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MECHANISM IN THE RADIOLYTIC DEGRADATION OF THE PEPTIDE MAIN-CHAIN IN DILUTE AQUEOUS SOLUTION CONTAINING OXYGEN[1]

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Abstract

In the γ-radiolysis of peptides, $RCONHCHR_2$, in dilute, oxygen-saturated solution, reaction of OH radicals at the main-chain leads to formation of peptide peroxy radicals, $RCONHC(\dot{O}_2)R_2$. These react preferentially via: $2RCONHC(\dot{O}_2)R_2 \longrightarrow 2RCONHC(\dot{O})R_2 + O_2$. The alkoxy radicals are removed through the step: $O_2 + RCONHC(\dot{O})R_2 \longrightarrow \text{products} + HO_2$. 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
(2,3) products which yield free ammonia on mild hydrolysis.[2] Although recent work[3]
(4-7) has shown that the OH radical formed in the radiation-induced step[4-7]

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

initiates peptide oxidation via

$$OH + RCONHCHR_2 \longrightarrow H_2O + RCONH\dot{C}R_2 \qquad (2)$$

the nature of subsequent reactions in oxygenated solution has not been clearly formulated.[2,3] 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 1×10^{18} eV/gm-min as determined by the Fricke dosimeter [$G(Fe^{3+}) = 15.5$, $\varepsilon_{305} = 2180$ at 24°C].

Amide ammonia was determined by the micro-diffusion method of
(8) Conway.[8] 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.[9] The pyruvic acid and acetaldehyde were assayed by the methods of Friedemann
(10,11) and Haugen[10] and Johnson and Scholes[11] respectively.

The acetic acid was separated through lyophilization of the sample solutions after acidification with sulfuric acid. Assay was by vapor-phase
(12) 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
(13) method of Johnson and Weiss.[13]

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 γ-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 $G(NH_3) \simeq 2.9 \simeq G_{OH}$ over the concentration range 0.02 M to 0.1 M. At N-acetylalanine concentrations above 0.1 M other reaction modes begin to contribute to the observed $G(NH_3)$ values. The chemistry of these other degradation modes in the more concentrated solutions is of quite a different nature as has been described
(14) elsewhere.[14]

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

$$O_2 + e^-_{aq}(H) \longrightarrow O_2^-(HO_2) \qquad (3)$$

(15) where the products of reaction 3 are related by the equilibrium,[15]
$HO_2 \rightleftarrows H^+ + O_2^-$. The peptide radicals $RCONHCR_2$ formed by OH attack via reaction 2 are also scavenged by oxygen i.e.

$$O_2 + RCONH\dot{C}R_2 \longrightarrow RCONHC(\dot{O}_2)R_2 \qquad (4)$$

In earlier work[2] we suggested that the simplest reaction scheme for the subsequent chemistry involves

$$HO_2 + RCONHC(\dot{O}_2)R_2 \longrightarrow RCONHC(OOH)R_2 + O_2 \quad (5)$$

$$H_2O + RCONHC(OOH)R_2 \longrightarrow RCONHC(OH)R_2 + H_2O_2 \quad (6)$$

where the dehydropeptide derivative $RCONHC(OH)R_2$ is labile and yields ammonia and carbonyl on mild hydrolysis

$$RCONHC(OH)R_2 \longrightarrow RCONH_2 + R_2CO \quad (7)$$

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

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, $G(NH_3) \simeq G(R_2CO) \simeq G_{OH} \simeq 2.9$. Quantitative assay of the carbonyl fraction from irradiated N-acetylalanine solutions show however that the combined yield of carbonyl products, pyruvic acid and acetaldehyde, is quite low with $G(R_2CO) \simeq 0.4$ as shown in Figure 1.

Further study of the oxidation products derived from N-acetylalanine 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 $RCONHC(OOH)R_2$ via the hydrolytic reaction 6 occurs in competition with a second degradation mode which in the specific case of N-acetylalanine

may be formulated in terms of the intramolecular rearrangement

$$\mathrm{RCONH{-}\overset{HO}{\underset{R}{C}}{-}\overset{OH}{C}{=}O} \longrightarrow \mathrm{RCONHCOR} + H_2O + CO_2 \qquad (9)$$

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.

$$H_2O + \mathrm{RCONHCOR} \longrightarrow \mathrm{RCONH_2} + \mathrm{RCOOH} \qquad (10)$$

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 $G(H_2O_2) \simeq G_{H_2O_2} \simeq 1.0$. Experimentally, however, we find that $G(H_2O_2) \simeq 2.2$ 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,
(16) $\mathrm{RCONHC}(\dot{O}_2)R_2$, are removed predominantly via[16]

$$2\mathrm{RCONHC}(\dot{O}_2)R_2 \longrightarrow 2\mathrm{RCONHC}(\dot{O})R_2 + O_2 \qquad (11)$$

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

$$O_2 + \mathrm{RCONH\overset{\dot{O}}{\underset{R}{C}}{-}\overset{OH}{C}{=}O} \longrightarrow \mathrm{RCONHCOR} + CO_2 + HO_2 \qquad (12)$$

to yield the labile diacetamide derivative. The HO_2 radicals formed in

reactions 3 and 12 are removed via

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

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 $RCONHC(\dot{O}_2)R_2$ so formed are then removed as shown in reaction 11 to yield the alkoxy radical, $RCONHC(\dot{O})R_2$. We envisage the next step i.e. the analogue of reaction 12 as involving the enol form of the adjacent peptide linkage.

$$RCONH\underset{\underset{R}{|}}{\overset{\dot{O}}{C}}-\overset{OH}{\overset{|}{C}}=N-R + O_2 \longrightarrow RCONHCOR + O=C=N-R + HO_2 \qquad (14)$$

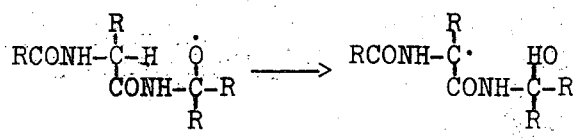
where

$$O=C=N-R + H_2O \longrightarrow CO_2 + RNH_2 \qquad (15)$$

follows essentially instantaneously.

We note in Table 1 that the oxidation yields in the case of polyalanine are appreciably greater than G_{OH} . For example, with polyalanine $G(NH_3) \simeq 3.9 > G_{OH}$ whereas with acetylalanine $G(NH_3) \simeq 2.9 \simeq G_{OH}$ in quantitative accord with the reaction scheme given in equations 1-4 and

11-13. 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 <u>intramolecularly</u> with other C-H linkages along the chain e.g.



(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-acetyl-alanine) the equivalent chemistry can only occur <u>intermolecularly</u> 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[17,18] (17,18)

$$RCONHC(\dot{O}_2)R_2 + 3Fe(II) + 2H_2O \longrightarrow RCONHC(OH)R_2 + 3Fe(III) + 3OH^- \quad (17)$$

$$RCONHC(OH)R_2 \longrightarrow RCONH_2 + R_2CO \quad (18)$$

$$HO_2 + Fe(II) + H_2O \longrightarrow H_2O_2 + Fe(III) + OH^- \quad (19)$$

Hydrogen peroxide formed in Step 19 is removed via the Fenton reaction[19] (19)

$$H_2O_2 + Fe(II) \longrightarrow OH + OH^- + Fe(III) \quad (20)$$

For oxygen-saturated, 0.05 <u>M</u> N-acetylalanine solutions we observe the

optimum yield at 10^{-3} M Fe(II) as shown in Figure 2. Under this condition $G(NH_3) \simeq G(R_2CO) \simeq 7.5$. The carbonyl yield (R_2CO) is made up of G(pyruvic) ≃ 5.7, G(acetaldehyde) ≃ 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
(20,21) kinetics[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
(22) 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(2×10^{-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.

(2) For a recent review see W. M. Garrison, "Current Topics in Radiation Research," ed. by M. Ebert and A. Howard, Vol IV, North Holland Publishing Co. Amsterdam, 1968, p. 45-94.

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(8) E. J. Conway, "Microdiffusion Analysis," Crosby Lockwood and Sons, Ltd. London, 1962.

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(12) E. M. Emery and W. E. Koerner, Anal. Chem., 33, 146 (1961).

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(b) W. M. Garrison, M. E. Jayko, M. A. J. Rodgers, H. A. Sokol, and W. Bennett-Corniea, Advances in Chemistry Series, No. 81, American Chemical Society, Washington, D. C., 1968, p. 384.

(15) G. Czapski and H.J. Bielski, J. Phys. Chem., 67, 2180 (1963).

(16) Reaction of the type $2RO_2 \longrightarrow 2RO + O_2$ 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) $RO_2 + Fe(II) + H_2O \longrightarrow ROOH + Fe(III) + OH^-$, (b) $ROOH + Fe(II) \longrightarrow RO + Fe(III) + OH^-$, (c) $RO + Fe(II) + H_2O \longrightarrow ROH + Fe(III) + OH^-$. See reference 18.

(18) C. Walling, "Free Radicals In Solution," John Wiley and Sons, Inc., New York, 1957.

(19) F. Haber and J. Weiss, Proc. Roy. Soc. (London), A147, 333 (1934).

(20) The rate constants for reaction of OH with N-acetylalanine and Fe(II) are both about $2\times10^8\ M^{-1}\ sec^{-1}$. See references 14 and 21.

(21) M. Anbar and P. Neta, Int. J. Appl. Radiat. Isotop., 17, 493 (1967).

(22) Cf. T. Shiga, J. Phys. Chem., 69, 3805 (1965).

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

Product	Yield (G)[a]	
	0.05 M N-acetylalanine	0.5% polyalanine
NH_3	2.9	3.9
CH_3COOH	3.0	∿3.9
CO_2	2.0	2.4
H_2O_2	2.2	2.2
ROOH[b]	0.5	---
$CH_3COCOOH$	∿0.2	1.2
CH_3CHO	∿0.2	0.4

[a]Product yields are independent of dose up to $\sim 2\times10^{19}$ eV/ml.

[b]Unspecified.

Figure Captions

Figure 1 Effect of solute concentration in the γ-radiolysis of N-acetylalanine in oxygenated solution: $G(NH_3)$, pH 7 (O), pH 3 (●); $G(CH_3COCOOH + CH_3CHO)$, pH 3 (Δ).

Figure 2 Effect of Fe(II) in the γ-radiolysis of oxygenated 0.05 M N-acetylalanine solutions at pH 3: $G(NH_3)$, (●); $G(CH_3COCOOH)$, (Δ); $G(CH_3CHO)$, (▲).

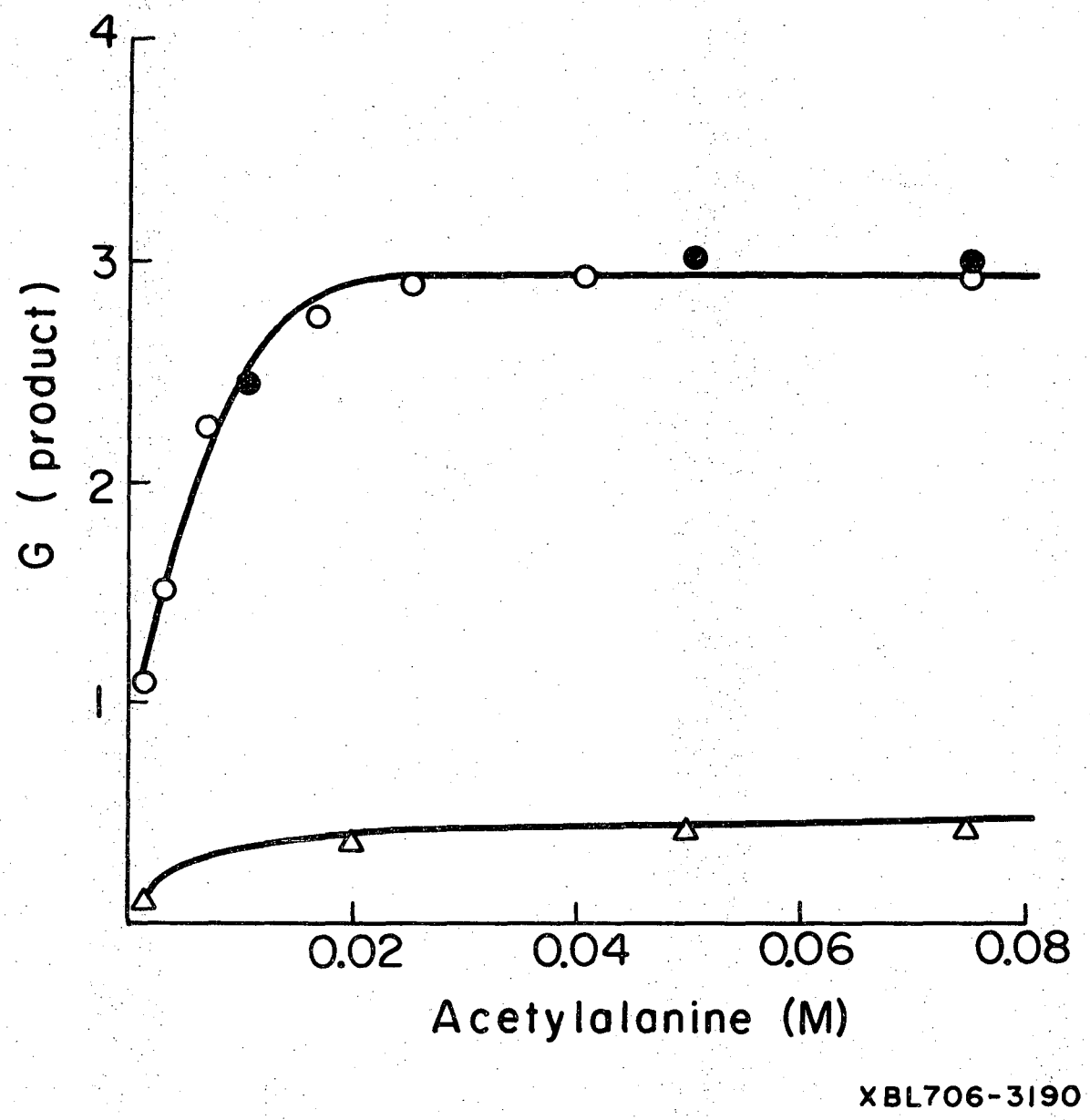

Figure 1

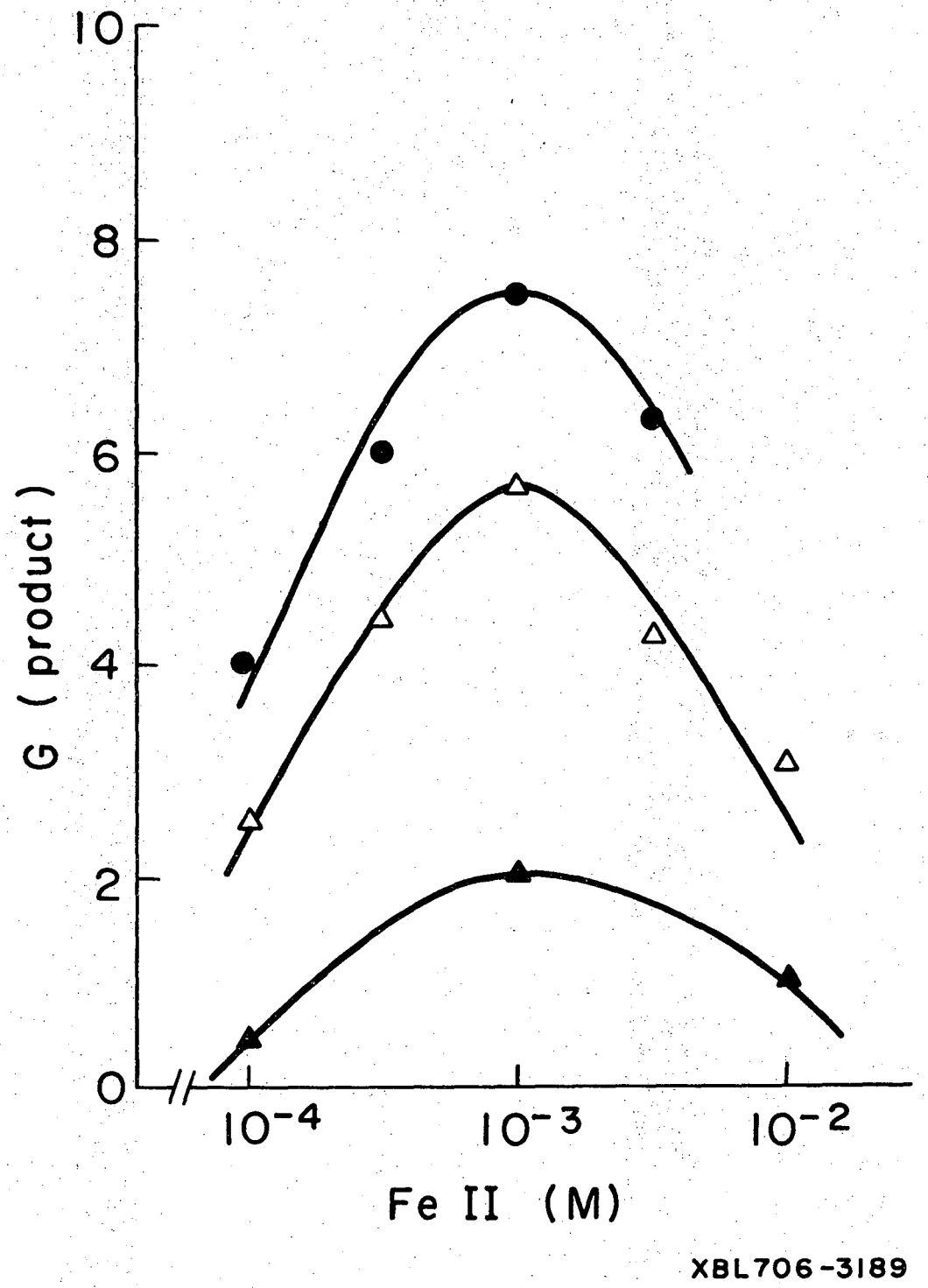

Figure 2

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