inhibition of its oxidation exerted by the phosphorus-containing compound would cease, and  $\text{Fe}^{2+}$  would be readily oxidized.

The data obtained suggest that the polyamine, by contrast, participates in the formation of the complex. The polyamine may form, for example with AMP and iron, a ternary complex more efficient than the binary complex AMP-iron in decreasing  $\text{Fe}^{2+}$  oxidation. It may be also able to transform an inefficient binary complex (for example cyclic AMP-iron) into an efficient ternary complex (cyclic AMP-iron-spermine). The concentrations of spermine ( $C_{0.5}$  1–10  $\mu\text{M}$ ) and effective nucleotide (10  $\mu\text{M}$ ) that are able to decrease the oxidation of 150  $\mu\text{M}$ - $\text{Fe}^{2+}$  lead one to wonder about the nature of the iron taking part in the formation of the complex.

$\text{Fe}^{2+}$  autoxidation in Mops buffer is characterized by a lag phase (Lambeth *et al.*, 1982; Tadolini, 1987a) that is decreased by  $\text{Fe}^{3+}$  addition (Tadolini, 1987b). A rate-limiting reaction producing a  $\text{Fe}^{3+}$  catalyst thus appears to occur. Phosphorus-containing compounds are able to decrease  $\text{Fe}^{2+}$  oxidation, and their  $C_{0.5}$  is related to the concentration of  $\text{Fe}^{3+}$  in the assay (Tadolini & Sechi, 1987). We proposed that phosphorus-containing compounds might decrease  $\text{Fe}^{2+}$  oxidation by complexing  $\text{Fe}^{3+}$  and thus decreasing its reactivity as a catalyst. The demonstration that spermine increases the inhibition by ATP of  $\text{Fe}^{2+}$  oxidation accelerated by  $\text{Fe}^{3+}$ , either added or generated by  $\text{Fe}^{2+}$  oxidation by  $\text{H}_2\text{O}_2$  and EDTA, indicates  $\text{Fe}^{3+}$  as the iron form participating in the formation of the proposed ternary complex. In this complex,  $\text{Fe}^{3+}$  would greatly decrease its catalytic activity and  $\text{Fe}^{2+}$  oxidation would be inhibited. This hypothesis is also supported by the time course of the inhibition of  $\text{Fe}^{2+}$  oxidation by AMP, either alone or complexed with spermine. An increased lag phase is observed, corresponding to the binding to the inhibitor of  $\text{Fe}^{3+}$  generated by the rate-limiting step. This is followed by an increased rate of  $\text{Fe}^{2+}$  oxidation, corresponding to the production of a concentration of the  $\text{Fe}^{3+}$  catalyst exceeding the inhibitor concentration. The hypothesis is further supported by the observation that higher concentrations of AMP, either alone or complexed with spermine, proportionally increase the lag phase (results not shown).

In summary, the results presented in this paper are consistent with the hypothesis that the inhibition of lipoperoxidation by polyamine is due to its ability to form a ternary complex with the phospholipid polar head and the  $\text{Fe}^{3+}$  catalyst of  $\text{Fe}^{2+}$  oxidation. The decreased  $\text{Fe}^{2+}$  oxidation would result in a decreased generation of oxygen free radicals. The lack of spermine inhibition of lipoperoxidation of certain vesicles types may be due either to a low affinity of the polyamine for the polar head of such phospholipids or to a low ability of the polyamine-acidic-phospholipid complex to bind and/or affect the  $\text{Fe}^{3+}$  catalyst. The lack of inhibition by spermine of the peroxidation of phosphatidylcholine-containing vesicles appears to belong to the former case, as these vesicles were shown not to interact significantly with spermine (Schuber *et al.*, 1983; Tadolini *et al.*,

1985a). On the contrary, phosphatidylinositol-containing vesicles bind spermine (Tadolini *et al.*, 1985a), but this complex does not interfere with  $\text{Fe}^{2+}$ -dependent peroxidation. The result obtained by studying the glycerophosphoinositol effect on  $\text{Fe}^{2+}$  autoxidation (Tadolini & Sechi, 1987) indicates that, in this case, it is the interaction between the phospholipid polar head and iron that does not appear to be suitable in affecting the  $\text{Fe}^{3+}$  catalyst.

The results presented, besides suggesting a possible molecular mechanism for the protection by polyamines of lipid peroxidation, proposed polyamines as part of the passive cellular defence mechanism against the oxidative damage caused by  $\text{Fe}^{2+}$ . When bound to suitable phosphorus-containing compounds, they may decrease  $\text{Fe}^{2+}$  oxidation and thus oxygen free-radical production.

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