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**Authors**
Garrison, Warren M.
Kland-English, Mathilde
Sokol, Harvey A.
[et al.]

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MECHANISM IN THE RADIOLYTIC DEGRADATION OF THE PEPTIDE MAIN-CHAIN IN DILUTE AQUEOUS SOLUTION CONTAINING OXYGEN

Warren M. Garrison, Mathilde Kland-English
Harvey A. Sokol, and Michael E. Jayko

Lawrence Radiation Laboratory
University of California
Berkeley, California 94720

June 1970

Abstract

In the γ-radiolysis of peptides, RCONHCHR₂, in dilute, oxygen-saturated solution, reaction of OH radicals at the main-chain leads to formation of peptide peroxy radicals, RCONHC(•O)R₂. These react preferentially via: 

\[ 2 \text{RCONHC}(\cdot\text{O})\text{R}_2 \rightarrow 2\text{RCONHCO} + \text{O}_2 \]

The alkoxy radicals are removed through the step:

\[ \text{O}_2 + \text{RCONHC}(\cdot\text{O})\text{R}_2 \rightarrow \text{products} + \text{HO}_2 \]

Experimental evidence for these oxidation modes is derived from a detailed study of reaction stoichiometry in the γ-ray induced oxidation of aqueous N-acetyl-DL-alanine and poly-DL-alanine.
Introduction

Radiolytic oxidation of the peptide main-chain in dilute, oxygenated solution is characterized by the formation of labile "amide-like" degradation products which yield free ammonia on mild hydrolysis. Although recent work has shown that the OH radical formed in the radiation-induced step initiates peptide oxidation via

\[ H_2O \rightarrow H_2O_2, H_2, OH, H, e_{aq}, H^+ \]  

the nature of subsequent reactions in oxygenated solution has not been clearly formulated. The purpose of the present work is to elucidate the mechanism of such reactions in dilute aqueous solutions of typical peptide derivatives of alanine viz. N-acetyl-DL-alanine and poly-DL-alanine.

Experimental

The N-acetylalanine (Cyclo Chemical Corp. NRC Grade I) was recrystallized from water. The polyalanine (Miles-Yeda Ltd.) as received contained traces of ammonia which were removed through lyophilization of a 1 per cent polyalanine solution after addition of sodium hydroxide to pH 9; the alkaline residue was redissolved to 1 per cent, acidified to pH 4 with sulfuric acid and then dialyzed to neutrality against redistilled water.

Water used in preparation of solutions was from a Barnstead still and was redistilled in pyrex first from alkaline permanganate and then from phosphoric acid. The pH adjustments of solutions to be irradiated were made with sodium...
hydroxide or sulfuric acid.

Solutions were irradiated under one atmosphere of oxygen in sealed pyrex tubes. These were removed from the $^{60}$Co source periodically and the contents were mixed to insure that the solution contained excess oxygen throughout the irradiation. A 10 kc $^{60}$Co γ-ray source was used to give a dose-rate of $1 \times 10^{18}$ eV/gm-min as determined by the Fricke dosimeter $[G(Fe^{3+}) = 15.5, \epsilon_{305} = 2180$ at $24^\circ C]$. 

Amide ammonia was determined by the micro-diffusion method of (8) Conway. The samples were made 2 N in sodium hydroxide in the outer compartment of the diffusion cell; hydrolysis and the transfer of free ammonia to the acid compartment (0.1 N sulfuric acid) is complete in 24 hr. Diffusates were assayed by means of Nessler reagent.

(9) Carbonyl products were identified by filter-paper chromatography. The pyruvic acid and acetaldehyde were assayed by the methods of Friedemann and Haugen (10,11) and Johnson and Scholes respectively.

(10,11) The acetic acid was separated through lyophilization of the sample solutions after acidification with sulfuric acid. Assay was by vapor-phase chromatography (12) (Aerograph 600C). The maximal acetic acid yield from acetylalanine was obtained after the irradiated solutions were made 1 N in sodium hydroxide and allowed to stand at room temperature for 15 min prior to separation and assay. Acetylalanine hydrolysis is negligible under these conditions.

Gaseous products were pumped off on the vacuum line through a dry-ice trap. The carbon dioxide yield corresponds to that fraction removed on contact with sodium hydroxide. Analysis was confirmed by gas-chromatography (Aerograph A90-P3).
Hydrogen peroxide and organic peroxide were determined after the method of Johnson and Weiss.\(^1\)

Appropriate control and blank runs confirmed the applicability of the above analytical methods to the present systems.

Results and Discussion

The production of amide-like ammonia in the \(\gamma\)-ray radiolysis of N-acetylalanine in oxygenated solution is shown in Figure 1 as a function of peptide concentration. The ammonia yield increases abruptly with increasing solute concentration and levels off at \(G(\text{NH}_3) \approx 2.9 \approx G_{\text{OH}}\) over the concentration range \(0.02 \text{ M}\) to \(0.1 \text{ M}\). At N-acetylalanine concentrations above \(0.1 \text{ M}\) other reaction modes begin to contribute to the observed \(G(\text{NH}_3)\) values. The chemistry of these other degradation modes in the more concentrated solutions is of quite a different nature as has been described elsewhere.\(^1\)

Now, in dilute oxygenated solutions of N-acetylalanine the reducing species \(H^\cdot\) and \(\text{e}^-_{\text{aq}}\), formed in the radiation induced step 1 are preferentially scavenged via

\[
\text{O}_2 + \text{e}^-_{\text{aq}}(H) \longrightarrow \text{O}_2^-(\text{HO}_2) \quad (3)
\]

where the products of reaction 3 are related by the equilibrium,\(^1\)

\[
\text{HO}_2 \leftrightarrow \text{H}^\cdot + \text{O}_2^- \].

The peptide radicals \(\text{RCONHCR}_2\) formed by OH attack via reaction 2 are also scavenged by oxygen i.e.

\[
\text{O}_2 + \text{RCONHCR}_2 \longrightarrow \text{RCONHC}(\text{O}_2)\text{R}_2 \quad (4)
\]
In earlier work we suggested that the simplest reaction scheme for the subsequent chemistry involves

\[
\text{HO}_2 + \text{RCONH}C(O\cdot)R_2 \rightarrow \text{RCONH}C(O\cdot)H_2R_2 + O_2 \tag{5}
\]

\[
\text{H}_2\text{O} + \text{RCONH}C(O\cdot)H_2R_2 \rightarrow \text{RCONH}C(O\cdot)H_2R_2 + \text{H}_2\text{O}_2 \tag{6}
\]

where the dehydropeptide derivative \( \text{RCONH}C(O\cdot)H_2R_2 \) is labile and yields ammonia and carbonyl on mild hydrolysis

\[
\text{RCONH}C(O\cdot)H_2R_2 \rightarrow \text{RCONH}_2 + \text{R}_2\text{CO} \tag{7}
\]

\[
\text{H}_2\text{O} + \text{RCONH}_2 \rightarrow \text{RCOOH} + \text{NH}_3 \tag{8}
\]

If degradation of the peptide main-chain does occur predominantly through the scheme formulated in equations 1-8 then it is clear that the ammonia and carbonyl yields should be in the relationship,

\[
G(\text{NH}_3) \simeq G(\text{R}_2\text{CO}) \simeq G_{\text{OH}} \simeq 2.9.
\]

Quantitative assay of the carbonyl fraction from irradiated N-acetylaspartic acid solutions show however that the combined yield of carbonyl products, pyruvic acid and acetaldehyde, is quite low with \( G(\text{R}_2\text{CO}) \approx 0.4 \) as shown in Figure 1.

Further study of the oxidation products derived from N-acetylaspartic acid reveals that the principal nitrogen-free organic compounds produced in this system are acetic acid and carbon dioxide. Yield data are summarized in Table 1. The finding that acetic acid and carbon dioxide are formed as major initial products in this system suggested to us that removal of \( \text{RCONHC}C(O\cdot)H_2R_2 \) via the hydrolytic reaction 6 occurs in competition with a second degradation mode which in the specific case of N-acetylaspartic acid
may be formulated in terms of the intramolecular rearrangement

$$\text{HO}_2\text{OH} \rightarrow \text{RCONH}\text{C} = \text{O} \rightarrow \text{RCONHOCR} + \text{H}_2\text{O} + \text{CO}_2$$  \hspace{1cm} (9)

The diacetamide configuration, RCONHCOR, is hydrolytically labile. Mild differential hydrolysis in 1 N sodium hydroxide at room temperature for 15 min (see experimental) converts diacetamide to acetamide and acetic acid.

$$\text{H}_2\text{O} + \text{RCONHCOR} \rightarrow \text{RCONH}_2 + \text{RCOOH}$$  \hspace{1cm} (10)

Both N-acetylalanine and acetamide are stable under this treatment.

There is, however, a difficulty in the above interpretation in that it does not account for the fact that hydrogen peroxide is also formed as major initial product in the radiolysis of oxygenated solutions of N-acetylalanine. The over-all stoichiometry of reactions 1-5 followed by 9 requires that $G(\text{H}_2\text{O}_2) = G_{\text{H}_2\text{O}_2} = 1.0$. Experimentally, however, we find that $G(\text{H}_2\text{O}_2) \approx 2.2$ as shown in Table 1.

To satisfy both the qualitative and quantitative requirements of the present system, we must conclude that the peptide peroxy radicals,

$$\text{RCONH(O}_2\text{)}_{\text{R}}$$  \hspace{1cm} (16)

are removed predominantly via

$$2\text{RCONH(O}_2\text{)}_{\text{R}} \rightarrow 2\text{RCONH(O)}_{\text{R}} + \text{O}_2$$  \hspace{1cm} (11)

In the case of N-acetylalanine, reaction 11 is followed by

$$\text{O}_2 + \text{RCONH(O)}_{\text{R}} \rightarrow \text{RCONHCOR} + \text{CO}_2 + \text{HO}_2$$  \hspace{1cm} (12)

to yield the labile diacetamide derivative. The HO$_2$ radicals formed in
reactions 3 and 12 are removed via

\[ 2\text{HO}_2 \longrightarrow \text{H}_2\text{O}_2 + \text{O}_2 \]  \hspace{1cm} (13)

The sequence of reactions 1-4 followed by reactions 11-13 yield an overall stoichiometry in good agreement with the N-acetylalanine data given in Table 1.

The similarity in the nature of the oxidation products derived from N-acetylalanine and polyalanine as observed in Table 1 indicates that the scheme of reactions 1-4 and 11-13 also applies to the radiolytic oxidation of the polypeptide main-chain. With polyalanine (molecular weight 2000) we must assume that OH removal through reaction 2 occurs at random along the chain. The peroxy radicals RCONHC(02)R2 so formed are then removed as shown in reaction 11 to yield the alkoxy radical, RCONHC(0)R2. We envisage the next step i.e. the analogue of reaction 12 as involving the enol form of the adjacent peptide linkage.

\[ \text{RCONH}-\text{C}=\text{N}-\text{R} + \text{O}_2 \longrightarrow \text{RCONHCOR} + \text{O}=\text{C}=\text{N}-\text{R} + \text{HO}_2 \]  \hspace{1cm} (14)

where

\[ \text{O}=\text{C}=\text{N}-\text{R} + \text{H}_2\text{O} \longrightarrow \text{CO}_2 + \text{RNH}_2 \]  \hspace{1cm} (15)

follows essentially instantaneously.

We note in Table 1 that the oxidation yields in the case of polyalanine are appreciably greater than \( G_{\text{OH}} \). For example, with polyalanine \( G(\text{NH}_3) \approx 3.9 > G_{\text{OH}} \) whereas with acetylalanine \( G(\text{NH}_3) \approx 2.9 \approx G_{\text{OH}} \) in quantitative accord with the reaction scheme given in equations 1-4 and
ll-13. Our interpretation of this effect is that with linear peptide molecules containing more than one amino acid residue, a fraction of the alkoxy radicals react intramolecularly with other C-H linkages along the chain e.g.

\[
\begin{align*}
RCONH\cdot C\cdot \cdot H \cdot O & \rightarrow RCONH\cdot C\cdot \cdot \cdot OH \\
& \rightarrow RCONH\cdot C\cdot \cdot \cdot \cdot R
\end{align*}
\] (16)

This leads to an enhancement in the observed yield for amide production. With small molecules containing a single amino acid residue (e.g. N-acetylalanine) the equivalent chemistry can only occur intermolecularly and is of negligible importance in dilute solution in competition with reaction 12.

We find in substantiation of the above formulations that addition of small amounts of reducing solute such as Fe(II) to these oxygenated peptide solutions leads to the formation of amide ammonia and carbonyl in equal yield. At sufficiently low concentrations, Fe(II) does not interfere with reactions 1-4 but does intercept the peroxy radicals via\textsuperscript{17,18}

\[
RCONH(O\cdot O\cdot O)_{\cdot \cdot \cdot 2} + 3Fe(\cdot \cdot \cdot II) + 2H_{\cdot \cdot \cdot 2}O \rightarrow RCONH(OH)_{\cdot \cdot \cdot 2} + 3Fe(\cdot \cdot \cdot III) + 3OH^{-} \] (17)

\[
RCONH(OH)_{\cdot \cdot \cdot 2} \rightarrow RCONH_{\cdot \cdot \cdot 2} + R_{\cdot \cdot \cdot 2}CO \] (18)

\[
HO_{\cdot \cdot \cdot 2} + Fe(\cdot \cdot \cdot II) + H_{\cdot \cdot \cdot 2}O \rightarrow H_{\cdot \cdot \cdot 2}O_{\cdot \cdot \cdot 2} + Fe(\cdot \cdot \cdot III) + OH^{-} \] (19)

(19) Hydrogen peroxide formed in Step 19 is removed via the Fenton reaction\textsuperscript{19}

\[
H_{\cdot \cdot \cdot 2}O_{\cdot \cdot \cdot 2} + Fe(\cdot \cdot \cdot II) \rightarrow OH + OH^{-} + Fe(\cdot \cdot \cdot III) \] (20)

For oxygen-saturated, 0.05 M N-acetylalanine solutions we observe the
optimum yield at $10^{-3}$ M Fe(II) as shown in Figure 2. Under this condition

$$G(NH_3) = G(R_2CO) \approx 7.5$$

The carbonyl yield ($R_2CO$) is made up of

$$G(pyruvic) \approx 5.7, \ G(acetaldehyde) \approx 2.0.$$ These findings are in quantitative

agreement with the yields of the radiation-induced reaction and the reaction

sequence formulated here. As would be anticipated on the basis of competition

kine

20,21 the oxidation yield goes through a pronounced maximum as the

Fe(II) concentration is increased from zero to $10^{-2}$ M as observed in Figure 2.

There is no evidence from these radiation-chemical studies that the

oxidizing species formed in reaction 20 is other than the free OH radical.22

In a separate control study of peptide oxidation by Fenton reagent, a dilute

solution of $H_2O_2 (2 \times 10^{-4}$ M) was added drop-wise with oxygen stirring to

0.05 M N-acetylalanine solution containing $10^{-3}$ M Fe(II). We obtained the

molecular stoichiometry $-[H_2O_2] = [RCONH_2] = [R_2CO]$ which is in accord

with the requirements of the radiolytic studies described above.
Footnotes and References

(1) This work was performed under the auspices of the U. S. Atomic Energy Commission.


(7) B. H. J. Bielski and A. O. Allen, Int. J. Radiat. Phys. Chem., 1, 153, (1969), report the following 100 eV yields for the γ-ray induced decomposition of water: \( G_{\text{OH}} \approx 2.74 \), \( G_{\text{aq}} \approx 2.76 \), \( G_{\text{aq}} \approx 0.55 \), \( G_{\text{aq}} \approx 0.40 \), \( G_{\text{aq}} \approx 1.00 \).


(14) (a) M. A. J. Rodgers and W. M. Garrison, J. Phys. Chem., 72, 758 (1968);


(16) Reaction of the type $2\text{RO}_2 \rightarrow 2\text{RO} + \text{O}_2$ was proposed as an intermediate step in the radical-initiated oxidation of gaseous hydrocarbons by Vaughan and co-workers [*J. Am. Chem. Soc.*, 73, 15 (1951)].

(17) Reaction 17 represents a composite reaction which for the general case includes the steps: (a) $\text{RO}_2 + \text{Fe(II)} + \text{H}_2\text{O} \rightarrow \text{ROOH} + \text{Fe(III)} + \text{OH}^-$, (b) $\text{ROOH} + \text{Fe(II)} \rightarrow \text{RO} + \text{Fe(III)} + \text{OH}^-$,

(c) $\text{RO} + \text{Fe(II)} + \text{H}_2\text{O} \rightarrow \text{ROH} + \text{Fe(III)} + \text{OH}^-$. See reference 18.


(20) The rate constants for reaction of OH with N-acetylalanine and Fe(II) are both about $2 \times 10^8 \text{ M}^{-1} \text{ sec}^{-1}$. See references 14 and 21.


Table I: Product Yields in the γ-Radiolysis of N-Acetylanaline and Polyalanine in Oxygenated Solution

<table>
<thead>
<tr>
<th>Product</th>
<th>Yield (G)(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.05 M N-acetylanaline</td>
</tr>
<tr>
<td>(\text{NH}_3)</td>
<td>2.9</td>
</tr>
<tr>
<td>(\text{CH}_3\text{COOH})</td>
<td>3.0</td>
</tr>
<tr>
<td>(\text{CO}_2)</td>
<td>2.0</td>
</tr>
<tr>
<td>(\text{H}_2\text{O}_2)</td>
<td>2.2</td>
</tr>
<tr>
<td>ROOH(^b)</td>
<td>0.5</td>
</tr>
<tr>
<td>(\text{CH}_3\text{COCOOH})</td>
<td>(\approx 0.2)</td>
</tr>
<tr>
<td>(\text{CH}_3\text{CHO})</td>
<td>(\approx 0.2)</td>
</tr>
</tbody>
</table>

\(^{a}\)Product yields are independent of dose up to \(2\times 10^{19}\) eV/ml.

\(^{b}\)Unspecified.
Figure Captions

Figure 1. Effect of solute concentration in the $\gamma$-radiolysis of N-acetylalanine in oxygenated solution: $G(\text{NH}_3)$, pH 7 ($\circ$), pH 3 ($\bullet$); $G(\text{CH}_3\text{COO}^- + \text{CH}_3\text{CHO})$, pH 3 ($\Delta$).

Figure 2. Effect of Fe(II) in the $\gamma$-radiolysis of oxygenated 0.05 M N-acetylalanine solutions at pH 3: $G(\text{NH}_3)$, ($\circ$); $G(\text{CH}_3\text{COO}^-)$, ($\Delta$); $G(\text{CH}_3\text{CHO})$, ($\Delta$).
Figure 1

Acetylalanine (M) vs. G (product)
Figure 2
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