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RADIATION-INDUCED OXIDATION OF PROTEIN IN AQUEOUS SOLUTION

Warren M. Garrison, Michael E. Jayko, and Winifred Bennett

August 1961

1. INTRODUCTION

1.1 General

Development of a comprehensive radiation chemistry of aqueous protein systems has been limited by a number of experimental factors. One of the major problems arises from the fact that the complex chemical composition of protein offers a multiplicity of loci for parallel reaction. The magnitude of the analytical problems involved in characterizing these reactions is indicated most directly by studies of the amino acid composition of hydrolyzates prepared from irradiated protein solutions. Although the aromatic and heterocyclic constituents and, in particular, the sulfur moieties appear to be the most susceptible to indirect action, the evidence is that the over-all reaction of protein involves a diversity of amino acid residues even at the lowest irradiation levels. (1-3) In fact, quite recent studies of the radiation-induced inactivation of enzymes containing a maximal number of free -SH groups show that an appreciable fraction of the absorbed energy is not accounted for simply in terms of the destruction of sulfhydryl function (4,5). While such studies of the loss of components and functions of protein structure have provided empirical estimates of the "radiation sensitivity" of amino acid residues, still, most concepts regarding the nature of the products and the mechanism of the reactions involved are largely based on various types of indirect evidence (6).

The present paper describes a direct approach to the study and measurement of radiation-induced modifications in the co-valent structure of protein. The reactions to be considered are largely those of the N-C bond which, in its various chemical forms, represents the single most ^{CHARACTERISTIC} recurrent configuration of protein structure.

1.2 Development of the Present Approach

Of the various types of compounds containing the N-C linkage, certainly the

α -amino acids have received the greatest attention in radiation chemistry. With the exception of cysteine, all amino acids of the type $\text{NH}_2\text{CH}(\text{R})\text{COOH}$ undergo oxidative deamination in aqueous solution to yield ammonia, α -keto acid, and hydrogen peroxide (6). Primary aliphatic amines undergo analogous reactions with formation of ammonia and the corresponding aldehyde or ketone. The net reaction for cleavage of the N-C linkage of primary amino compounds is represented to a first approximation by the equation:



On the basis of a detailed study of mechanism in the radiolysis of the α -amino acids (7), we were prompted several years ago to investigate the radiolytic oxidation of certain of the simpler secondary amines. We found in the case of diethylamine, for example, that a principle over-all reaction in oxygenated solution yields ethylamine and acetaldehyde (8):



and subsequent studies of various N-alkyl amino acids have established that the above reaction is generally characteristic of this class of compounds. For example, sarcosine (N-methyl glycine) and proline (pyrrolidine-2-carboxylic acid), both undergo degradation as represented by equation 2. Such reaction is chemically quite analogous to the radiation-induced deamination of primary amines, as given by equation 1 and, as will be discussed, presumably involves similar intermediate processes.

Now, in our initial communication (8) on radiation-induced degradation of secondary amines via reaction 2, we also suggested that a corresponding reaction viz



could be of importance in the radiolysis of aqueous peptide systems notwithstanding earlier reports that various N-substituted amines had been found to be

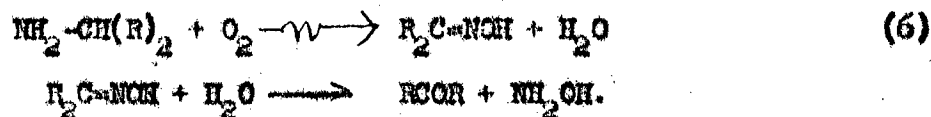
relatively inert to the action of ionizing radiation in aqueous solution. The latter conclusion was based primarily on the finding that ammonia production in oxygenated solutions of such compounds represents only a fraction of the ammonia yield obtained from free α -amino acids under identical conditions (9). However, in terms of equations 1-3, measurements of "free" ammonia would not provide a basis for estimating radiation-chemical reaction at the peptide linkage, for example, unless the "amide" products had been quantitatively hydrolyzed prior to analysis. We have investigated the effect of post-irradiation hydrolysis on a variety of simple peptide systems, and find that the total yield of liberated ammonia does in fact approach that obtained from the free α -amino acid under the same experimental conditions (10).

By analogy with reactions 1-3, we consider it likely that the radiation-induced decomposition of tertiary amines and quaternary nitrogen compounds involves reaction of type 4,5 respectively



The carbonyl product, CH_3CHO , from triethyl amine has been observed (11), but the nature of the products formed in the decomposition of choline derivatives in oxygenated solution (12) has not been determined.

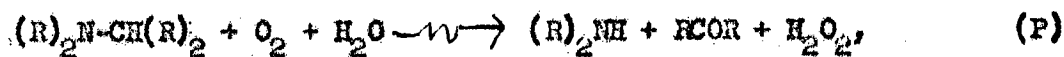
Finally, reference should be made to the fact that at pH values above 7, a competing path for degradation of the N-C bond involves formation of an oxime intermediate. For primary amines, such reaction may be represented by the stoichiometry.



Yields of "total hydroxylamine" (hydroxylamine + oxime) from primary and

secondary amines increase abruptly with pH over the range 8 to 10 and then level off above pH 10 to 11; however, the maximum yield of reaction 6 is relatively low even at the highest pH values (11).

The basis of the present approach to the elucidation of mechanism in the radiolysis of oxygenated solutions of protein is that reaction at the loci of N-C linkages may be generalized in terms of the over-all equation:



where $(R)_2N-CH(R)_2$ represents any of the variously substituted primary, secondary, and tertiary amino configurations of protein molecular structures. Note that regardless of the type or extent of substitution, a reactive carbonyl function is obtained as one of the products of reaction P as written. The chemical form of the nitrogen-containing product is specified, however, by the nature of the N-C linkage. As will be shown in subsequent sections of this paper, reactions of type P, together with other closely related reactions, account for a principal fraction of the energy absorbed in solutions of certain proteins. Preliminary chemical evidence of the validity of this general approach to the radiation-chemical study of aqueous protein systems has been reported on briefly from time to time (8,13-15); the present paper amplifies and extends these observations and offers the detailed experimental procedures.

2. EXPERIMENTAL PART

2.1 Materials and Irradiation Conditions

Solutions of pepsin (Worthington Biochemical Corp., twice recrystallized) were prepared by adding solid in successive small amounts to distilled water that had been adjusted to pH 5 or below with H_2SO_4 ; a magnetic stirrer provided adequate mixing without causing undue foaming. In certain experiments the pepsin solutions were dialyzed in cellophane against acidified water (pH 4 to 5). Aliquots of the stock solutions were adjusted to a particular pH value by addition of H_2SO_4 or NaOH immediately prior to irradiation (16).

The gelatin used in these studies (Eastman, lime processed) was selected because of its low amide and carbonyl content. The stock solutions (approximately 5%) were routinely dialyzed against running water at 30°C for 18 to 24 hours to remove split products and traces of free ammonia; final concentration was determined by drying an aliquot to constant weight at 100°C. The poly-dl-glutamic acid (Pilot chemicals) was treated in a similar manner. Solutions of yeast alcohol dehydrogenase, β -lactoglobulin, and α -chymotrypsin (Nutritional Biochemical Corp.) were prepared by adding the materials as received directly to triply distilled water immediately prior to irradiation.

Reagent 2,4-dinitrophenylhydrazine (Eastman Organic Chemicals) was used at a concentration of 1 mg/ml in 2 N hydrochloric acid. Irradiations were made with γ -rays from either a 2000 curie or a 100 curie source. Dose-rates for the geometries used were 2.6×10^{16} ev/ml/min and 7.2×10^{16} ev/ml/min respectively.² The solutions (10 ml) were exposed under oxygen (5 ml) at 1 atmosphere in sealed Pyrex tubes. Contents were mixed at intervals to prevent depletion of oxygen in the solution during exposure.

2.2 Total Carbonyl Production

The 2,4-dinitrophenylhydrazones of most carbonyl compounds show essentially the same absorption spectrum in dilute sodium hydroxide and have very similar extinction coefficients (ϵ) at the wave length of maximum absorption (λ_{\max}). Exceptions include the 1,2-dicarbonyls and certain other forms containing α - β unsaturation (17). The fact that the spectral properties of the chromophore $(R)_2C=N-NH-C_6H_4(NO_2)_2$ in alkaline solution are essentially independent of the nature of R (with the exceptions noted) has been variously applied in the elucidation of structure of organic compounds and, in the present instance, provides the basis for a sensitive and direct measurement of total carbonyl production in irradiated protein systems.

2.2.1 Formation of Protein Phenylhydrazones: Phenylhydrazone derivatives of the high-molecular-weight products were formed by treating the irradiated protein solution with 2,4-dinitrophenylhydrazone-hydrochloric acid solution after removal of hydrogen peroxide as described in Section 2.3. The protein phenylhydrazones could be quantitatively determined in amounts as small as 10^{-8} mole after removal of excess reagent (2,4-DNPH) by solvent extraction or by dialysis.

In the extraction procedure, the protein solutions were made approximately 0.1 N in hydrochloric acid and treated with 2,4-DNPH to give a two-fold to five-fold excess of reagent over the estimated carbonyl concentration (A dose of 1×10^{18} ev/ml, for example, gives a carbonyl concentration of 1.6×10^{-5} M for a G(>C=O) of 1). The solutions were allowed to stand for 3 to 4 hours at room temperature to insure complete reaction, and were then extracted with a series of equal volumes of diethyl ether. Some 4 to 6 extractions were required to remove all unreacted 2,4-DNPH. In the dialysis procedure the initial hydrochloric acid concentration was increased to 1 N to insure that the solutions were acidic as long as unreacted 2,4-DNPH was present. The solutions were dialyzed in cellophane against running water at 30°C for a period of 15 to 18 hours. It was found that if the initial solutions were made up to contain a five to ten-fold excess of 2,4-DNPH, no pre-standing prior to dialysis was required for complete reaction. Higher concentrations of 2,4-DNPH were avoided for reasons noted in Section 2.2.2. After removal of unreacted reagent by extraction or dialysis, an appropriate aliquot of the protein solution was made approximately 1.25 N in potassium hydroxide, and the absorption spectra of the protein phenylhydrazones were measured over the range 400 m μ to 650 m μ with a Beckman model DU spectrophotometer (17). Aliquots of the unirradiated solutions were always carried through the entire procedure in parallel with corresponding irradiated samples.

2.2.2 Dark-reaction of 2,4-dinitrophenylhydrazine with proteins: Of the proteins studied, pepsin, chymotrypsin, and β -lactoglobulin in unirradiated solution show negligible retention of 2,4-DNPH after dialysis or extraction as outlined in Section 2.2.1. Unirradiated gelatin solutions, particularly at the higher concentrations (i.e., above 0.1%) exhibit measurable readings at 450 m μ even after prolonged dialysis or exhaustive extraction of the gelatin-2,4-dinitrophenylhydrazine solutions. However, for a particular stock solution, the background correction is reproducible and introduced an uncertainty of less than 5%. Aliquots of the unirradiated solutions (in all cases) were carried through the described procedures in parallel with the irradiated samples, and calculations of carbonyl yields are based on the differential spectra.

All of the proteins studied undergo a slow "dark-reaction" with 2,4-DNPH if the reagent is present in large excess, or if the reaction mixture is allowed to stand for long periods (24 hours) prior to dialysis or extraction. Heating accelerates the reaction, and temperatures above 35°C were avoided. We have not inquired into the nature of the dark reaction, although oxidation of protein by 2,4-DNPH and(or) formation of hydrazone derivatives would seem to be likely processes. Figure 1 illustrates the effect of concentration on reactions of 2,4-DNPH with irradiated and unirradiated solutions of gelatin; these data were obtained through use of the extraction procedure.

2.2.3 Quantitative measurements: The absorption spectra of the "protein phenylhydrazone(s)" obtained from pepsin and gelatin are shown in Fig. 2. That these are predominantly unconjugated phenylhydrazones is evidenced by the shape of the absorption curve. The presence of appreciable amounts of the phenylhydrazones of conjugated carbonyls, particularly the 1,2-dicarbonyls, would be manifested by a pronounced shift in λ_{max} to longer wave lengths (17).

Most of our quantitative studies on the yield of total carbonyl [$C(>C=O)_t$]

involved the pepsin and gelatin systems and, unless otherwise noted, the values of $G(>C=O)_t$ herein reported were calculated, using 2.4×10^4 as the extinction coefficient of the $(R)_2C=N-NHC_6H_4(NO_2)_2$ configuration at 450 m μ in 1.25 N NaOH. This value represents an average of the molecular extinction coefficients reported for a wide variety of authentic monocarbonyl derivatives (17,18). Values of $G(>C=O)_t$ for several different proteins under a standard set of irradiation conditions are given in Table IA. In subsequent references to the effect of a particular experimental parameter on $G(>C=O)_t$ it may be assumed, unless otherwise stated, that all other variables conform to these standard reference conditions.

Values of $G(>C=O)_t$ were found to be essentially independent of dose at levels below 10^{20} ev/ml, provided the solvent extraction procedure was used. Pertinent data for gelatin are shown in Fig. 3. If dialysis was employed to remove excess 2,4-DNPH reagent, then the measured $G(>C=O)_t$ value for gelatin was found to decrease gradually with increasing dose above approximately 3×10^{19} ev/ml. Solutions of pepsin showed the same effect but at a somewhat higher dose. Presumably, after prolonged exposures of these solutions, the molecular weight distribution of the product fragments is such that a measurable fraction of the phenylhydrazone derivatives are removed in the dialysis step along with the excess reagent. Although the effect of protein concentration on $G(>C=O)_t$ was not studied in great detail, we did establish that (initial) carbonyl yields from pepsin and gelatin do not decrease appreciably with decreasing protein concentration until concentrations below 0.1% are reached. Carbonyl yields from gelatin are essentially constant over the range 0.1% to 3.0%.

Table IB shows the effects of various added solutes on carbonyl yields from pepsin. Neither Na_2SO_4 nor NaCl at concentrations as high as 0.1 M have any appreciable effect on carbonyl production. The absence of an attenuation by

chloride ion is of particular interest from the radiation biological standpoint. In regard to the effects of added cysteine, we observe that a molar concentration approximately ten times that of the protein is required to decrease $G(>C=O)_t$ by 50%. Large differences in the effect of pH on carbonyl yields from pepsin and gelatin solutions were found as shown in Table IC. It is to be noted that pepsin is stable in acid solution and inactivated rapidly at pH values above neutrality (16).

Reference should be made to the fact that the $G(>C=O)_t$ value of 0.5 reported for alcohol dehydrogenase (Table IA) is subject to considerable uncertainty. We consistently found with this particular system that the (differential) absorption spectrum of the product phenylhydrazones indicated appreciable amounts of 1,2-dicarbonyl or conjugated carbonyl derivatives (with unknown extinction coefficients). The nature of this phenomenon is not understood, but it seems likely that it is related to the finding that the (unirradiated) alcohol dehydrogenase contains some 4 to 5 unconjugated carbonyl groups as evidenced by reaction with 2,4-DNPH reaction.

2.3 Constituent Carbonyl Compounds

In the initial stages of this work various attempts were made to hydrolyze the protein-hydrazones directly to the constituent hydrazone derivatives but, unlike the classical case of the *N*-dinitrophenyl derivatives (19), the dinitrophenylhydrazones (as might be expected) undergo extensive decomposition and rearrangement under conditions required to bring about complete hydrolytic cleavage of protein. However, as will be described in a following paragraph, we found in a series of quantitative control studies that the constituent carbonyl product compounds are themselves relatively stable under the conditions of conventional acid hydrolysis. Accordingly, the constituent carbonyl compounds were first liberated by hydrolysis in 2-4 *N* HCl and then treated with 2,4-DNPH to obtain the individual hydrazone derivatives for subsequent

manipulation.

The irradiated solutions were dialyzed until hydrogen peroxide³ (in an aliquot) could not be detected with titanium sulfate reagent, made 4 N in HCl, and then hydrolyzed in vacuo at 95°C for a period of 18 to 24 hours. The hydrolyzate was neutralized with NaOH in a minimum volume and placed on a column of Dowex-50 (hydrogen form). This was then washed with water to effect a simple separation of two carbonyl-product fractions. Aldehydes, ketones, and carbonyl acids (fraction I) pass directly through the column with little or no retention, and were collected together for subsequent analysis. The nitrogen-containing carbonyls, e.g., aldehyde α -amino acids, amino α -keto acids, etc., (fraction II) are retained on the column along with the unchanged amino acids. Fraction I was treated with excess 2,4-DNPH and, after standing for 3 to 4 hours (to allow for complete reaction), hydrazone products plus unreacted reagent were quantitatively extracted with chloroform or ether. Separate aliquots of the extracted mixture were chromatographed on filter paper (Whatman No. 1) by use of the solvent systems (a) butanol saturated with 3% aqueous ammonia (b) heptane (pract.) saturated with methanol (20). The first method separates the carbonyl acid hydrazones; the second method separates the "neutral" hydrazones, i.e., those of formaldehyde, acetaldehyde, etc.

All the irradiated protein solutions examined in this way gave a complex mixture of α -keto acids as the principal component of fraction I. However, most of our detailed studies of α -keto acid products were confined to the gelatin system because of its low serine-threonine content. These β -hydroxy α -amino acids yield small amounts of pyruvic acid and α -ketobutyric acid respectively during conventional acid hydrolysis of protein (21) and, in the case of pepsin, the yields are such as to seriously interfere with the measurement of α -keto acids derived from radiation-chemical reactions.

The upper curve of Fig. 4 shows a densitometric tracing of a typical chromatogram of the α -keto acid phenylhydrazones isolated from an irradiated gelatin solution. The lower curve was obtained with an equal volume of the unirradiated control solution under identical conditions. These tracings are presented here primarily to illustrate the orders of magnitude of the principal peaks. The actual chromatograms, particularly those of the hydrazones isolated from irradiated solutions, are considerably more complex when scanned visually.

This complexity arises not only from the fact that fraction I contains a mixture of α -keto acid hydrazones, but also from the fact that each individual α -keto acid yields two isomeric (cis-trans) hydrazones with different R_f values. Although one form usually predominates, the relative yields of the two are influenced by temperature, solvent, concentration, time of standing, etc. (22). Therefore, identifications could not be based wholly on co-chromatographic studies of product and authentic hydrazone derivatives. As a correlative procedure, the α -keto acid hydrazones were eluted with methanol and then hydrogenated over PtO_2 under 40 lb. pressure for 16 hours to obtain the corresponding α -amino acids which were then re-chromatographed (23). Through obvious combinations of these two procedures, we succeeded in identifying the following acids among the radiation-chemical products of fraction I from gelatin: oxalacetic,⁴ α -ketoglutaric, glyoxylic, pyruvic,⁴ and phenylpyruvic. Although pyruvic acid (and α -ketobutyric) is observed in the control, it is readily apparent even qualitatively from Fig. 4 that the amount is considerably increased by irradiation. Incidentally, the yield of α -ketobutyric acid provides an internal control on the hydrolysis conditions since this particular carbonyl acid appears to be derived wholly from the hydrolytic degradation of threonine. Although G values for individual α -keto acids in the product mixture were not determined because of the complications introduced by cis-trans isomerism,⁵

the combined yield of α -keto acid function was measured by a modification of the spectrophotometric procedure outlined in Section 2.2. A modified procedure is required since the cis-isomers of the α -keto acid hydrazones, unlike the trans-isomers, do not show the characteristic hydrazone color-reaction in dilute (1.25 N) sodium hydroxide. However, it has been shown elsewhere (22) that hydrazones of the various α -keto acids do have very similar absorption spectra in 0.25 M NaHCO_3 with values of λ_{max} (380 $\text{m}\mu$) and E that are essentially independent of the relative concentrations of the cis-trans isomers.⁶ Application of this modified procedure to the α -keto acid hydrazones derived from gelatin gives $G(>\text{C}=\text{O})_{\alpha} = .4 G(>\text{C}=\text{O})_t$. This relationship was found to hold constant in the case of gelatin (1% solution) for dosages up to approximately 1×10^{20} ev/ml.

In the later stages of this work we found that α -ketoglutaric acid (unlike all other α -keto acids studied) gives but one hydrazone (trans-isomer) in the aforementioned extraction procedure. This finding, together with the fact that the hydrazone of α -ketoglutaric acid is well separated chromatographically from other product species enabled us to measure the effect of dosage and other variables on the yield of this particular carbonyl acid. As shown in Fig. 3, α -ketoglutaric acid production from gelatin (1% solution) is directly proportional to dose over the range studied. Similar results were obtained with β -lactoglobulin (0.1% solution); no preferential oxidation of the N-terminal amino acid leucine could be detected even at doses as low as 1×10^{18} ev/ml.

In the introductory paragraph of this section we stated that the identity of the α -keto acid products is retained during acid hydrolysis. Evidence for this is as follows: (a) pyruvic and α -ketobutyric acids are the only α -keto acids observed in hydrolyzates of unirradiated controls; (b) addition of any one

of the product acids to irradiated or unirradiated solutions just prior to hydrolysis does not result in any significant change in the spectrum of the other components of fraction I;⁷ (c) no evidence was found for the formation of α -keto acids by reactions of added formaldehyde or acetaldehyde; (d) an increase in the time of hydrolysis from 18 hours to 36 hours does not introduce a measurable change in $G(>C=O)_{\alpha}$.⁸ It should also be noted here that addition of various amino acids, glycine, glutamic acid, etc., to irradiated protein solution (1 mg amino acid/mg protein) does not result in any preferential change in the carbonyl product distribution.

2.4 Ammonia and Amide Groups

Study of nitrogen products was confined largely to the gelatin system. Lime processed gelatin (Section 2.1) was found to be particularly suitable, since most of the amide groups of the glutamine and asparagine residues of the parent collagen are removed through hydrolysis in the manufacturing process (24). Unmodified proteins generally contain amide groups in sufficient number to mask the radiation-chemical production of "amide" function at the lower irradiation dosages.

Analyses were made for free and "amide" ammonia, both before and after irradiation. An appropriate aliquot of each solution was made alkaline to phenolphthalein, chilled, and then distilled in vacuo into a receiver containing 1 ml of 0.1 N H_2SO_4 at the temperature of liquid nitrogen (control analysis established that amides are not hydrolyzed appreciably during this manipulation). The distillate was then isolated, thawed, and assayed for ammonia by means of the Nessler reaction. A second aliquot of each solution was made 1 N in HCl and heated for 90 minutes at 95°C to liberate ammonia from amide linkages (25); subsequent treatment was then as described for ammonia. Figure 5 gives typical hydrolysis data for control and irradiated solutions of gelatin, and Fig. 6 shows the dose-yield relationships for the

"amide" and free ammonia. The corresponding G values are included in Table II. The possibility that part of the "amide" fraction as measured in the above procedure arises from a Strecker degradation of N-C linkages during the hydrolysis was investigated in some detail since both peroxides and carbonyls under certain conditions are known to degrade amino acids with formation of ammonia. (26). However, it was found that the addition of hydrogen peroxide⁹ and α -keto acids in amounts equivalent to five times the respective product G values does not change the observed amide yield. Also, there is no effect of added free amino acids in large excess (1 mg amino acid/mg protein).

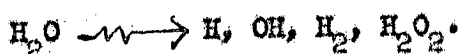
2.5 Peroxides

Hydrogen peroxide was determined by the titanium sulfate method; total peroxide ($H_2O_2 + ROOH$) was measured iodometrically. The detailed procedures employed were those recently given in a series of reports on the production of organic peroxides in the radiolysis of aqueous solutions of amino acids, peptides, and related compounds (27). In the present application some difficulty was encountered in the use of the iodide method for measurement of total peroxide in irradiated gelatin solutions. Whereas most of the systems studied in reference 27 give reasonably stable solutions of I_3^- on addition of KI in excess, we found in the case of gelatin that absorption of I_3^- at 350 m μ decreased on standing with a half-time of approximately 30 minutes. However, since the reaction of I^- with peroxides in these systems is rapid compared to the fading reaction, extrapolation could be employed to obtain the initial I_3^- concentrations. Peroxide data are included in Table II.

3. GENERAL CONSIDERATIONS

3.1 Elementary Processes

The over-all chemical change produced in water by absorption of high-energy radiation is represented here by



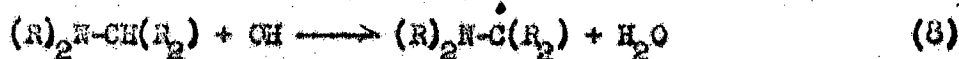
(w)

We assume for γ -rays the 100-ev yields as given by the approximations

$G(H) \approx G(OH) \approx 3$, $G(H_2) \approx G(H_2O_2) \approx 0.5$ (7). Recent proposals that the solvated

electron $e_{H_2O}^-$ may represent an alternate form of the H atom (28,29) ^{are} is not considered in any detail here, since the systems under consideration both $e_{H_2O}^-$ and H react preferentially with O_2 to yield the equilibrium system $H^+ +$

$O_2^- \rightleftharpoons HO_2$. The generalized cleavage reaction as represented by equation 1 may be interpreted in terms of the primary step (W) followed by the intermediate processes:



It is to be noted that step 9 as written gives only a stoichiometric representative of the reaction of O_2 with $(R)_2N-\dot{C}(R_2)$; the nature of the intermediates likely to be involved in this step is considered in a following discussion on the role of organic peroxides.

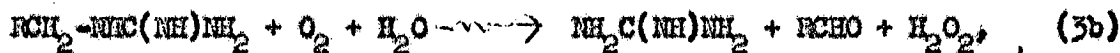
3.2 Loop of Reaction

Of the data given in the experimental part, certainly the most direct evidence for major involvement of reaction 1 in the radiolysis of oxygenated protein solutions are the results obtained on ammonia and amide production as summarized in Table II. The fact that $G(NH_3)_t^{10} = 1.25$ indicates on the basis of the reaction sequence 7-10 that at least half of the available OH radicals are removed by reaction at N-C linkages under the described condition. And, since "free" ammonia is formed as an initial product in the cleavage of primary amino linkages, it is apparent that the value $G(NH_3)_f = .30$ represents an upper limit for the collective contribution of side-chain (and terminal) amino groups. The major fraction of the total ammonia yield, $G(NH_3)_a = .95$ is attributed primarily to reaction at the peptide bond as given by equation 3.

It might be anticipated on the basis of reaction 3 that the combined yield of α -keto acids should approximate the "amide" ammonia yield more closely than is indicated by Table II which shows $G(>C=O)_{\alpha} = 0.42 G(NH_2)_a$. We note, however, that the reported $G(>C=O)_{\alpha}$ value is based on analysis of carbonyl fraction I (Section 2.3) and does not include contributions of the possible nitrogen-containing carbonyl derivatives. Oxidation via reaction 3 of the peptide chain at the loci of lysine and arginine residues, for example, would yield nitrogen-containing α -keto acids, and these would appear in carbonyl fraction II.¹¹ As mentioned in the experimental part, methods are being developed for the detailed analysis of fraction II, but at the present time the most that can be said is that carbonyl products are definitely present. Of course, part of the discrepancy between $G(>C=O)_{\alpha}$ and $G(NH_2)_a$ must be attributed to the fact that there is some loss of carbonyl acids during hydrolysis of the irradiated solutions (footnote 7). However, there appear to be other more basic considerations involved. For example, there is evidence that oxidative cleavage of the peptide bond at the locus of the glycine residue is more complicated than is shown by equation 3. Studies of the oxidation of glycine anhydride indicate (10) that parallel reaction of the type:



is involved. Analogous reactions have been invoked in interpreting radiation-induced oxidations of various organic species (20). Also, (as a corollary of reaction 3), it must be assumed that reaction of the guanidino group of arginine



may contribute to the yield of "amide" ammonia through hydrolysis of guanidine under the analytical conditions employed. Evidence that chemical change corresponding to 3b occurs in the radiolysis of oxygenated solutions of arginine is

to be found in the older literature (30), although we have not as yet determined specific contributions of reactions of this type in protein systems.

The fact that aldehyde α -amino acids may be formed in reaction 3b and in other cleavage reactions of side-chain N-C linkages (of lysine, for example) emphasizes the importance of extending these studies to include all of the nitrogen-containing carbonyl products. There are of course other possible oxidation reactions which do not involve the N-C linkage, but which do yield amino aldehyde and amino ketone derivatives: Oxidation of the alcohol function of serine and threonine would be of this type. Oxidative reactions of aromatic and heterocyclic side-chains (31) may also contribute to the carbonyl content of fraction II. A fairly detailed study of the contributions of the various nitrogen-containing carbonyl products is now being carried on as an outgrowth of the present work.

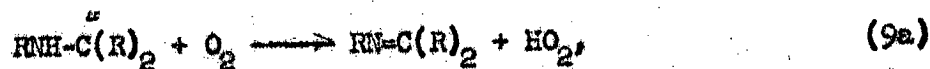
3.3 Reaction Specificity

Apropos of this general subject of the multiplicity of reaction loci in protein radiolysis, there is the interesting question of whether or not certain of these loci undergo preferential attack by OH radicals before other loci become involved. From the dose-yield data of Figs. 5 and 6, it is seen that the production of ammonia, amide, and the several carbonyl fractions from gelatin are strictly proportional to dose over the ranges studied, and that each of the curves extrapolates through the origin. Since the first experimental point in Fig. 5 corresponds to the formation from water of less than one OH radical per protein molecule, we conclude that the observed chemical changes arise from initial reactions that occur in parallel. Data obtained with β -lactoglobulin (Section 2.3) also lead to a similar conclusion, in that chromatograms of the product α -keto acid hydrazones show (a) no measurable variation in relative yield with dose, and (b) no evidence for preferential oxidation of leucine, the N-terminal acid.

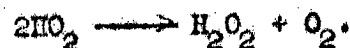
Although the relative contributions of various reaction loci of protein would be expected on a strictly chemical basis to vary with pH, it is interesting to find (Table IC) that the magnitude of the effect of pH on $G(>C=O)_t$ for pepsin is considerably greater than for gelatin at pH values above neutrality. While gelatin maintains a relatively extended configuration over the pH range studied, pepsin is denatured at pH 7 and above to give a random coil-like configuration (16). The comparative effects of pH on $G(>C=O)_t$ in these two systems would suggest that radiation-chemical studies may have useful applications in the study of protein structures in aqueous solution.

3.4 Role of Oxygen

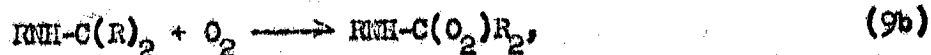
Reference has been made to the fact that step 9 of the mechanism outlined above does not specify the nature of the intermediate formed on reaction of $(R)_2N-\overset{\cdot}{C}R_2$ radicals with O_2 . For N-C linkages containing at least one H atom attached to nitrogen, two alternate paths for step 9 can be written. The simplest formulation involves hydrogen abstraction with formation of a dehydro product¹²



followed by



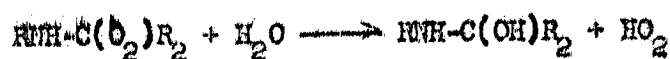
On the other hand, an organic peroxy radical may be involved



and subsequently lead to the same product stoichiometry via



or possibly through

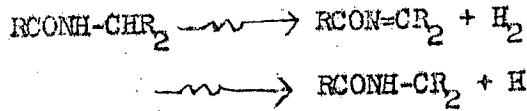


With primary amines ($R = H$), it is difficult to differentiate reactions of type 9a, 9b, since both the dehydro intermediate and the intermediates derived from the peroxy radical represent extremely labile chemical forms. On the other hand, it does appear possible to distinguish such intermediates in reactions involving the peptide bond, ($R = RCO$). In this case, the dehydropeptides, $RCON=CR_2$, are moderately stable in aqueous solution (32), and it appears that they can be differentiated kinetically from intermediates derived from the organic hydroperoxides. On the basis of this approach, we have obtained preliminary results on certain of the simpler acylaminoacids and cyclic dipeptides which indicate that both reactions can be of importance, and that the relative contributions of steps 9a and 9b are determined by the chemical properties of the amino acid (residue) involved.

Although relatively long-lived hydroperoxides are known to be produced in the radiolysis of various amino acids, peptides and related compounds (27), the evidence is that most of the peroxy groups detected in such cases are not at the α -carbon position, and are not directly involved as major intermediates in oxidative cleavage of the N-C bond. Those amino acids and simple peptides that have been found to give relatively high yields of organic hydroperoxides have side-chains that possess either a tertiary carbon atom or at least two adjacent methylene groups. The relatively stable hydroperoxide(s) that is formed in the radiolysis of oxygenated gelatin solutions with $G \approx 0.3$ (Section 2.5) is presumed, therefore, to be associated with such side-chain configurations.

3.5 Applications to Solid State Studies

On the basis of the initial proposals regarding the reactivity of the peptide linkage (8), it was apparent that the same intermediate species could be of importance in the radiolysis of protein in the solid state



and in a subsequent paper (13) it was shown that irradiated solid pepsin on dissolution in water yields high-molecular-weight products containing the carbonyl function. Similar observations have been reported from other laboratories (33,34). At the present time we are applying the analytical procedures developed in the present work to a detailed study of constituent carbonyl products derived from "dry" proteins following γ -irradiation. Of the preliminary results obtained to date with gelatin, perhaps the most interesting is the observation that (a) the relative yields of the various individual α -keto acid products are strongly influenced by the presence of oxygen, and that (b) the spectra of carbonyl acids from both evacuated and oxygenated systems differ strikingly from the tracing shown in Fig. 4. A preliminary report on this work is in preparation.

4. SUMMARY

The action of ionizing radiations on compounds containing primary amine, secondary amine, N-alkylamide and peptide bond configurations are correlated in terms of related mechanisms involving the N-C linkage as the locus of chemical change. It is shown that major radiation-chemical changes in aqueous protein systems may be interpreted in terms of a general over-all reaction:

$$\text{R}_2\text{N-CHR}_2 + \text{O}_2 + \text{H}_2\text{O} \xrightarrow{\sim} \text{R}_2\text{NH} + \text{RCOR} + \text{H}_2\text{O}_2$$

where $\text{R}_2\text{N-CHR}_2$ may represent any of the variously substituted N-C linkages of protein molecular structure. Detailed experimental studies of oxidation products derived from various such loci are reported; correlations with studies of "dry" protein are given.

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TABLE I

Production of Reactive Carbonyl Function in the γ -ray
Radiolysis of Oxygenated Protein Solutions^{a, b}

IA: Values of $G(>C=O)_t$ for various proteins

| | |
|-----------------------------|------------------|
| Pepsin | 1.20 |
| Gelatin | .89 |
| β -lactoglobulin | .85 ^c |
| α -chymotrypsin | 1.25 |
| Yeast alcohol dehydrogenase | ~ .5 |

IB: Effect of added solutes on $G(>C=O)_t$ for pepsin

| | |
|---|------------------|
| NaCl, 0.1 M | 1.15 |
| Na ₂ SO ₄ , 0.1 M | 1.27 |
| Cysteine, 5×10^{-5} M | 1.15 |
| 5×10^{-4} M | .85 |
| 5×10^{-3} M | .40 ^d |
| O ₂ -free | .25 ^d |

IC: Effect of pH on $G(>C=O)_t$ for pepsin and gelatin

| | Gelatin | Pepsin |
|--------|-----------------|--------|
| pH 1.3 | -- ^e | 1.34 |
| 3.0 | .87 | 1.25 |
| 5.0 | .89 | 1.20 |
| 7.0 | -- ^e | 1.52 |
| 9.0 | 1.04 | 2.21 |

^a Unless otherwise stated, dose= 3×10^{18} ev/ml, protein concentration=0.5%, pH \approx 5.

^b Each of the reported values represents an average of at least two independent measurements.

^c For a 0.3% solution.

^d Carbonyl production in oxygen-free solution is attributed to disproportionation reactions of the type $2RNH-CR_2 \longrightarrow RNH-CHR_2 + RN=CR_2$ and subsequent hydrolysis of the dehydro product (Cf. ref. 7).

^e Not measured.

TABLE II

Product Yields in the γ -ray Radiolysis
of Oxygenated Solutions of Gelatin^a

| <u>Product</u> | <u>100-ev yield, G</u> |
|---------------------------|------------------------|
| Total carbonyl | .89 |
| α -ketoacids | .40 |
| (α -ketoglutaric) | |
| Total ammonia | 1.25 |
| Amide | .95 |
| Free | .30 |
| Hydrogen peroxide | 1.24 |
| Organic peroxide | .38 |

^a Gelatin concentration=1%, dose= 2.5×10^{19} ev/ml.

FOOTNOTES

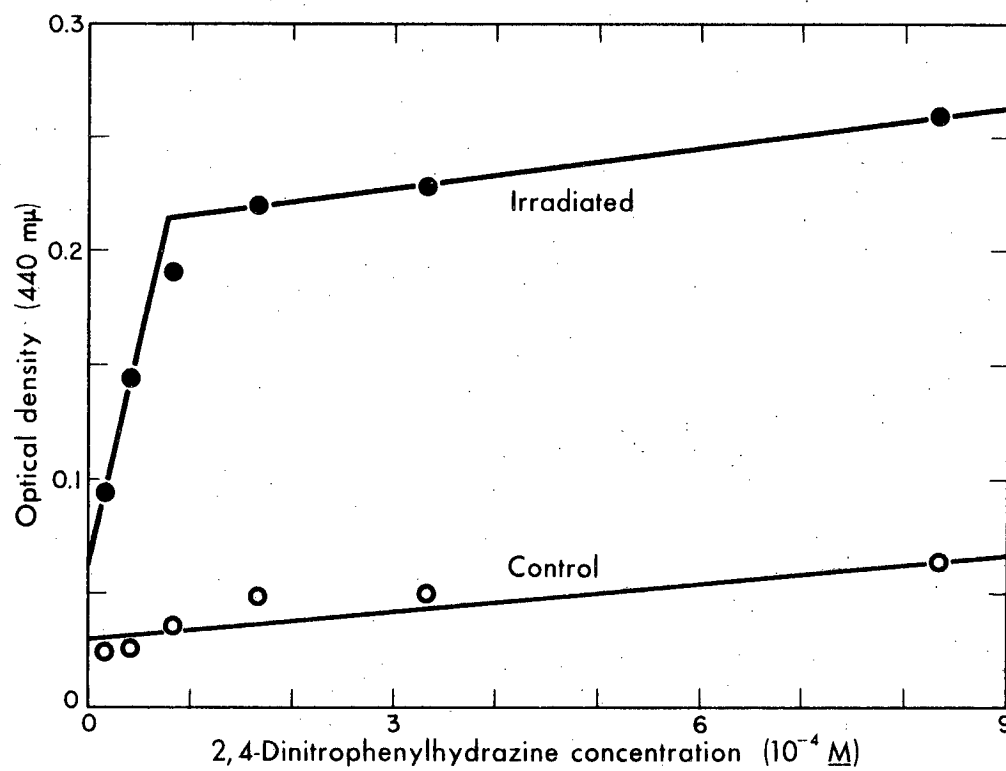
- ¹ This work was performed under the auspices of the U. S. Atomic Energy Commission.
- ² Yields are reported as 100-ev yields or G values. Dose was determined by conventional techniques using the value $G(\text{Fe}^{+3})=15.6$ for the Fricke dosimeter.
- ³ Platinum black was used to remove hydrogen peroxide in a number of runs. Control experiments established that if the solutions were centrifuged prior to hydrolysis, no untoward effects were introduced by the platinum black treatment.
- ⁴ Oxalacetic acid undergoes decarboxylation during the hydrolysis step and hence contributes to the pyruvic acid yield. However, in several of the runs oxalacetic acid was isolated separately (as the hydrazone) in minute amounts.
- ⁵ We are presently investigating the use of 1,2-diamino-4-nitrobenzene (35) in the analysis of these complex mixtures of α -keto acids.
- ⁶ Reference 22 shows this to be the case for pyruvic, oxalacetic and α -keto glutaric acids. We have found that the same values also apply within $\pm 5\%$ to the isomers of glyoxylic, α -ketobutyric and mesoxalic acids.
- ⁷ Recovery of added α -ketoacids (glyoxylic and α -ketoglutaric) from both irradiated and unirradiated systems was approximately 85%.
- ⁸ This indicates also that the observed α -ketoacids are not produced in any appreciable amount through hydrolytic decomposition of radiation-produced β -hydroxy acids.
- ⁹ Hydrogen peroxide does, however, decrease the observed yield of α -ketoacids and for this reason was routinely removed from the irradiated solutions prior to hydrolysis as described in Section 2.3.

FOOTNOTES (continued)

- 10 The 100-ev yields for total, "amide", and free ammonia are designated $G(\text{NH}_3)_t$, $G(\text{NH}_3)_a$ and $G(\text{NH}_3)_f$ respectively.
- 11 The proline residue represents an important example of a tertiary amino configuration which through reaction 4 may also contribute to the amino carbonyl content of fraction II.
- 12 Qualitative data on ammonia formation in the photolysis and (cathode ray) radiolysis of acylamino acids and simple peptides in aqueous solution are given in the older literature (ref. 36). Although the distinction between direct and indirect action was not made in these early studies, it is of interest to note that dehydro products of the type $\text{RCON}=\text{CR}_2$ ^{were} included among the possible reaction intermediates.

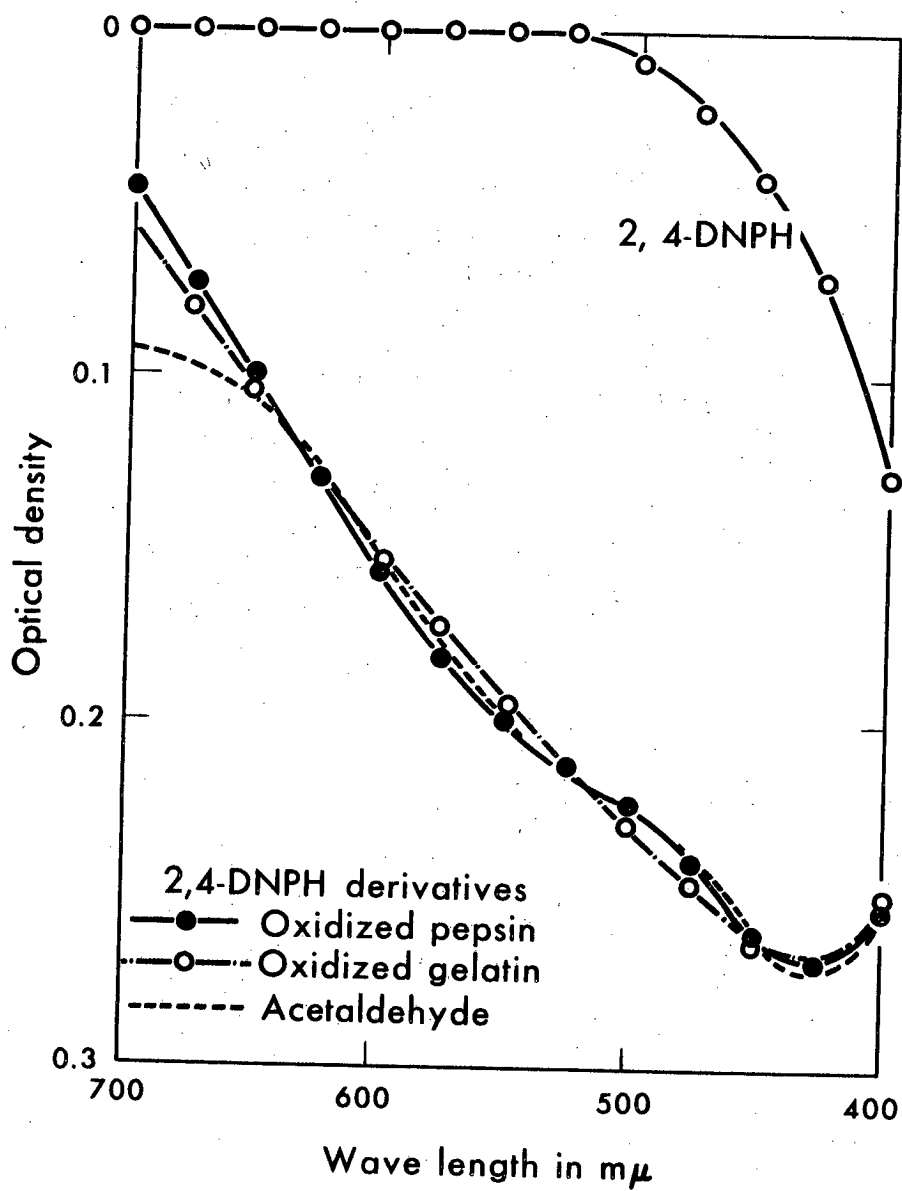
FIGURE LEGENDS

- Fig. 1. Effect of irradiation on the reactions of 2,4-dinitrophenylhydrazine with gelatin.
- Fig. 2. Absorption spectra of 2,4-dinitrophenylhydrazine and derivatives.
- Fig. 3. Carbonyl production from 1% gelatin as a function of time of irradiation.
- Fig. 4. Densitometric tracing of typical chromatogram of α -ketoacid 2,4-dinitrophenylhydrazones from gelatin: (1) α -ketoglutaric acid; (2) glyoxylic acid; (3) pyruvic acid; (4) α -ketobutyric acid; (5) phenylpyruvic acid.
- Fig. 5. Typical hydrolysis data for control and irradiated 1% gelatin.
- Fig. 6. Ammonia production from 1% gelatin as a function of time of irradiation.



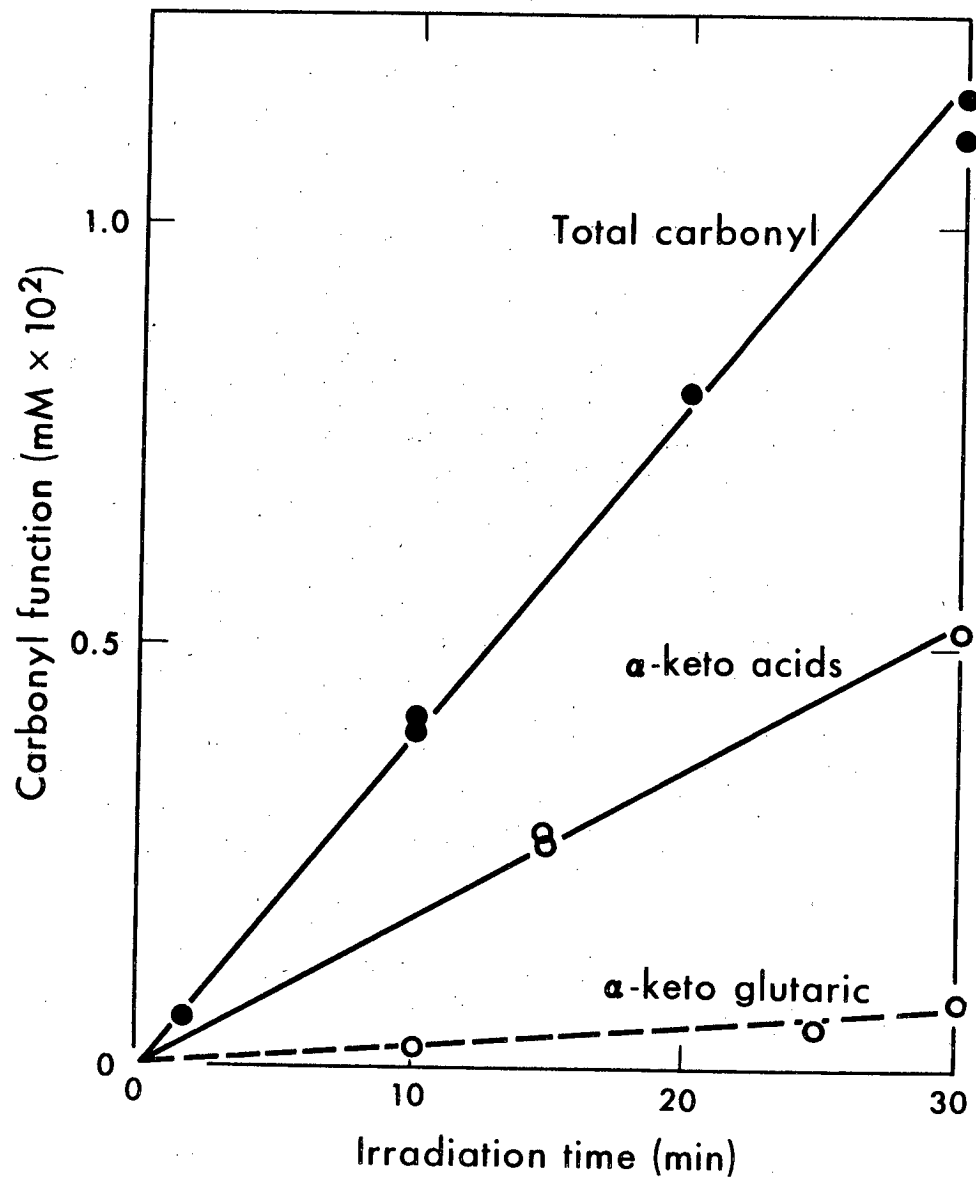
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Fig. 1



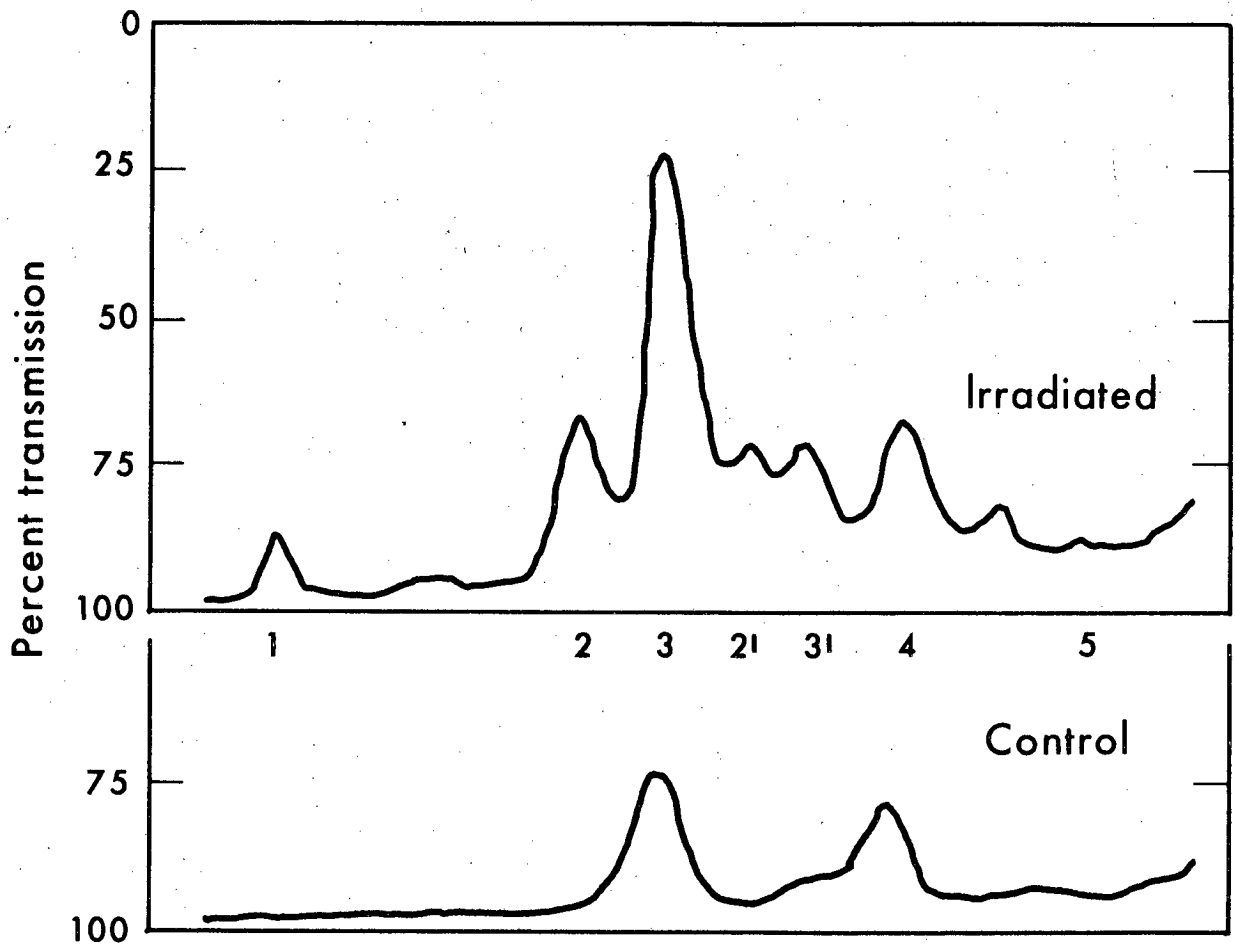
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Fig. 2



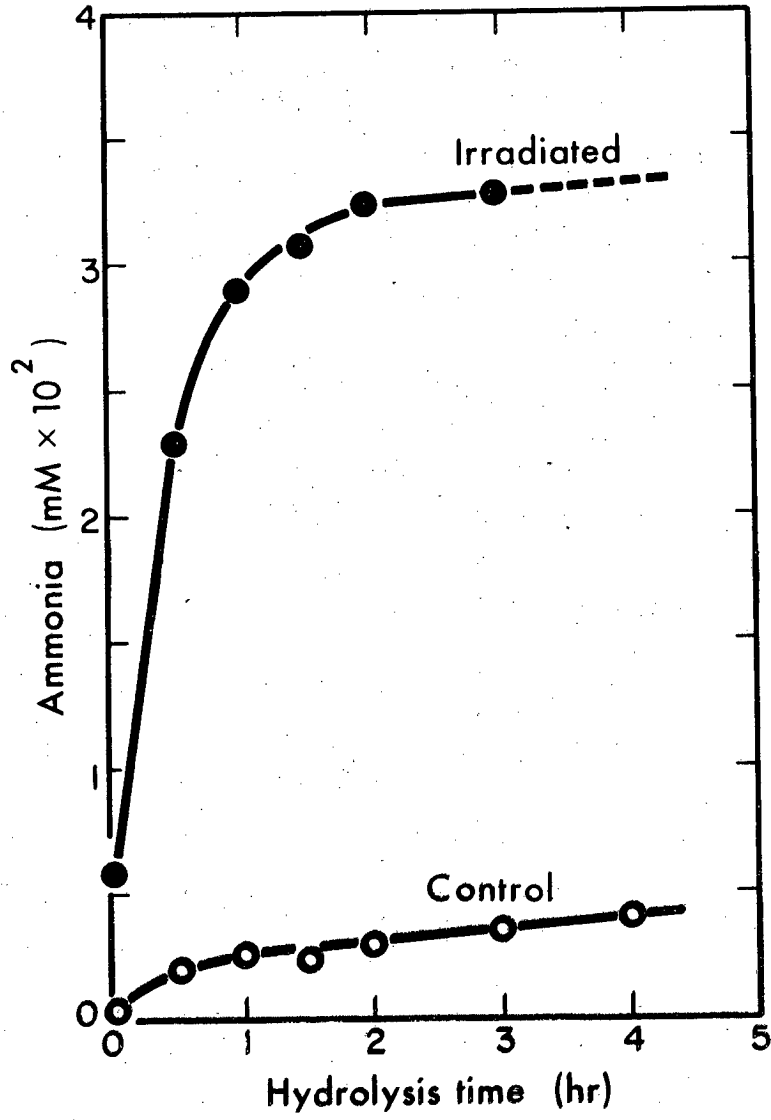
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Fig. 3



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Fig. 4



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Fig. 5

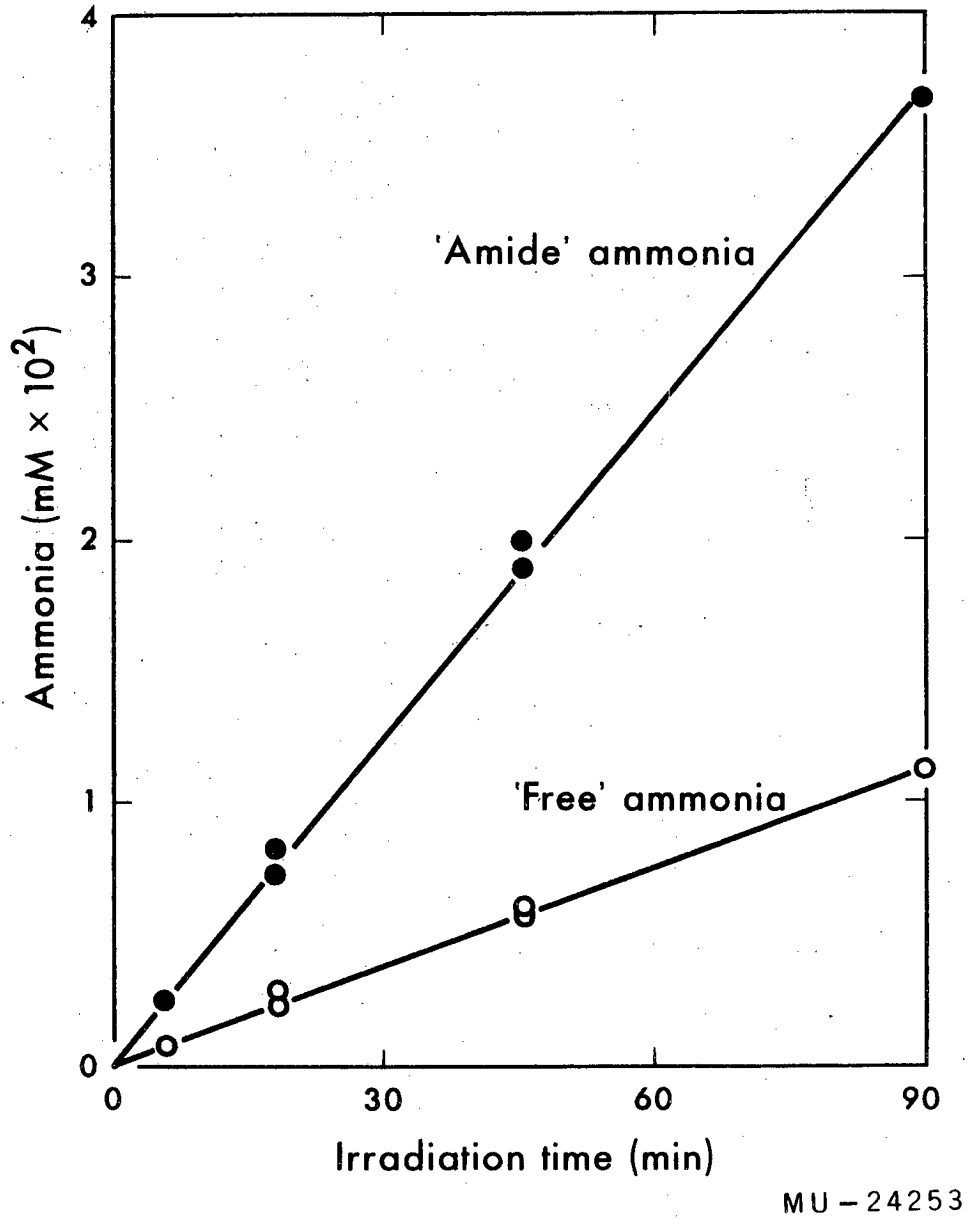


Fig. 6

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