ESR Spin-trapping Studies on the Reaction of Fe\textsuperscript{2+} Ions with H\textsubscript{2}O\textsubscript{2}-reactive Species in Oxygen Toxicity in Biology*

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Using ESR spin-trapping techniques with 5,5-di methyl-1-pyrroline-N-oxide (DMPO), we confirmed the 1:1 stoichiometry for the formation of hydroxyl radicals with Fe\textsuperscript{3+} in the Fenton reaction under experimental conditions wherein [H\textsubscript{2}O\textsubscript{2}] is 90 \textmu M and [Fe\textsuperscript{3+}] is very low, 1 \textmu M or less. The stoichiometry decreased markedly as the Fe\textsuperscript{3+} concentration was increased. The efficiency of hydroxyl radical generation varied with the nature of the iron chelators used and increased in the order of phosphate alone \approx ADP < EDTA < diethylenetriaminepentaacetic acid (DETAPAC). The second order rate constant for the Fenton reaction was measured to be \(2.0 \times 10^4 \text{ M}^{-1} \text{s}^{-1}\) for phosphate alone, \(8.2 \times 10^3 \text{ M}^{-1} \text{s}^{-1}\) for ADP, \(1.4 \times 10^4 \text{ M}^{-1} \text{s}^{-1}\) for EDTA, and \(4.1 \times 10^4 \text{ M}^{-1} \text{s}^{-1}\) for DETAPAC. Measuring the radicals formed as spins trapped in the presence of ethanol, we confirmed the amount of total oxidizing intermediates formed in the Fenton reaction, which we concluded consists of hydroxyl radicals and an iron species. The oxidizing species of iron which might be assigned as ferryl, FeO\textsuperscript{2+}, or Fe(IV) = O was generated effectively in the presence of ADP even at low Fe\textsuperscript{3+} concentrations. In general, as the Fe\textsuperscript{3+} concentration was increased, the ferryl species predominated over the hydroxyl radical except for the case of Fe(II)-DETAPAC, which generated only hydroxyl radicals as the oxidizing species. Three possible pathways are proposed for the Fenton reaction, the cases depending very much on the nature of the iron chelator being used.

A variety of studies have supported a hypothesis that the hydroxyl radical is a major species for oxygen toxicity in biology (1). The most important source of the radical in tissues has been assumed to be the Fenton reaction involving a ferrous ion and hydrogen peroxide.

\[
\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{HO}^+ + \text{HO}^-
\]

The formation of hydroxyl radicals in Reaction 1 has been directly confirmed by the ESR spin-trapping technique (2-6). Despite numerous reports on the above ESR detection of hydroxyl radicals, the nature of the Fenton reaction as represented in Reaction 1 has not yet been elucidated because of the lack of carefully designed quantitative measurements involving the production of hydroxyl radicals. An alternative interpretation of the Fenton reaction which postulates the formation of oxidizing iron species such as ferryl, FeO\textsuperscript{2+}, or Fe(IV) = O cannot be excluded (7-9).

\[
\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{HO}^+ + \text{HO}^-
\]

Fe(IV) = O cannot be excluded (7-9).

The ferryl ion is supposed to be kinetically equivalent to the hydroxyl radical in many respects (7-9).

The ambiguity of oxidizing species generated in the Fenton reaction may arise also from the fact that the mechanism of the reaction varies with the nature of the iron chelator (1). Although several papers have reported that EDTA accelerates and DETAPAC\textsuperscript{1} inhibits the iron-catalyzed formation of hydroxyl radicals (10-13), others have reported that the DETAPAC-chelated iron is as active as the EDTA-chelated iron for the generation of hydroxyl radicals (14-19). Hydroxyl radicals are also formed in the reaction of ADP-chelated iron with hydrogen peroxide (20), but it has been reported that the participation of the radical is not important particularly in lipid peroxidation induced by ADP-chelated iron (21, 22). At the moment it seems difficult to explain the discrepancy between the above results because the fundamental nature of the Fenton reaction in terms of hydroxyl radical production has not been analyzed.

The reactions caused by Fenton reagents have been analyzed quantitatively in the presence of aromatic compounds (23, 24) and deoxyribose (25, 26), but the stoichiometry of the oxidizing intermediates formed in the reaction is still uncertain. There is no doubt that the ESR spin-trapping technique is the most direct method to measure the hydroxyl radical (2). Floyd and Lewis (16), for instance, reported that the yield of hydroxyl radicals in the Fenton reaction was 20% in the presence of 2 mM ADP. If so, the Fenton reaction cannot be expressed simply as Reaction 1. In our previous report (27), we have described kinetic and stoichiometric analysis of spin-trapping of oxygen radicals generated in NADPH-cytochrome P-450 reductase-paraoxon systems. In this work, we have improved the previous method by using a flow apparatus and applied it to the analysis of the Fenton reaction.

MATERIALS AND METHODS

DETAPAC and ADP were obtained from Sigma and EDTA from MCB Manufacturing Chemicals, Inc. DMPO (5,5-di methyl-1-pyrroline-N-oxide) and Tempol (2,2,6,6-tetramethyl-1-hydroxypiperidine) were obtained from Aldrich Chemical Co. DMPO was used after redistilling.

ESR assays were performed in a flat cell using a Varian E-9 spectrometer. The flat cell was connected with a flow apparatus (Model RX 1000, Applied Photophysics Ltd., England). Reactions were carried out at 25 °C in 20 mM potassium phosphate buffer (pH 7.4) containing 150 mM KCl (control system). Reactions were started with the flow apparatus by mixing an aerobic solution containing 150 mM KCl, 40 mM phosphate buffer, 40 mM DMPO (standard experiments).

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\(1\) The abbreviations used are: DETAPAC, diethylenetriaminepentaacetic acid; DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; DMPO-OH, hydroxyl radical adduct of DMPO; Tempol, 2,2,6,6-tetramethyl-1-hydroxypiperidine-1-oxyl.
RESULTS

Since hydroxyl radicals are trapped by DMPO at a high rate with a rate constant of $3.4 \times 10^9 \text{ M}^{-1} \text{s}^{-1}$, they form a relatively stable spin adduct, DMPO-OH (29), the formation of hydroxyl radicals in the Fenton reaction can be followed by measuring DMPO-OH. By fixing the magnetic field at a specified ESR signal peak of DMPO-OH, a time course of DMPO-OH formation was measured in the reaction of Fe(II)-DETAPAC with $\text{H}_2\text{O}_2$ (Fig. 1A). When $[\text{H}_2\text{O}_2] > 10 \times [\text{Fe}^{2+}]$, the reaction obeyed first order kinetics, and the apparent first order rate constant was proportional to $[\text{H}_2\text{O}_2]$ but independent of $[\text{Fe}^{2+}\text{-DETAPAC}]$. Although the molar ratio of accumulated DMPO-OH to Fe(II)-DETAPAC added was less than unity, the second order rate constant was tentatively estimated at $4.1 \times 10^9 \text{ M}^{-1} \text{s}^{-1}$. When DETAPAC was replaced by EDTA or ADP, the reaction became much faster and the DMPO-OH formation was completed in a few seconds (Fig. 1B). The DMPO-OH level remained constant after the reaction was over. The reaction pattern was nearly the same in a 20 mM phosphate buffer solution (control reaction system). In these cases, the second order rate constants were measured at low concentrations of reactants as shown in Fig. 1C. Rate constants thus obtained are listed in Table I. The respective rate constants for the Fenton reaction in the presence of DETAPAC and EDTA were not much different from those reported by Sutton and Winterbourn (30) who measured the rate constants as the sum of $k_1$ and $k_2$ using a recycling reaction system containing paraquat radicals, and by Rush and Koppenol (31) who measured the rate of iron oxidation in the presence of scavengers (Table I).

We thought it important to know whether or not the 1:1 stoichiometry of Reaction 1 could be obtained under certain experimental conditions. Fig. 2 shows the effect of $\text{Fe}^{2+}$ concentration on the efficiency of hydroxyl radical formation in the Fenton reaction. It was found that the molar ratio of DMPO-OH to $\text{Fe}^{2+}$ in the presence of excess $\text{H}_2\text{O}_2$ was greatly dependent on the concentration of $\text{Fe}^{2+}$ initially added. Although the ratio became smaller with the increase in $\text{Fe}^{2+}$ concentration, the inset in Fig. 2 clearly indicated that the ratio approached nearly unity at low $\text{Fe}^{2+}$ concentrations. The relatively higher ratio was observed in the presence of DETAPAC or EDTA. In the absence of these chelators, the ratio decreased drastically as the $\text{Fe}^{2+}$ concentration was increased. The $[\text{DMPO-OH}]/[\text{Fe}^{2+}]$ ratio also depended on $[\text{H}_2\text{O}_2]$ even when $[\text{H}_2\text{O}_2] > [\text{Fe}^{2+}]$ (Fig. 3). Under these conditions, however, the increase in the DMPO concentration above 20 mM had no effect on the amount of trapped spin obtained. We concluded from Figs. 2 and 3 that the stoichiometry shown in Reaction 1 was achieved at $\text{H}_2\text{O}_2$ concentrations around 100 $\mu$M and at low $\text{Fe}^{2+}$ concentration (below 1 $\mu$M), not only in the presence of DETAPAC and EDTA but also in the presence of ADP and phosphate alone. With DETAPAC, the stoichiometry was achieved at even higher $\text{Fe}^{2+}$ concentrations (Fig. 2). The question was how to explain the deviation from the 1:1 stoichiometry at high $\text{Fe}^{2+}$ concentrations. To answer this, we tried to measure the amount of total oxidizing species generated from the Fenton reaction.

Ethanol is known to be oxidized by a hydroxyl radical (29, 32) and an oxidizing species of iron (7, 31, 33, 34), both to an ethanol radical, which can then be trapped by DMPO (29).

$$\text{HO} + \text{CH}_2\text{CH}_2\text{OH} \rightarrow \text{H}_2\text{O} + \text{CH}_2\text{CHOH} \quad (3)$$

$$\text{Fe}^{2+} + \text{CH}_2\text{CH}_2\text{OH} \rightarrow \text{Fe}^{3+} + \text{HO}^- + \text{CH}_2\text{CHOH} \quad (4)$$

$$\text{DMPO} + \text{CH}_2\text{CHOH} \rightarrow \text{DMPO-CH}_2\text{CHOH} \quad (5)$$

If one assumes that the primary products of the Fenton reaction are not changed by the ethanol addition and there is no side reaction causing the loss of spins during Reactions 3, 4, and 5, the amount of increase of total spin adducts due to the presence of ethanol should equal that derived from Reaction 4. As the efficiency of hydroxyl radical formation depended on the $\text{Fe}^{2+}$ concentration, the quantitative assay was carried out at $\text{Fe}^{3+}$ concentrations of 4 $\mu$M (Fig. 4A) and 10 $\mu$M (Fig. 4B). Because the FSR sensitivity was lower for DMPO-CH$_2$CHOH than for DMPO-OH, the minimum $\text{Fe}^{2+}$ concentration necessary to get reliable quantitative data for this purpose appeared to be about 4 $\mu$M. In Fig. 4, the amounts of DMPO-OH and DMPO-CH$_2$CHOH and their sum were plotted against the ethanol concentration. There was a remarkable increase in the total spin adducts detected with ethanol in the presence of ADP even at a $\text{Fe}^{2+}$ concentration of 4 $\mu$M (Fig. 4A). When the $\text{Fe}^{2+}$ concentration was increased up to 10 $\mu$M, such an increase was also observed in the presence of EDTA (Fig. 4B). In the case of ADP, the total spin adducts detected by the ethanol addition increased more than 2-fold (Fig. 4B). Fig. 4 suggests that an oxidizing species of iron was formed at higher $\text{Fe}^{2+}$ concentrations. Only in the case of DETAPAC were the total spin adducts somewhat decreased by ethanol, probably because of loss of the spin during the conversion from hydroxyl radical to ethanol radical to DMPO-CH$_2$CHOH.

Although numerous papers have reported that the Fenton reagents generate hydroxyl radicals to cause biochemical re-
Quantitative Analysis of the Fenton Reaction

TABLE I

<table>
<thead>
<tr>
<th></th>
<th>EDTA</th>
<th>DETAPAC</th>
<th>ADP</th>
<th>Control</th>
<th>Ref.</th>
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<tr>
<td>Fenton reaction</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(M$^{-1}$ s$^{-1})$</td>
<td>1.4 x 10$^4$</td>
<td>4.1 x 10$^3$</td>
<td>8.2 x 10$^3$</td>
<td>2.0 x 10$^4$</td>
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</tr>
<tr>
<td></td>
<td>7 x 10$^4$</td>
<td>8 x 10$^3$</td>
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<td>(30)</td>
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<tr>
<td></td>
<td>5 x 10$^4$</td>
<td>1.37 x 10$^3$</td>
<td>4 x 10$^3$</td>
<td>2.0 x 10$^3$</td>
<td>(31)</td>
</tr>
<tr>
<td>Reduction of DMPO-OH by Fe$^{3+}$ (M$^{-1}$ s$^{-1})$</td>
<td>3 x 10$^3$</td>
<td>1 x 10$^3$</td>
<td></td>
<td></td>
<td>(55)</td>
</tr>
</tbody>
</table>

*If the Fenton reaction takes place mainly via Reactions 1 and 2 in parallel (see Fig. 7), the second order rate constants estimated from the apparent first order rate constants for the DMPO-OH formation at [H$_2$O$_2$] > 10 × [Fe$^{3+}$] (see Fig. 1) are nearly identical with those for the overall Fenton reaction.

These values were measured from the decay curves observed at increasing concentrations of Fe$^{3+}$ above that equimolar to H$_2$O$_2$.

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**FIG. 2.** Dependence of the maximum level of accumulated DMPO-OH on the Fe$^{3+}$ concentration. Reactions were carried out as described in Fig. 1 at 90 μM H$_2$O$_2$ and varied concentrations of Fe$^{3+}$. The inset shows a different series of experiments carried out at low concentrations of Fe$^{3+}$. Phosphate control and ADP gave essentially the same results, so only the ADP result is shown. DETAPAC (200 μM), EDTA (200 μM), or ADP (2 mM) was added to the control system (20 mM phosphate (pH = 7.4) and 150 mM KCl). Broken lines show the 1:1 stoichiometry of Reaction 1.

**FIG. 3.** The effect of H$_2$O$_2$ concentration on the maximum level of accumulated DMPO-OH. Reactions were carried out as described in Fig. 2 in the presence of 1 μM Fe$^{3+}$ and varied amounts of H$_2$O$_2$.

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actions related to tissue damage (1, 10–17, 35–37), this idea has not always been accepted (4, 8, 9, 38–41). An oxidizing iron intermediate formed in the reaction of Fe$^{3+}$ with H$_2$O$_2$ was suggested by Rush and Koppenol (8), and an Fe(II)-O$_2$-Fe(III) complex was suggested by Minotti and Aust (4). In their experiments, high concentrations of Fe$^{2+}$ (100–200 μM) were used where the [Fe$^{3+}$]/[H$_2$O$_2$] ratio might also become critical. In connection with these reports, two series of experiments were undertaken. Fig. 5A shows that the amount of DMPO-trapped spins increased 2.6-fold in the presence of ethanol when 100 μM Fe(II)-EDTA reacted with 200 μM hydrogen peroxide under experimental conditions similar to those used by Rush and Koppenol (8). Our results may not contradict their conclusion that an intermediate other than the hydroxyl radical is an important oxidizing species in the Fenton reaction under these conditions, and it is of interest to compare this result with that of the Fe(II)-DETAPAC reaction. In this latter reaction, the efficiency of hydroxyl radical formation was very high, and the addition of ethanol rather decreased the total spin adducts (Fig. 5B). Although the amount of DMPO-CH$_2$CHOH detected was about the same in the presence of EDTA and DETAPAC, the contaminating DMPO-OH was about 10 times larger in the case of DETAPAC than in that of EDTA. This difference can be explained by assuming that the ethanol radical was formed...
only via hydroxyl radicals in the former (DETA-PAC) and mostly from other sources in the latter (EDTA). Similar experiments were carried out in the presence of ADP and phosphate alone. These results including the effect of Fe3+ are summarized in Table II. We can see in this table that the addition of Fe3+ increased the amount of trapped spins in the phosphate system and also that the addition of ethanol increased the total spin adducts by 4.2 times in the presence of ADP.

One more series of experiments was performed under experimental conditions similar to those used by Minotti and Aust (4). In Fig. 6, the amount of spin adducts was plotted against the Fe2+ concentration at a fixed H2O2 concentration of 100 μM and in the presence of phosphate buffer alone. We observed broad maxima of the spin adduct accumulation at a Fe2+ concentration equimolar to H2O2. The decreases observed above that concentration can be explained in terms of reduction of the spin adducts by excess Fe3+ ions. In the presence of 200 μM Fe3+, DMPO-OH decayed in about 1 min nearly to zero, and its concentration plotted in Fig. 6 was a maximum accumulated value. Under these conditions, the addition of 100 μM Fe3+ increased the spin adducts nearly 2-fold both in the presence and absence of ethanol. It is well known that the reductive decomposition of DMPO-OH by mitochondria (42) and superoxide anion (43) can take place. We found that excess Fe2+ ion was also able to reduce DMPO-OH at fairly high rates, the approximate rate constants being listed in Table I. Under the experimental conditions where [H2O2] ≫ [Fe2+], the decay of DMPO-OH by Fe2+ appeared to be negligibly small in most cases.

Our conclusion that Fe(II)-DETA-PAC is an efficient Fenton reagent in producing hydroxyl radicals clearly contradicts that of Rahhal and Richter (9), who suggest no hydroxyl radicals are formed in this system. Analyzing the effects of methanol and t-butylalcohol in the reaction of Fe(II)-DETA-PAC with H2O2, they have concluded that the oxidizing species produced in the reaction is not the hydroxyl radical, but an iron-oxo species such as the ferryl ion.

This difference in conclusion is serious since their experimental conditions are very similar to ours. Their method of analysis for this reaction system is, however, quite different from ours. They derive their conclusion indirectly from a kinetic and stoichiometric analysis of the overall reaction assuming a variety of steps that might be involved in the Fenton reaction. Therefore, the analysis becomes inevitably complicated and assumptive. On the other hand, the ESR spin trapping method we have employed involves a direct measurement of hydroxyl radical production that can result in only one conclusion that indeed hydroxyl radicals are formed in this Fenton reaction as discussed below.

**DISCUSSION**

The advantages of the ESR spin-trapping technique in the analysis of the Fenton reaction are: 1) it is the most direct assay of hydroxyl radicals, providing unequivocal detection and identification of hydroxyl radicals, 2) the assay is very sensitive, and 3) as the trapping rate is very fast, being 3.4 × 10^6 M^-1 s^-1 for DMPO (29) and the trapped spin is relatively stable, the analysis of reaction mechanism becomes simple because the propagation of free radical chain reactions can be eliminated. Therefore, it seems curious why this technique has never been applied in the analysis of the mechanism of the Fenton reaction, which it is believed plays a vital role in our survival and has been disputed for decades as to its primary products (7). The main features of the Fenton reaction disclosed in this study are: 1) the stoichiometry of Reaction 1 can be attained at low Fe2+ concentrations, 2) as the Fe3+ concentration is increased, the efficiency of hydroxyl formation decreases drastically while the oxidizing species of iron predominates except when the iron is chelated with DETA-PAC, 3) a reaction path yielding nonoxidizing species also increases with Fe3+ concentration, 4) the addition of Fe3+ augments the oxidizing species at least in the control phosphate buffer system, and 5) these reaction patterns greatly depend on the nature of iron chelators.

### Table II

<table>
<thead>
<tr>
<th>Chelator</th>
<th>Ethanol</th>
<th>Fe3+</th>
<th>DMPO-OH</th>
<th>DMPO-CH2CHOH</th>
<th>Sum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>100</td>
<td>6.2</td>
<td>10.5</td>
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<tr>
<td></td>
<td>0.165</td>
<td>0</td>
<td>0.43</td>
<td>0.64</td>
<td>1.07</td>
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<tr>
<td>ADP (2 mM)</td>
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<td>100</td>
<td>6.2</td>
<td>14.8</td>
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</tr>
<tr>
<td></td>
<td>0.165</td>
<td>0</td>
<td>0.64</td>
<td>14.8</td>
<td>15.4</td>
</tr>
<tr>
<td>EDTA (200 μM)</td>
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<td>100</td>
<td>5.8</td>
<td>24.2</td>
<td>29.0</td>
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<tr>
<td></td>
<td>0.165</td>
<td>0</td>
<td>0.21</td>
<td>24.2</td>
<td>24.4</td>
</tr>
<tr>
<td>DETAPAC (200 μM)</td>
<td>0</td>
<td>0</td>
<td>0.21</td>
<td>27.1</td>
<td>27.2</td>
</tr>
<tr>
<td></td>
<td>0.165</td>
<td>0</td>
<td>0.21</td>
<td>44.6</td>
<td>44.8</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>2.1</td>
<td>45.6</td>
<td>47.7</td>
</tr>
</tbody>
</table>
some controversial data (51–53). This difference in the rates between \( \text{O}_2 \) and \( \text{H}_2\text{O}_2 \) reduction by one-electron reducing agents will be valid whenever the electron transfer takes place through the outer-sphere mechanism. On the other hand, Fe(II)-chelates reduce \( \text{H}_2\text{O}_2 \) much faster than \( \text{O}_2 \) although the difference is not so large in the case of Fe(II)-EDTA (compare Table I with Fig. 2 of Ref. 54). The relative high rate of the reduction of \( \text{H}_2\text{O}_2 \) by Fe(II)-chelates may be explained in terms of the activation via intermediate complexes shown in Fig. 7. Fe(III)-EDTA forms an intensely purple complex with \( \text{H}_2\text{O}_2 \) (7, 55), but intermediate complexes in Fig. 7 are still assumptive, most of them being cited from Rush and Koppenol (31).

The effect of iron chelators on the Fenton reaction is of particular interest. As the effect varies with the Fe** concentration, the ratios of Reactions 1, 2, and 6 shown in Table III are approximately estimated at Fe** concentrations of 4 to 10 \( \mu \text{M} \). The difference between DETAPAC and EDTA is now very clear. Fe(II)-DETAPAC is a typical Fenton reagent which generates hydroxyl radicals, while Fe(II)-EDTA generates both hydroxyl radicals and ferryl species, particularly at high Fe** concentrations. Another significant difference appears in the rate of the Fenton reaction (Table I). Furthermore, if the reaction starts from the Fe(III)-chelates as is the case in biochemical systems, the rate of their reduction should be taken into consideration. Controversial data on DETAPAC and EDTA effects can mostly be explained on the basis of these features. The redox potential, \( E_c(\text{Fe}^{**}/\text{Fe}^{**}) \) is affected by the presence of chelators (56, 57), but we cannot correlate these data with the mechanistic difference. A structural factor in the formation of complexes with \( \text{H}_2\text{O}_2 \) is probably more important in the selection of reaction paths shown in Fig. 7.

## REFERENCES

Quantitative Analysis of the Fenton Reaction