# Electron Spin Resonance Study of Amino Acid Radicals Produced by Fenton's Reagent[1]

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Free-radical intermediates in the reaction of the hydroxyl radical with amino acids and structurally related compounds such as alcohols, amine, and carboxylic acids have been studied by ESR spectroscopy using a continuous-flow method. The hydroxyl radical is generated by a ferrous ion-hydrogen peroxide system (Fenton's reagent). ESR spectra with fairly good signal-to-noise ratios are obtained for the first time in a Fenton's reagent system from glycine, valine, leucine, glutamic acid, lysine, methionine, tyrosine, ethylamine, acetic acid, propionic acid, *n*-butyric acid, and succinic acid. Structures, *g*-values, and hyperfine coupling constants of these radicals are tabulated. The analysis of ESR spectra shows that the same radical species are produced mainly in a Fenton's reagent system as in a titanous chloride-hydrogen peroxide system. This is consistent with the existence of the hydroxyl radical in both systems. The observed ratio of $C_2$–H to $C_1$–H proton hyperfine coupling constants[2] gives information concerning steric conformations, freedom in the internal rotations, and *s*-character of the intermediate radicals.

The hydroxyl radical, generated by a ferrous ($Fe^{2+}$) ion-hydrogen peroxide ($H_2O_2$) system (Fenton's reagent),[3] has been found to oxidize a variety of organic substrates.[4,5] A kinetic examination and product analysis have elucidated the various mechanisms of these oxidations of aliphatic[4] and aromatic[5] compounds. Unstable organic radicals produced by Fenton's reagent have been detected successfully by electron spin resonance (ESR) spectroscopy using a continuous-flow method. Much is already known about the unstable radicals from alcohols,[6] aliphatic ethers,[7] alicyclic compounds,[7] and furans.[8] However, little is known about the free radicals obtained by Fenton's reagent from biomolecules other than the amino acids such as α-alanine,[2] β-alanine,[2] serine, aspartic acid, cystine, and cysteine.[9,10]

In the course of investigations on the oxidation of biomolecules, intermediate radicals in the reaction of the hydroxyl radical generated by a titanous ($Ti^{3+}$) chloride–hydrogen peroxide system with amino acids,[10] amino acid derivatives,[11] nitrogen heterocyclic compounds,[12] and dialkylsulfoxides[13] were reported in previous papers. Our experimental study of the oxidation involving the hydroxyl radical has now been extended in the present work to include also the oxidation by Fenton's reagent.

Since the hydroxyl radical is one of the main products of water radiolysis,[14] a chemical reaction system generating the hydroxyl radical could be used to simulate the effects of ionizing radiation on aqueous organic solutions. Recently, Neta and Fessenden[15] reported an ESR spectrum of $NH_2\dot{C}HCOOH$ radical from aqueous acidic solution of glycine irradiated directly in the ESR cavity with high-energy electrons, and the structure of glycine radical is identical with that obtained in this work. Therefore, a Fenton's reagent system might be a good model system of the radiolysis of aqueous solutions of biomolecules.

## Experimental

The experimental arrangements and procedures for the observation of intermediate radicals produced in a Fenton's reagent system were essentially the same as described previously in a $Ti^{3+}$–$H_2O_2$ system.[10-13] The ESR spectra of intermediate radicals were recorded on an X-band spectrometer (JEOL, Model JES-ME-3X) at room temperature within 7 to 13 msec after mixing two reactants. The hyperfine (hf) coupling constants and *g*-values were calibrated by comparison with a sample of potassium peroxylamine disulfonate in aqueous sodium carbonate solution (nitrogen hf splitting, $13.0\pm0.1$ G; $g=2.00550\pm0.00005$).[16] Probable errors in hf coupling constants and *g*-values are 0.1 G and 0.0001, respectively.

All materials were obtained commercially and used without further purification. The concentrations of ferrous sulfate ($FeSO_4\cdot 7H_2O$) and hydrogen peroxide were 0.005M and 0.1M, respectively. Organic substrates were added to both reactants usually in 0.1 to 1.0M concentration according to their reactivity and solubility. Reactant solutions of cystine and

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1) This work was presented at the 10th Symposium on Electron Spin Resonance under the auspices of the Chemical Society of Japan, Osaka, Japan, October 1971.

2) The designations $C_1$ and $C_2$ refer to the position relative to the carbon atom which bears the unpaired electron, while α and β refer to the position relative to that of the carboxyl or hydroxyl group.

3) H. J. H. Fenton, *J. Chem. Soc.*, **65**, 899 (1894).

4) C. Walling and S. Kato, *J. Amer. Chem. Soc.*, **93**, 4275 (1971), and references therein.

5) J. R. L. Smith and R. O. C. Norman, *J. Chem. Soc.*, **1963**, 2897, and references therein.

6) T. Shiga, *J. Phys. Chem.*, **69**, 3805 (1965).

7) T. Shiga, A. Boukhors, and P. Douzou, *ibid.*, **71**, 3559, 4264 (1967).

8) T. Shiga and A. Isomoto, *ibid.*, **73**, 1139 (1969).

9) W. A. Armstrong and W. G. Humphreys, *Can. J. Chem.*, **45**, 2589 (1967).

10) H. Taniguchi, K. Fukui, S. Ohnishi, H. Hatano, H. Hasegawa, and T. Maruyama, *J. Phys. Chem.*, **72**, 1926 (1968).

11) H. Taniguchi, H. Hatano, H. Hasegawa, and T. Maruyama, *ibid.*, **74**, 3063 (1970).

12) H. Taniguchi, *ibid.*, **74**, 3143 (1970).

13) H. Taniguchi, H. Takagi, and H. Hatano, *ibid.*, **76**, 135 (1972).

14) See, for example, B. H. J. Bielski, and A. O. Allen, *Int. J. Radiat. Phys. Chem.*, **1**, 153 (1969).

15) P. Neta and R. W. Fessenden, *J. Phys. Chem.*, **75**, 738 (1971).

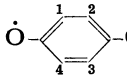
16) J. S. Hyde, unpublished result quoted by J. Q. Adams, J. W. Nicksic, and J. R. Thomas in *J. Chem. Phys.*, **45**, 654 (1966).

aspartic acid with comparatively low solubility at room temperature were prepared and flowed through the cavity of ESR spectrometer at 40 to 50°C. Both reactant solutions were acidified to about pH 2 with dilute sulfuric acid and deoxygenated by bubbling nitrogen gas in order to prevent the oxidation of ferrous ion. In the experiment conducted within a pH region of approximate neutrality, the pH of each reactant solution being adjusted with sodium hydroxide, ethylenediaminetetraacetic acid was added to the reactant solution of ferrous sulfate so as to avoid the precipitation of ferric salt.

## Results and Discussion

*Identification of the Intermediate Radicals.* ESR spectra with sufficient signal-to-noise ratios were obtained from glycine, α-alanine, valine, leucine, aspartic acid, glutamic acid, lysine, cystine, methionine, tyrosine, methyl alcohol, ethyl alcohol, isopropyl alcohol, ethylamine, acetic acid, propionic acid, *n*-butyric acid, and succinic acid. The structures of amino acid radicals deduced from the analysis of the spectra, and the

Table 1. Structures, *g*-values, and hyperfine coupling constants of the intermediate radicals in the reaction of Fenton's reagent with amino acids[a)]

| Substrate | Radical[b)] | Ratio | *g*[c)] | Coupling constant, *G*[c)] $C_1$–H | $C_2$–H | N | $\frac{a_{C_2-H}}{a_{C_1-H}}$ |
|---|---|---|---|---|---|---|---|
| Glycine | $\dot{C}H(NH_2)COOH$ | | 2.0034 | 11.7 | 5.4[d)] | 6.3 | |
| DL-α-Alanine | $\dot{C}H_2$–R | | 2.0026 | 22.3 | 26.2 | 3.4 | 1.17 |
| β-Alanine | $\dot{C}H(COOH)CH_2NH_3^+$ | | (2.0029) | (21.7) | (24.3) | (3.7) | 1.12 |
| DL-Serine | $\dot{C}H(OH)$–R | | (2.0028) | (17.5) | (8.9) | (7.9) | 0.51 |
| L-Valine | $\dot{C}(CH_3)_2$–R | 1.6 | 2.0027 | | (23.4[e)] 7.2 | 7.2 | |
| | $\dot{C}H_2CH(CH_3)$–R | 1 | 2.0025 | 21.7 | 29.9 | | 1.38 |
| L-Leucine | $\dot{C}H_2CH(CH_3)CH_2$–R | | 2.0026 | 21.9 | 21.9 | | 1.00 |
| L-Aspartic acid | $\dot{C}H(COOH)$–R | | 2.0028 | 20.6 | 14.3 | 3.4 | 0.69 |
| L-Glutamic acid | $\dot{C}H(COOH)CH_2$–R | | 2.0032 | 22.5 | 20.5 | | 0.91 |
| L-Lysine | $\dot{C}H(CH_2CH_2NH_3^+)CH_2$–R[f)] | | 2.0026 | 21.9 | 23.9 | | 1.09 |
| L-Cystine | $\dot{S}CH_2$–R | | 2.0102 | | 9.4[g)] | | |
| L-Cysteine | $\dot{S}CH_2$–R | | (2.0108) | | (9.5)[g)] | | |
| DL-Methionine | $\dot{C}H_2CH_2$–R | | 2.0026 | 22.0 | 24.7 | | 1.12 |
| | $\dot{C}H_3$ | | 2.0025 | 22.1 | | | |
| L-Tyrosine |  | | 2.0041 | 6.4[h)] | 1.6[i)] | 7.5[j)] | |

a) See text and Ref. 2 for the notations in columns 1, 5, and 6.
b) R represents $H_3N^+CH(COOH)$–.
c) The values in parentheses were obtained by Armstrong and Humphreys.[9)]
d) $a_{NH}$. e) $a_{C_1-CH_3}$.
f) More than two kinds of radicals exist, but only one can be assigned.
g) $a_{S-CH}$. h) $a_{H_1}=a_{H_4}$. i) $a_{H_2}=a_{H_3}$. j) $a_{CH_2}$.

Table 2. Structures, *g*-values, and hyperfine coupling constants of the intermediate radicals in the reaction of Fenton's reagent with organic compounds structurally related to amino acids[a)]

| Substrate | Radical | Ratio | *g* | Coupling constant, *G* $C_1$–H | $C_2$–H | N | $\frac{a_{C_2-H}}{a_{C_1-H}}$ |
|---|---|---|---|---|---|---|---|
| Methyl alcohol | $\dot{C}H_2OH$ | | 2.0032 | 17.3 | | | |
| Ethyl alcohol | $\dot{C}H_2CH_2OH$ | 1.3 | 2.0027 | 21.8 | 27.4 | | 1.26 |
| | $CH_3\dot{C}HOH$ | 1 | 2.0032 | 15.3 | 22.7 | | 1.48 |
| Isopropyl alcohol | $\dot{C}H_2CH(CH_3)OH$ | | 2.0024 | 22.1 | 22.1 | | 1.00 |
| Ethylamine | $\dot{C}H_2CH_2NH_3^+$ | | 2.0026 | 21.5 | 26.1 | 4.6 | 1.21 |
| Acetic acid | $\dot{C}H_2COOH$ | | 2.0032 | 21.2 | | | |
| Propionic acid | $CH_3\dot{C}HCOOH$[b)] | | 2.0033 | 20.1 | 24.3 | | 1.21 |
| *n*-Butyric acid | $CH_3\dot{C}HCH_2COOH$ | 4.0 | 2.0028 | 21.8 | (25.3[c)] 21.8) | | (1.16 1.00) |
| | $\dot{C}H_2CH_2CH_2COOH$ | 1.3 | 2.0026 | 21.8 | 27.0 | | 1.24 |
| | $CH_3CH_2\dot{C}HCOOH$ | 1 | 2.0034 | 20.1 | 23.7 | | 1.18 |
| Succinic acid | $\dot{C}H(COOH)CH_2COOH$ | | 2.0030 | 20.5 | 22.1 | | 1.08 |

a) See text and Ref. 2 for the notations in columns 5 and 6.
b) See text concerning an unidentified component of propionic acid radicals.
c) $a_{C_1-CH_3}$.

estimated $g$-values and hf coupling constants are summarized in Table 1; $g$-values and hf coupling data of $\beta$-alanine, serine, and cysteine radicals obtained by Armstrong and Humphreys[9] are also shown in parentheses. For comparison, structures, $g$-values, and hf coupling constants of radicals from organic substrates structurally related to amino acids are tabulated in Table 2. The concentration ratio is also given for the substrates where two or three kinds of radicals were observed, although the ratio might depend upon the kinetic conditions. Radicals from twelve substrates listed in the synopsis section were not previously studied by ESR spectroscopy in a Fenton's reagent system. ESR spectra of the intermediate radicals from glutamic acid and tyrosine are reproduced in Figs. 1 and 2. L-Phenylalanine gave an ESR signal with poor signal-to-noise ratio.

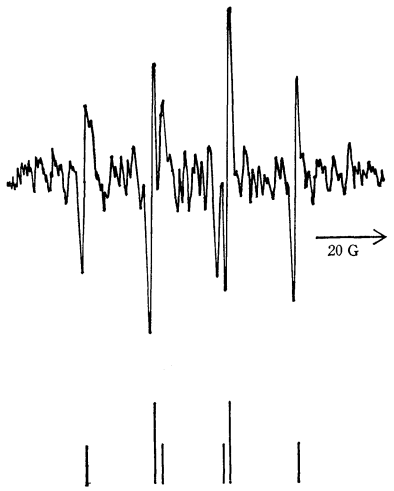



Fig. 1. Electron spin resonance spectrum of the intermediate radical from 0.15M L-glutamic acid with reconstruction. Microwave power, 20 mW; pH, 2.2.

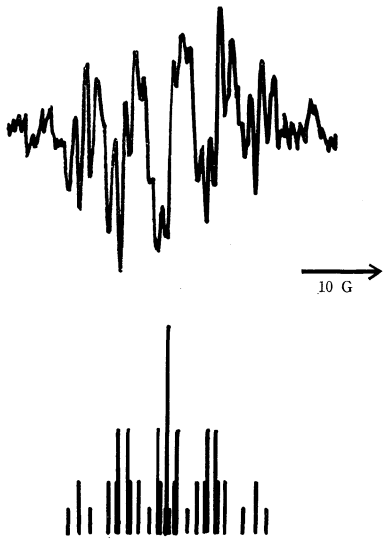



Fig. 2. Electron spin resonance spectrum of the intermediate radical from 0.015M L-tyrosine with reconstruction. Microwave power, 20 mW; pH, 1.7.

Almost all substrates, except aspartic acid, glutamic acid, lysine, and tyrosine which had not been examined in an acidic $Ti^{3+}$–$H_2O_2$ system, were found to give the same radical species as those formed in a $Ti^{3+}$–$H_2O_2$ system.[9,10,13,17,18] This supports the view that the hydroxyl radical is the active species formed in both $Fe^{2+}$–$H_2O_2$ and $Ti^{3+}$–$H_2O_2$ systems. However, there were some differences in ESR spectra between the two systems; minor component of $\alpha$-alanine radicals, $NH_2\dot{C}(CH_3)COOH$,[19] $\alpha$-hydroxy[2] radical of isopropyl alcohol,[17] methyl radical from acetic acid,[20] and $\dot{C}H_2$-$CH_2COOH$ radical from propionic acid[10] which were observed in a $Ti^{3+}$–$H_2O_2$ system, were not recorded in a Fenton's reagent system, while $\beta$-hydroxy[2] radical of ethyl alcohol with poor signal-to-noise ratio in a $Ti^{3+}$–$H_2O_2$ system and an unidentified component of propionic acid radicals consisting apparently of quartet (splitting, 20.0 G) of triplet (22.7 G) were observed in this Fenton's reagent system.

From ethyl alcohol, Shiga[6] detected only the $\beta$-hydroxy radical in a Fenton's reagent system. In contrast, he detected only the $\alpha$-hydroxy radical from ethyl alcohol oxidized with a $Ti^{3+}$–$H_2O_2$ system. He claimed that the active species in Fenton's reagent had different character from that in a $Ti^{3+}$–$H_2O_2$ system. We also reexamined intermediate radicals in the reaction of Fenton's reagent with ethyl alcohol, and an ESR spectrum due to two kinds of radicals was observed, in which the hydroxyl radical abstracted a hydrogen atom from different C–H bonds; *i.e.*, from $C_\alpha$–H and $C_\beta$–H bonds.[2] The apparent concentration ratio of $\alpha$- to $\beta$-hydroxy radicals was 1 : 1.3. Consequently, there is no essential difference in reactivities between the Fenton's reagent and a $Ti^{3+}$–$H_2O_2$ system, and it is supposed that the active species is the hydroxyl radical in both systems. The relative concentrations of the various organic radicals would depend on the one-electron oxidation of the resulting radicals by both metal ions and hydrogen peroxide as Norman and West[21] stated and recently Czapski *et al.*[22] confirmed; *i.e.*, ferric ($Fe^{3+}$) ion is a much stronger oxidizing agent than titanic ($Ti^{4+}$) ion for organic radicals, and the $\alpha$-hydroxy radical is supposed to be oxidized more readily than the $\beta$-hydroxy radical with $Fe^{3+}$ ion. This is applicable to the case of isopropyl alcohol in which an $\alpha$-hydroxy radical was not observed in a Fenton's reagent system. Walling and Kato[4] developed a new kinetic analysis for the oxidation of ethyl alcohol with Fenton's reagent. They showed also that the hydroxyl radical attacked both $\alpha$- and $\beta$-hydrogens of ethyl alcohol, and the $\beta$-hydroxy radical was not oxidized by $Fe^{3+}$ ion but dimerized, while the $\alpha$-hydroxy radical was oxidized.

The $g$-values of radicals with $C_1$–OH and $C_1$–COOH groups such as methyl alcohol, ethyl alcohol, acetic acid, glutamic acid, propionic acid, $n$-butyric acid, and

17) W. T. Dixon and R.O.C. Norman, *J. Chem. Soc.*, **1963**, 3119.
18) H. Fischer, K. H. Hellwege, and M. Lehnig, *Ber. Bunsenges. Phys. Chem.*, **72**, 1166 (1968).
19) P. Smith, W. M. Fox, D. J. McGinty, and R. D. Stevens, *Can. J. Chem.*, **48**, 480 (1970).
20) W. T. Dixon, R. O. C. Norman, and A. L. Buley, *J. Chem. Soc.*, **1964**, 3625.
21) R. O. C. Norman and P. R. West, *J. Chem. Soc., B*, **1969**, 389.
22) G. Czapski, A. Samuni, and D. Meisel, *J. Phys. Chem.*, **75**, 3271 (1971).

glycine, and those of radicals from tyrosine, cystine, and cysteine are higher than the $g$-values of other radicals and in the range of 2.0032—2.0108. This shows that those radicals are $\pi$ electron radicals in which an unpaired electron is delocalized on oxygen or sulfur atom which has a fairly large spin-orbit coupling constant and lone pairs of electrons.

ESR spectra with good signal-to-noise ratios were obtained from 0.01M cystine and 0.015M tyrosine, whose concentrations were much lower than in the case of the other organic substrates. It is suggested that the reaction mechanism and reactivity of the hydroxyl radical with cystine and tyrosine are different from those with other amino acids. As for tyrosine, the ESR spectrum of the corresponding phenoxy radical was the only one observed as shown in Fig. 2. It was not observed in the absence of ferrous ion. Therefore, tyrosine radical is postulated to be formed in the reaction with the hydroxyl radical and/or ferric ion, either abstracting a hydrogen atom from the hydroxyl group or removing an electron from tyrosine itself. The coupling constants of ring protons in the tyrosine radical are in good agreement with those of $p$-cresol radical obtained by the oxidation with $Ce^{4+}$ ion.[23] Cystine radical might be formed by the addition of the hydroxyl radical to sulfur atom and consequent cleavage of sulfur-sulfur bond. This is a similar mechanism to the case of methionine radicals. From methionine, ethyl type radical and methyl radical were observed resulting from the cleavage of carbon-sulfur bond, as is the case in a $Ti^{3+}$–$H_2O_2$ system.[13]

In the case of lysine, ESR spectrum was obtained at pH 4, which was similar to that observed at pH 2. Since two protonated amino groups deactivate adjacent C–H bonds towards the electrophilic hydroxyl radical,[10] the hydrogen abstraction with the hydroxyl radical is thought to occur mainly at the $C_\gamma$–H bond in the side chain.

*Hyperfine Coupling Constants.* It is well established that the $C_1$–H coupling constant, $a_{C_1-H}$, of a $\pi$ electron radical with a planar configuration of bonding orbitals around the $C_1$ carbon atom is proportional to the unpaired spin density on the $C_1$ atom, $\rho_{C_1}$.[24] The

$$a_{C_1-H} = Q_{C_1-H}\rho_{C_1} \quad (1)$$

$C_2$–H coupling arises from the hyperconjugative interaction between the unpaired $2p_z$ orbital and the $C_2$–$H_{3-n}R_n$ group, and the semi-empirical relationship (2) has been used to correlate the $C_2$–H coupling constant, $a_{C_2-H}$, with the conformation of free radicals.[25]

$$a_{C_1-H} = \rho_{C_1}(B_0 + B_2\cos^2\theta) \quad (2)$$

The angle $\theta$ is the dihedral one between the $p$-orbital containing the unpaired electron and the $C_1$–$C_2$–H plane. In most cases,[26] $B_0$ is assumed to be negligible relative to $B_2$.

$$a_{C_1-H} = \rho_{C_1}B_2\cos^2\theta \quad (2)'$$

From the above Eqs. (1) and (2)′, the ratio of $C_2$–H to $C_1$–H coupling constants is expressed as Eq. (3).

$$a_{C_2-H}/a_{C_1-H} = B_2\cos^2\theta/Q_{C_1-H} \quad (3)$$

The value of $|B_2/Q_{C_1-H}|$ for an aliphatic $\pi$ electron radical is estimated experimentally to be 1.20 based on the proton hf coupling constants of the ethyl radical[26] in which the methyl group rotates freely around the $C_1$–$C_2$ bond so that $\cos^2\theta = 1/2$. Therefore, the radical with the ratio of $a_{C_2-H}$ to $a_{C_1-H}$ deviating much from 1.20 is supposed to take a restricted conformation in which the internal rotation of the $C_2$–H bond around the $C_1$–$C_2$ axis is hindered much, or is assumed to have some $s$-character in the orbital of an unpaired electron on $C_1$ atom.

The ratios of $a_{C_2-H}$ to $a_{C_1-H}$ obtained in the present investigation are shown in column 6 of Tables 1 and 2. Looking at the structures of radicals with ratios more than 1.32 or less than 1.08, we notice that in a group consisting of radicals from aspartic acid (ratio, 0.69), glutamic acid (0.91), $n$-butyric acid (1.00), isopropyl alcohol (1.00), leucine (1.00), and valine (1.38), the bulky methyl groups, a protonated amino group, and a carboxyl group will severely restrict the internal rotation of $C_2$–H bond around the $C_1$–$C_2$ axis. In the α-hydroxy radical of ethyl alcohol (ratio, 1.48), a large value of the ratio may be ascribed to a somewhat nonplanar configuration around $C_1$ and the consequent $s$-character giving small $C_1$–H coupling constant.[27] For the serine radical (ratio, 0.51), a small value of the ratio might be due to the hindered internal rotation probably because of the formation of an intramolecular hydrogen bond between the hydroxy group and $NH_3^+$ and/or COOH.

Thus, the ratio of $a_{C_2-H}$ to $a_{C_1-H}$ is expected to become an additional measure concerning steric conformations, freedom in the internal rotations, and planarities of organic radicals.

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23) T. J. Stone and W. A. Waters, *Proc. Chem. Soc.*, **1962**, 253.
24) H. M. McConnell, *J. Chem. Phys.*, **24**, 632, 764 (1956).
25) C. Heller and H. M. McConnell, *ibid.*, **32**, 1535 (1960).
26) R. W. Fessenden and R. H. Schuler, *ibid.*, **39**, 2147 (1963).
27) A. J. Dobbs, B. C. Gilbert, and R. O. C. Norman, *J. Chem. Soc., A*, **1971**, 124.