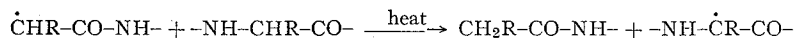


Electron Paramagnetic Resonance of Several Polyamino Acids Gamma-Irradiated at 77 K

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Effects of Co-60 γ -ray irradiation on several polyamino acids have been studied in the solid state by electron paramagnetic resonance spectroscopy. Spectra observed at 77 K in polyglycine, polyalanine, and polyglutamic acid are ascribed to radicals of a type $\dot{\text{C}}\text{HR-CO-NH-}$ (R: an amino acid side chain) produced by the N-C bond scission in polypeptide chains, on the basis of investigation on dipeptides. When warmed to higher temperatures, the spectra begin to change, resulting in those originating from radicals of a type $-\text{NH-}\dot{\text{C}}\text{R-CO-}$. Changes in the radical concentration indicate the conversion of the radicals according to a following scheme:



INTRODUCTION

It seems to be well established that generally two kinds of stable radicals are observed in the EPR (electron paramagnetic resonance) spectra of proteins irradiated in solid state. One is $-\text{NH-CH}(\text{CH}_2\text{-}\dot{\text{S}})\text{-CO-}$ (classified as Group I in a preceding paper¹⁾), and the other is $-\text{NH-}\dot{\text{C}}\text{H-CO-}$ (Group II). In order to reveal this selectivity of radiation effects, the study of the primary process may give some important clues. On the other hand, investigations on simplified systems, such as dipeptides and amino acid copolymers, are thought to be prior steps to those conducted on proteins themselves.

The preceding paper¹⁾ reports the presence of the unstable radical in a γ -irradiated single crystal of glycylglycine and its conversion to the stable radical. The most probable structure of the former is $\text{NH}_3^+\text{-CH}_2\text{-CO-NH-}\dot{\text{C}}\text{H}_2$, and the latter is identified as $\text{NH}_3^+\text{-CH}_2\text{-CO-NH-}\dot{\text{C}}\text{H-COO}^-$. Drew and Gordy²⁾ have found, from systematic EPR studies on irradiated polyamino acids, that the stable radicals of most polyamino acids are of Group I, $-\text{NH-CR-CO-}$ (R: an amino acid side chain), the same type as that produced secondarily in glycylglycine. They also found that spectra obtained at 77K are generally different from those at room temperature, although their structures have not been identified.

This paper presents the structures of these unstable free radicals and their conversion to the stable radicals on the basis of quantitative EPR studies on irradiated several polyamino acids and dipeptides.

MATERIALS AND METHODS

Polyalanine, polyglutamic acid, and polyglycine were supplied through the courtesy of Prof. J. Noguchi, Hokkaido University. Alanyl glycine, alanylvaline, and glycyvaline were purchased from Nakarai Chemicals, Co. Ltd., Kyoto. The polycrystalline samples

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were sealed at a pressure lower than 10^{-3} mm Hg in glass tubes with a 4 mm diameter, and irradiated in liquid nitrogen with γ -rays from a Co-60 source at a dose rate of 6×10^4 rad per hour to the total dose of 1×10^6 rad.

EPR spectra were recorded at 77K as the first derivative of the absorption with a JEOL 3 BX X-band ESR spectrometer and a JEOL X-band Radical Detector P-10 equipped with 100 Kc/sec modulation. Standard samples of POA (peroxylamine disulfonate) solutions were used to determine g -values and hyperfine (hf) splitting widths of the spectra. Temperature-dependent changes were observed at 77K after successive stepwise heat treatments at dry ice and room temperatures. The total concentration of radicals was evaluated by numerical integration.

RESULTS

Polyglycine

Figure 1 shows EPR spectra of irradiated polyglycine at 77 K and those after succes-

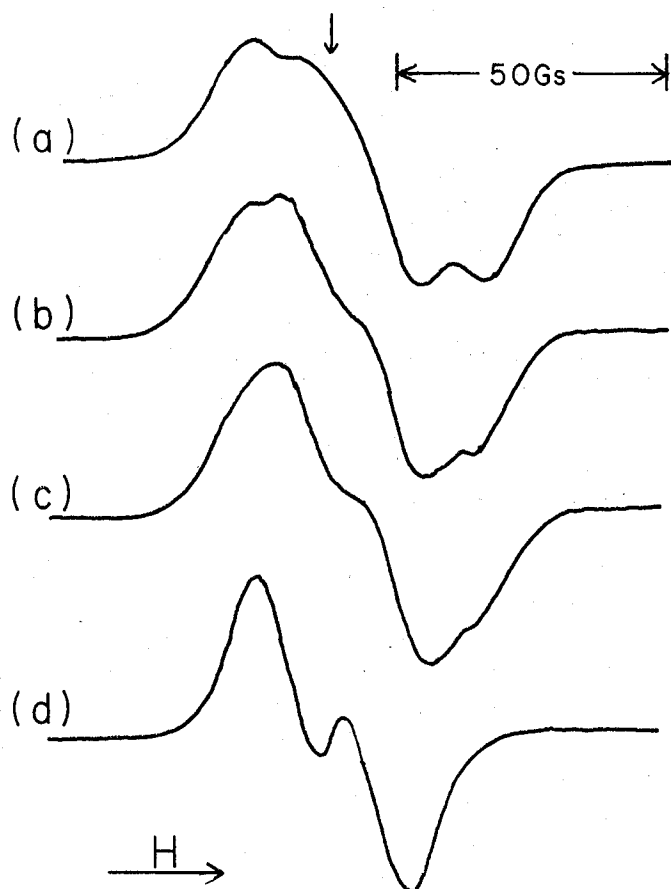


Fig. 1. EPR spectra of polycrystalline polyglycine irradiated and observed at 77 K. Irradiation dose: 1×10^6 rad. (a) before heat treatment, (b) after heat treatment for one minute at -70°C , (c) 90 minutes at -70°C , and (d) additional 90 minutes at 25°C . The arrow indicates the position of the DPPH signal ($g=2.0036$).

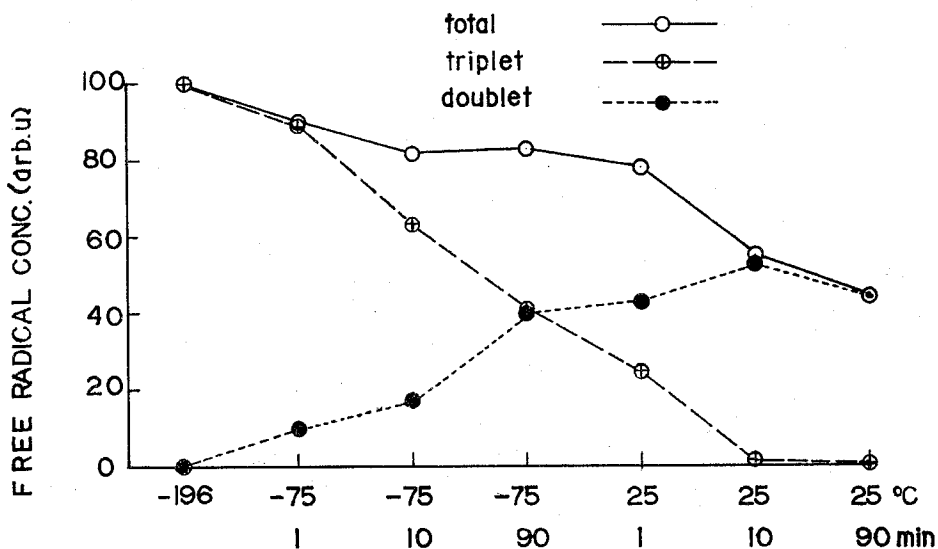


Fig. 2. Changes in the radical concentrations of polycrystalline polyglycine irradiated and observed at 77 K after successive heat treatment at increasing temperatures.

sive heat treatments at dry ice and room temperatures. The spectrum at 77 K is a broad triplet, which begins to change to a doublet spectrum on standing at dry ice and room temperatures. The triplet has spacing 22 G centered at g -value of 2.002 and the doublet has 17 G splitting with a g -value of 2.0035. The doublet spectrum at room temperature has been ascribed by Drew and Gordy²⁾ to a radical $-\text{NH}-\dot{\text{C}}\text{H}-\text{CO}-$ with an unpaired electron interacting with one proton, the same type of radical as that observed at room temperature in an irradiated single crystal of glycylglycine.^{1,3,4)} The triplet is ascribable to $-\text{NH}-\dot{\text{C}}\text{H}_2$ or $\dot{\text{C}}\text{H}_2-\text{CO}-$, produced by a scission of peptide chains, with an unpaired electron interacting with two α -protons, the same type of radicals observed at 77 K in a single crystal of glycylglycine irradiated at 77 K.¹⁾

The relative changes in the signal intensities accompanying the successive heat treatments are shown in Fig. 2. The concentration of total radicals is calculated by numerical integration, that of the triplet is estimated by the height of the outermost peak of the triplet spectrum where the overlapping to the doublet component is negligible, and that of the doublet is estimated as the difference between that of the total and the triplet. The result suggests that the doublet spectrum increases at the expense of the triplet spectrum by the heat treatment, while the total concentrations do not decrease so much.

Polyalanine

As shown in Fig. 3, the EPR spectrum of polyalanine irradiated at 77 K is a quintet with the approximate intensity ratio of 1 : 4 : 6 : 4 : 1 and with the hf splitting of about 22 G centered at $g=2.002$. After standing at dryice temperature, a quartet begins to appear overlapping with the quintet, and at room temperature only a quartet spectrum appears with the approximate intensity ratio of 1 : 3 : 3 : 1 with a 16 G splitting centered at $g=2.003$. The quartet spectrum at room temperature has been ascribed by Drew and Gordy²⁾ to $-\text{NH}-\dot{\text{C}}(\text{CH}_3)-\text{CO}-$ with an unpaired electron interacting with three equivalent

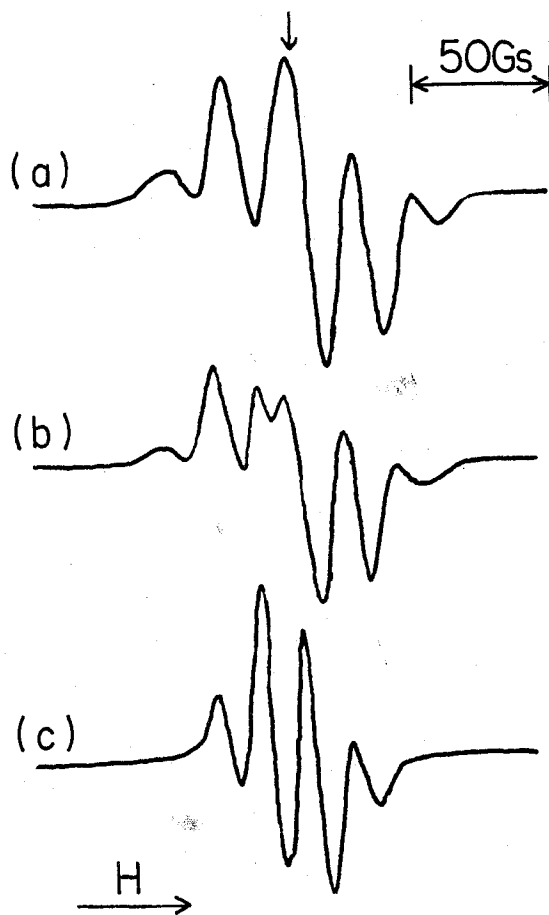


Fig. 3. EPR spectra of polycrystalline polyalanine irradiated and observed at 77 K. Irradiation dose: 1×10^6 rad. (a) before heat treatments, (b) after heat treatment for 10 minutes at -70°C , and (c) 60 minutes at -70°C followed by 90 minutes at 15°C . The arrow indicates the position of DPPH signal ($g=2.0036$).

methyl protons. The quintet can be attributed to a radical $-\text{NH}-\dot{\text{C}}\text{H}-\text{CH}_3$ or $\dot{\text{C}}\text{H}-$
 $(\text{CH}_3)-\text{CO}-\text{NH}-$ in which an unpaired electron interacts with four protons on the α - and β -carbons, as has been proposed by Drew and Gordy.²⁾

Changes in the radical concentrations are shown in Fig. 4. The concentrations of the total, the quintet, and the quartet are evaluated in a similar way as in the case of polyglycine. The result indicates that the radical conversion from the quintet to the quartet occurs as in the case of polyglycine.

Polyglutamic acid

Irradiated polyglutamic acid shows incompletely resolved hf structures as shown in Fig. 5, and it was impossible to calculate hf coupling constants. A major structure at 77 K is triplet-like and that at room temperature is doublet-like with some substructures on the major peaks. The g -value changes from 2.002 at 77 K to 2.004 at room temperature.

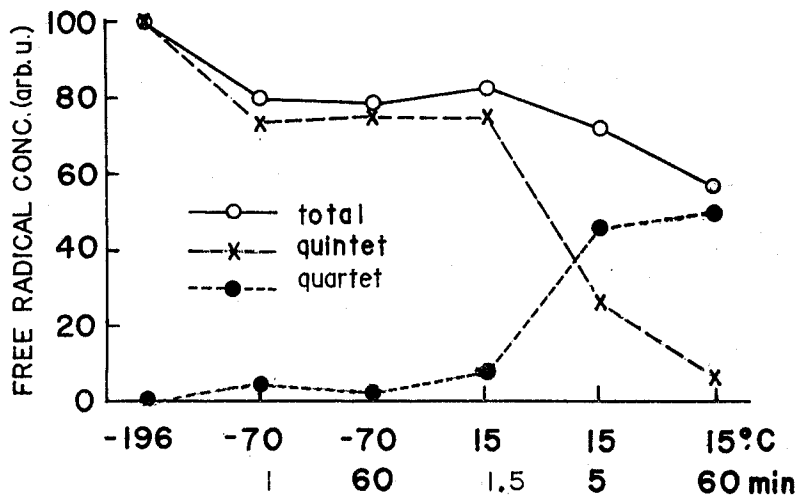


Fig. 4. Change in the radical concentrations of polycrystalline polyalanine irradiated and observed at 77 K after successive heat treatment at increasing temperatures.

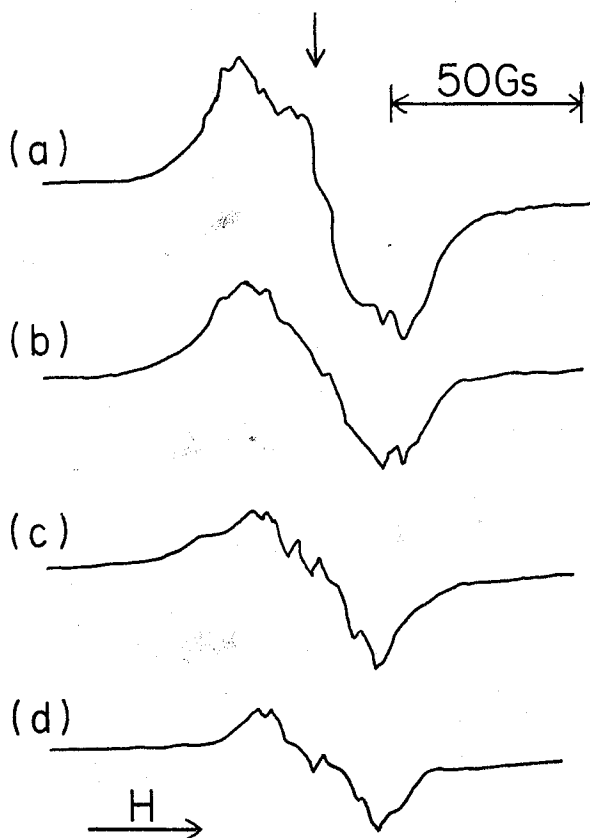


Fig. 5. EPR spectra of polycrystalline polyglutamic acid irradiated and observed at 77 K. Irradiation dose: 1×10^6 rad. (a) before treatment, (b) after heat treatment for 5 minutes at -70°C , (c) 90 minutes at -70°C , and (d) additional 60 minutes at 20°C . The arrow indicates the position of DPPH singal ($g=2.0036$).

Table I. Hyperfine Structures and Coupling Constants of Radicals Produced in Dipeptides Containing Alanine and Glycine in their Carboxyl- or Amino-Terminals

dipeptide	hf structure	possible radical structure	coupling constant G
glycylvaline	1:2:1	$\dot{\text{C}}\text{H}_2\text{CONHCHCOO}^- \text{CH}(\text{CH}_3)_2$	23
alanylvaline	1:4:6:4:1	$\dot{\text{C}}\text{H}(\text{CH}_3)\text{CONHCHCOO}^- \text{CH}(\text{CH}_3)_2$	23
alanylglycine	1:2:1	$\text{NH}_3^+\text{CH}(\text{CH}_3)\text{CONH}\dot{\text{C}}\text{H}_2$	17
glycylalanine	1:4:6:4:1	$\text{NH}_3^+\text{CH}_2\text{CONH}\dot{\text{C}}\text{HCH}_3$	18.5 ^a

a. Johns *et al.* (see ref. 5)

The spectrum at room temperature has been ascribed by Drew and Gordy²⁾ to $-\text{NH}-\dot{\text{C}}-(\text{CH}_2-\text{COOH})-\text{CO}-$ in which an unpaired electron interacts strongly with one of two β -protons giving the major doublet and weakly with other protons giving the substructures. The spectrum at 77 K can be attributed to $-\text{NH}-\dot{\text{C}}\text{H}-\text{CH}_2-\text{COOH}$ or $\dot{\text{C}}\text{H}(\text{CH}_2-\text{COOH})-\text{CO}-$ in which an unpaired electron interacts almost equally with an α -proton and one of the two β -protons giving the major triplet and interacts weakly with the other β -proton giving the substructures. The radical at 77 K is produced by main chain scission as those of the above two polyamino acids. Therefore, the stable radical is probably the secondary product converted from the unstable one, similarly to the case of the above two polyamino acids, although the concentration change could not be measured due to the overlapping of two spectra.

Dipeptides

Table I summarizes the hf couplings of alanylglycine, alanylvaline, and glycylvaline γ -irradiated and observed at 77 K, together with those reported on glycyl-L-alanine by Johns *et al.*⁵⁾ The spectrum is either a 1 : 2 : 1 triplet or a 1 : 4 : 6 : 4 : 1 quintet and ascribed to the corresponding radicals noted in the table. Similar radicals to the above have been reported for various dipeptides containing glycine or alanine,⁵⁻⁷⁾ although the coupling constants have not been described except those studied by Johns *et al.* The glycine and alanine residues seem to be more sensitive to radiation than valine, and carboxyl-terminal than amino-terminal amino acids.

The obtained coupling constants will be discussed in the next section in conjunction with the identification of unstable radicals in polyamino acids.

DISCUSSION

Identification of unstable radicals

For the radicals at 77 K, two structures produced by the main chain scission are to be considered.

Radical I. $\dot{\text{C}}\text{HR}-\text{CO}-$

Radical II. $-\text{NH}-\dot{\text{C}}\text{HR}$

As described in RESULTS, both structures explain the observed hf structures. The identification may, however, be possible through the consideration of the coupling constants.

The spin density on the α -carbon differs between that bonded to the carbon atom of the peptide group and that bonded to its nitrogen atom, as shown theoretically and experimentally in the preceding paper.¹⁾ The former for Radical I is appreciably larger than the latter for Radical II.

In order to obtain the hf coupling constants of Radical I and II, dipeptides containing either glycine or alanine in its N- or C-terminal have been investigated (see Table I). For both glycine and alanine, the coupling constants of Radical I are larger than 20 G and those of Radical II are smaller than 20 G, representing the difference in the spin-density distributions. Hence, the unstable radical in polyglycine (coupling constant 22 G) and that in polyalanine (22 G) are $\dot{\text{C}}\text{H}_2\text{-CO-}$ and $\dot{\text{C}}\text{H}(\text{CH}_3)\text{-CO-}$, respectively, both of Group I. Although the coupling constants for polyglutamic acid have not been determined because of the substructures, the triplet-like spectrum at 77 K may be ascribed to Radical I when the similar changes of the g -value and of the hf structure to those for polyglycine and polyalanine upon heat treatment are considered.

The production of Radical I, rather than Radical II, seems to be reasonable, since the bond energies for N-C and C-C are 64.7 and 78.8 Kcal/mole⁸⁾ respectively, which indicate the N-C is an easier bond to break than the C-C, if the other conditions are same as expected in high polymers. Short peptides may not follow such a law because of some effects from the terminals, as shown in Table I.

Mechanisms of the radical conversion

The results obtained with three polyamino acids are summarized as follows.

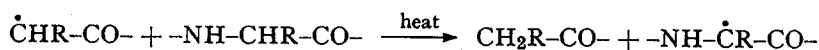
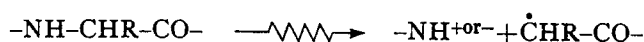
(a) A spectrum observed by irradiation and measurements at 77 K is ascribable to $\dot{\text{C}}\text{HR-CO-}$ (Radical I) produced by N-C bond scission.

(b) The radicals at 77 K change irreversibly into the stable species by warming to room temperature. Quantitative studies show that this process involves radical conversion in addition to decay of the radicals.

(c) The stable radical after standing at room temperature is $-\text{NH}-\dot{\text{C}}\text{R-CO-}$, as has been already proposed by Drew and Gordy.²⁾

(d) Spectroscopic splitting factors, g -values, change irreversibly from 2.002 at 77 K to 2.003 at room temperature.

These common features must have their bases on the common structure of the polyamino acids, that is the peptide main chain, and could reasonably be explained by the following scheme, as



where the peptide main chain is temporally broken at its N-C bond, and thermal excitation causes intermolecular hydrogen abstraction from the undamaged polymer by the radical fragment, similarly to that proposed for irradiated dipeptides.^{1,6)} If the main chain scission were homolysis, there must be a counterpart of $\dot{\text{C}}\text{HR-CO-}$, a radical of a type $-\text{CO}-\dot{\text{N}}\text{H}$, which would show some hyperfine structures coming from the interactions with nuclei N and H. Since these are not observed in the EPR spectra, the counter fragment must be positively or negatively charged to be nonparamagnetic.

Whereas the unstable radical has its $2p\pi$ system spreading over four atoms C-CO-N, the secondary produced radical has a seven-membered system over CO-N-C-CO-N. Because, in general the stability of a $p\pi$ -radical is proportional to the range of the π -orbital, it seems reasonable that the primary radical is converted to the secondary one when warmed to room temperature.

The partial decay of the total radical concentration observed during heat treatment may be due to the recombination of the radicals.

Relation to the radiation effects on proteins

Drew and Gordy²⁾ have reported that nine of fifteen polyamino acids, including three investigated this time, show spectra ascribable to radicals of Group II, $-\text{NH}-\dot{\text{C}}\text{R}-\text{CO}-$, when irradiated and observed at room temperature. They are polyglycine, polysarcosine, poly-L-alanine, polyleucine, polyvaline, polyaspartic acid, polyglutamic acid, and polyphenylalanine, and they show different spectra with more hf peaks when irradiated and observed at 77 K, although they have not been identified. When their spectra at 77 K are compared to the present results, they can all ascribed to Radical I.

From the above discussion, it is most probable that the free radicals of Group II in irradiated proteins are the secondary products from Radical I converted through the process proposed above. It is expected that the selective radical formation on glycine residues in irradiated proteins at room temperature can be explained on the basis of the present investigation.

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