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RADIOLYTIC OXIDATION OF PEPTIDE DERIVATIVES OF GLYCINE IN AQUEOUS SOLUTION

Authors
Makada, Hashim A.
Garrison, Warren M.

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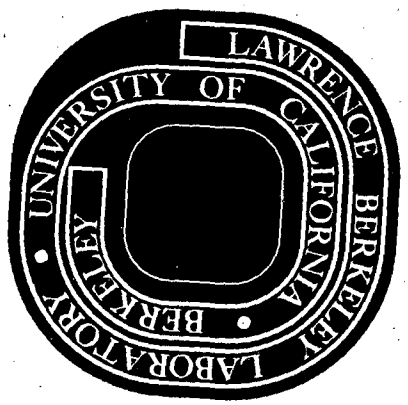

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Hashim A. Makada and Warren M. Garrison

Lawrence Berkeley Laboratory
University of California
Berkeley, California 94720

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RADIOLYTIC OXIDATION OF PEPTIDES

Send proof to

Warren M. Garrison

Lawrence Berkeley Laboratory
University of California
Berkeley, California 94720

RADIOLYTIC OXIDATION OF PEPTIDE DERIVATIVES OF GLYCINE IN AQUEOUS SOLUTION

Hashim A. Makada and Warren M. Garrison

Abstract

A study has been made of the γ-ray induced reactions of the peptide derivatives N-acetylglycine and glycylglycine in oxygenated solution. In these systems the OH radical is removed through reaction at the C-H position of the main-chain $OH + RCONHCH_2R \longrightarrow RCONHCHR + H_2O$ to yield the corresponding peroxy radical via $O_2 + RCONHCHR \longrightarrow RCONNCH(O_2)R$. The subsequent chemistry has been examined in detail. The proposed reaction schemes account for the yields of ammonia, glyoxylic acid, formic acid and hydrogen peroxide observed in both systems.

Radiolysis

Peptides

Oxidation

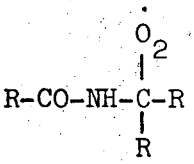
RADIOLYTIC OXIDATION OF PEPTIDE DERIVATIVES OF GLYCINE IN AQUEOUS SOLUTION[1]

Hashim A. Makada and Warren M. Garrison

Lawrence Berkeley Laboratory
University of California
Berkeley, California 94720

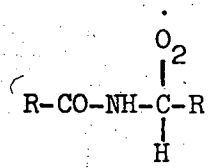
October 1971

Radiolytic oxidation of peptide derivatives of the simpler α-amino acids in aqueous solution is initiated by OH attack at the C-H position of the peptide main-chain (1). With peptide derivatives of alanine, for example, such attack in the presence of oxygen leads to formation of peroxy radicals of the type:



I

We have recently completed a study of the chemistry of such radicals as formed in the radiolytic oxidation of aqueous N-acetylanine and polyalanine (2). In the present work we consider the nature of the intermediate processes involved in the radiolytic oxidation of peptide derivatives of glycine. In this case the degradation involves the radical species



II

It was expected that the presence of the C-H linkage of radical II in place of the C-R linkage of radical I would lead to differences in the subsequent chemistry.

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[1]Work performed under the auspices of the U. S. Atomic Energy Commission.

We present here a detailed study of the γ-ray induced oxidation of the simple peptide derivatives, N-acetylglycine and glycylglycine.

EXPERIMENTAL

The N-acetylglycine and the glycylglycine were obtained from Cyclo Chemical Corp (NRC grade 1) and were recrystallized from distilled 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. Solutions which were irradiated under one atmosphere of oxygen were contained in sealed pyrex tubes. These were removed from the source at frequent intervals and shaken to insure that the solution contained excess oxygen during the irradiation period. Solutions under eight to ten atmospheres of oxygen were irradiated in open tubes enclosed in a small metal bomb. Dosage was determined through use of the Fricke dosimeter [$G(Fe^{+++}) = 15.5$, $\epsilon_{305} = 2180$ at 24°C].

Amide ammonia was determined by the microdiffusion method of Conway (3). Samples of the irradiated solution were made 2N in sodium hydroxide in the outer compartment of the diffusion cell; hydrolysis of the amide and the transfer of free ammonia to the acid compartment (0.1 N sulfuric acid) is complete in 24 hours. The diffusates were assayed with Nessler reagent. In the estimation of free ammonia, magnesium oxide slurry (in excess) was used in place of sodium hydroxide in the outer cell (4). Acetamide which is the major degradation product of N-acetylglycine is stable for prolonged periods of time in the presence of magnesium oxide; the diffusion of free ammonia is complete in approximately eight hours. Application of this magnesium oxide technique to the determination of free ammonia in the irradiated glycylglycine solutions was not as satisfactory.

The difficulty here stems from the fact that the amide products formed in this system, i.e., glycine amide and glyoxylamide, are quite labile and hydrolyze slowly even in the mildly basic magnesium oxide solutions. The free ammonia data reported in Table II is based on initial rates of ammonia transfer and must be considered semi-quantitative in nature. We were unable to devise a precise method for determination of free ammonia in solutions of labile amides.

Hydrogen peroxide and organic peroxide were assayed according to the method of Johnson and Weiss (5). In all cases, the organic peroxide yields were found to be negligible, $G(ROOH) \leq 0.1$.

Prior to the determination of other organic products, the irradiated solution was treated with platinum black for 30 minutes at room temperature to remove hydrogen peroxide. This step was developed in earlier work (6) to eliminate the possibility of post-irradiation oxidation during the acid hydrolysis step. The platinum black was removed with centrifugation, the solution was made 0.1 N in sulfuric acid, evacuated, and then hydrolyzed for $\sim$ 20 hours at 95°C.

The carbonyl products, glyoxylic acid and formaldehyde were identified through filter paper chromatography of the 2,4-dinitrophenylhydrazone derivatives (7). The quantitative determinations of glyoxylic acid and formaldehyde were made after the methods of Friedemann and Haugen (8) and Johnson and Scholes (9) respectively, with the modifications introduced by Sokol.[2]

Formic acid was separated from the hydrolysate through lyophilization. The lyophilizate was neutralized with sodium hydroxide, evaporated to dryness and treated with methanol saturated with anhydrous hydrogen chloride. The

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[2]H. A. Sokol, Lawrence Berkeley Laboratory Report LBL-241.

methyl formate so formed was identified by vapor-phase chromatography (10). Formic acid in the hydrolyzate was quantitatively determined after the colorimetric method of Grant (11). In this procedure, the acid is reduced to formaldehyde which is then assayed through use of chromotropic acid. Appropriate corrections for the contribution of formaldehyde in the initial sample were made.

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

RESULTS AND DISCUSSION

Radiolytic oxidation of the peptide main-chain in dilute oxygenated solution is characterized by the formation of labile amide-like degradation products (1,2,6). The yield of amide ammonia in the γ-radiolysis of N-acetylglycine in oxygenated solution corresponds to $G(NH_3) = 2.8$ over the concentration range 0.005 M to 0.1 M. A summary of the yields of inorganic and organic products observed in the radiolysis of 0.05 M N-acetylglycine solutions under one atmosphere of oxygen is given in Table I.

The evidence from previous radiation-chemical studies (1) in which Fe III was used as a radical scavenger is that the OH radical formed in the radiation-induced step 1 (12-14)

$$H_2O \longrightarrow H_2O_2, H_2, OH, H, e^-_{aq}, H^+ \quad , \qquad (1)$$

reacts with simple peptide derivatives such as N-acetylglycine and N-acetylalanine at the α-carbon position i.e. for N-acetylglycine

$$OH + RCONHCH_2COOH \longrightarrow H_2O + RCONH\dot{C}HCOOH \quad . \qquad (2)$$

In the presence of oxygen, the peptide radicals, $RCONH\dot{C}HCOOH$, are scavenged via

$$O_2 + RCONH\dot{C}HCOOH \longrightarrow RCONHCH(\dot{O}_2)COOH \quad . \qquad (3)$$

The reducing species formed in the radiation-induced step are also scavenged by oxygen

$$e^-_{aq}(H) + O_2 \longrightarrow O_2^-(HO_2) \quad , \qquad (4)$$

where the products of reaction 4 are related by the equilibrium $HO_2 \rightleftarrows H^+ + O_2^-$ (15). We attribute the production of glyoxylic acid with G = 0.3 as given in Table I to the steps

$$HO_2 + RCONHCH(\dot{O}_2)COOH \longrightarrow RCONHCH(OOH)COOH + O_2 \qquad (5)$$

$$H_2O + RCONHCH(OOH)COOH \longrightarrow RCONHCH(OH)COOH + H_2O_2 \quad , \qquad (6)$$

where the dehydropeptide derivative, RCONHCH(OH)COOH, is unstable and yields amide and glyoxylic acid via

$$RCONHCH(OH)COOH \longrightarrow RCONH_2 + CHOCOOH \quad . \qquad (7)$$

Then on acid hydrolysis

$$H_2O + RCONH_2 \longrightarrow RCOOH + NH_3 \quad . \qquad (8)$$

The finding (Table I) that formic acid is the major organic product in this system suggested here, as in the earlier study of alanine peptides (2) that the

removal of the hydroperoxide via step 6 occurs in competition with a second degradation mode

$$RCONHCH(OOH)COOH \longrightarrow RCONHCHO + H_2O + CO_2 \quad , \qquad (9)$$

where the diamide, RCONHCHO, yields ammonia and formic acid on hydrolysis.

$$2H_2O + RCONHCHO \longrightarrow RCOOH + NH_3 + HCOOH \quad . \qquad (10)$$

However, if steps 5, 9, 10 do represent a major oxidation pathway in the present system then it is clear from the incident stoichiometry that the observed hydrogen peroxide yield should not be appreciably greater than the "molecular" yield of the radiation-induced step 1, i.e., $G \simeq 0.8$ (14). We find experimentally, though, as shown in Table I, that the actual hydrogen peroxide yield is appreciably greater than this, viz, $G(H_2O_2) = 2.1$. To account for the observed yields of hydrogen peroxide and formic acid we conclude that the peroxy radicals, $RCONHCH(\dot{O}_2)COOH$, are removed not only through step 5 but also through

$$2RCONHCH(\dot{O}_2)COOH \longrightarrow 2RCONHCH(\dot{O})COOH + O_2 \quad , \qquad (11)$$

which reaction is followed by

$$O_2 + RCONHCH(\dot{O})COOH \longrightarrow RCONHCHO + CO_2 + HO_2 \quad , \qquad (12)$$

and by

$$2HO_2 \longrightarrow H_2O_2 + O_2 \quad . \qquad (13)$$

Reactions akin to steps 11-13 were invoked in the earlier study of the radiation-induced oxidation of peptide derivatives of alanine (2).

In the particular case of the glycine residue it is clear that reaction 12 could be accompanied by the parallel reaction

$$O_2 + RCONHCH(\dot{O})COOH \longrightarrow RCONHCOCOOH + HO_2 \quad , \qquad (14)$$

which step would lead to the liberation of oxalic acid on hydrolysis

$$2H_2O + RCONHCOCOOH \longrightarrow RCOOH + NH_3 + (COOH)_2 \quad . \qquad (15)$$

However, the yield of reaction 14 is apparently quite low since we find as shown in Table I that $G((COOH)_2) \leqslant 0.1$ for the N-acetylglycine system following acid hydrolysis.

Turning our attention now to the glycylglycine system, we consider first of all the remarkably high ammonia yield, $G(NH_3) = 4.8$, obtained in the γ-radiolysis of 0.05 M glycylglycine solutions saturated with oxygen as shown in Table II. This value is almost twice the yield for OH production ($G_{OH} = 2.8$) in reaction 1. Our preliminary conclusion was that either (a) both e^-_{aq} and OH react with glycylglycine to yield ammonia or (b) a short chain reaction is involved in the radiolysis of this system.

With reference to point (a) we note that e^-_{aq} in evacuated solution reacts essentially quantitatively with glycylglycine via (16)[3]

$$e^-_{aq} + NH_2CH_2CONHCH_2COOH \longrightarrow NH_3 + \dot{C}H_2CONHCH_2COOH + OH^- \quad . \qquad (16)$$

[3] We represent glycylglycine here in the uncharged form rather than the zwitterion form ($NH_3^+CH_2CONHCH_2COO^-$) for the purpose of simplicity in notation.

However, the rate constants for reaction of e^-_{aq} with glycylglycine and with oxygen are 2×10^8 M^{-1} sec^{-1} and 2×10^{10} M^{-1} sec^{-1} respectively (17). Hence, in 0.01 M glycylglycine solution under one atmosphere of oxygen the electrons are preferentially scavenged by O_2. We also find that increasing the oxygen pressure to 8 atmospheres has essentially no effect on the ammonia yield. We conclude then that reaction 16 does not contribute appreciably to the production of ammonia in the radiolysis of these glycylglycine solutions. As regards point (b) above, we find experimentally that there is no appreciable effect of dose-rate on $G(NH_3)$ from oxygen-saturated 0.05 M solutions of glycylglycine over the range 1×10^{18} eV/ml to 1×10^{19} eV/ml. While this observation does not wholly exclude the possibility of a chain reaction, still, the above findings en toto suggest we should consider other possible explanations for the high ammonia yields observed in the radiolysis of the glycylglycine system.

Now, previous work (16) has shown that glycylglycine undergoes the characteristic peptide reaction with the OH radical i.e.

$$OH + NH_2CH_2CONHCH_2COOH \longrightarrow H_2O + NH_2CH_2CONH\dot{C}HCOOH \quad , \qquad (17)$$

which step in oxygenated solution is followed by

$$O_2 + NH_2CH_2CONH\dot{C}HCOOH \longrightarrow NH_2CH_2CONHCH(\dot{O}_2)COOH \quad . \qquad (18)$$

As observed in the acetylglycine case, we find as shown in Table II that formic acid and hydrogen peroxide are major products of the radiolytic oxidation of glycylglycine. By analogy with reactions 11, 12 we formulate steps 19, 20 as major paths in the chemistry of the peroxy radicals $NH_2CH_2CONHCH(\dot{O}_2)COOH$

$$2NH_2CH_2CONHCH(\dot{O}_2)COOH \longrightarrow 2NH_2CH_2CONH(\dot{O})COOH + O_2 \qquad (19)$$

$$O_2 + NH_2CH_2CONHCH(\dot{O})COOH \longrightarrow NH_2CH_2CONHCHO + HO_2 + CO_2 \quad , \qquad (20)$$

where the HO_2 radicals formed in step 20 yield hydrogen peroxide via the disproportionation reaction 13. The diamide derivative $NH_2CH_2CONHCHO$ is labile and yields ammonia and formic acid on hydrolysis

$$2H_2O + NH_2CH_2CONHCHO \longrightarrow NH_2CH_2COOH + NH_3 + HCOOH \quad . \qquad (21)$$

In explaining the remarkably high yield of total ammonia, $G(NH_3) = 4.58$, from glycylglycine, we note that in the earlier study of the radiolytic oxidation of alanine peptides (2) there was some evidence that the alkoxy radical sites formed in reaction of the type given in equations 11, 19 can react intramolecularly with other C-H linkages along the chain. In the case of the alkoxy radicals formed from glycylglycine via step 19, the subsequent chemistry would be of the form:

$$NH_2CH_2CONHCH(\dot{O})COOH \longrightarrow NH_2\dot{C}HCONHCH(OH)COOH \quad . \qquad (22)$$

The radical product of reaction 22 reacts in turn with oxygen

$$O_2 + NH_2\dot{C}HCONHCH(OH)COOH \longrightarrow NH = CHCONHCH(OH)COOH + HO_2 \qquad (23)$$

$$H_2O + NH = CHCONHCH(OH)COOH \longrightarrow NH_3 + CHOCONH_2 + CHOCOOH \quad . \qquad (24)$$

The glyoxylamide, $CHOCONH_2$, then yields additional ammonia and glyoxylic acid on acid hydrolysis

$$H_2O + CHOCONH_2 \longrightarrow CHOCOOH + NH_3 \quad . \qquad (25)$$

The formulations of reactions 19-25 provide a self-consistent explanation for both the qualitative and quantitative on glycylglycine oxidation as given in Table II. In the case of N-acetylglycine, the equivalent chemistry of reactions 19-25 can only occur intermolecularly and such reaction is of negligible importance in competition with reaction 12 in dilute solutions of N-acetylglycine.

It is clear that direct identification of glyoxylamide, $CHOCONH_2$, as a major oxidation product in the glycylglycine system would provide convincing supporting evidence for the intramolecular oxidation steps formulated in the reaction scheme of equations 19-25. Accordingly we analyzed chromatographically the 2,4-dinitrophenylhydrazone derivatives of the carbonyl products present in the irradiated glycylglycine solutions prior to acid hydrolysis. We separated to major hydrazone components: (1) glyoxylic acid hydrazone (cis and trans forms) and (2) a second hydrazone fraction which yields glyoxylic acid on mild acid hydrolysis. Additional evidence that the second component is the hydrazone derivative of glyoxylamide is to be found in the fact that this same component is observed as a major carbonyl product in the γ-ray induced oxidation of acetamide, CH_3CONH_2, in oxygenated solution. The OH radical attacks acetamide preferentially at the methyl group to give $\dot{C}H_2CONH_2$ (18). In the presence of oxygen, radicals of this type are oxidized to the corresponding glyoxylic acid derivative which in the case of $\dot{C}H_2CONH_2$ would be glyoxylamide, $CHOCONH_2$.

And finally we note that implicit in the formulation of the intramolecular step 22 is the prediction that the γ-ray induced oxidation of a mixed dipeptide

such as glycylalanine should yield both glyoxylic acid and pyruvic acid. Examination of the carbonyl fraction isolated from an irradiated glycylalanine-oxygen solution reveals that both keto acids are indeed formed in approximately equal yield.

ACKNOWLEDGMENTS

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References

(1) H. L. Atkins, W. Bennett-Corniea and W. M. Garrison, The radiation-induced oxidation of peptides in aqueous solution, J. Phys. Chem., 71 772-774 (1967).

(2) W. M. Garrison, M. Kland-English, H. A. Sokol and M. E. Jayko, Radiolytic degradation of the peptide main-chain in dilute aqueous solution containing oxygen, J. Phys. Chem., 74 4506-4509 (1970).

(3) E. J. Conway, Microdiffusion Analysis, Crosby, Lockwood and Son, London 1962.

(4) D. B. Peterson, J. Holian and W. M. Garrison, Radiation chemistry of the α-amino acids: γ radiolysis of solid cysteine, J. Phys. Chem., 73 1568-1572 (1969).

(5) G. R. A. Johnson and J. Weiss, Formation of methyl hydroperoxide from methane irradiated by x-rays in aqueous solution in the presence of dissolved oxygen, Chem. and Ind., 358-359 (1955).

(6) W. M. Garrison, M. E. Jayko and W. Bennett-Corniea, Radiation-induced oxidation of protein in aqueous solution, Radiation Res., 16 483-502 (1962).

(7) D. Cavallini, N. Frontali and G. Toschi, Determination of keto acids by partition chromatography, Nature, 163 568-569 (1949).

(8) T. E. Friedemann and G. E. Haugen, Determination of keto acids in blood and urine, J. Biol. Chem., 147 415-420 (1943).

(9) G. R. A. Johnson and G. Scholes, Micro-determination of acetaldehyde as its 2,4-dinitrophenyl hydrazone, Analyst, 79 217-219 (1954).

(10) E. T. Oakley, L. Weissbecker and F. E. Resnik, Gas chromatographic determination of free formic and acetic acids in cigarette smoke, Anal. Chem., 37 380-382 (1965).

(11) W. M. Grant, Colorimetric micro-determination of formic acid based on reduction to formaldehyde, Anal. Chem., 20 267-269 (1948).

(12) G. Czapski and H. A. Schwarz, The nature of the reducing species formed in water radiolysis, J. Phys. Chem., 66 471-474 (1962).

(13) E. J. Hart and J. W. Boag, Absorption spectrum of the hydrated electron, J. Am. Chem. Soc., 84 4090 (1962).

(14) A. O. Allen, Radiation yields and reactions in dilute inorganic solutions, Radiation Res. Suppl., 4 54-73 (1964).

(15) G. Czapski and H. J. Bielski, Formation and decay of H_2O_3 and HO_2 in electron-irradiated aqeuous solution, J. Phys. Chem., 67 2180-2184 (1963).

(16) R. L. S. Willix and W. M. Garrison, Chemistry of the hydrated electron in oxygen-free solutions of amino acids, peptides and related compounds, Radiation Res., 32 452-462 (1967).

(17) M. Anbar and P. Neta, A compilation of specific bimolecular rate constants for the reactions of hydrated electrons, hydrogen atoms, and hydroxyl radicals with inorganic and organic compounds in aqueous solution, Int. J. Appl. Radiat. Isotopes, 18 493 (1967).

(18) J. Holian and W. M. Garrison, On the radiation-induced reduction of peptide and amide functions in aquoorganic systems, J. Phys. Chem., 72 4721-4723 (1968).

Table I. Product Yields in the γ-Radiolysis of N-acetylglycine (.01M) in Oxygenated Solution

Product	Yield, G
NH_3(total)[a]	2.9
NH_3(free)	< 0.2
CHOCOOH	0.3
CH_2O	0.1
HCOOH	2.9
$(COOH)_2$	< 0.1
H_2O_2	2.1
ROOH[b]	< 0.1

[a]Amide plus free ammonia.

[b]Total organic peroxide, unspecified.

Table II. Product Yields in the γ-Radiolysis of Glycylglycine (0.01M) in Oxygenated Solution

Product	Yield, G
NH_3(total)[a]	4.8
NH_3(free)	~ 2[b]
CHOCOOH	1.9
CH_2O	0.1
HCOOH	1.6
$(COOH)_2$	< 0.1
H_2O_2	2.1
ROOH	< 0.1

[a]Amide plus free ammonia.

[b]For the basis of this estimation see experimental section.

[c]Total organic peroxide, unspecified.

TECHNICAL INFORMATION DIVISION
LAWRENCE BERKELEY LABORATORY
UNIVERSITY OF CALIFORNIA
BERKELEY, CALIFORNIA 94720