

## Chemical Reactions in Proteins Irradiated at Subfreezing Temperatures

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*The course of chemical reactions in irradiated proteins is determined by factors that influence the reactivity of the primary free radicals, the kind of protein radicals formed, and the decay of these protein radicals to stable products. To understand these reactions, basic radiation chemical concepts are considered, chemical changes in several representative proteins irradiated under different conditions are compared, and results from optical and electron spin resonance studies on model systems are presented. Among the reactions described are those involving cation, anion, and  $\alpha$ -carbon radicals of amino acids and peptides. Analogous reactions common to proteins are then summarized. These mechanistic considerations have important implications for the irradiation of hydrated muscle proteins at  $-40^{\circ}\text{C}$  and for radiation sterilization of foods.*

The chemical reactions occurring in a protein system exposed to ionizing radiation are affected by several factors. The nature of the protein, its state of hydration, the phase and temperature of the system, and the presence of reactive compounds are particularly important factors. Proteins containing disulfide groups, metal ions, or large proportions of aromatic or heterocyclic amino acids will undergo reactions differing from those without these constituents. Proteins that are hydrated

undergo different chemical and physical processes than proteins that are desiccated. Most important, however, is the physical state: fluid solutions are much more susceptible to radiation induced changes than frozen aqueous systems. Temperature, particularly as it affects the viscosity of the medium, can change the chemical consequences markedly. Solutes in these systems, such as oxygen or metal ions, can alter the course of the reactions as well.

Irradiation initiates a series of reactions as a consequence of electrons being ejected and bonds in the protein being ruptured. Various ionic and free radical intermediates are generated that ultimately become stabilized by the formation of covalently bonded compounds. Reactions of the primary radicals can generate other radicals and these in turn combine to form the final products. Any factor that affects the rates and routes of these intermediates will influence which products are formed. For the proteins of interest here, the final effects might be observable as valence change, deamination, decarboxylation, disulfide loss or formation, chain degradation or aggregation, or modification of individual amino acid moieties.

An understanding of these reactions and how they are affected, particularly by irradiation at subfreezing temperatures, can be achieved by considering certain basic concepts and major experimental observations. Consequently, the basic radiation chemical concepts, the techniques used to discern intermediate species and their eventual products, the major findings on such proteins as myoglobin and myosin, and the generalized reactions of primary and secondary radicals as gleaned from studies on amino acid and peptides will be considered herein. The implications of these findings, though generally relevant to radiation biology, redox processes in biochemistry, and protein dynamics, will be discussed in relation to low temperature radiation sterilization of high protein foods.

### *Basic Radiation Chemical Concepts*

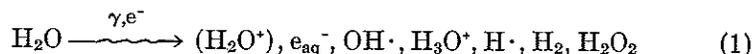
**Energy Deposition and Free Radical Distribution.** The interaction of penetrating gamma rays or high energy electrons with the valence shells of the atoms comprising the molecules of the condensed medium results in energy being deposited in the medium. These electrons can be produced directly in machine sources such as a linear accelerator or a Van de Graaff accelerator, and would have energies typically in the range of 2-10 MeV. Gamma rays, such as those from cobalt-60 or cesium-137 sources, as a consequence of interacting via the Compton process produce energetic electrons as well. Because of its charge, it is the electron that is particularly effective in excitation and ionization processes.

By successive ionization acts, the primary electron gradually becomes degraded in energy and concomitantly produces secondary electrons, also capable of ionizing the molecules. Ultimately, these primary, secondary, and tertiary, etc. electrons can no longer cause ionizations, and lose their remaining energy via electronic, vibrational, and rotational excitation. An energetically degraded electron might be drawn back to the positive ion formed upon ionization, might be trapped if the medium is polar and become solvated, or might react with an impurity of high electron affinity. Some of the excited molecules formed directly or produced upon electron-positive ion reaction will dissociate into free radicals. The overall effect is the initial formation of ions and free radicals nonuniformly distributed in regions called spurs along the track of the ionizing particle.

The distribution of these primary species and their eventual fate is determined by the nature and state of the medium. If the viscosity is extremely high as in solids or glasses, the distribution remains nonuniform and the reactions that occur involve species within the same, or closely related, spur. If the viscosity is low, such as in a fluid system, these species tend to diffuse apart and lead to a uniform distribution throughout the medium. In this case, the reactions conform to kinetic laws for a homogeneous system.

The yield of any species formed as a direct consequence of the energy being absorbed by the component molecules is given in terms of a G-value, which is defined as the number of ions, free radicals, or excited molecules formed for every 100 eV of energy absorbed. G-values may also be given for the stable products that are eventually formed.

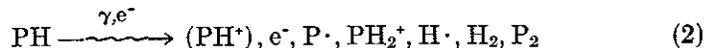
**Direct Effect on Water.** For water, radiolysis leads to the formation of species with rather special features that have been the subject of considerable investigation (1, 2, 3). Equation 1 describes the overall effect:



The molecular ion of water is shown in parenthesis because it rapidly converts to  $\text{OH}\cdot$  and  $\text{H}_3\text{O}^+$ . The solvated electron (2), corresponding to an electron bound to several water molecules in a fluid system, is designated here as  $e_{\text{aq}}^-$ , but will also be denoted as  $e_s^-$  for an electron bound in other polar media. It is highly mobile, has a broad, intense absorption spectrum with a maximum at 720 nm, and is a powerful reductant. The hydroxyl radical,  $\text{OH}\cdot$ , is also very mobile and is a strong oxidant; it exhibits a weak absorption in the 240 nm region. The hydrogen atom,  $\text{H}\cdot$ , is a reductant and exhibits only a weak absorption in the ultraviolet

region. All three radicals have been detected and characterized by virtue of their electron spin resonance (ESR) spectra. Evidence for an unsolvated electron is extensive and is based on chemical and conductometric measurements, which will be mentioned below. G-values for the  $e_{aq}^-$ ,  $OH\cdot$ , and  $H\cdot$  uniformly distributed in water at room temperature are 2.8, 2.7, and 0.55, respectively; G-values for these same species in ice at  $-5^\circ C$  are approximately 0.3, 1.0, and 0.7, respectively.

For proteins or other organic constituents, radiolysis presumably leads to analogous species, although these have not been unequivocally established. Equation 2 describes the overall effect:



The generalized protein molecule is shown as PH. The  $e^-$  is left unspecified; its fate would depend on the medium and other conditions. As will be described later, there are several free radicals that have been detected, but the general designation  $P\cdot$  is all that is needed here.

**Effect of Phase, Temperature, Solutes, and Dose on the Radiolysis.**

Since the primary radical species must diffuse to other radical species or molecules in the system to transfer or share electrons and become stable, the phase and temperature, because they affect viscosity, determine which reactions will occur. Those reactions involving the primary species and the molecules not directly affected by the radiation give rise to new species—secondary radicals—which are considered as being an indirect consequence of the radiolysis. In fluid aqueous systems,  $e_{aq}^-$ ,  $OH\cdot$ , and  $H\cdot$  readily react with each other and with solutes, even those present at low concentrations ( $\sim 10^{-4} M$ ). In frozen systems, such reactions are impeded by the rigidity of the medium. Indirect consequences are limited to systems with very high solute concentrations ( $\sim 1 M$ ) and at temperatures near  $0^\circ C$ , both factors contributing to formation of amorphous or fluid-like regions. Studies of the reaction of  $e_s^-$  with various solutes in polycrystalline systems at  $-40^\circ C$  have shown that G-values for products are only about 1% to 10% of the corresponding values found for fluid systems (4, 5, 6). When chloroacetic acid was used as a probe for the electrons, the G-value for  $Cl^-$  formation increased from approximately 0.03 to 0.8 as the chloroacetic acid concentration was increased from  $10^{-2} M$  to  $1 M$ . Similarly, studies of reactions of  $OH\cdot$  in polycrystalline ice at  $-40^\circ C$  have shown that this species is even more restricted than  $e_s^-$  in its ability to migrate and react (4). These experiments involved using ferrocyanide as a probe for  $OH\cdot$ . It has been estimated that only 4% of the available  $OH\cdot$  can be scavenged using

0.5 M ferrocyanide, the highest practical concentration obtainable. Consequently, the overall chemical change in a frozen or solid system is significantly reduced by limiting the indirect formation of other species.

The course of primary species reactions in a fluid system, which is dependent upon the rate constants for reaction (see Table I) and the concentrations of reactive solutes, determines the nature of the indirect effects. The solutes could be introduced directly or could be formed as a consequence of the radiolysis. For systems in which homogeneous kinetic laws apply, the predominant reaction is determined by competition principles: the reaction involving the highest product of rate constant  $k$  times concentration predominates. If a reactive product is formed in the radiolysis with a reasonably high G-value, it will tend to compete as the dose is increased.

Table I. Rate Constants for Reaction of Primary Water Radicals with Some Amino Acids and Peptides in Fluid Aqueous Solutions<sup>a</sup>

Amino Acid/Peptide	$k, M^{-1} s^{-1} (pH)$		
	$e_{aq}^-$	$OH\cdot$	$H\cdot$
Glycine	$8.2 \times 10^6$ (6.4)	$1.6 \times 10^7$ (5.2)	$9 \times 10^4$ (7)
Glycylglycine	$3.7 \times 10^8$ (6.4)	$4.4 \times 10^8$ (5.2)	$2.6 \times 10^6$
Alanine	$5.9 \times 10^6$ (6.4)	$4.7 \times 10^7$ (6)	$2.9 \times 10^5$
Lysine	$2 \times 10^7$ (7)	$6.0 \times 10^8$ (2)	$1.6 \times 10^6$
Arginine	$1.8 \times 10^8$ (6)	$3.5 \times 10^9$ (6.5-7.5)	$4.9 \times 10^6$
Aspartic acid	$1 \times 10^7$ (7.3)	$2.1 \times 10^7$ (6.8)	$2.9 \times 10^6$ (7)
Histidine	$6 \times 10^7$ (7)	$5.0 \times 10^9$ (6-7)	$2.5 \times 10^8$ (7)
Phenylalanine	$1.5 \times 10^8$ (6.8)	$6.6 \times 10^9$	$8.0 \times 10^8$
Tryptophan	$4.0 \times 10^8$ (6.8)	$1.4 \times 10^{10}$ (6.1)	$2.3 \times 10^9$
Methionine	$3.5 \times 10^7$ (6.0)	—	—
Cysteine	$8.7 \times 10^9$ (6.3)	—	$4 \times 10^9$
Cystine	$1.3 \times 10^{10}$ (6.1)	—	$8 \times 10^9$

<sup>a</sup> From Refs. 2, 3, 52, 56.

#### Major Physicochemical and Analytical Techniques

**Techniques for Examining and Characterizing Irradiated Proteins.** Several techniques have been employed to detect the presence of intermediate species in the irradiated protein or to characterize the changes brought about in its structure or composition. Optical techniques have been used both for detecting intermediates as well as for determining final changes. Because only species with unpaired electrons can be detected by ESR, this technique has been applied for detecting intermediates directly or, most recently, for discerning their nature after trapping with stable free radicals (7). Among the more standard bio-

chemical approaches for characterizing the permanent modifications in the protein are the electrophoretic methods, chemical analyses of products or constituent moieties, and several structure-related methods. Electrophoresis, isoelectric focusing, and chromatographic separations are useful for discerning changes due to loss or modification of moieties that influence size, shape, and charge on the protein. Amino acid analyses, SH group analysis, and determination of amide and fatty acid products pertain to similar modifications. Experiments involving enzymatic activity, sedimentation rates, and binding of radioactive labels also pertain to structural alterations or specific moiety modifications. In general, the analysis of permanent changes is relatively insensitive, and very high doses, often in the range of 3000 kGy (1 Mrad = 10 kGy), must be used. Considerably greater sensitivity is available and a more direct understanding of the chemistry is possible if one uses the techniques recently developed for studying the intermediates.

**Detection of Transient (Short-Lived) Intermediates.** By pulse irradiating (8) a system, it is possible to detect the presence of short-lived intermediates and to characterize their structural and kinetic properties. The technique involves the use of electron accelerators capable of delivering a high dose to a system in a time that is short compared to the lifetime of the species being studied. Typically, such machines deliver doses of about 200 Gy (1 krad = 10 Gy) in a square wave pulse lasting about 1  $\mu$ s. For systems in which intermediate is formed with a G-value of 3, the instantaneous concentration after the pulse is  $6 \times 10^{-5}$  M. If the species has a relatively high extinction coefficient ( $\sim 10^3$  M $^{-1}$  cm $^{-1}$ ), it can be monitored with fast spectrophotometric techniques. This optical approach has been used primarily for aqueous solutions; but it has also been applied to aqueous glasses (9). Where the magnetic resonance properties of the radicals do not lead to broad lines, ESR can also be used (10, 11, 12). Provided a particular species has a reasonably high or sufficiently different conductivity, electrochemical detection is especially effective for charged intermediates (13). A recent variation on these techniques has been described by Warman (14), in which microwave devices are used to detect high-mobility unsolvated electrons in ice. Although not considered a pulsed or fast reaction approach, the technique developed by Eiben and Fessenden (15), involving continuous irradiation and in situ ESR detection, is especially effective. With this technique, radicals that have attained a requisite steady-state concentration can be examined, and a substantial catalogue of spectra for free radicals in fluid aqueous solutions has been compiled.

**Detection of Trapped or Stabilized Intermediates.** In systems of high viscosity, the free radicals of interest in protein radiolysis can be

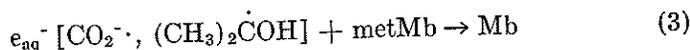
immobilized and examined with standard optical or ESR techniques. Transparent media must be used for examining the radicals optically and for generating them photolytically (16). Aqueous glasses made from hydroxide, perchlorate, or ethanediol solutions are used at low temperatures ( $< -90^{\circ}\text{C}$ ). For a glass equimolar in water and ethanediol and containing a solute of interest, the procedure involves irradiating at less than  $-130^{\circ}\text{C}$  with  $\gamma$  rays, photobleaching using visible light the electrons that are trapped in the matrix, and then scanning the spectrum of the species derived from the electron-solute reaction. For generating the radicals photolytically and examining them with ESR, the procedure involves either direct photoionization (17) of the solute or first photoejecting electrons from ferrocyanide ion with uv light and then photobleaching the trapped electrons to obtain the desired electron-solute reaction (18, 19, 20). Opaque media such as polycrystalline ice plugs can be used if the radicals are generated radiolytically and then examined with ESR as described. In systems such as powders or crystals, the radicals formed upon  $\gamma$  irradiation are also relatively immobile and can be studied (21). Since lowering the viscosity in all of these cases allows various rotational processes and diffusional processes to take place, different radicals can be formed and observed as the viscosity and/or temperature is changed. These techniques have been used to identify and characterize many of the free radicals from proteins, amino acids, and peptides that will be discussed here.

#### *Major Effects on Representative Proteins*

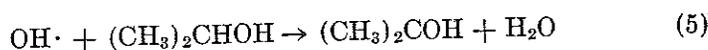
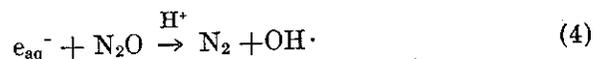
Before summarizing the reactions generally occurring in proteins irradiated at low temperatures, it is instructive to review some of the major observations that have been made for certain representative proteins. Those selected for illustration are myoglobin, ribonuclease (RNase), the myofibrillar proteins myosin and actomyosin, and gelatin. Wherever possible, comparisons will be made between results for fluid and frozen systems.

**Myoglobin: A Representative Metallo-protein.** Because the iron in myoglobin can exist in two stable valence states, +3 for metmyoglobin (metMb) and +2 for deoxymyoglobin (Mb), the radiation chemistry of myoglobin in fluid aqueous solution is dominated by oxidation and reduction reactions. Accordingly, Satterlee (22), who was prompted by the work of others (23, 24) to study color changes in irradiated meats and myoglobin solutions, showed by proper selection of conditions that  $\text{OH}\cdot$  was not responsible for the reduction of metMb. Subsequently, Simic and coworkers (25, 26) and Shieh and coworkers (27, 28) systematically

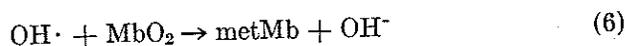
examined the pertinent reactions, and demonstrated that metMb can be reduced by a variety of free radicals. Reaction 3 applies for both native and denatured metMb:



Rate constants for the reduction by  $e_{aq}^-$  and  $\text{CO}_2^{\cdot-}$  (a reductant derived from formate ion) are  $2.5 \times 10^{10}$  and  $2.0 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$  respectively, which approach diffusion-controlled limits. The reduction of metMb by  $(\text{CH}_3)_2\dot{\text{C}}\text{OH}$  indicated in Figure 1 is typical, being linearly dependent upon dose until almost all the metMb is depleted. In this experiment  $e_{aq}^-$  is converted to  $\text{OH}\cdot$  by reaction 4 which, in turn, is converted to  $(\text{CH}_3)_2\dot{\text{C}}\text{OH}$  by reaction 5:



Conversely, the divalent ion in oxymyoglobin ( $\text{MbO}_2$ ) is readily oxidized to metMb, as reaction 6 indicates:



Since metMb is not easily (if at all) further oxidized to a higher valent iron, its reaction with  $\text{OH}\cdot$  does not appear to affect the iron, but apparently involves the amino acid moieties on the surface of the protein. Their involvement is supported by the formation of dimeric metMb at high doses.

If the irradiation is carried out in a frozen system, however, these reactions are either minimized or eliminated. Reduction by  $e_s^-$  is possible but restricted. Experiments involving metMb in water-ethanediol glasses at  $-130^\circ\text{C}$  show that if electrons could interact with this compound, reduction would take place. Figure 2 shows the development of Mb in the glass following irradiation and photobleaching of trapped electrons, which moves the electron to the immobile protein. The implication of these results is confirmed by experiments (29) on polycrystalline solutions of metMb irradiated over a range of temperatures from  $-80$  to  $0^\circ\text{C}$ . The G-value for reduction, which is 3.1 at room temperature, is only 0.07 at  $-45^\circ\text{C}$ . There is a slight increase in this value in going from  $-45$  to  $0^\circ\text{C}$ , which is consistent with other studies on frozen ices (4, 5). Reaction with  $\text{OH}\cdot$  is even less probable, and chromatographic analyses of metMb irradiated at  $-45^\circ\text{C}$  to doses as high as 80 kGy show no high molecular weight myoglobin oligomers.

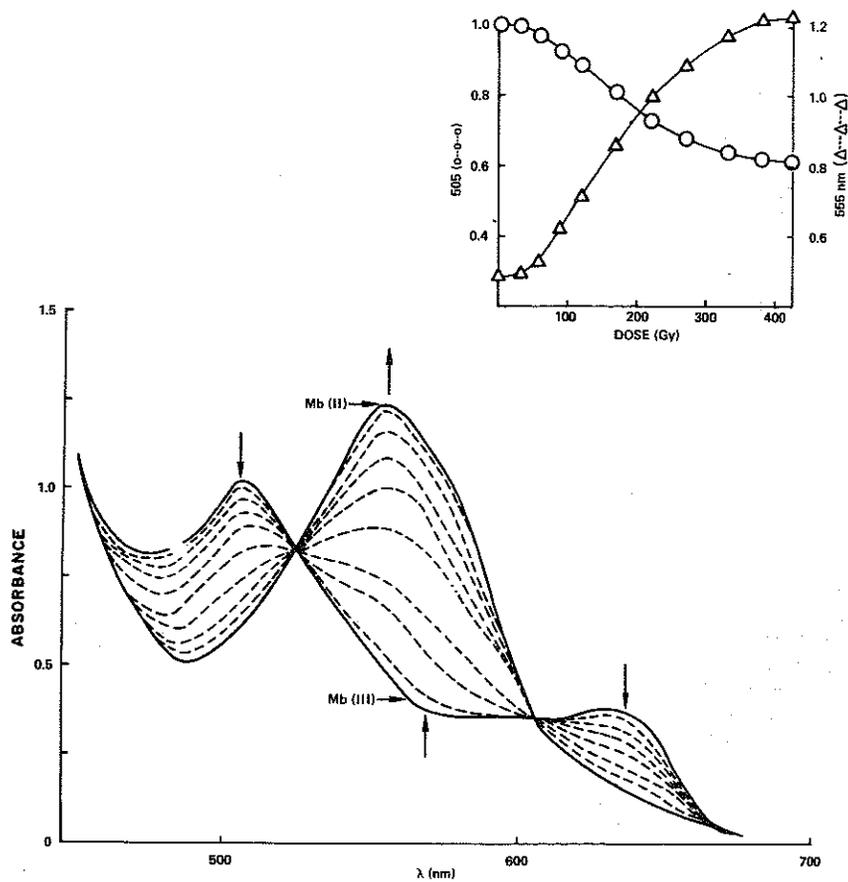


Figure 1. Spectral changes associated with reduction of metmyoglobin by isopropanol radicals at 20°C. Solution contained  $1 \times 10^{-4}$ M metmyoglobin, 0.05M isopropanol, and  $10^{-2}$ M phosphate buffer (pH = 7.5), and was saturated with  $N_2O$ . Spectrum before irradiation is designated by Mb(III), and after completion of irradiation, by Mb(II); spectra for intermediate cases are dotted. (Inset) Plot of absorbance at 555 and 630 nm vs. dose.

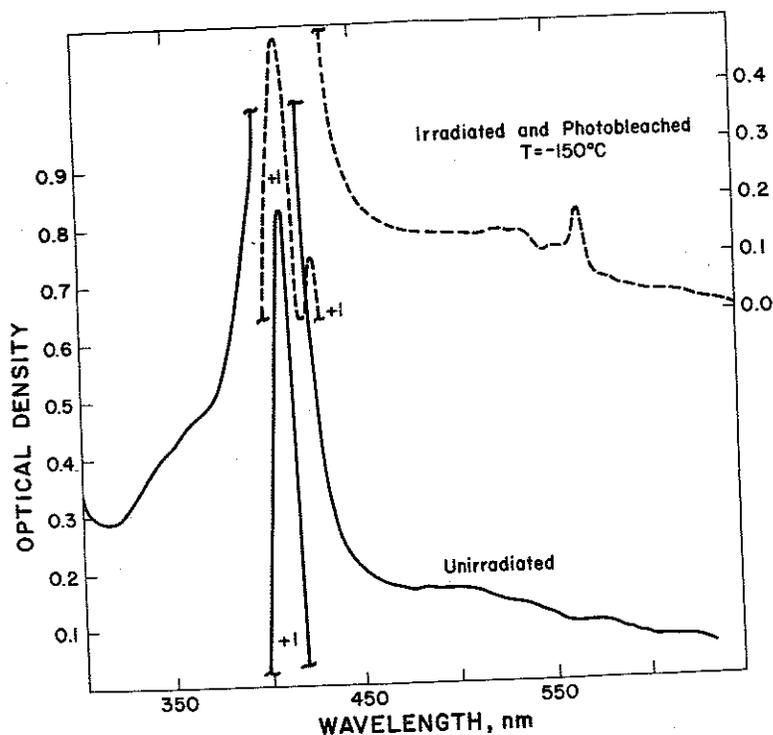


Figure 2. Spectral changes associated with electron reduction of metmyoglobin in an ethanediol-water glass at  $-150^{\circ}\text{C}$ . Glass was formed from an equimolar mixture of ethanediol and water containing 1 g/liter of metMb, irradiated to approximately 8 kGy at  $-196^{\circ}\text{C}$ , and exposed to visible light to bleach the trapped electrons at  $-150^{\circ}\text{C}$ . Solid curve corresponds to the unirradiated system; dotted curve, which is displaced vertically for clarity, corresponds to the final spectrum. Change of scale is indicated by the +1 designation.

**RNase: A Representative Enzyme.** Both  $\text{H}\cdot$  and  $\text{OH}\cdot$  radicals in fluid, aqueous solutions react with RNase in ways that lead to inactivation. Mee and coworkers (30) have found that  $\text{H}\cdot$  leads to aggregation, and that cystine, methionine, and tyrosine are mainly affected. Adams and coworkers (31), investigating the specificity of free radical reaction with RNase, found that  $\text{OH}\cdot$  and  $\text{Br}_2\cdot^-$  are effective inactivators because they rapidly react with histidine, which is associated with the active site.

Structural changes can be discerned in RNase irradiated in solution or in the dry state. Delincée and Radola (32) could show from isoelectric focusing measurements on irradiated 0.1 and 1% RNase solutions that several active components having lower isoelectric points are produced. Their gel chromatographic results showed that aggregates are formed stepwise from monomer to dimer to higher polymers. Experiments on dry

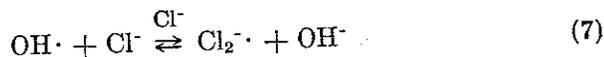
RNase gave similar results but required much higher doses: the gel chromatographic pattern obtained from a 0.1% RNase solution irradiated to 0.5 kGy could be matched by irradiating a dry preparation to 150 kGy. Friedberg (33) also studied dry RNase and found that its apparent average molecular weight is increased upon irradiation to 300 kGy. He concluded that the structure of globular proteins imposes constraints favoring combination of radicals.

The effectiveness of irradiation in inactivating dry RNase depends on the temperature, as shown by Fluke (34). He determined the doses needed to reduce the activity to 37% of its original value ( $D_{37}$ ) at temperatures from  $-160$  to  $182^{\circ}\text{C}$ . Arrhenius-type plots of  $D_{37}^{-1}$  vs.  $T^{-1}$  are found to be nonlinear and were interpreted in terms of a temperature-independent term and two temperature-dependent terms. The inactivation becomes relatively effective above about  $60^{\circ}\text{C}$ .

The significance of structure and temperature on the types of free radicals formed in irradiated, dry RNase has been demonstrated by Riesz and White (35). Using a labelling technique in which tritium becomes distributed among the amino acids as a result of free radicals reacting with tritiated hydrogen sulfide, HST, they could show different sets of radicals and reactions occurring at  $-78^{\circ}\text{C}$  and at  $25^{\circ}\text{C}$ . They found, for example, that the distribution of tritium in native RNase at  $-78^{\circ}\text{C}$  is the same as in denatured RNase at  $25^{\circ}\text{C}$ , but is different than in native RNase at  $25^{\circ}\text{C}$ . Apparently, at the lower temperature the radicals are formed randomly and independent of conformation, but their conversion at higher temperature is specific and depends on the particular protein conformation.

**Actomyosin and Myosin: Representative Myofibrillar Proteins.** The radiolytic effects on solutions of either actomyosin or myosin above and below the temperature of freezing cannot easily be compared. So few studies on these proteins in fluid systems have been conducted, presumably because of their low solubility. However, results from a study by Coelho (36) on actomyosin in solutions containing  $\text{CaCl}_2$  and  $\text{MgCl}_2$ , in which both activity (ATPase) and chemical analyses were made, can serve as reference. Of interest here was whether any effect of radiolysis on enzyme activity could be correlated with chemical and/or structural changes. Coelho found that as the dose was increased, the activity increased up to a maximum (achieved at 2.5 kGy) and then decreased monotonically thereafter. The number of accessible and/or buried SH groups, in contrast, decreased steadily with increasing dose. Ultracentrifugation measurements were also made and these showed that the developing reticulation might be related to the changes in enzyme activity. There is no definite conclusion that can be reached about the reactions responsible for either the change in activity or the overall

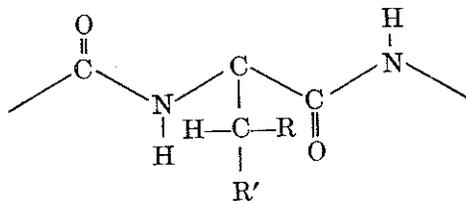
indications of chemical change. Since  $\text{Cl}^-$  was used in high concentrations,  $\text{Cl}_2^{\cdot-}$ , formed through reaction 7, would have been the predominant reactive species:



Consequently, many amino acid sites on the actomyosin would have been susceptible to oxidation by this radical.

Considerably more information on the radiolysis of frozen, hydrated actomyosin and myosin has recently become available (5, 6, 37, 38) showing that these proteins are relatively stable towards radiolytic decomposition. The experimental procedures involved isolating the proteins from prerigor beef, dialyzing to remove  $\text{Cl}^-$ , denaturing some of the samples with a mild heat treatment (70°C, 30 min), preparing the samples either as suspensions or precipitates, freezing them, irradiating them, analyzing some by ESR or electrophoresis, and determining the amino acid composition in others after desiccating and hydrolyzing. In this way the influence, if any, of structure could be ascertained and the formation and fate of free radicals as they relate to protein degradation or aggregation and to amino acid modification could be discerned.

**ELECTRON SPIN RESONANCE RESULTS.** Irradiation of both actomyosin and myosin in either the native or heat-denatured state at  $-40^\circ\text{C}$  leads to the formation of the same spin centers. Figure 3 shows a high resolution spectrum for actomyosin. It is characterized by a broad doublet signal, having a peak-to-peak separation of 28 gauss, and eight other, weaker lines extending about 130 gauss. The absence of signals attributable to N-centered or S-centered radicals rules out contributions from other than C-centered radicals. Based on comparisons with spectra from dipeptide radicals (to be discussed below), this spectrum is attributed to contributions primarily from different  $\alpha$ -carbon radicals on the peptide backbone and secondarily from side chain radicals. Signals for backbone radicals of the type



correspond to interaction of the unpaired electron with protons on the associated methylene carbon. Depending on the nature of the R and R'

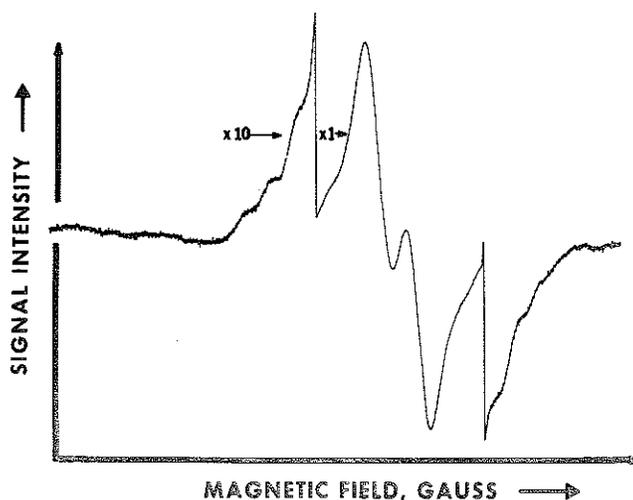
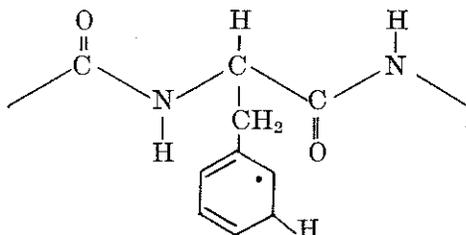


Figure 3. High resolution electron spin resonance spectrum of an irradiated, frozen suspension of actomyosin. Sample was irradiated to 50 kGy at  $-40^{\circ}\text{C}$  and spectrum was recorded at  $-40^{\circ}\text{C}$ . The central portion of the spectrum corresponding to a gain of  $\times 1$  shows the doublet feature with a shoulder on either side. At a gain of  $\times 10$ , an additional three lines are clearly discernible on both the high and low field portions of the spectrum.

groups, sets of doublet and quartet lines would be produced. Of the side group radicals that could contribute, analyses show that the H atom adduct of the phenylalanine ring



is responsible for the extreme lines and accounts for about 4% of the total radicals. In general, the spectra obtained are very similar to those obtained from other proteins (39, 40).

On the basis of conventional ESR measurements, the radicals observed at  $-40^{\circ}\text{C}$  appear stable at this temperature. Such backbone radicals on so large a protein with two helically entwined main chains would be immobile in this matrix. More recently, however, pulse ESR experiments on *beef muscle* samples (41) indicate that a portion of the radicals formed at  $-40^{\circ}\text{C}$  do react within several minutes following

irradiation. The portion remaining thereafter corresponds to radicals that have been observed before. The general trend in these results can be seen in Figure 4 in which the intensity of the main doublet signal, recorded within 10 sec after each pulse, is shown as a function of the number of pulses. At certain points more time has elapsed between pulses and the decrease in intensity is noticeable. The curvature in the intensity-dose plot also indicates a contribution from transient species. The signal intensity corresponding to the stable protein radicals had been found previously to increase linearly with dose at low doses, and then to reach a saturation limit at doses above 200 kGy.

That the protein radicals are not indefinitely stable in a frozen system is shown by raising the sample temperature to  $-10^{\circ}\text{C}$  (5). The decay is slow, the half-life being about 8 hours. If the sample is thawed,

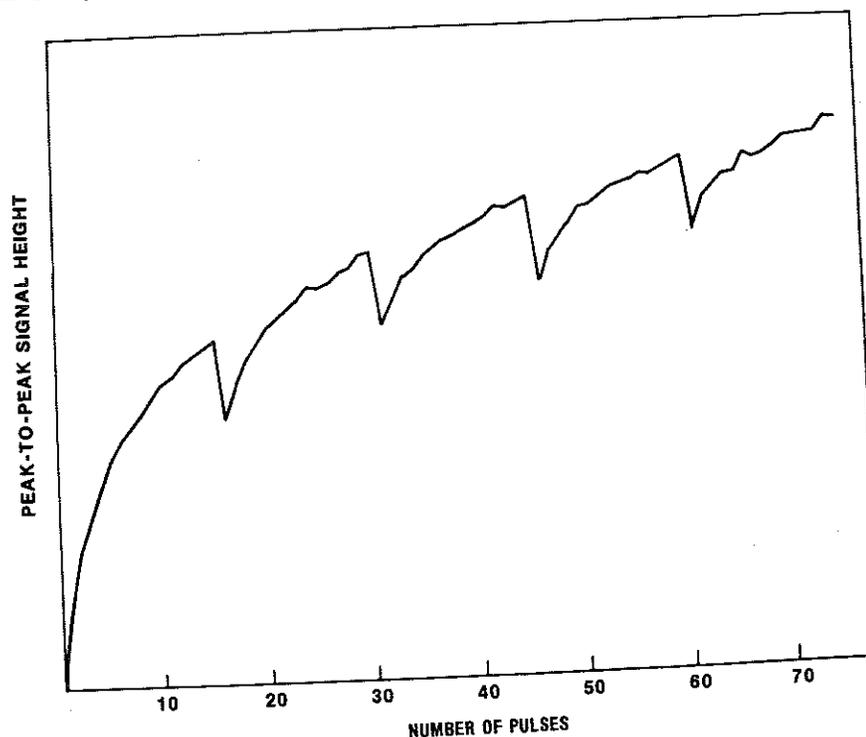


Figure 4. Effect of pulses of irradiation on the ESR signal intensity for radicals in beef at  $-40^{\circ}\text{C}$ . Beef samples contained NaCl and tripolyphosphate and had been heat treated to approximately  $73^{\circ}\text{C}$ . Signal intensity corresponds to the peak-to-peak height of the two most intense lines in the spectrum (38). Each spectrum was scanned within 10 seconds after each 5  $\mu\text{sec}$  pulse from a 10 MeV linear accelerator. After every 15 pulses, several minutes elapsed before the pulsing was reinitiated. The dose per pulse was approximately 1 kGy.

refrozen, and re-examined within 30 min, no signal can be detected. Reaction, therefore, is possible provided that certain relaxation processes or short translational motions are promoted at the higher temperatures in the ice. Reaction readily occurs when the constraints of the medium are removed, that is, upon thawing.

**SOLUBILITY AND ELECTROPHORETIC RESULTS.** Irradiation of these proteins at  $-40^{\circ}\text{C}$  over a dose range of about 200 kGy causes a decrease in their overall solubility that is associated with noncovalent aggregation of the main myosin chains (42). This decrease in the amount of protein extracted into a solution 8 M in urea and  $10^{-3}$  M in dithiothreitol or  $\beta$ -mercaptoethanol is nonlinear with dose and reaches a limit at about 80 kGy. The loss in soluble protein is matched by the recoverable protein in the residue. Electrophoretic separations (using SDS and 5% polyacrylamide gels) of extracts of samples receiving successively higher doses show no new bands, no increase in the low molecular weight fragments, a slight development of a very diffuse region centered at about 100,000 daltons, and the gradual loss of the 210,000–250,000 dalton main chain bands. Solubilizing the residue with a guanidine hydrochloride-urea mixture and dialyzing the solution against urea make it possible to examine the protein pattern. Electrophoresis of this material (also with SDS) shows that the main myosin chains have been retained intact. Despite the high doses, no significant aggregation or degradation of actomyosin or myosin occurs under these conditions.

**AMINO ACID ANALYSIS RESULTS.** Irradiations for these analyses were done with considerably higher doses, 0–400 kGy, to compensate for the limitations in detecting small changes in amino acid composition. All samples were desiccated and then hydrolyzed with *p*-toluenesulfonic acid. The number of residues determined for a particular amino acid was normalized per 1000 residues of all amino acids analyzed. Plots of this ratio for each amino acid against dose are straight lines with zero slope. Such results indicate that, within the limits of reproducibility of about 2%, none of the amino acids is discernibly modified (37, 42) under these conditions.

**Gelatin: Additional Comparisons.** Several experiments on gelatin irradiated as a gel and in a dry system provide an interesting comparison of medium effects.

Stein and co-workers (43) irradiated solutions of gelatin ranging in concentration from 1% to 20% and containing  $4 \times 10^{-4}$  M of ferricyanide ion at  $25^{\circ}\text{C}$ . Reaction of the gelatin free radicals with ferricyanide would produce ferrocyanide. G-values for this reduction in nitrogen-purged, 1% and 10% gelatin solutions are approximately 2.6 and 5.3, respectively. Since the gels become "stiffer" at higher gelatin concentrations, reaction

between gelatin radicals becomes less likely and reduction of the more freely diffusing ferricyanide ion predominates.

Bachman and coworkers (44), using ESR techniques, monitored the lifetime of free radicals in dry gelatin irradiated to approximately 50 kGy and stored in air at 20°C. Unlike the radicals in the gel, these are relatively immobile, and required several days to decay appreciably. Some were still detectable after about a month. The work of Friedberg and coworkers (45) indicates that the formation of these gelatin radicals in the dry state involves rupture of peptide bonds and that their decay does not involve combination of long chains. They found a decrease in the viscosity of solutions made from dry gelatin irradiated to a dose of 155 kGy.

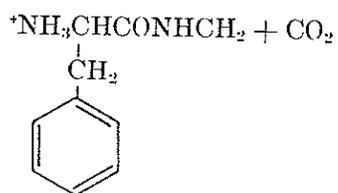
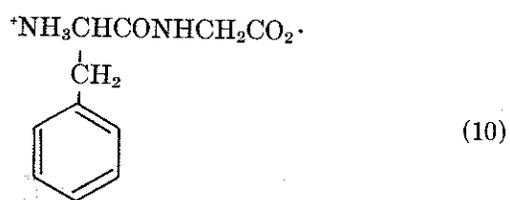
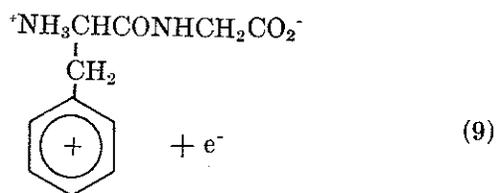
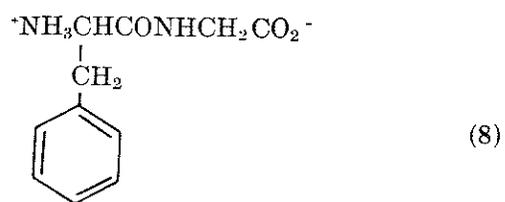
**Other Proteins.** In general, the findings described above for these proteins are common to many proteins. More specific information about other proteins can be obtained from available reviews (46, 47, 48, 49).

#### *Formation and Conversion Reactions of Amino Acid and Peptide Radicals*

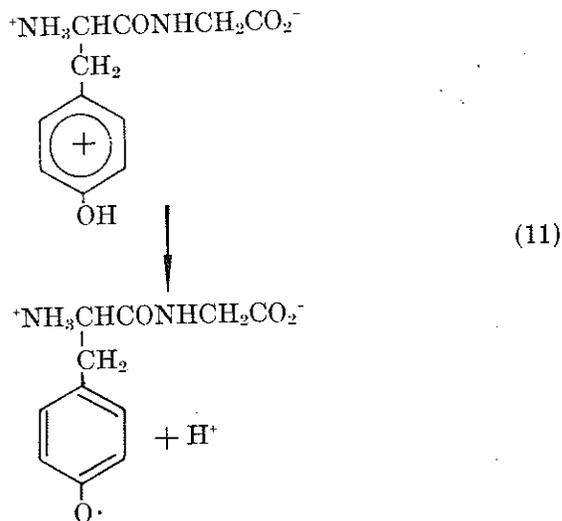
Some understanding of the chemical reactions responsible for changes observed in proteins irradiated in fluid and frozen systems can be achieved by considering results from studies on amino acids and peptides. For the sake of simplicity, these reactions are classified according to whether formation of the radical formally involves donating or accepting an electron, whether formation or decay involves conversion from or to another radical, or whether decay involves bimolecular radical interaction. Results from fluid aqueous solutions will be used for reference and those from frozen systems will be emphasized. The implications of these findings to observations on proteins described above will be given where possible.

**Electron Donation: Ionization, OH-Adduct Formation, and Hydrogen Abstraction.** Ionization of amino acids or peptides either through photolysis or radiolysis leads to cation radicals, the fate of which will be influenced by the nature of the compound and the medium.

Photolytic studies of aromatic dipeptides and tripeptides in NaClO<sub>4</sub> and NaOD glasses (50) show that  $\pi$ -cation radicals of phenylalanine, tyrosine, and tryptophan can undergo different reactions depending on their disposition in the molecule and the molecular conformation. (Electrons produced upon photolysis of the perchlorate system are converted to O<sup>-</sup>, for which corrections can be made). For PheAla and PheGly charge transfer from the -COO<sup>-</sup> occurs, followed by decarboxylation, as shown by reactions 8, 9, and 10:



For TyrAla, TyrGly, TrpAla, and TrpGly, radicals on the ring group are observed, along with the decarboxylated radical. Deprotonation of tyrosine leading to the phenoxyl radical is shown in reaction 11:



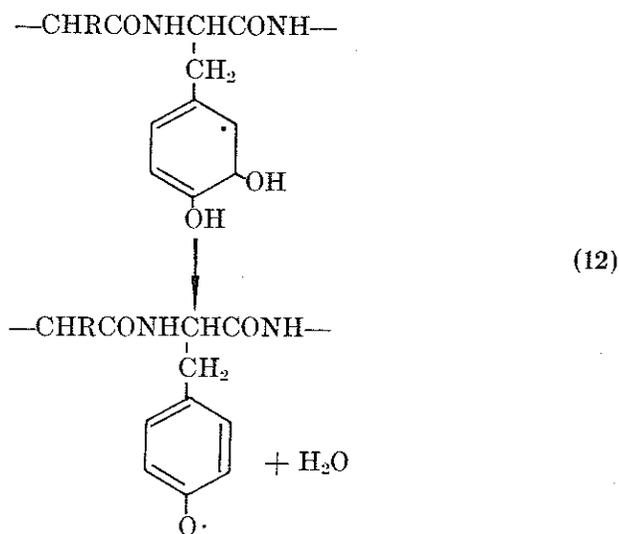
For the tripeptide, PheGlyGly, in which the cation is not favorably disposed for charge transfer, only the  $\pi$ -cation radical of phenylalanine is observed (this species would readily hydrolyze in water to form the OH-adduct radical of phenylalanine). These results have a direct bearing on the reactions in an irradiated system.

Radiolytic studies on ices containing peptides and *N*-acetyl amino acids have been conducted recently (51) that provide evidence for cationic processes. (Electrons generated concurrently with the solute cations participate in reactions described below, but those formed in the ice do not contribute to the observed reactions.) The appearance of the decarboxylated species, the yield of which depends on the nature of the compound, provides this evidence. At  $-196^\circ\text{C}$ , approximately 25% of the radicals observed for *N*-acetylalanine corresponds to  $\text{CH}_3\text{CONDC}\dot{\text{C}}\text{H}_3$ , indicating that  $\text{CO}_2$  has been lost. For the dipeptide GlyAla, the decarboxylated radical is observed to be equivalent in yield to the product from the electron reaction, indicating an efficient mechanism for decarboxylation. The findings for other dipeptides are very similar. Consistent with the photolytic results, the extent of decarboxylation is pH dependent, reflecting an influence of charge state on the mechanism.

These competitive pathways for reaction of cationic species imply that several different radicals could be formed in proteins, depending on

their structure and the conditions. The evolution of  $\text{CO}_2$  that has been observed can be understood as arising from such cationic precursors. The formation of OH-adducts of phenylalanine moieties and formation of the phenoxy radicals from tyrosine moieties might also result from such cations and need to be investigated.

Oxidation of the amino acid moieties in irradiated aqueous systems by reaction with  $\text{OH}\cdot$  is well established for fluid systems, but it is not likely to be encountered in frozen systems. Being a strong oxidant, the  $\text{OH}\cdot$  reacts by electron transfer. It also adds readily to double bonds and abstracts H from C—H, N—H, and S—H bonds, but with lower reaction rate constants. A compendium of rate constants for aqueous solution has been published (52) and a few representative values for amino acids are shown in Table I. As discussed by Simic (53), the predominant sites for reaction in amino acids and peptides can be inferred from these values, which indicate that the ring groups are favored, while abstraction from the peptide backbone is less likely. Hydroxylation of the phenylalanine ring also occurs as was found for the prototype reaction with benzene (54). Formation of phenoxy radical following  $\text{OH}\cdot$  addition to tyrosine should be similar to the mechanism established for phenol (55) in which elimination of water occurs as is shown in reaction 12:



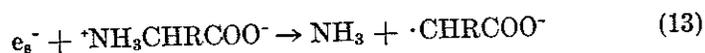
However, these adduct formation reactions are not expected to occur with much efficiency in frozen, hydrated proteins, because of the limited mobility of  $\text{OH}\cdot$  in the rigid matrix.

Formation of  $\alpha$ -carbon radicals either from cation decomposition or through abstraction of H by  $\text{OH}\cdot$  is considered here because it can be construed formally as loss of an electron followed by proton transfer. If the cation deprotonates from the peptide chain by transfer to components of high proton affinity then the  $\alpha$ -carbon radical is formed directly. Abstraction by  $\text{OH}\cdot$  in fluid systems gives the same result.

(Abstraction of H by  $\text{H}\cdot$  is also possible in both fluid and frozen systems. Rate constants for  $\text{H}\cdot$  with amino acids are known (56, 57) and the intermediates formed have been characterized (53).)

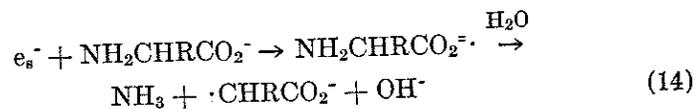
**Electron Acceptance: Reduction and Adduct Formation.** Acceptance of electrons at specific sites on amino acids and peptides depends on their reactivities and produces different chemical consequences. Among the sites of particular importance are the terminal amino and carboxyl groups, the ring groups, the peptide carbonyl, and the sulfur bonds. Reactivities of these are reflected in the rate constants for reaction of solvated electrons with individual amino acids in aqueous solutions, as shown in Table I and as discussed by Simic (53). More detailed information, however, regarding the stepwise progression from attachment to specific radical formation has been obtained from low temperature studies.

**ATTACHMENT TO AMINO GROUP.** The electron reacts with the protonated terminal amino group in aliphatic amino acids, leading to deamination (53, 58). The rate constants in solution depend on pH,  $k$  decreasing as pH is increased. ESR studies on electron reaction with amino acids in neutral glasses at  $-196^\circ\text{C}$  indicate that dissociative attachment readily occurs producing a fatty acid radical:



The formation of ammonia does not necessarily imply direct reaction with the  $\text{}^+\text{NH}_3$  group.

**ATTACHMENT TO THE CARBOXYLATE GROUP.** In basic systems where the amino group is not protonated, attachment occurs on the carboxylate ion, as evidenced from ESR studies on hydroxide glasses (16). At  $-196^\circ\text{C}$  spectra are observed for the dianion radical of several amino acids. At higher temperatures, the fatty acid radicals are formed, indicating that deamination has taken place:



The two-step sequence demonstrated for frozen systems could also occur in fluid systems, but at rates too fast to observe.

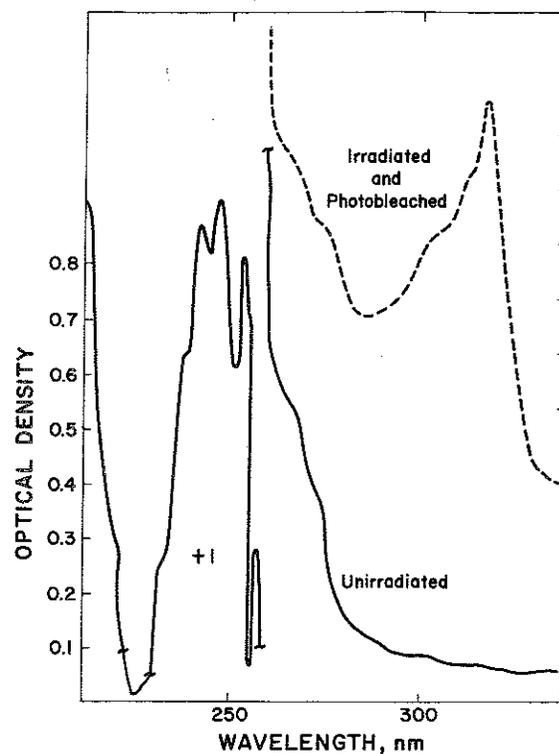
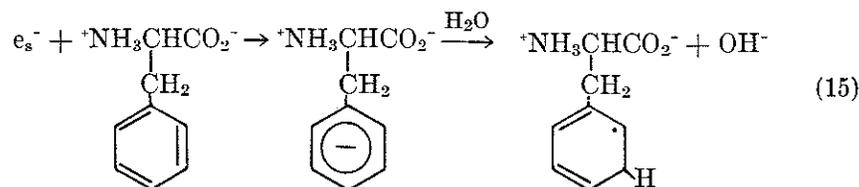


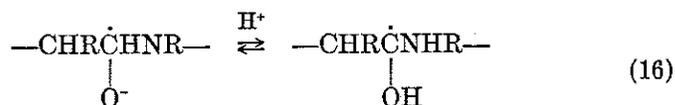
Figure 5. Optical spectrum of hydrogen adduct of phenylalanine in an ethanediol-water glass at  $-196^{\circ}\text{C}$ . Glass was formed from an equimolar mixture of ethanediol and water containing  $4 \times 10^{-2}\text{M}$  phenylalanine, irradiated to approximately 4 kGy, and exposed at  $-196^{\circ}\text{C}$  to visible light to bleach trapped electrons. Solid curve corresponds to phenylalanine in the unirradiated glass; dotted curve is displaced vertically for clarity and corresponds to the  $^{\bullet}\text{NH}_3\text{CH}(\text{CH}_2(\text{C}_6\text{H}_5))\text{CO}_2^-$  radical.

**ATTACHMENT TO AROMATIC AND HETEROCYCLIC GROUPS.** Electrons react more rapidly with histidine, tryptophan, phenylalanine, and tyrosine than with the aliphatic amino acids, forming radicals involving the ring groups. Such electron-adduct radicals readily protonate in aqueous systems (59) giving the equivalent of an H-adduct radical. Similar reactions occur in frozen systems for which extensive ESR evidence has been obtained (18). Optical evidence for the H-adduct of phenylalanine in a water-ethanediol glass (60) is shown in Figure 5. Reaction 15 indicates the sequence:



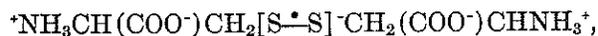
Attachment to the ring is not the exclusive fate of the electron, but it is particularly competitive with other pathways for reaction.

**ATTACHMENT TO THE PEPTIDE CARBONYL.** As the peptide length increases, the rate constant for electron reaction increases (61), indicating that reaction occurs at a peptide carbonyl. The resulting radical can be protonated depending on the pK for the following equilibrium (62):



ESR evidence for this electron adduct of the carbonyl group in frozen solutions of acetylamino acids and di- and tripeptides is extensive (50). A typical spectrum is shown in Figure 6 for the anion radical of  $\beta$ -alanylglycine at  $-153^\circ\text{C}$ . Depending on the peptide and pH, deamination can occur by a process involving either inter- or intramolecular electron transfer. Dissociation at other C—N bonds is also possible, leading to an amide and a "fatty acid" radical (20, 63); this process will be referred to as "deamidation" or "secondary deamination." As will be mentioned below, this reaction and the chemistry that follows are important for peptides and proteins.

**ATTACHMENT TO SULFUR GROUPS.** Of all the amino acids, the two most reactive in solution toward the electron are cystine and cysteine. Reaction with the former leads to the disulfide anion radical,



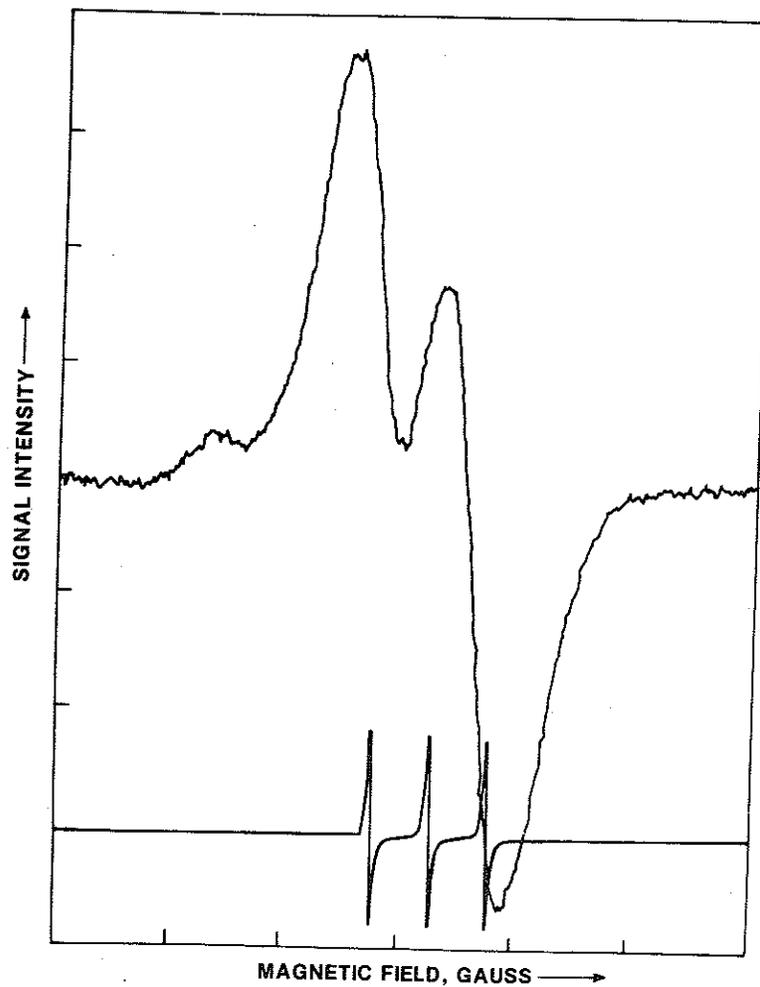
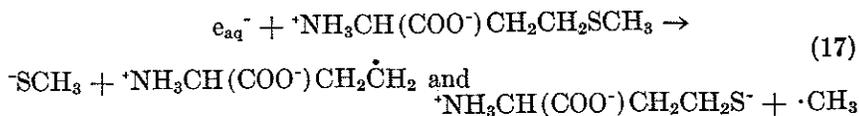


Figure 6. ESR spectrum of the peptide anion radical derived from electron attachment to  $\beta$ -alanylglycine at  $-196^\circ\text{C}$  in a LiCl glass. Sample contained  $5 \times 10^{-2}\text{M}$  of  $\beta$ -alanylglycine and the electrons were generated photolytically (64). Spectrum was recorded at  $-153^\circ\text{C}$  and is attributed to the radical  $^+\text{ND}_s\text{-CH}_2\text{CH}_2\text{CO}^-\text{NDCH}_2\text{CO}_2^-$ .

and with the latter, to  $^{\cdot}\text{NH}_3\text{CH}(\text{COO}^-)\text{CH}_2\cdot$  and  $\text{HS}^-$ . Reaction with the  $-\text{CH}_2\text{SCH}_3$  group in methionine also occurs and appears to produce two types of radicals:



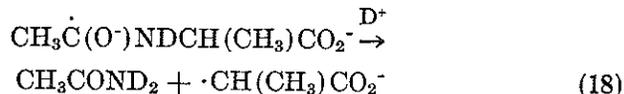
ESR studies of the amino acids in glasses confirm the formation of the disulfide anion (20), which is especially stable, and the methyl radical (20). Similar reactions may be expected to occur directly or indirectly on peptides.

**COMPETITION FOR ELECTRONS BY REACTIVE GROUPS ON PEPTIDES.** Since there are many sites for reaction on peptides, the fate of the electron will be influenced by the specific moieties and their disposition. Several illustrations can be given. For peptides with an aromatic group, deamination competes with ring attachment, but peptide carbonyl attachment predominates as the number of peptide groups increases. In the series (a) GlyPhe, (b) PheGly, and (c) PheGlyGly: deamination is preferred for (a), ring attachment and deamination are equivalent for (b), and peptide attachment begins to compete with these two processes in (c). Furthermore, ring attachment is exclusive in HisGly, but about 40% of the electrons react with GlyHis to deaminate it. In acetylpeptides, for which no sites for deamination exist, electron attachment to the peptide carbonyl predominates, but competition by methionine, cysteine, phenylalanine, tyrosine, tryptophan, glutamic acid, and aspartic acid is extensive. Detailed comparisons of these processes will be reported elsewhere (64).

**Reaction of Peptide Radicals.** On the basis of the kind of physico-chemical evidence presented here and of the chemical evidence described elsewhere (46, 47), it is apparent that the various radicals formed initially in the irradiation of peptides and proteins convert to other radicals that subsequently react to form products. Conversion of cation radicals has already been mentioned. Conversions of the peptide  $\alpha$ -carbon radicals are especially important to understanding the radiolysis of proteins, so some illustrative examples will be given. The eventual reaction of the  $\alpha$ -carbon radicals is not well understood, but certain assumptions can be made relevant to the systems studied.

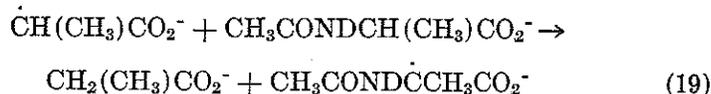
**DECOMPOSITION OF THE PEPTIDE CARBONYL RADICALS.** Depending on the nature of the constituent groups, these radicals can decompose by transferring the electron to the terminal amino group or by splitting off an amide. Both processes, deamination and deamidation, lead to the formation of  $\cdot\text{CHRCONH}-$  radicals, deamination corresponding to chain

scission. As an illustration, Figure 7 shows the spectra for D<sub>2</sub>O ices of *N*-acetylalanine (190 mg/ml) irradiated at -196°C and then annealed (64). The carbonyl anion predominates in the spectrum after annealing at -153°C (to eliminate OD·) and amounts to about 65% of the radicals; the next most abundant radical is the decarboxylated species. Upon annealing to -80°C, the anion converts by deamidation to the amide and fatty acid radical:



The spectrum shown can be compared with that of the propionic acid radical formed independently or by the deamination of alanine. This sequence is representative of several acetylamino acids studied.

**ABSTRACTION OF H FROM THE PEPTIDE BY CARBON RADICALS.** The carbon radicals derived from deamination, deamidation, and decarboxylation react subsequently with the peptides to abstract hydrogen, forming the more stable  $\alpha$ -carbon peptide radicals (63). This reaction can be demonstrated in irradiated ices containing acetylamino acids. Continuing with the *N*-acetylalanine example, one can also see in Figure 7 that upon further annealing to -50°C, another radical appears corresponding to reaction 19:



The quartet spectrum shown is assigned to the radical from acetylalanine. The same type of reaction occurs with dipeptides. Consequently, a series of aliphatic dipeptides in D<sub>2</sub>O ices were irradiated and the resultant radicals at approximately -50°C were examined to obtain detailed spectral data for comparison with proteins. Table II shows the substituents on the model peptide radical,  $\cdot\text{ND}_3\text{CH}(\text{R}')\text{COND}\dot{\text{C}}\text{RCO}_2^-$ , and the type of spectra observed. Because peptides with R = H or CH<sub>2</sub>R'' predominate in proteins and because these would give rise to doublet splittings at low temperatures, it is understandable why the irradiated protein spectra for actomyosin, myosin, and others are characterized by a broad doublet signal. Computer simulation of the protein signal by properly combining the peptide spectra is underway.

**BIMOLECULAR REACTION OF THE  $\alpha$ -CARBON RADICALS.** The eventual formation of stable covalent bonds requires that protein  $\alpha$ -carbon radicals (whose distribution is determined by molecular conformation and the conditions) combine or disproportionate with similar or other radicals.

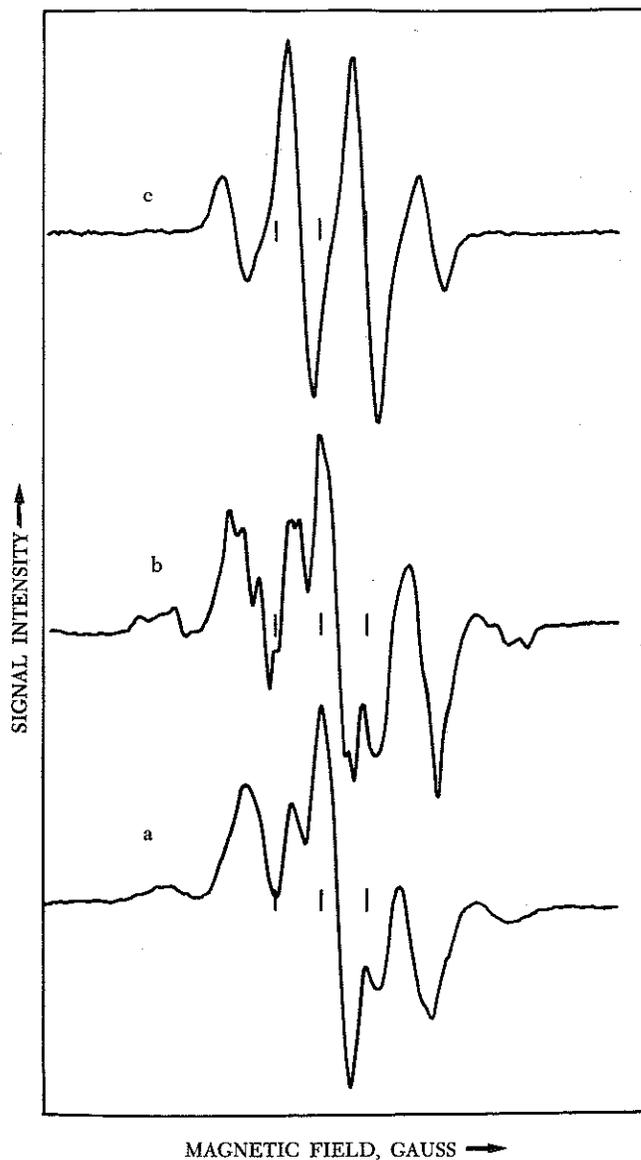


Figure 7. ESR spectra of radicals derived from N-acetyl-L-alanine in an irradiated  $D_2O$  ice plug. Sample contained 190 mg/ml of N-acetylalanine and was irradiated to 5 kGy at  $-196^\circ C$ . Spectrum a was recorded at  $-196^\circ C$ ; spectra b and c at  $-135^\circ C$ . All spectra have markers from Fremy's salt superimposed. (a) Composite spectrum of radicals present after annealing at  $-153^\circ C$  (approximately 65% corresponds to the anion). (b) Composite spectrum of the radicals formed upon annealing the ice plug to  $-80^\circ C$ , of which 55% corresponds to the fatty acid radical,  $\cdot CH(CH_3)CO_2^-$ . (c) Spectrum of the peptide radical,  $CH_3CONDC(CH_3)CO_2^-$ , formed upon further annealing the ice plug to  $-50^\circ C$ .

Table II. ESR Spectral Features for Different  
 ${}^1\text{ND}_3\text{CH}(\text{R}')\text{CONDC}(\text{R})\text{CO}_2^-$  Radicals<sup>a</sup>

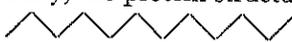
Parent Dipeptide	R'	R	Number of Lines <sup>b</sup>
Glycylglycine	H	H	2
Alanylglycine	CH <sub>3</sub>	H	2
Glycylalanine	H	CH <sub>3</sub>	4
Alanylalanine	CH <sub>3</sub>	CH <sub>3</sub>	4
Glycylglutamic acid	H	CH <sub>2</sub> CH <sub>2</sub> CO <sub>2</sub> <sup>-</sup>	2
Glutmylglutamic acid	CH <sub>2</sub> CH <sub>2</sub> CO <sub>2</sub> <sup>-</sup>	CH <sub>2</sub> CH <sub>2</sub> CO <sub>2</sub> <sup>-</sup>	2
Glycylaspartic acid	H	CH <sub>2</sub> CO <sub>2</sub> <sup>-</sup>	2
Glycylmethionine	H	(CH <sub>2</sub> ) <sub>2</sub> SCH <sub>3</sub>	2
Glycylserine	H	CH <sub>2</sub> OH	2
Lysyllsine	(CH <sub>2</sub> ) <sub>4</sub> ND <sub>3</sub> <sup>+</sup>	(CH <sub>2</sub> ) <sub>4</sub> ND <sub>3</sub> <sup>+</sup>	2

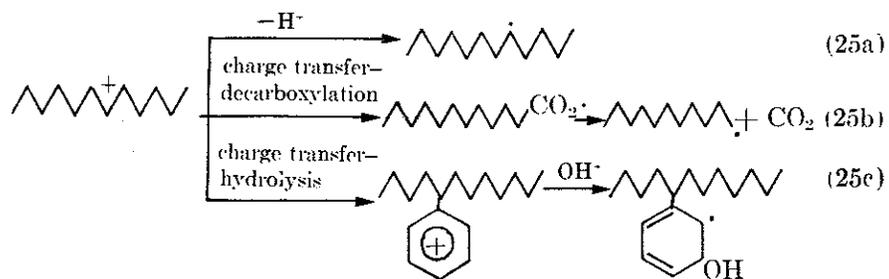
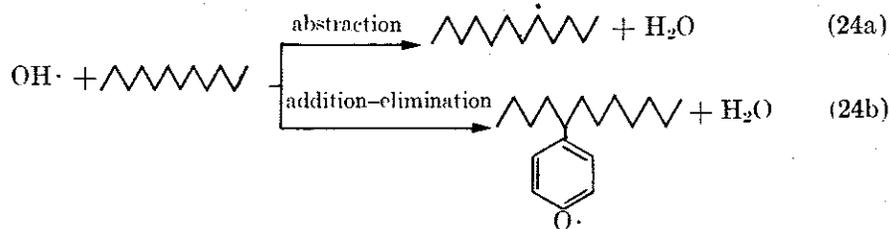
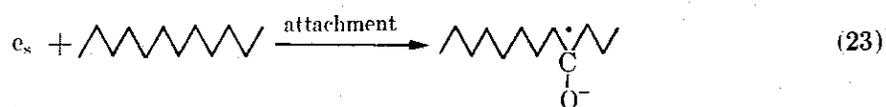
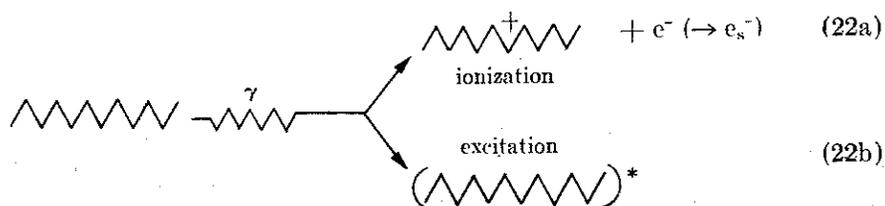
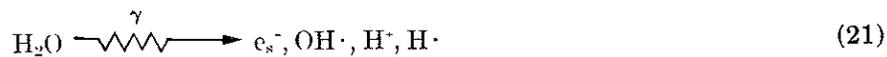
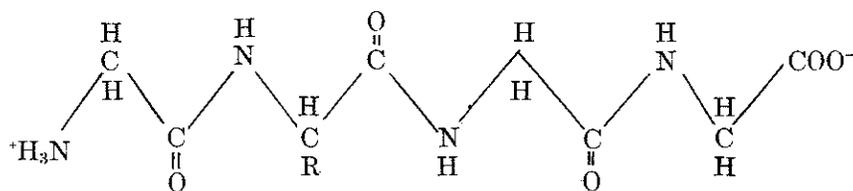
<sup>a</sup> Species stable after  $\gamma$ -irradiating approximately 200 mg/ml of the corresponding dipeptide in ice plugs at  $-196^\circ\text{C}$  to a dose of about 5 kGy and annealing to  $-50^\circ\text{C}$ .

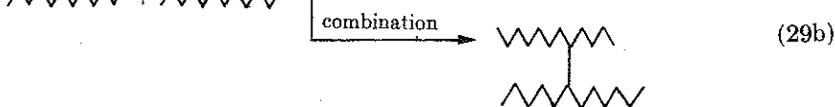
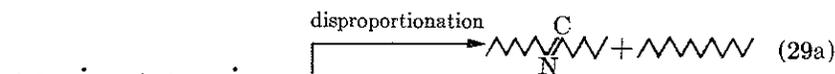
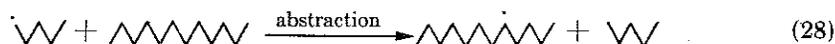
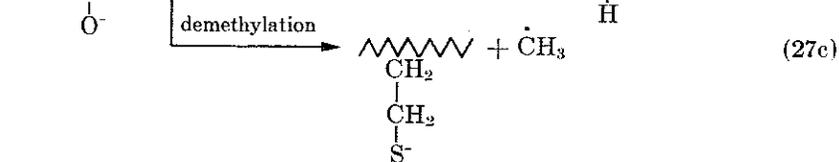
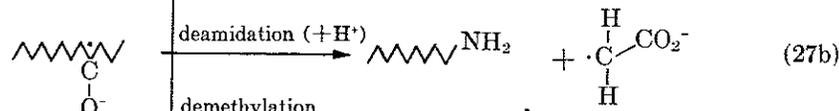
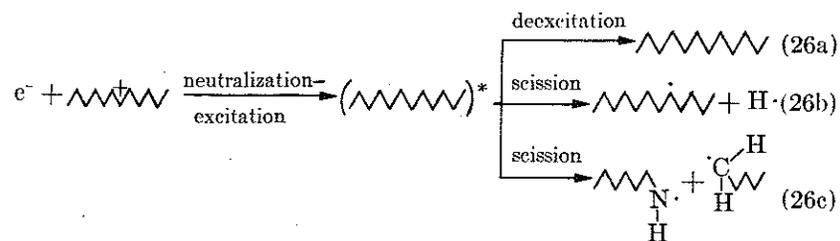
<sup>b</sup> Hyperfine splittings are found to be 18–20 gauss for all the radicals observed.

Their large size and relative immobility would render such reactions slow in fluid media and improbable in viscous or rigid media. Reaction in dry systems and in frozen systems would involve either small mobile radicals diffusing to the larger radicals or free radical sites on the large molecules being in proximity to each other. Fragment radicals such as CH<sub>3</sub>,  $\dot{\text{C}}\text{HRCO}_2^-$ , or  $\dot{\text{S}}\text{CH}_3$  could account for some of the decay. For large, globular polypeptides or proteins, the radicals on folded-over chains could combine if close enough. For the long, fibrous molecules, the radicals on neighboring chains might be able to react if sufficient bending occurs to place these sites in an appropriate disposition. Since myosin has two chains entwined about each other, some cross reaction might be expected. If the proteins are desiccated there should be some unraveling in the structure, making reaction even less likely. Significantly more kinetic information is needed before these final steps in the radiolytically initiated sequence of reactions are understood.

### Summary

Many of the reactions that occur in the specific systems described are common to most irradiated proteins. These reactions are summarized in the generalized scheme given below. No attempt is made to show all possible pathways for reaction or to explain all of the observations noted for proteins. For the sake of clarity, the protein structure is idealized and schematically represented by , which is equivalent to (20) (details in the structure are included only for specific cases):





As is implied in this scheme, there will be certain modifications in the protein resulting from irradiation that depend on specific conditions. The indirect effects of reactions of primary water radical, as well as those resulting from unimpeded diffusion of secondary radicals, will be minimized or eliminated in dry systems or frozen aqueous systems. Formation of the peptide radical by long chain, fatty acid-type radicals abstracting hydrogen could occur in fluid, concentrated solutions, but would be difficult in rigid media, being limited to either neighboring molecules or proximal parts of the same molecule. Effects on globular and fibrous

proteins will differ for this reason. Final reaction of the large peptide radicals, in turn, would be affected by the constraints of the medium on their flexing and diffusional motions. Such reactions in frozen, hydrated, fibrous proteins might involve only neighboring radicals brought close enough together by flexing and torsional modes of motion. In dry systems, the interaction of these would be more difficult because of a less favorable disposition of the chains.

Since the proteins in food preserved by irradiation to approximately 40 kGy at  $-40^{\circ}\text{C}$  are hydrated and fixed in a rigid medium, the observation (6) that these proteins are minimally affected is consistent with the implications of this scheme. The major free radicals derived from the protein are of the peptide backbone type, and they do not persist in the thawed product. These radicals apparently undergo reconstitutive recombination reactions, so no significant degradation or covalent aggregation of the long molecular chain is observed. Other free radicals from side chain groups represent a small contribution to the total, and consequently, none of the amino acids is discernibly affected. The limited extent to which changes occur in the proteins, as well as in the equally important lipid components (65, 66, 67), explains the high quality of the low-temperature irradiated meat, fish, and poultry products.

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