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イオン化放射線および紫外光により生成したラジカルとチトクロムCの反応の研究

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STUDIES ON REACTIONS OF
CYTOCHROME C WITH
RADICAL SPECIES
PRODUCED BY IONIZING RADIATION
AND ULTRAVIOLET LIGHT

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Studies on Reactions of Cytochrome c with Radical Species
Produced by Ionizing Radiation and Ultraviolet Light

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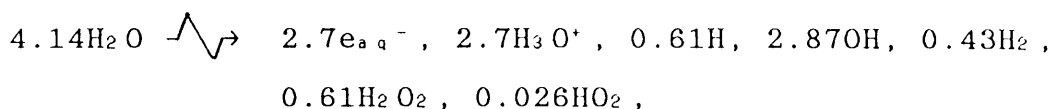
General Introduction

Nowadays the probability of an exposure to radiation increases with increasing usage of ionizing radiation in various situations and quite a few people who are troubled by radiation-induced lesion are waiting for the essential treatment. So radiation-induced lesion in biological systems is an important subject to be studied in detail.

Living systems usually consist of 60~80% water. This water content is somewhat delicate for evaluating the relative contribution of direct or indirect action of radiation. A cell is a heterogeneous multiple component system, and the precise analysis of the reactions taking place in a cell is quite difficult. Therefore a certain model system should be used to estimate the fundamental information on radiation-induced damage of biological systems. Dilute aqueous solutions of various biological substances may be used as model systems for the indirect action. In the systems,

a reaction between a certain biomolecule and an active species produced in radiolysis of water can be accurately analyzed on the basis of reaction kinetics.

The radiolysis of water have been extensively studied by many research groups since the earliest stage of radiation chemistry and a lot of literatures are available on the subject. Recently Burns and Marsh (1981) summarized those studies and proposed the energy yields (G-value) of radiolytic products from water as follows¹⁾:



where the figures before each chemical species represent G-values (molecules per 100 eV). These G-values are relevant in deaerated neutral water irradiated by 1 MeV γ -rays and at 10^{-7} to 10^{-6} s after irradiation. The reactions between active species and biomolecules in dilute aqueous solutions take place usually in the time scale. The G-values also indicate that hydrated electron (e_{aq}^-) and hydroxyl radical (OH) are major active products in aqueous media irradiated by low LET radiation. Therefore their reactions with biomolecules have been mainly studied.

In this study, horse heart cytochrome c is taken as a model. Rate constants for the reactions of cytochrome c with hydrated electron and OH radical were determined by competition kinetics with some radical scavengers.^{2, 3)} The radiation damage of the protein was estimated by spectroscopic

analysis and biochemical techniques.^{4, 5)}

The mechanism of the reduction of ferricytochrome c induced by ultraviolet light was studied by flash photolysis and also by kinetic analysis of continuous irradiation experiments using a low pressure mercury lamp. The results indicate that ferricytochrome c is directly reduced by UV light and the process consists of photoionization of tryptophyl residue.^{6, 7)}

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Chapter 1. Radiation-Induced Reduction of Horse Heart Cytochrome c in Gamma-Irradiated Aqueous Solution.

Cytochrome c is a protein that has heme as a prosthetic group and transfers electrons in a respiratory system. It produces ATP from ADP and inorganic phosphate through the electron transfer, namely redox reaction of the heme. In irradiated aqueous media, redox reaction of cytochrome c is expected to occur, since reducing or oxidizing species such as hydrated electron or OH radical are produced in the system. To clarify the chemical changes induced by radiation and also to determine the rate constants for the reaction of cytochrome c with OH and hydrated electron, continuous irradiation of gamma rays was carried out. The G-values for reduction of ferricytochrome c or oxidation of ferrocytochrome c have been determined in the presence of suitable radical scavenger and kinetically analyzed by competition kinetics.

EXPERIMENTAL

Materials. Cytochrome c was prepared from horse heart muscle which was kindly supplied by Teikoku Hormone Co. Ltd. according to the method of Hagiwara et al.¹⁾ The preparation was desalted by passing through a Sephadex G-25 column and stored in refrigerator after lyophilization.

Preparation of Irradiation-Sample. The sample was dissolved

in triply distilled water and rechromatographed on a column of Amberlite CG-50 to eliminate a small amount of ferrocytochrome c contained in the sample. Subsequently the sample solution was purified on a Sephadex G-25 column. Ferrocytochrome c was obtained by reduction with cysteine and purification on a Sephadex G-25 column. Degassing of the sample for irradiation was carried out by the conventional freeze-thaw method on vacuum line.

Irradiation. Irradiations were carried out with ^{60}Co gamma source at ambient room temperature (15°C – 20°C). The dose rate were determined by Fricke dosimeter taking 15.5 as $G(\text{Fe}^{3+})$.

Determination of G-value for reduction. Absorbance of the sample at 550 nm was measured at frequent intervals during the course of irradiation by removing the irradiation vessel from the radiation source and pouring the solution into a side arm to which was attached a 1 x 1 cm silica cell. The initial reduction yield (G_r^0) was obtained by extrapolating G_r^D to zero dose as reported by Rabani and Stein.²⁾ G_r^D is given by the following relation.

$$G_r^D = \frac{(A_2 - A_1)N}{(\epsilon_r - \epsilon_o)(D_2 - D_1) \times 10^{-2}}$$

where ϵ_r and ϵ_o are absorption coefficient of ferro and ferricytochrome c at 550 nm, namely $2.77 \times 10^4 \text{ dm}^3 \text{ mol}^{-1}$

cm^{-1} and $9.2 \times 10^3 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$, respectively. A_1 and A_2 are absorbance of the sample at 550 nm at dose D_1 and D_2 , respectively. N represents Avogadro constant.

RESULTS AND DISCUSSION

Initial Reduction Yields in Solutions Containing Ferricytochrome c.

The increase in absorbance at 550 nm was observed during irradiation of ferricytochrome c. Initial reduction yield of ferricytochrome c, G_r^0 increases with increasing initial concentration of ferricytochrome c and approaches to a limiting value of ca. 5 above the concentration of $8 \times 10^{-5} \text{ mol dm}^{-3}$, as shown in Fig. 1-1. The limited G-value for reduction of ferricytochrome c to ferrocytochrome c cannot be explained by initial G-values of reducing radical species, hydrated electron and hydrogen atom, since in deaerated neutral aqueous media 2.7 and 0.61 are accepted as G-values of hydrated electron and hydrogen atom, respectively (see p.2). In addition to these reducing radicals, contribution of other radicals should be taken into the reduction mechanism. Rabani and Stein (1962)²⁾ suggested that OH radical reacts with protein part of cytochrome c to produce organic radical which reduces other ferricytochrome c. To confirm the contribution of OH

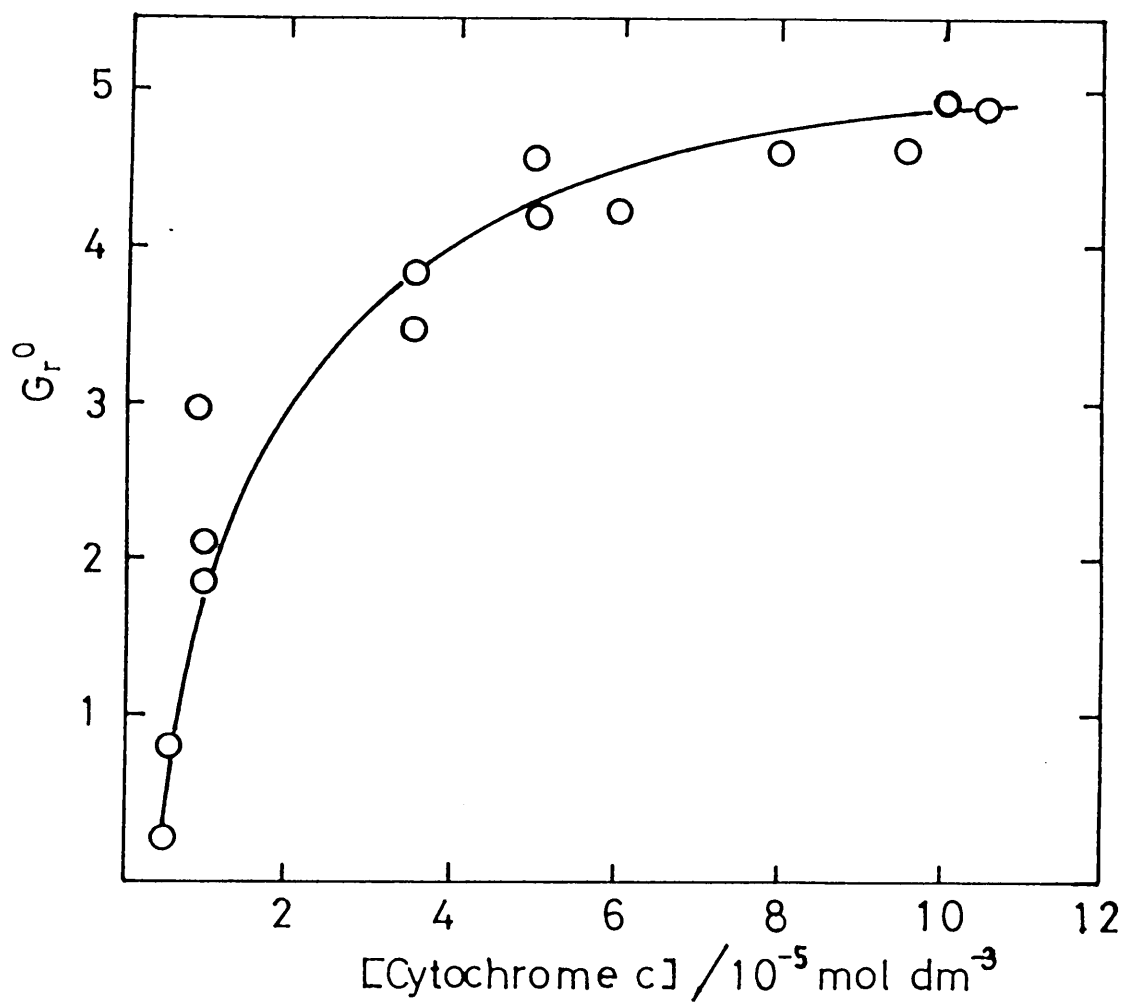


Fig. 1-1. Dependence of G_r^0 on concentration of ferricytochrome c.

pH = 7.0 (0.05 mol dm⁻³ phosphate buffer).

radical, effect of OH radical scavenger was examined on the reduction of ferricytochrome c.

Effect of Thallous Ion on Initial Reduction Yield.

It was reported by Sworski³⁾ and Bednar⁴⁾ that reduction yields of ceric ion and ferric $\alpha\alpha'$ -dipyridyl complex in aqueous solution were largely increased by addition of thallous ion and the increase could be explained by OH-scavenging action of thallous ion. As shown in Fig. 1-2, initial G-value of reduction decreases from 5 to ca. 3 with increasing concentration of thallous ion in contrast to the results of Sworski and Bednar. This implies that thallous ion reacts with OH to form unstable Tl^{2+} which may oxidize ferrocytochrome c or reducing species. No thallic ion was detected in the irradiated sample solution. When thallic ion prepared by oxidation with ozone was added into solution of ferrocytochrome c, instant oxidation of ferrocytochrome c to ferricytochrome c was observed. Excess ozone was cautiously removed from thallic solution before addition.

Initial Reduction Yields in Solution Containing Ferri- and Ferrocytochrome c.

In order to estimate a ratio of rate constants for the reactions of OH with ferrocytochrome c and ferricytochrome c (probably with its protein part), the initial reduction yields were determined for several mixed solution of

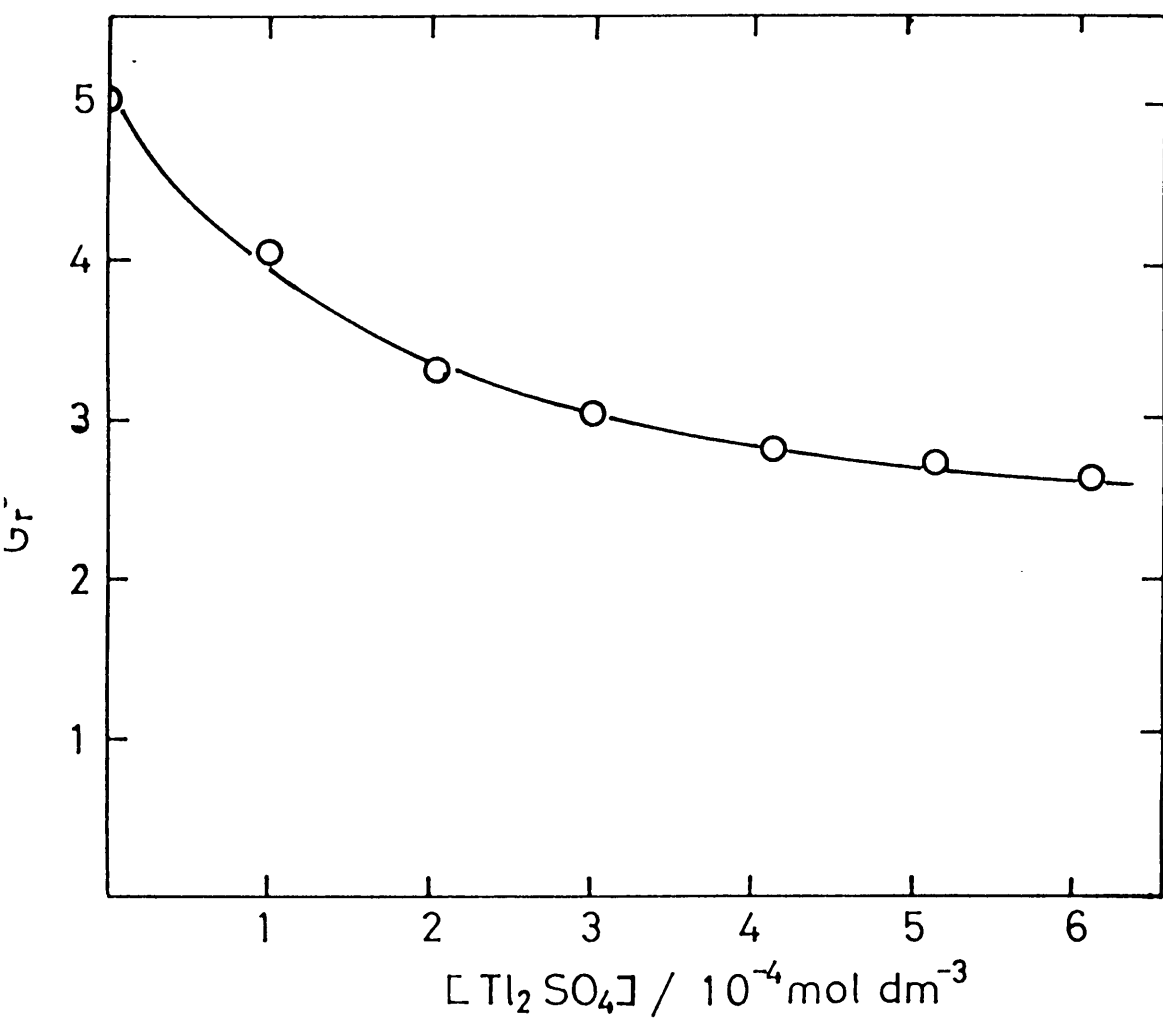
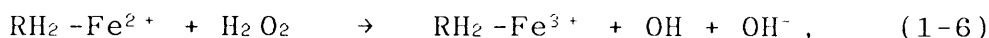
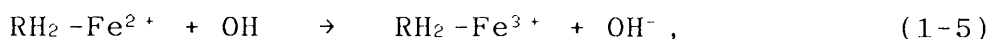
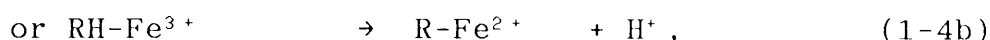
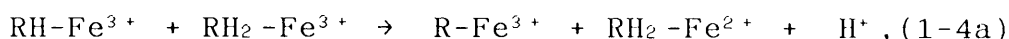
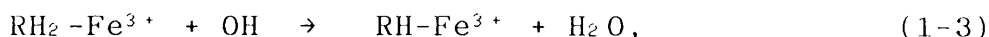
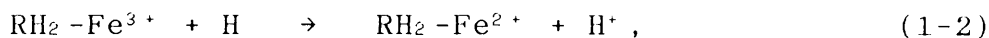
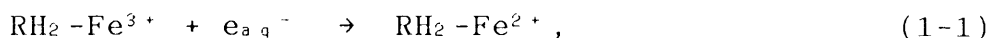


Fig. 1-2. G_r^0 as a function of thallous sulfate concentration.

Concentration of ferricytochrome c: $1.03 \times 10^{-4} \text{ mol dm}^{-3}$. Dose rate : $1.87 \times 10^{17} \text{ eV cm}^{-3} \text{ h}^{-1}$. pH: 7.0(0.05 mol dm^{-3} phosphate buffer).

different ratio of concentration. The result is shown in Fig. 1-3. The total concentration of cytochrome c used in this run was higher than $8 \times 10^{-5} \text{ mol dm}^{-3}$. The following reduction mechanism will be relevant in the system.



where $\text{RH}_2\text{-Fe}^{3+}$ represents ferricytochrome c and RH-Fe^{3+} is ferricytochrome protein radical. Reaction (1-4) may not always proceed efficiently. A factor f is introduced into the mechanism so that it may indicate the ratio of RH-Fe^{3+} that contributes to the reduction of ferricytochrome c through reactions (1-4).

On the basis of the mechanism, the initial reduction yield is expressed by the following relation:

$$G_r^0 = (G_e + G_H) + (G_{OH} + G_{H_2O_2}) \frac{f - \frac{k_5 (\text{RH}_2\text{-Fe}^{2+})}{k_3 (\text{RH}_2\text{-Fe}^{3+})}}{1 + \frac{k_5 (\text{RH}_2\text{-Fe}^{2+})}{k_3 (\text{RH}_2\text{-Fe}^{3+})}} - G_{H_2O_2} \quad (1-I).$$

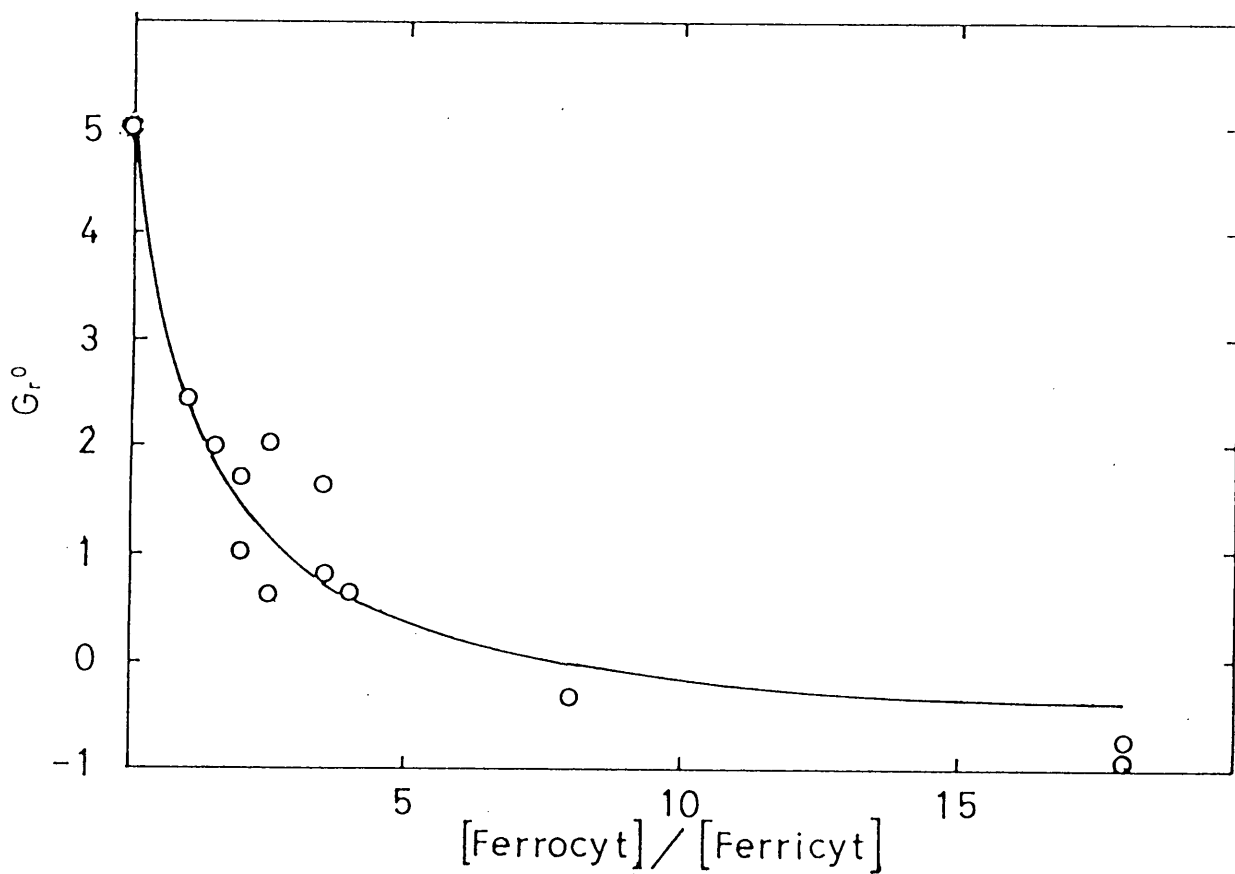


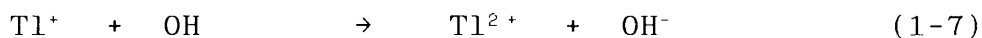
Fig. 1-3. G_r^0 as a function of $[\text{Ferrocyclochrome } c]/[\text{Ferricyclore } c]$.

At zero concentration of ferrocytochrome c, equation 1-I is reduced to $G_{r^0} = G_e + G_H + f(G_{OH} + G_{H_2O_2}) - G_{H_2O_2}$. Taking $G_e = 2.7$, $G_H = 0.61$, $G_{OH} = 2.87$, $G_{H_2O_2} = 0.61$, and $G_{r^0} = 5.0$, a value 0.66 is obtained as f .

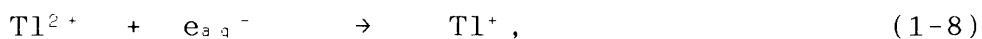
On the other hand, equation 1-I is reduced to $G_{r^0} = G_e + G_H - G_{OH} - 2G_{H_2O_2}$, where the ratio of concentration of ferro- to ferri-cytochrome c is infinity. Then G_{r^0} comes close to -0.78. The negative G-value means oxidation, namely a decrease in absorbance at 550 nm. Simulation of G_{r^0} against $[RH_2-Fe^{2+}]/[RH_2-Fe^{3+}]$ is shown in Fig.1-3 as a curve taking $f = 0.66$ and $k_5/k_3 = 0.8$. In the region of high ferro-/ferri- ratio, the agreement with observed values is not good. However, the simulation is excellent in the region where the change of G_{r^0} is most sensitive to the ratio of $[RH_2-Fe^{2+}]/[RH_2-Fe^{3+}]$, and it is acceptable as a whole.

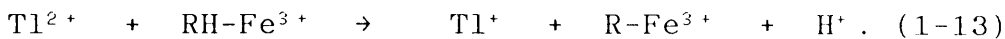
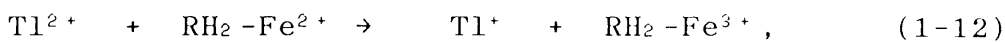
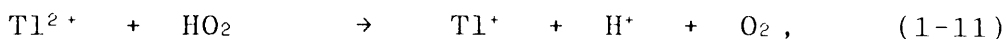
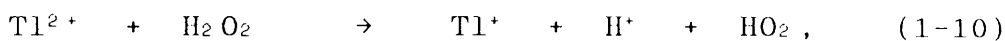
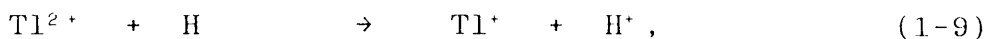
Kinetic Analysis of Thallous Ion and Ferricytochrome c Systems.

As mentioned above, addition of thallous ions into solutions of ferricytochrome c induces marked decrease in G_{r^0} . In the system, OH radical oxidizes thallous ion to divalent thallium ion as reported by Sworski³⁾.



The divalent thallium ion may decay through the following reactions:





On the basis of reactions 1-1 to 1-13, the following relation can be derived.

$$G_{r^0} = G_{e^0} + G_H$$

$$+ G_{0H} \frac{1}{k_3 [\text{RH}_2\text{-Fe}^{3+}] + k_5 [\text{RH}_2\text{-Fe}^{2+}] + k_7 [\text{Tl}^+]}$$

$$\star \{ k_3 [\text{RH}_2\text{-Fe}^{3+}] \frac{f \star k_4 [\text{RH}_2\text{-Fe}^{3+}] + k_{13} [\text{Tl}^{2+}]}{k_4 [\text{RH}_2\text{-Fe}^{3+}] + k_{13} [\text{Tl}^{2+}]}$$

$$- k_5 [\text{RH}_2\text{-Fe}^{2+}] - k_7 [\text{Tl}^+] \}$$

$$+ G_{H_2O_2} \{ \frac{k_6 [\text{RH}_2\text{-Fe}^{2+}]}{k_6 [\text{RH}_2\text{-Fe}^{2+}] + k_{10} [\text{Tl}^{2+}]}$$

$$\frac{k_3 [\text{RH}_2\text{-Fe}^{3+}] \frac{f \star k_4 [\text{RH}_2\text{-Fe}^{3+}] + k_{13} [\text{Tl}^{2+}]}{k_4 [\text{RH}_2\text{-Fe}^{3+}] + k_{13} [\text{Tl}^{2+}]} - k_5 [\text{RH}_2\text{-Fe}^{2+}] - k_7 [\text{Tl}^+]}{\star \frac{k_3 [\text{RH}_2\text{-Fe}^{3+}] + k_5 [\text{RH}_2\text{-Fe}^{2+}] + k_7 [\text{Tl}^+]}$$

$$- \frac{k_6 [\text{RH}_2\text{-Fe}^{2+}] - 2k_{10} [\text{Tl}^{2+}]}{k_6 [\text{RH}_2\text{-Fe}^{2+}] + k_{10} [\text{Tl}^{2+}]} \} \quad (1-11)$$

$$(14)$$

Since G_r^0 is obtained as an extrapolated value at time 0, the intermediate unstable species can be assumed to be negligible. Therefore the equation 1-II can be reduced to the following equation:

$$G_r^0 = G_e + G_H$$

$$f = \frac{k_7 [Tl^+]}{k_3 [RH_2 - Fe^{3+}]} + (G_{0H} + G_{H2O2}) \frac{k_7 [Tl^+]}{1 + \frac{k_7 [Tl^+]}{k_3 [RH_2 - Fe^{3+}]}} - G_{H2O2} \quad (1-III)$$

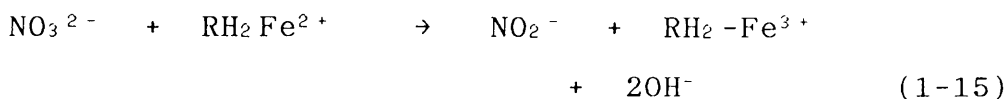
Taking zero as $[Tl^+]/[RH_2 - Fe^{3+}]$, a value 0.66 is obtained as f . If it is assumed that the ratio of rate constants, k_7/k_3 , is 0.21, the curve in Fig. 1-4 is obtained.

Kinetic Analysis of Nitrate Ion and Ferricytochrome c Systems.

A similar kinetic treatment was carried out in systems containing ferricytochrome c and potassium nitrate. In Fig. 1-5, G_r^0 is plotted against the ratio of concentration $[NO_3^-]/[RH_2 - Fe^{3+}]$. Nitrate ions reacts with hydrated electrons to produce an unstable intermediate as follows:



The intermediate species oxidizes ferrocyclochrome c.



From reactions 1 to 6, 14, and 15, equation 1-IV can be derived.

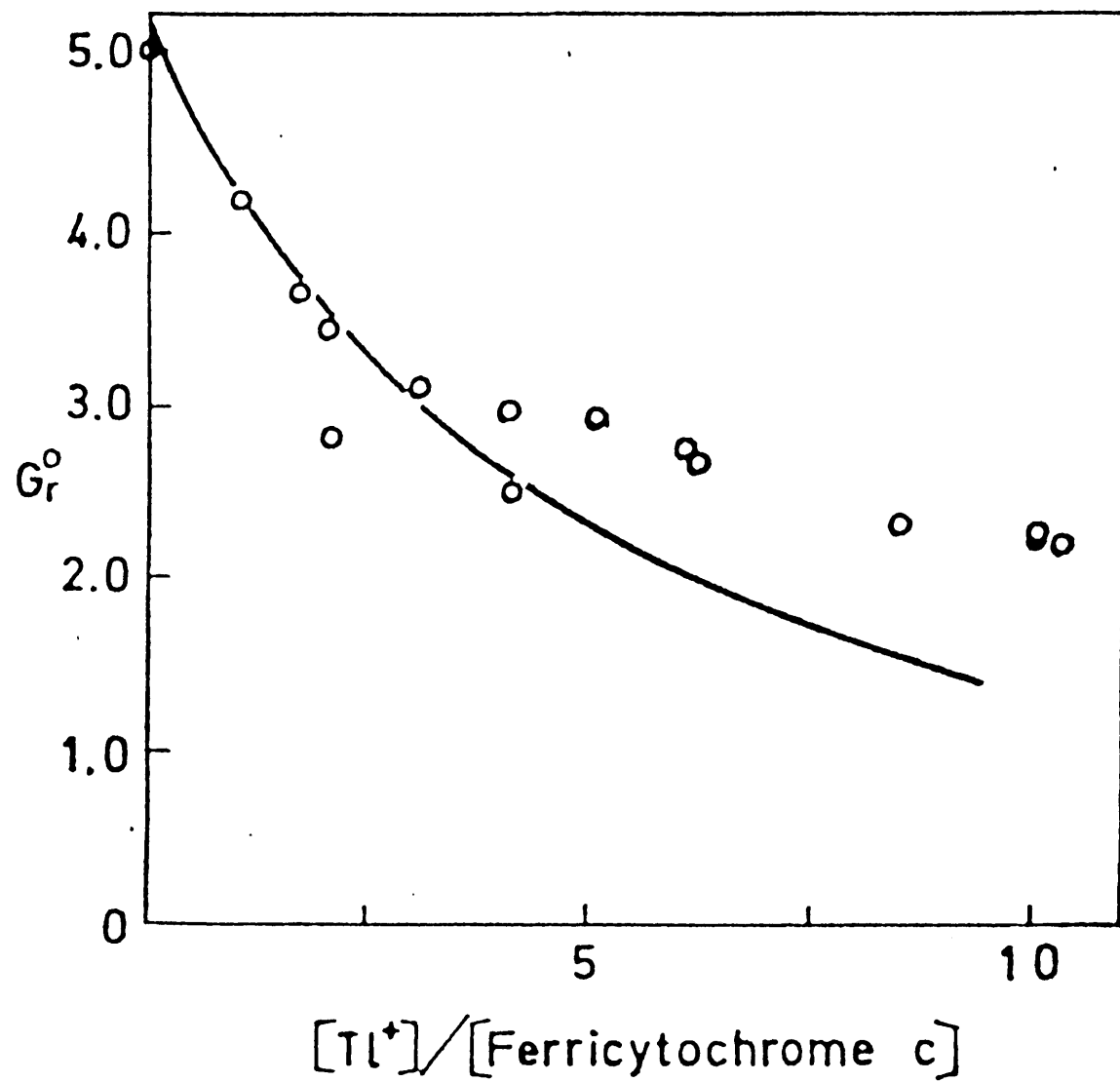


Fig. 1-4. Gr^0 as a function of $[Tl^+]/[Ferricytochrome\ c]$.

$$G_{r0} = G_e \frac{1 - \frac{k_{14} [\text{NO}_3^-]}{k_1 [\text{RH}_2 - \text{Fe}^{3+}]}}{1 + \frac{k_{14} [\text{NO}_3^-]}{k_1 [\text{RH}_2 - \text{Fe}^{3+}]}} + G_H + f(G_{OH} + G_{H_2O_2}) - G_{H_2O_2} \quad (1-IV)$$

At $[\text{NO}_3^-] = 0$, 0.66 is obtained as f . Taking $k_{14}/k_1 = 0.15$, the curve in Fig. 1-5 is obtained.

Estimation of Rate Constants For The Reactions of Ferri- and Ferrocytochrome c With hydrated electrons and OH radicals.

Using rate constants found in literatures, k_1 , k_3 , and k_5 can be estimated. As k_7 , $(7.6 \pm 1) \times 10^9 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$ was reported by Cercek *et al.* (1966)⁵⁾ and $1.0 \times 10^{10} \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$ was estimated as k_{14} (Hentz *et al.* 1972, Peled and Czapski 1970).^{6, 7)} When 0.8, 0.21, and 0.15 are applied to the ratios of rate constants, k_5/k_3 , k_7/k_3 , and k_{14}/k_1 , respectively, the rate constants for reactions 1, 3, and 5 are given as follows:

$$k_1 = 6.7 \times 10^{10} \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1},$$

$$k_3 = 3.6 \times 10^{10} \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1},$$

$$k_5 = 2.9 \times 10^{10} \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}.$$

Smoluchowski⁸⁾ had given the following relation to the specific collision number between neutral reactants in liquid phase.

$$\Sigma = 4\pi N R_{AB} D_{AB} \times 10^{-3} \quad (1-V)$$

where N , R_{AB} , and D_{AB} represent Avogadro constant, collision radius, and relative diffusion coefficient, respectively.

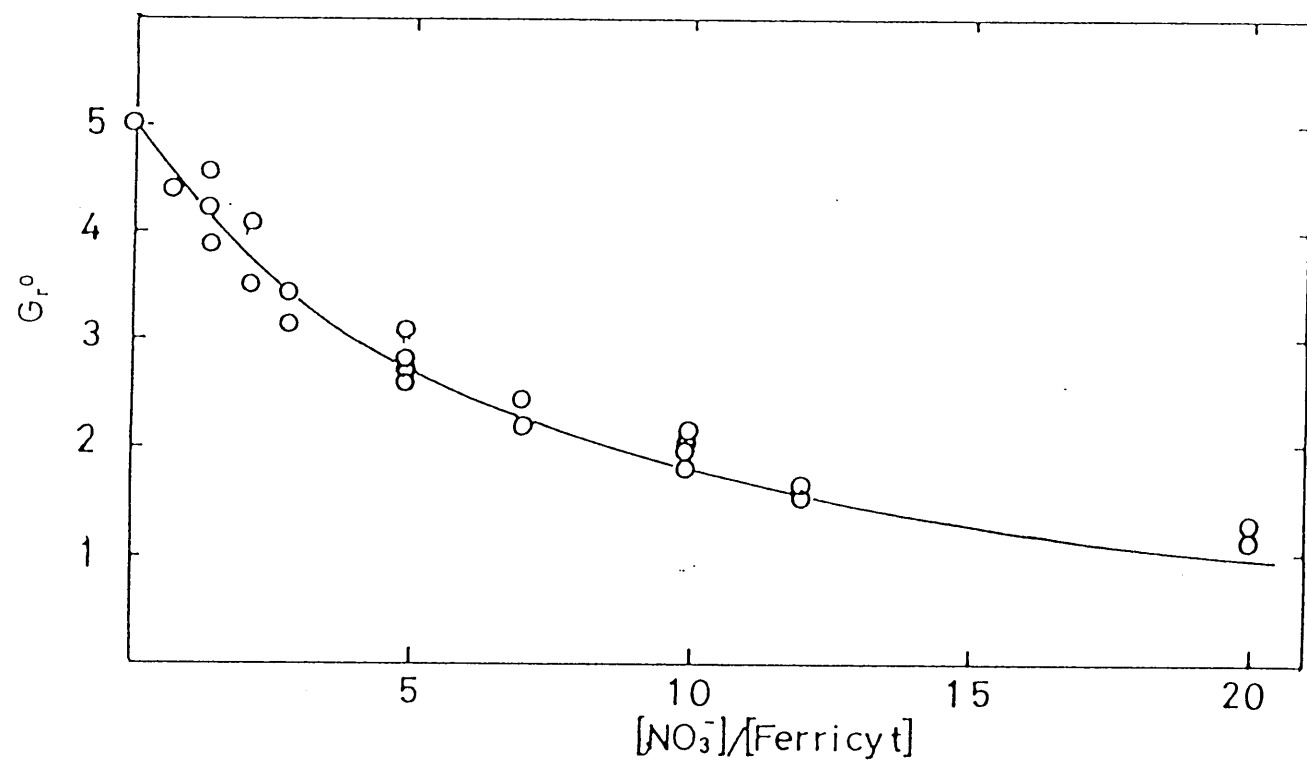


Fig. 1-5. G_r^0 as a function of $[\text{NO}_3^-]/[\text{Ferricytochrome c}]$.

The size of cytochrome c molecule was determined by Levin⁹⁾ to be 4 x 2.8 nm from the electron microscopic data. The diffusion coefficient of cytochrome c was reported by Ehrenberg and Paleus to be $9.5 \times 10^{-11} \text{ m}^2 \text{ s}^{-1}$.¹⁰⁾ The value for OH radical was reported to be $2.3 \times 10^{-9} \text{ m}^2 \text{ s}^{-1}$ (Dorfman and Adams 1973).¹¹⁾ If the collision radius between OH and ferricytochrome c is approximated by the radius of ferricytochrome c, namely 2 nm, Σ is obtained to be $3.5 \times 10^{10} \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$. This is almost the same with the observed rate constant for the reaction of OH radical with ferricytochrome c, k_3 . For reaction between charged species such as the reaction between ferricytochrome c and hydrated electron, a reaction rate constant can be expressed by the following equation:^{12, 13)}

$$k = 4\pi q_A q_B N^2 D / 10^3 \epsilon RT \quad (1-VI)$$

where q_A and q_B are the electric charges of reactant A and B, respectively. D represents the relative diffusion coefficient and ϵ is the dielectric constant of medium. At 300 K ϵ is 78.5 and D is approximated by the diffusion coefficient of hydrated electron, $4.9 \times 10^{-9} \text{ m}^2 \text{ s}^{-1}$.¹⁴⁾ If +3e is taken as the electric charge of ferricytochrome c, $7.9 \times 10^{10} \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$ is calculated as the rate constant for the reaction of ferricytochrome c with hydrated electron. Observed value is $6.7 \times 10^{10} \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$. Total net charge of ferricytochrome c is +12e. Therefore, partial charge around heme may contribute reaction rate.

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Chapter 2. Conformational Degradation of Cytochrome c in Aqueous Solution During Gamma-Irradiation.

Extensive work has been published on chemical action of ionizing radiation on cytochrome c in aqueous solution. Chemical changes of component amino acids, oxido-reduction of heme, an appearance of CO-affinity, and an increase in absorption at 280 nm and 600 nm have been reported.¹⁻⁶⁾

Since Margoliash et al. (1959) concluded that an appearance of CO-affinity is due to conformational change of cytochrome c molecule,⁷⁾ CO-affinity is a good mark for examining conformational change of cytochrome c. On the other hand, susceptibility to proteinase was also used to reveal the conformational change of protein.⁸⁾ Spectrophotometric titration is another good tool to investigate the conformational change.⁹⁻¹¹⁾ It gives an information on the state of tyrosyl residues embedded in protein.

In this chapter, studies on conformational change of cytochrome c induced by gamma-irradiation will be described. In addition to the above-mentioned methods, difference spectra method in UV-region has been also applied to obtain the information of conformational change of cytochrome c.

12, 13)

EXPERIMENTAL

Preparation of horse heart cytochrome c was described in Chapter 1. Trypsin was purchased from Worthington Biochemical Corp. in three times recrystallized and free from salt type. α -chymotrypsin was obtained from Sigma Biochemical Co. as three times recrystallized and free from salt type. Nagarse(three times recrystallized) was purchased from Nagase Co. Ltd.

Gamma-irradiation was carried out at 17°C with ^{60}Co gamma rays at dose rates 8.85×10^{17} to 2.06×10^{18} eV cm⁻³ h⁻¹. Irradiation samples were saturated with air. In the experiments about CO-affinity all solutions were irradiated in 0.05 mol dm⁻³ phosphate buffer at pH 7. Initial concentration of cytochrome c was calculated from the absorbance at 550 nm taking 27.7×10^3 dm³ mol⁻¹ cm⁻¹ as its molar absorption coefficient. Affinity of cytochrome c to carbon monoxide was assayed by the method of Tsou.¹⁴⁾

Susceptibility of cytochrome c against proteinase was determined by the following procedure. One cm³ of trypsin Nagarse, or α -chymotrypsin solution, which was adjusted to the concentration showing an appropriate activity in phosphate buffer(pH 7.0), was added to 1 cm³ of irradiated solution including 5.82×10^{-5} mol dm⁻³ of cytochrome c. The ratio of proteinase to cytochrome c in the solution was taken to be 1:75.3 in weight. The solution was incubated at 30°C for 10 minutes. Then 2 cm³ of 0.44 mol dm⁻³ trichloroacetic acid was added into the solution and the mixture was

incubated at 30°C for 30 minutes. The mixture was filtered and the absorbance at 280 nm (aromatic amino acids) and 395 nm (heme peptide) of the filtrate was measured by spectrophotometer.

Difference spectra were measured by Cary M-14 recording spectrophotometer. For spectrophotometric titration and the difference spectra measurement, the solutions were prepared by the following procedure: 2 cm³ of irradiated or unirradiated solution was transferred into 10 cm³ measuring flask and pH and the ionic strength of the solution were adjusted with 0.1, 1, 10 mol dm⁻³ KOH, 1 mol dm⁻³ HCl and /or KCl.

RESULTS AND DISCUSSION

Change in CO-affinity of cytochrome c

As indicated by Margoliash et al.⁷⁾ native cytochrome c does not react either with oxygen or carbon monoxide, because the heme is embedded in protein molecule. As shown in Fig. 2-1, CO-affinitive cytochrome c increased with increasing dose in initial stage of irradiation and then decreased. The increase in CO-affinity can be explained in terms of formation of partly modified molecule and the exposure of embedded heme. The decrease in higher dose region would be interpreted by the destruction of heme exposed directly to active species. The ordinate in Fig. 2-1

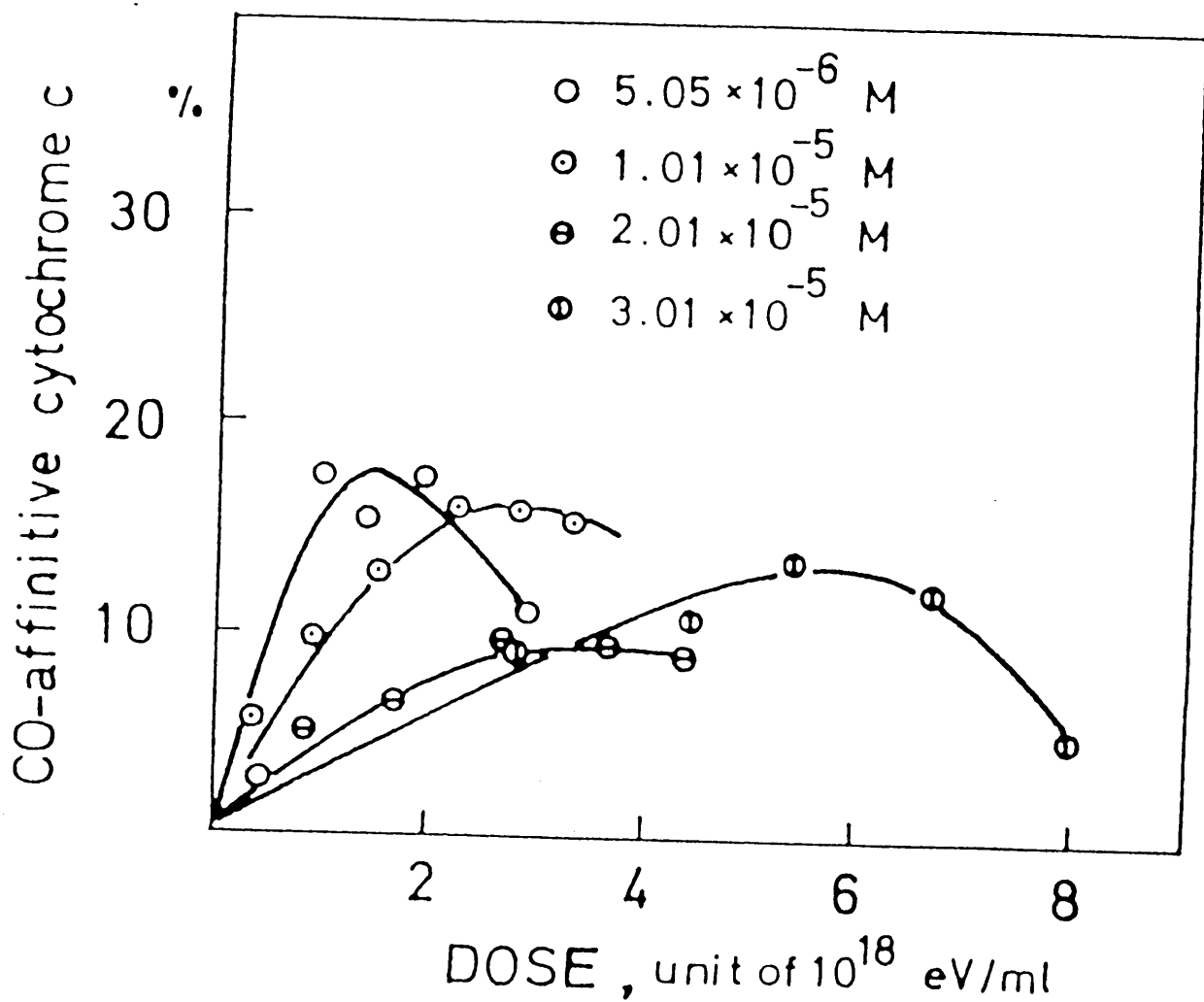


Fig. 2-1. Increase in CO-affinity of cytochrome c.

is corrected for the decrease in absorbance at 550 nm due to simultaneous decomposition of heme.

Susceptibility to Proteinase

Yamanaka et al. reported that native cytochrome c is fairly resistant to digestion by proteinase, although there is a slight difference in susceptibility between ferri- and ferrocytochrome c.⁸⁾ As shown in Fig. 2-2, the amount of digestible molecule increased with increasing dose. In the present experimental condition, unirradiated cytochrome c was hardly digested. Absorbance at 395 nm of the filtrate had a maximum at about 8×10^{18} eV cm⁻³. These results indicate that the active species produced in radiolysis of water induce firstly the change in conformation of cytochrome c molecule (so-called denaturation) and then the destruction of heme. Dose-dependence in absorbance at 395 nm seems to be similar to that of CO-affinity. This suggests that CO-affinity demands a modified but not destructed structure of vicinity of heme. Probably an appearance of CO-affinity needs exposure of embedded heme and release of a ligand from heme iron. Susceptibility to α -chymotrypsin above 10^{19} eV cm⁻³ seems to differ from that to other proteinases. This probably reflects the degradation of aromatic amino acids which are essential to specificity of the enzyme action.

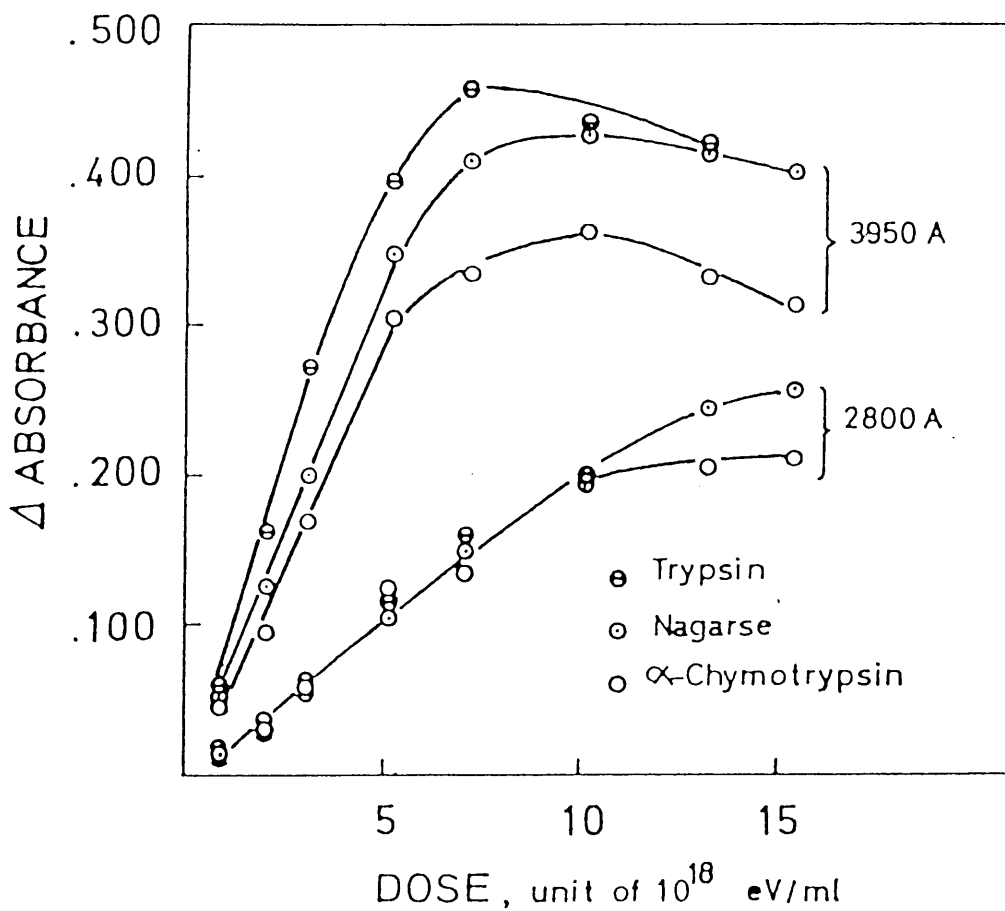


Fig. 2-2. Increase in susceptibility of cytochrome c against some proteinases.

Spectrophotometric titration of tyrosyl residues.

In Fig. 2-3, difference spectra of cytochrome c in alkaline solution vs in neutral solution are shown, where the latter was used as a reference. Increase in absorbance at 243 and 295 nm caused by alkaline environment was assigned to ionization of phenoxy group of tyrosyl residue.¹³⁾ The difference spectrum of irradiated cytochrome c solution was somewhat different from that of unirradiated one in the region of 260-330 nm. The broad absorption observed for irradiated solution may be caused by some products from degradation of heme. Therefore, the difference absorbance at 243 nm was used for spectrophotometric titration. Fig. 2-4 shows the titration curves obtained for the solutions irradiated with various doses. A midpoint of the titration curve of unirradiated sample was seen at pH 12.4. This is in close agreement with that reported by Stellwagen.⁹⁾ The value is extraordinarily high compared with the usual midpoint for free tyrosine and indicates that all tyrosine residues are embedded in the inner part of the protein. The midpoint of the titration curve moved to lower pH with increasing dose. The midpoint of 10.8 for the sample irradiated with dose of $13.4 \times 10^{18} \text{ eV cm}^{-3}$ was approximately the same with that for tryptic digest of cytochrome c (10.4) or cytochrome c denatured with 8 mol dm^{-3} urea (11.0). This indicates that the circumstance of the embedded tyrosyl residues gradually changes to unfolded state.

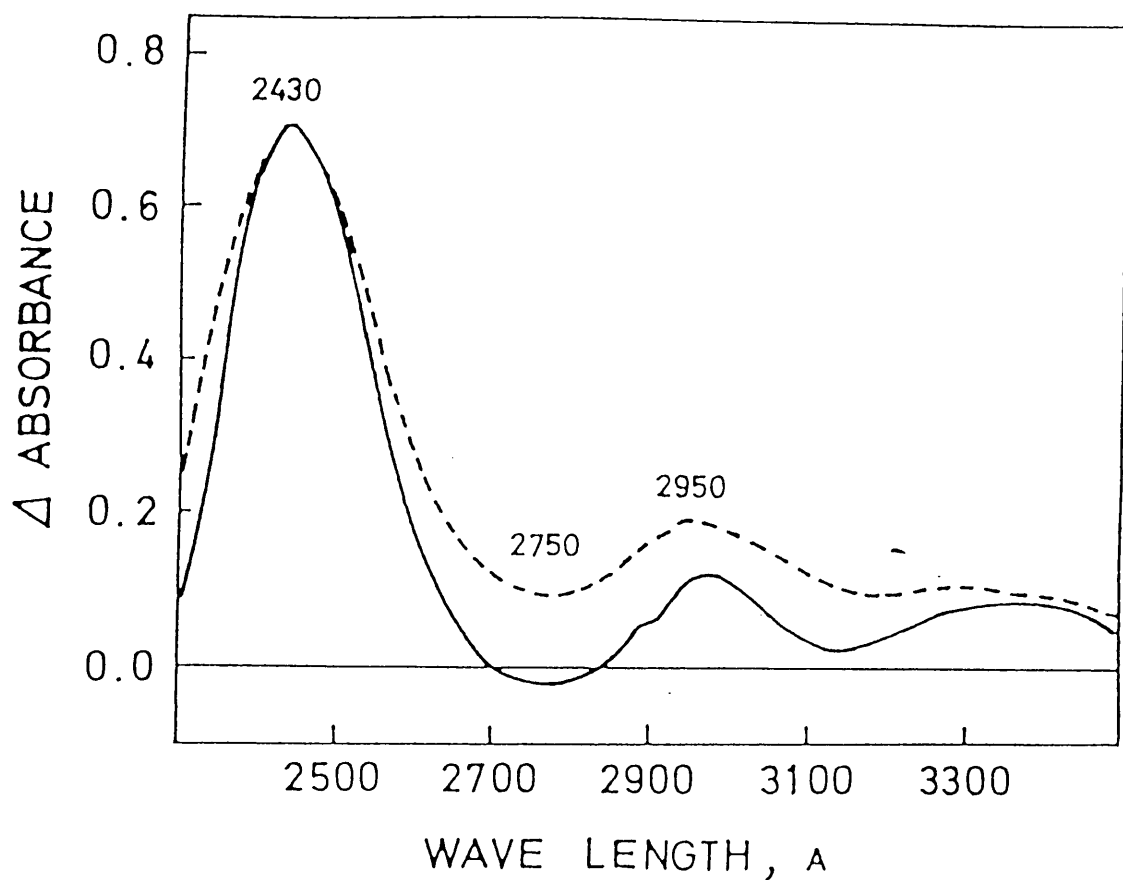


Fig. 2-3. UV difference spectra of cytochrome c in alkaline solution(pH 13.1) vs. in neutral solution(pH 6.4). Concentration of cytochrome c: 1.6×10^{-5} mol dm^{-3} . Ionic strength was adjusted to 0.25. Solid line: unirradiated. Broken line: 8.01×10^{-5} mol dm^{-3} solution of cytochrome c was irradiated to 1.35×10^{19} eV cm^{-3} .

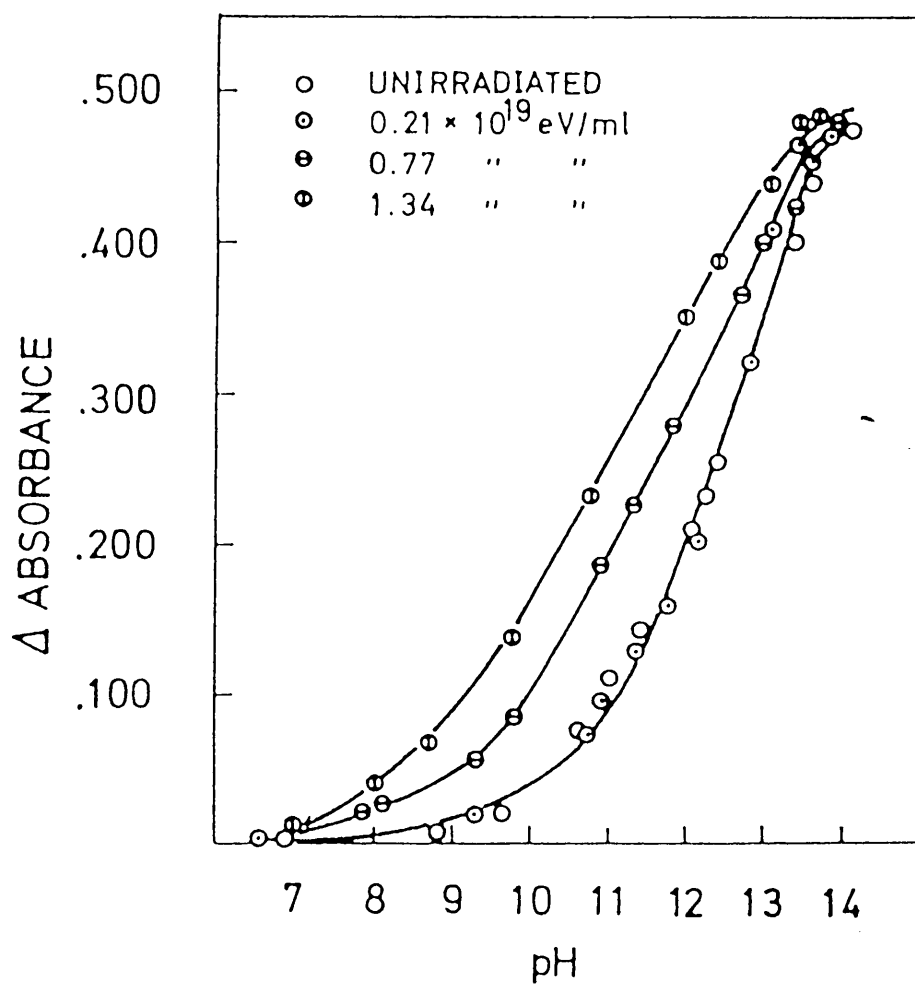


Fig. 2-4. Spectrophotometric titration curves of cytochrome c.

Difference Spectra of Untreated vs Acid- or Urea-Treated Cytochrome c.

The difference spectra of untreated vs acid- or 8 mol dm^{-3} urea-treated cytochrome c indicated two peaks at 230 and 290 nm as shown in Figs. 2-5 and 2-6. The peaks are considered to be characteristic for the difference spectrum of native vs denatured protein.^{1,2,13)} The peak at 290 nm may be assigned to the environmental change of tyrosyl or tryptophyl residues owing to denaturation of protein and the peak at 230 nm can be attributed to ionization of carboxyl groups, change in aromatic absorption, and conformational change of polypeptide backbone, as reported by Glazer and Smith^{1,2)} and Wetlaufer.¹³⁾ As shown in Fig. 2-5, both peaks were virtually lost after irradiation in the difference spectrum of untreated vs urea-treated cytochrome c. In the case of the difference spectrum of untreated vs acid-treated cytochrome c, the peaks were seen after irradiation, although the peak at 230 nm was slightly reduced (Fig. 2-6). These difference spectra imply that irradiation of about 10^{19} eV cm^{-3} causes the conformational change in cytochrome c molecule to the similar extent with urea treatment.

As a rough profile of radiation-induced degradation of cytochrome c molecule, this study can present the following steps;

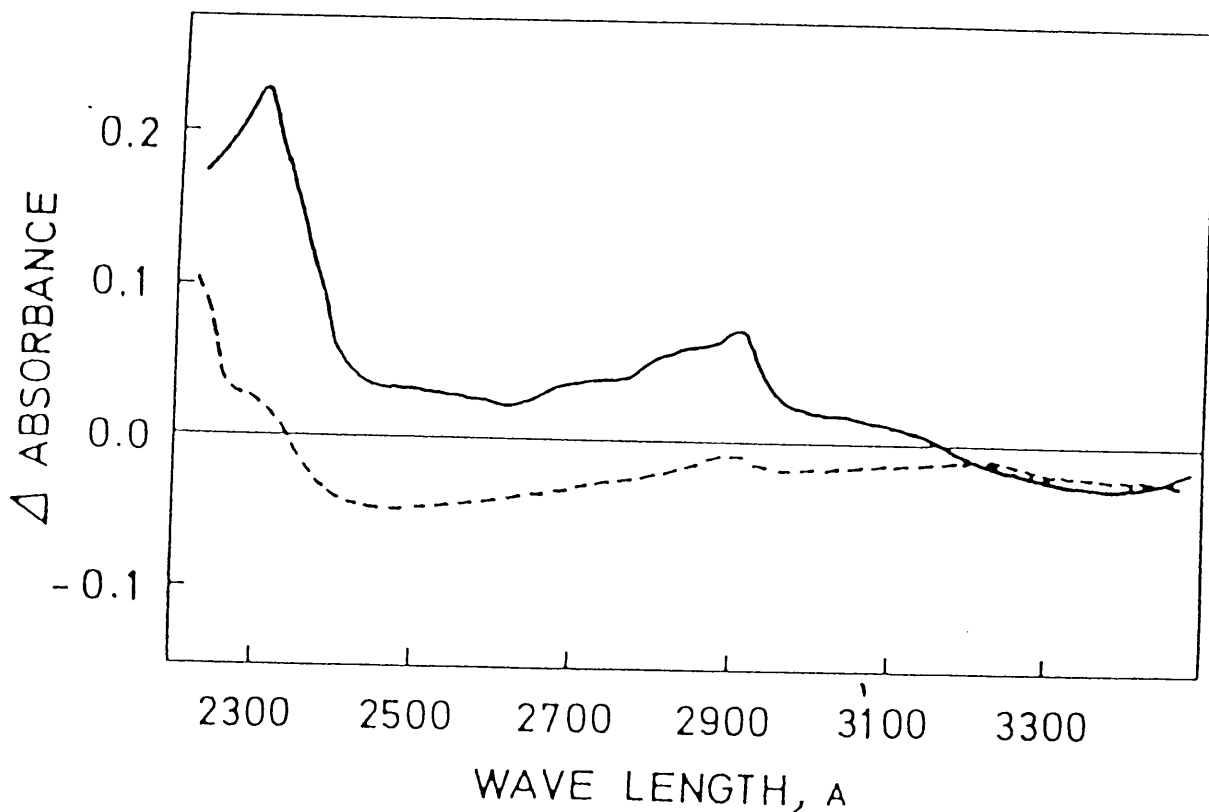


Fig. 2-5. UV difference spectra of untreated vs. urea-treated cytochrome c.

Concentration of cytochrome c: $1.6 \times 10^{-5} \text{ mol dm}^{-3}$. Sample without urea(pH 6.1) vs. Sample containing 8 mol dm^{-3} urea(pH 6.7). Ionic strength was adjusted to 0.25. Solid line: unirradiated. Broken line: irradiated at a concentration of $8 \times 10^{-5} \text{ mol dm}^{-3}$ to a dose of $1.33 \times 10^{19} \text{ eV cm}^{-3}$.

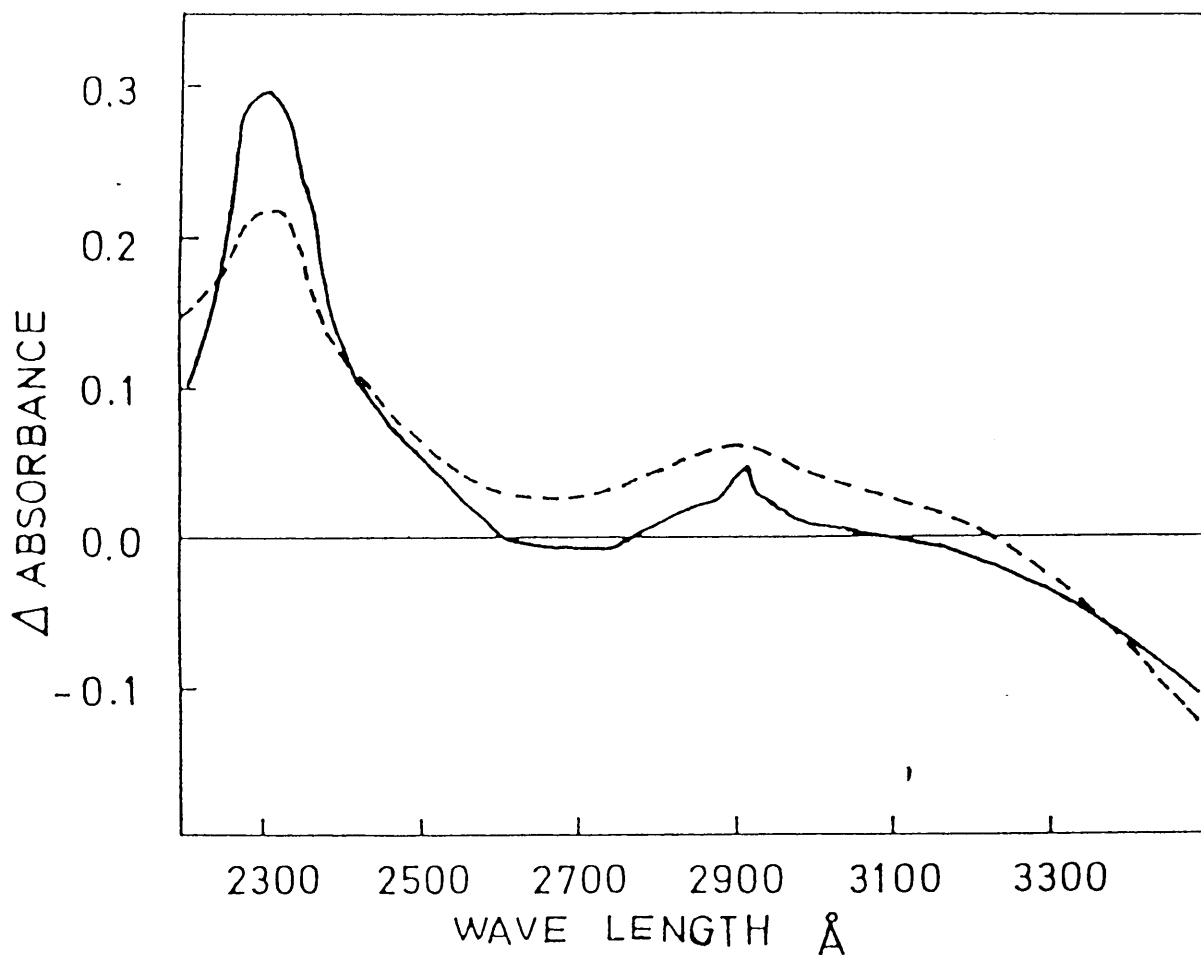


Fig. 2-6. UV difference spectra of untreated vs. acid treated cytochrome c.

pH 6.1 vs. pH 0.2(ionic strength: 0.25).

Concentration of cytochrome c: 1.6×10^{-5} mol dm^{-3} . Solid line: unirradiated. Broken line: irradiated at a concentration of 8×10^{-5} mol dm^{-3} to a dose of 1.33×10^{19} eV cm^{-3} .

- 1) unfolding of peptide chain in the vicinity of heme and exposure of embedded heme.
- 2) release of a ligand from heme and appearance of CO-affinity,
- 3) unfolding of peptide chain and exposure of embedded aromatic amino acids such as tyrosine or tryptophan to outside where they are attacked by proteinases,
- 4) destruction of heme and amino acid residues which were initially interior protein molecule.

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Chapter 3. Ultraviolet Absorption Spectra of γ -irradiated Cytochrome c.

It has been reported that the absorbance at 280 nm of oxidized horse heart cytochrome c increases in aqueous solution following exposure to ionizing radiation.¹⁾ The similar findings have been also reported by Barron *et al.*²⁾ In this chapter, more detailed study will be described on the UV-spectral changes in γ -irradiated cytochrome c, native cytochrome c, mixture of component amino acids of cytochrome c, and aromatic amino acids.

EXPERIMENTAL

The method of preparation of irradiation sample and spectrophotometric study have been already described in Chapter 2. The aqueous mixtures of constituent amino acids of cytochrome c were prepared with free L-amino acids except D,L-methionine and L-lysine hydrochloride based on the published amino acids composition of horse heart cytochrome c.³⁾ Concentration of component amino acids for spectrophotometric measurements corresponds to 1.6×10^{-5} mol dm⁻³ of cytochrome c. Difference spectra were measured by Cary M-14 spectrophotometer with 1 x 1 cm quartz cell.

Irradiation was carried out with ^{60}Co γ -source and the total dose lies on 1.33 to 1.35×10^{19} eV cm⁻³.

RESULTS

Figure 3-1 shows the ultraviolet absorption spectra of unirradiated (solid line) and irradiated aqueous cytochrome c (broken line). For the irradiated solution, very broad increase in absorbance was observed in the range of wavelength from 230 to 330 nm. The shoulder at 290 nm, which has been assigned to tryptophyl residue, was found to disappear. No shift of a peak at 280 nm was observed. These are in accordance with the results reported by Barron *et al.*²⁾ The similar increase in UV-absorbance has been observed by several researchers in irradiated solution of tyrosine.⁷⁻⁹⁾ The same change in spectrum has been reported in aqueous solution of tyrosine or various proteins after treatment with tyrosinase.^{7, 10, 11)} Therefore, Barron *et al.* attributed the increase in UV-absorbance to oxidation of tyrosine.⁷⁾ On the other hand, Drake *et al.*¹²⁾ and Rosen¹³⁾ explained it in terms of light scattering due to aggregation of protein molecule. In Fig. 3-2, difference spectra of the irradiated vs. unirradiated solution of ferricytochrome c are shown. At neutral pH, a broad increase with tiny peaks at 242, 287, and 297 nm was observed. This is almost the same with the difference spectra obtained irradiated aqueous ribonuclease^{14, 15)} and yellowfin tuna metmyoglobin¹⁶⁾. Brown *et al.*¹⁶⁾ attempted to explain the causes of the

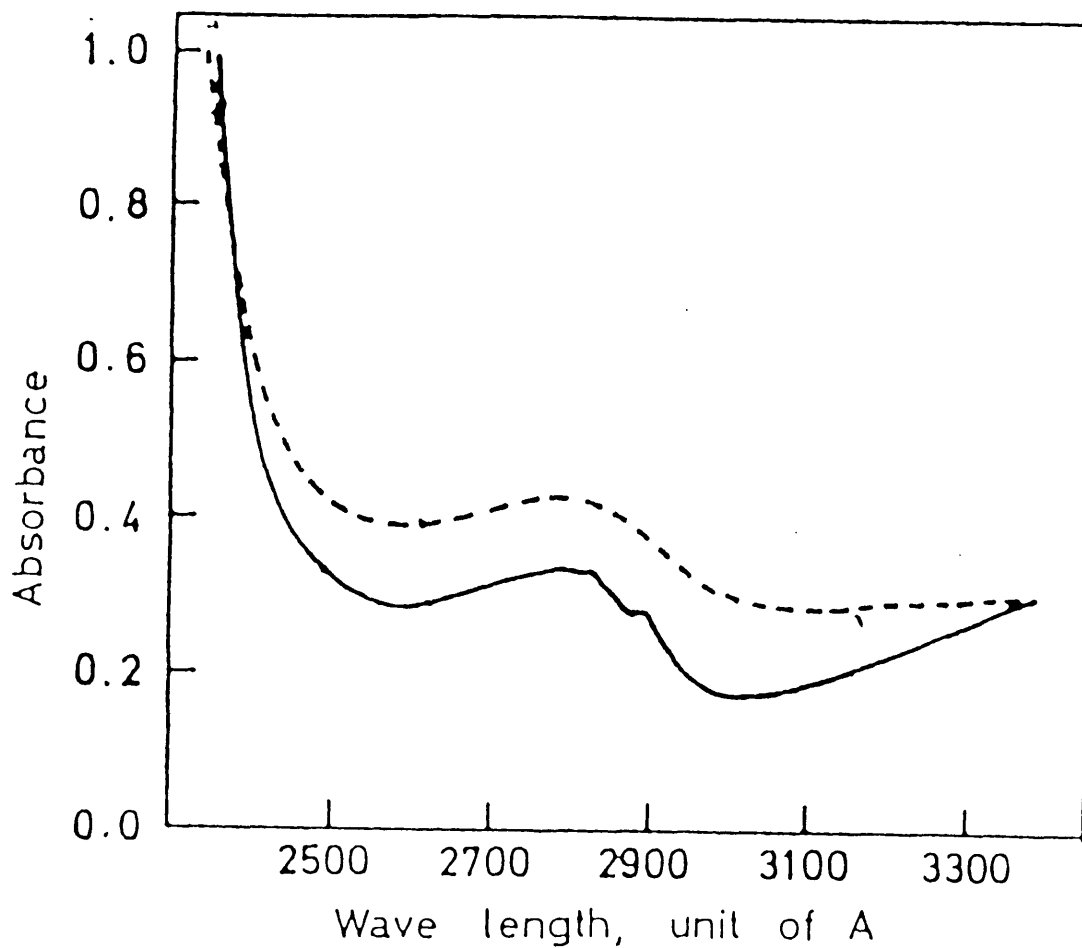


Fig. 3-1. UV absorption spectra of unirradiated and irradiated solutions of cytochrome c(pH 5.6).
Concentration of cytochrome c: 1.59×10^{-5} mol dm^{-3} (7.94×10^{-5} mol dm^{-3} on irradiation).
Solid line: unirradiated. Broken line: irradiated.

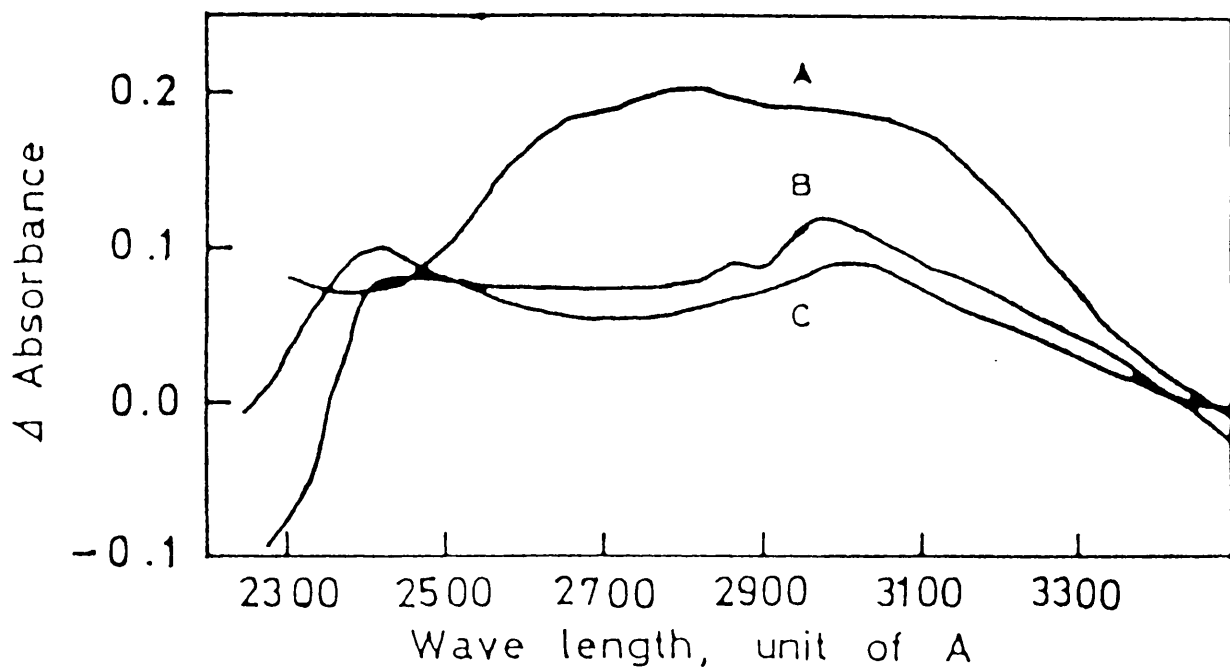


Fig. 3-2. UV difference spectra of irradiated vs. unirradiated aqueous solution of cytochrome c. Concentration of cytochrome c: 1.6×10^{-5} mol dm^{-3} (8.01×10^{-5} mol dm^{-3} on irradiation). A: pH 13.1. B: pH 6.4. C: pH 0.3.

increase in UV-absorbance in terms of 1) hydroxylation on aromatic rings, 2) formation of cyclic structure, and/or 3) light scattering due to an increase in size of particle or molecule (Tyndall phenomenon). The last possibility was denied by their finding that sedimentation coefficient of myoglobin was not affected by irradiation. Smith *et al.*¹⁵⁾ attributed the broad increase in UV-absorbance of the irradiated aqueous ribonuclease to new chromophore produced in the reaction of tyrosyl residue with OH radical based on the following experimental results; 1) inapplicability of Rayleigh scattering function to the observed increase in absorbance, 2) close similarity of the difference spectrum of irradiated vs. unirradiated 6N-HCl hydrolysate of ribonuclease with that observed for irradiated vs. unirradiated tyrosine, and 3) decrease in fluorescence of tyrosine induced by irradiation. They also concluded that the change in spectrum due to alteration of environment of tyrosyl residues is superimposed on the broad increase in absorbance, because several troughs around 235, 280, and 287 nm, which are characteristic to difference spectrum caused by denaturation of protein, were found in the difference spectrum of irradiated vs. unirradiated ribonuclease. Rosen¹³⁾ revealed that the fourth power of wavelength times difference in absorbance, $\lambda^4 \times \Delta A$, is kept constant, if the broad increase in absorbance is solely caused by Rayleigh scattering of light due to aggregation of protein. On the

other hand, the relation does not hold when a certain intramolecular structural change takes place. In the case of X-irradiated human serum albumin, the difference spectrum of the irradiated vs. unirradiated sample shows small peaks at 295 and 300 nm. However the spectrum of irradiated vs. unirradiated aqueous tyrosine had an intense absorption peak at 285 nm. Based on these results, the author concluded that the intramolecular change produced in irradiated serum albumin is not the alteration of tyrosine itself. In Fig. 3-3, $\lambda^4 \times \Delta A$ is plotted against wavelength λ for cytochrome c. It indicated the formation of new chromophore that strongly absorbs light around 297 nm. The difference spectrum of aqueous cytochrome c acidified with HCl after irradiation was almost similar with that measured at neutral pH except the disappearance of a peak at 287 nm and a trough at 290 nm as shown in Fig. 3-2. When the solutions were alkalified with KOH after irradiation, the peaks at 240, 287, and 297 nm diminished. Furthermore, the difference spectrum of the alkalified solution changed entirely its shape and showed a broad maximum around 280 nm and a minimum around 240 nm. Fig. 3-4 shows the difference spectra measured at various pH after irradiated and unirradiated solutions were kept over night in the presence of 8 mol dm^{-3} urea. For the difference spectrum measured at neutral pH, a peak at 287 nm and a trough at 290 nm were not seen after urea treatment. For alkalified solution, the difference

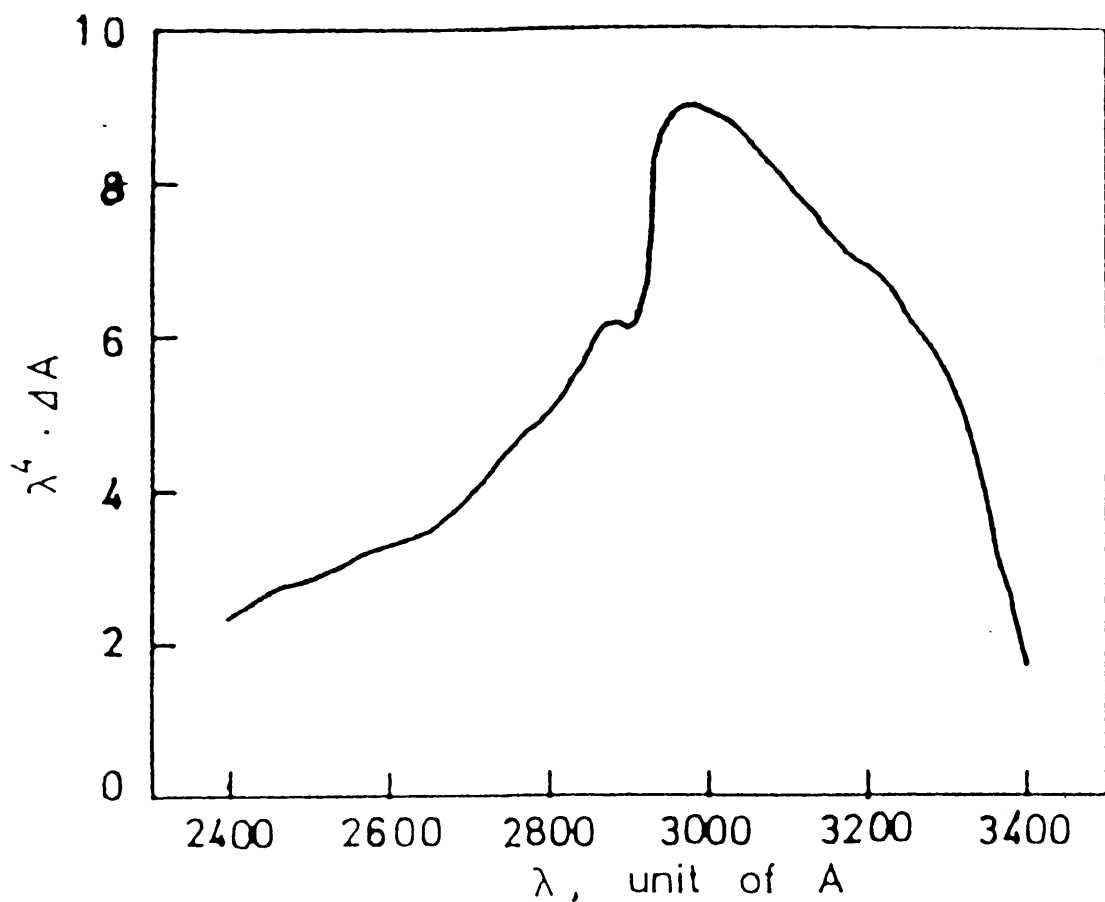


Fig. 3-3. $\lambda^4 \Delta A$ against λ (wavelength).

ΔA is difference of absorbance between irradiated and unirradiated solutions. The ordinate is expressed in $10^{-12} \times (\text{wavelength in } \text{\AA})^4 \times \text{absorbance difference}$).

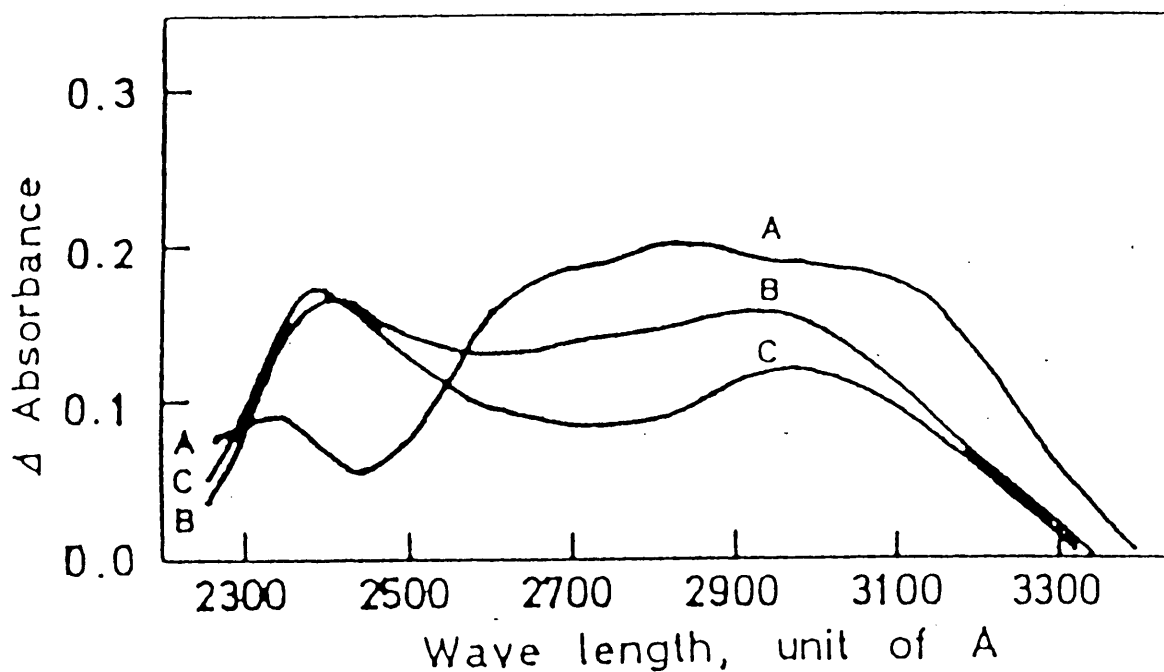


Fig. 3-4. Difference spectra of irradiated vs. unirradiated aqueous solution of cytochrome c in the presence of 8 mol dm⁻³ urea.

A: pH13.0. B: pH6.7. C:pH 1.6. Other conditions: See the legend in Fig. 3-2.

spectra resembled each other irrespective of urea treatment.

. In order to clarify the increase in absorbance of irradiated cytochrome c, an attempt was made to measure the difference spectrum of irradiated vs. unirradiated solution of aromatic amino acids, because these amino acid residues mainly contribute to the light absorption of protein in UV-region. Furthermore, the difference spectra of 3,4-dihydroxyphenylalanine (3,4-dopa) vs. tyrosine were measured, because 3,4-dopa is a most probable candidate of a product formed from tyrosine after irradiation. The difference spectra of irradiated vs. unirradiated aromatic amino acids are shown in Fig. 3-5. Broad increase in absorbance was observed in the difference spectra of phenylalanine and tyrosine. Peaks at 222 and 272 nm were observed for phenylalanine and those at 238 and 287 nm for tyrosine. On the other hand, the absorbance of tryptophan below 300 nm decreased after irradiation, as the difference spectrum of tryptophan indicated. The difference spectrum of unirradiated 3,4-dopa vs. unirradiated tyrosine showed peaks at 236 and 286 nm. They are very similar to those of irradiated tyrosine vs. unirradiated tyrosine. The difference spectra of acidified solutions of aromatic amino acids were almost the same with those of neutral solutions. Figure 3-6 shows the difference spectra of aqueous aromatic amino acids measured in alkaline condition. They gave little information on structural changes except broad

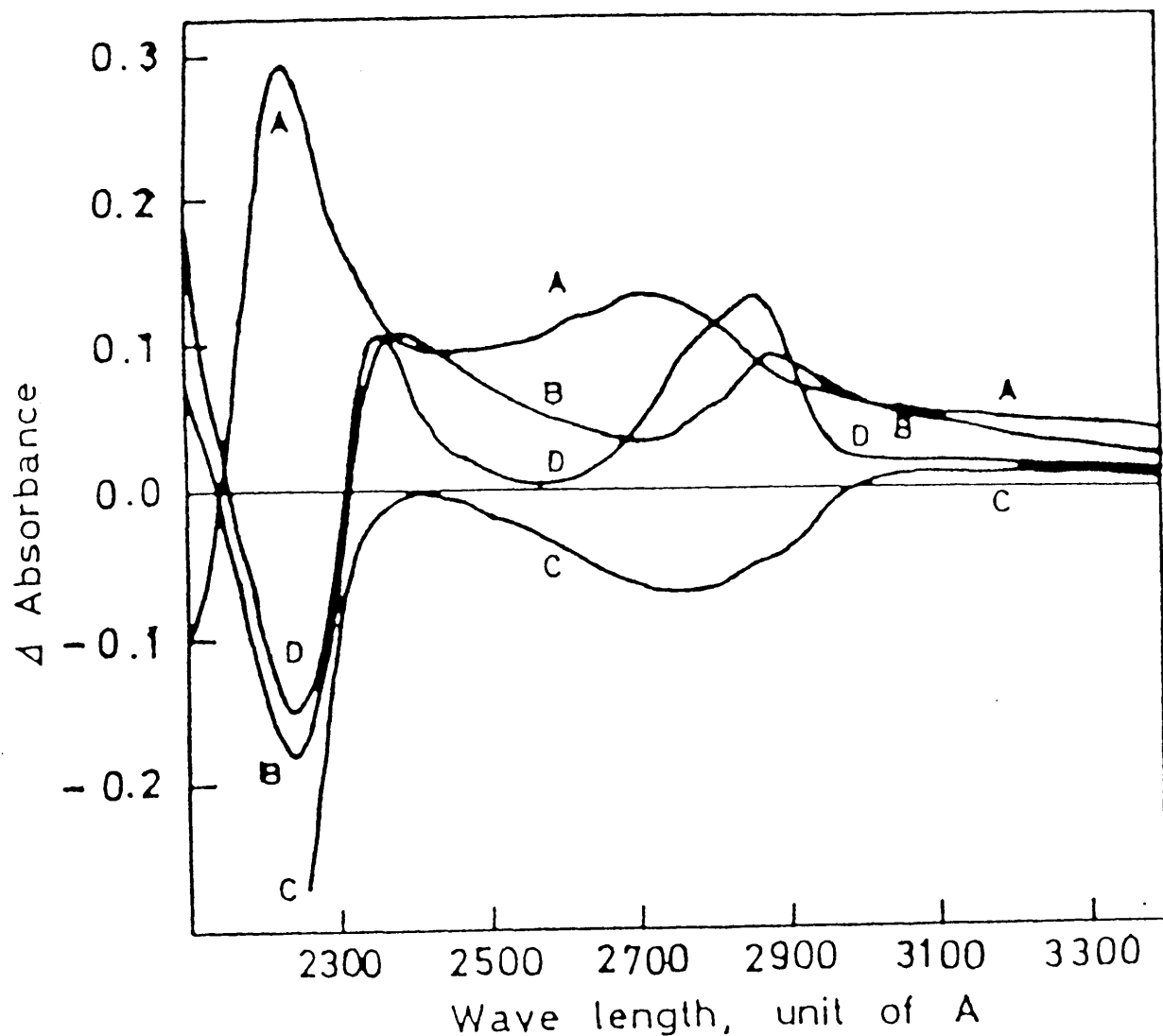


Fig. 3-5. Difference spectra of irradiated vs. unirradiated neutral solutions of aromatic amino acids (pH 5.5-6.1).

A; phenylalanine. B; tyrosine. C; tryptophan.
D; unirradiated 3,4-dopa vs. unirradiated tyrosine.

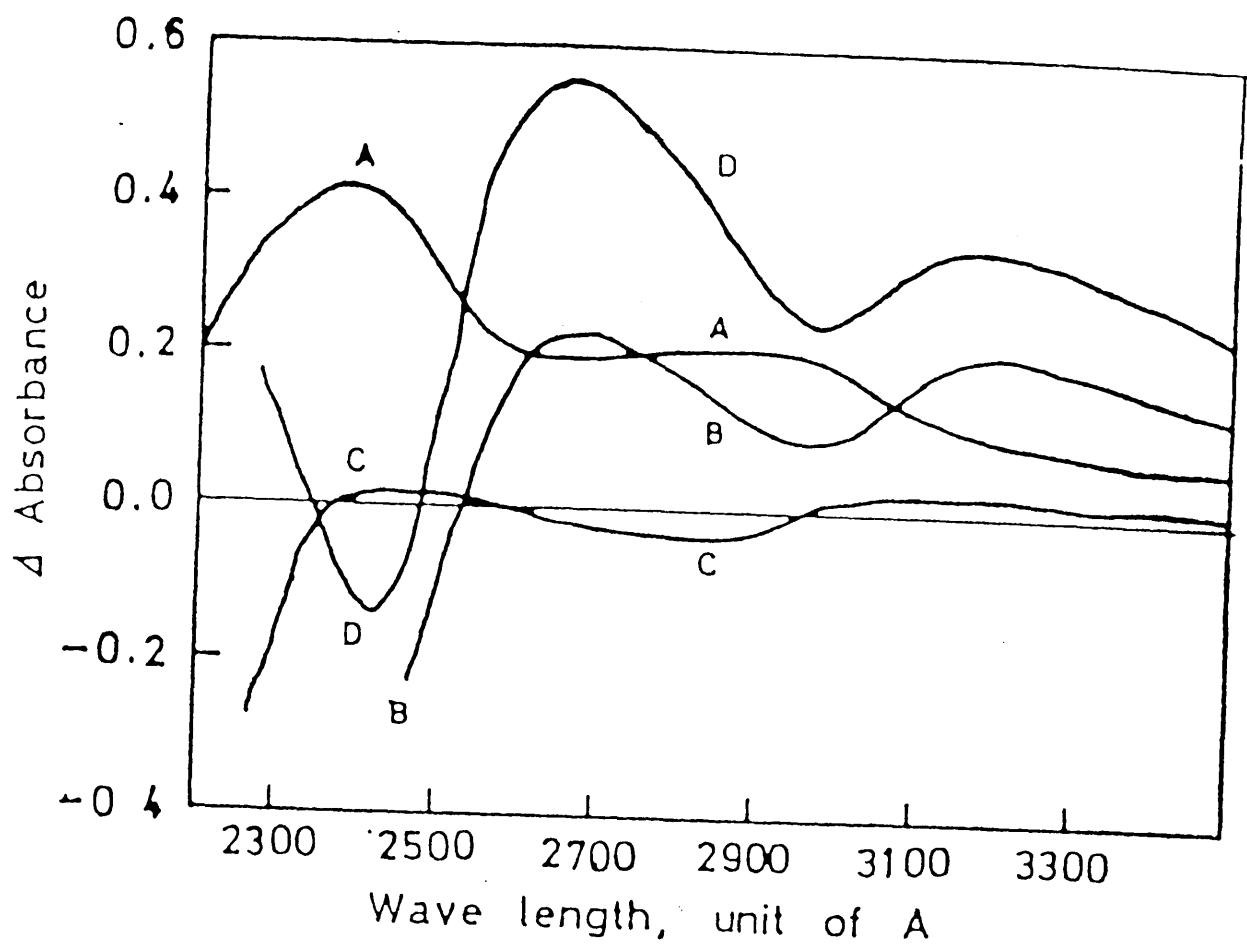


Fig. 3-6. Difference spectra of irradiated vs. unirradiated KOH-alkalified solutions of aromatic amino acids (pH13.0-13.3). Other conditions; see the legend in Fig. 3-5.

increase in absorbance of irradiated solutions.

In neutral or acid condition, the difference spectrum of cytochrome c appeared to resemble the spectrum of tyrosine. Therefore, chemical change of tyrosine to dopa, which may be produced through the reaction with OH radical, contributes mostly to the difference spectrum.

In Fig. 3-7, the difference spectra of irradiated vs. unirradiated aqueous mixture of the constituent amino acids of cytochrome c are given. Spectrum D corresponds to a mixture of constituent amino acids except phenylalanine, tyrosine, and tryptophan. In alkaline condition, it showed a broad and tiny increase in absorbance but the increase was hardly seen in neutral and acid condition. The difference spectra of a complete mixture measured at neutral and acid pH show the peaks around 235 nm and around 295-310 nm, but those in longer wavelength were not distinct. Since the equivalent concentration of constituent amino acids was taken to cytochrome c, small peak in longer wavelength suggests further destruction of dopa once produced from tyrosine or phenylalanine. In protein molecule it may be protected by neighbor residues against further destruction.

As shown in Fig. 3-8, the difference spectrum of alkaline vs. neutral solution of tyrosine were similar to that of dopa after irradiation. Radiation-induced change in peak height at 242 nm was larger than that at 295 nm.

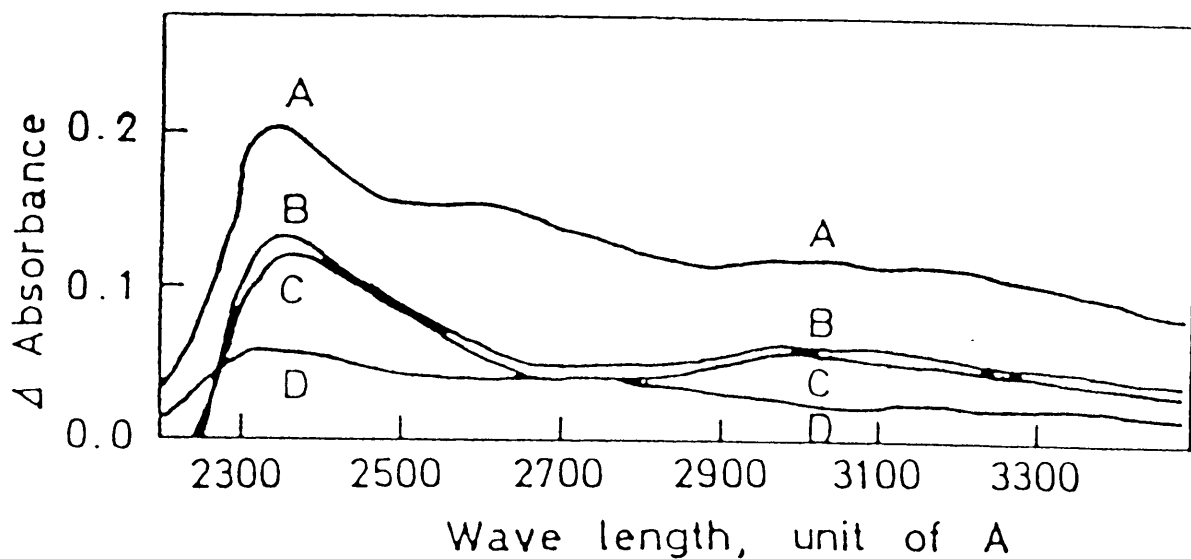


Fig. 3-7. Difference spectra of irradiated vs. unirradiated aqueous solutions of the constituent amino acids of cytochrome c.

A; pH 12.5. B; pH 4.5. C; pH 0.7. D; solution without tyrosine phenylalanine, and tryptophan(pH 10.0).

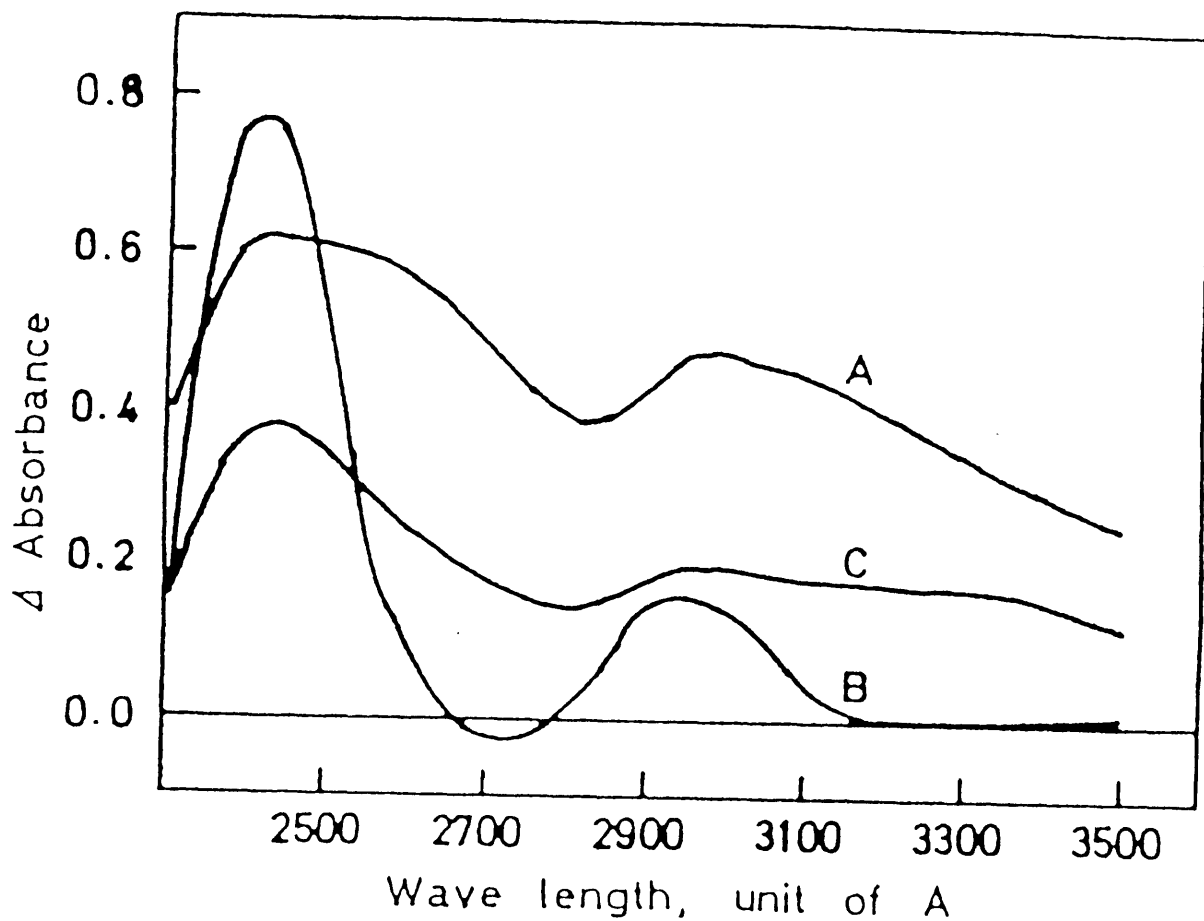


Fig. 3-8. Difference spectra of alkalified vs. neutral solutions of tyrosine or 3,4-dopa.

A; unirradiated 3,4-dopa(pH 12.5 vs. pH 5.3). B; unirradiated tyrosine(pH 13.3 vs. pH 6.1). C; irradiated tyrosine(pH 13.1 vs. pH 6.4).

However this difference was not observed in the difference spectrum of alkaline vs. neutral mixture of constituent amino acids of cytochrome c (Fig. 3-9).

DISCUSSION

The results obtained in the present experiments suggest that the increase in absorbance of irradiated cytochrome c in UV region is most attributable to the formation of new chromophore absorbing light around 297 nm. However, Tappel *et al.* indicated polymer formation in irradiated cytochrome c solution by means of Sephadex gel filtration.¹⁷⁾ The difference spectrum of irradiated vs. unirradiated aqueous cytochrome c implies that the possibility of light scattering by aggregated protein molecules cannot be excluded. For other irradiated proteins, the light scattering due to aggregation of protein is accepted as a major cause of the broad increase in UV-absorbance.^{12, 13)} The present experiments is not sufficient to provide the information about formation of new chromophore. However, dopa, which is produced from tyrosyl residues through reaction with OH radical, is a most probable candidate for change of absorption spectrum. This is consistent with the results reported by Barron *et al.*⁷⁾ There still remains a difference between the difference spectrum of cytochrome c and that of tyrosine,

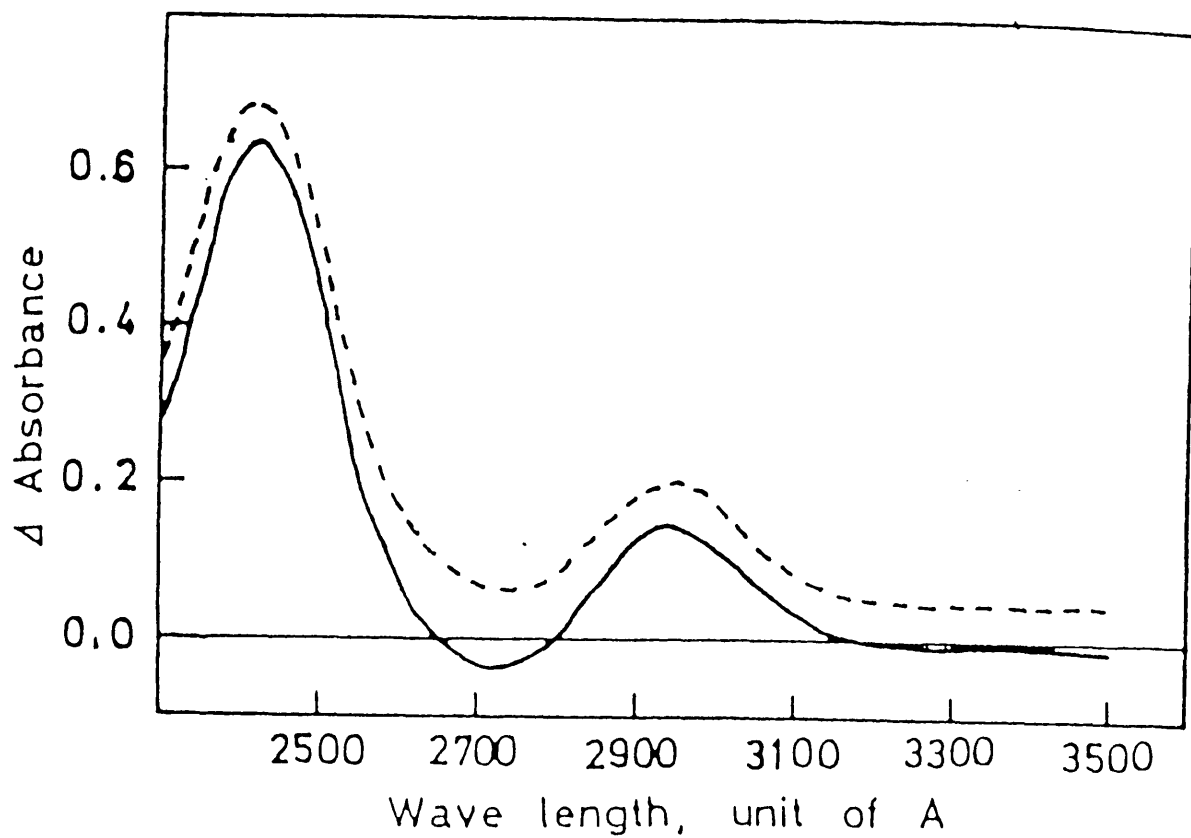


Fig. 3-9. Difference spectra of alkalified vs. neutral solutions of the constituent amino acids of cytochrome c. (pH 12.5 vs. pH 4.5).

Solid line: unirradiated solution. Broken line: irradiated solution.

when their peak positions are compared with each other. The former had a peak at 297 nm and the latter 286 nm. The difference spectrum of alkaline vs. neutral solution of free tyrosine showed that a peak at 242 nm was remarkably reduced by irradiation. However, such large decrease in absorbance at 242 nm was not observed for the difference spectrum of cytochrome c (Fig. 2-3)⁵⁾ or constituent amino acids mixture (Fig. 3-9). These indicate that the environment of tyrosyl residues affects the reaction of tyrosyl residue with OH radical and also light absorption. Contribution of destructed heme should be also taken into the explanation of absorption change in UV region. It is noteworthy that the location of the trough at 290 nm in the difference spectrum of irradiated vs. unirradiated neutral solution of cytochrome c coincides with that of peak in the difference spectrum of native vs. denatured cytochrome c.⁵⁾

Finally the overall pattern of the difference spectrum of irradiated vs. unirradiated aqueous cytochrome c can be explained as follows; 1) absorption change caused by conformational change of cytochrome c molecule and 2) formation of new chromophore(s) absorbing light at around 297 nm. The overlap of two events is most likely to occur.

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Chapter 4. Photoreduction of Ferricytochrome c in the Presence and Absence of Electron-Donor

As described in chapter 1, ferricytochrome c is easily reduced to ferrocytochrome c by hydrated electrons. Numerous studies have been published on the reduction in connection with an interest in energy trapping mechanism of terminal oxidation system in living cells.¹⁾

On the other hand, it is well known that indole and its derivatives are photoionized to give hydrated electrons.²⁾ The hydrated electrons are reasonably expected to reduce ferricytochrome c in the same manner with those in radiolysis of water.

In this chapter, flash photolysis experiment carried out in aqueous solution of cytochrome c containing indole or *o*-dimethoxybenzene will be described. The formation of ferrocytochrome c was observed not only in nitrogen-saturated solution of ferricytochrome c containing indole or *o*-dimethoxybenzene but also in solution saturated by dinitrogen monoxide, which is known as a typical electron scavenger. Furthermore, the reduction was observed in the absence of electron donor, although the yield was less than that observed in the presence of the electron donor. The direct photoreduction of ferricytochrome c was completely inhibited by an optical filter using 10^{-3} mol dm⁻³ of indole. It was also depressed by oxygen but not completely

by dinitrogen monoxide. The results suggest that the considerable direct photoreduction takes place through an intramolecular process such as an electron transfer from an excited aromatic amino acid residue to ferric ion in heme. Furthermore, hydrogen peroxide was found to photoreduce ferricytochrome c even in oxygen saturated aqueous solution. It suggests that the hydroxyl radical produced photochemically from hydrogen peroxide reacts with ferricytochrome c to give ferrocytochrome c. A mechanism of the direct photoreduction will be discussed in terms of conformation of horse heart ferricytochrome c and photoionization of aromatic amino acid residues.

EXPERIMENTAL

Cytochrome c was prepared from horse heart muscle according to a method of Hagihara et al.³⁾

Indole, o-dimethoxybenzene and other reagents were commercially obtained as chemicals of the highest purity available.

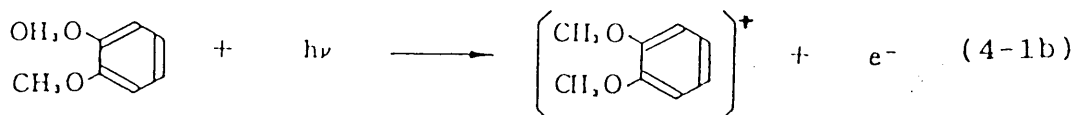
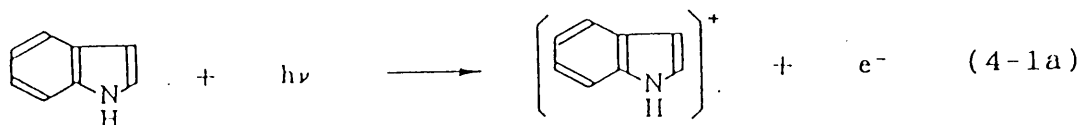
Lyophilized preparation of cytochrome c was dissolved in a small amount of triply distilled water and desalted by filtration through a column of Sephadex G-25. Solutions for irradiation were adjusted to the desired pH by adding 0.1 mol dm⁻³ NaOH or 0.1 mol dm⁻³ H₂SO₄. The solutions were equilibrated with the commercially available nitrogen,

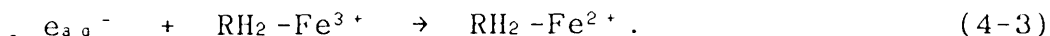
oxygen, or dinitrogen monoxide by bubbling for about ten minutes.

A conventional flash apparatus was used. A pair of xenon flash lamps (USHIO FS-225 HMA) were fired with an input energy of 200 J (12 μ s of 1/e duration). The monitoring system was composed of a xenon arc lamp (USHIO UXL-150D), a monochrometer (NARUMI RM-23-I) and a synchroscope (IWATSU SS-112); a photomultiplier was HAMAMATSU R 136. A cylindrical quartz cell of 10 cm long and 1cm in diameter was used for photoreaction.

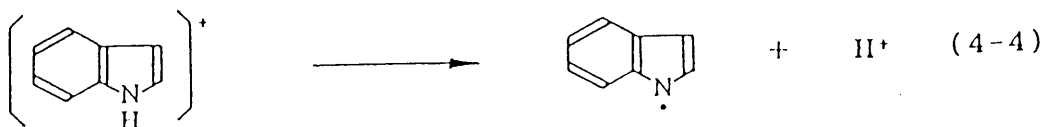
RESULTS AND DISCUSSION

Increase in absorbance at 550 nm was observed in nitrogen saturated solutions of ferricytochrome c containing indole or *o*-dimethoxybenzene after flash as shown in Fig.4-1. The increase suggests the reduction of ferricytochrome c to ferrocytochrome c by photo-produced hydrated electrons. The reduction process can be represented by the following set of reactions:

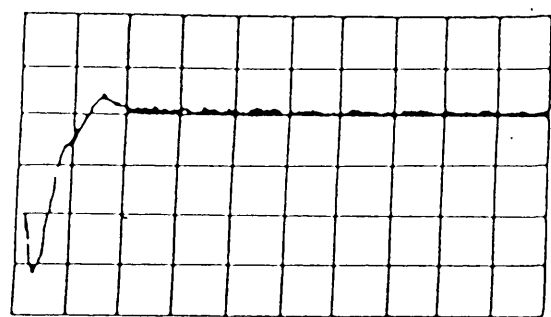




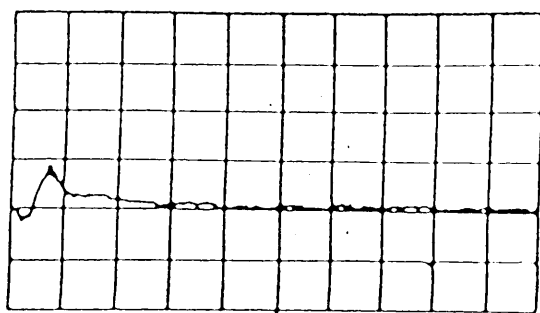
where RH_2-Fe^{3+} and RH_2-Fe^{2+} represent ferri- and ferrocytochrome c, respectively. In the solution containing indole slow decay of the absorption was noticed during initial several hundreds microseconds. It is attributable to a neutral radical of indole, because an absorption near 510 nm, which was observed in photolyzed indole at neutral pH, has been assigned to the neutral radical produced by reaction 4-4.⁴⁾



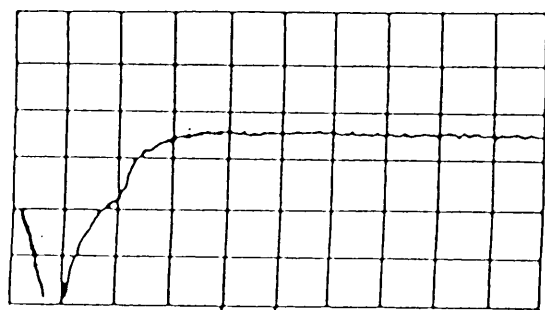
Similar decay of the absorption at 550 nm was observed in nitrogen saturated solutions of indole (Fig.4-1b). Since o-dimethoxybenzene gives no transient absorption in visible region of light,²⁾ optical absorption observed after flash is due to ferrocytochrome c (Fig.4-1c). In order to confirm the formation of ferrocytochrome c, an absorption spectrum of the solution was measured after several flashes. As shown in Fig. 4-2, the spectrum is characteristic of a mixture of ferro- and ferricytochrome c. Figure 4-3 shows the increase in absorbance at 550 nm per flash plotted against concentration of cytochrome c. The increase in



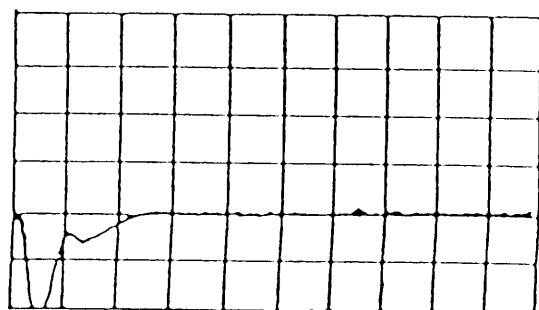
50 μs
(a)



50 μs
(b)



20 μs
(c)



20 μs
(d)

Fig. 4-1. Oscilloscope traces indicating increases in optical absorption at 550 nm.

a; 10^{-4} mol dm $^{-3}$ indole and 6.3×10^{-6} mol dm $^{-3}$ ferricytochrome c.

b; 10^{-4} mol dm $^{-3}$ indole.

c; 10^{-4} mol dm $^{-3}$ o-dimethoxybenzene and 6.3×10^{-6} mol dm $^{-3}$ ferricytochrome c.

d; 4×10^{-4} mol dm $^{-3}$ o-dimethoxybenzene.

Ordinate: Three divisions correspond to 100 per cent of relative transmittance, where the transmittance of solution before flash is taken as 100 percent.

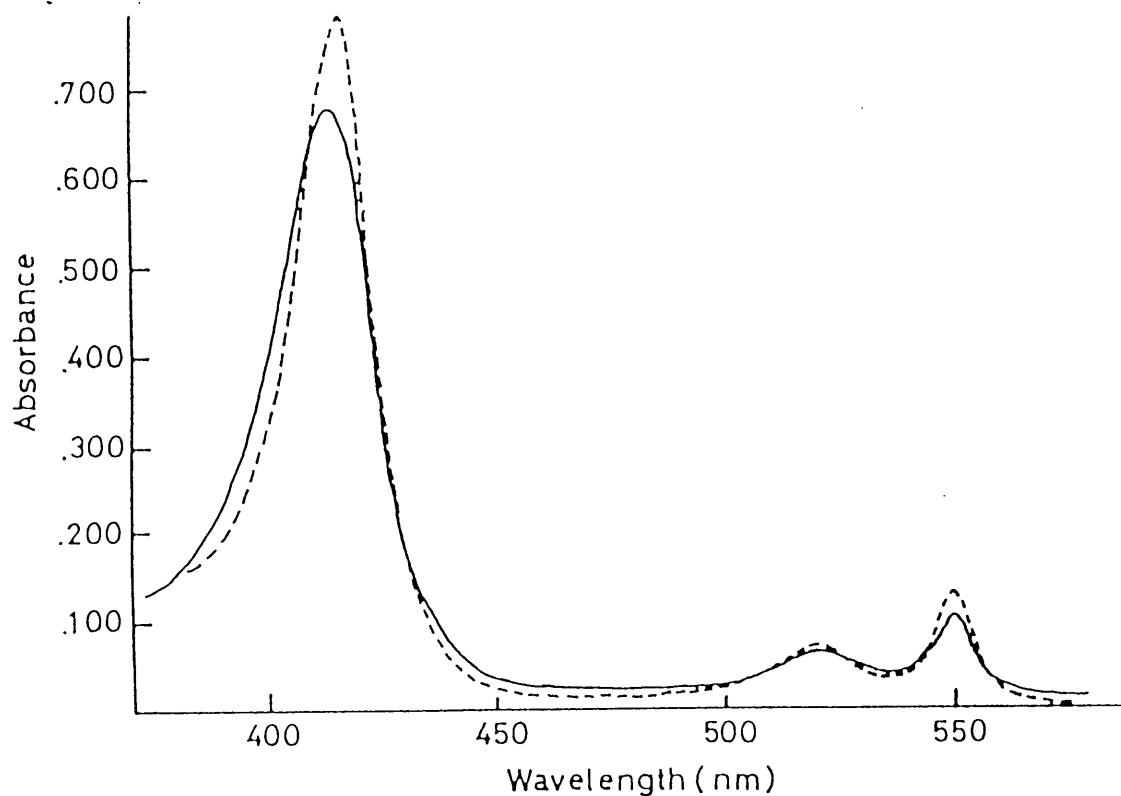


Fig. 4-2. Absorption spectra of photoreduced and chemically reduced cytochrome c.

[cytochrome c]: $2.6 \times 10^{-6} \text{ mol dm}^{-3}$.

Solid line; deaerated solution of ferricytochrome c flashed three times in the presence of $10^{-3} \text{ mol dm}^{-3}$ indole.

Broken line; cytochrome c reduced by adding of a small amount of $\text{Na}_2\text{S}_2\text{O}_3$.

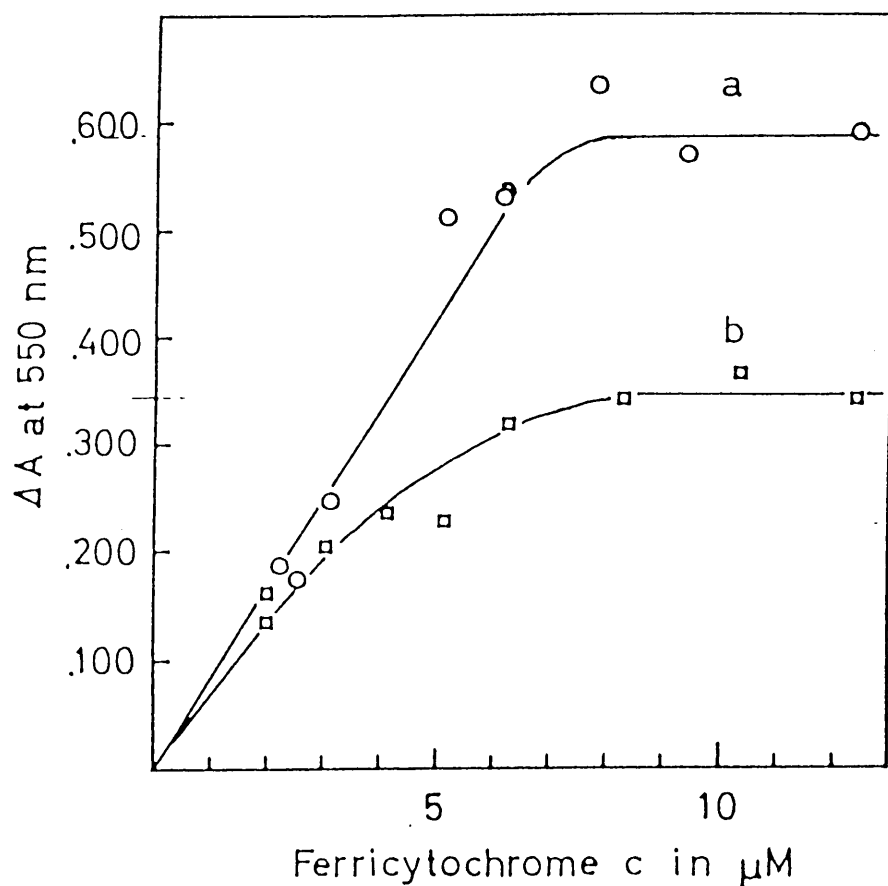


Fig. 4-3. Light induced increase in absorbance at 550 nm plotted against concentration of ferricytochrome c.

Absorbance was measured at 0.5 ms after flash.

Solutions were saturated by nitrogen. a; 10^{-4} mol dm^{-3} indole present. b: 4×10^{-4} mol dm^{-3} o-dimethoxybenzene present.

absorbance measured at 0.5 milliseconds after flash was taken as ΔA on ordinate, because the absorption due to the neutral radical of indole almost diminished after that time scale. Based on the concentration of ferrocytochrome c calculated from the plateau value of the curve in Fig. 4-3, the concentration of hydrated electrons produced in flash photolysis of indole and *o*-dimethoxybenzene could be estimated to be $3.2 \times 10^{-6} \text{ mol dm}^{-3}$ and $1.9 \times 10^{-6} \text{ mol dm}^{-3}$, respectively, by taking $18.5 \times 10^3 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$ as a difference in molar absorption coefficient at 550 nm between ferro- and ferricytochrome c. The estimation is based on the assumption that all hydrated electrons are consumed by reaction with ferricytochrome c. When $1800 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$ is taken as the molar absorption coefficient at 510 nm⁵⁾, the concentration of neutral radical of indole is calculated from the absorbance of neutral radical at initial stage to be $3.3 \times 10^{-6} \text{ mol dm}^{-3}$. Although it includes the small yield due to direct photoreduction of ferricytochrome c, the yield of hydrated electrons is approximately equal to that of neutral radical of indole (cf. reaction 4-1 and 4-4). In order to confirm the participation of photoproducts hydrated electrons in reduction, flash photolysis experiments were carried out in dinitrogen monoxide saturated solutions of ferricytochrome c containing indole or *o*-dimethoxybenzene. The yield of reduction in the presence of dinitrogen monoxide (electron scavenger) was found to be

Table 4-1

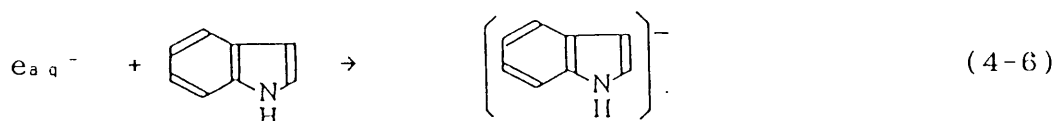
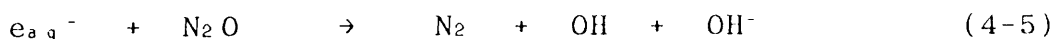
Photoreduction of ferricytochrome c in the presence and absence of electron-donor, hydrogen peroxide and/or radical scavenger.

[cytochrome c]	Additive	gas phase	Increase in absorbance at 550 nm
$10^{-6} \text{ mol dm}^{-3}$	mol dm^{-3}		
8.3	4×10^{-4} ODMB ^a	N ₂	0.354
8.2	4×10^{-4} ODMB ^a	N ₂ O	0.056 (15.8%)
9.5	10^{-4} indole	N ₂	0.566
8.6	10^{-4} indole	N ₂ O	0.118 (20.8%)
7.2		N ₂	0.051
7.2	10^{-4} H ₂ O ₂	N ₂	0.075
8.0		N ₂	0.056
8.0		N ₂ O	0.032
8.0		air	0.032 ^b
8.0		O ₂	0.000 ^b
8.0	10^{-4} H ₂ O ₂	air	0.042 ^b
8.0	10^{-4} H ₂ O ₂	O ₂	0.024 ^b
8.0	solution filter	N ₂	0.000

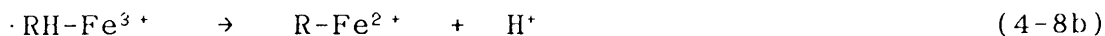
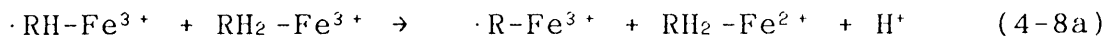
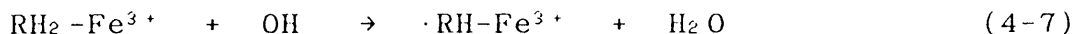
a. o-dimethoxybenzene.

b. 0.1 mol dm^{-3} phosphate buffer (pH 6.9) was used.

lowered to about twenty percent of that in nitrogen saturated solution (Table 1). Since 8.7×10^9 ,^{6,9)} 6.7×10^{10} ,⁷⁾ and $1.9 \times 10^8 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$ ⁸⁾ were reported as the rate constants for reactions 5, 3, and 6, respectively, concentration of dinitrogen monoxide (about 24 m mol dm^{-3}) is sufficient to scavenge hydrated electrons (99.7 %) under the condition where $8.6 \times 10^{-6} \text{ mol dm}^{-3}$ of ferricytochrome c and $10^{-4} \text{ mol dm}^{-3}$ of indole were used.



There are two ways of possible explanation about appreciable residual yield of reduction observed in the presence of dinitrogen monoxide. The first explanation is that ferricytochrome c is reduced by OH radicals converted from hydrated electrons in reaction 4-5. The process has been proposed in Chapter 1 in connection with steady state kinetics for reduction of ferricytochrome c.



$\cdot RH-Fe^{3+}$ is an intermediate radical formed by reaction of OH

with a protein moiety of cytochrome c. The intermediate radical may give ferrocytochrome c through an inter- (reaction 4-8a) or intramolecular process (reaction 4-8b). The second is direct photoreduction. Incident flash light is absorbed by a certain chromophore in ferricytochrome c and reduces the ferric ion of heme through energy transfer process or electron transfer process. To confirm that ferricytochrome c is directly reduced by light, nitrogen saturated solution of ferricytochrome c was flashed in the absence of electron-donor. As shown in Fig. 4-4, apparent increase in absorbance at 550 nm was observed and absorption spectrum of the solution after several flashes was found to be characteristic of the mixture of ferri- and ferro-cytochrome c. The direct photoreduction is lowered by dinitrogen monoxide (electron scavenger) to about sixty percent and completely inhibited by oxygen, quencher (Table 1 and Fig. 4-4). Optical filters of 10^{-3} mol dm⁻³ indole was found to suppress the photoreduction completely by setting them between cell and two main flash lamps. Since the depressive effect of dinitrogen monoxide is not sufficient on the photoreduction, the remaining reduction may proceed mostly through intramolecular process.

The complete inhibition by optical filter of indole indicates that the direct photoreduction of cytochrome c is not induced by excitation of heme, which was reported by

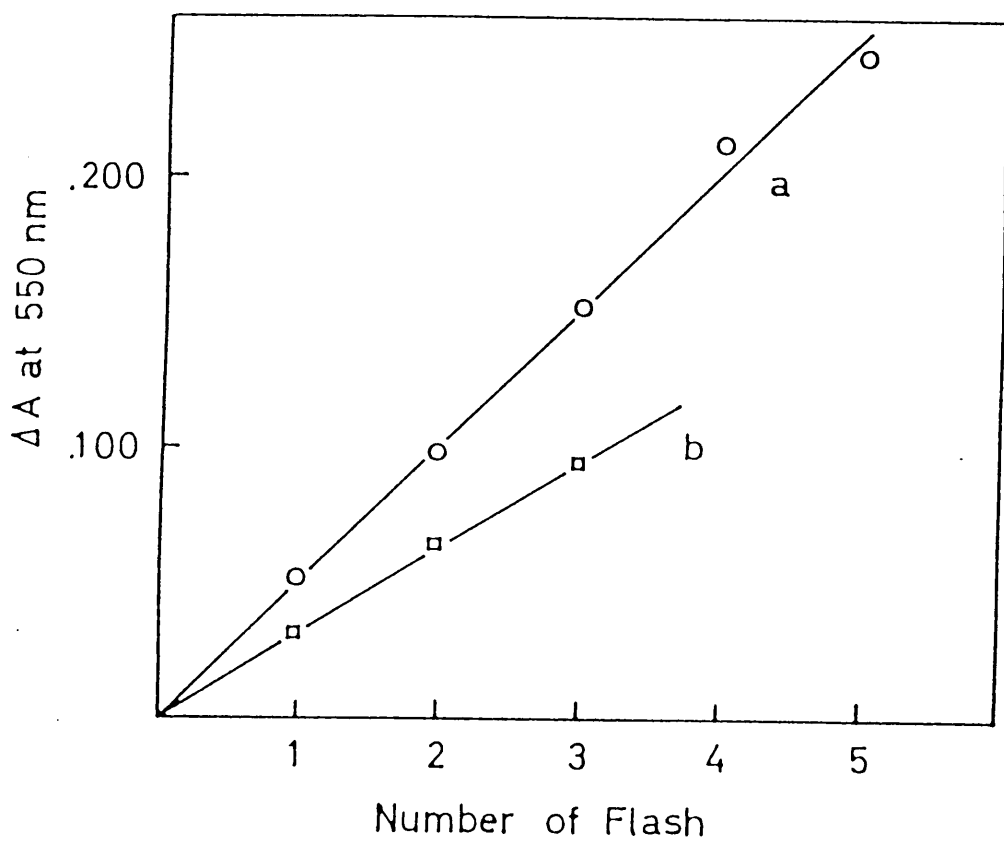


Fig. 4-4. Photoreduction of ferricytochrome c in the absence of electron-donor.

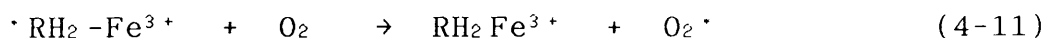
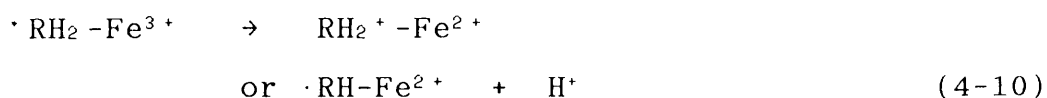
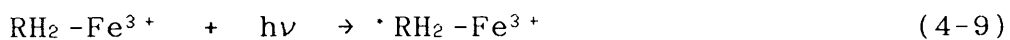
a; $7.2 \times 10^{-6} \text{ mol dm}^{-3}$ saturated by N_2 .

b; $8 \times 10^{-6} \text{ mol dm}^{-3}$ saturated by N_2O .

Vorkink and Cusanovich⁹⁾ on reduction by visible light. Horse heart cytochrome c contains a tryptophyl, four tyrosyl, and four phenylalanyl residues. Bent and Hayon reported that the quantum yields of photoionization by 265 nm light are 0.04, 0.035, and 0.038 for glycyltryptophylglycine, glycyltyrosylglycine, and glycylphenylalanylglycine, respectively.^{4, 10, 11)} Trp-59 residue is located in the vicinity of heme and bound to propionic acid group of the heme with hydrogen bonds.¹²⁾ Therefore, excitation or ionization of the tryptophyl residue will be a most probable intermediate step of reduction of ferric ion in heme. Tyr-48 residue is also bound to the heme with hydrogen bond.¹²⁾ Tyr-67, Phe-10, and Phe-82 are found within about 1 nm from heme. The other aromatic amino acid residues are also found within 1.5 nm or so from the heme.

In the presence of 10^{-4} mol dm⁻³ hydrogen peroxide, ferricytochrome c was reduced by flash light and 0.075 was observed as the increase in absorbance at 550 nm (Table 1). The increase in absorbance due to the direct photoreduction is 0.051 and hence 0.024 is the increase caused by hydrogen peroxide. The reduction is probably caused by OH radicals photochemically produced from hydrogen peroxide (reactions 4-7 and 4-8). The reduction observed in dinitrogen monoxide saturated solution of ferricytochrome c containing indole or *o*-dimethoxybenzene may, therefore, include that induced by OH radicals. In oxygen saturated solution, the photoreduc-

tion of ferricytochrome c was observed in the presence of hydrogen peroxide, while it was completely suppressed in the absence of hydrogen peroxide. The yield, 0.024, is in good agreement with increment due to hydrogen peroxide in nitrogen saturated solution as described above. Since oxygen quenches effectively triplet state of tryptophan, tyrosine, or phenylalanine, complete inhibition of the direct photo-reduction in the presence of oxygen can be easily understood in terms of quenching the triplet state of aromatic amino acid residues of cytochrome c. Lifetimes of triplet states of aromatic amino acid residues were well investigated by Bent and Hayon, ^{4,10,11}) and the rate constants for the decay processes were reported to be $2.7 \times 10^5 \text{ s}^{-1}$, $5.7 \times 10^5 \text{ s}^{-1}$, and $8.5 \times 10^4 \text{ s}^{-1}$ for glycylytyrosylglycine, glycylyphenylalanylglycine, and glycylytryptophylglycine, respectively. Rate constants for quenching of triplet states of three tripeptides by oxygen were also determined by them to be $3.9 \times 10^9 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$, $3.8 \times 10^9 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$, and $4.5 \times 10^9 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$, respectively. Process from excited cytochrome c to ferrocytochrome c will compete with quenching by oxygen.



where $\cdot\text{RH}_2\text{-Fe}^{3+}$ represents ferricytochrome c having a

certain aromatic amino acid residue in excited state. $\text{RH}_2^+ - \text{Fe}^{2+}$ is ferrocytochrome c which possesses a positive charge resulted from photoionization of aromatic amino acid residue and $\cdot\text{RH} - \text{Fe}^{2+}$ represents ferrocytochrome c including a neutral radical of aromatic amino acid residue (see reaction 4-4). Smoluchowski's equation¹³⁾ gives $5.9 \times 10^{10} \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$ as an estimated encounter between cytochrome c and oxygen in aqueous media, if $2.6 \times 10^{-4} \text{ cm}^2 \text{ s}^{-1}$ is taken as the relative diffusion coefficient and 3 nm as the reaction radius. Since 57.1 percent of reduction was observed in air saturated solution of ferricytochrome c, competition kinetics between reactions 10 and 11 gives $2.0 \times 10^7 \text{ s}^{-1}$ as a rate constant for reaction 10.

$$\frac{[\text{RH}_2^+ - \text{Fe}^{2+}]_{\text{N}_2}}{[\text{RH}_2^+ - \text{Fe}^{2+}]_{\text{air}}} = 1 + \frac{k_{11} [\text{O}_2]}{k_{10}} \quad (4-1)$$

In this estimation, we take $2.5 \times 10^{-4} \text{ mol dm}^{-3}$ as the concentration of oxygen in air saturated aqueous solution and $5.9 \times 10^{10} \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$ as rate constant for reaction 4-11. If $4.5 \times 10^9 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$ is taken as the rate constant for quenching by oxygen as reported for triplet state of glycytryptophylglycine by Bent and Hayon⁴⁾, $1.5 \times 10^6 \text{ s}^{-1}$ is obtained as the rate constant for reaction 4-10. The intramolecular reduction process, namely reaction 4-10, is sufficiently fast as compared with decay of triplet state of any aromatic amino acid residue, even if lower value of rate constant is taken for reaction 10.

In conclusion, the results described above suggest the following mechanisms. In nitrogen saturated solution, indirect reduction is given by hydrated electrons. In dinitrogen monoxide saturated solution, it is done by OH radicals converted from hydrated electrons, with the efficiency factor f of reaction 4-8 smaller than chapter 1 ($f \simeq 0.2$). The half of direct reduction results from hydrated electron path and the remains from intramolecular one. In dinitrogen monoxide saturated solution, the path of direct reduction (ratio $\simeq 0.6$) consists of intramolecular one (0.5) and OH radical one ($0.5 \times 0.2 = 0.1$). Oxygen inhibits both intramolecular and hydrated electron one.

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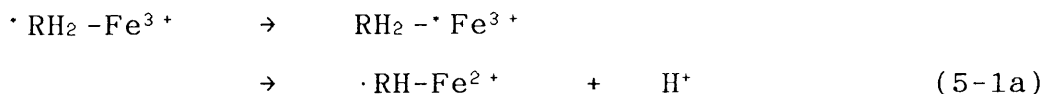
- 1) A perfect citation is impossible on this subject, because so many papers have been published. Several papers are cited here. Others will be seen in the following review; T. Masuda, *Seibutsubutsuri*, **14**, 56(1974).
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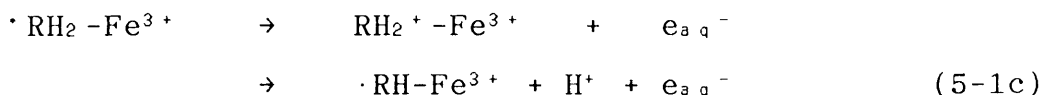
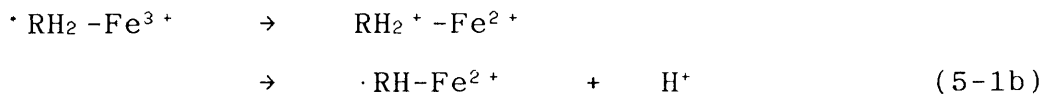
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Chapter 5. Studies on The Mechanism of Direct Photoreduction of Ferricytochrome c.

In the preceding chapter, ferricytochrome c can be reduced by flash light from xenon lamp in deaerated solution in the absence of electron-donor. Participation of tryptophyl residue was presumed on the basis of the fact that optical filter of indole solution effectively suppresses the reduction. However in the experiment, oxygen was found to inhibit the reduction perfectly. This implies that an excitation energy transfer from an amino acid residue, which absorbs UV-light, to heme moiety is possible to take place.



where $\cdot \text{RH}_2 - \text{Fe}^{3+}$ represents ferricytochrome c including an excited state of an amino acid residue, probably aromatic one and $\text{RH}_2 - \cdot \text{Fe}^{3+}$ indicates an excitation of heme moiety of ferricytochrome c. However, there still remains another possibility of participation of an intramolecular electron transfer (5-1b) or rapid intermolecular process including hydrated electron (5-1c) in addition to the energy transfer process mentioned above.



This study has been performed to clarify the mechanism of the direct photoreduction more decisively. The continuous irradiation experiments using a low-pressure mercury lamp revealed that the direct photoreduction occurs according to biphotonic process and oxygen could not completely suppress the photoreduction. Dinitrogen monoxide considerably inhibits the photoreduction and, in particular, complete suppression was observed in the presence of 4 mol dm⁻³ urea. Based on these results, a mechanism will be proposed to explain the photoreduction.

EXPERIMENTAL

Horse heart cytochrome c (type IV) was purchased from Sigma Chemical Co. and used without further purification. Tryptophan, tyrosine, and phenylalanine were obtained from Ajinomoto Co. Ltd. Ferrocycytochrome c was prepared by the following procedure; ferricytochrome c was dissolved in triply distilled water and then the solution was filtered through Sephadex G-25 column after reduction by cysteine.

Solutions for irradiation were prepared by dissolving ferricytochrome c in triply distilled water and bubbling with nitrogen, oxygen, or dinitrogen monoxide for ten minutes. The pH of the solution was not adjusted because the natural pH of the solution was about 7.

UV-irradiation was carried out with a low-pressure

mercury lamp (Toshiba 19W sterilizing lamp), using a silica cuvette(1 x 1 x 4 cm) equipped with a stop cock. The solution was stirred with a small magnetic stirrer during UV-irradiation. Actinometry was carried out using potassium trioxaloferrate (III). The flash photolysis apparatus was described in the preceding chapter.

RESULTS AND DISCUSSION

Absorbance at 550 nm of aqueous solution of ferricytochrome c saturated with nitrogen has been observed to increase with irradiation time as shown in Fig. 5-1. The formation of ferrocytochrome c was confirmed with the absorption spectrum characteristic to ferrocytochrome c. Light intensity was determined to be $1.8 \times 10^4 \text{ erg cm}^{-2} \text{ s}^{-1}$ by using potassium trioxaloferrate. The reduction follows the first-order kinetics within the initial change up to 25 percent. Flash photolysis experiments indicated that tryptophan^{1 a)} or tyrosine^{1 b)} reduced ferricytochrome c in aqueous solutions indirectly with photoejected electrons but phenylalanine did not effectively^{1 b)}. In the experiment with continuous irradiation, the effect of aromatic amino acids differs from that in flash experiment. As shown in Table 5-1, tryptophan is the only amino acid effective on the indirect photoreduction. Low-pressure mercury lamp used in this study delivers light at 253.7 nm principally and

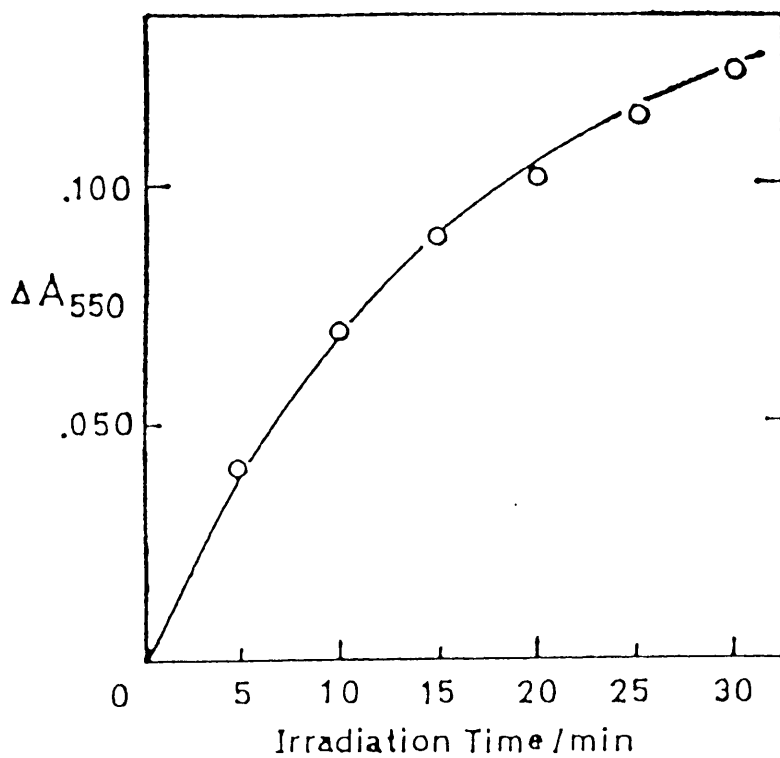


Fig. 5-1. Direct photoreduction of ferricytochrome c by UV light delivered from a low-pressure mercury lamp.
[ferricytochrome c]: $2 \times 10^{-5} \text{ mol dm}^{-3}$.
Saturated by N_2 .

Table 5-1

Photoreduction of ferricytochrome c in the presence of aromatic amino acids.

Amino acid	Ratio of reduction rate
—	1.00
10^{-4} mol dm^{-3} tryptophan	3.77
10^{-4} mol dm^{-3} tyrosine	1.00
10^{-4} mol dm^{-3} phenylalanine	1.22

[ferricytochrome c]: 8×10^{-6} mol dm^{-3} .

tyrosine or phenylalanine does not effectively absorb the light at this wavelength. This may be the possible explanation of their ineffectiveness. Consequently, tryptophyl residue is a most probable candidate for an electron donor in the direct photoreduction, at least, in the continuous irradiation with the low-pressure mercury lamp.

Effect of light intensity on the relative yield of photoreduction was examined by using cyclohexane solutions of carbon tetrachloride as an optical filter. As shown in Fig. 5-2, the result indicates that the relative yield of the photoreduction is proportional to the square of light intensity. This suggests that the photoreduction is a biphotonic process.

In N_2O -saturated solution, considerable amount of reduction was depressed but small increase in absorbance at 550 nm was still observed as shown in Fig. 5-3a. Concentration of dinitrogen monoxide ($2.4 \times 10^{-2} \text{ mol dm}^{-3}$) is enough to completely depress the reduction by hydrated electrons, because the rate constants for reactions of hydrated electrons with dinitrogen monoxide and with ferricytochrome c are $8.7 \times 10^9 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$ and $6.7 \times 10^{10} \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$, respectively.^{6, 7)} Therefore, the remaining reduction of ferricytochrome c is not due to hydrated electrons, as indicated by a simple competition between reactions 5-2 and 5-3.

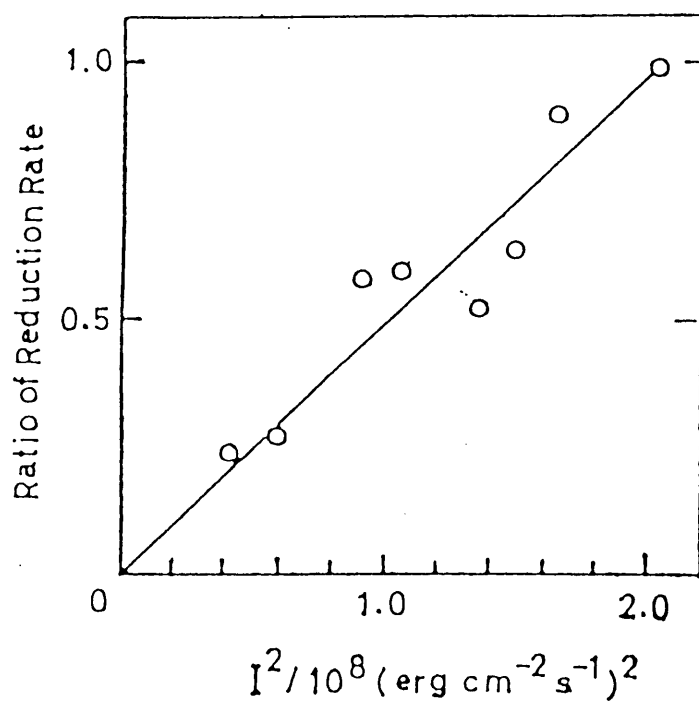


Fig. 5-2. Effect of light intensity on the direct photoreduction.

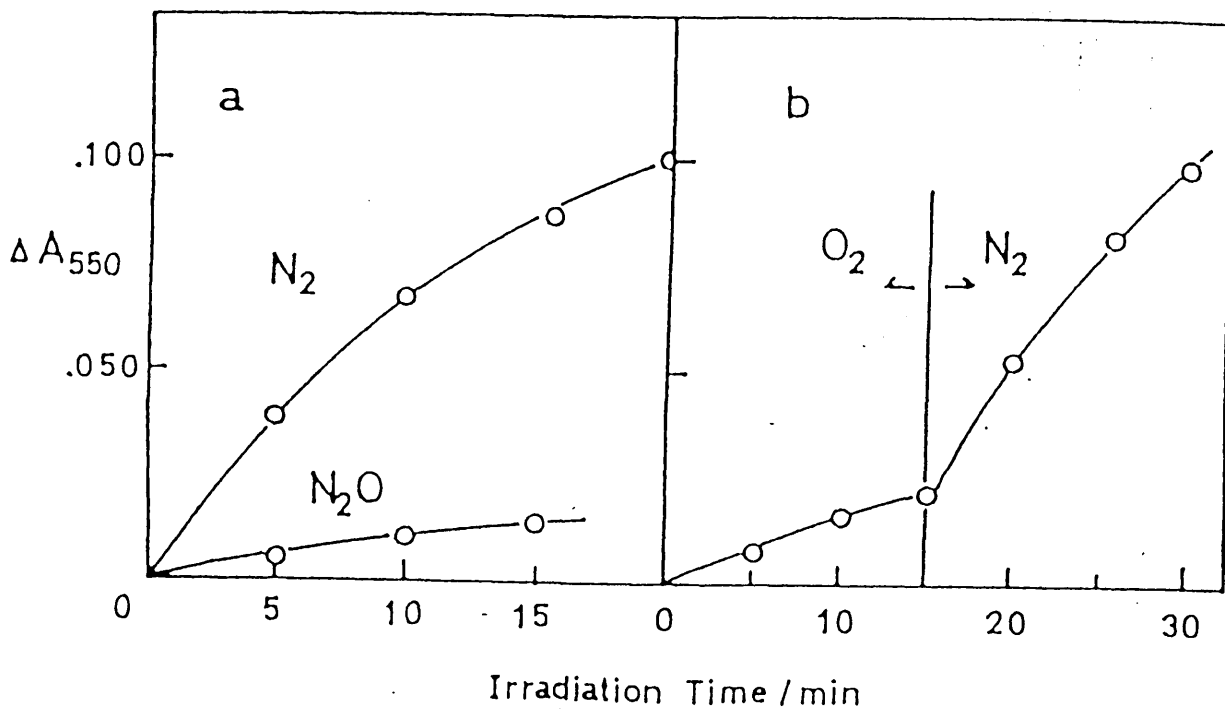
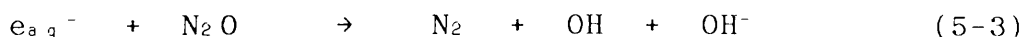
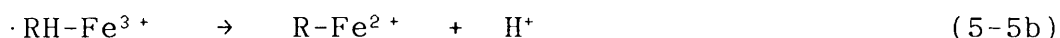
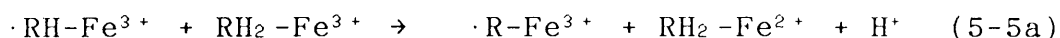


Fig. 5-3. Effect of dinitrogen monoxide and oxygen on direct photoreduction of ferricytochrome c.

[ferricytochrome c]: $2 \times 10^{-5} \text{ mol dm}^{-3}$.



As a possible mechanism of the photoreduction in N_2O -saturated solution, reactions 5-4, 5-5a and 5-5b are conceivable.



Since oxygen is well known as an effective quencher of the excited aromatics, photoreduction was examined in oxygen-saturated solution of cytochrome c. As shown in Fig. 5-3b, a slight increase in absorbance at 550 nm was observed. The rate of the photoreduction increased after replacing oxygen with nitrogen. Since $4.5 \times 10^9 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$ was reported as the rate constant for the reaction of oxygen with triplet state of glycyltryptophylglycine,⁸⁾ $1.25 \times 10^{-3} \text{ mol dm}^{-3}$ oxygen may sufficiently quench the triplet state of aromatic amino acid residue, unless the residue is buried in the protein molecule or an effective energy transfer operates. It was found in ferricytochrome c modified by urea that fluorescence from tryptophy residue is enhanced.⁹⁾ The enhancement is understood in terms of disappearance of quenching by heme. The effect of urea treatment on the direct photoreduction is summarized in Table 5-2. Yield of the photoreduction increases with increasing concentration of urea. It suggests that a close contact of tryptophyl

Table 5-2

Effect of urea on rate of photoreduction

[Urea]/ mol dm ⁻³	Ratio of reaction rate
0	1.00
1.0	2.13
2.0	2.75
3.0	3.90
4.0	4.00

[Ferricytochrome c]: 8.2×10^{-6} mol dm⁻³.

residue with the heme interferes with the photoreduction in intact molecule and conformational change induced by urea causes loss of the contact between tryptophyl residue and heme to enhance the photoreduction. The result also indicates that an excitation energy transfer from tryptophyl residue to heme is not the cause of photoreduction. When the solution saturated with oxygen was irradiated in the presence of 4 mol dm^{-3} urea, the increase in absorbance at 550 nm is almost the same with that observed in the absence of urea (Figs. 5-3b and 5-4a). However, the reduction is completely suppressed by urea treatment in the case of N_2O -saturated solution (Fig. 5-4b). These results indicate that the reduction observed in N_2O -saturated solution of intact cytochrome c is attributable to reactions 5-4 and 5-5. The rate constant for the reaction of OH radical with urea cannot be found in literature and has been determined to be $7.9 \times 10^5 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$ in our laboratory according to the method of Kraljic and Trumbore.¹⁰⁾ Considering competition between urea and ferricytochrome c against OH radical, about 26 percent reduction is expected. However the result shows complete suppression. To ascertain whether conformational change of molecule by urea is responsible for the complete suppression or not, photoreduction was examined in N_2O -saturated solution containing $6.8 \times 10^{-3} \text{ mol dm}^{-3}$ *t*-butyl alcohol, which is well known as an effective scavenger of OH radical (rate constant is $5.8 \times 10^8 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$).¹¹⁾

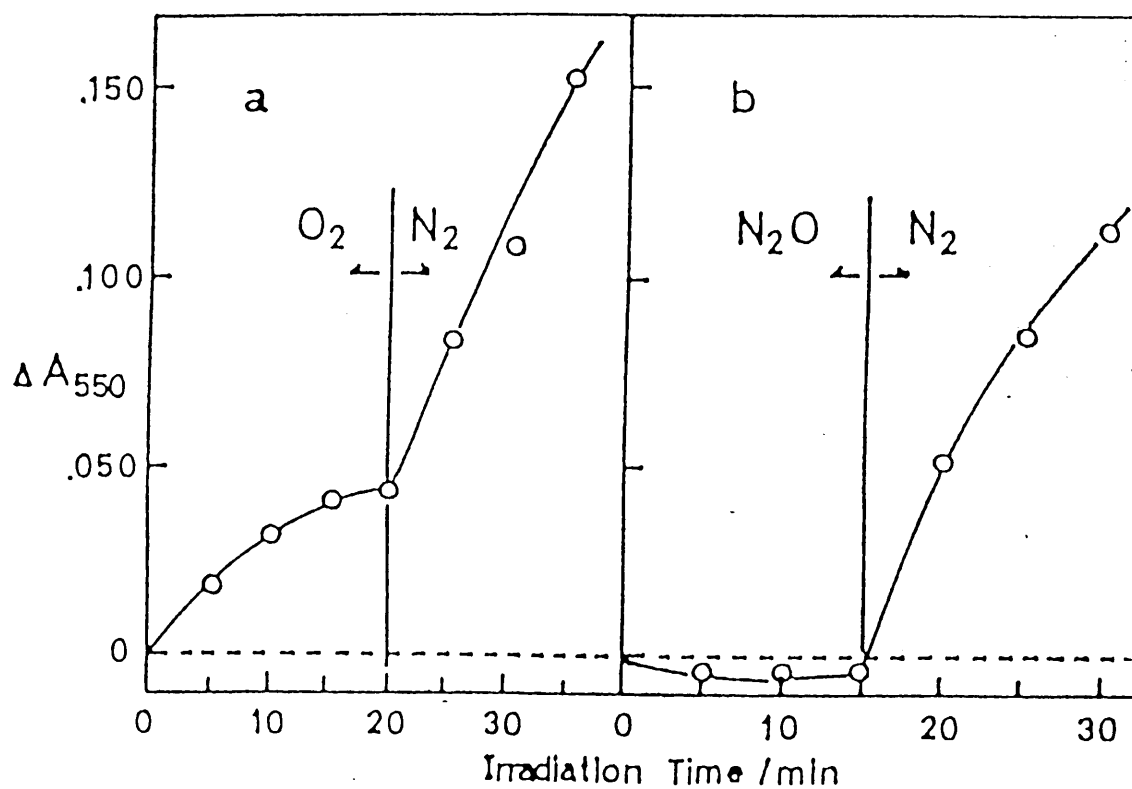


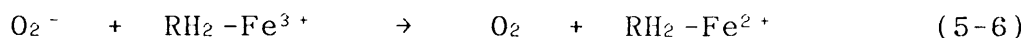
Fig. 5-4. Effects of oxygen and dinitrogen monoxide on direct photoreduction of ferricytochrome c in the presence of 4 mol dm^{-3} urea.

[ferricytochrome c]: $3.5 \times 10^{-5} \text{ mol dm}^{-3}$.

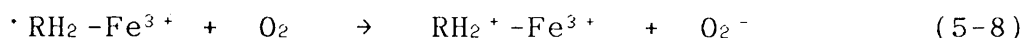
A slight oxidation, which is probably due to contaminated ferrocytochrome c, was observed to take place similarly to that in the presence of urea. Based on competition between ferricytochrome c and *t*-butylalcohol against OH radical, about 24 percent of reduction is expected. However, the reduction is completely suppressed. The discrepancy may be understood in terms of low efficiency of reduction through reactions 5-4 and 5-5¹²⁾ as discussed in Chapter 4. That is, the efficiency factors of reduction *f* in the absence and the presence of urea are 0.2 and 0, respectively.

The UV-induced oxidation of ferrocytochrome c was observed in nitrogen saturated solution of ferrocytochrome c. The oxidation process, however, is complicated and follows neither first nor second order kinetics.

The photoreduction takes place in oxygen saturated solution independently of the presence of urea. It can be attributable to reaction of O_2^- , because O_2^- was found to reduce ferricytochrome c with the rate constant of $1.4-2.4 \times 10^6 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$.¹³⁾



The following two processes probably contribute to the formation of O_2^- .

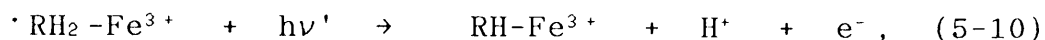


The reduction in the presence of urea may considerably proceed through reactions 5-8 and 5-6, because an effective

quenching of the excited state of tryptophyl residue by oxygen can be reasonably expected in modified cytochrome c molecule. The efficiency of the reduction through reaction 5-6 is probably as low as that through reactions 5-4 and 5-5.

Participation of tryptophyl residue in the direct photoreduction is supported by the transient absorption observed in flash photolysis of ferricytochrome c. Especially a transient absorption around 530 nm was apparently observed in the presence of urea. The absorption peak was found to shift to 510 nm in the absence of urea and the same shift was observed in the flash photolysis of aqueous indole solution. Therefore the transient absorption can be assigned to neutral radical of indole ring of tryptophyl residue.^{8, 14)}

Finally quantum yield of photoionization (reactions 5-9 and 5-10) will be discussed. Since the effect of light intensity on the yield of photoreduction indicates the biphotonic process, formation of hydrated electrons will be expressed by the following reactions:



The mechanism of the direct photoreduction may be composed of reactions 5-9, 5-10, 5-11, and 5-2. On the initial stage of the direct photoreduction, the rate of the formation of ferrocytochrome c is expressed by the following equation:

$$\frac{d[\text{RH}_2 - \text{Fe}^{2+}]}{dt} = k_2 [\text{e}_{\text{aq}}^-] [\text{RH}_2 - \text{Fe}^{3+}] \quad (5-1)$$

For hydrated electrons, equation 5-II can be given if it is assumed that reactions 5-9 and 5-10 can be regarded as one process because of biphotonic process.

$$\frac{d[\text{e}_{\text{aq}}^-]}{dt} = \varepsilon_1 \phi_1 I_0 l [\text{RH}_2 - \text{Fe}^{3+}] - k_2 [\text{e}_{\text{aq}}^-] [\text{RH}_2 - \text{Fe}^{3+}] \quad (5-II)$$

where ε_1 and ϕ_1 represent a molar absorption coefficient of ferricytochrome c at 253.7 nm and quantum yield of biphotonic process, namely photoionization of cytochrome c. l is light path and 1 cm in this case. I_0 is light intensity. Assuming the steady state for hydrated electrons, we obtain the steady state concentration of hydrated electrons.

$$[\text{e}_{\text{aq}}^-]_{\text{ss}} = \frac{\varepsilon_1 \phi_1 I_0}{k_2} \quad (5-III)$$

By substituting equation 5-III into 5-I, equation 5-IV is obtained.

$$-\frac{d[\text{RH}_2 - \text{Fe}^{3+}]}{dt} = \varepsilon_1 \phi_1 I_0 [\text{RH}_2 - \text{Fe}^{3+}] \quad (5-IV)$$

By integrating equation 5-IV, the following relation is obtained.

$$\ln \frac{[\text{RH}_2 - \text{Fe}^{3+}]}{[\text{RH}_2 - \text{Fe}^{3+}]_0} = -\varepsilon_1 \phi_1 I_0 t \quad (5-V)$$

Plots of $\ln([RH_2-Fe^{3+}]/[RH_2-Fe^{3+}]_0)$ against time t give the value for $\epsilon_1 \phi_1 I_0$ as a slope. The molar absorption coefficient of ferricytochrome c at 253.7 nm was experimentally determined to be $2.05 \times 10^4 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$. Light intensity was also experimentally determined to be $1.8 \times 10^4 \text{ erg cm}^{-2} \text{ s}^{-1}$, which corresponds to 2.30×10^{15} photons $\text{cm}^{-2} \text{ s}^{-1}$ for light of 253.7 nm. The quantum yield of photoionization of ferricytochrome c is calculated to be 3.4×10^{-3} . The value of 0.04 was reported for photoionization of glycytryptophylglycine at pH 5.2 by laser-pulse (265 nm) experiment. The value obtained in this study is about one order of magnitude less than that of tripeptide. However, Grossweiner pointed out that quantum yield for photoionization of tryptophan varies with light source.¹⁵⁾ The value determined by xenon flash lamp is 0.008¹⁶⁾ and 0.08-0.11 by laser-pulse experiment.^{8, 14)} Tryptophyl residue of cytochrome c (Trp-59) is connected with heme and the quenching by heme may lower the quantum yield for photoionization of indole ring. Conformational change of cytochrome c molecule induced by urea increases the yield of direct photoreduction as seen in Table 5-2. Simultaneously dinitrogen monoxide completely inhibits the photoreduction of ferricytochrome when the protein is treated by urea. This indicates that reaction 5-5 is intramolecular process and needs some conformation for electron transfer.

In conclusion, the results described above suggest the following mechanisms. In nitrogen saturated solution, the direct photoreduction of ferricytochrome c by the continuous irradiation with the low-pressure mercury lamp is mostly given through hydrated electrons from photoionization of tryptophyl residue. In dinitrogen monoxide saturated solution, it is intramolecularly done with low efficiency through intermediate radicals of ferricytochrome c produced by OH radicals converted from hydrated electrons. The intramolecular process is completely suppressed by urea-denaturation of cytochrome c. Irrespective of the existence of urea, oxygen suppresses the reduction but resulting O_2^- in turn reduces ferricytochrome c though the efficiency is low.

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Chapter 6. General Discussion

1 Oxidation and Reduction

Since cytochrome c is a member of respiratory chain in living cells and works as a machine to transfer electron, oxidation or reduction is easily expected to occur in aqueous solution of cytochrome c when it is irradiated by ionizing radiation. In fact, reduction can be observed in irradiated aqueous solution of ferricytochrome c. The formed ferrocytochrome c can be oxidized by liver homogenate of mouse.¹⁾ Kinetic analysis has given a detailed profile of the reduction of ferricytochrome c, namely rate constants for three reactions as follows:

$$k(e_{aq}^- + \text{ferricytochrome c}) = 6.7 \times 10^{10} \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1},$$

$$k(\text{OH} + \text{ferricytochrome c}) = 3.6 \times 10^{10} \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1},$$

and

$$k(\text{OH} + \text{ferrocytochrome c}) = 2.9 \times 10^{10} \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}.$$

After our study, many researchers have reported the rate constants determined by pulse radiolysis technique.²⁻⁶⁾ They support the values obtained in this study, although the rate constants for the reaction of OH radical with ferri and ferrocytochrome c are not available in literatures as relevant value. Obtained values indicate that oxidation of ferrocytochrome c is a less effective process compared with other two. The value is slightly smaller than that calculated by Smoluchowski's equation(see Chapter 1).

2 Changes in Structure

Increase in CO-affinity, appearance of susceptibility to proteinases, and spectrophotometric titration curves indicate the gradual modification of three-dimensional structure (conformation) of cytochrome c during irradiation. Obviously the change of structure is caused by active species produced in radiolysis of water (indirect action). Subsequently destruction of the exposed heme and change of aromatic amino acid residues take place. The difference in behavior of proteinase susceptibility and optical absorption spectra indicate those processes.

3 Photochemical Changes

Ferricytochrome c is indirectly reduced by electrons produced from indole or o-dimethoxybenzene by UV irradiation. In the presence of dinitrogen monoxide, electron scavenger, considerable reduction of ferricytochrome c can still be observed(20.8 %), although the concentration of saturated dinitrogen monoxide is enough to scavenge almost whole electrons(99.7 %). This implies that OH radicals converted from hydrated electrons by dinitrogen monoxide contribute to the reduction similarly to the case of gamma irradiation experiment. To confirm the estimation, reduction of cytochrome c was investigated in photolyzed aqueous solution of cytochrome c containing hydrogen peroxide. The experiment revealed that the yield of the reduction increases about 50 percent and the increment of reduction is not

inhibited by saturated oxygen. It is supposed that the reduction is caused by OH radicals from the photolysis of hydrogen peroxide.

In the absence of electron donor, direct photoreduction of cytochrome c can be observed. In the presence of dinitrogen monoxide, considerable reduction still takes place. This is understood in terms of reduction through OH radicals. Optical filter of aqueous indole suppresses the direct reduction completely. This indicates that tryptophyl or other aromatic amino acid residue participates in the direct reduction by UV light. Dinitrogen monoxide or oxygen cannot fully inhibit the direct reduction. Urea treatment enhances the rate of reduction. These results suggest that UV light photoionizes aromatic amino acid residue, probably tryptophyl residue to give hydrated electron. In intact molecule tryptophyl residue contacts with heme and excitation of the residue is easily quenched by heme. Therefore, urea treatment enhances the direct reduction. Photoejected electron is captured by dinitrogen monoxide, but resulting OH radical reduces ferricytochrome c. Since the reduction of urea-treated ferricytochrome c is perfectly suppressed by dinitrogen monoxide, the reduction through reaction with OH radical may be an intramolecular process abolished by urea-denaturation of cytochrome c. Oxygen inhibits the reduction but the resulting O_2^- reduces ferricytochrome c as reported by Seki and Imamura.²⁾

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