Effects of Buffer Solutions and Chelators on the Generation of Hydroxyl Radical and the Lipid Peroxidation in the Fenton Reaction System

Yoshihiro Yoshimura,* Yumi Matsuzaki, Takaho Watanabe, Katsumi Uchiyama, Keiko Ohsawa, and Kazuo Imaeda

Department of Analytical Chemistry, Faculty of Pharmaceutical Sciences, Hoshi University, Shinagawa-ku, Tokyo 142, Japan

(Received July 25, 1992)

Summary Oxygen free radicals, in particular the hydroxyl radical (·OH), which is readily formed by the Fenton reaction, are known to initiate lipid peroxidation. In this study the effects of several buffer solutions and chelators on the generation of ·OH and lipid peroxidation were examined. The generation of ·OH was decreased significantly in phosphate buffer solution, and slightly in Tris-HCl buffer solution, compared to that in HEPES or MOPS buffer solution, both of which have low affinity for iron ions. Phosphate and Tris-HCl buffer solutions also decreased the formation of lipid peroxides. The generation of ·OH varied by the addition of several kinds of chelators at different ratios of chelator/Fe(II), 1/10–10/1. And conflicting effects of EDTA or diethyleneetriamine pentaacetic acid (DETAPAC) were observed in HEPES buffer and in phosphate buffer. At the concentration ratios of chelator/Fe(II) at which EDTA or DETAPAC inhibited ·OH generation in HEPES buffer, ·OH generation was promoted by EDTA or DETAPAC in phosphate buffer. On the other hand ADP slightly enhanced ·OH generation at the ratios of ADP/Fe(II) of 5/1 and 10/1 in HEPES buffer, but did not affect ·OH generation in phosphate buffer. Lipid peroxidation was completely inhibited by the addition of DETAPAC and EDTA, while ADP promoted ·OH generation at the chelator/Fe(II) ratios of 5/1 and 10/1.

Key Words: hydroxyl radicals, Fenton reaction, Fe(II), chelators

Active oxygen species, such as ·OH, 1O₂, O₂⁻, and H₂O₂, have been implicated

---

*To whom correspondence should be addressed.
as being important causative agents of aging and of several diseases including cancer, atherosclerosis and inflammation, because of their high reactivity with biological molecules. Above all, •OH has the highest reactivity and is believed to be responsible for many lesions in vivo. •OH is generated by the reduction of H$_2$O$_2$ in the presence of traces of ferrous ions intracellularly (Fenton reaction).

$$\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \cdot\text{OH} + \text{OH}^-$$

Almost all the Fe(II) in vivo is bound to proteins such as ferritin or transferrin, so that Fe(II) does not participate in the Fenton reaction. However, it is reported that O$_2^-$ is able to release Fe(II) from ferritin [1], and a trace amount of Fe(II) in body fluids does participate in the Fenton reaction and generate •OH [2, 3].

Fe(II) readily binds to low-molecular-weight chelators and generates •OH by the Fenton reaction [4]. Several investigators have reported the ability of many kinds of Fe(II) complexes to generate •OH. For example, EDTA-Fe(II) and diethylenetriamine pentaacetic acid (DETAPAC)-Fe(II) complexes react readily with H$_2$O$_2$ to form •OH [5-8], but the Fenton reaction is prevented by the chelation of iron by phytate [9]. Meanwhile, DETAPAC-Fe(III) is hardly reduced by O$_2^-$, so that DETAPAC-Fe is believed not to be a good catalyst of the Haber-Weiss cycle, and fails to support •OH generation [10]. Another report suggested the requirement for at least one coordination site of iron that is open or occupied by readily dissociable ligand such as water for catalytic activity; EDTA-Fe, nitrilotriacetic acid (NTA)-Fe, ADP-Fe, etc. have the activity, while DETAPAC-Fe, phytate-Fe, Desferal-Fe, etc. do not [11].

In many experimental systems, phosphate buffer, which can ligate metals and thus might affect the reaction of Fe-complexes, is in wide use. Therefore we have investigated the effects of this buffer and several other kinds of buffer solutions and chelators (DETAPAC, EDTA, ADP, Desferal) on the generation of •OH and the lipid peroxidation in the Fenton reaction system.

MATERIALS AND METHODS

Materials. 1,3-Diphenyl-2-thiobarbituric acid (DPTBA) was prepared from 1,3-diphenyl-2-thiourea, malonic acid and acetyl chloride, obtained from Nacalai Tesque Inc., Kyoto. Ammonium ferrous sulfate, hydrogen peroxide, disodium hydrogen phosphate, sodium dihydrogen phosphate, tris(hydroxymethyl)aminomethane (Tris), N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 3-(N-morpholino)propanesulfonic acid (MOPS), egg lecithin, DETAPAC, EDTA were purchased from Wako Pure Chemical Co., Osaka. Desferal was purchased from Ciba-Geigy Co., Ltd., Hyogo, Japan. 5,5-Dimethyl-1-pyrroline-N-oxide (DMPO) was obtained from Labotec Co., Ltd., Tokyo.

Assay for •OH. The generation of •OH was detected by E.S.R., with DMPO used as a spin trap. Reaction mixtures contained the following reagents, added in the order stated: 50 $\mu$L of Fe(II) solution (0.1 mM), 50 $\mu$L of pH 7.5 buffer solution.
EFFECTS OF CHELATORS IN THE FENTON SYSTEM

(0.1 M), 50 μl of chelator solution (0.01–1.0 mM), 10 μl of DMPO, and 50 μl of H₂O₂ solution (1 mM). ESR spectra were recorded 1 min after the addition of H₂O₂ on a JEOL JES-REIX X-band spectrometer with a 100 kHz magnetic field. The relative intensity of ·OH was represented by the ratio of the intensity of the first peak in the spectrum of the DMPO-OH spin adduct to the intensity of the third peak in the spectrum of Mn²⁺.

Assay for lipid peroxides. Lipid peroxides were measured by the fluorescence detection method with DPTBA [12]. The following reagents were added to 1 ml of 0.1% lecithin solution in the order stated: 50 μl of Fe(II) solution (0.1 mM), 50 μl of pH 7.5 buffer solution (0.1 M), 50 μl of chelator solution (0.01–1.0 mM), 10 μl of DMPO, and 50 μl of H₂O₂ solution (1 mM). After the reaction mixture had been shaken for 5 min at room temperature, 1.5 ml of pH 2.5 HCl-sodium acetate buffer solution (0.5 M) and 100 μl of DPTBA-dimethylsulfoxide solution (0.12 M) were added, and the mixture was then heated in a water bath at 95°C for 40 min. Next, the mixture was cooled in ice water for 5 min, after which the reaction products were extracted with 1-butanol. The fluorescence intensity of the 1-butanol layer was measured at 556 nm with excitation at 520 nm. The level of lipid peroxides was represented as malondialdehyde value in 1 mg lecithin (nmol/mg).

RESULTS AND DISCUSSION

Effects of buffer solutions on the generation of ·OH

Figure 1 shows the generation of ·OH via the Fenton reaction in four different buffer solutions (pH 7.5, 0.1 M).

The ·OH signal intensity rates in the solutions of HEPES, MOPS, Tris-HCl, and phosphate buffers were 14.75, 13.75, 11.25, and 1.24, respectively. HEPES and

![Fig. 1. Effects of several kinds of buffer solutions on the generation of ·OH by the Fenton reaction. The reaction mixture contained the following reagents, added in the order stated: 23.8 μM Fe²⁺, 23.8 mM buffer, DMPO, 238 μM H₂O₂. ·OH/Mn²⁺ represents the ratio of the intensity of the first peak of DMPO-OH spin adduct to the intensity of the third peak of Mn²⁺.](image)

Vol. 13, No. 3, 1992
MOPS buffers are Good’s buffers that have little interaction with metal ions [13]. The generation of •OH was lowered by 21% in Tris buffer solution, and by 91% in phosphate buffer solution. Tris has the ability of weakly chelating iron [14] and a •OH scavenging effect [15, 16]. And phosphate has relatively high binding effect on iron and also catalyzes Fe(II) auto-oxidation [17]. Thus, it is clear that both Tris and phosphate can interfere with the generation of •OH by the Fenton reaction.

Our data also suggest that HEPES and MOPS buffers can be suitable buffers for use in experimental systems involving iron complexes, such as the Fenton reaction system.

Effects of chelators on the generation of •OH

Figure 2 shows the generation of •OH via the Fenton reaction in HEPES buffer solution (pH 7.5, 0.1 M) containing several kinds of chelators at different concentration ratios of chelator to Fe(II), 1/10–10/1.

The addition of DETAPAC caused a decrease in •OH generation by 10, 33, and 24% at the concentration ratios of DETAPAC to Fe(II), 1/10, 1/2, and 1/1, respectively. On the other hand, it caused a slight increase at the ratios of 2/1, 5/1, and 10/1. DETAPAC inhibited the generation of •OH only at the optimum concentration ratio, 1/2–1/1.

When EDTA was added, a significant decrease in •OH generation was observed depending upon the increase in the ratio of EDTA to Fe(II), and the inhibition rates were 15, 49, 59, 49, and 55% at the ratios of 1/2, 1/1, 2/1, 5/1, and 10/1, respectively. ADP exerted no effect at the ratios from 1/10 to 2/1. But at the ratios of 5/1 and 10/1, the addition of ADP resulted in •OH generation 1.3-fold.

![Fig. 2. Effects of chelators on the generation of •OH by the Fenton reaction in HEPES buffer solution. The reaction mixture contained the following reagents, added in the order stated: 23.8 μM Fe²⁺, 23.8 mM HEPES buffer, 2.38–238 μM chelator or 2.5/5.0 μg Desferal, DMPO, 238 μM H₂O₂. •OH/Mn²⁺ represents the ratio of the intensity of the first peak of DMPO-OH spin adduct to the intensity of the third peak of Mn²⁺.](image)
over that of the control. Desferal, which is clinically used as an iron excreter, decreased the generation of •OH by 65 and 96%, when added in amounts of 2.5 and 5.0 μg, respectively.

Figure 3 shows the generation of •OH via the Fenton reaction in phosphate buffer solution (pH 7.5, 0.1 M) containing several kinds of chelators at different concentration ratios of chelator to Fe(II), 1/10–10/1.

The addition of DETAPAC resulted in an increase in •OH generation to 1.5 times and twice as high as that of the control, at the ratios of 1/10 and 1/2, respectively. Meanwhile, it did not have any effect on the generation of •OH at the ratios of 1/1–10/1. EDTA added at higher ratios caused a considerable increase in the generation of •OH, giving values 2.8, 3.3, 3.6, and 4.3-fold over that of the control at the ratios of 1/1, 2/1, 5/1, and 10/1, respectively. The addition of ADP did not exert any effect at all over the ratios examined. Desferal decreased the •OH generation by 68 and 77% when added in amount of 2.5 and 5.0 μg, respectively.

In HEPES buffer solution Fe(II) seems to exist as a free ion to which DETAPAC and EDTA can bind strongly to form stable DETAPAC-Fe(II) and EDTA-Fe(II) complexes. Such complexes can be presumed to inhibit the binding of H₂O₂ to Fe(II) stereochemically, so that H₂O₂ fails to be reduced to form •OH. Otherwise, it is possible the redox potential of DETAPAC-Fe(II) or EDTA-Fe(II) is too high to allow reduction of H₂O₂. ADP added at a more than 5-fold excess of Fe(II) seems to form ADP-Fe(II) complexes that can promote the Fenton reaction. On the other hand, in phosphate buffer solution, which is able to bind iron, DETAPAC and EDTA exerted the opposite effect. They promoted the generation of •OH at the ratios at which they inhibited it in HEPES buffer.

---

**Fig. 3.** Effects of chelators on the generation of •OH by the Fenton reaction in phosphate buffer solution. The reaction mixture contained the following reagents, added in the order stated: 23.8 μM Fe²⁺, 23.8 mM phosphate buffer, 2.38–238 μM chelator or 2.5/5.0 μg Desferal, DMPO, 238 μM H₂O₂. •OH/Mn²⁺ represents the ratio of the intensity of the first peak of DMPO-OH spin adduct to the intensity of the third peak of Mn²⁺.
solution. But ADP did not affect the generation of •OH in HEPES buffer solution.

The opposite results obtained with the phosphate buffer indicated that the chelator-Fe(II) complexes formed in the presence of phosphate ions are not binary complexes, but ternary ones, i.e., chelator-Fe(II)-phosphate, which have a different catalytic character in the Fenton reaction. In the circumstance in which there is an excess of phosphate salt, the binding of ADP to Fe(II) is possibly weakened by phosphate and ADP-Fe(II) can be hardly formed. Desferal could inhibit the generation of •OH in both HEPES and phosphate buffer solutions, and this inhibition is considered to be due to the strong ligation of Fe(II) to Desferal to form inert iron complexes in the Fenton reaction system.

Effects of buffer solutions on the lipid peroxidation

Figure 4 shows the amount of lipid peroxides formed via the Fenton reaction in different buffer solutions (pH 7.5, 0.1 M).

The amount of lipid peroxides produced in HEPES, MOPS, Tris-HCl, and phosphate buffer solutions was 1.59, 1.50, 1.32, and 1.15 (nmol MDA/mg), respectively. When phosphate or Tris-HCl buffer solution was used, lipid peroxidation was inhibited by 28% or 17%, respectively, due to their interaction with Fe(II).

Effects of chelators on the lipid peroxidation

Figure 5 shows the amount of lipid peroxides formed via the Fenton reaction in HEPES buffer solution (pH 7.5, 0.1 M) containing several kinds of chelators at different concentration ratios of chelator to Fe(II), 1/10–10/1.

The addition of DETAPAC decreased the lipid peroxidation at higher ratios; 20, 33, and 89% decreases were observed at the ratios of 1/2, 1/1, and 2/1, respectively. And at the ratios of 5/1 and 10/1, lipid peroxidation was completely inhibited. The inhibition of lipid peroxidation was also seen after the addition of EDTA. Inhibition rates were 20, 34, 74, and 76% at the ratios of 1/10, 1/2, 1/1, and

EFFECTS OF CHELATORS IN THE FENTON SYSTEM

2/1, respectively; and at the ratios of 5/1 and 10/1, the lipid peroxidation was completely inhibited. ADP did not affect the lipid peroxidation added at the ratios from 1/10 to 2/1, but at the ratios of 5/1 and 10/1, its addition caused formation of lipid peroxides to a level 1.3-fold over that of the control. The addition of 5.0 µg of Desferal resulted in a slight decrease in the formation of lipid peroxides.

DETAPAC and EDTA added at the high concentration ratios of DETAPAC/EDTA to Fe(II) inhibited the lipid peroxidation effectively. DETAPAC and EDTA can ligate to Fe(II) and form DETAPAC-Fe(II) and EDTA-Fe(II), which can hardly be partitioned into the lipid phase [18], and thus prevent the Fenton reaction in the lipid phase. However, ADP readily partitions into the lipid phase [18], and also ADP-Fe(II) can participate in the Fenton reaction, so that lipid peroxidation could be promoted. Desferal inhibited the generation of •OH significantly, whereas it hardly inhibited the lipid peroxidation. It appears that Desferal-Fe(II) does not participate in the Fenton reaction but facilitates the lipid peroxidation by some means other than by generating •OH.

Phosphate buffer is widely used in biological experimental systems. But phosphate ions readily bind to iron ions, thereby significantly decreasing the generation of •OH. We recommend, therefore, the use of HEPES or MOPS buffer solution, either of which has little interaction with iron ions. By the addition of DETAPAC and EDTA, the generation of •OH was inhibited in HEPES buffer, but enhanced in phosphate buffer. The opposing effects of DETAPAC/EDTA in phosphate buffer and in HEPES buffer can be attributed to the difference in the formation of the complexes, that is, binary complex vs. ternary complex. Graf et al. [11] postulated that for catalytic activity, iron requires at least one coordination site that is open or occupied by a readily dissociable ligand such as water.
under the condition of Tris used as a buffer. Our results, however, indicate that the condition of iron coordination site must vary in the presence of buffer components that have high affinity for iron ions, and also by the ratio of chelator/Fe(II) concentration.

Our results suggest that phosphate ions, which are contained in biological fluids, especially in intracellular fluids, have a protective effect on biological systems by inhibiting •OH generation and lipid peroxidation.

REFERENCES