# Electron Spin Resonance Studies on the Mechanism of Enzyme Inactivation by Direct Action of Gamma Radiation*

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Electron spin resonance spectra of a sulfhydryl enzyme, urease, and a non-sulfhydryl enzyme, bacterial proteinase, were observed after exposure to irradiation of $\gamma$-rays. Urease showed a characteristic pattern to a sulfhydryl protein and bacterial proteinase to a non-sulfhydryl protein.

A quantitative relationship among inactivation of the proteinase, intensity of ESR signal of the enzyme and irradiation dose of $\gamma$-rays, was measured. Whereas in an early process of the inactivation any significant increase of the intensity of ESR pattern was not observed, a parallel relationship between the inactivation of the enzyme and the increase of free radicals was found in a range from 100 kiloroentgen dose to 10 megaroentgen dose of $\gamma$-rays and no significant increase of the free radicals was found contrary to further progress of the inactivation of the enzyme in the course of the irradiation of larger doses than 10 megaroentgens. Possibility of conduction of electron vacancy from a damaged area of the enzyme molecule to an active center on the enzyme, and a inactivating mechanism by direct action of gamma radiation, were proposed and discussed.

## INTRODUCTION

It has been proposed that in the primary process of radiation effects on biological materials, electron vacancies were resulted on the molecules of constituent compounds of the materials and could be conducted along chemical bonds to other area of the materials. Resulting unpaired electrons in its structural entities have been demonstrated in the electron spin resonance studies about many biochemical compounds and biological materials after exposure to irradiation of ionizing radiations.

Many kinds of free radicals which have unpaired electrons, were reported on irradiated molecules of amino acids[1,2], peptides[3], proteins[1,4], carbohydrates[5,6], steroids[7], vitamins[7,8,9], nucleic acids[10], enzymes[4] and coenzymes[8], and also includings cellular particles[11] and even tissues[11].

The mechanism of chemical protection from direct effects of ionizing radiation was investigated by Gordy *et al.*[12] and electron spin resonance studies on the sulfur-containing protectors gave an experimental evidence to the protecting

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mechanism to radiation-induced damages of certain proteins.

It has been established that inactivation of enzymes in solution by ionizing radiations is caused by the indirect action of the radiation in aqueous solutions[13~17]. The mechanism of inactivation of enzymes in solid by irradiation, however, has been left obscure except to a treatment by the direct hit theories of Lea and of Pollard.

The present paper deals with the evidences that in the primary stage of enzyme inactivation by $\gamma$-irradiation, the effect was attributed to the electron vacancies which were produced in irradiated enzyme molecules in solid and were detectable by electron spin resonance spectroscopy.

## EXPERIMENTAL

**Materials.** Enzyme preparations of urease were used in this experiment as a specimen of a sulfhydryl enzyme. The urease was prepared in crystalline from Japanese Jack Beans using the method described by Kobashi *et al.*[18]. Specific activity of the urease preparation was $6.8\times10^4$ Sumner units per gram of protein. The crystalline urease was liophylized in vacuo. An another specimen of a non-sulfhydryl enzyme was crystalline bacterial proteinase prepared from *Batillus subtillis* and supplied by Daiwa Kasei Co. Ltd., Osaka. This enzyme was characterized as a non-sulfhydryl enzyme by Ganno[19]. The proteinase had its proteolitic activity of $1.3\times10^6$ $[PU]_{\gamma Ty\gamma}^{Cas,FRB}$. Preparation of "solid solution of an enzyme and a protector" was preformed according to the method of Gordy *et al.*[12].

**Methods.** Irradiation of $\gamma$-rays: For $\gamma$-irradiation the two-kilocurie cobalt-60 gamma ray irradiation facility[20] was used in this experiment. The dose rate was approximately 140 kiloroentgens per hour. The liophylized preparation of the enzyme was put into the $4\times200$ mm glass tubes for an electron spin resonance measurement described below, and sealed in vacuo. The glass tubes were exposured to $\gamma$-irradiation of apropriate doses of $\gamma$-rays at room temperature.

Measurement of electron spin resonance: An electron spin resonance spectrometer Model JES-3B of Japan Electron Optics Co. Ltd., Tokyo, was used in this experiment. All measurements were made at a frequency of 9,000 megacycle per sec. (X-band) and all resonances were shown as the curves of first derivatives of the actual absorption (ordinate) versus the magnetic field variation (abscissa) at a constant frequency. Electron spin density of the irradiated preparations was calculated relatively from that of DPPH or $\gamma$-irradiated polyethylene under the same experimental conditions.

## RESULTS AND DISCUSSION

A typical ESR spectrum of $\gamma$-irradiated sulfhydryl enzyme, urease, was presented in Fig. 1, and that of non-sulfhydryl enzyme, proteinase, in Fig. 2. Fig. 1 illustrates an asymetric pattern like that of powdered cystine which is appeared to be a characteristic of sulfur-containing amino acids and proteins, and much more spread out at about 120 gauss, owing to anisotropy on the g-factor. Fig. 2

shows a symetric doublet resonance with about 83 gauss width, which seems to be a characteristic of non-sulfur containing proteins.

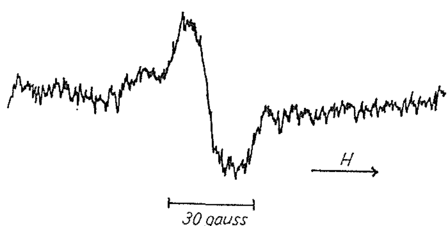



Fig. 1. An electron spin resonance spectrum (a first derivative curve) of $\gamma$-irradiated urease, at 9,000 Mc.

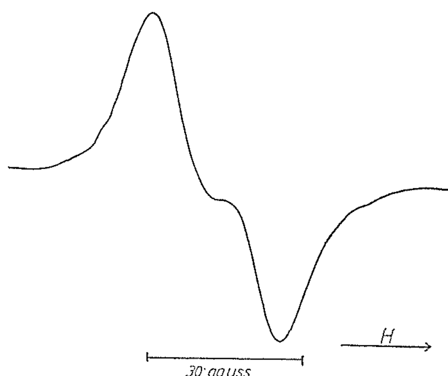
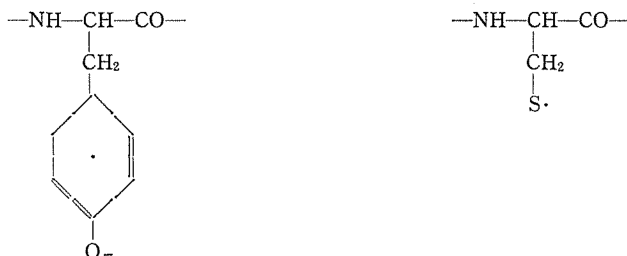



Fig. 2. An electron spin resonance spectrum (a first derivative curve) of $\gamma$-irradiated bacterial proteinase, at 9,000 Mc.

Sulfhydryl enzymes were different from non-sulfhydryl enzymes in many biological properties and especially in the behaviors to irradiation, such as radiation sensitivity and inactivating mechanisms[13~17]. The urease preparation used in this experiment has been established to be a typical sulfhydryl enzyme[14,26] and the bacterial proteinase preparation characterized to be a non-sulfhydryl enzyme which contained no cystine or cysteine residue. Electron spin resonance patterns showed an evidence of characteristic behaviors of sulfhydryl and non-sulfhydryl enzymes in solid to irradiation.

There are two different kinds of regions which form electron traps, in the enzyme proteins as evidenced by the cystine like resonance and a doublet resonance. The electron vacancy produced by $\gamma$-irradiation may be migrated to these traps.

In the previous paper[21] it was shown that the constituent tyrosine in protein was most radiosensitive among the constituent amino acids of the protein contrary to the free tyrosine in solution. From this fact it is considered that the tyrosine residue may be the trap in the damaged protein as rsemble as cysteine residue, where may perhaps be produced the following radicals:




Any electron spin resonance evidence, however, has not yet been shown on the resonance pattern of tyrosine or tyrosine containing protein. It has been discussed[4] that the damaged area which formed traps showing the cystine-like

ESR signal, is probably in an S-S or an S-H bond of a side chain of the SH-enzyme. Another traps to which is due the doublet signal, is possibly in an -N-C-C- linkage along the back bone structure of the enzyme protein.

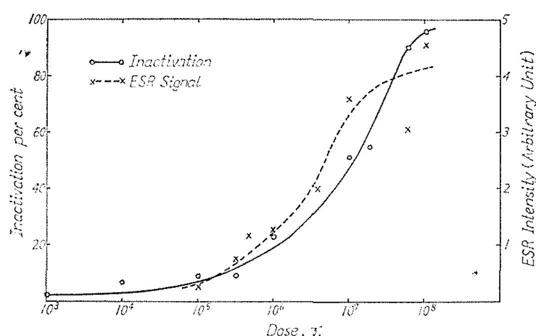



Fig. 3. A quantitative relationship among the inactivation of Bacterial proteinase, the intensity of the ESR signal, and the dose of $\gamma$-rays.

Intensity of the signal shown in Fig. 2, increased with the dose of $\gamma$-irradiation. This means the increase of a radiation damage on the enzyme. Table 1 and Fig. 3 present a quantitative relationship among the inactivation of the enzyme, the intensity of the ESR signal and the radiation dose. In the early stage of the inactivation of the enzyme, significant appearance of ESR signal could not be observed owing to not enough sensitivity of the measurements. In a range of doses from 100 kiloroentgens to 10 megaroentgens a parallel relationship between increase of the ESR signal intensity and the radiation dose, was observed showing that an electron vacancy was conducted to the active area of the enzyme to cause inactivation, though the migration of charge of the molecule has not yet been characterized. There must be effective traps spaced at the active area of the enzyme molecule where the electron vacancy becomes localized or lodged. In their studies of Gordy *et al.*[12], it was presented that when certain proteins in solid solution with an appropriate protector were exposured to X-radiation they showed a characteristic ESR pattern quite different from either that of the protein or that of a protector. In the experiments protecting properties of the compounds used were proposed without any measurements of the protecting powers. Table 1 presents that cysteine and even alanine are possible

Table 1. Protecting effect of several compounds in solid solution to $\gamma$-radiation induced inactivation of crystalline urease.

| Protecting compound | $\gamma$-Ray dose X $10^{-5}$r. | Enzyme activity, Sumner unit | | Inhibition % |
|---|---|---|---|---|
| | | Before irrad. | After irrad. | |
| None | 1.3 | 14.81 | 6.92 | 52.8 |
| Cysteine | 1.2 | 8.20 | 7.27 | 11.3 |
| None | 1.1 | 97.56 | 53.76 | 44.9 |
| Alanine | 1.1 | 86.00 | 60.30 | 29.9 |

to have certain protecting properties to $\gamma$-radiation when they were put in the solid solution of the enzyme. A few per cent protection shown by alanine appeared to be different from the large protection of cystine.

In the stage of irradiation dose of more than 10 megaroentgens no more increase of ESR intensity was observed in spite of further progressing of the inactivation and of the $\gamma$-irradiation. In this process it is probable that wheather recombination and disappearance of free radicals owing to their extreme instability or no more formation of electron vacancies on the enzyme molecules may happen.

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