Free Radicals of Gamma-Ray Irradiated Amino Acids and Some Substances of Biological Interest Studied by Electron Spin Resonance Absorption.

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In this experiment, investigations on the ESR signals of the gamma-irradiated amino aids, peptides and proteins are chieifly made, the results obtained being as follows:

(a) ESR absorption of $10^4 \sim 10^7$ r irradiated amino acids, peptides and proteins show characteristic curves respectively.

(b) The signals of those which contain sulfhydryl or disulfide groups show essentially the same pattern as those of $S^{\cdot}$ or $S\overset{\cdot}{\cdots}S$.

(c) The concentration of the free radical electron in the irradiated protein (about 10^{18} spins per gram) is lower than those of amino acids and peptides (abont 10^{19} spins per gram).

(d) When irradiation was made in the presence of oxygen, the signals of irradiated protein show marked difference from those of irradiated *in vacuo* or in the presence of nitrogen.

(e) The protein irradiated in the presence of water shows rapid decaying ESR signal.

(f) UV irradiation also produces in protein and nucleic acids considerable amount of free radicals.

Some discussions were made on the radiation damage of the biologically interesting materials from these reults.

INTRODUCTION

As pointed out by Ingram[1], electron spin resonance (ESR) absorption is one of the most direct method for studying the breakdown processes in living system produced by irradiation and a systematic investigation of radicals formed by irradiation of substances of biological interest seems very important to obtain basic physical knowledges in this field. Indeed, some studies[2-4] along such an investigative approach have already appeared. We have also sttempted to observe the ESR spectra of gamma-ray irradiated amino acid, peptides, proteins and nucleic

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acids to examine the nature of radiation-induced free radicals of these biologically important substances. This brief report is chiefly concerned with such an observation of ESR spectra, its detailed account will be published later. To determine the exact trapping portion or distribution of unpaired electron of free radicals, however, observations on the single crystals are necessary. These experiments are now in progress on some compounds in our laboratory.

METHODS

Samples tested: Crystalline bovine serum albumin was Armour's product, while human serum albmin was prepared and crystalized by Cohn's method[5]. Samples of DNA and RNA were prepared from pig's semen[6] and calf liver microsome[7], respectively. Other samples were commercial ones, mainly obtained from Azinomoto Co.

Irradiation: The samples, whicn were in a powdered form, were sealed in the long glass tube evacuated to 10^{-3}mmHg and irradiated by 2 kilo-Curie Co^{60} gamma-ray source at the Institute for Chemical Research, Kyoto University, its intensity being 3×10^{3} r per minutes. Total dosis of gamma-ray irradiation were $10^{4}\sim10^{7}$ r. Irradiation was made under vacuum to avoid the possible oxygen effect, except that albumin samples were irradiated in the presence of oxygen or water to observe their effect upon free radical formation.

ESR absorption: After irradiation, one end of the glass tube was annealed to remove undesirable signals from free radicals yielded in glass and then a sample contained in the other end was transfered to the annealed portion. For strong signals from some amino acids, the background signals from the glass tube were negligible. For weak signals, however, such a background signal should be taken into account. Hence the sealed glass tube was opened, the sample was transfered into another unirradiated tube and ESR signal was observed. No appreciable oxygen effect was observed in our all sample tested at least immediately after exposure to air, but considerable changes in the ESR signal was obtained several hours after exposure to air (see leucine in Table 1).

ESR spectra was observed by a hand made ESR apparatus in earlier experiments. But Varian V-4500 spectrometer at Resources Research Institute was chiefly used in later observations because of its stability to compare the relative characteristics of all tested samples. The conditions for signal measurement were tried to be the same for all samples compared as far as possible, which were as follows:

Microwave frequency		9450 MC/sec
Modulation frequency of magnetic field		400 C/sec.
Its amplitude	3.5 Gauss	for nearly all samples.
	12 Gauss	for samples of weak signals.
	0.22 Gauss	for samples of strong signal with sharp h. f. s.
Microwave r. f. field		H_1=about 0.1 Gauss.
Field sweep late		22 Gauss/minute.
Integrating time constant		0.3~0.8 sec.
Temperature		15°C.

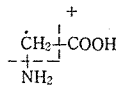
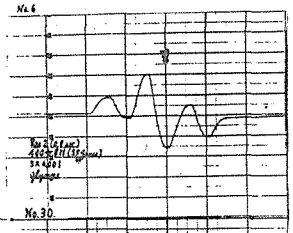
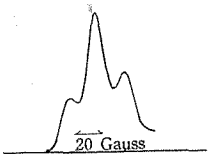
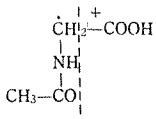
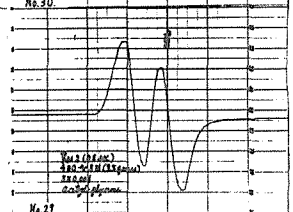
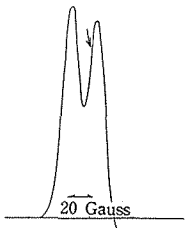
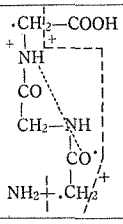
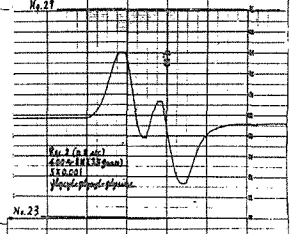
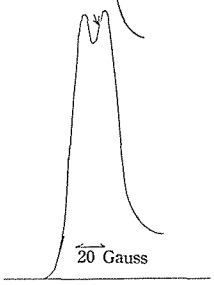
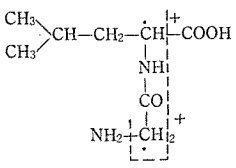
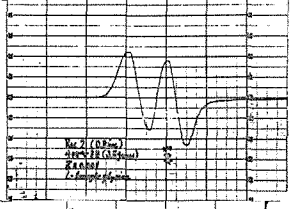
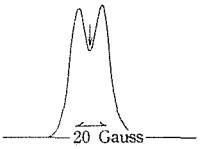
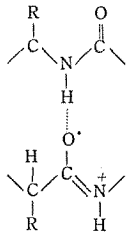
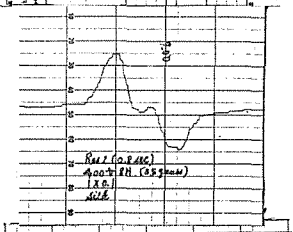
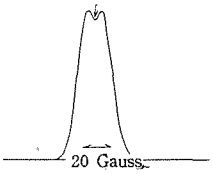
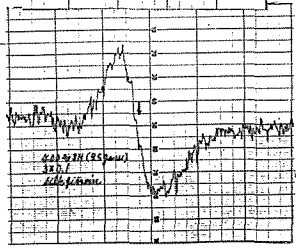
Table 1. Amino acids, peptides and protein mainly consisting of aliphatic carbon chain irradiated by 10^7r gammaray in the absence of oxygen.

A : possible trapping portion of unpaired electron, and breaking positions are shown by a dot and broken lines respectively.

B : The concentration of unpaired electrons per gram sample. estimated by comparing them with that of the standard carbon radical.

C : The first derivative EPR signals.
The difference between the lines in the recorder chart is 22 Gauss each.

D : Integrated absorption signals, the position of g≈2.003 being indicatea by the arrow,

(A)	(B)	(C)	(D)
(1) 	2×10^{19}		
(2) 	3×10^{19}		
(3) 	9×10^{19}		
(4) 	9×10^{19}		
(5)  silk fibre	2×10^{18}		
(6) Silk fibroine powder.			

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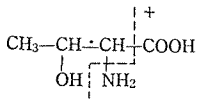

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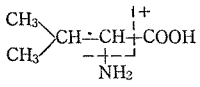

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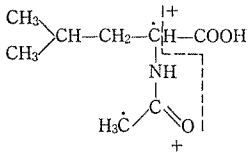

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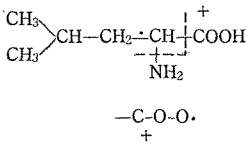

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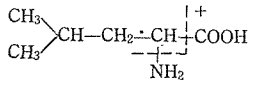

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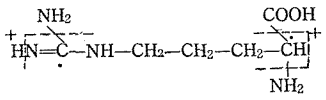

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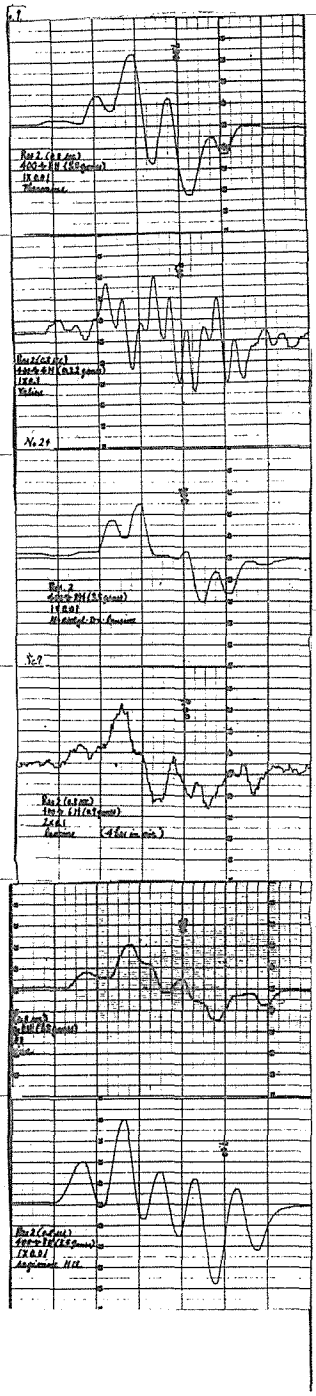

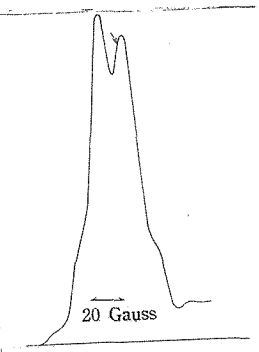

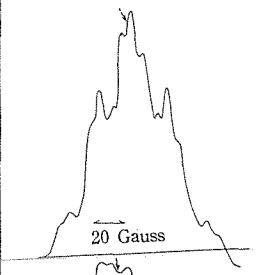

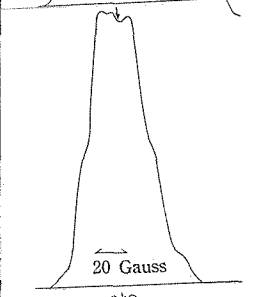

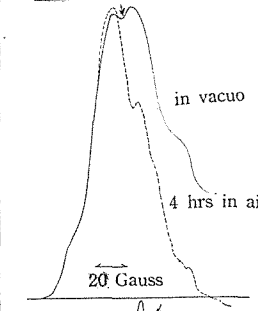

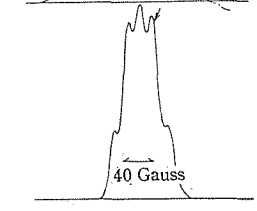

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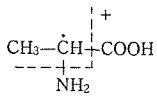

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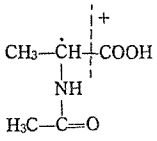

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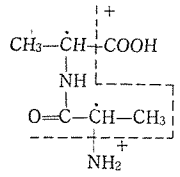

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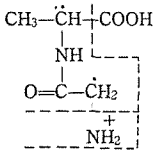

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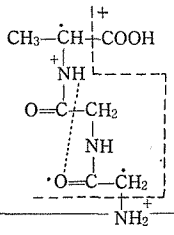

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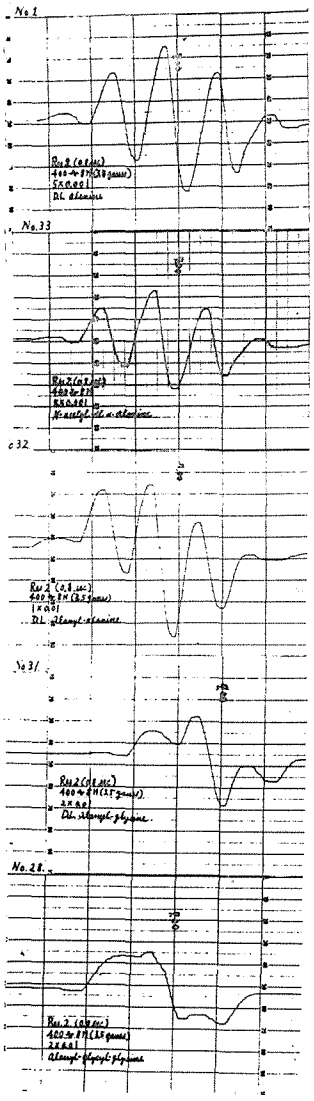

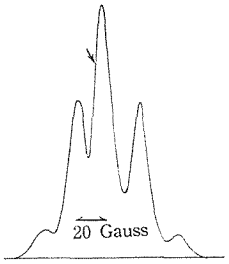

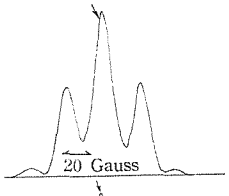

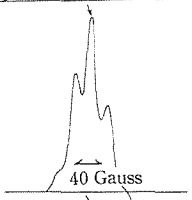

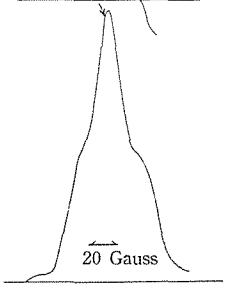

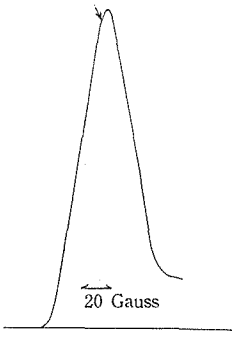

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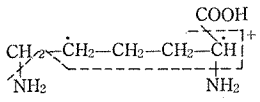

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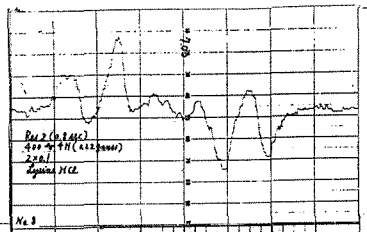

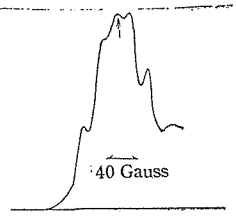

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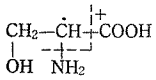

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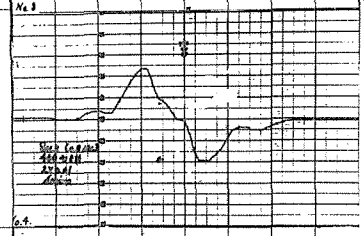
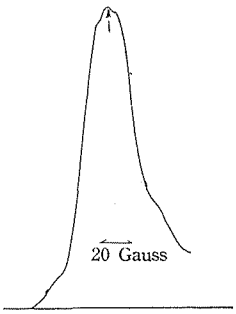
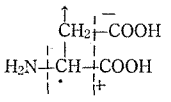




(19)

combine with adjacent molecule



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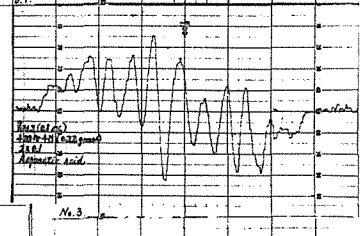

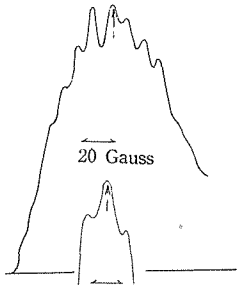

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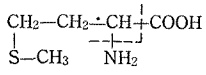
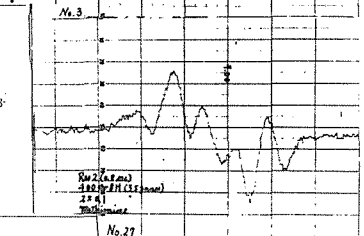
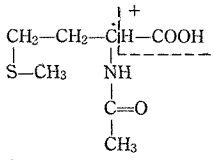


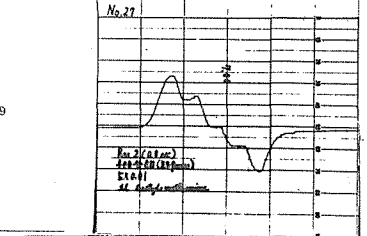
2×10^{18}

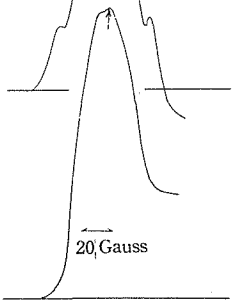


(21)

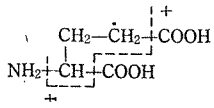


10^{19}

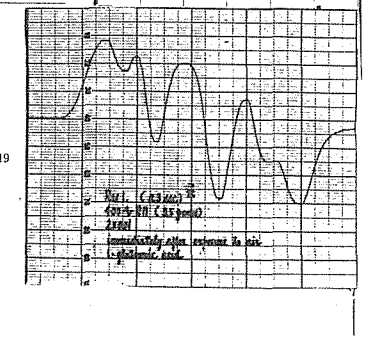


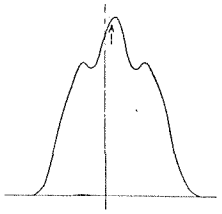


(22)



1.8×10^{19}





RESULTS

Amino acids and peptides. ESR absorption signals recorded were presented in Tables 1, 2 and 3, in which a supposed (probable) position of unpared electron of free radicals produced by ionization, decarboxylation and deamination as suggested by previous investigators[8] is shown by the dot, while position of breakdown of bond is represented by broken lines. From these results, ESR signals seem to be classified into three types according to g-values, over-all splitting and line width.

1. Aliphatic amino acids and peptides composed of them. Their g value is near 2.00 over-all splitting is more than 90 Gauss except glycine and its peptides and line wibth is also wide.

2. Amino acids and peptides containing aromatic carbon ring. Their g value is also near 2.00, but over-all splitting is narrower than the former (less than 80 Gauss) and line width is narrower.

3. Cystine, cystein and peptides containing them in types of sulfhydryl or disulfide groups. Their g-value markedly deviates from 2.00, asymmetry of absorption curve is remarkable.

It seems worthy to note here that doublet splitting of peptides containing glycin summarized in Tables 1-2, 3, 4, 5, 6 is different between the small molecules and the high polymer, unpaired electrons in the former case mainly localized on carbon atom coupling with one bonded hydrogen nucleus and in the latter mainly localized on an O atom experiencing dipolar interaction with bridging hydrogen nucleus, when dipolar broadening and inhomogeneous broadening are taken into account, and that peptide bonds are considerably resisting for radiation damage as shown in acetylated amino acids and peptides.

Protein. The results obtained are illustrated in Tables 1-5, 6, and Table 4. The signal of silk fibre was observed on the portion perpendicular to the static magnetic field. Comparing the absorption pattern of fibroin powder, orientation-dependet doublet was observed in the former.

Radiation effect on bovine serum albumin, human serum albumin, fibrin, fibroin and silk fibre itself were observed. Cystine and cysteine content in these samples

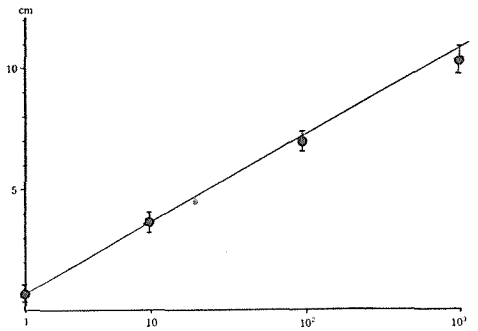
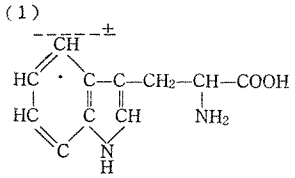
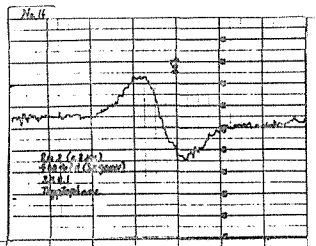
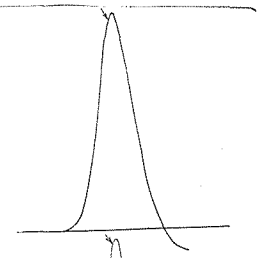
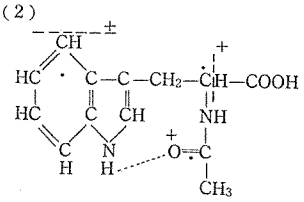
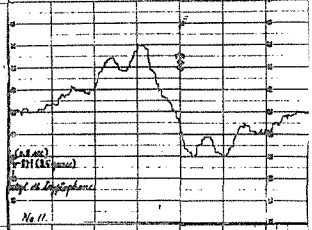
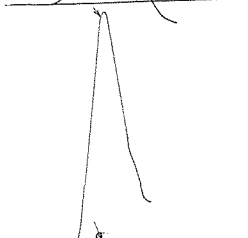
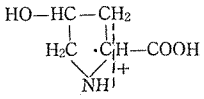
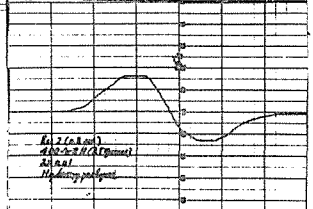
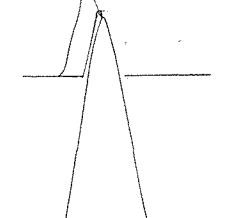
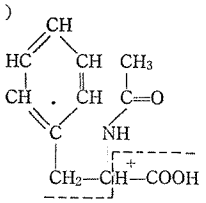
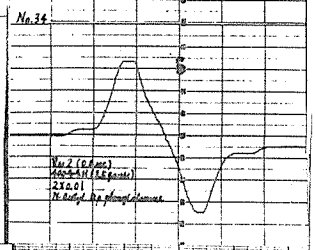
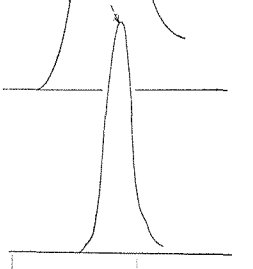
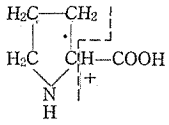
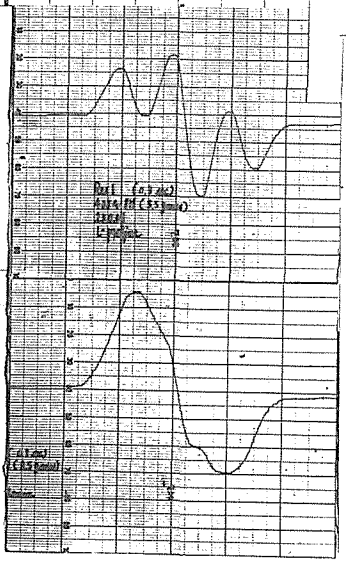
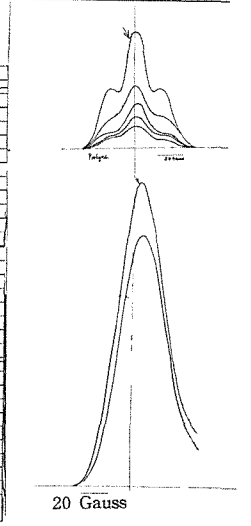
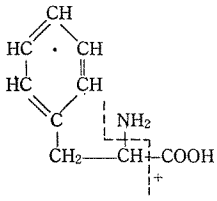
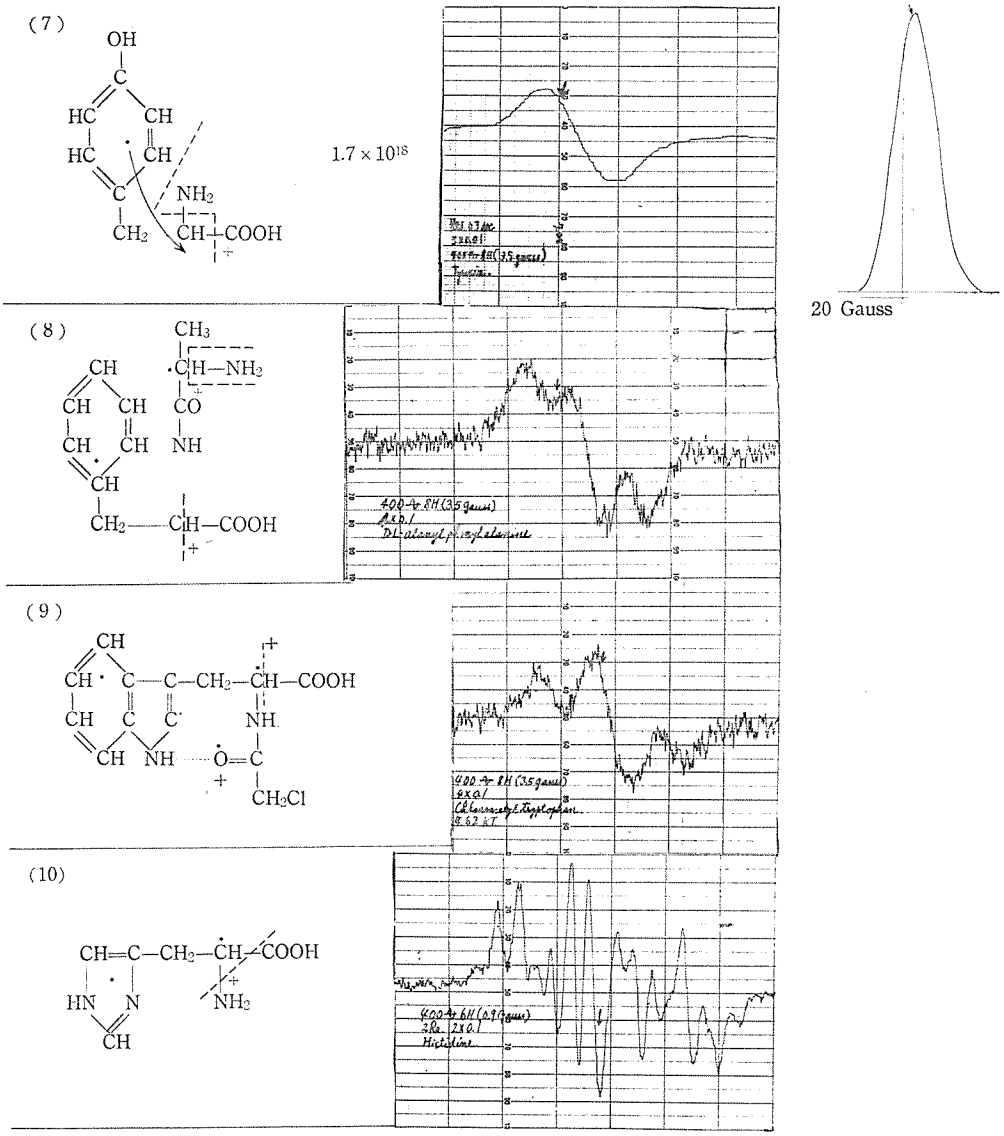


Fig. 1. Corelation between the dosis of gamma irradiation and the signal height of protein.
Absissa : dosis of gamma-rays in kilo-roentogen.
Ordinate : heights of ESR signals.

Table 2. Amino acids and peptides containing aromatic carbon ring.
A, B, C, D are the same as in Table 1.

(A)	(B)	(C)	(D)
(1) 	1.4×10^{18}		
(2) 	6×10^{18}		
(3) 	1.3×10^{19}		
(4) 	1.8×10^{19}		
(5) 	1.3×10^{19}		
(6) 	7.5×10^{19}		

20 Gauss



are graded in several to zero percent (bovine serum albumin≧human serum albumin>fibrin>silk=0.)

The pattern of ESR absorption curve was not affected by change in dose of gamma-rays, only effect being increase in the signal height (Fig. 1). Such a circumstance is quite similar to the dose-effect of amino acids.

DISCUSSION

Aliphatic amino acids as well as peptides and silk protein mainly consisting of aliphatic carbon chain give, as illustrated in Table 1, widely spread signal (90~150 Gauss) with g value of approximately 2.00. Such a pattern would be interpreted by unpaired electron of free radicals trapped on the carbon atom which has dipole-

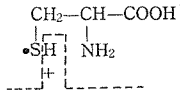
Table 3. Cystine, cysteine and peptides containing them.

A, B, C, D are the same as in Table 1, except for the numbered arrows presented in the absorption curve of reduced glutatione.

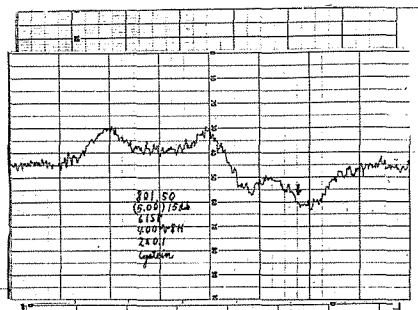
1, 2, 3, 4 indicates the positions of g~2.04, 2.027, 2.017 and 2.003 respectively.

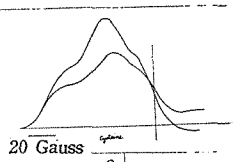
(A) (B) (C) (D)

(1)



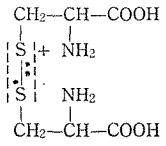
1.6×10^{19}



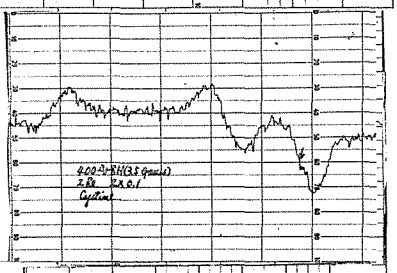


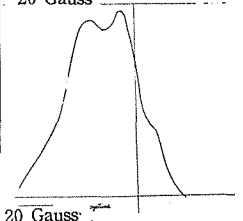
20 Gauss

(2)



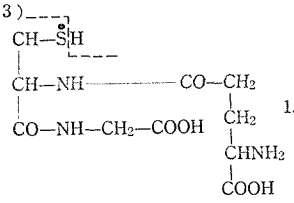
1.5×10^{19}



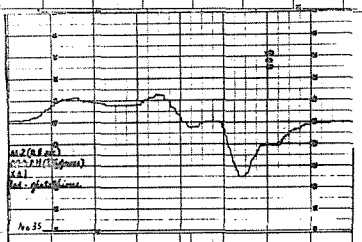


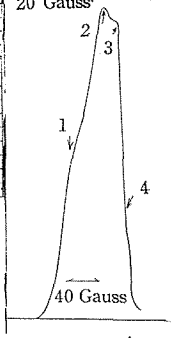
20 Gauss

(3)



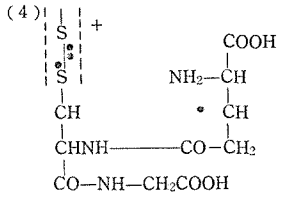
1.3×10^{19}



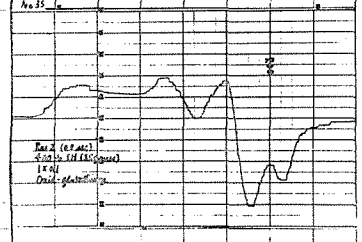


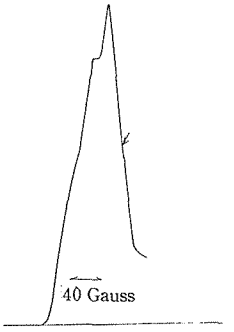
40 Gauss

(4)



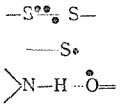
1×10^{19}



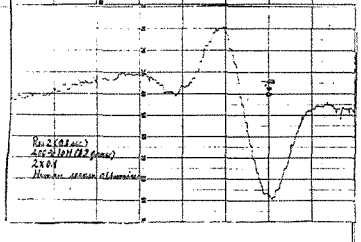


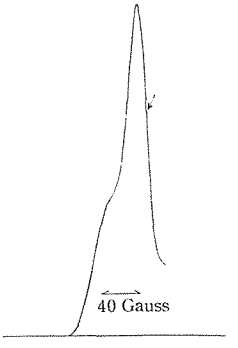
40 Gauss

(5)



4×10^{18}



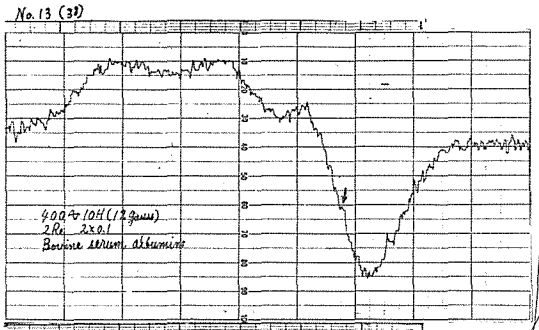


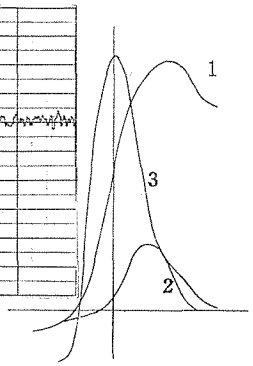
40 Gauss

Table 4. ESR signals of 10^7 irradiated protein in the presence or absence of onxyge and water.

(1)

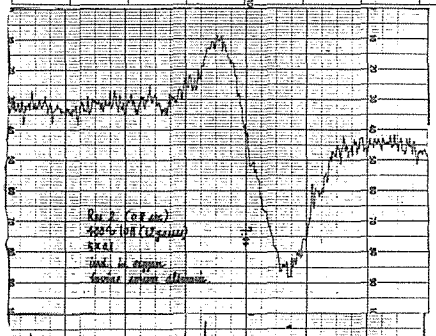
bovine serum albumin irradiated *in vacuo*





(2)

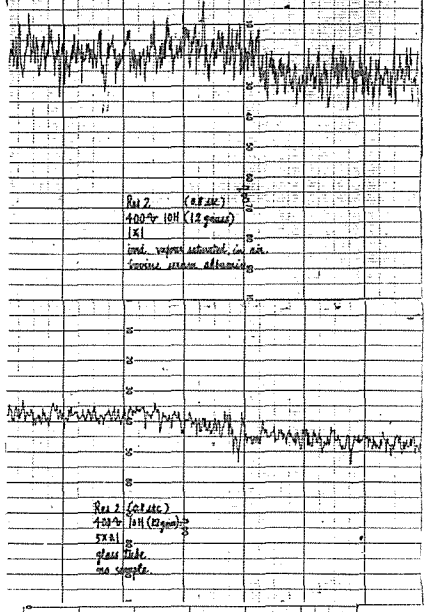
bovine serum albumin irradiated in the presence of oxygen



1. 10^7 r irradiated *in vacuo*.
2. 14^4 r irradiated *in vacuo*.
3. 10^7 r irradiated in oxygen.

(3)

bovine serum albumin irradiated in the presence of water

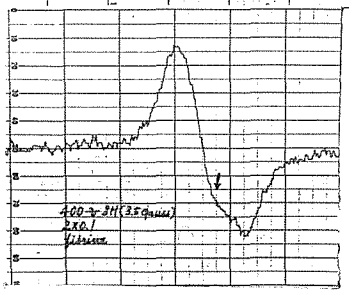


(4)

sample tube (glass) bakground

(5)

fibrin irradiated *in vacuo*



dipole coupling with adjacent protons.

Signals of irradiated glycine and its peptides are somewhat narrower (about 50 Gauss), the splitting width of their triplet and doublet being about 22 Gauss which is not so unreasonable for CH_2 and CH radicals.

The s orbital is spherically symmetrical, so that its wave function at the H nucleus does not vanish and orientation independent (Fermi type) coupling which is directly proportional to the density $(\psi_s\psi_s^*)_0$ at the nucleus arises.

Since the doublet caused by pure s-state electron of H· has spacing of about 500 Gauss[9], it is understandable on the case of amino acids and peptides having narrower spacing than that of pure s-state electron that the unpaired electron has the contribution of p character and considerable exchange with adjacent bonding electron at this temperature (15°C).

In other words, if we consider the unpaired electron possesses carbon π-orbital, metyl group or others having two hydrogen symmetric to this π-orbital give additional coupling of hyperconjugation (alanine, threonine, valine *etc.*).

It might be said, therefore, that the decarboxylation, deamination and CH bond breakage are the main damage of aliphatic amino acids or peptides produced by ionizing radiation as suggested by previous workers[10], and peptide bond is considerably resistive when the results of irradiated glycine-silk series and others are taken into account.

The ESR signals of the amino acids and peptides containing ring carbons are different from those of aliphatic carbon chain. The narrower spacing might be caused by motional narrowing of ring π-electron system but for peptides which has long chain (acetyl tryptophan, alanyl phenylalanine). In the latter case, ESR signals of free radical electron in ring system are superimposed on those of aliphatic chain.

The influence of hydroxyl group is shown in the case of phenylalanine and tyrosine. Since π-electron density in the aromatic ring shifts to the hydroxyl oxygen atom by its high electronegativity, namely density of the free radical electron is lower in side chain carbon atom than ring carbon and this effect would produce only weak hyperconjugational interaction of π-electron with side chain CH_2, it seems not so unexpectable that ESR signal of irradiated tyrosine is monotonous singlet.

Prolyne and hydroxyplolyne are somewhat different because these have no conjugated double bond and no mobile electron. Since the π-orbital of unpaired electron is aproximately vertical to the ring plane (Table 2-4) and wave function of -CH bond of adjacent $-CH_2$ group is symmetric with the orbital of unpaired electron, it would be expected that hyperconjugational interaction with these two proton produces triplet line in the case of prolyne but singlet in hydroxyprolyne by high electronegativity of oxygen atom.

Sulfer containing amino acids, peptides and proteins show the same character as the polymeric sulfer radical whose ESR absorption are also studied by Ingram[11] in dilute oleum.

As shown in Table 3 ESR signals of these samples have widely spread (120 Gauss) asymmetric curves. According to Ingram's results made by radiation with

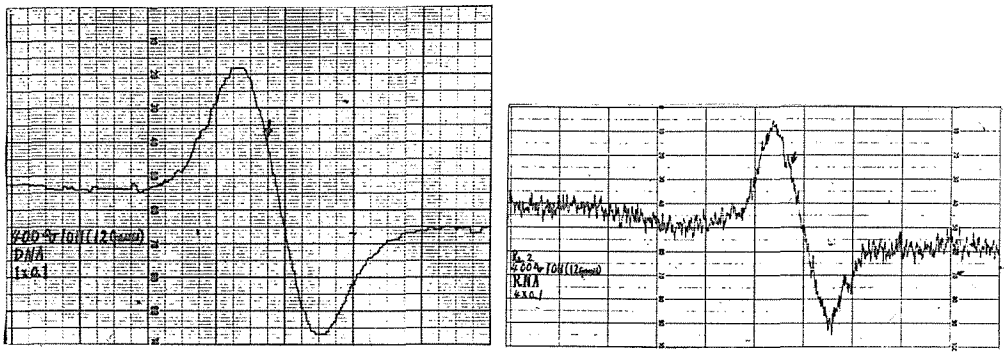


Fig. 2 ESR signals of 10^6r irradiated DNA and RNA Arrow indicates the position of $g \approx 2.003$.

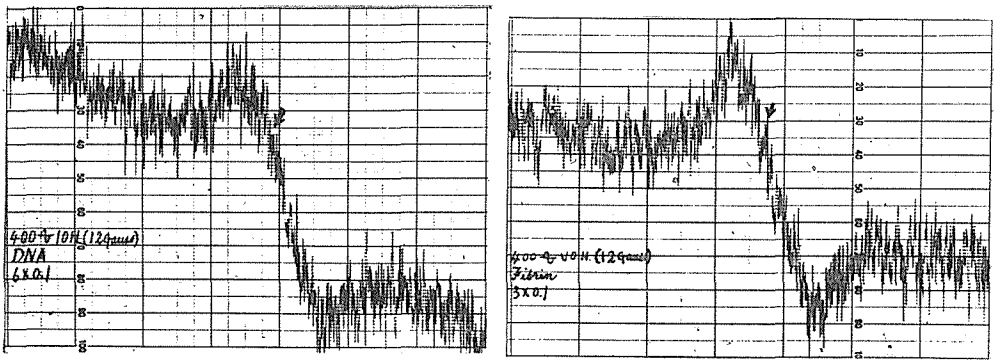


Fig. 3 ESR signals of UV irradiated DNA and fibrine in the presence of oxygen for 5 hours.

different microwave length, these complex curves are not hyperfine structure but caused by different g values. The g-values observed in the present experiment were 1 : 2.04, 2 : 2.027, 3 : 2.017, 4 : 2.003, respectively, their agreement with those of polymeric S radical in dilute oleum (20% SO_3) being fairly well.

This concept was also assured by another experiment with single crystal of cystein, since g values was orientation depeudent. (to be published).

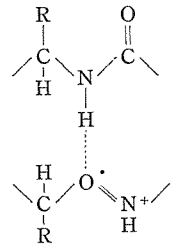
These data indicate that unpaired electron produced by ionizing radiation locarizes at the lone pair orbitals in -S or -S-S-. The results obtained by irradiation studies on the aqueous solution indicated that in -SH or -S-S- containing amino acids and peptides chemical degradation such as deamination is much smaller than those containing no sulfhydryl and disulfide groups, except for such a reaction of oxidation of -SH and -S-S-[12].

It may be said, therefore, that -S or -S-S- group regarded as electron reserver by Gordy protect molecules from the degradation such as deamination, decarboxylation or ionization produced by ionizing radiation, though some doubts remains concerning its mechanism postulated by Gordy[10].

Proteins : -

As shown in Table 1-5, Tables 3-5, 6, Table 4-1, ESR absorption of silk fibre shows orientation dependent doublet which is ascribed to an odd electron, localized

on an O but experiencing direct dipole-dipole interaction with the bridging proton in the ionized structure, as suggested by Gordy *et al.*[13],



while that of silk fibroin powder gives only asymmetric singlet for random orientation. In contrast to the above two, ESR absorption of irradiated proteins containing sulfhydryl and disulfide groups gives mixed patterns of two species of radicals depending on the cystine and cysteine content (-S· or -S∴S- and O· interacting with hydrogen nuleus by direct-dipole dipole coupling).

Yielded free radical concentration was lower in porteins than amino acids and peptides (probably by radical recombination), and its decay time was also fast in protein.

The effect of oxygen and water observed with bovine serum albumin is shown in Table 4.

ESR signals of irradiated protein in the presence of oxygen (Table 4 : 2) is the same as that of oxide radical observed on Teflon[14]. It may be said that one of the oxygen diradical electron combines abruptly with induced free radical at any position, and that free radical electron localizes on the secondarily combined oxygen atom.

In the presence of water, the ESR signal decays very rapidly by chemical degradation through reactions with water molecules. (Table 4-3). In this case radiation effect on protein is mainly that of irradiated water molecules[15].

Nucleic Acids: ESR signal of gamma-ray irradiated DNA, RNA and mononucleotides are investigated by Gordy *et al*[16]. We also examined 10^6 r irradiated DNA and RNA (Fig. 2), and results are the same as theirs. However, it may be worthy to note here that 5 hours illumination by 500 W high pressure Hg-lamp produces free radical considerably, which seems to be same as the radical produced by gamma irradiation (Fig. 3).

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