Studies on Radiolysis of Amino Acids and Proteins

III. On Radiolysis of Peptides and Proteins in Aqueous Solutions by Gamma Irradiation*

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ABSTRACT

Several peptides and proteins were irradiated by γ-rays in aqueous solutions. Radiolytic deamination of peptides and related nitrogenous compounds were found to occur in the air-containing solutions by γ-irradiation. To the liberation of ammonia, not only free amino group but also peptide bond were proved to contribute. Moreover, in the course of radiolysis of the peptides, corresponding keto acids to constituent amino acids of peptides, were produced in the γ-irradiated peptide solutions. They were determined quantitatively by column chromatography of their 2, 4-dinitrophenylhydrazones. It was shown that aspartic acid formed from glycine in acetic acid solution by -irradiation under oxygen-free condition. To the mechanism of radiolysis of peptides and proteins, a valid evidence, that organic free radical produced by γ-irradiation might play an important role in the progress of reactions, was presented. From these facts, together with previous results, a scheme of radiolysis of proteins, was proposed and considered in discussion.

INTRODUCTION

In the previous paper it has been reported that several amino acids are deaminated oxidatively to give the corresponding keto acids when they are irradiated with γ-rays in aerated aqueous solutions. In the course of radiolysis of proteins by X-rays, carbonyl compounds are likewise seen to produce in the irradiated solutions. In the latter case, splitting of peptide linkage is to be expected to form organic free radicals by irradiation.

The present paper is concerned with studies on radiolysis of simple peptides to see how peptide linkage is broken in an aerated aqueous solution by γ-irradiation.

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EXPERIMENTAL

Materials Seven dipeptides, a tripeptide, other related nitrogeneous compounds, and five protein preparations were used in this experiment. Serum albumin used was a Behringwerke's product. Egg albumin and trypsin were merchandises of E. Merck, Darmstadt. Crystalline bacterial amylase was obtained from Daiwa Kasei Co., Ltd. and crystalline bacterial Proteinase, "Nagarse", was kind donation by Nagase & Co., Ltd.

Irradiation A 2 kilo-curie cobalt-60 γ-ray irradiation facility* was used for irradiation. The dose rate was determined by a physical measurement† and a Fricke's ferrous-ferric chemical dosimeter‡ to be 1.97 × 10⁵ r. (± 5 %) per hr. The peptide or protein solutions containing molecular oxygen, were irradiated with γ-rays in glass tubes (5 cm. length, 1 cm. dia.) at a room temperature (18°-25°). The procedure was quite identical with those adopted in the experiments of the previous report§.

Methods Amounts of ammonia liberated from peptide in the γ-irradiated solutions were caught by Conway's diffusion method¶ and determined photometrically at 625 mμ by indophenol method** tempered with author's modifications***. Keto acids produced from constituent amino acids of the parent peptides in the γ-irradiated solutions, were changed into their 2,4-dinitrophenylhydrazones and were separated each other by paper chromatography or by column chromatography using a Hyflo-Super-Cel column. The procedure for the paper chromatography adopted was quite identical with that in the previous paper¶. The column chromatographic determination of α-ketoisovaleric acid 2,4-dinitrophenylhydrazones and of cis- and trans-isomers of pyruvic acid 2,4-dinitrophenylhydrazones by the following modification of Egashira's method***. Fifteen g. of Hyflo-Super-Cel was suspended in 9 ml. of a 0.1 N sodium carbonate solution, which was buffered with 0.01 N sodium bicarbonate and was saturated with purified ethyl acetate. A column of Hyflo-Super-Cel, 18 cm. length, 1.6 cm. dia., was prepared from the suspension immersed in purified ethyl acetate buffered with 0.1 N sodium bicarbonate, by packing in a glass tube under pressure of 40 cm. Hg. On the top of the column, 0.5 ml. (or 1.0 ml.) of an ethyl acetate solution of the mixture of keto acid 2,4-dinitrophenylhydrazones was added. The absorbed column was poured with 0.5 ml. (or 1.0 ml.) of ethyl acetate twice. Then developing with ethyl acetate was carried out under the pressure of 40 cm. Hg. Efluent was separated into fraction in volume 1.5 ml. by using a fraction collector. In the progress of developing, α-ketoisovaleric acid 2,4-dinitrophenylhydrazone was eluted with buffered ethyl acetate in the 10-20th fractions. cis-Isomer of pyruvic acid derivative was eluted in the 25-35th fractions. After completion of the eluation of cis-isomer at the 50 fraction, the solvent was changed to a mixture of an equal volume of n-butanol and buffered ethyl acetate. In 60-70th fraction the trans-isomer of pyruvic acid derivative was eluted. A typical elution curve was shown in Fig. 1. The amount of keto acids was determined from the optical density of the eluted solution, comparing it with the following molecular extinction coefficient of the authentic materials; cis-pyruvic acid derivative, ε₃₈₀ mμ = 2.43 × 10⁴, trans-isomer, ε₃₆₈ mμ = 2.14 × 10⁴, and α-ketoisovaleric acid derivative, ε₃₇₈ mμ = 2.39 × 10⁴. The recovery of this method was proved to be 95 ± 5 per cent. Other measuring techniques for keto acids were identical with the previous paper¶.

Determination of Amino Acid and Peptide Glycylalanine and liberated glycine were

* The equipment in Prof. Shimizu's Laboratory, The Institute for Chemical Research, Kyoto University§, was used; The author expresses his sincere gratitude to Prof. S. Shimizu, Kyoto University, for his kind permission to use it.
separated by column chromatography using a potato starch column as described by Stein and Moore\textsuperscript{11}). Determination of amino acid was carried out colorimetrically by Moore and Stein's method\textsuperscript{12)} with some modifications\textsuperscript{13)}.

RESULTS

(1) Ammonia yield of radiolytic deamination from peptides and related compounds in aerated aqueous solutions.

When the oxygen containing aqueous solutions of peptides or related compounds with peptide linkage in their molecule, were irradiated with a $\gamma$-ray dose of 197 kr., liberation of ammonia was always observed. The names and chemical structures of the materials irradiated and ammonia yield were summarized in Table I. Ammonia yield of five protein preparations was determined under the similar conditions and the results were presented in Table II. Similar results were considered in the discussion.

(2) Determination of keto acid formed in the $\gamma$-irradiated peptide solutions.

It was shown that when an aerated aqueous solution of alanylvaline was irradiated with

<table>
<thead>
<tr>
<th>TABLE I</th>
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<tr>
<td>Ammonia yield from peptides and related nitrogenous compounds by $\gamma$-irradiation of 197 kr.</td>
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</table>

<table>
<thead>
<tr>
<th>Compound</th>
<th>Chemical structure</th>
<th>G value moles/100 eV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dipeptide</td>
<td>Glycylglycine</td>
<td>H$_2$NCH,C=O-NHCH$_2$COOH</td>
</tr>
<tr>
<td></td>
<td>Glycyltyrosine</td>
<td>H$_2$NCH,C=O-NHCH(C$_6$H$_5$OH)COOH</td>
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<tr>
<td></td>
<td>Alanylglucose</td>
<td>H$_2$NCH(CH$_2$CO=O-NHCH$_2$COOH</td>
</tr>
<tr>
<td></td>
<td>Alanylbalanine</td>
<td>H$_2$NCH(CH$_3$)CO=O-NHCH$_2$COOH</td>
</tr>
<tr>
<td></td>
<td>Alanylvaline</td>
<td>H$_2$NCH(CH$_3$)CO=O-NHCH(C$_6$H$_5$)COOH</td>
</tr>
<tr>
<td></td>
<td>Alanylleucine</td>
<td>H$_2$NCH(CH$_3$)CO=O-NHCH(C$_6$H$_5$)COOH</td>
</tr>
<tr>
<td>Tripeptide</td>
<td>Glutathione</td>
<td>H$_2$NCH$_2$CH$_2$CO=O-NHCH$_2$COOH</td>
</tr>
<tr>
<td></td>
<td>Acetyl-amino acid</td>
<td>Acetylglucose</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Acetylalanine</td>
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<tr>
<td></td>
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<td>Acetylvaline</td>
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<td></td>
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<td>Acetylalacine</td>
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<tr>
<td></td>
<td></td>
<td>Acetylmethionine</td>
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<tr>
<td>Benzoyl amino acid</td>
<td>Hippuric acid</td>
<td>C$_6$H$_5$CO=O-NHCH$_2$COOH</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TABLE II</th>
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<tr>
<td>Ammonia yield from protein preparation in 0.5% protein solution in 10 mM solution by $\gamma$-irradiation of 197 kr.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Protein</th>
<th>G-value, moles/100 eV</th>
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</thead>
<tbody>
<tr>
<td>Egg albumin</td>
<td>1.06</td>
</tr>
<tr>
<td>Serum albumin</td>
<td>1.55</td>
</tr>
<tr>
<td>Bacterial amylase</td>
<td>1.38</td>
</tr>
<tr>
<td>Bacterial proteinase</td>
<td>1.47</td>
</tr>
<tr>
<td>Trypsin</td>
<td>2.05</td>
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y-ray, pyruvic acid and α-ketoisovaleric acid were produced in the solution. By chromato-
graphic separation of 2,4-dinitrophenylhydrazones of pyruvic acid and α-ketoisovaleric acid produced in the γ-irradiated alanylvaline solutions and of the authentic mixture.
The 10 mM alanylvaline solutions were irradiated with γ-ray dose of; A, 985kr. and B, 4331kr. S, synthetic mixture of the authentic specimens. Details about chromatographic procedure were presented in the experimental part. Peak d: derivative of α-ketoisovaleric acid; c: cis-isomer of pyruvic acid derivative; t: trans-isomer of pyruvic acid derivative; and x₁-x₅: derivatives of unknown materials produced by irradiation.

γ-ray, pyruvic acid and α-ketoisovaleric acid were produced in the solution. By chromato-
graphic separation of 2,4-dinitrophenylhydrazones of the keto acids produced, the presence of α-ketoisovaleric acid derivative and of cis-and trans-isomers of pyruvic acid derivative was confirmed successfully. The results of column chromatographic separation of their derivatives were shown in Fig. 1. Quantitative analysis of pyruvic acid and α-ketoisovaleric acid in the γ-irradiated alanylvaline solutions was carried out colorimetrically with fractions of 2, 4-dinitrophenylhydrazones separated by column chromatography. The results obtained were shown in Table III.

(3) Radiolytic Splitting of Peptide Bond by γ-Irradiation.

Presence of constituent amino acids splitted from the parent peptides, was proved by
TABLE III

Reaction yield of keto acids formation from peptides in the 10 mM alanylvaline solution by γ-irradiation

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<tr>
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<tbody>
<tr>
<td>197</td>
<td>8.67</td>
<td>0.39</td>
<td>11.87</td>
<td>0.71</td>
</tr>
<tr>
<td>394</td>
<td>6.09</td>
<td>0.14</td>
<td>16.20</td>
<td>0.48</td>
</tr>
<tr>
<td>788</td>
<td>6.36</td>
<td>0.07</td>
<td>17.46</td>
<td>0.26</td>
</tr>
<tr>
<td>1379</td>
<td>3.96</td>
<td>0.03</td>
<td>19.62</td>
<td>0.17</td>
</tr>
</tbody>
</table>

Paper chromatographic analysis of the solutions when the peptides were irradiated with γ-rays in the aerated aqueous solution. The results obtained were shown in Fig. 2. Glycine from alanylglucose, alanine from alanylvaline, glycine from glycylleucine, glycine from glycylytyrosine, and cystine from glutathione, were found in irradiated solution of the respective peptides.

Glycine liberated from glycylleucine was separated successfully from its parent peptide by starch column chromatography, though another constituent amino acid, leucine, was hardly proved in the irradiated solution of the parent peptide. Fig. 3. showed the result of chromatographic separation of glycine from glycylalanine. Reaction yield, G-value, of the radiolysis of glycylleucine was determined, from this result, to be 0.97 moles per 100 eV.
Formation of Aspartic Acid by Radiolysis of Glycine in an Oxygen Free Acetic Acid Solution.

When a 1 M solution of glycine in 50% acetic acid was irradiated with 591 kr. dose of \( \gamma \)-rays, under an oxygen-free condition, formation of aspartic acid was proved by paper chromatography as shown in Fig. 4. This amino acid was never found in radiolytic products under an aerated condition. The results elucidated were as follows: Intermediary radicals would be produced by radiolysis of glycine and acetic acid and the combination of the radicals followed to form aspartic acid.

DISCUSSION

As shown in the previous paper\(^1\) several \( \alpha \)-amino acids were deaminated oxidatively in aerated aqueous solutions to produce the corresponding \( \alpha \)-keto acids by \( \gamma \)-irradiation. The reaction yield was seen to depend closely upon chemical structure of the amino acids. An amino group in \( \alpha \)-position appeared to be more sensitive to deamination than that in \( \gamma \)-or in \( \beta \)-position. The similar deamination were also proved to take place in the case of peptides and related compounds. As observed by Dale with glycylglycine\(^{14} \), glycyl amino acids were found to give a larger yield of ammonia than other amino acyl dipeptides. It was not made clear yet whether free amino group in glycy peptides would be particularly sensitive to radiolytic deamination, or peptide linkage between N-terminal glycine and other amino acids would be especially inclined to split by \( \gamma \)-irradiation following to deamination of amino acid splitted. The C-N bond in acetyl and benzoyl amino acid was proved to be more stable to deamination than the free amino group in amino acid by \( \gamma \)-irradiation. Especially a relatively smaller yield of deamination found in hippric acid appeared to be due to the radio-resistancy of aromatic compounds. The C-N bond between aromatic acids and other amino acids, seemed to be very stable against radiolytic deamination and attributed to the stability of structure of aromatic ring. Sulfur-containing peptides such as glutathione and acetylmethionine, did not show any characteristic behaviour against radiolytic deamination.

Deamination of similar degree was also observed in radiolysis of several crystalline pro-
teins. Formation of carbonyl compounds from peptide, in the course of radiolysis, would take place through the following two pathways: One of them was that the keto acid would be produced oxidatively from the corresponding amino acid which had been produced as hydrolytic splitting products from the parent peptide by radiolysis as follows:

\[
\begin{align*}
&H_2NR'CHCOOH + 1/2 \text{O}_2 \rightarrow H_2NRCHCOOH + H_2NR'CHCOOH \\
&H_2NRCHCOOH + 1/2 \text{O}_2 \rightarrow H_2N + RCOCOOH \\
&H_2R'CHCOOH + 1/2 \text{O}_2 \rightarrow H_2N + R'COOCOOH
\end{align*}
\]

Second was a direct formation of keto acid from the parent peptide oxidatively by the way of:

\[
\begin{align*}
&H_2NRCHCO-NHR'CHCOOH + 1/2 \text{O}_2 \rightarrow H_2NRCHCOOH + H_2N + R'COOCOOH \\
&H_2RCHCOOH + 1/2 \text{O}_2 \rightarrow H_2N + RCOCOOH
\end{align*}
\]

Either of the two phenomena or both of them were uncertain to occur in radiolysis of peptides in the aerated aqueous solutions.

Difficulty of identification of both constituent amino acids from dipeptide in the irradiated solution, suggested the predominance of a second pathway in the course of radiolysis. N-terminal amino acid dipeptide, i.e. alanine of alanylvaline, and glycine of glycyleucine and glycyltyrosine, were always proved in the radiolyzed solution, while C-terminal amino acids were scarcely found excepting the case of alanylglycine.

As presented in Fig. 5, the formation of aspartic acid from glycine in acetic acid solution was observed to occur by γ-irradiation under oxygen-free condition. This fact supported also occurrence of a second way. The following mechanism, including formation of dehydrogenated radicals of glycine and acetic acid by radiolysis and combination of these radicals, would be offered to explain the production of aspartic acid:

\[
\begin{align*}
&H_2NCH_2COOH + CH_3COOH \rightarrow H_2NCHCOOH + CH_2COOH \\
&H_2NCHCOOH + CH_2COOH \rightarrow H_2NCHCOOH + CH_2COOH
\end{align*}
\]

over all:

\[
H_2NCH_2COOH + CH_2COOH \rightarrow H_2NCHCOOH + CH_2COOH
\]

By combining with oxygen in the irradiated solution, these radicals should be oxidized spontaneously and the corresponding carbonyl compounds obtained as radiolysate.

From these results of radiolytic break down of peptide and the previous experimental results, decomposition of protein molecule by γ-irradiation would take place as shown under:

ACKNOWLEDGEMENT

The author expresses his thanks to Prof. Shozo Tanaka, Kyoto University, for his useful suggestions and encouragement throughout this study. The author also appreciates Mr. Shigetake Ganno of the laboratory for his technical assistance in these experiments.
REFERENCES