DAEHAN HWAHAK HWOEJEE
(Journal of the Korean Chemical Society)
Vol. 15, Number 1, 1971
Printed in Republic of Korea

AN ESR STUDY OF AMINO ACID AND PROTEIN FREE RADICALS IN SOLUTION*

PART I. Reaction Mechanism of Free Radical Production in the Ti-H_2O_2 Flow System

by

Sun-Joo Hong and L. H. Piette**

Department of Biochemistry and Biophysics, University of Hawaii, Honolulu, Hawaii, 96822, U. S. A

(Received Feb. 1, 1971)

용액에서의 아미노산 및 단백질 자유기에 관한 ESR 연구

제 1 보 : Ti-H_2O_2 Flow System에서 자유기를 만드는 반응기구

홍 순 주 · L. H. Piette

미국 하와이 대학교 생화학 및 생물리학과

(1971. 2. 1 접수)

ABSTRACT

The reaction of amino acids and the reactive hydroxyl radical generated by $Ti^{3+}-H_2O_2$ system was studied using fast flow techniques coupled with ESR.

Upon adding methionine to the 0.2M H_2O_2 solution (0.05M methionine after addition) and mixing with 0.01M $TiCl_3$, the low field component of the two incompletely resolved peaks, in the spectrum of $Ti^{3+}-H_2O_2$ system alone, vanished completely whereas the high field component remained almost constant and superimposed on the secondary spectrum of the methionine free radical. Similar results were obtained for other amino acids and proteins. The results strongly demonstrate that the $T^{3+}-H_2O_2$ flow system generates two different radical species, only one of which, giving rise to the low field component, is alone responsible for abstracting hydrogen atoms from substrate molecules.

The effects of HCl, H_2SO_4 and NaOH on the system were also studied with widely varying results.

INTRODUCTION

The development of fast flow techniques has opened a new experimental approach for ESR study of short-lived free radicals in the liquid state.

* This work was supported by U. S. Public Health Service research grant.

** Work reported is a portion of M. S. dissertation. Present address; Department of Agricultural Chemistry, Kang-Won National University, Chun-Chon, Korea.

The first attempt to adapt a continuous flow method[1] to obtain ESR of free radicals in aqueous solutions was reported by Yamasaki, Mason and Piette[2] in 1959. They demonstrated that free redicals in enzyme catalysed oxidations of substrates can be identified[2)-5)] by mixing two reactants immediately before they flow through an aqueous cell situated in the cavity of the spectrometer. Successively, Piette[6),7)] and Borg[8),9)] developed an improved flow system of the type first used, which required high consumption of materials[10)-16)] and the modifications resulted in reduced dead time and provided greater signal-to-noise resolution from free radical products of very short life times.

Dixon and Norman[10),17)], on the other hand, have shown that radicals produced during the rapid reduction of H_2O_2 by Ti^{3+} can be used to generate organic free radicals by abstracting a hydrogen atom from the molecules. The reaction likely to produce primary radical is,

$$Ti^{3+} + H_2O_2 \rightarrow Ti^{4+} + OH^- + \cdot OH \quad (1)$$

The identity of the radicals produced by this Ti-H_2O_2 flow system, and the effect of acid on this radicals have been objects of much debate[6)-10),11),12),18)], but the formation of two different radical species has been confirmed[6),11),12),19)]. However, which of these two radical species is responsible for abstraction of a hydrogen atom from organic molecules has not yet been conclusively determined.

It was the aim of this investigation[20)] to ascertain, by the use of newly modified Piette's flow-mixing chamber and reaction cell (Fig. 1), the radical species responsible for the hydrogen atom abstraction from organic molecules. In addition, the effects of acid and alkali on the system were also studied with widely varying results.

EXPERIMENTAL

MATERIAL: $TiCl_3$, 20% solution in HCl, was obtained from K & K Laboratories, Hollywood, California. Hydrogen peroxide was Baker analysed reagent. All amino acids were obtained as crystalline powders of the highest purity available from Sigma Chemical Company., St. Louis, Missouri, and used without further purification.

IN SITU GENERATION OF FREE RADICALS: The flow system used by Piette[3)] was applied, in this experiment, to a Varian ESR spectrometer with a newly designed mixing cell. It was capable of both stopped and continuous flow operation. Continuous flow was obtained by using nitrogen gas as the driving force which was connected to a mercury pressure gauge which directly read the applied pressure. Stopped flow was achieved with three microsolenoid valves, one placed at the exit of the spectrometer cavity and the other two at the entrance arms of the mixing cell.

The two reactants are housed in reservoir liter bottles which are connected through valves to air powered syringes, and at the bottom of each of these is an electrically operated valve. From these bottles, the separate liquids pass through manually operated valves which serve to fix the rationing of reactants into the mixing chamber where the reaction takes place. The newly formed products and intermediates then flow through the quartz mixing cell which is fixed in the detection cavity of the ESR spectro meter.

Fig. 1 shows the quartz, liquid-flow, mixing chamber and reaction cell which is modified Piette's cell[17)]. The mixing chamber with two tangent jets and flow cell is of one piece construction eliminating the necessity for flexible

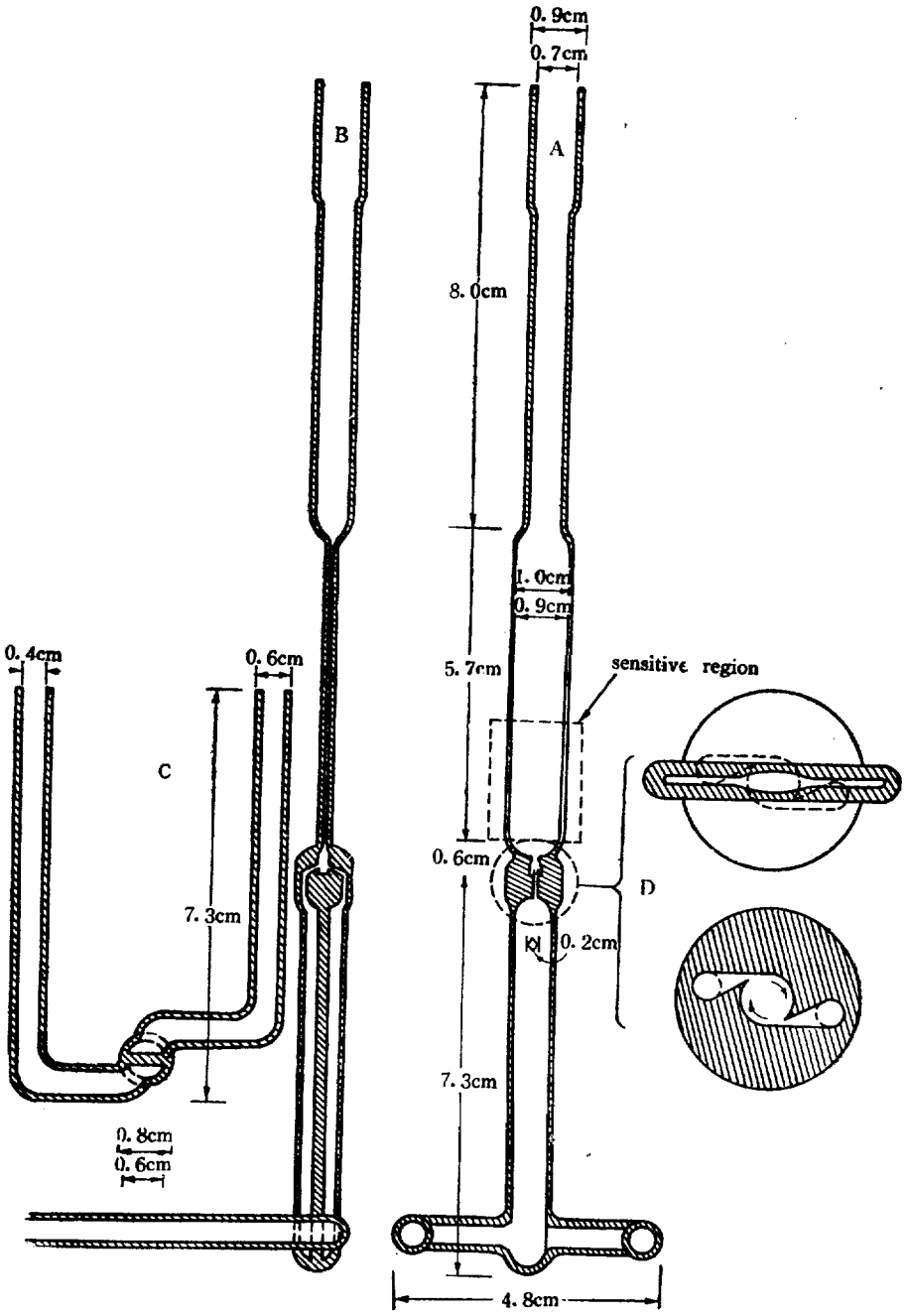

Fig. 1 DRAWING OF QUARTZ, FLOW-MIXING CELL: (A) front view; (B) side view; (C) bottom view; (D) cross-section of the entrance of the flat cell (above) and the mixing-chamber (below).

connections. The jets are off set so as to impart a swirling motion to the reactant streams as they mix. The reaction chamber itself is fashioned as close as possible to the bottom of the flat cell. The mixing cell is easily positioned in the V-4531 cavity using the Varian V-4548 aqueous sample cell holder.

The flow rate was varied between 1-11 ml. sec.$^{-1}$ as measured at the exit tube, by pressuring the reactant reservoirs with nitrogen and adjusting the manually operated valves. The dead volume from mixing to observation was less than 0.05 ml.

The sample handling operations consisted of preparing dilute solutions of H_2O_2 and $TiCl_3$, dissolving amino acids of interest in the solutions, and then flowing the solutions at room temperature through a mixing cell located in the microwave cavity. In the course of flow, OH radicals generated by the reaction of $TiCl_3$ with H_2O_2 react with the solute, forming solute free radicals.

ESR SPECTROSCOPY: The ESR measurements were made using a Varian model V-4500 spectrometer. The spectrometer operates at a nominal frequency of 9.5kMc./sec., employs 100kc./sec. modulation and phase sensitive detection, and records the first derivative of the absorption signal on Varian F-80 XY recorder.

RESULTS AND DISCUSSION

The spectrum obtained by mixing equal volumes of 0.01M $TiCl_3$ and 0.2M H_2O_2 aqueous solution and flowing directly into the ESR cavity is shown in Fig. 2a. The spectrum consists of two incompletely resolved peaks instead of the one reported by Dixon and Norman[10]. A two peak ESR spectrum obtained by a similar technique has been reported[6),11),18)] but the relative intensity of the two peaks and shape of the signals were somewhat different.

It is now generally understood that the spectrum can be changed by varying the experimental factors such as pH of the system, flow rate, relative concentration of the reactive species in the system, and the temperature.

In the present work, it was found that addition of a small amount of H_2SO_4 (0.05ml of concentrated H_2SO_4/l of 0.2M-H_2O_2 solution) before mixing enhances the low field component of the spectrum (Fig. 2a) nearly two-fold whereas the high field component was reduced rapidly. Further addition of the acid (0.5ml/l) produced a singlet with a maximum intensity which was followed by a gradual decrease in signal intensity. On the other hand, the intensity of the original spectrum (Fig. 2a) is diminished by approximately one-third (Fig. 2b) after adding 10ml/l of concentrated HCl to a 0.2M H_2O_2 solution. This effect was also observed by Turkevich et al[18)] and was explained by Dixon and Norman[10)] as a complex formation by chloride ion. On the contrary, wall et al[11)] reported that the high field component, which was attributed to OH-Ti^{4+} complex, is independent of chloride concentration. The successive addition of HCl to the H_2O_2 solution before mixing in the present experiments, however, caused a gradual diminishing of the total spectral intensity with the high field component dropping faster than the low field peak.

Upon adding methionine to the H_2O_2 solution (0.05M methionine after addition), the low field component (see trough P) in the original spectrum (Fig. 2a) vanished completely whereas the high field component (see trough q) remained almost constant and superimposed on the secondary spectrum of the methionine free radical (Fig. 2c).

This superimposed spectrum was also observed

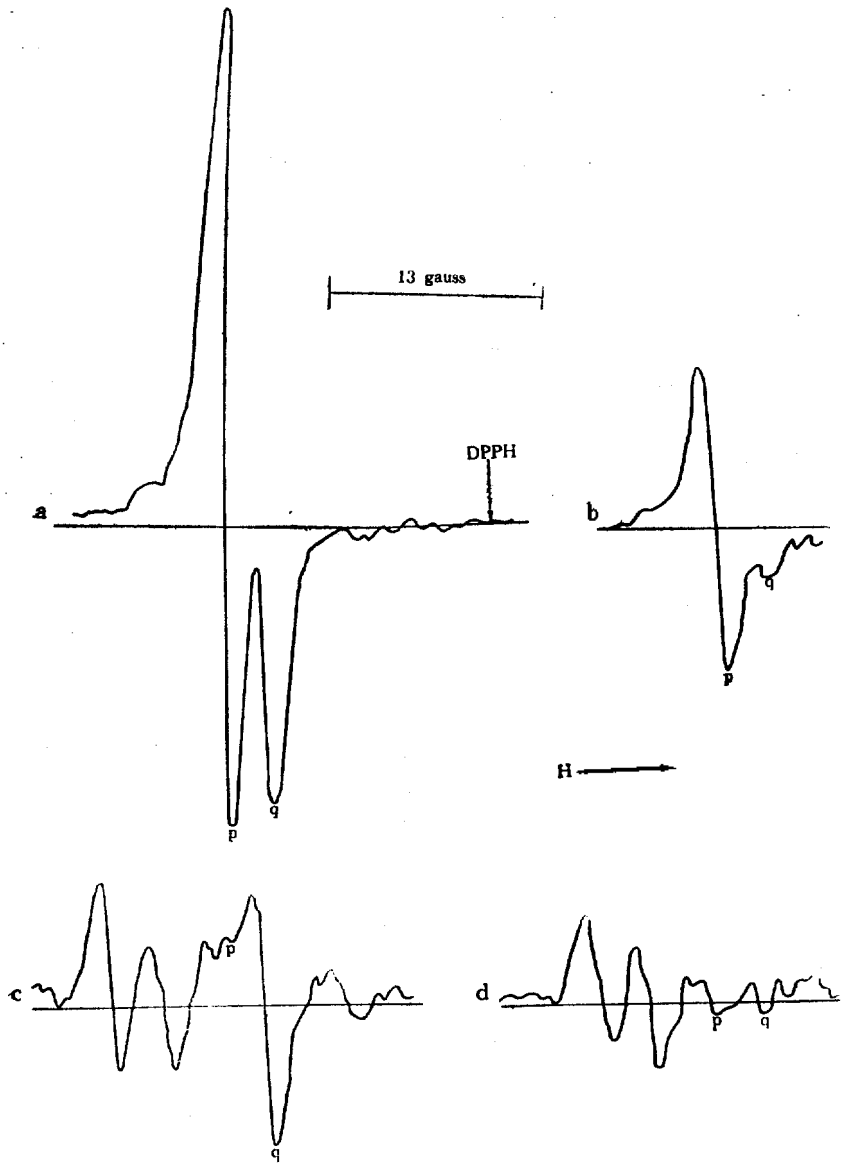

Fig. 2. SPECTRA OBTAINED BY FLOW-MIXING OF TITANIUM CHLORIDE AND HYDROGEN PEROXIDE:

(a) original spectrum obtained by mixing equal volumes of 0.01M $TiCl_3$ and 0.2M H_2O_2 aqueous solution; (b). spectrum obtained by mixing original solution pair where H_2O_2 solution contains 10ml/l of concentrated HCl; (c). spectrum obtained by mixing original solution pair where H_2O_2 solution contains 0.05M methionine; (d). spectrum obtained by mixing solution pair (b) where H_2O_2 solution contains 0.05M methionine. g-values of the low and high field component in the original composite spectrum are 2.0128 and 2.0114, respectively.

for the other amino acids and proteins which were investigated in the present experiments, by adjusting the relative concentrations of each component of the system.

Although the present experiments fail to identify the oxidizing species, the results strongly demonstrate that the Ti-H_2O_2 flow system generates two different radical species, only one of which, giving rise to the low field component, is responsible for producing the amino acid free radical. The behavior of the other radical species was found to be much more complicated in agreement with the previous results[11]. It will therefore be tentatively assumed that the low field component is due to a reactive OH-radical complex and the high filed component is the spectrum of a non-reactive OH-associated titanic complex.

The most plausible explanation for the effect of acid employed would then seem to be destruction of the nonreactive OH-Ti^{4+} complex. Destruction of the complex by H_2SO_4 addition may result in the release of the reactive OH-radical complex and causes the improvement of the low field peak intensity assigned to that species. For the case of HCl addition, on the other hand, there seems to be a strong reducing effect on the reactive species presumably due to the reaction[21]:

$$\cdot OH + H^+ + Cl^- \longrightarrow \cdot Cl + H_2O \qquad (2)$$

in addition to the complex destruction effect. If the two reactions are assumed to proceed at approximately the same rate, the reactive OH-radical complex concentration may not be changed ver much up to a certain extent, and the radical can be used to produce a constant amount of substrate free radical. Spectrum (d) in Fig. 2 strongly supports this assumption. The signal intensity of the methionine free radical in (d) is not changed from that of spectrum (c) in spite of the signal intensity decrease of the primary radical in (b) to almost one-third of that in (a). Furthermore, no low field component of the primary spectrum was superimposed on the secondary spectrum of the methionine free radical, but trough (q) remained. Chlorine atoms in solution, in contrast to the reactive OH-radical, cannot abstract H-atoms from saturated compounds.[21]

Low concentrations of NaOH were found to enhance the total signal intensity without any observable changes in shape. However, upon increasing the amount of NaOH, the trough (q) was gradually smeared out with reduced total signal intensity. Finally the signal became a singlet. NaOH could facilitate the formation of OH-Ti^{4+} complex but a higher production of OH-radical by adding NaOH can hardly be expected due to the reaction (1).

A low $[Ti^{3+}]/[H_2O_2]$ ratio improved the intensity of the low-field component while that of the high field component was enhanced under the condition of high $[Ti^{3+}]/[H_2O_2]$ ratio. A similar result was obtained by others[6,11]. In order to investigate this effect more completely, an expriment was carried out in which the two extreme cases could be examined. When $[Ti^{3+}]/[H_2O_2]$ is far higher (0.01 M $TiCl_3$—0.01M H_2O_2), than usual (0.01M $TiCl_3$—0.2M H_2O_2), a narrow, sharp single line was observed only after stopping the flow, whereas in the usual case the spectrum in Fig. 2a is seen only during flow at the dead time of 0.01 second. The appearance of this singlet occurs in a short time after stopping flow and was followed by a slow decay. On the other hand, a very low $[Ti^{3+}]/[H_2O_2]$ ratio in the system, 0.002M $TiCl_3$—0.2 M H_2O_2, presented the singlet only during steady flow mixing. If the previously discussed $[Ti^{3+}]/[H_2O_2]$ ratio effect on the spectrum is extended further to these extreme cases, the radical species generated under very high $[Ti^{3+}]$

$/(H_2O_2]$ ratio could primarily correspond to the high field component and that under very low $[Ti^{3+}]/(H_2O_2)$ to the low field component. To investigate this, the spectra under the two extreme conditions of $[Ti^{3+}]/(H_2O_2]$ were recorded with the same instrumental setting. The singlet in each case was found to occur at the same magnetic field position with similar line widths and intensities. No change in the magnetic field position of absorption seems to run counter to the previous assumptions that the two radical species are not identical. However, the addition of methionine to the high $[Ti^{3+}]/[H_2O_2]$ system presented neither the spectrum of methionine nor the primary radical during or after stopping flow. Whereas, methionine enhances thes inglet intensity in the low $[Ti^{3+}]/(H_2O_2]$ system. This fact is consistent with the previous work[18] that adding small amounts of the substrate increased the intensity of the low field peak (see Fig. 2a). This was occasionally observed for all amino acids in the present work when the relative concentration of the reactive species are not sufficient to produce a secondary spectrum of the substrate free radical. This result presents further evidence that the radical species generated by very low $[Ti^{3+}]/(H_2O_2]$ primarily consists of the low field component (Fig. 2a). The explanation of the results in the high $[Ti^{3+}]/[H_2O_2]$ system seems to involve a more complicated mechanism. This complexity was also observed for the case of other amino acids which will be discussed later.

The amount of experimental information is too small to give a more detailed description of the identity of the radical species generated by the $Ti-H_2O_2$ flow system and the reaction mechanisms in this system. However, it is now clear that the $Ti-H_2O_2$ flow system generates two different kinds of radical species the low field component of which is alone responsible for abstracting H-atoms from substrate molecules.

REFERENCES

1) H. Hartridge and F. J. W. Roughton, *Proc. Roy. Soc.*, *A*, **104**, 376 (1923).
2) I, Yamazaki, H. S. Mason, and L. H. Piette, *Biochem. Biophys. Research Commun.*, **1**, 336 (1959).
3) I. Yamazaki, H. S. Mason, and L. H. Piette, *J. Biol. Chem.*, **235**, 2444 (1960).
4) H. S. Mason, E. Spencer, and I. Yamazaki, *Biochem. Biophys. Research Commun.*, **4**, 236 (1961).
5) I. Yamazaki, and L. H. Piette, *Biochem. Biophys. Acta.*, **50**, 62 (1961).
6) L. H. Piette, G. Bulow and K. Loeffler, Preprint of the *Am. Chem. Soc.*, **9**, No. 2-c, P. c-9 (1964)
7) L. H. Piette in "Rapid Mixing and Sampling Techniques in Biochemistry" B. Chance et al (eds), Academic Press, New York (1964).
8) D. C. Borg, Nature, 201, 1087 (1964).
9) D. C. Borg, in "Rapid Mixing and Sampling Techniques in Biochemistry" B. Chance et al (eds), Academic Press, New York (1964).
10) W. T. Dixon and R. O. C. Norman, *J. Chem. Soc.*, **572**, 3119 (1963).
11) F. Sicilio, R. E. Florin, and L. A, Wall, *J. Phys. Chem.*, **70**, 47 (1966).
12) T. Shiga, *ibid.*, **69**, 3805 (1965).
13) P. Smith, J. T. Pearson, P. B. Wood, and T. C. Smith, *J. Chem. Phys.*, **43**, 1535(1965).
14) T. Stone and W. A. Waters, *Proc. Chem. Soc.*, 253 (1962).
15) P. L. Kolker, *J. Chem. Soc.*, 5929 (1964).
16) M. F. R. Muleahy, J. R. Stenven, and J. C. Ward, Aust. *J. Chem.*, **18**, 1177 (1965)
17) W. T. Dixon and R. O. C. Norman, *Nature*, **196**, 891 (1962).
18) Y. S. Chiang, J. Craddock, D. Mickewich,

and J. Turkevich, *J. Phys. Chem.*, **70**, 3509 (1966).

19) D. C. Borg and J. J. Elmore, Jr. "Magnetic Resonance in Biological System" Wenner-Gren Center International Symposium Series, **9**, 383 (1967).

20) Sun-Joo Hong, Abstracts, 1st ACS. Hawaiian Section Annual Meeting-In-Miniature, Honolulu, Hawaii, Paper No. 4 (1968).

21) J. F. Ward, and L. S. Myers, Jr. *Radiation Res.*, **26**, 483 (1965).