Title

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ABSTRACT

Studies of the reactions of irradiated pepsin solutions with 2, 4 - dinitrophenylhydrazine are described. The data indicate that formation of the >C=O bond is a principal chemical effect of the attack of hydroxyl radicals on pepsin in oxygenated solution. Dialysis and precipitation studies show (a) that the carbonyl products are of high molecular weight and (b) that more than one oxidation path is involved. Parallel studies of the action of Fenton's reagent on aqueous gelatin indicate that similar oxidation processes occur.

Preliminary investigations of irradiated tissue homogenates suggest that carbonyl formation may represent a major process in the radiation chemistry of biological systems. It is pointed out that oxidation of substances other than protein may be involved.

FORMATION OF >C=O BONDS IN THE RADIATION-INDUCED OXIDATION OF PROTEIN IN AQUEOUS SYSTEMS*

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A recent series of observations indicate that formation of the >C = 0 bond is a principal chemical effect of the attack of hydroxyl radicals on protein in oxygenated solution. Irradiations were made in a neutron flux produced by bombardment of beryllium with 24-Mev deuterons in the Crocker Laboratory 60-inch cyclotron. Solutions were exposed under one atmosphere of oxygen in sealed Pyrex cells mounted in a motor-driven reel situated 15 cm from the beryllium target. One of the cells in each exposure contained a formic acid–oxygen dosimeter.[1, 2]

The following procedures were found applicable to the study of carbonyl formation in aqueous pepsin[3] solutions (6.0 mg/ml, 0.5 M in Na_2SO_4, 10^{-3} M in H_2SO_4).[4, 2] Immediately after irradiation of the solution, we added platinum black to destroy hydrogen peroxide. The solution was then heated and two fractions, a precipitate (I) and a supernatant (II), were obtained. Each was treated with 2, 4-dinitrophenylhydrazine–hydrochloric acid solution[5] and dialyzed to remove excess reagent and any products of low molecular weight. An appropriate aliquot of the dialyzed protein was then added to

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*This work was performed under the auspices of the U. S. Atomic Energy Commission.

[1] E. J. Hart, Radiation Research 1, 53 (1954).

[2] We employed the formic acid-oxygen dosimeter to obtain an estimate of the relative number of water molecules decomposed via (1) $H_2O = H + OH$ and (2) $H_2O = 1/2(H_2) + 1/2(H_2O_2)$. Absolute yield values could then be calculated in terms of G (molecules/100 ev) because, in dilute solutions at pH values above 2.5, the sum $G(1) + G(2) = G(H_2O)$ is to a first approximation independent of the quality of the absorbed radiation [Ref. 1]. With our standard geometry for neutron exposures, we obtained G(1) = 2.68, G(2) = 0.97 on the basis of $G(H_2O) = 3.65$ (Garrison, Weeks, Ward, and Bennett, J. Chem. Phys., in press.)

[3] Pepsin crystallized (2x), Lot No. 9477, Nutritional Biochemicals Corporation, Cleveland, Ohio.

[4] See Northrop, Kunitz, and Herriott, Crystalline Enzymes, (Columbia University Press, New York, N. Y., 1948), p. 28.

[5] H. A. Iddles and C. E. Jackson, Ind. Eng. Chem., Anal. Ed., 6, 454 (1934).

methanol-potassium hydroxide solution for spectrophotometric analysis after the method of Lappin and Clark.[6] These authors have shown for monocarbonyl 2, 4-dinitrophenylhydrazones that the position of the absorption maximum, as well as the value E_{max}, is nearly independent of the structure of the carbonyl compound. We found that the absorption spectrum of fraction I ($E_{max} \simeq 480$ mμ) corresponded to the spectrum of authentic monocarbonyl hydrazones and, by using the formaldehyde derivative as a standard, obtained the value $G \simeq 1.2$ for monocarbonyl production at a dose equivalent to 2.5×10^{18} ev/ml.[3] Fraction II showed a blue color and absorption spectrum ($E_{max} = 550$ mμ) that can be attributed to 1, 2-dicarbonylhydrazones. Using an authenic glyoxal derivative as a standard, we obtained $G \simeq 0.02$ for dicarbonyl production. The pepsin in unirradiated control solutions showed no retention of 2, 4-dinitrophenylhydrazine.

Independent evidence for formation of carbonyl groups through processes induced by reaction of hydroxyl radicals with protein has been obtained in a parallel study of the oxidation of gelatin[7] (40 mg/ml in air-saturated solution) by Fenton's reagent, in which case hydroxyl radicals are formed by the reaction $Fe^{+2} + H_2O_2 = Fe^{+3} + OH^- + OH$.[8] We have found in this system (a) that the dialyzed protein contains approximately one carbonyl group for every two hydroxyl radicals formed and (b) that glyoxylic, pyruvic, and α-ketoglutaric acids are among the carbonyl compounds liberated on hydrolysis of the dialyzed fraction with 2 N hydrochloric acid solution under oxygen-free conditions. Identification of the keto acids[9] is based on the observation that the 2, 4-dinitrophenylhydrazone derivatives could not be distinguished chromatographically[10] from the corresponding authentic material.

The possible biological importance of carbonyl formation is indicated by the observation that the addition of 2, 4-dinitrophenylhydrazine to irradiated (4×10^{17} ev/ml) rat liver homogenate (50 mg/ml in water)[11] gave a

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[6] G. R. Lappin and L. C. Clark, Anal. Chem., 23, 541 (1951).

[7] Gelatin (purified pigskin) Lot No. 60-6657, Eastman Organic Chemicals.

[8] F. Haber, and J. Weiss, Proc. Roy. Soc. (London) A147, 332 (1934).

[9] Formation of these products is consistent with a previously suggested mechanism that involves the intermediate formation of imino linkage: $RCONHCR_2 \longrightarrow RCON=CR_2 \xrightarrow{H_2O} RCONH_2 + R_2CO$ [M. E. Jayko and W. M. Garrison, J. Chem. Phys., 25, 1084 (1956)]. We have recently found that irradiated solid pepsin on dissolution in water also yields high-molecular-weight products containing carbonyl groups and suggest that imino intermediates are also involved in direct action: $RCONHCR_2 \longrightarrow RCON=CR_2 + H_2$.

[10] Cavallini, Fronlali, and Toschi, Nature 163, 568 (1949).

[11] V. R. Potter and C. A. Elvehjem, J. Biol. Chem., 114, 495 (1936).

non-dialyzable hydrazone fraction that exhibited an intense magenta color on mixing with methanol–potassium hydroxide.[12] The absorption spectrum indicated that monocarbonyls were also present. The corresponding control tests with unirradiated homogenate gave negative results. The literature indicates that radiation-induced oxidation of a number of different types of chemical linkages may contribute to carbonyl production in a biological system.[13]

A detailed report of this work will appear in a forthcoming paper.

[12] We estimate that this technique, with slight modification, could be used to measure an x-ray dose of a few hundred roentgen.

[13] See (a) E. Collinson and A. J. Swallow, Chem. Rev. 56, 471 (1956) and (b) W. M. Garrison, Ann. Rev. Phys. Chem. 8, (1957).