<table>
<thead>
<tr>
<th><strong>Title</strong></th>
<th>Reaction Rate Constant as Indicator of Antioxidative Activity of Substances against Hydroxyl Radicals Produced by Fenton’s System</th>
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<tr>
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<tr>
<td><strong>Citation</strong></td>
<td>Science Journal of Kanagawa University, 21: 01-06</td>
</tr>
<tr>
<td><strong>Date</strong></td>
<td>2010-06-30</td>
</tr>
<tr>
<td><strong>Type</strong></td>
<td>Departmental Bulletin Paper</td>
</tr>
<tr>
<td><strong>Rights</strong></td>
<td>publisher</td>
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Introduction

About two decades ago, the hazardous effects of excess amounts of active oxygen species (singlet molecular oxygen, superoxide, hydrogen peroxide, hydroxyl radical) in the human body were first observed. For example, Totter reported that cancer may be caused through the denaturation of DNA by superoxide ions or hydroxyl radicals that are not inactivated in tissues\(^1\). Martin et al. and Boyce et al. found that lung damage was caused by hydroxyl radicals\(^2,3\). They also found that desferrioxamine or dimethylthiourea could prevent such damage. At about the same time, it was discovered that some chemical reagents were able to eliminate active oxygen species: metallothionein eliminates superoxide ions and hydroxyl radicals\(^4\); and ascorbic acid is highly effective at eliminating them\(^5\). At present, research is being carried out to find active substances that can eliminate hydroxyl radicals efficiently and also to examine the mechanisms involved in their eliminative effects\(^6-11\). Such substances are sometimes called antioxidants.

Direct electron spin resonance (ESR) observation of hydroxyl radicals is considered difficult due to their unique electronic structure. Indirect ESR observation became possible, however, by the discovery of spin-trapping reagents\(^12,13\). Harbour and Bolton employed 5,5-dimethyl-1-pyrroline 1-oxide (DMPO) in spin-trapping experiments and observed hydroxyl radicals produced by the decomposition of hydrogen peroxide with UV light indirectly as DMPO-OH\(^14\). They also observed the ESR spectrum of DMPO-OOH produced from OOH radicals. By use of the same spin-trapping reagent, Buettner and Oberley studied superoxide ions and hydroxyl radicals produced via an enzyme reaction, and hydroxyl radicals produced in Fenton’s system\(^15\).

These spin-trapping experiments indicated that the activity of a chemical reagent as an antioxidant was related to the reaction rate constant. Marriott et al. determined the rate constant for the formation of DMPO-OH through competitive reactions using the reaction of methanol with hydroxyl radicals\(^16\). Finkelstein et al. also determined the rate constant of the same reaction using the reaction of ethanol with hydroxyl radicals as a reference\(^17\). However,
the expressions employed by these authors involved reaction rates, which are functions of time and difficult to determine experimentally. In photo-chemistry and radiation chemistry, an expression was employed for the same purpose to determine the rate constant through competitive reactions\textsuperscript{18-20}. This expression appears in several reports, for example, that of Bors\textsuperscript{19}. Since its derivation is not given in those reports, however, its reliability and applicability are rather unclear.

For some years we have been studying the antioxidative activity of substance against hydroxyl radicals by spin-trapping ESR, in the hope of being able to represent antioxidative activity quantitatively using the rate constant. We approached this from first principles and obtained an exact expression to determine the rate constant by means of two competitive reactions. An approximation of the expression leads to the well-known expression in Bors’ report. The objectives of our study were to: (1) determine feasibility of representing the antioxidative activity of a substance using the rate constant; (2) elucidate the relationship between antioxidative activity and molecular structure, for example, the existence of specific functional groups; (3) derive an additive rule for antioxidative activity, namely, the representation of the rate constant of a molecule by the sum of the reaction rates of its functional groups; and (4) identify superior antioxidants or ways in which to design them. In this paper, we present our findings pertaining to the (1) above.

**Materials and Methods**

**Theory**

We used an improved Fenton’s system to generate hydroxyl radicals\textsuperscript{21-23}, namely, a reductive decomposition of hydrogen peroxide with a diethylenetriaminepentaacetic acid (DTPA) chelate complex of ferrous ions \( \text{Fe(II)DTPA} \) being used in place of the original ferrous aqua-ion:

\[
\text{H}_2\text{O}_2 + \text{Fe(II)DTPA} \rightarrow \text{OH}^\cdot + \text{OH}^- + \text{Fe(III)DTPA}^+ \quad (1)
\]

Since the direct ESR observation of hydroxyl radicals is difficult, adduct radicals with a spin-trapping reagent, for example, DMPO, were observed indirectly. When an antioxidative substance (SODL) was added to the system, it reacted with hydroxyl radicals competing with DMPO, decreasing the signal intensity of the adduct. The amount of decrease indicates the strength of the antioxidative activity; SODLs with high antioxidative activity caused a much greater decrease in intensity even at low concentrations.

We analyzed this measure of antioxidative activity, that is, representing it using the reaction rate constant. The experiments were carried out in two steps:

1. The first step was carried out without SODL (control experiment) and involved the reaction of hydroxyl radicals with DMPO. It can be expressed by the following two-body reaction:

\[
\text{OH} + \text{DMPO} \rightarrow \text{DMPO-OH} ; \text{rate constant } k_1 \quad (2)
\]

Here, DMPO-OH represents the adduct radical of OH with DMPO, giving a quartet spectrum with an intensity ratio of 1:2:2:1. The reaction formula (2) leads to the rate equation:

\[
\frac{d[\text{DMPO-OH}]}{dt} = k_1[\text{OH}][\text{DMPO}] \quad (3)
\]

It is clear that DMPO-OH after the completion of the reaction is equal to OH produced, unless other substances that react with hydroxyl radicals are present in the system. This can be proved if satisfactory assumptions are made concerning the reaction. One assumption is that the production rate of OH is fast. In that case, assuming that the initial concentrations are such that \( \text{OH}_0 = a \text{ mol/l} \) and \( \text{DMPO}_0 = b \text{ mol/l} \), and that the concentration at a later time \( t \) is \( \text{DMPO-OH} = x(t) \text{ mol/l} \), rate equation (3) leads to:

\[
\frac{dx}{dt} = k_1(a - x)(b - x) \quad (4)
\]

This differential equation can be solved exactly giving the analytical solution \( x(t) \). When the reaction is completed, it holds that \( x(\infty) = a \) for \( a \leq b \), or \( x(\infty) = b \) for \( b \leq a \). In order to measure
the total concentration of OH generated, the condition \( a \leq b \) is required.

(2) The second step was carried out with SODL. In this step, SODL reacted with hydroxyl radicals competitively with DMPO:

\[
\text{OH} + \text{DMPO} \rightarrow \text{DMPO-OH}; \\
\text{rate constant of } k_1 \text{ (5a)} \\
\text{OH} + \text{SODL} \rightarrow \text{SODL/OH}; \\
\text{rate constant of } k_2 \text{ (5b)}
\]

In the reaction of hydroxyl radicals with SODL, several different end products are possible. All these are shown collectively as SODL/OH. The competitive reactions (5a) and (5b) may lead to the rate equations:

\[
\frac{d}{dt} [\text{DMPO-OH}] = k_1 [\text{OH}][\text{DMPO}] \quad (6a)
\]

\[
\frac{d}{dt} [\text{SODL/OH}] = k_2 [\text{OH}][\text{SODL}] \quad (6b)
\]

Assuming the initial concentrations of DMPO and SODL are such that \( \text{DMPO}_0 = b \text{ mol/l, SODL}_0 = c \text{ mol/l,} \) and the concentrations of OH, DMPO-OH, and SODL/OH at a time \( t \) are such that \( \text{OH}_0 = a(t) \text{ mol/l, DMPO-OH}_0 = x(t) \text{ mol/l, and SODL/OH}_0 = y(t) \text{ mol/l,} \) the rate equations (6a) and (6b) lead to:

\[
\frac{dx}{dt} = k_1 a (b - x) \quad (7a)
\]

\[
\frac{dy}{dt} = k_2 a (c - y) \quad (7b)
\]

Taking the ratio of (7a) to (7b), and integrating it with respect to \( dy \), we obtain the following expression without approximation:

\[
\frac{k_2}{k_1} = \frac{\ln(1 - \frac{y}{c})}{\ln(1 - \frac{x}{b})} \quad (8)
\]

It can be shown that this expression holds true even when substances that react with hydroxyl radicals other than DMPO and SODL are included in the system. While expression (8) always holds true, the concentrations at the end of the reactions, \( x(\infty) \) and \( y(\infty) \), were measured in standard experiments. If \( y(\infty) \) could be measured directly, then the first step control experiment may be unnecessary. Since it is difficult to measure \( y(\infty) \) directly, it is evaluated by using the relationship \( y(\infty) = a(\infty) - x(\infty) \), where \( a(\infty) \) represents the total concentration of OH generated. This is equal to DMPO-OH in the control experiment. Then, we have

\[
\frac{k_2}{k_1} = \frac{\ln(1 - \frac{y(\infty)}{c})}{\ln(1 - \frac{x(\infty)}{b})} \quad (9)
\]

Since in standard experiments the initial concentrations of SODL and DMPO are set to much higher values than those of DMPO-OH or SODL/OH, the inequalities \( y(\infty)/c \ll 1 \) and \( x(\infty)/b \ll 1 \) hold true. Then expression (9) can be approximated by:

\[
\frac{k_2}{k_1} \approx \frac{b}{c} \frac{f}{x(\infty)} \quad (10)
\]

Here \( f \), defined by \( x(\infty)/a(\infty) \), is the depletion ratio of DMPO-OH due to the addition of SODL. Expressions (9) and (10) may be interpreted as representing the antioxidative activity by the relative rate constant: high activity results in small \( f \) at low \( c \), giving a large value of the relative rate constant. In order to obtain the rate constant \( k_1 \) through the expressions, the value of \( k_1 \) is necessary as a standard.

Although the derivation of expression (8) is probably not novel, we were unable to find it in the existing literature. An approximate expression (10), on the other hand, can be easily found in reference 19, usually derived by using the steady-state approximation.

**Experimental procedure**

Hydroxyl radicals were generated using an improved Fenton’s system, which is the decomposition of hydrogen peroxide by Fe(II)DTPA\(^{21-23}\). DMPO was used as a spin-trapping reagent (purchased from Labotec, Aldrich, and Sigma Co.). The experimental procedures previously described in reference 23...
were used: (1) In the control experiment, 0.1 mM of an aqueous solution of iron(II) sulfate heptahydrate (50 μl) was mixed with 0.1 mM aqueous DTPA (50 μl) to produce an aqueous solution of Fe(II)(DTPA). Then 50 μl pure water, to adjust concentration, and 50 μl of an aqueous solution of DMPO were added. The pH of the solution was adjusted to 6.4 using hydrochloric acid or aqueous sodium hydroxide. Finally, 1 mM of an aqueous solution of hydrogen peroxide (50 μl) was added. (2) In the measurement with SODL, almost the same procedure as that used in the control experiment was employed, except for the use of a 50 μl aqueous solution of SODL instead of pure water. As SODL, the common organic compounds summarized in Table 1 were used. ESR measurements were started two minutes after mixing. A thin rectangular-type sample cell for an aqueous solution was employed (60 mm × 10 mm × 2 mm, 150 μl in volume). In order to confirm the validity of the analysis described in the previous section, the dependence of the rate constant on the concentration of SODL was examined. The concentration range examined depended on the type of SODL, as is summarized in Table 1. The concentration of DMPO-OH in the control experiments \( a(\infty) \) was determined using the aqueous solution of 4-hydroxy-TEMPO (0.01 mM) as a reference to confirm the applicability of the approximate expression (10).

In order to evaluate \( k_2 \) by expression (10) or (9), a \( k_1 \) value of \( 5.8 \times 10^9 M^{-1} s^{-1} \) was employed. This was determined so as to make the \( k_2 \) value of methanol equal to the recommended one of \( 0.97 \times 10^9 M^{-1} s^{-1} \) previously reported. \(^{17} \) \( k_1 \) values of \( 3.6 \times 10^9 M^{-1} s^{-1} \) and \( 4.3 \times 10^9 M^{-1} s^{-1} \) were also previously reported in references 24 and 25.

**Results and Discussion**

DMPO purchased from the three manufacturers produced almost the same results. The concentration of OH in the control experiment \( a(\infty) \) was determined to be 0.018 mM. This value confirmed that two inequalities \( y(\infty) / c \ll 1 \) and \( x(\infty) / b \ll 1 \) hold true and that the approximate expression (10) can be used for nearly all substances except citric acid, methylamine, and ethylamine. For the latter substances in especially low concentrations, the exact expression (9) was employed in the evaluation of the rate constant.

The rate constants obtained in our experiments are summarized in Table 1 along with those reported in the literature. Almost all of the latter values were taken from previously reported rate constant data\(^{20} \), compiled up to 1986. They were obtained mainly by pulse radiolysis experiments, and none of them used ESR experiments. A few reference data obtained after 1986 have since been added\(^{26} \). The reported values for each substance are shown as a range, since several different values due to the difference in pH or other experimental conditions are published. Our measured rate constant values of many substances fell roughly within the reported ranges. The value for formamide is new. It was reported that the rate constants for acids and bases are dependent on pH: formic acid and acetic acid have greater values at high pH, which suggests that the anions have greater values than the neutral molecules. In the present study we did not carry out pH control nor measure pH dependence to eliminate the possible interference of buffer substances.

**Antioxidative activity**

In this study, we define and characterize effective antioxidants. Considering that rate constants for common substances, for example alcohols, are in the order of \( 10^9 M^{-1} s^{-1} \), we may define effective antioxidants as substances with a rate constant of more than \( 10^{10} M^{-1} s^{-1} \). By this definition, none of the substances in Table 1 is an effective antioxidant. Recently, a rate constant value of in \( 4.62 \times 10^{10} M^{-1} s^{-1} \) has been reported for a substance the catechin group\(^{27} \). A rate constant on the order of \( 10^{11} M^{-1} s^{-1} \) seems unusual. By comparing the antioxidative activity among substances with the same functional group, it may be observed that a substance with more hydrogen atoms has higher activity. This observation suggests a mechanism in which hydrogen abstraction is one of the main reactions of antioxidants with hydroxyl radicals. By accumulating more rate constant data, we hope to determine an additive rule for antioxidative activity.
IC₅₀ has been employed as an index of antioxidative activity, especially when the concentration of an antioxidant is unknown. It is defined as the concentration of antioxidant required to reduce the amount of an active oxygen species by half. Although it is useful for the comparison of activities of antioxidants at a constant concentration of an active oxygen species, it is unsuitable for the comparison of activities at different concentrations of an active oxygen species.

This shortcoming can be seen by analyzing IC₅₀ first by using the approximate expression (10). Applying the definition of IC₅₀ to the expression (10), we have:

$$IC_{50} = \frac{b k_k}{k_2}$$  \quad (11)

This expression shows the dependence of IC₅₀ on the concentration of the spin-trapping reagent b. We analyzed IC₅₀ next by using the exact expression (9). By the same procedure used to obtain formula (11), we obtain the expression:

$$IC_{50} = \frac{a}{2 \left[ 1 - \exp \left\{ \ln \left( 1 - \frac{a}{2b} \right) \frac{k_2}{k_1} \right\} \right]}$$  \quad (12)

This expression shows that IC₅₀ depends upon the concentration of the active oxygen species a in addition to that of the spin-trapping reagent b. In contrast, the rate constant is, by definition, independent of concentration.

### Conclusion

The activity of substances as antioxidants against hydroxyl radicals was studied by electron spin resonance spin-trapping experiments. An exact expression was derived from first principles to represent the antioxidative activity using the reaction rate constant. An approximation to the expression leads to a well-known expression for competitive reactions. Using these expressions, we were able to determine some rate constants for reactions of common organic substances with hydroxyl radicals. The rate constants obtained in our experiments are summarized in Table 1 along with those reported in the literature. Our measured rate constant values of many substances fell roughly within the reported ranges. Two expressions revealed that the IC₅₀ index, an indicator of antioxidative activity, depended on the concentrations of both the antioxidant and a spin-trapping reagent.

### Table 1. Rate constants for the reactions of organic substances or reducing agents with hydroxyl radicals

<table>
<thead>
<tr>
<th>Substance</th>
<th>Concentration /mM</th>
<th>(k) (this work)/(10^9) (M^{-1}) s(^{-1})</th>
<th>(k) (reference)/(10^9) (M^{-1}) s(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>methanol</td>
<td>35-570</td>
<td>0.97</td>
<td>0.78-1.0 (pH 6-10.7) [20]</td>
</tr>
<tr>
<td>ethanol</td>
<td>35-570</td>
<td>1.9</td>
<td>1.7-2.2 (pH 6-11) [20]; 2.1 [26]</td>
</tr>
<tr>
<td>formaldehyde</td>
<td>35-570</td>
<td>0.65</td>
<td>1.0 (pH 1) [20]</td>
</tr>
<tr>
<td>acetone</td>
<td>35-570</td>
<td>0.18</td>
<td>0.083-0.14 (pH 6-7) [20]; 0.21 [26]</td>
</tr>
<tr>
<td>methyl ethyl ketone</td>
<td>18-290</td>
<td>1.9</td>
<td>0.90 (pH 6-7) [20]</td>
</tr>
<tr>
<td>formic acid</td>
<td>35-570</td>
<td>5.1</td>
<td>0.13-4.1 (pH 1-11) [20]</td>
</tr>
<tr>
<td>acetic acid</td>
<td>14-910</td>
<td>0.10</td>
<td>0.0092-0.85 (pH 1-10.7) [20]</td>
</tr>
<tr>
<td>methyl acetate</td>
<td>140-480</td>
<td>0.13</td>
<td>0.12 (pH 6-7) [20]</td>
</tr>
<tr>
<td>ethyl acetate</td>
<td>21-710</td>
<td>0.64</td>
<td>0.40 (pH 6-7) [20]</td>
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<tr>
<td>formamide</td>
<td>35-570</td>
<td>0.22</td>
<td>—</td>
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<tr>
<td>acetamide</td>
<td>8.9-570</td>
<td>0.51</td>
<td>0.19 (pH 5.5) [20]</td>
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References


