ADVANCES IN FREE RADICAL BIOLOGY AND MEDICINE

Volume 1

Edited by
Fang Yunzhong

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The monograph consists of the review articles and research papers written by 139 scientists in the field of free radical biology and medicine.

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PREFACE

Since J. M. McCord and I. Fridovich discovered superoxide dismutase and its function in the dismutation of superoxide anion radical in 1969, studies in the field of free radical biology and medicine have made rapid progress. Nearly 10, 000 Chinese researchers and students in several hundred universities, research institutes and hospitals are working in this field and have made many substantial contributions. From 1979 to 1989, the First and Second Nationwide Conferences on Free Radical Biology and Medicine, the Symposium on Lipid Peroxidation and Disease, Symposium on Nutrition, Trace Elements and Free Radical and the International Workship-Symposium on Biological and Medical Aspects of Free Radicals were held in China. Many original papers, reviews and other articles in this field have been published in scientific periodicals. They were, however, almost all written in Chinese and translated versions are not available. In order to facilitate interational academic exchanges, it is highly necessary to translate all these works into English, which, however, will involve too much effort and is almost impossible to be accomplished. At the suggestion of some authors, we decide to publish review articles and research papers written in English in a series of volumes entitled "Advances in Free Radical Biology and Medicine" to meet international academic exchanges in this field. The first volume, published by the Atomic Energy Press, includes review articles based on systematic studies and original research papers of the authors.

It is a great pleasure to express my sincere appreciation and deep gratitude to the contributors who are working in many scientific fields, such as chemistry, physics, biochemistry, biophysics, biology, pharmacology, pharmacy and medicine and making prominent contributions to the study of free radical biology and medicine. I am indebted to professor Zhang Shuxian, senior editor of the Atomic Energy Press, for her support and assistance in editting this monograph. I would also like to thank Dr. Wang Zangong for his correction and revision of the English version of all the manuscripts. Finally I wish to express my sincere hope that academic papers contributed from foreign authors are welcome. They may be published in our subsequent volumes. We hope the monograph will become an international endeavor in the future.

Fang Yunzhong

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EFFECT OF IONIZING RADIATION ON SUPEROXIDE DISMUTASE in vitro and in vivo

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(*Received on February* 20, 1990)

Abstract: A series of investigations on the effects of ionizing radiation on superoxide dismutase (SOD) in vitro and in vivo have been made, Based on these reports, some discussions were presented and the headlines were shown as follows: (1) effect of irradiation in vitro on the activities of Cu, Zn-SOD and Mn-SOD, (2) effect of irradiation in vitro on some physicochemical properties of Cu, Zn-SOD, (3) relationship between the inactivation of Cu, Zn-SOD and the action of free radicals induced by irradiation in vitro, (4) inhibition of the reconstitution and reactivation of Cu, Zn-SOD by the action of irradiation in vitro, (5) effect of irradiaion in vitro on spin labelled Cu, Zn-SOD, (6) effect of the whole body irradiation on the activities of Cu, Zn-SOD and Mn-SOD in the liver and spleen of mice, (7) effect of the whole body irradiation on the activities of Cu, Zn-SOD and Mn-SOD in mitochondria and microsome of liver and spleen of mice.

Key words: ionizing radiation, superoxide dismutase, Cu, Zn-SOD, Mn-SOD, activity, physicochemical property, effect of irradiation inactivation, reconstitution, spin labelled Cu, Zn-SOD, mitochondria, microsome, liver, spleen, mice

By the action of ionizing radiation on superoxide dismutase (SOD) in vitro in the presence of water and oxygen, several reactive oxygen species including $O_2^{-\cdot}$, $^{\cdot}OH$ and H_2O_2 are produced, and the latter can be subsequently eliminated by catalase, but the others may attack SOD. There are two kinds of SOD in mammalia; one is Cu, Zn-SOD, existing in the cellular fluid and the other, Mn-SOD, mainly in the mitochondria. It is very interesting to find that the effects of ionizing radiation on the two enzymes in vitro may reflect the action of some oxygen reactive species on their structures and catalytic activities. Although the immediate effect of ionizing radiation in vivo is likely to be similar to that in

vitro ,after irradiation radiation damage might give rise to many indirect,complex effects such as abnormal imbalance between reactive oxygen species production and their elimination.

Great attention must be paid to the effects of endogenous oxygen reactive species on Cu,Zn-SOD and Mn-SOD. In connection with the results obtained in a series of studies in vitro and in vivo,some discussions were presented as follows:

1. Effect of Irradiation in vitro on the Activities of Cu,Zn-SOD and Mn-SOD[1,2]

Cu, Zn-SOD purified from bovine erythrocyte by the method of McCord and Fridovich[3] was identified to be homogeneously pure as shown by the criteria for enzyme activity and its physicochemical properties. The lyophilized enzyme was dissolved in pH 7.8,0.05 mol/L phosphate buffer. While it was being irradiated in vitro,its activity decreased with the increase of irradiation dose (Fig. 1) as well as with the dilution of the enzyme concentration (Table 1).

Table 1 D_{37} **and** G **value of different solutions of Cu,Zn-SOD**

Cu,Zn-SOD (mg/ml)	D_{37} (Gy)	G value
0.01	17	0.17
0.25	27	0.26
0.50	49	0.29

D_{37} —— Irradiation dose that will cause 37% of enzyme activity to be retained.

G value—— Number of molecules inactivated per 100 eV of absorbed radiation energy.

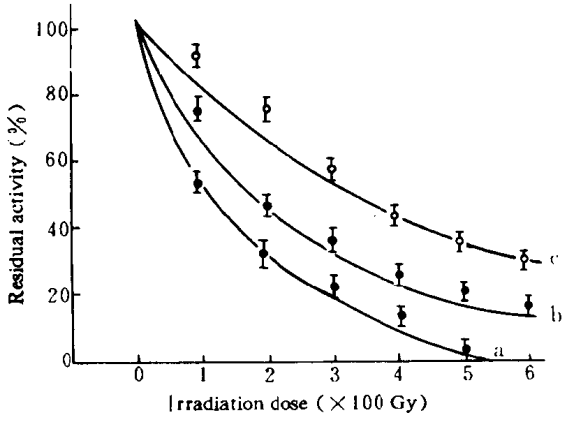

Fig. 1 The effect of irradiation on Cu,Zn-SOD activity

a:0.05 mg/ml

b:0.25 mg/ml

c:0.50 mg/ml

Manganese superoxide dismutase (Mn-SOD) has been purified from the pig liver, which proved to be perfectly pure as shown by a single band in the polyacrylamide gel electrophoresis pattern and its specific activity of 3760 units per mg protein. Irradiation in vitro caused Mn-SOD activity to decrease with the increase of irradiation dose,but its sensitivity was lower than that of Cu,Zn-SOD. Although the structure of the active center of the two enzymes and their catalytic activity are almost identical,the metals as well as apoenzymes are quite different. It is proposed that the oxygen reactive species can affect the catalytic activity of Cu,Zn-SOD

and Mn-SOD by the same mechanism but their irradiation sensitivity are different due to the above-mentioned differences of the two enzymes. Since the structure and active center of enzyme are strictly related to its activity, the decrease of SOD activity on exposure to γ rays may reflect the damage on conformation and active center of the enzyme.

2. Effect of Irradiation in vitro on Some Physicochemical Properties of Cu, Zn-SOD[1]

Pure Cu, Zn-SOD dissolved in pH 7.8, 0.05 mol/L phosphate buffer was irradiated with 100 — 1000 Gy. This treatment gave rise to the change of some physicochemical properties of Cu, Zn-SOD.

(1) Alteration of polyacrylamide gel electrophoresis pattern

The unirradiated enzyme exhibited itself typical Cu, Zn-SOD protein band as well as on the corresponding position achromatic band stained for its activity in the polyacrylamide gel electrophoresis at 20, 40, 60 or 80 min intervals, whereas the electrophoresis pattern for 1000 Gy irradiated enzyme developed the distinct but wider band as in the electrophoresis at 20 min interval, or the diffused band as in that at 80 min interval. It was suggested that either net electric charge or molecular size of enzyme could cause the alteration of electrophoresis pattern, so that the diffused bands indicated that these properties might be affected.

(2) Alteration of ultraviolet absorbance spectra

The ultraviolet absorbance spectra of Cu, Zn-SOD (Fig. 2) showed that the absorbance was increased with the increase of irradiation dose. The irradiated enzyme had such characteristic property of hyperchromism, which was relevant to the Cu, Zn-SOD concentration. The ratio of optical density to the Cu, Zn-SOD concentration (0.05 — 1.00 mg/ml) is a constant, i.e. molar extinction coefficient, but irradiation

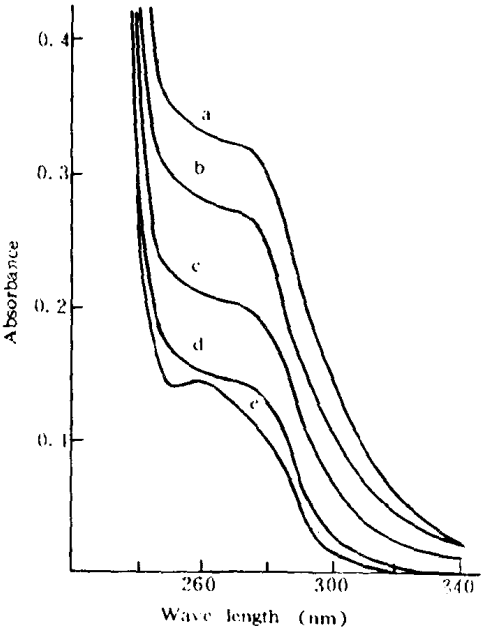

Fig. 2 Ultraviolet absorbance spectra of Cu, Zn-SOD
Cu, Zn-SOD (0.5 mg/ml); in pH 7.8, 0.05 mol/L phosphate buffer
a: 1000 Gy; b: 800 Gy;
c: 400 Gy; d: 100 Gy; e: 0 Gy.

with 100 or 1000 Gy caused the ratio to be increased following the dilution of Cu,Zn-SOD concentration as illustrated in Table 2. The results indicated that the irradiation caused the enzyme possessing characteristic property of hyperchromism, resulting from the disorderliness of enzyme structure that showed hydrophobic aromatic amino acid residues exposed from inner region into the surface. Such a tendency was enhanced with the increase of the irradiation dose.

Table 2 The ratio of optical density to Cu,Zn-SOD concentration

Cu,Zn-SOD (mg/ml)	OD_{258}/concentration		
	0 Gy	100 Gy	1000 Gy
0.05	10460	17782	73400
0.10	10460	15365	56100
0.50	10460	11213	24820
1.00	10460	10816	18122

(3) Change of fluorescence intensity

The fluorescence spectra of excitation and emission of Cu,Zn-SOD were shown in Fig. 3 and 4. The λ_{max} of excitation spectrum was 280 nm, whereas that of emission spectrum 310 nm. Their features were all similar on exposure to 100—600 Gy, but the fluorescence intensity at 310 nm emitted was decreased with the increase of irradiation dose.

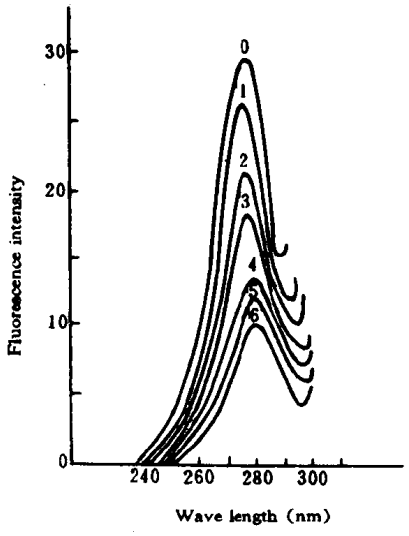

Fig. 3 Fluorescence excitation spectra of Cu,Zn-SOD
0,1,2,3,4,5,6 represent irradiation dose (× 100 Gy).

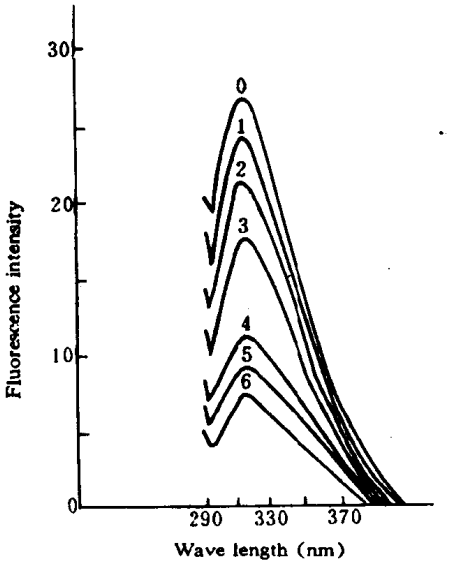

Fig. 4 Fluorescence emission spectra of Cu,Zn-SOD
0,1,2,3,4,5,6 represent irradiation dose (× 100 Gy).

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The effect of irradiation in vitro on the change of fluorescence intensity implicated that the molecular structure had been changed with the alteration of fluorescence-producing groups in Cu, Zn-SOD.

(4) Changes of infrared absorbance spectra, circular dichroism spectra and electron spin resonance of Cu, Zn-SOD

Irradiation with 1000 Gy caused the destruction of Cu, Zn-SOD structure as shown by the change of infrared absorbance spectra, circular dichroism spectra and electron spin resonance (ESR) spectra of the enzyme.

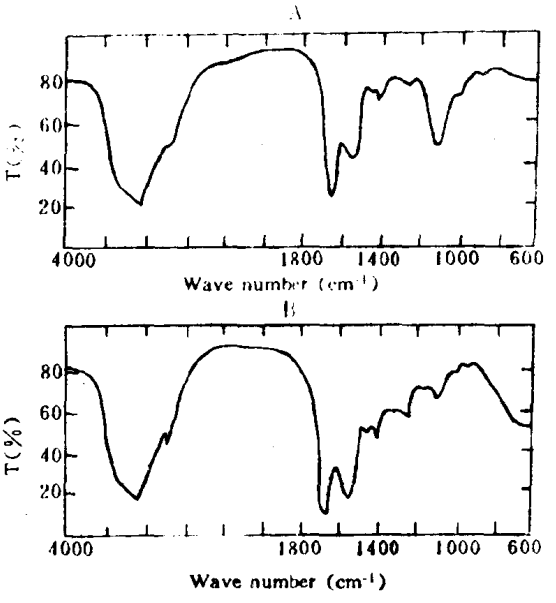

Fig. 5 Infrared absorbance spectra of Cu, Zn-SOD

Above: Cu, Zn-SOD irradiated with 1000 Gy;

Below: Cu, Zn-SOD unirradiated.

The infrared absorbance spectrum of Cu, Zn-SOD irradiated with 1000 Gy was very similar to that of the enzyme unirradiated, but Cu, Zn-SOD that was irradiated in pH 7.8, 0.05 mol/L phosphate buffer, desalted by dialysis against water and finally lyophilized had infrared absorbance spectrum altered obviously as shown in Fig. 5. The result indicated that the enzyme structure underwent irreversible change.

The circular dichroism (CD) spectra of unirradiated Cu, Zn-SOD were quite different from those of the enzyme irradiated with 1000 Gy (Fig. 6 and 7). The former had absorbance at the region of 240—320 nm, which showed a peak at 255 nm, but the latter exhibited no distinct absorbance at that region. The CD spectrum of irradiated Cu, Zn-SOD was very similar to that of its apoenzyme, Cu, Zn-SOD devoid of copper and zinc, in 8 mol/L urea[4]. Since CD is extraordinarily sensitive to the change of conformation of biomacromolecule, the irradiation caused the effect of altering the conformation or structure of the enzyme.

Purified Cu, Zn-SOD was dissolved in pH 7.8, 0.05 mol/L phosphate buffer, its concentration being 0.10 mg/ml. It was irradiated with 1000 Gy and treated as irradiated Cu, Zn-SOD solution for measurement of infrared absorbance spectra. Irradiated and unirradiated Cu, Zn-SOD were weighed 3.82 mg, respectively and then placed into quartz tubes with inner diameter of 5 mm. The ESR spectrum of unirradiated Cu, Zn-SOD was quite different from that of irradiated enzyme. For instance, the ESR spectrum

of Cu,Zn-SOD irradiated with 1000 Gy showed $g_{\perp}$ signal amplitude decreased by 68%. This fact indicated the obvious loss of Cu^{2+}. The feature of ESR spectrum of irradiated Cu,Zn-SOD was corresponding to that of Cu,Zn-SOD denatured or deactivated as well as to that of small Cu^{2+}-peptide complex.

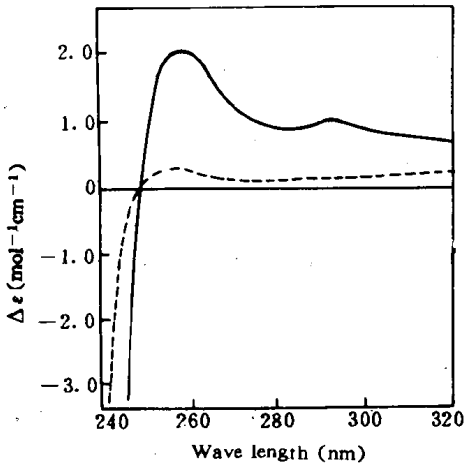

Fig. 6 CD spectra of Cu,Zn-SOD (240—320 nm)
——Unirradiated Cu,Zn-SOD;
-----Irradiated Cu,Zn-SOD;
Cu,Zn-SOD solution (1 mg/ml).

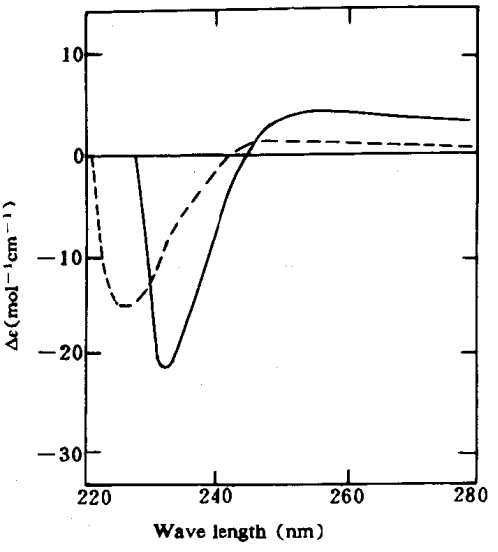

Fig. 7 CD spectra of Cu,Zn-SOD (220—280 nm)
——Unirradiated Cu,Zn-SOD;
-----Irradiated Cu,Zn-SOD;
Cu,Zn-SOD solution (1 mg/ml).

(5) Decrease of copper and zinc contents

The purified Cu,Zn-SOD was found to contain 0.339% of copper and 0.320% of zinc as estimated by the atomic absorption spectrophotometry, but Cu,Zn-SOD in pH 7.8,0.05 mol/L phosphate buffer was irradiated with 1000 Gy and then treated through dialysis against water. The finally lyophilized Cu,Zn-SOD had much lower contents of copper and zinc as 0.142% and 0.156%, respectively.

It was suggested that after irradiation of Cu,Zn-SOD in vitro, the binding of copper-zinc and apoenzyme became much looser, giving rise to the loss of the metals. Its mechanism was contributed to the change of conformation of the active center. In a series of studies, other physicochemical properties affected by irradiation in vitro resulted from the destruction of Cu,Zn-SOD structure as we proposed.

3. Relationship between the Inactivation of Cu,Zn-SOD and the Action of Free Radicals Induced by Irradiation in vitro[5]

Cu,Zn-SOD is known as an important enzyme in the living bodies. The decrease of the enzyme activity by irradiation in vitro was suggested to be the effect of free radicals and the alteration of its physicochemical properties might be caused by the same mechanism. The scavenger of free radicals might give rise to the protection of SOD from the effect of irradiation, but no investigation regarding that was made before. In this study ·OH was found to attack Cu,Zn-SOD since 85—90% of the enzyme activity was protected by the formate, one of ·OH scavengers, in the irradiation of 200—800 Gy in air as shown in Fig. 8.

Although the catalase, the scavenger of H_2O_2, was added before irradiation, only the addition of formate almost made the hyperchromism effect eliminated and the original spectra of the ultraviolet region were nearly restored (Fig. 9). The hyperchromism effect on ultraviolet absorbance by irradiation in vitro reflected ·OH action on the structure of Cu,Zn-SOD but formate played a principal role in the protection of enzyme activity and the elimination of hyperchromism. The important result implicated that by irradiation in vitro ·OH was the main oxygen reactive species to cause the inactivation of Cu,Zn-SOD as well as the change of physicochemical properties of the enzyme.

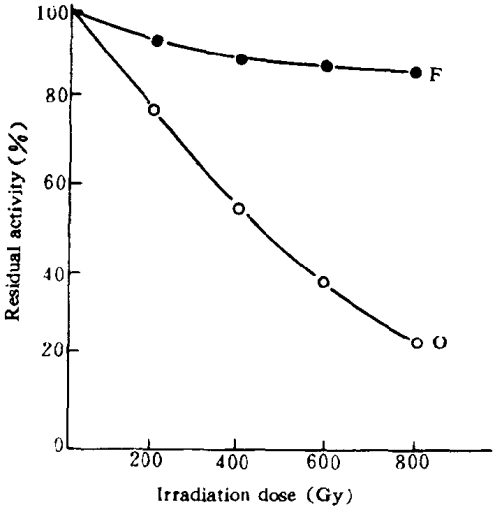

Fig. 8 Protective effect of formate on the irradiated inactivation of Cu,Zn-SOD
O—— Cu,Zn-SOD (0. 1 mg/ml): in pH 7. 8, 0. 05 mol/L phosohate buffer;
F—— Sodium formate (mmol/L): added before irradiation.

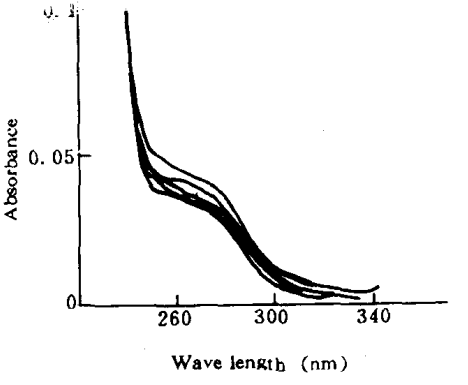

Fig. 9 Effect of addition of formate before irradiation on the ultraviolet spectra of Cu, Zn-SOD
Curved lines (from below to above): 0, 200, 400,600,800 Gy;
Cu,Zn-SOD solution (0. 1 mg/ml).

4. Inhibition of the Reconstitution and Reactivation of Cu,Zn-SOD by the Action of Irradiation in vitro[6,7]

We have observed that removal of copper and zinc gave rise to the damage of catalytic activity of Cu,Zn-SOD as well as the change of some physicochemical properties,such as absorption spectra,circular dichroism and ESR. It indicated that copper and zinc were indispensable to the stability of structure and the maintenance of catalytic activity of Cu,Zn-SOD,but the addition of Cu^{2+} and Zn^{2+} to epoenzyme might reconstitute Cu,Zn-SOD as shown by the recovery of enzyme activity and some characteristics of absorption spectrum. Although the dissociation and association between copper-zinc and apoenzyme are reversible,whether the apoenzyme obtained from Cu,Zn-SOD irradiated by large dose can associate copper and zinc ion as usual,whether ionizing radiation gives rise to the profound damage to the active center of Cu,Zn-SOD and which free radical is the most important factor to attack the enzyme,are not definitely cleared up. In our laboratory the inhibition of reconstitution of copper and zinc in apo-SOD was specially investigated.

The Fee's method modified by us was applied to prepare apo-SOD (E_2E_2-SOD). The main procedure was that lyophilized powder of Cu,Zn-SOD was dissolved in pH7. 8, 0. 05 mol/L potassium phosphate buffer containing 0. 1 mol/L $NaClO_4$ and centrifuged by 40000×g for 30 minutes,and the supernatant obtained was dialysed against pH 4. 2, 0. 0025 mol/L acetate buffer and then double-distilled water for 24 h. All manipulation was performed at 4℃,lyophilized powder of E_2E_2-SOD being finally obtained.

For observing the effect of ionizing radiation on reconstitution of copper-zinc and apoenzyme in Cu,Zn-SOD,lyophilized powder of E_2E_2-SOD dissolved in double-distilled water making the comcentration of 0. 1 mg/ml was irradiated by different doses of γ-ray emitted from ^{60}Co and then $CuSO_4$ and $ZnSO_4$ solution were added to it slowly and with constant stirring to make the final concentration of Cu^{2+} and Zn^{2+} twice those contents in Cu,Zn-SOD. Lyophilized powder was analyzed for enzyme activity,which was calculated as percentage of that of original Cu,Zn-SOD.

As shown in Fig. 10,irradiation of 20—80 Gy caused holo-SOD to lose activity not more than 5%,but the loss of activity of apo-SOD reconstitution reactivated with Cu^{2+} and Zn^{2+} was decreased with the increase of irradiation dose. For instance, the residual activity of hole-SOD (0. 1 mg/ml) irradiated by 80 Gy was 95% as that of the unirradiated enzyme ,whereas apo-SOD irradiated with the same dose and reconstituted with copper and zinc was only 58%. It was indicated that apo-SOD is more sensitive to

the exposure to γ-ray than Cu,Zn-SOD. Since the addition of formate (50 mol/L) before irradiation gave obvious protection to reconstitution and reactivation of Cu,Zn-SOD, ·OH was suggested to be the major species for inhibiting the reconstitution and reactivation of Cu,Zn-SOD.

The effect of ionizing radiation on the ultraviolet spectra of apo-SOD was comparatively studied. The ultraviolet absorption of apo-SOD irradiated with 5 — 50 Gy was increased with the increase of irradiation dose. Although the hyperchromism effect was also observed in the irradiation of native Cu,Zn-SOD,the dose required in the irradiation was 10 times as large as that in the irradiation of apo-SOD. This fact implicated that copper and zinc play an important role in the stability of enzyme structure, and the removal of them may increase the sensitivity to irradiation in vitro. It is very interesting to find that the addition of formate might eliminate the hyperchromism effect induced by ionizing radiation both in native Cu,Zn-SOD and in apo-enzyme,The effect of ionizing radiation on CD spectra of apo-SOD was also observed. There were three peaks of positive absorbsnce of 350,290 and 255 nm in the ultraviolet region (240—400 nm) of CD spectrum of holo-SOD, but the removal of copper made the peak of 350 and 255 nm vanished and further removal of zinc,290 nm vanished as well. Addition of formate and catalase made the spectra of holo-SOD kept in native state in spite of irradiation in vitro.

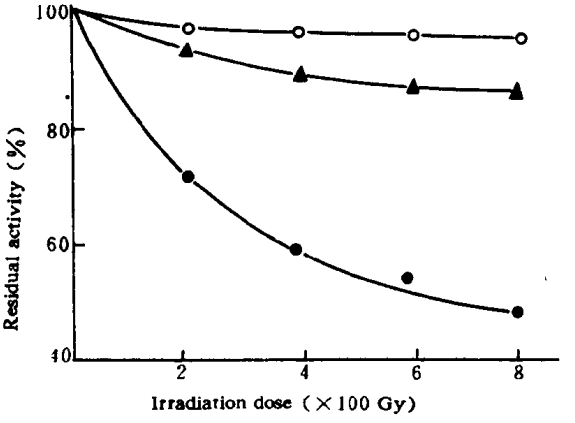

Fig. 10 Effect of irradiation on the residual activity of reactivation of apoenzyme repleted with copper and zinc
○——○ Cu,Zn-SOD
●——● apo-SOD (irradiated)+Cu & Zn
▲——▲ apo-SOD and formate (irradiated)+Cu & Zn
Concentration of enzyme or apoenzyme: 0.1 mg/ml; sodium formate:50 mol/L.

Owing to the prensence of copper and zinc,Cu,Zn-SOD is stable to the effects of many factors,such as inactivation by heat,depolymerization by SDS and denaturation by photosensitization,The reverse is true for SOD in the removal of copper and zinc. Our experiment proved that apo-SOD had much weaker ability to resist the damaged action than holo-SOD,that is to say,if apo-SOD concentration was the same as that of holo-SOD and the irradiation conditions for apo-SOD and for holo-SOD were identical,the reconstitution and reactivation of apo-SOD inhibited by irradiation became much severer than those of holo-SOD. Since copper and zinc were connected by coordinate bond through the nitrogen atom on the imidazole ring of histidine residue in SOD molecule showing that an imidazole

bridge existed between copper and zinc, it was reasonable to propose that imidazole bridge constitution might be the sensitive site for the damage of irradiation on Cu, Zn-SOD and that the free radicals produced in the irradiation were the direct factors determining the breakage of imidazle bridge by attaking. As shown in Fig. 11, on the side of copper ion connected to imidazole bridge there was a molecule of water, which was transformed into ·OH and others by the action of ionizing radiation. As ·OH life is very fleeting, it moves within the limit of diffusion so as to attack imidazole bridge, and in this manner H_2O_2 was produced by the interaction of ionizing radiation and water molecule, which might destroy Cu, Zn-SOD by the following mechamism:

$$E\text{-}Cu^{2+} + H_2O_2 \longrightarrow E\text{-}Cu^{+} + O_2^{-}\cdot + 2H^{+} \quad (1)$$

$$E\text{-}Cu^{+} + H_2O_2 \longrightarrow E\text{-}Cu^{2+}\text{----}OH + OH^{-} \quad (2)$$

$$E\text{-}Cu^{2+}\text{----}OH + ImH \longrightarrow \text{Inactivated } E\text{-}Cu^{2+} + Im\cdot + H_2O \quad (3)$$

Formate is one of the ·OH scavengers, therefore the addition of formate before irradiation gave rise to protective action on Cu, Zn-SOD or its apo-enzyme, indicating that ·OH produced by the action of ionizing radiation on H_2O was the main factor determining the radiation damage of Cu, Zn-SOD.

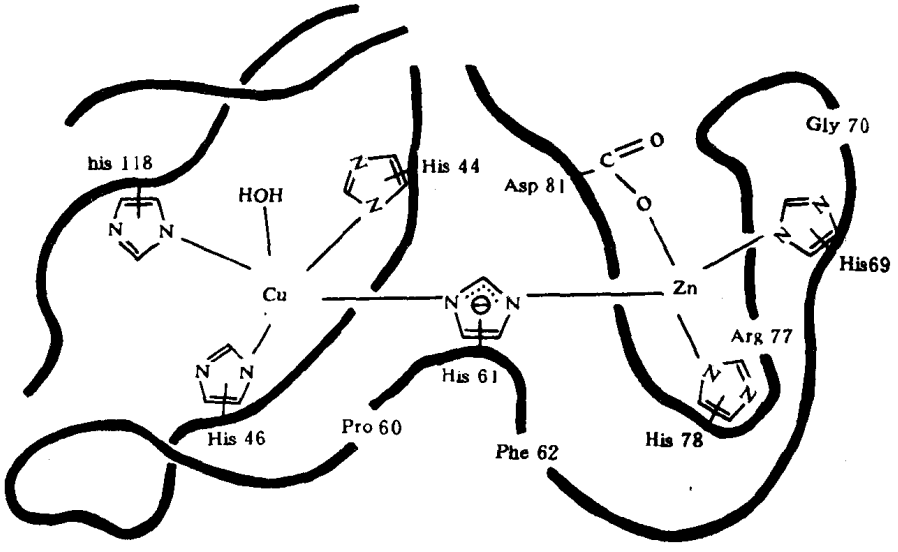

Fig. 11 Scheme for "imidazole bridge" of active center of bovine Cu, Zn-SOD

5. Effect of Irradiation in vitro on Spin Labelled Cu, Zn-SOD[8]

Steinman et al.[9] had reported that there are three cysteine residues in the amino acid sequences of bovine Cu, Zn-SOD subunit, two of which form disulfide linkage as-S-S-group in the inner region of the enzyme subunit but the other cycteine (cys_6) remains

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