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Publication Date
1958-03-01

UCRL-8195

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BERKELEY, CALIFORNIA

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UCRL-8195

MECHANISM IN THE RADIOLYSIS OF AQUEOUS PROTEIN SOLUTIONS

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The indirect action of radiation on protein in aqueous solution could presumably involve any of the various bonds and linkages which comprise the structure of the macromolecule. However, not many purely chemical changes in irradiated protein systems have been established. There is good evidence that sulfhydryl groups can be oxidized,[1] and there are results which indicate that the tyrosine, tryptophane, and other aromatic residues are radiosensitive.[2,3] Ammonia has been shown to be liberated,[4] and organic peroxides have been detected.[5]

According to accepted theory of water radiolysis, the indirect action of radiation on solutes is initiated by H and/or OH in oxygen-free solutions and by HO_2 and/or OH in oxygen-saturated solutions. Kinetic studies of chemical change in solutions of the simpler organic compounds are generally consistent with the concept that reaction of these intermediates with solute produces organic free radicals which in secondary reactions yield observed products. Detailed free-radical mechanisms have been proposed for a number of the simpler organic solutes containing chemical bonds and linkages common to protein.[6] As an extension of work of this type now in progress in this Laboratory, we have initiated studies to obtain information on specific loci of indirect action on protein. Preliminary findings are reported in the present paper.

OXYGENATED SOLUTIONS

Oxidation of organic compounds generally involves formation of the >C=O function. Radiation-induced oxidation of organic solutes in oxygenated aqueous solution is no exception. Aldehyde and keto derivatives, although not necessarily the main products, are formed in reactions initiated by OH attack at (for example) methyl, hydroxy, methylene, phenyl, alkyl phosphate, and amine groups.[6] Oxidation at the $-CH_2-NH_2$ linkage can be interpreted most simply in terms of the intermediate formation and subsequent hydrolysis of the imino derivative -CH=NH. We recently found that the $-CH_2-NH-$ bond in secondary amines undergoes an analogous reaction to give primary amine and aldehyde. This radiation-induced rupture of the N-C bond was suggested as a possible path for cleavage of the peptide chain.[7] Now, since sensitive

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analytical methods are available for study of carbonyl compounds in amounts as small as 10^{-7} mole, we concluded that study of >C=O formation in irradiated protein solution could provide detailed information on the locus of OH attack on both main and side-chain bonds.[8]

Information on the indirect action of Co^{60} γ-rays on pepsin and gelatin has been obtained. Irradiations were made at a dose rate of ~ 2 x 10^{17} ev/ml, min. The solutions (3ml) were exposed under one atmosphere of oxygen in sealed pyrex tubes mounted in a motor-driven reel, situated a standard distance from the source.

Total Carbonyl Production

Immediately after irradiation, the protein solution (5 mg/ml) was treated with platinum black to destroy hydrogen peroxide and then treated with 2,4-dinitrophenylhydrazine-hydrochloric acid solution to form the hydrazone derivatives.[9] (Control runs established that protein oxidation is not induced by the addition of platinum black to these systems). The solution was dialyzed to remove excess reagent and possible reaction products of low molecular weight. An appropriate aliquot of the dialyzed protein was then added to methanol-potassium hydroxide solution for spectrophotometric analysis after the method of Lappin and Clark.[10] A typical absorption curve (filled circles) for irradiated pepsin solution treated in this manner is shown in Fig. 1. Similar curves were obtained with gelatin. The absorption spectrum of a representative monocarbonyl derivative (acetaldehyde 2,4-dinitrophenylhydrazone) is shown for purposes of comparison. The upper curve (open circles) shows the absorption spectrum of 2,4-dinitrophenylhydrazine reagent under identical conditions. The absorption curve of irradiated pepsin treated with 2,4-dinitrophenylhydrazine is seen to be characteristic of the >C=N-NH-$C_6H_4(NO_2)_2$ chromophore. Lappin and Clark[10] 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. If the acetaldehyde derivative is used as a standard, an initial yield of 1.2 carbonyl groups is obtained per 100 ev absorbed in the pepsin solution. Pepsin in unirradiated controls showed negligible retention of 2,4-dinitrophenylhydrazine.

The effects of a number of different variables on the carbonyl yield in irradiated pepsin solution are summarized in Table I. Included are data on the effects of (a) dose (b) oxygen concentration (c) pH (d) added solutes including chloride ion, sulfate ion, and a typical protective agent for indirect action viz the sulfhydryl derivative cysteine.

Locus of Carbonyl Production

Preliminary information on one of the processes of carbonyl formation has been obtained. The evidence is derived from studies of the carbonyl products found to be liberated on hydrolysis of the irradiated (and dialyzed) protein. The irradiated protein solutions were hydrolyzed in 4 N HCl (in vacuo) for 24 hours prior to treatment with 2,4-dinitrophenylhydrazine reagent. The hydrazone derivatives were extracted with chloroform and examined chromatographically.[11] It was found that the irradiated samples yield a series of α-keto acids. It is of interest to note that control runs with unirradiated pepsin showed the presence of measurable amounts of pyruvic acid. This apparently arises from the decomposition of serine during acid hydrolysis[12] but was not observed in unirradiated gelatin hydrolysates, presumably because of the lower serine content of the latter. Most of the keto

acid identification studies were therefore made on the irradiated gelatin system. The following keto acid products have been identified: oxaloacetic, α-ketoglutaric, glyoxylic, pyruvic, and phenylpyruvic. Identification was based on the fact that the 2,4-dinitrophenylhydrazone derivatives could not be distinguished chromatographically from the corresponding authentic material.

Semiquantitative data indicate that although the α-keto acid fraction does not represent the total carbonyl production, it nevertheless appears that these products represent a major chemical effect of the indirect action of radiation on gelatin and pepsin in oxygenated aqueous solution. This result is interpreted as substantiating the earlier suggestion[7] that the peptide link may be involved in the radiolysis of aqueous protein, and if so, would give rise to terminal carbonyl functions. This proposal was based on the observation[7] that the indirect action of ionizing radiation on diethylamine in oxygenated aqueous solution gives rise to ethylamine and acetaldehyde as principal products. The mechanism of this reaction is written

$$H_2O \longrightarrow H,\ OH,\ H_2,\ H_2O_2$$

$$H + O_2 \longrightarrow HO_2 \qquad (1)$$

$$OH + RNHCH_2R' \longrightarrow RNHCHR' + H_2O \qquad (2)$$

$$O_2 + RNHCHR' \longrightarrow RN{=}CHR' + HO_2 \qquad (3)$$

$$H_2O + RN{=}CHR' \longrightarrow RNH_2 + R'CHO, \qquad (4)$$

$$2\ HO_2 \longrightarrow H_2O_2 + O_2, \qquad (5)$$

where RN=CHR represents an imino intermediate which is rapidly removed through the hydrolytic reaction.[4] The corresponding reaction at the peptide chain would give rise to the imino derivative ~~CO-N=C(R)-CO~~ which on hydrolysis would yield the keto acid RCOCOOH.

The radiation-induced oxidation of other chemical groups and bonds in pepsin and gelatin also contribute to the over-all carbonyl production. Evidence has been obtained which indicates that this fraction is more complex and less susceptible to analytical investigation, at least with the identification methods now at hand.

OXYGEN-FREE SOLUTIONS

The work of the preceding section represents a specific case in the general use of radical scavengers to obtain information on labile intermediates derived from protein molecules. Oxygen, of course, is of particular interest in view of the radiation biological inference. However, other second solutes could conceivably provide as much or more information on the intermediate processes. Moreover, it is not necessary that the second solute itself react with the protein radicals. For example, consider the radiation chemistry of oxygen-free solution containing protein molecules RH and a simple organic solute R'H. Reaction of H and/or OH with solute would yield the intermediates R and R'. Regardless of other considerations, there is a certain probability that combination reaction R + R' = RR' ensues. We have investigated

the radiation chemistry of the system in which RH = pepsin, and $\acute{R}H$ = $CH_3COOH(C^{14}H_3COOH)$. The latter has been shown to yield the intermediate CH_2COOH on H and OH attack.[13] Reaction of $C^{14}H_2COOH$ with pepsin radicals R could yield C^{14}-labelled protein derivatives $R\text{-}C^{14}H_2COOH$. Conventional hydrolysis of this modified protein would yield simple C^{14}-labelled organic compounds. Identification of these would, in turn, provide information on the nature of the free-radical intermediates derived from protein.

Oxygen-free solutions containing acetic acid and pepsin, approximately 10^{-2} M and 10^{-5} M, respectively, were irradiated with 40-Mev helium ions from the Crocker Laboratory 60-inch cyclotron using previously described procedures.[14] The irradiated solutions were distilled to dryness *in vacuo* to recover C^{14}-labelled acetic acid. The residue was re-dissolved in 1 N hydrochloric acid and dialyzed for 3 hours in cellophane against running water in the cold. (Using our dialysis procedure, we find that C^{14} activity in the irradiated protein solution decreases rapidly to a limiting value which does not change appreciably after about 2 hours). Retention was essentially proportional to dose over the range 2×10^{18} - 2×10^{19} ev/ml. There was negligible retention of C^{14}-activity in a duplicate manipulation of an unirradiated pepsin-$C^{14}H_3COOH$ target solution. The dialyzed material was made 4 N in hydrochloric acid, evacuated, and hydrolyzed on the steam bath for 24-36 hours. The hydrolysate was distilled to dryness *in vacuo*, and chromatographed on a Dowex-50 ion exchange column using a modification[15] of the method developed by Stein and Moore[16] for separation of amino acids in protein hydrolysates. The elution curve showed a series of distinct C^{14} activity peaks. A principal one of these (peak A fig.2), rechromatographed with added amino acid carriers, showed an exact correspondence with authentic aspartic acid. The co-chromatogram is shown in Fig. 3.

The simplest explanation for the formation of C^{14}-labelled aspartic acid under the conditions of this study is that $C^{14}H_2COOH$ radicals combine with protein radicals containing the -NH-CH-CO-configuration. That is, aspartic acid is derived through substitution of CH_2COOH for H in the glycine units of the peptide chain. Of course, it might be argued that glycine molecules were introduced into the target solution through protein hydrolysis, and that free aspartic acid formed by combination of $NH_2CHCOOH$ and CH_2COOH radicals was not adequately removed in the dialysis step. However, results of a series of control dialysis experiments with protein-CH_3COOH solutions containing added authentic C^{14}-labelled aspartic acid were found to negate this possibility.

These studies on oxygen-free pepsin-$C^{14}H_3COOH$ solutions lead to a conclusion similar to that arrived at on the basis of the carbonyl measurements in oxygenated solution. That is, the peptide link is involved as a locus of indirect action, and the various processes can be subjected to detailed inquiry by use of the techniques described. It is anticipated that study of the remaining unidentified products will provide further insight into the mechanism of radiolysis in aqueous protein systems.

ACKNOWLEDGMENTS

The authors wish to thank the staff of the Crocker Laboratory 60-inch Cyclotron for their assistance, and Mrs. Grace Walpole for help in the preparation of this manuscript. The cobalt source was made available through the courtesy of the Bio-Organic Chemistry Group of the University of California Radiation Laboratory.

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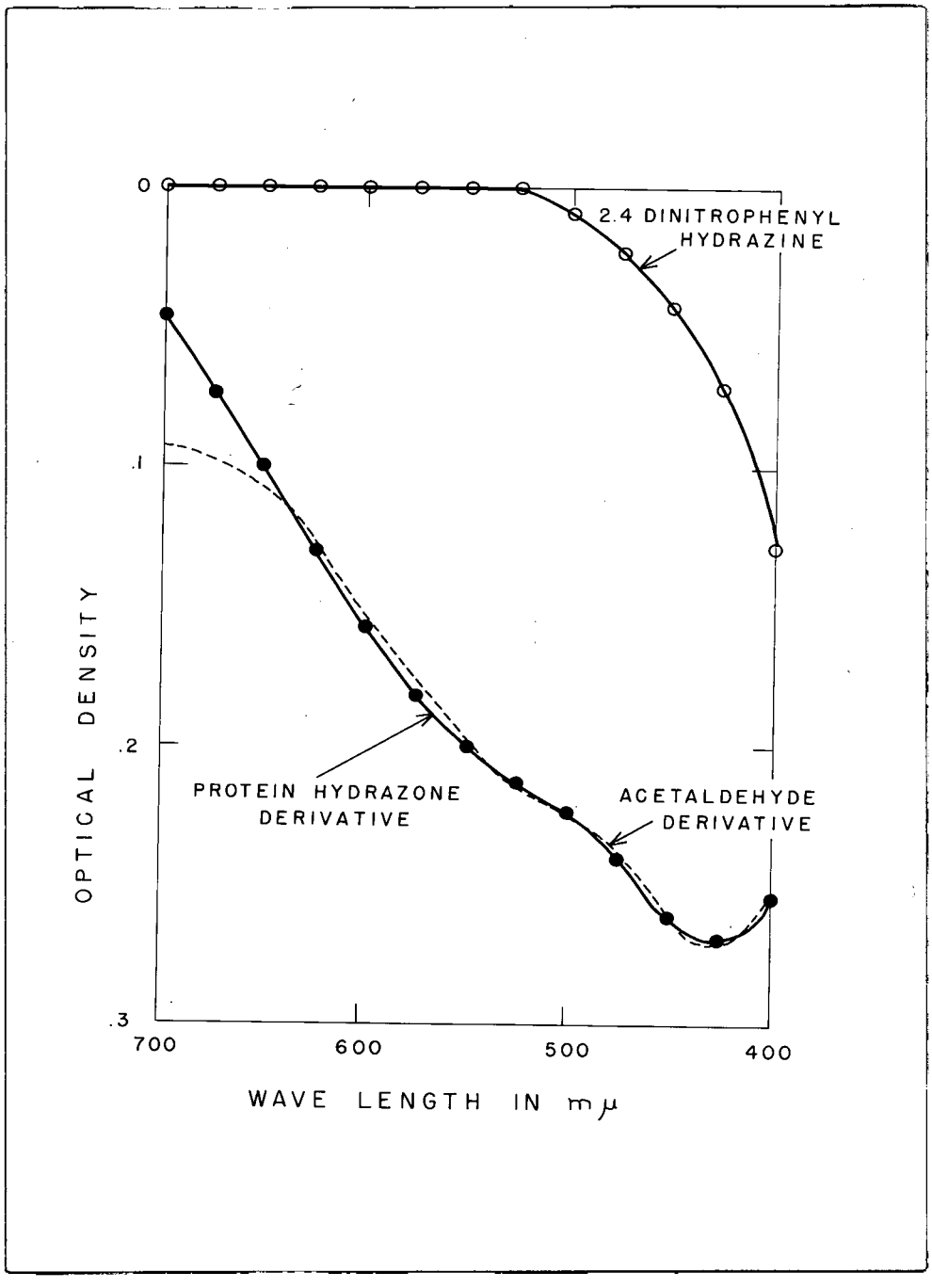

Figure 1. Absorption spectra of hydrazone derivatives

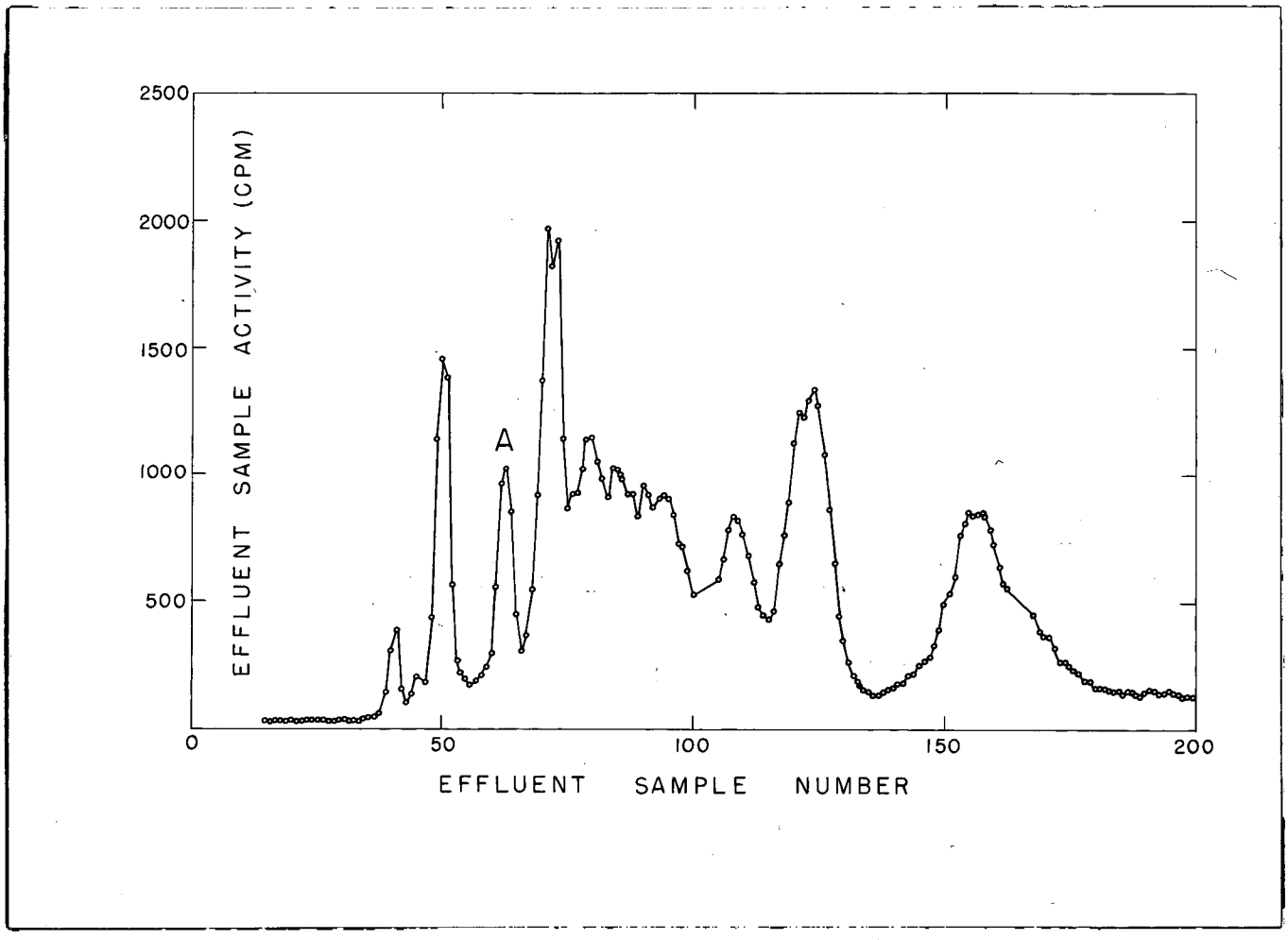

Figure 2. Typical elution curve for hydrolyzed pepsin - $C^{14}H_2COOH$ derivative.

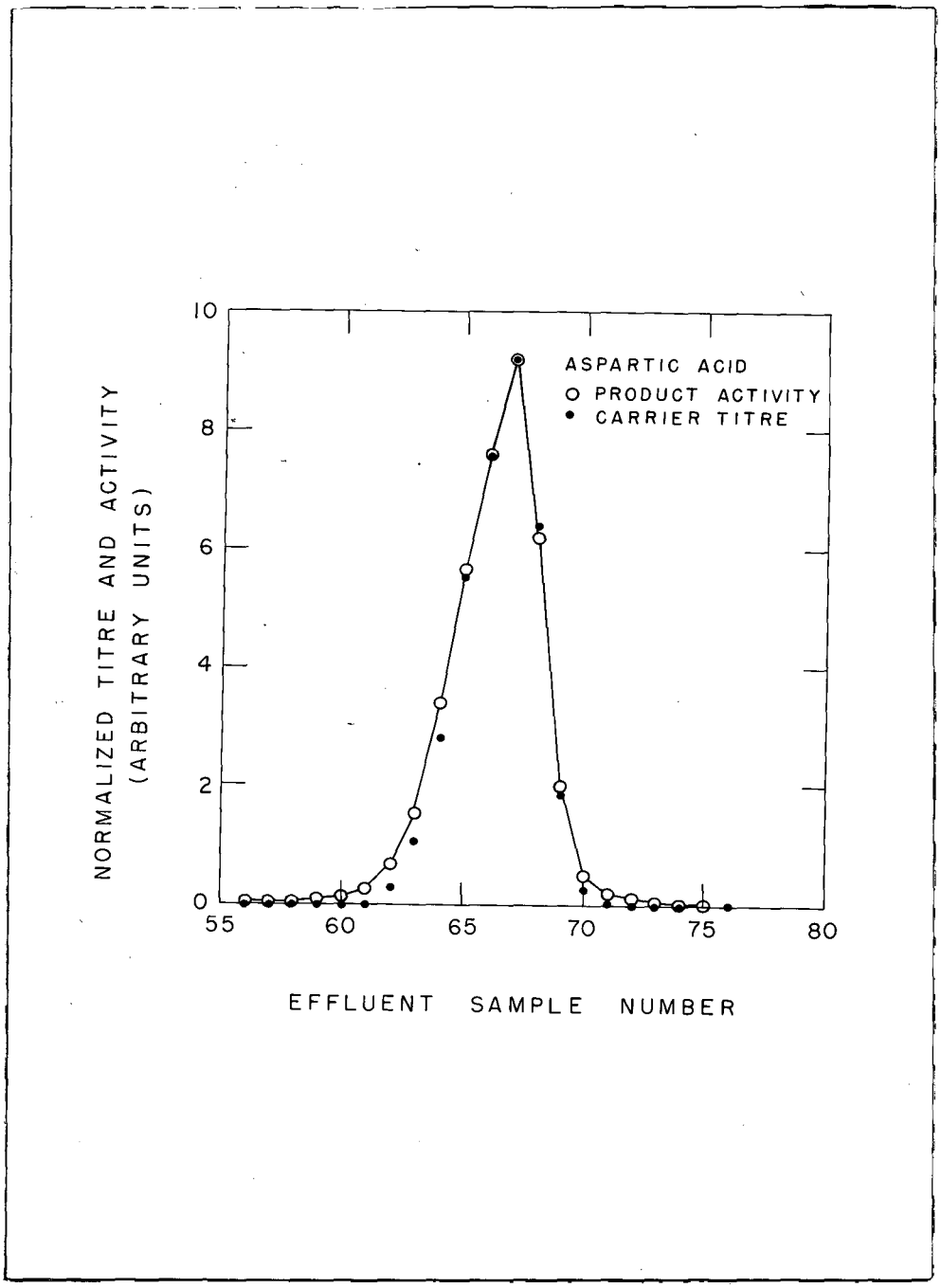

Figure 3. Co-elution curve of authentic aspartic acid with product A (Fig.2).

Table I

Carbonyl production in the Co^{60} γ-ray radiolysis of aqueous pepsin solution. Pepsin concentration = 5 mg/ml. irradiated volume = 3 ml.

Solution	Dose (ev/ml)	Optical density of product hydrazone solution at 430 mμ[a]
O_2 sat., pH 5	1.35×10^{18}	0.068
O_2 sat., pH 5	4.05×10^{18}	0.197
O_2 sat., pH 5	8.10×10^{18}	0.296
O_2 sat., pH 5	8.10×10^{18}	0.311
O_2 sat., pH 5	13.50×10^{18}	0.485
evac pH 5	8.10×10^{18}	0.108
evac pH 5	8.10×10^{18}	0.116
O_2 sat., pH 5, 4.8×10^{-5} M cysteine	8.10×10^{18}	0.287
O_2 sat., pH 5, 4.8×10^{-4} M cysteine	8.10×10^{18}	0.224
O_2 sat., pH 5, 4.8×10^{-3} M cysteine	8.10×10^{18}	0.101
O_2 sat., pH 5, 0.1 M NaCl	8.10×10^{18}	0.288
O_2 sat., pH 5, 0.1 M Na_2SO_4	8.10×10^{18}	0.300
O_2 sat., pH 1.3	8.10×10^{18}	0.292
O_2 sat., pH 3.6	8.10×10^{18}	0.269
O_2 sat., pH 5.2	8.10×10^{18}	0.301
O_2 sat., pH 6.4	8.10×10^{18}	0.312
O_2 sat., pH 7.5	8.10×10^{18}	0.342
O_2 sat., pH 9.4	8.10×10^{18}	0.526

[a] As obtained by using the analytical procedure of Lappin and Clark (Ref. 6). Irradiated volumes and dilution factors were uniform throughout the several series of runs.