

Oxidation of Ascorbic Acid and Metal Ions As Affected by NaCl[†]

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Ascorbic acid is one of the biological reductants that reduce ferric or cupric ions to ferrous or cuprous forms, inducing in the presence of oxygen a "metal redox cycle" which initiates a cascade of reactions producing reactive oxygen species. NaCl inhibits ascorbic acid oxidation and the reactions that produce $O_2^{\cdot-}$, H_2O_2 , and HO^{\cdot} by preventing the interaction of the transition metal (Fe, Cu) with oxygen. If the transition metal is liganded by a weak ligand such as acetate or ADP, NaCl prevents the interaction between oxygen and the transition metal. However, if the metal ion is chelated by a strong ligand such as citrate or EDTA, the effect of NaCl on ferrous oxidation is weak or nonexistent. NaCl does not inhibit the interaction of the metal ions (Fe, Cu) with H_2O_2 and the generation of hydroxyl radicals from preformed H_2O_2 .

Keywords: Oxidation; ascorbic acid; metal ions; NaCl

INTRODUCTION

Sodium and chloride ions are present in all living cells (Mathews and van Holde, 1990). Researchers include NaCl in buffers or solutions to simulate its concentration in blood plasma and to provide an "inert environment" for reactions (Minotti and Aust, 1992).

The chloride anion, a weak nucleophile, was found to enhance hemoglobin autoxidation (Wallace et al., 1974). The chloride anion in the presence of H_2O_2 is a substrate for chloroperoxidase and myeloperoxidase, producing hypochlorite, as a part of plant and animal defense mechanisms against microorganisms (Morrison and Schonbaum, 1976).

Sodium chloride, an important additive in the food industry, has been reported to act as a pro-oxidant of muscle lipid peroxidation (Lea, 1937; Chang and Watts, 1950; Tappel, 1952; Banks, 1961; Ellis et al., 1968; Powers and Mast, 1980; Kanner and Kinsella, 1983; Kanner et al., 1991) or as an inhibitor of this reaction (Chang and Watts, 1950; Mabrouk and Dugan, 1960).

Recently, the pro-oxidative effect of NaCl was studied in model systems, using an extract of turkey muscle tissue (Kanner et al., 1991) or fish muscle (Osinchak et al., 1992). Earlier publications (Mapson, 1941, 1945) showed that NaCl prevents ascorbic acid oxidation by cupric ions, especially at a low pH.

The purpose of this study was to elucidate the effect of NaCl on autoxidation of ferrous and cuprous ions in the presence of ascorbic acid and several iron chelators.

MATERIALS AND METHODS

Materials. Ascorbic acid, sodium chloride, potassium chloride, and sodium citrate were purchased from Merck (Darmstadt, Germany); ferrous sulfate, Ferrozine, scopoletin, and horseradish peroxidase (HRP) from Sigma Chemical Co. (St. Louis, MO); and ethylenediaminetetraacetic acid disodium salt (EDTA) and sodium benzoate from BDH Chemicals Ltd. (Poole, England). Adenosine 5'-diphosphate disodium salt (ADP) was obtained from Boehringer (Mannheim, Germany) and ferric sulfate from Riedel-de Haen (Seelze, Holland). All chemicals used were of AR quality and met ACS requirements.

Methods. Hydroxyl radical generation in the model systems was determined by benzoate hydroxylation, using a method developed by Baker and Gebicki (1984). The addition of HO^{\cdot} radicals to benzoate, at pH 7.3, produces 2-monohydroxy benzoate, which was separated by HPLC from an RP-18 column by 0.05 M acetate buffer, pH 4.5, and acetonitrile (8:2 v/v) and detected fluorometrically using excitation at 300 nm and emission at 410 nm. The amount of monohydroxy benzoate produced was calculated from a salicylate standard curve.

Chelatable iron ions were assayed by using ferric chloride for standard calibration. Iron ions were determined by a method developed by Stookey (1970) using Ferrozine reagent. Ferrozine forms with ferrous ions a complex with a high extinction coefficient of $E_{562} = 2.79 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$.

Ascorbic acid oxidation by ferric ions, with or without chelators, was monitored by its absorbance at 265 nm.

Oxygen absorption in a reaction system containing 0.05 M acetate, pH 7, was determined using an oxygen monitor (Yellow Springs Instrument Co., Model 53) with a Clark electrode. The aqueous solutions of ferrous or cuprous ions were prepared by passing nitrogen free of oxygen through the distilled water and through the solutions. Anaerobic oxidation was done by passing a continuous stream of nitrogen through the solutions. The solubility of oxygen in distilled water at 25 °C and 1 atm is 8.26 mg/L, while in a 0.5 M NaCl solution it is 6.75 mg/L. A linear interpolation was used for calibration values. The solubility of oxygen in each sample was taken into account during calculations.

Generation of H_2O_2 was determined by the HRP-dependent reaction of H_2O_2 with the fluorescent dye scopoletin and detected by fluorescence measurements (excitation 350 nm, emission 460 nm) (Loschen and Flohe, 1971). A decrease in fluorescence intensity demonstrates oxidation of scopoletin by H_2O_2 -activated HRP.

During the reactions and analysis the pH was taken into account and corrected.

RESULTS AND DISCUSSION

Ascorbic acid is one of the biological reductants that may reduce ferric or cupric ions to ferrous or cuprous forms and by this induce in the presence of molecular oxygen a "metal redox cycle" which initiates a cascade of reactions producing superoxide anion radical ($O_2^{\cdot-}$), H_2O_2 , and hydroxyl radicals (HO^{\cdot}) (Barber, 1966; Scarpa et al., 1983; Kanner et al., 1986, 1987; Miller and Aust, 1989; Miller et al., 1990; Minotti and Aust, 1992). This cascade of reactions, which in the end produces the

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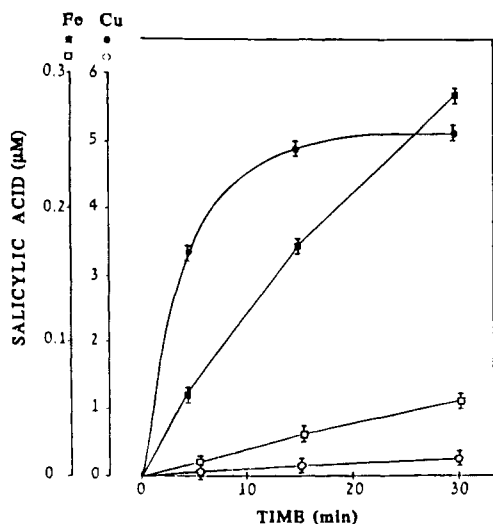
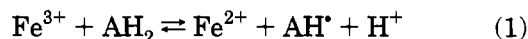


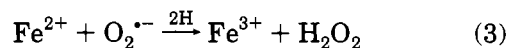
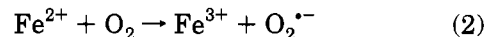
Figure 1. Effect of NaCl on the generation of hydroxyl radicals by ascorbic acid and metal ions, detected by benzoate hydroxylation: (■) 13 μM FeCl_3 ; (●) 5 μM CuSO_4 ; (□) 13 μM FeCl_3 + 0.5 M NaCl; (○) 5 μM CuSO_4 + 0.5 M NaCl. The reaction was performed in 0.05 M acetate, pH 7, at 37 °C with 200 μM ascorbic acid.

hydroxyl radical, known to be the biologically most oxidizing compound, was found to be inhibited by NaCl (Figure 1). Oxidation of ascorbic acid estimated by oxygen absorption (Figure 2a) or spectrophotometrically at 265 nm (Figure 2b) (Buettner, 1986, 1988; Aronovitch et al., 1987) was inhibited by NaCl. To understand this inhibition, several of the reactions that participate in the oxygen metal redox cycle were determined.

The first reaction in this system is the chelation of iron by ascorbic acid and the reduction of the ferric to ferrous ion reaction (eq 1), which was estimated by the Ferrozine method (Stokey, 1970). This reaction was affected only partially by NaCl (~10%) at pH 7.0 (results not shown).

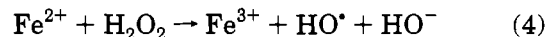


Ferrous ions interact with oxygen, producing superoxide and ferric ions (Kanner et al., 1986). Superoxide could further interact with ferrous ion, producing H_2O_2 (Cohen and Sinet, 1980; Aust et al., 1990; Miller et al., 1990) by the following reactions:



The reactants and products of these proposed reactions were determined by (a) oxygen absorption (Figures 3 and 4), (b) accumulation of H_2O_2 (Figure 5), and (c) oxidation of ferrous ion (Figure 6). The methods used to estimate reactions 2 and 3 showed that NaCl inhibits these reactions, most probably by preventing the interaction of the transition metal with oxygen. If the transition metal is liganded by a weak ligand such as acetate or ADP, NaCl prevents the interaction between oxygen and the transition metal (Figures 3 and 4). However, if the metal ion is chelated by a strong ligand such as citrate or EDTA, the effect of NaCl on ferrous oxidation is weak or nonexistent.

It was also assumed that NaCl could affect the reaction of ferrous ion with H_2O_2 .



This reaction was demonstrated by measuring the decrease of ferrous ion and hydroxylation of benzoate to hydroxylated compounds. The results demonstrate no effects of NaCl on these reactions (Figure 7) (results of benzoate hydroxylation not shown).

The results in Figure 7 demonstrate a very rapid oxidation of ferrous ion (200 μM) in the presence of H_2O_2 (50 μM) with or without NaCl (0.5 M). The stoichiom-

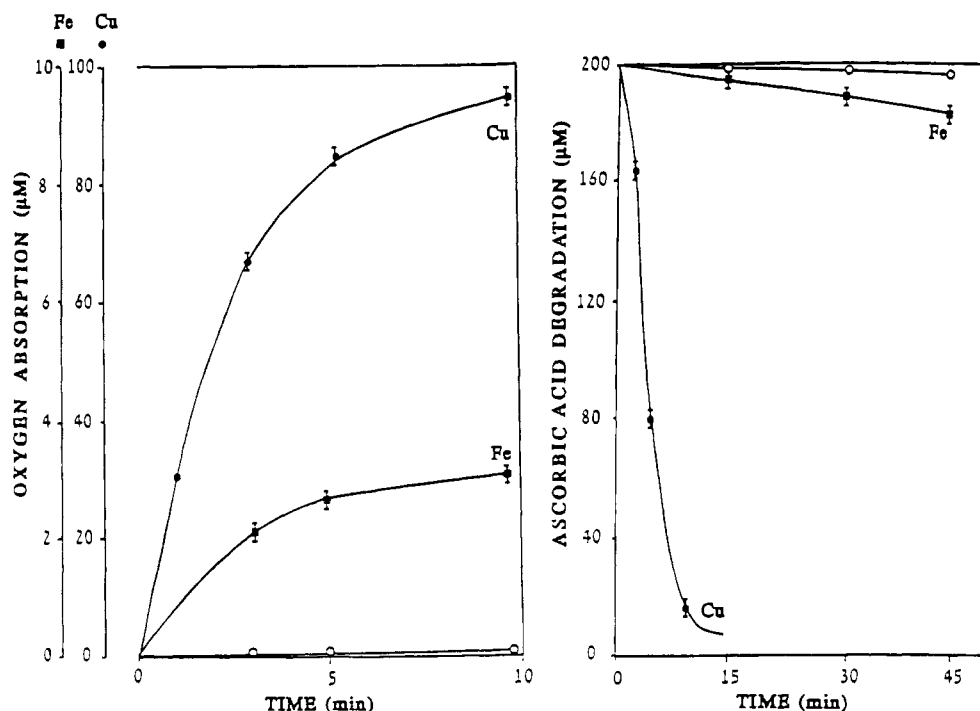


Figure 2. Inhibition by NaCl of ascorbic acid oxidation in the presence of metal ions: (a, left) reactions determined by oxygen absorption; (b, right) reactions determined spectrophotometrically at 265 nm; (●) 5 μM CuSO_4 ; (■) 13 μM FeCl_3 ; (○) CuSO_4 or FeCl_3 + 0.5 M NaCl. The reaction was performed in 0.05 M acetate, pH 7, at 25 °C and with 200 μM ascorbic acid.

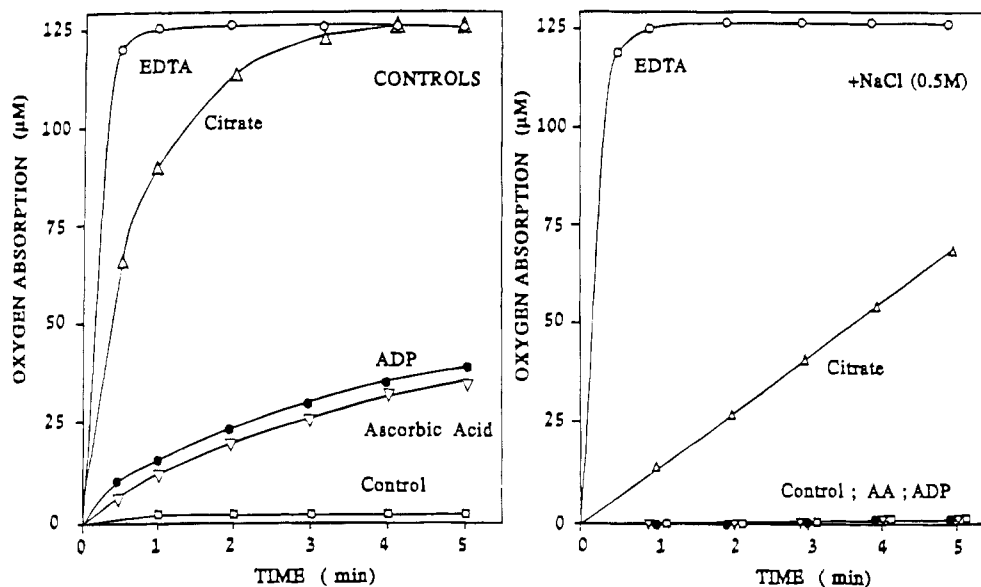
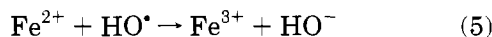


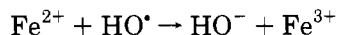
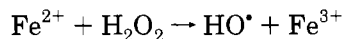
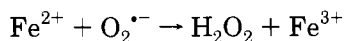
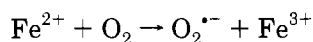
Figure 3. Inhibition by NaCl of oxygen consumption during the oxidation of ferrous ion: (a, left) (□) control (250 μM FeSO₄ in 0.05 M acetate, pH 7); (○) control + 330 μM EDTA; (△) control + 330 μM sodium citrate; (●) control + 330 μM ADP; (▽) control + 330 μM ascorbic acid; (b, right) in the presence of 0.5 M NaCl.

etry indicates that it is consistent with reactions 4 and 5 which show oxidation of ferrous ion by H₂O₂ and HO•.



At the end of both reactions 4 and 5, the remaining ferrous ion was oxidized by oxygen and this reaction was found, as previously, to be affected by NaCl.

Determination of ferrous oxidation by the Ferrozine method is much more sensitive than oxygen absorption in this model system. One of the reasons is because for every 1 mol of oxygen, 4 mol of ferrous ion is oxidized by O₂•⁻, H₂O₂, and HO• produced during reactions 2–5.



The inhibition of ferrous ion oxidation by NaCl stems from the interaction of the chloride anion with the iron ion. Different chloride salts, such as CaCl₂, MgCl₂, and LiCl, affect the reaction very similarly (results not shown).

The anionic ligands interfere with chloride ions and prevent the interaction between metal ions and oxygen. If the metal is liganded to relatively strong chelators such as EDTA or citrate, the effect of the chloride ion is very poor or only moderate (Figure 3).

It was found that NaCl could affect the stability of oxyhemoglobin and accelerate decomposition of the oxyhemoglobin to methemoglobin (Wallace et al., 1974). It seems that NaCl prevents or disturbs the interaction between iron or iron–heme and oxygen in a similar manner.

Citrate, phosphate, and EDTA ions increase iron reactivity with oxygen to produce active oxygen species (Miller et al., 1990). Chelation of iron to different

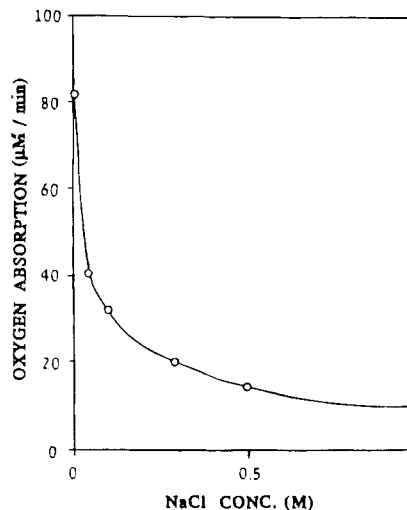


Figure 4. Effect of NaCl concentration on oxygen consumption during oxidation of ferrous ion. The reaction was performed with 250 μM FeSO₄ and 330 μM sodium citrate in 0.05 M acetate, pH 7, at 25 °C.

compounds affects iron reactivity by changing its redox potential and also by changing its potential to interact with oxygen, H₂O₂, or ligands. If iron is liganded by all of its six coordination positions, as occurs with cytochrome *c*, ligation of oxygen is prevented but interaction of iron with H₂O₂ or hydroperoxides is not (Harel et al., 1988).

In blood and tissues the concentration of NaCl may reduce autoxidation of ferrous or cuprous ions liganded by weak chelators such as ADP or ascorbic acid, thereby preventing the autoxidation of several important reducing compounds and the formation of oxygen reactive species. These effects are also relevant to several foods. However, since NaCl does not affect the reactivity of ferrous ions with previously formed H₂O₂ or hydroperoxides, it may turn the reactivity of metal ions to these pathways and thereby contribute to and affect (*in vivo* or *in situ*) the generation of hydroperoxides and lipid free radicals.

The increase of lipid peroxidation by NaCl in several model systems and *in situ* in muscle foods was at-

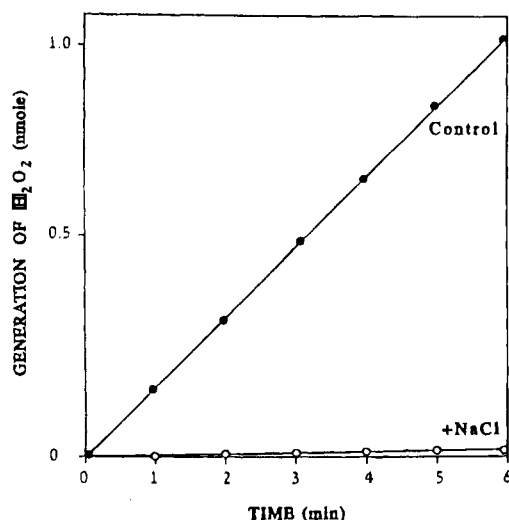


Figure 5. Effect of NaCl on ferrous ion oxidation and the generation of H_2O_2 . The reaction was performed in $250 \mu M$ $FeSO_4$ in $0.05 M$ acetate, pH 7, at $25^\circ C$ with and without $0.5 M$ NaCl: (●) control; (○) $0.5 M$ NaCl.

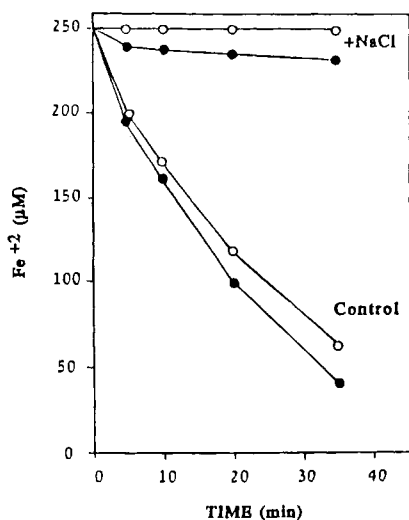


Figure 6. Effect of NaCl ($0.5 M$) on ferrous ion oxidation as determined by the ferrozine method. The reaction was performed in $0.05 M$ acetate, pH 7, at $25^\circ C$: (○) $250 \mu M$ $FeSO_4$; (●) $250 \mu M$ $FeSO_4$ + $330 \mu M$ ADP.

tributed to the possible effect of NaCl to elevate the amount of free iron in tissues (Kanner et al., 1991). On the basis of our recent results we suggest that, in addition, NaCl may play a role in lipid peroxidation by shifting ferrous ions from interaction with oxygen to reaction with previously formed H_2O_2 or hydroperoxides and decomposing these compounds to free radicals, accelerating in particular the peroxidation process.

In conclusion, the cascade reactions generated during the interaction of ascorbic acid with ferric or cupric ions in the presence of oxygen are prevented by NaCl. Sodium chloride prevents only partly the interaction of the metal ions with ascorbic acid but completely prevents the interaction of the metal with oxygen and by this the formation of superoxide, H_2O_2 , and hydroxyl radicals. In this way the oxidation and autoxidation of ascorbic acid and the reduced metals by oxygen are prevented by NaCl. The effect of NaCl is significant if the metal ion is chelated by weak chelators such as acetate, ADP, or ascorbic acid, only moderate with citrate, and nonexistent with strong chelators such EDTA.

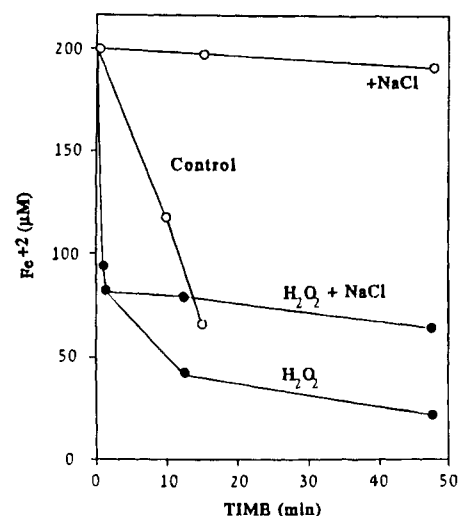


Figure 7. Effect of NaCl ($0.5 M$) on ferrous ion oxidation by H_2O_2 , as determined by the ferrozine method. The reaction was performed in $0.05 M$ acetate, pH 7: (○) $200 \mu M$ $FeSO_4$; (●) $200 \mu M$ $FeSO_4$ + $50 \mu M$ H_2O_2 .

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