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Reaction Mechanisms in the Radiolysis of Peptides, Polypeptides and Proteins[1]

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Abstract v

I. Introduction. 1

II. Main-chain Chemistry of Peptides in Aqueous Solution. 3

A. Background chemistry. 3

B. Glycine and alanine peptides. 6

III. Side-chain Chemistry of Peptides in Aqueous Solution. 17

A. Aliphatic residues. 17

B. Aromatic-unsaturated residues 23

C. Sulfur-containing residues. 29

IV. Main-chain Chemistry of Proteins in Aqueous Solution. 34

V. Side-chain Chemistry of Proteins (and Nucleoproteins) in Aqueous Solution. 39

A. Sulfur-containing residues. 39

B. Aromatic-unsaturated residues 45

C. Aliphatic residues. 47

VI. Solid Peptides, Polypeptides and Proteins 50

A. Peptides and polypeptides 50

B. Proteins. 53

References. 56

Acknowledgement 65

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Abstract

The purpose of this review is to bring together and to correlate the wide variety of experimental studies that provide information on the reaction products and reaction mechanisms involved in the radiolysis of peptides, polypeptides and proteins (including chromosomal proteins) in both aqueous and solid-state systems. The comparative radiation chemistry of these systems is developed in terms of specific reactions of the peptide main-chain and the aliphatic, aromatic-unsaturated and sulfur-containing side-chains. Information obtained with the various experimental techniques of product analysis, competition kinetics, spin-trapping, pulse radiolysis and ESR spectroscopy is included.

I. Introduction

The major emphasis in radiation biology at the molecular level has been on the nucleic acid component of the chromosome because of its primary genetic importance (Hütterman et al., 1978; Ward, 1975). But there is increasing evidence that radiation damage to the conjugated nuclear proteins also has important biological implications. It has been shown that radiation damage to the deoxynucleoprotein complex in dilute aqueous solution (oxygenated) is confined almost exclusively to the protein component as evidenced by quantitative measurements of amino acid loss (Robinson et al., 1966 a, b) Damage to capsid protein now appears to be an important factor in the inactivation of phage and other viruses by both the indirect and the direct actions of ionizing radiations (Ward, 1980; Becker et al., 1978; Clarkson and Dewey, 1973). Other studies have established that covalent DNA-protein crosslinks are formed when whole cells or mixtures of DNA and cell proteins (histones etc.) are exposed to γ and UV radiation (Smith, 1976; Mee and Adelstein, 1979; Mee and Adelstein, 1981; Cress and Bowen, 1983). The histone nuclear-proteins are the most highly conserved of all proteins studied so far; the evidence is that the H3, H4 sequences have been essentially unchanged through the evolution of the eukaryotes (Bradbury et al., 1981). The implication is that radiation chemical damage to a single amino acid residue of histone could lead to a modification of a biological function of the DNA-histone complex.

Knowledge of the radiation chemistry of proteins and related compounds is also of basic importance in other fields such as in

studies of the mechanism of enzyme function (Adams and Wardman, 1977), electron migration in proteins (Klapper and Faraggi, 1981; Schafferman and Stein, 1975) and in the application of radiation sterilization to foods, drugs and other medical products. Recent findings that a class of compounds, the α,α'-diaminodicarboxylic acids, not normally present in food proteins, are produced in protein radiolysis (Boguta and Dancewicz, 1981) has re-emphasized the need for more extensive information on the production of amino acid dimers in the radiation sterilization of high-protein foods as proposed several years ago in a radiation-chemical evaluation of the food irradiation program (Garrison, 1979, 1981). The α,α'-diamino acids occur naturally in the glycoprotein (peptidoglycan, chemotype components I and II) of the bacterial cell wall (Ghuysen and Shockman, 1973; Azuma et al., 1976). Simple peptide derivatives of certain α,α'-diaminodicarboxylic acids have recently been shown to have immunological activity (Kitaura, 1979).

The purpose of this review is to bring together and to correlate the wide variety of experimental studies that provide information on the reaction products and reaction mechanisms involved in the radiolysis of peptides, polypeptides and proteins (including chromosomal proteins) in both aqueous and solid-state systems. The comparative radiation chemistry of these systems is developed in terms of specific reactions of the peptide main-chain and the aliphatic, aromatic-unsaturated and sulfur-containing side-chains. Information obtained with the various experimental techniques of product analysis, competition kinetics, spin-trapping, pulse radiolysis and ESR spectroscopy is included.

II. Main-chain Chemistry of Peptides in Aqueous Solution

A. Background Chemistry

Studies of the chemical actions of ionizing radiations on the peptide main-chain have evolved from earlier studies of the radiation chemistry of the simpler α-amino acids as monomers both in aqueous and solid state systems.

Chemical change in dilute aqueous solution is initiated by the radiation induced decomposition of water (Draganic and Draganic, 1971; Spinks and Woods, 1976)

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

where e^-_{aq} represents the hydrated electron. For γ rays and fast electrons the 100 eV yields (G) of the radical products correspond to $G(OH) = 2.8$, $G(e^-_{aq}) \simeq 2.7$, $G(H) \simeq 0.55$. The reactions of the major radical products e^-_{aq} and OH with the amino acids glycine and alanine in oxygen free solution yields ammonia, keto acid and fatty acid as major products. Detailed chemical studies of these systems including the use of second solutes for the preferential scavenging of e^-_{aq} and OH led to identification of the principal reaction modes (Weeks et al., 1965; Garrison, 1968)

$$e_{aq} + NH_3^+CH(R)COO^- \longrightarrow NH_3 + \dot{C}H(R)COO^- \qquad (2)$$

$$OH + NH_3^+CH(R)COO^- \longrightarrow H_2O + NH_3^{+\bullet}C(R)COO^- \ ; \qquad (3)$$

subsequent reactions include

$$\dot{C}H(R)COO^- + NH_3^+CH(R)COO^- \longrightarrow CH_2(R)COO^- + NH_3^{+\cdot}C(R)COO^- \qquad (4)$$

$$\dot{C}H(R)COO^- + NH_3^{+\cdot}C(R)COO^- \longrightarrow CH_2(R)COO^- + NH_2^+{=}C(R)COO^- \qquad (5)$$

$$2\ NH_3^{+\cdot}C(R)COO^- \longrightarrow NH_2^+{=}C(R)COO^- + NH_3^+CH(R)COO^- \quad . \qquad (6)$$

A small fraction of the $NH_3^{+\cdot}C(R)COO^-$ radicals undergo dimerization to yield α,α'-diaminosuccinic acid. The labile imino acid derivative produced in the disproportionation steps 5,6 hydrolyzes spontaneously

$$H_2O + NH_2^+{=}C(R)COO^- \longrightarrow NH_4^+ + RCOCOO^- \qquad (7)$$

The overall stoichiometry of reactions 2-7 gives

$$G(NH_3) \simeq G(RCOCOOH) + G(CH_2RCOOH) \simeq 5$$

which corresponds very closely to the experimentally observed values.

In an extension of these studies (Willix and Garrison, 1967; Garrison, 1968) it was found that amino acids such as β-alanine and ε-aminocaproic do not undergo the reductive deamination reaction 2. Nor do the simpler aliphatic amines such as ethylamine. It was concluded that e^-_{aq} adds to the C=O bond of the simpler α-amino acids

$$e^-_{aq} + NH_3^+CH(R)COO^- \longrightarrow NH_3^+CH(R)\dot{C}\begin{matrix} O^- \\ O^- \end{matrix} \qquad (2a)$$

and that the radical cation intermediate then dissociates

$$NH_3^+CH(R)\dot{C}\begin{matrix} O^- \\ O^- \end{matrix} \longrightarrow NH_3 + \dot{C}H(R)COO^- \qquad (2b)$$

If there is more than one carbon unit between the amino and carbonyl groups reductive deamination does not occur.

The radical products of reactions 2,3 have since been studied quite extensively by the pulse radiolysis technique (Neta and Hayon, 1970). Reactions 2a, 2b have also been observed in ESR studies of the reactions of photogenerated electrons with amino acids in aqueous glasses at low temperatures (Sevilla, 1970).

With the aliphatic α-amino acids of higher molecular weight, i.e., with α-amino butyric, valine, leucine, etc., the reductive deamination reaction 2 continues to represent a major path for removal of e^-_{aq} (Garrison, 1972). However, with the longer aliphatic side chains the attack of OH via reaction 3 is no longer confined to the C-H bond at the α-carbon position, other C-H bonds along the side chain also become involved. With the unsaturated α-amino acids such as phenylalanine, tyrosine and histidine, the side chain represents a major competing locus for reaction of both e^-_{aq} and OH (Chrysochoos, 1968; Armstrong and Swallow, 1969; Bansal and Sellers, 1975; Simic and Hayon, 1975). With the amino acid, cysteine, the reactions of e^-_{aq} and OH occur exclusively at the SH function (Wilkening et al., 1968). Detailed reaction mechanisms involved in the radiolysis of the various amino acid side chains are treated in detail in Part III.

The presence of dissolved O_2 at a sufficiently high relative concentration results in the blocking of the reductive deamination reaction 2 since the hydrated electron, e^-_{aq}, is preferentially scavenged to yield the hydroperoxy radical, HO_2

$$e^-_{aq} + O_2 \longrightarrow O_2^- \tag{8}$$

$$O_2^- + H_2O \rightleftharpoons HO_2 + OH^- \tag{9}$$

$$2HO_2 \longrightarrow H_2O_2 + O_2 \tag{10}$$

The OH reaction is not inhibited by O_2 and in the case of glycine and alanine the α-carbon radicals $NH_3^+\dot{C}(R)COO^-$ formed in reaction 3 reacts with O_2 to yield ammonia and keto acid

$$O_2 + NH_3^+\dot{C}(R)COO \longrightarrow NH_2^+{=}C(R)COO^- + HO_2 \tag{11}$$

$$H_2O + NH_2^+{=}C(R)COO^- \longrightarrow NH_4^+ + RCOCOO^- \tag{12}$$

The product stoichiometry in oxygenated solution is approximated by $G(NH_3) \simeq G(\text{Carbonyl}) \simeq G(OH) \simeq 3$ (Garrison, 1968).

B. Glycine and Alanine Peptides

1. Oxygenated Solutions.

Specific chemical evidence for oxidative degradation of the peptide main chain through OH attack at a C-H bonds along the chain to yield amide and keto acid functions was first represented in terms of the overall stoichiometry (Jayko et al., 1958; Garrison et al., 1962; Garrison et al., 1970).

$$RCONHCHR_2 + O_2 + H_2O \longrightarrow RCONH_2 + R_2CO + H_2O_2 \tag{13}$$

The proposed reaction mechanism includes the radiation induced step 1 followed by:

$$OH + RCONHCHR_2 \dashrightarrow H_2O + RCONH\dot{C}R_2 \quad (14)$$

$$O_2 + RCONH\dot{C}R_2 \dashrightarrow RCONHC(\dot{O}_2)R_2 \quad (15)$$

The reducing species e^-_{aq} and H are scavenged preferentially by O_2 to give O_2^- and HO_2 which are related by the equilibrium $HO_2 \rightleftarrows H^+ + O_2^-$. The subsequent steps were written:

$$HO_2 + RCONHC(\dot{O}_2)R_2 \dashrightarrow RCONHC(OOH)R_2 + O_2 \quad (16)$$

$$H_2O + RCONHC(OOH)R_2 \dashrightarrow RCONHC(OH)R_2 + H_2O_2 \quad (17)$$

$$RCONHC(OH)R_2 \underset{\longleftarrow}{\dashrightarrow} RCON{=}CR_2 + H_2O \quad (17a)$$

$$H_2O + RCONHC(OH)R_2 \dashrightarrow RCOOH + NH_3 + R_2CO \quad (18)$$

Much of the subsequent information on the detailed mechanisms of oxidative main chain degradation has been derived from studies involving the the peptide derivatives of the simpler α-amino acids glycine and alanine. Radiolysis of N-acetylglycine and N-acetylalanine in oxygenated solution results in the formation of labile peptide derivatives which are readily degraded on mild hydrolysis to yield ammonia and carbonyl products (keto acid plus aldehyde). Free ammonia is not a major initial product in the radiolysis of these systems. If the radiation degradation of the peptide main chain does occur predominantly through

the sequence formulated in eq. 1, 14-18, then it is clear that ammonia and carbonyl yields with γ-rays should be in the relationship $G(NH_3) \simeq G(R_2CO) \simeq G(OH) \simeq 3$. Quantitative assays of the ammonia and carbonyl yields from a series of model peptide derivatives including acetylglycine, acetylalanine, glycine anhydride, etc., consistently show $G(NH_3) \simeq 3$ for each system (Atkins et al., 1967; Garrison et al., 1970). However, it was also found that the yield of carbonyl products from these simple peptide systems is not in accord with the quantitative requirements of the reaction sequence 14-18. The carbonyl yields (keto acid plus aldehyde) are consistently low with $G(R_2CO) \simeq 1$. There was, then, the question as to whether this apparent discrepancy arises from (a) an incorrect formulation of the locus of initial OH attack or from (b) an unspecified complexity in the chemistry of removal of the peroxy radicals $RCONHC(\dot{O}_2)R_2$.

To obtain specific information on these questions, the radiolysis was carried out using ferric ion instead of O_2 as the scavenger of intermediate radicals formed by OH attack on N-acetylglycine and N-acetylalanine (Atkins et al., 1967). Heavy metal ions FeIII and CuII oxidize organic free-radicals in aqueous solution by electron transfer and by ligand transfer. Such reactions in the case of the peptide radical $RCONH\dot{C}(R_2)$ would correspond to

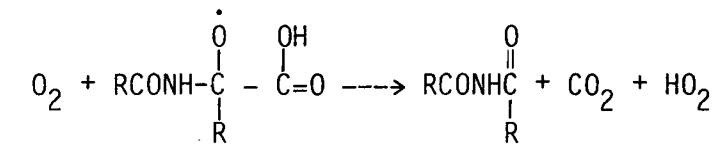
$$FeIII(H_2O) + RCONH\dot{C}R_2 \begin{cases} \nearrow RCON{=}CR_2 + FeII + H^+ + H_2O \\ \searrow RCONHC(OH)R_2 + FeII + H^+ \end{cases} \qquad (19)$$

The oxidation product of these reactions are identical to the postulated product of reactions 16-18 and would then yield amide and carbonyl on hydrolysis. It was found that the γ-radiolysis of 0.1M N-acetylglycine and N-acetylalanine in O_2-free solution containing 0.05 M FeIII gives the product stoichiometry $-G(\text{peptide}) \simeq G(NH_3) \simeq G(RCOCOOH) \simeq 3.2 \simeq G(OH) + G(H_2O_2)$. In the presence of FeIII the values $G(NH)_3$ and G(RCOCOOH) are greater than G(OH) since molecular hydrogen peroxide formed in the radiation step 1, yields additional OH through reaction with FeII (generated in step 19) via $FeII + H_2O_2 \longrightarrow FeIII + OH + OH^-$. The fact that much lower carbonyl yields are obtained with O_2 in place of FeIII shows then that the chemistry of removal of the peroxy radicals $RCONHC(\dot{O}_2)R_2$ is indeed more complicated than that represented in reactions 15-18.

More complete and detailed examination of the oxidation products formed in the γ-radiolysis of the peptides in oxygenated solutions have established that organic products in addition to keto acid and aldehyde are present (Garrison et al., 1970). In the case of N-acetylalanine the organic products identified include pyruvic acid, acetaldehyde, acetic acid and carbon dioxide. The evidence is that the reaction of the peroxy radicals $RC\dot{O}NHC(O_2)R_2$ with HO_2 via step 16 occurs in competition with

$$2\ RCONHC(\dot{O}_2)R_2 \longrightarrow 2\ RCONHC(\dot{O})R_2 + O_2 \qquad (20)$$

In the case of the N-acetyl amino acids, the alkoxy radicals formed in step 20 are removed in turn via



(21)

to yield a diacetamide derivative. In the case of N-acetylalanine the diacetamide product RCONHCOR is hydrolytically labile, and under mild differential hydrolysis in dilute base at room temperature is converted to acetamide and acetic acid.

$$H_2O + RCONHCOR \longrightarrow RCONH_2 + RCOOH$$

The parent N-acetylalanine is stable under this condition. In this way, it was possible to quantitatively separate and measure all of the products formed in the γ-radiolysis of O_2-saturated N-acetylalanine solutions:

$$G(CH_3COCOOH + CH_3CHO) \simeq 1,\ G(CH_3COOH) \simeq 2.5,\ G(CO_2) \simeq 2.$$

The formation of several compounds with different oxidation states as <u>initial</u> products is frequently observed in oxidation processes involving peroxy radicals (Howard, 1966).

The reactions of OH with the dipeptide derivatives of glycine and alanine occur preferentially at the peptide C-H linkage:

$$OH + NH_3^+CH(R)CONHCH(R)COO^- \longrightarrow H_2O + NH_3^+CH(R)CONH\dot{C}(R)COO^- \quad (22)$$

The chemical evidence is that ~90 percent of the OH radicals are so removed (Makada and Garrison, 1972). With the tri, tetra, and poly derivatives the reaction of OH at peptide C-H linkages is essentially quantitative. The formation of "peptide" radicals ~CONHC(R)$\dot{C}$ONH~ through the reaction of OH with oligopeptides has been extensively studied by pulse radiolysis and spin-trapping techniques (Rao and Hayon, 1975; Joshi et al., 1978).

In the presence of oxygen

$$O_2 + NH_3^+CH(R)CONH\dot{C}(R)COO^- \longrightarrow NH_3^+CH(R)CONHC(\dot{O}_2)(R)COOH \qquad (23)$$

The subsequent chemistry is, in part, quite analogous to that observed in the radiolytic oxidation of N-acetylamino acids via the sequence 14-18, 20, 21 to yield amide and the lower fatty acid. However, with the oligopeptides, the amide yield approaches $G(NH_3) \simeq 5$ which is considerably higher than the value $G(NH_3) \simeq 3 \simeq G(OH)$ observed with the N-acetylamino acids. The chemical evidence is that with oligo and polypeptides an <u>intramolecular</u> reaction occurs.

$$\begin{array}{ccc} \sim(R)CH & & \dot{O}C(R)\sim \\ & \diagdown\ CONH\ \diagup & \end{array} \longrightarrow \begin{array}{ccc} \sim(R)\dot{C} & & HOC(R)\sim \\ & \diagdown\ CONH\ \diagup & \end{array} \qquad (24)$$

This leads to formation of additional ammonia and keto acid. With diglycine the major product stoichiometries correspond to $G(NH_3) \simeq 4.8$, $G(HCOOH) \simeq 1.7$, $G(CHOCOOH) \simeq 1.9$ (Makada and Garrison, 1972).

With the N-acetylamino acids reaction of type 24 can only occur intermolecularly and is of negligible importance in competition with reaction 21. In agreement with reaction 24 as formulated, an analysis of the carbonyl fraction from a mixed dipeptide, glycylalanine, shows that both keto acids, glyoxylic and pyruvic are indeed formed in approximately equal amounts with a combined yield of $G(>CO) \simeq 2$ with $G(NH_3) \simeq 4.8$ (Makada and Garrison, 1972).

Product yields in the γ-radiolysis of polyalanine are consistent with the reaction sequence formulated above, i.e., $G(NH_3) \simeq 4.0$ $G(RCOCOOH) \simeq 1.2$ $G(RCOOH) \simeq 3.0$, $G(CO_2) \simeq 2.4$ (Makada and Garrison, 1972). With polyalanine at molecular weight $\simeq$3000 it must be assumed that alkoxy-radical formation via reaction 14, 15, 21 must occur more or less at random along the peptide chain. The equivalent of reaction 21 then must involve an adjacent peptide bond (enol form), i.e.,

$$O_2 + RCONH-\overset{\dot{O}}{\underset{R}{C}}-\overset{OH}{C}=N-CHR_2 \longrightarrow RCONH-\overset{O}{\underset{R}{C}} + O=C=N-CHR_2 + HO_2 \qquad (25)$$

where

$$H_2O + O=C=N-CHR_2 \longrightarrow CO_2 + NH_2CHR_2 \qquad (26)$$

follows essentially instantaneously.

2. Oxygen-free Solutions

The carbonyl group of the peptide bond represents the principle trapping center for e^-_{aq} in oxygen free solutions of peptide derivatives of glycine, alanine and most other aliphatic amino acids (Garrison, 1972; D'Arcy and Sevilla, 1979; Rao and Hayon, 1974).

$$e^-_{aq} + RCONHCHR_2 \xrightarrow{H_2O} R\dot{C}(OH)NHCHR_2 + OH^- \qquad (27)$$

Chemistry of $R\dot{C}(OH)NHCHR_2$ radicals formed in reaction 27 has been studied under a number of experimental conditions. Detailed product-analysis studies of γ-irradiated N-acetylalanine solutions at pH 7 indicate that the major process for removal of $R\dot{C}(OH)NHCHR_2$ radicals involves the back reaction (reconstitution)

$$R\dot{C}(OH)NHCHR_2 + RCONH\dot{C}R_2 \longrightarrow 2\ RCONHCHR_2 \qquad (28)$$

where $RCONH\dot{C}R_2$ corresponds to the product of OH attack via reaction 14. It was concluded that main chain cleavage via dissociative deamidation

$$RC(OH)NHCHR_2 \longrightarrow RCONH_2 + \dot{C}HR_2 \qquad (29)$$

was relatively unimportant with $G \leq 0.3$ under the experimental conditions employed. Pulse radiolysis studies of the addition of e^-_{aq} to the carbonyl group of the peptide bond do not show any

evidence for main chain cleavage via reaction 29 (Rao and Hayon, 1974; Klapper and Faraggi, 1981).

However, ESR studies of the reactions of photogenerated electrons with simple peptides in aqueous glasses at low temperatures show the addition reaction 27 which is followed by the dissociation reaction 29 as the system is warmed (Sevilla, 1970; D'Arcy and Sevilla, 1979). Reaction 29 has also been observed in a wide variety of aqueous peptide systems through use of spin trapping techniques involving t-nitroso-butane as the spin trap, i.e., (Rustgi and Riesz, 1978)

$$\dot{C}HR_2 + tBu\text{-}N{=}O \longrightarrow tBu\text{-}\overset{\overset{CHR_2}{|}}{N}\text{-}O\cdot \quad . \tag{30}$$

The differences in these findings probably involves a dose rate effect. If the rate of the first order dissociation reaction 29 is relatively slow, then the second order reconstitution reaction 28 would be favored at the higher dose rates. Both the product analysis studies and the pulse radiolysis studies were done at dose rates $\geq 10^{18}$ eV/gm min. The spin trapping studies were done at a dose rate a factor of 10 lower. In the low temperature glasses, dissociation of the "matrix isolated" $R\dot{C}(OH)NHCHR_2$ radical would be greatly favored over any diffusion controlled recombination reaction.

Addition of electron scavengers such as N_2O and H_3O^+

$$e^-_{aq} + N_2O + H_2O \longrightarrow OH + OH^- + N_2 \tag{31}$$

$$e^-_{aq} + H_3O^+ \longrightarrow H + H_2O \tag{32}$$

eliminates the possibility of the back reaction and results in the formation of the α,α'-diamino succinic acid derivatives through cross-linking at the main chain (Garrison, 1968)

$$2RCONHC\dot{R}_2 \longrightarrow \begin{array}{c} RCONHCR_2 \\ | \\ RCONHCR_2 \end{array} \qquad (33)$$

High yields of main chain cross linking with formation of α,α'-diamino acid derivatives are also observed in the radiolysis of the low molecular-weight linear oligopeptide derivatives of glycine (Bennett-Corniea et al., 1973) and alanine (Dizdaroglu et al., 1982; Dizdaroglu and Simic, 1983). In these systems, the addition of e^-_{aq} occurs preferentially at the C=O bond of the N-terminal residue.

$$e^-_{aq} + NH_3^+CH(R)CONHCR_2 \longrightarrow NH_3^+CH(R)\underset{\underset{OH}{|}}{\dot{C}}NHCR_2 + OH^- \qquad (34)$$

and this leads to deamination via

$$NH_3^+CH(R)\underset{\underset{OH}{|}}{\dot{C}}NHCR_2 \longrightarrow NH_4^+ + \dot{C}H(R)CONHCHR_2 \qquad (35)$$

Reactions 34, 35 are characteristic of compounds containing the amino group in the α-position to the carbonyl function i.e., $NH_3^+CH(R)COX$ where X = O^-, OH, OR, NHR etc (Willix and Garrison, 1967; Garrison, 1968; Sevilla, 1970: Rao and Hayon, 1974). Subsequent steps include

$$NH_3^+CH(R)CONHCHR_2 + \dot{C}H(R)CONHCHR_2 \longrightarrow$$

$$NH_3^+CH(R)CONH\dot{C}R_2 + CH_2(R)CONHCH(R_2) \qquad (36)$$

and

$$2\ NH_3^+CH(R)CONH\dot{C}R_2 \longrightarrow \begin{array}{l} NH_3^+CH(R)CONHCR_2 \\ \qquad\qquad\qquad\quad | \\ NH_3^+CH(R)CONHCR_2 \end{array} \qquad (37)$$

The yields of the reductive deamination reaction 34 with di and triglycine corresponds to $G(NH_3) \simeq 3 \simeq e^-_{aq}$. The free ammonia yield from tetraglycine is somewhat less, $G(NH_3) \simeq 2.4$. With polyalanine the free ammonia yield decreases to $G(NH_3) \simeq 0.3$. Electron addition along the peptide bond (away from the N-terminal C=O leads to the chemistry of reactions 27-29.

III. Side-Chain Chemistry of Peptides in Aqueous Solution

A. Aliphatic Residues

1. Oxygenated Solutions

Although OH attack at the glycine and alanine residues occurs almost exclusively at the α C-H position along the peptide main chain (reaction 14), with all other aliphatic amino acids the side chain represents a major competing locus of OH reaction (Joshi et al., 1978; Liebster and Kapoldova, 1966). With the alkyl series, α-aminobutyric, valine, leucine, etc., the yield for oxidative degradation of the main chain to yield amide, keto acid and fatty acid functions (Sec. II.B) decreases with increasing number of C-H bonds in the hydrocarbon chain (Garrison, 1972). Competing side-chain chemistry leads to hydroxyl and carbonyl substitution via

$$\overset{\diagup}{\underset{\diagdown}{HCH}} + OH \longrightarrow \overset{\diagup}{\underset{\diagdown}{HC}} + H_2O \tag{38}$$

$$\overset{\diagup}{\underset{\diagdown}{HC}}\cdot + O_2 \longrightarrow HC\dot{O}_2 \tag{39}$$

followed by the characteristic reactions of alkyl peroxy radicals (Garrison et al., 1970; Howard, 1966)

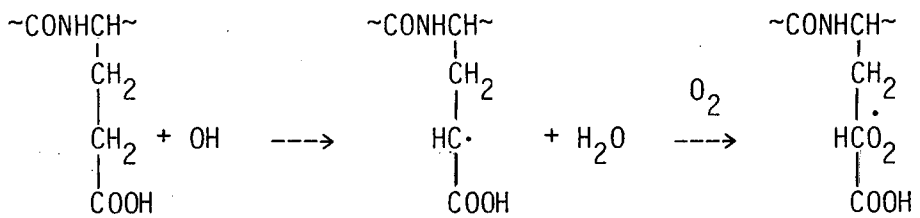
$$\overset{\diagup}{\underset{\diagdown}{HC}}\dot{O}_2 + HO_2 \xrightarrow{H_2O} \overset{\diagup}{\underset{\diagdown}{HC}}OH + H_2O_2 + O_2 \tag{40}$$

$$\overset{\diagup}{\underset{\diagdown}{HC}}\dot{O}_2 + \overset{\diagup}{\underset{\diagdown}{HC}}\dot{O}_2 \longrightarrow 2\ \overset{\diagup}{\underset{\diagdown}{HC}}\dot{O} + O_2 \tag{41}$$

$$\overset{\diagup}{\underset{\diagdown}{HC}}\dot{O} + O_2 \longrightarrow \overset{\diagup}{\underset{\diagdown}{C}}=O + HO_2 \tag{42}$$

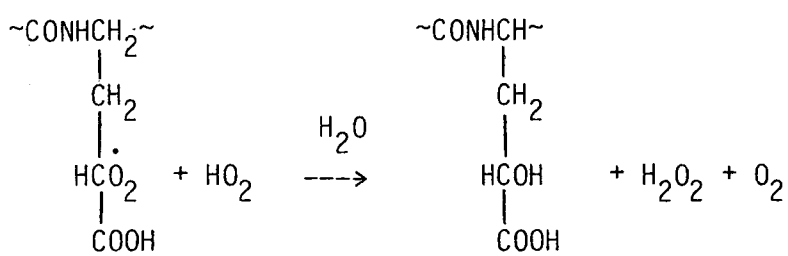
Oxidation can occur at any C-H position along the chain. Valine and leucine yield 3-hydroxy valine and 3-hydroxy plus 4-hydroxy leucine respectively as major products. Detailed chemical identifications and quantitative determinations of the various hydroxy and carbonyl products formed through OH attack at side chain loci of α-aminobutyric, valine and leucine have been made (Liebster and Kopoldova, 1966).

With the dicarboxylic amino acids aspartic and glutamic the C-H bond α to the side-chain carboxyl group represents the principal locus of OH attack in competition with the main-chain reaction 14 as observed by both product analysis and spin trapping methods (Sokol et al., 1965; Joshi et al., 1978). With N-acetylglutamic acid in oxygenated solution, main chain degradation via reactions 14-18 to yield amide and α-ketoglutaric acid functions accounts for ~30 percent of the OH radicals (Sokol et al., 1965). The remainder attack at the side chain via



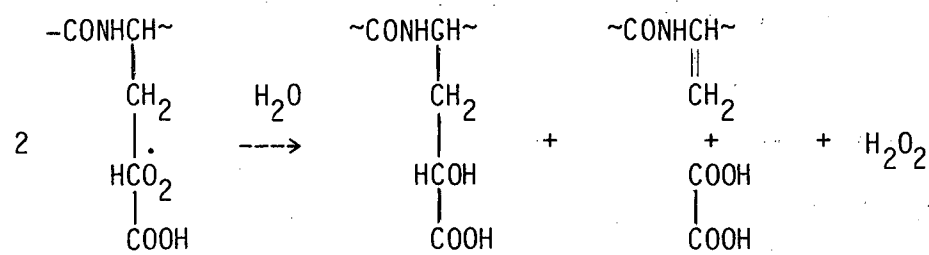
(43)

Part of the subsequent chemistry ($RO_2 + HO_2$ ---->) is similar to that formulated in equation 40 i.e.,



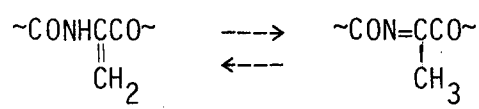
(44)

However, the analogues of the competing reactions ($2RO_2$ ---->) takes a more complicated form than that shown in equations 41,42 i.e.,



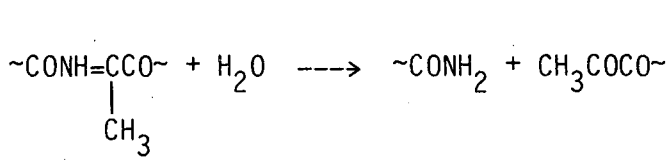
(45)

Reactions akin to the degradation reaction 45 have been observed in other systems (Howard, 1966). The unsaturated degradation product formed in reaction 45 corresponds to a class of compounds referred to as dehydropeptides.



(46)

These compounds are easily hydrolized to yield amide and keto acid functions (cf Eq. 17a)



(47)

We have here an example of a case in which OH attack at a side chain locus can lead to oxidative degradation of the peptide main chain.

Product yields in the γ-radiolysis of N-acetylglutamic acid (0.1 M, O_2-sat) are essentially independent of pH over the range pH 3 to 8 with G(amide) $\simeq$ 2.3, G(α-ketoglutaric) $\simeq$ 0.8, G(pyruvic) $\simeq$ 0.9.

Similar product yields are obtained with polyglutamic acid solutions (0.15%, O_2-sat) over the pH range 6-8. But, the amide and pyruvic acids yields from PGA decrease abruptly as the pH is lowered from 6 to 4 whereas the α-ketoglutaric yield remains essentially constant over the entire pH range 8 to 3.

In interpreting these pronounced pH effects in the polyglutamic system, it has been pointed out that one of the unique characteristics of the radiation chemistry of macromolecular substances in aqueous solution is that each (macro)molecule undergoes reaction with a relatively large number of OH radicals even at the lowest practicable dosages (Garrison, 1972). For example, with a 0.15% solution of PGA, a γ-ray dose of 3×10^{18} eV/g produces only one OH per 100 glutamic acid residues, but, at the same time this corresponds to about 20 OH radicals per PGA molecule (MW 140,000). Since PGA above pH 6 has the random coil configuration, the various segments of the macromolecule are free to interact both intermolecularly and intramolecularly, and we find at pH > 6 no essential differences between the macromolecule and the low molecular weight model from the standpoint of product yields. But, as the pH of the solution is decreased, PGA undergoes a coil $\longrightarrow$ helix transition over the pH range 6 to 4.5. This is the range over which there is an abrupt decrease in the amide and pyruvic acid yields. With PGA in the helix form, the peroxy radicals $R\dot{O}_2$ are frozen in a fixed spatial arrangement and it is obvious that the probability of reaction 45 ($2R\dot{O}_2 \longrightarrow$) is greatly reduced, reaction 44 ($RO_2 + HO_2 \longrightarrow$) is favored and as a result the yield of main chain degradation decreases as observed experimentally.

The loci of OH attack with asparagine and glutamine residues are analogous to those observed with peptide derivatives of the parent compounds aspartic acid and glutamine acid as formulated above (Joshi et al., 1978). Serine, threonine, lysine and arginine undergo oxidation at the α C-H position via reaction 14 etc., in competition with their respective side-chain reactions which also yield carbonyl products:

$$\sim CH(R)OH + OH \xrightarrow{O_2} \sim CO(R) + H_2O + HO_2 \qquad (48)$$

$$\sim CH_2NH_2 + OH \xrightarrow{O_2} \sim CHO + NH_3 + HO_2 \qquad (49)$$

$$\sim CH_2NH(NH)NH_2 + OH \xrightarrow{O_2} \sim CHO + NH_2(NH)NH_2 + HO_2 \qquad (50)$$

The reactions of OH radicals with peptide derivatives of the aliphatic amino acids outlined above, all involve the formation of carbon centered radicals through H abstraction. Such reactions are of intermediate velocity with $k \approx 10^8 - 10^9\ M^{-1}\ sec^{-1}$ (Klapper and Faraggi, 1981; Farhataziz and Ross, 1977).

2. Evacuated Solutions

The available data from product analysis, (Garrison, 1972) pulse radiolysis (Klapper and Faraggi 1981) and spin trapping studies (Rustgi and Riesz 1978) in irradiated aqueous solutions and from ESR studies (D'Arcy and Sevilla, 1979) of photogenerated electrons in aqueous glasses indicate that the carbonyl bond of most aliphatic amino acid

residues represents the major trapping center for e^-_{aq} via the addition reaction 27. This applies to (a) the alkyl amino acids, glycine, alanine, valine, leucine etc., (b) the dicarboxylic acids, aspartic, glutamic and their respective amides, asparagine and glutamine, (c) the basic amino acids, lysine and arginine, (d) the hydroxy containing amino acids, serine and threonine. These addition reactions are of intermediate velocity with k values in the 10^8 - 10^9 M^{-1} sec^{-1} (Anbar et al., 1973; Klapper and Faraggi, 1981).

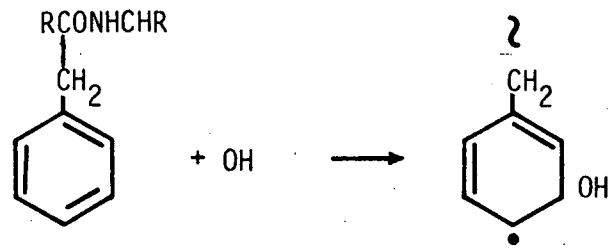
Both main chain and side chain radicals

formed through OH attacks in these systems undergo dimerization to yield, α,α'-diaminodicarboxylic derivatives. The dimerization of type I radicals from glycine and alanine residues to give α,α'-diaminosuccinic and α,α'-diaminodimethyl succinic acids respectively has been discussed. Higher molecular weight dimers such as α,α'-diaminopimelic and α,α'-diaminosuberic formed through dimerization of type II radicals have also been identified (Liebster and Kopoldova, 1966). The formation of unsaturated dimers through combination of side chain radicals derived from aromatic-unsaturated amino acid residues is discussed below.

B. Aromatic Unsaturated Residues

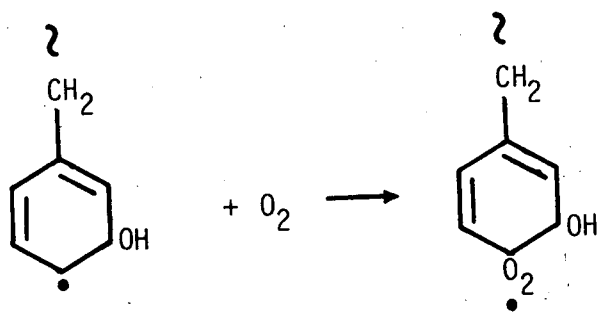
1. Oxygenated Solutions

Reactions of OH radicals with the phenylalanine residue include: (1) H-abstraction at the main chain via reaction 14 to give RCONHĊ(ϕ)R radicals (Joshi et al., 1978) and (2) addition to the aromatic side chain (Brodskaya and Sharpatyi, 1967; Dizdaroglu and Simic, 1981) e.g.,



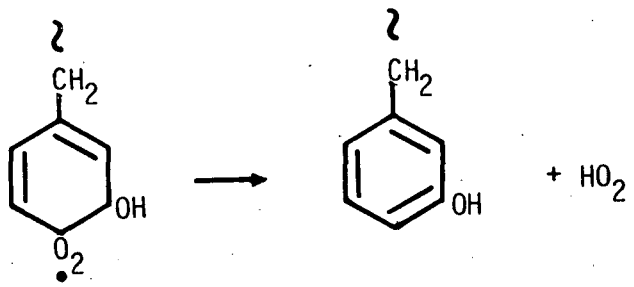
(51)

where reaction 51 represents the principal path for OH removal. In the presence of O_2, the main chain radicals, RCONHĊ(ϕ)R undergo oxidative degradation with formation of amide, phenylpyruvic acid and products of higher oxidation through reactions analogous to those formulated in equations 14-21. The hydroxycyclohexadienyl radicals formed through the OH addition reaction 51 react with O_2 to yield peroxy radical intermediates

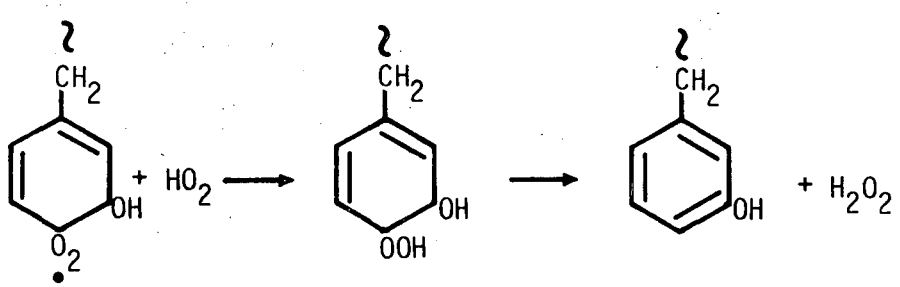


(52)

which undergo the subsequent reactions



(53)



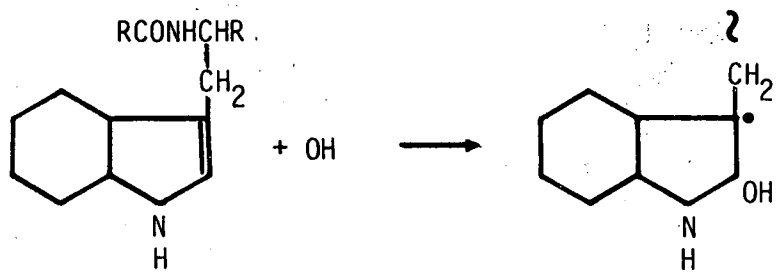
(54)

to yield tyrosine (ortho, meta, para) as the major products (Brodskaya and Sharpatyi, 1967; Balakrishnan and Reddy, 1970). In the radiolysis of aqueous benzene a fraction of the peroxy radicals formed in reaction 52 undergo rearrangement and further oxidation to yield β-hydroxy-mucondialdehyde (Balakrishan and Reddy, 1970).

The radiolytic oxidation of tyrosine residues in O_2 saturated solution appears to involve reactions analogous to those given in equations 51-54 to yield dopa, 3,4-dihydroxyphenylalanine, plus other unidentifed products (Fletcher and Okada, 1961; Lynn and Purdie, 1976).

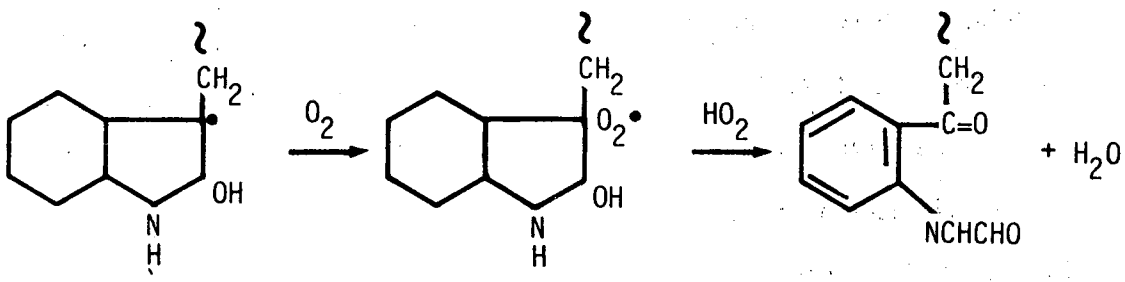
The radiolytic oxidation of the tryptophan residue in oxygenated solution arises predominantly through reactions initiated by OH

addition to unsaturated bonds of the indole moiety as evidenced by both product analysis and pulse radiolysis studies (Armstrong and Swallow, 1969; Winchester and Lynn, 1970; A. Singh et al., 1981). The addition of OH to the C2-C3 double bond of the indole heterocyclic ring e.g.,



(55)

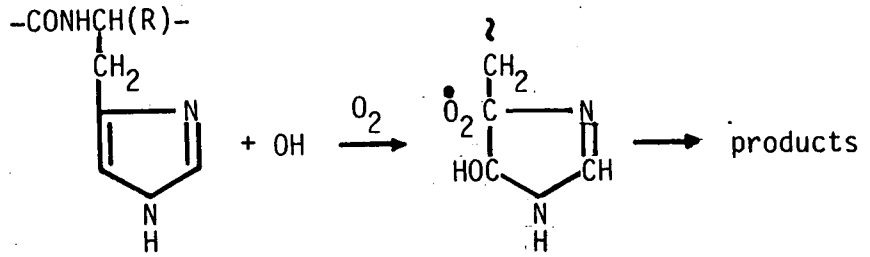
leads to formation of formylkinurenine as a major degradation product in oxygenated solution (Winchester and Lynn, 1970).



(56)

The addition of OH to the benzenoid ring leads to formation of phenolic products in lesser yield. The attack of OH radicals at the peptide main chain appears to be minimal in this system.

Product analysis, (Kopoldova and Hrneir, 1977) ESR, (Samuri and Neta, 1973) and pulse radiolysis (Rao et al., 1975; Mittal and Hayon, 1974) studies all show that the major mechanism for OH attack at the histidine residue involves addition to the imidazole ring e.g.,



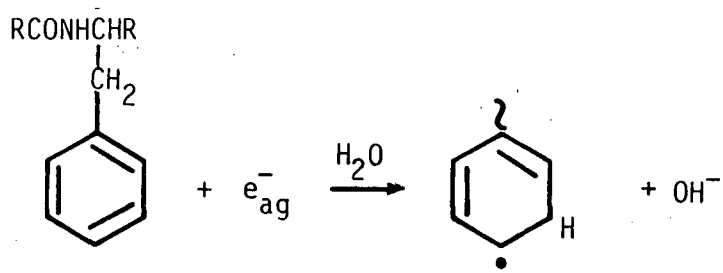
(57)

The yield for oxidative degradation of the histidine (imidazole) ring corresponds to G(-His) ~ 4 in dilute O_2-saturated solutions under γ-rays and a complexity of degradation products are observed (Kopoldova and Hrneir, 1977). Among the major products of reaction (57) are asparagine and aspartic acid. The identification of imidazolylpyruvic and imidazolylacetic acids as lesser products indicates that OH attack of the main chain via reaction 14 (followed by the analogues of reactions 14-21) is also involved in the reaction of OH radicals at the histidine residue.

The reactions of OH radicals with the phenylalanine, tyrosine, tryptophan and histidine residues are relatively fast with k-values in the range 10^9 - 10^{10} M^{-1} sec^{-1} (Farhataziz and Ross, 1977; Klapper and Faraggi, 1981).

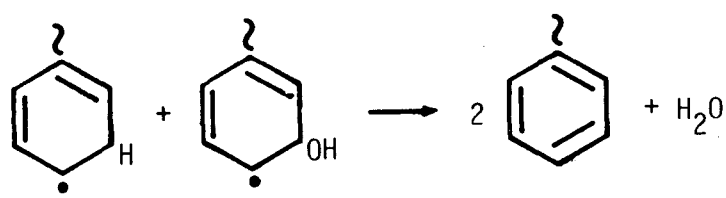
2. Oxygen free Solutions

In oxygen free solutions, both the peptide C=O bond and the benzene ring of the phenylalanine residue represent major trapping centers for the hydrated electron, e^-_{aq}, via reaction 27 and the reaction 58 (D'Arcy and Sevilla, 1979; Rustgi and Riesz, 1978; Mittal and Hayon, 1974)



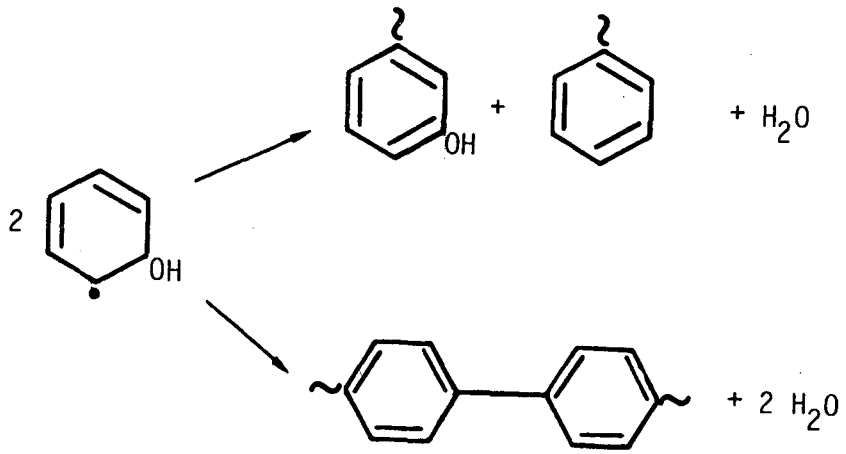
(58)

Pulse radiolysis studies of aqueous N-acetylphenylalanine indicate that ~50 percent of e^-_{aq} reacts via addition to the peptide C=O bond, the remainder add to the benzene ring via reaction 58 (Mittal and Hayon, 1974). Subsequent reactions of the e(H) adduct (Sevilla and D'Arcy, 1978) formed in step 58 and the OH adduct of step 51 above include the back-reaction



(59)

and



(60)

(61)

where the formation of tyrosine and the phenylalanine dimer via reactions 60,61 are written as analogues of the reaction scheme proposed for the production of phenol and diphenyl in the radiolysis of aqueous benzene (Eberhart, 1974; Gordon et al., 1977). Comparison of the observed yields of phenylalanine destruction G(-Phe) with the yields of phenylpropionic and tyrosine formed in the γ-radiolysis of O_2-free phenylalanine solutions indicate that the phenylalanine dimer is produced as a major product. Direct experimental evidence for the formation of phenylalanine dimers and other cross-linked products from phenylalanine peptides has been reported (Yamamoto, 1973; Yamamoto, 1977; Kim et al., 1982).

Chemistry similar to that given in reactions 58-61 appears to be involved in the radiolysis of tyrosine and tyrosine peptides in O_2-free solution. The large differences between G(-tyr) and the total yield of observed products, principally dihydroxyphenylalanine(dopa), has been interpreted as evidence for polymer(dimer)

formation in relatively high yield (Lynn and Purdie, 1976). In recent chemical and pulse radiolysis studies, a blue fluorescent product characteristic of dityrosine has been observed in irradiated solutions of tyrosine, glycyltyrosine, polytyrosine and protein (Prutz et al., 1983); Boguta and Dancewicz, 1981; Boguta and Dancewicz, 1982).

Pulse radiolysis and ESR studies indicate that e^-_{aq} reacts with tryptophan almost exclusively through addition to the indole moiety (Armstrong and Swallow, 1969; Winchester and Lynn, 1970; Moan and Kaalhus, 1974). The γ-ray yield for tryptophan destruction G(-Trp) is extremely low in O_2-free solution. The reconstruction reaction

$$\dot{trpH} + \dot{trpOH} \longrightarrow 2trp + H_2O$$

represents the principal radical-removal step in the absence of O_2.

C. Sulfur-Containing Residues

1. Oxygenated Solutions

The reaction of OH at the cysteine residue occurs preferentially at the sulfur moiety

$$RSH + OH \longrightarrow R\dot{S} + H_2O \qquad (62)$$

where reaction 62 is essentially diffusion controlled with $k \simeq 10^{10}$ M^{-1} sec^{-1} (Klapper and Faraggi, 1981; Farhataziz and Ross, 1977). In acidic oxygenated solution the overall stoichiometry corresponds to:

$$2RSH + O_2 \longrightarrow RSSR + H_2O_2 \qquad (63)$$

(Packer and Winchester, 1970; Barton and Packer, 1970; Lal, 1974;

Al-Thannon et al., 1974). In the γ-radiolysis of 10^{-3} M cysteine in O_2-saturated solution at pH 3, G(-RSH) ≃ 10, G(RSSR) ≃ 5, $G(H_2O_2)$ ≃ 5. The mechanism in acidic solution involves a short chain:

$$R\dot{S} + O_2 \longrightarrow R\dot{S}O_2 \tag{64}$$

$$R\dot{S}O_2 + RSH \longrightarrow RSOOH + R\dot{S} \tag{65}$$

The hydroperoxide radical, HO_2, appears to be unreactive towards RSH. Observed products are formed via the subsequent chemistry

$$RSOOH + RSH \longrightarrow RSSR + H_2O_2 \tag{66}$$

$$RSOOH + H_2O \longrightarrow RSOH + H_2O_2 \tag{67}$$

$$RSOH + RSH \longrightarrow RSSR + H_2O \tag{68}$$

A marked increase in the values of G(-RSH), G(RSSR), $G(H_2O_2)$ is observed with increasing pH above pH $\geq$ 5 (Barton and Packer, 1970; Al-Thannon et al., 1974). The evidence is that the thiolate ion

$$RSH + OH^- \longrightarrow RS^- + H_2O$$

competes with oxygen (reaction 64) for thiyl radicals via

$$RS^- + R\dot{S} \rightleftarrows (R\bar{S}SR) \tag{69}$$

The radical ion reacts in turn with oxygen

$$(R\bar{S}SR) + O_2 \dashrightarrow RSSR + O_2^- \tag{70}$$

which generates additional $R\dot{S}$ via[2]

$$RSH + O_2^- \dashrightarrow R\dot{S} + HO_2^- \tag{71}$$

Reactions 69-71 constitute a chain which gives G(-RSH) values as high as 50 in the γ-radiolysis of 3×10^{-3} M cysteine containing 2×10^{-4} M oxygen.

Oxidation of the disulfide linkage of cystine by the hydroxyl radical is also a fast reaction ($k \simeq 1 \times 10^{10}\ M^{-1}\ sec^{-1}$) which involves ion-pair formation and dissociative OH addition (Bonifacic et al., 1975; Bonifacic and Asmus, 1976; Elliot et al, 1980)

$$RSSR + OH \rightarrow (RSSR)^+ + OH^- \tag{72}$$

$$RSSR + OH \rightarrow RSOH + R\dot{S} \tag{73}$$

where reactions 72,73 occur with about equal probability. In the presence of O_2, the principle final oxidation products, the sulfuric and sulfonic acid derivatives, RSO_2H and RSO_3H are formed in the

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[2]Although HO_2 as noted above does not react with RSH, the conjugated base $HO_2 \rightleftharpoons H^+ + O_2^-$ does, with $k_{71} \simeq 1.8 \times 10^4\ M^{-1}\ sec^{-1}$ (Al-Thannon et al., 1974).

γ-radiolysis of 10^{-3} M cystine with G ≃ 1.7 and G ≃ 0.7 respectively (Purdie, 1967).

The reaction of OH with methionine yields an addition product ($k_{71} \simeq 10^{10}\ M^{-1}\ sec^{-1}$)

$$RSCH_3 + OH \longrightarrow R\overset{OH}{\underset{\bullet}{S}}CH_3 \qquad (74)$$

which breaks down in a complex series of reactions to yield a cation dimer and a sulfone (Bonifacic et al., 1975). In oxygenated solution the major final products of γ-radiolysis are methionine sulfone, $RS(O)CH_3$, and methionine sulfoxide, $RS(O_2)CH_3$, with a combined yield of G ≃ 3 (Kopoldova et al., 1967).

2. Oxygen-free Solutions

The reaction of e^-_{aq} at the cysteine residue

$$RSH + e^-_{aq} \longrightarrow R + SH^- \qquad (75)$$

is essentially quantitative with $k \simeq 10^{10}\ M^{-1}\ sec^{-1}$ in neutral solution. The e^-_{aq} reaction 75 and the OH reaction 62 lead to the subsequent chemistry

$$\begin{aligned} R + RSH &\longrightarrow R\dot{S} + RH \\ 2R\dot{S} &\longrightarrow RSSR \end{aligned} \qquad (76)$$

to give G(cystine) ≃ G(alanine) ≃ $G(H_2S)$ ≃ 3 in steady-state γ-radiolysis (Wilkening et al. 1968; Al-Thannon et al. 1968). Pulse

radiolysis studies (Hoffman and Hayon, 1973) are in accord with the above formulation.

The reactions of disulfides, cystamine, cystine etc. with e^-_{aq}

$$RSSR + e^-_{aq} \longrightarrow R\bar{S}\bar{S}R \tag{77}$$

are also fast with $k \approx 10^{10}\ M^{-1}\ sec^{-1}$. The anion radical product absorbs strongly with a band centered at ~410 nm with an extinction of $\sim 10^4\ M^{-1}\ sec^{-1}$. The first order constant for the decay reaction

$$R\bar{S}\bar{S}R \longrightarrow RS + RS^- \tag{78}$$

is independent of pH in the range 4-7.5 but rises sharply with decreasing pH (Adams et al., 1967; Hoffman and Hayon, 1972).

With methionine and its peptide derivatives the reaction of e^-_{aq} occurs preferentially at the main-chain C=O bond via the addition reaction 27. ESR studies (Rustgi and Riesz, 1978) of spin-trapped (tNB) radicals also indicate a small contribution of

$$RCH_2SCH_3 + e^-_{aq} \longrightarrow R\dot{C}H_2 + CH_3S^- \tag{79}$$

$$RCH_2SCH_3 + e^-_{aq} \longrightarrow RCH_2S^- + \dot{C}H_3 \tag{80}$$

IV. Main-Chain Chemistry of Proteins in Aqueous Solution

1. Oxygenated Solutions

The early studies of the oxidative degradation of the peptide chain in aqueous O_2-saturated solutions (Jayko et al.; Garrison et al., 1962) involved both protein and model peptides systems. The reactions of OH at C-H positions along the protein main-chain were found to lead to oxidative degradation via

$$\text{P-CONHCH(R)-P} + \text{OH} \longrightarrow \text{P-CONH}\dot{\text{C}}\text{(R)-P} + \text{H}_2\text{O} \qquad (81)$$

$$\text{P-CONH}\dot{\text{C}}\text{(R)-P} + \text{O}_2 \longrightarrow \text{P-CONHC}(\dot{\text{O}}_2)\text{-P} \qquad (82)$$

followed by analogues of reactions 14-18 to yield amide and keto acid functions. Reaction 81 was viewed as occurring in competition with OH attack at side-chain loci - with the yield of main-chain degradation being affected by both the amino acid composition and the conformational characteristics of the protein.

A detailed study of keto acid and amide production in the γ-radiolysis of soluble collagen (gelatin 1 percent in oxygenated solution) was included in this early study. The keto-acid products from gelatin include: glyoxylic, pyruvic, phenylpyruvic α-keto-glutaric, oxalacetic plus traces of other unidentified α-keto acids to give a combined keto-acid yeld of G(>CO) $\simeq$ 0.5. The combined amide yield is appreciably higher with G(amide) $\simeq$ 0.9. Later more detailed studies with model peptides (Garrison et al., 1970; Makada and Garrison, 1972) show that the observed difference between G(>CO) and G(amide) arises in

part from the fact that a second (parallel) stoichiometry is involved in the radiolytic degradation of the peptide chain. This second degradation mode yields the amide function and organic products of higher oxidation. The specific intermediate chemistry is given in Sec II B. On the basis of these chemical measurements, the yield for main-chain degradation in this system is set by the limits $G \geq 0.5 < 0.9$.

The separation and isolation of protein fragments formed by main-chain cleavage in the X-radiolysis of ribonuclease (RNase), bovine serum albumen (BSA), and lactate dehydrogenase (LDH) in oxygenated solution has recently been achieved (Scheussler and Schilling, 1984; Schuessler and Herget, 1980). The protein fragments become apparent only after reduction of S-S bonds in the irradiated proteins with mercapto ethanol (RSH)

$$\text{PSSP} + \text{RSH} \longrightarrow \text{PSH} + \text{PSSR} \tag{83}$$

$$\text{PSSR} + \text{RSH} \longrightarrow \text{PSH} + \text{RSSR} \tag{84}$$

in aqueous sodium dodecyl sulfate, SDS. Separation was achieved by a combination of gel filtration and gel electrophoresis. The observed fragmentation yield (G value) for RNase, BSA and LDH are ~ 0.19, 0.3, 0.1 respectively.[3] Proteins, such as LDH, which contain larger amounts of

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[3]These values could be somewhat low as a measure of OH radical attack at the protein main-chain in these systems. Main-chain cleavage as formulated in reactions 81,82 etc., involves the hydrolysis of dehydropeptide intermediates as outlined in the reaction sequence 14-18. While these intermediates are known to be labile, there is no evidence that the cleavage reaction 18 occurs spontaneously. Mild acid hydrolysis prior to the filtration and electrophoresis steps could result in fragmentation yields somewhat greater than those reported.

aromatic-unsaturated and sulfur-containing amino acids would be expected to show lower main-chain fragmentation yields.

Pulse radiolysis and kinetic spectroscopy studies have also provided evidence for OH reactions at the protein main-chain. The ~CONHC$\dot{}$(R)~ radical in model peptides has been shown to have a strong absorption band at 250 nm with a relatively high extinction coefficient (Rao and Hayon, 1974). Studies of the absorption spectra produced by OH attack on papain (Clement et al., 1972) and ribonuclease (Lichtin et al., 1972) suggest that ~20-30 percent of the OH radicals generated in these systems react at C-H positions along the protein main-chain.

Combined gel filtration and ultracentrifugation studies of proteins irradiated in oxygenated solution have shown the presence (before reduction with RSH) of conformationally charged monomers with a lower γ-helical content that the "native" protein (Schuessler and Jung, 1967; Aldrich and Cundall, 1969). This radiation-induced unfolding of the protein structure is observed only in oxygenated solution and has been followed in a number of protein systems by measuring changes in optical rotatory dispersion (Aldrich and Cundall, 1969), Amide I absorption (T.E. Pavlovskaya et al., 1975) and tryptophan fluorenscence (Berzrukova and Ostashevsky, 1977). These authors have suggested that this radiation induced disruption of the protein helix in oxygenated solution arises from chemical change at side-chain sites. It would seem that oxidative cleavage of the peptide chain via reaction 81,82 (which occurs in parallel with changes at side chain site) could also be an important factor in the destabilization of the α-helical structure of proteins irradiated in oxygenated solution.

2. Oxygen-Free Solutions

The carbonyl group of the peptide bond represents a major trapping center for e^-_{aq} via reaction 27 in oxygen free solutions of simple peptides (Holian and Garrison, 1968; Rao and Hayon, 1974) and more complex polypeptides (Adams et al.1973). The adduct products have weak absorptions in the spectral region below 300 nm and are relatively long lived.

Various types of evidence indicate that the addition of e^-_{aq} to the peptide carbonyls of proteins in oxygen free solutions

$$\text{P-CONH-P} + e^-_{aq} \longrightarrow \text{P-}\dot{\text{C}}(\text{O}^-)\text{NH-P} \qquad (85)$$

occurs in completion with e^-_{aq} addition to the disulfide bond of cystine (sec III C) and the protonated histine residue (sec III B). For example, pulse radiolysis studies of papain (Clement et al., 1972) and α-chymotrypsin (Adams et al., 1973) indicate that reaction 85 represents the major path for removal e^-_{aq} in these systems with the remainder being trapped predominantly at cystine disulfide linkages (eq. 77). On the other hand, with lysozyme and trypsin (Adams et al., 1972; Ovadia, 1972), e^-_{aq} is preferentially trapped at cystine residues.

It has been suggested (Faraggi and Tal, 1975; Klapper and Faraggi, 1981) that e^-_{aq} first adds to peptide carbonyl bonds located on the surface of the protein and that electron transfer from $-\dot{\text{C}}(\text{O}^-)\text{NH}-$ radicals to cystine and histidine residues occurs through hydrogen bonds between peptide units (Brillouin, 1962). Recent ESR studies of proteins in γ-irradiated aqueous glasses suggest that electron transfer from one

peptide unit to the next is a fast process (Rao et al., 1983). It is also of interest in this regard to note the finding that the mobilities of electrons and holes in "dry" polyglycine are ~1.5 cm^2/Vs and ~6 cm^2/Vs respectively whereas with "hydrated" polyglycine the corresponding values are ~150 cm^2/Vs and ~4 cm^2/Vs, indicating that electrons in the "hydrated" polyglycine can be considered as conventional delocalized charge carriers (Kertesz et al., 1978).

V. Side Chain Chemistry of Proteins (and Nucleoproteins) in Aqueous Solution

A. Sulfur-containing Residues

Studies with papain (Lin et al., 1975; Clement et al., 1972) and glyceraldehyde -3- phospate dehydrogenase, GAPDH, (Buchanan and Armstrong, 1978) have established three reaction modes for the radiation induced oxidation of protein SH groups in oxygenated solutions i.e.

$$PSH + OH \longrightarrow P\dot{S} + H_2O \tag{86}$$

$$PSH + O_2^- \longrightarrow P\dot{S} + HO_2^- \tag{87}$$

and the slower molecular process

$$PSH + H_2O_2 \longrightarrow PSOH + H_2O \tag{88}$$

The radical reactions 86,87 which form the protein $P\dot{S}$ radicals are analogues of reactions 62,71 which were identified in radiation chemical studies of cysteine and its simple model peptides (Sec III C). Subsequent reaction of macromolecular $P\dot{S}$ with O_2

$$P\dot{S} + O_2 \longrightarrow P\dot{S}O_2 \longrightarrow \text{products} \tag{89}$$

leads to formation of non-repairable products which are presumed to be the sulfinic (RSOOH) and sulfonic (RSO_2H) derivatives.

The oxidation of PSH by H_2O_2 via reaction 88 to yield the sulfenic acid derivative PSOH is repairable in that it can be reversed by addition of excess cysteine or other thiols (Lin and Armstrong, 1977).

$$PSOH + RSH \longrightarrow PSSR + H_2O \quad (90)$$

$$PSSR + RSH \longrightarrow PSH + RSSR \quad (91)$$

Inactivation yields for papain and GAPDH in oxygenated solutions are exceptionally high. For example, with air saturated 3×10^{-5} M papain under γ-radiolysis $G(inact)O_2 \simeq 4.8$ of which $G \simeq 3.5$ is repairable on treatment with excess cysteine (Lin et al., 1975). Similar high yields are observed with GAPDN (Buchanan and Armstrong, 1978). With papain which has a single SH group (which is essential for enzymatic activity) only ~ 10 percent of the available OH radicals are involved in reaction 86 to yield non repairable damage. The remainder are removed through reactions at other side-chain loci and at the protein main-chain (eq. 81). Most of the sulfur chemistry in these systems arises from reactions of O_2^- and H_2O_2 with PSH as formulated in reactions 87,88.

Similar studies have been made with lactate dehydrogenase (Buchanan and Armstrong, 1976). With LDH the loss of enzymatic activity in oxygenated solution also involves SH oxidation at the active site. However, the inactivation yield with LDH is much lower with $G(inact)O_2 \simeq 0.12$. This low inactivation yield, as compared to papain and GAPDH, is attributed to the fact that the SH groups of papain and GAPDH are involved in substrate binding and are highly nucleopleophilic. Hence, they are more reactive towards O_2^- and H_2O_2 than the SH groups of LDH which do not have this function but conserve three dimensional structure through H bond formation.

The inactivation yield $G(inact)O_2 \simeq 0.12$ obtained with LDH falls within the range of inactivation yields observed with most other enzymes $G(inact)O_2 \sim 0.1$ to ~ 0.5 in oxygenated solution (Sanner and Phil, 1962; Armstrong and Buchanan, 1978). There are two main reasons for the generally low values for enzyme inactivation by ionizing radiation (1) Only a few enzymes such as papain and GAPDH have active sites reactive towards O_2^- and H_2O_2 (2) The attack of OH radicals is relatively non-specific and is widely distributed over many loci both main chain and side chain. From the radiation chemical standpoint, chemical change at the active site represents only one of many chemical changes in the system which can ultimately affect enzymatic activity.

Major sites for reaction of e^-_{aq} with proteins in oxygen free solutions include disulfide linkages, peptide carbonyls and histidine side-chains. Electron trapping at disulfide bridges in proteins

$$PSSP + e^-_{aq} \longrightarrow P\bar{S}\bar{S}P \qquad (92)$$

yields the characteristic absorption band at ~ 400 nm observed with cystine and other simple disulfides (Adams, 1972). The chemical consequences of e^-_{aq} attachment to S-S bonds of proteins are not fully understood. There appear to be two types of disulfide traps in proteins. (Clement et al., 1972; Adams et al., 1973). In the one type, the $P\bar{S}\bar{S}P$ adduct is short-lived and undergo the dissociation

$$P\bar{S}\bar{S}P \longrightarrow PS + PS^- \qquad (93)$$

$$P\bar{S} + H_2O \longrightarrow PSH + OH^- \qquad (94)$$

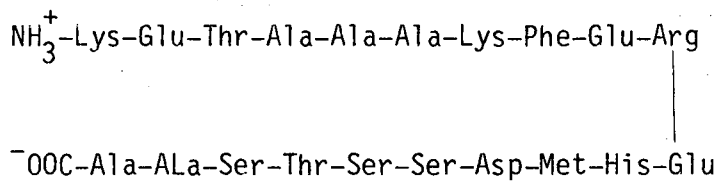
as is found with the simple disulfides, cystine etc. The production of PSH by the dissociation reaction 93,94 has been observed with a number of aqueous proteins including papain (Gaucher et al., 1977) and Bowman-Birk proteinase inhibitor (Wandell and Kay, 1977). With papain, the dissociation reaction 93 contributes to the overall inactivation yield. The $P\bar{S}\bar{S}P$ adducts of the second type are long lived and have the stability of $-S^{-}S-$ions observed in aqueous glasses (Gasyna et al., 1976; Rao et al. 1983). Apparently the long lives $P\bar{S}\bar{S}P$ adducts have structures and environments which prevent dissociation. Enzymes that show a high yield of long lived $P\bar{S}\bar{S}P$ radicals with little or no short lived component are not significantly inactivated by e^{-}_{aq} (Adams, 1972). It has been suggested that the long lived $P\bar{S}\bar{S}P$ species are ultimately removed through back reaction with radicals formed through OH attack at various side chain and main-chain loci (Ovadia, J., 1972).

The $P\dot{S}$ radicals formed in reaction 93 and in the OH reaction 86 can undergo combination reactions in O_2-free solutions to yield higher molecular-weight dimer and trimer products which contribute to the combined aggregate yield. Radiation aggregates arise from both covalent and non-covalent binding. The latter aggregate forms dissociate to monomer in concentrated aqueous solutions of sodium dodecyl sulfate (SDS) or urea (Yamamoto, O., 1977). Recently detailed studies have been made of the radiolytic formation of aggregate products in oxygen-free solutions of lactate dehydrogenase, LDH (Schuessler, H. and Herget, 1980), ribonuclease, RNase (Hajos and Delincee, 1983) and bovine serum albumen, BSA (Schuessler, and Schilling, 1984). Gel filtration and gel

electrophoresis techniques were employed in the separation of protein fractions in aqueous SDS solutions with and without added reducing agent, RSH.

The findings clearly show that two types of covalently bonded dimers are formed - those that contain disulfide cross links and those that contain other (non-reducible) covalent linkages. The combine yield of covalently linked dimers in these systems are in the range G ~ 0.1 to G ~ 0.5. Disulfide bridges appear to be less important (10 to 40 percent) than cross-links derived from other types of radical-radical reactions. These other modes of radiation crosslinking are treated in following sections.

The S peptide of ribonuclease (RNase-S) has an interesting structure from the radiation chemical standpoint because it is made up of a single chain of 20 amino acids of known sequence with a single sulfur-containing amino acid residue (methionine)



The combination of S-peptide with S-protein is enzymatically active and the single methionine residue appears to be involved in the binding of S-peptide to S-protein.

Studies of the radicals formed in the reactions of photo-generated OH radicals ($H_2O_2 \xrightarrow{h\nu} 2OH$) with S-peptide (using t-nitrosobutane

as the spin trap, (eg. 30) show the formation of both main-chain and side chain radicals (A. Joshi et al., 1978). The alanine main-chain and the methionine side chain radicals were identified as major products. However, each of these represented but one of a large number of radical products of each type. Earlier studies (Mee and Adelstein, 1967; Mee and Adelstein, 1974) of amino acid destruction in the γ-radiolysis of S-peptide in oxygenated solution appear to be in general agreement with the spin-trap data. The observed amino acid losses expressed as changed residues/mole S-peptide (at 17 percent residual activity) correspond to

Met ~0.65, Ala ~0.25, Ser ~0.25, Phe ~0.2, Lys ~0.2,

His ~0.18, Glu ~0.15, Arg ~0.13, Thr ~0.1, Asp ~0.1.

Column chromatography (on polyacrylamide gels) of the irradiated S-peptide (after mild hydrolysis at pH3 for 30 minutes) showed no new peaks and the authors concluded that main chain fragmentation resulting from OH attack (eq. 81,82 etc.) does not occur. However, the single main peak showed broadening and a pronounced shift in the maximum to the lower-molecular weight side. This would be consistent with a more or less random fragmentation of the S-peptide main chain as a consequence of OH attack. A re-examination of this sytem using the more sensitive SDS-polyacrylamide gel electrophoresis technique would be interesting as would a keto acid analysis of the unidentified carbonyl product fraction.

B. Aromatic-Unsaturated Residues

Most enzymes are inactivated by OH attack in oxygenated solutions and have $G(inact)O_2$ values in the range 0.1 to 1.0 (Sanner and Phil, 1969). The problem has been to identify the amino acid residues which if damaged lead to loss of enzymatic activity. Pulse radiolysis studies have shown that the spectra of transition products formed in the reaction of OH with proteins in N_2O saturated solution ($N_2O + e^-_{aq} \longrightarrow N_2 + OH^-$) are relatively featureless and reflect the non-specificity of the OH reactions which are widely distributed over many loci - both main-chain and side-chain (Lichtin et. al., 1972; Adams et al., 1973; Hashimoto et al., 1981). However, the finding that inorganic free radical ions generated via

$$X^- + OH \longrightarrow X + OH^- \tag{95}$$

$$X + X^- \longrightarrow X_2^- \quad (X = CNS^-, Br^-, I^-) \tag{96}$$

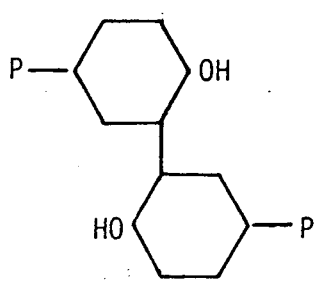
are relatively unreactive towards aliphatic amino acids but show specificity in their reactions with aromatic and sulfur containing residues has provided a new approach to the identification of residues crucial to the activity of a particular enzyme. In the early study (Adams et al., 1969) the specificity of the reaction of $(CNS)_2^-$ radicals with trypsin

$$TrpH + (CNS)_2^- \longrightarrow TrpH^+ + 2\ CNS^- \tag{97}$$

$$TrpH^+ \longrightarrow Tryp^{\cdot} + H^+ \tag{98}$$

was used to show that radiation damage to tryptophane leads directly to the loss of lysozyme activity. Combined pulse radiolysis and enzyme inactivation studies of the reactions of $(CNS)_2^-$, Br_2^- and I_2^- radicals with numerous enzymes have led to the identification of amino acid residues essential to the enzymatic activity of: lysozyme [tryp]; ribonuclease [his], chymotrypsin [his], trypsin [his], (Adams et al., 1972), pepsin [tryp] (Adams et al., 1979), papain [cys], lactate dehydrogenase [cys] (Buchanan and Armstrong, 1976).

The cross linking of the simple peptide derviatives of the unsaturated amino acids, phenylalanine, tyrosine, tryptophan and histidine in oxygen free solution has been discussed in Sec. III B. There now is definite evidence that similar processes are involved in the radiation induced dimerization of proteins. The di-tyrosyl cross link



can be readily detected because of its strong characteristic fluorescence spectrum with λ_{max} = 400 nm and has been identified as a product of the γ-radiolysis of aqueous insulin, ribonuclease, papin, collagen (Boguta and Dancewicz, 1983) histone (Prutz et al., 1983) and lysozyme (Hashimoto et al., 1982). Although the observed yields of di-tyrosyl cross links in these systems, G≈0.1, is quite low as compared to G(OH), their detection suggests that similar cross-linking reactions at other unsaturated residues are also involved but are less readily identifiable (Garrison, 1979, 1981).

C. Aliphatic Residues

In the radiolysis of most enzymic proteins the relative amino-acid contents of the protein are such that a major fraction of the OH radicals are removed at the aromatic-unsaturated and the sulfur-containing residues. However, with many other proteins such as the albumens and globins (Urbain, 1977; Duda, 1981) and chromosomal proteins (Robinson et al., 1966a) a higher relative fraction of the OH radicals is removed at reactive aliphatic residues e.g., serine threonine, proline, leucine, lysine and arginine.

The histone group of chromosomal proteins are highly basic with high contents of lysine and arginine. Another feature of histone composition is the absence of tryptophan, cysteine[4] and cystine and the relatively lower content of histidine, tyrosine and phenylalanine. Detailed studies of amino acid destruction in the γ-radiolysis of thymus chromosomal proteins in dilute oxygen-saturated solution have been made (Robinson et al., 1966a). The protein solutions were prepared by dissociating the DNA-protein complex at high ionic strength. After dialysis, the absorption spectra of the isolated proteins were recorded over the range 210 to 350 mμ and showed that no DNA was present. Analysis of the irradiated proteins established that the amino acid damage occurs largely in the aliphatic amino acid residues. The observed G-values for amino acid destruction are :

lys ~0.31, leu ~0.16, arg ~0.15, tyr ~0.16, ser ~0.13, his ~0.13, val ~0.10, Ile ~01.10, phe ~0.09.

---

[4]With the exception of histone H3 which contains one cysteine residue.

The yield for total amino acid destruction in the γ-radiolysis of the "free" protein in oxygen saturated solution corresponds to G ≃ 1.6

In a parallel study, the deoxyribonucleoprotein complex was irradiated under identical conditions., The total yield for amino acid destruction in this system was found to be the same (within experimental error) as that obtained in the radiolysis of the separated protein i.e. G ≃ 1.6 (Robinson et al., 1966a). The chromosomal proteins act as an efficient protective agent towards the DNA component i.e., most of the OH induced chemistry in the radiolysis of deoxyribonucleoprotein in oxygen saturated solution occurs at the protein moiety (Robinson et al., 1966b; Mee et al., 1978).

Since the amino acid sequences of the histones have been so rigidly conserved throughout the evolution of the eukaryotic cell, it has been suggested that the integrity of each and every amino acid residue is essential for the biological function of the DNA-histone complex (Bradbury et al., 1981). Hence, radiation damage to one or a few amino acid residues in the histone component could lead to a decrease in the stability of the DNA-histone complex. Oxidative cleavage of the histone main-chain by OH attack via reactions 81,82 would be a particulary destabilizing form of damage. Such radiation chemical damage could then lead to the exposure of genetic information which is not required by the cell and which could lead to cell dysfunction. A decrease in the stability of the DNA-protein complex after irradiation in oxygenated solution has been observed with a number of experimental procedures (Robinson et al., 1966b; Lloyd and Peacocke, 1968; Bauer et al., 1969; Juhasz et al., 1982).

Detailed studies of the radiation chemistry of histones and other protein components of chromatin in oxygenated systems both in vitro and in vivo are indicated.[5]

The γ-irradiation of deoxynucleoprotein in oxygen-free systems leads to the formation of DNA-protein crosslinks. These appear to be covalent in nature and are stable to salt and detergent treatment. (Smith, 1976; Mee and Adelstein, 1979). Recent work indicates that the core histones H2A, H2B, H3, H4 are the specific proteins involved in the radiation crosslinking reaction in chromatin. (Mee and Adelstein, 1981). Oxygen inhibits DNA histone crosslinking presumably by reacting preferentially with DNA and protein radicals.

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[5]Sensitive analytical procedures, which were developed in early studies of the radiolytic oxidation of protein in dilute aqueous solution (Garrison et al., 1962), could be useful in studies of histone oxidation both in-vitro and in-vivo at dosages in the kilo-rad range commonly used in biological studies. The analysis are based on the finding that reactive carbonyl groups are major products of protein oxidation initiated by OH attack as discussed in Sec. II B, III B. The method is of particular interest in relation to the radiolytic oxidation of histones since these proteins have high contents of aliphatic amino-acids - particularly serine, threonine proline, lysine and arginine which characteristically yield carbonyl products from both main-chain and side-chain oxidation (Sec. III B).

VI. Solid Peptides, Polypeptides and Proteins

A. Peptides and Polypeptides

Main-chain cleavage with formation of amide and fatty acid function was established some years ago as a major reaction in the radiolysis of peptides in the solid state (Garrison et al., 1967; Garrison et al., 1968). The overall chemistry was formulated in terms of the stoichiometry

$$3\ RCONHCHR_2 \rightsquigarrow RCONH_2 + CH_2R_2 + 2\ RCONH\dot{C}R_2 \qquad (99)$$

where $RCONH\dot{C}R_2$ corresponds to the long-lived radical products observed at room temperature by ESR spectroscopy (Drew and Gordy, 1963). Dimerization of these radical products occurs on dissolution of the irradiated solid in O_2-free water via reaction 33. Detailed chemical separations of products formed in the radiolysis of polyalanine and N-acetylanine give G(amide) ≃ 3, G(propionic acid) ≃ 2, G(pyruvic acid) ≃ 1 G(dimer) ~ 2 (Garrison et al., 1968).[6] The evidence is that the keto acid is produced through the dehydrogenation

$$RCONHCHR_2 \dashrightarrow RCON{=}CR_2 + H_2 \qquad (100)$$

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[6]The authors of a recent study (Hill et al., 1981) in which γ-irradiated solid polyalanine (and other aliphatic polyamino acids) were simply heated to 100°C for gas-chromatographic analysis have reported that propionic acid (and the corresponding fatty acid from the other aliphatic polyamino acids) are produced only in low yield, G < 0.1. These authors seem to have overlooked the fact that the chemistry of equation 99 gives an acyl end group which must undergo hydrolysis to yield the free fatty acid.

The dehydropeptide formed in reaction 79 is readily hydrolyzed to give keto acid and amide

$$RCON{=}CR_2 + H_2O \dashrightarrow RCONH_2 + R_2CO \tag{101}$$

ESR studies indicate that the observed stoichiometries given in equation 99 for polyamino acid derivatives of the simpler α-amino acids results from the intermediate ionic processes (Saxebol, 1973; Sinclair and Codella, 1973; Sevilla et al., 1979a)

$$RCONHCHR_2 \rightsquigarrow RCONH\dot{C}R_2 + H^+ + e^- \tag{102}$$

$$e^- + RCONHCHR_2 \dashrightarrow R\dot{C}(O^-)NHCHR_2 \xrightarrow{H^+} R\dot{C}(OH)NHCHR_2 \tag{103}$$

$$R\dot{C}(OH)NHCHR_2 \dashrightarrow RCONH_2 + \dot{C}HR_2 \tag{104}$$

$$\dot{C}HR_2 + RCONHCHR_2 \dashrightarrow CH_2R_2 + RCONH\dot{C}HR_2 \tag{105}$$

to give the overall stoichiometry given in equation 99.

The ESR work also shows that the radiation chemistry of the lower-molecular weight peptides such as the N-acetyl amino acids and the di and tripeptides is somewhat more complicated than that given in the scheme of reactions 102-105. In these systems, the carboxyl group represents the major locus of positive-hole formation i.e.,

$$RCONHCH(R)COOH \rightsquigarrow [RCONHCH(R)COOH]^+ \dashrightarrow RCONHCH(R)CO\dot{O} + H^+ \tag{106}$$

The ionization step 106 is then followed by

$$RCONHCH(R)C\dot{O}_2 \longrightarrow RCONH\dot{C}H(R) + CO_2 \qquad (107)$$

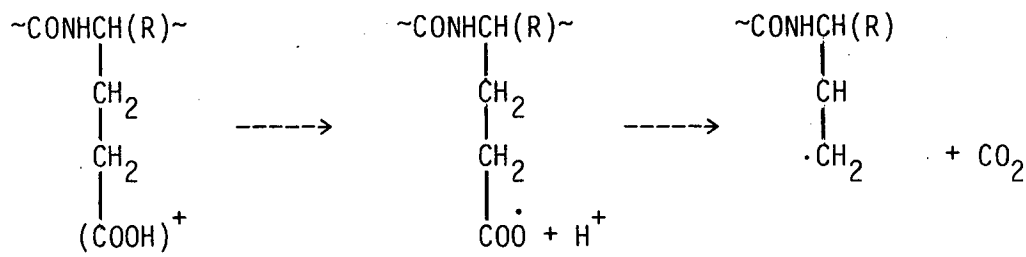
and the abstraction reaction

$$RCONH\dot{C}H(R) + RCONHCH(R)COOH \longrightarrow RCONHCH_2(R) + RCONH\dot{C}(R)COOH \qquad (108)$$

to yield the long-lived α-carbon radical. Both the decarboxylated radical, RCONHCHR, and the α-carbon radical, RCONH$\dot{C}$(R)COOH have been spin-trapped on dissolution of γ-irradiated N-acetyl amino acids and dipeptides in aqueous solution of t-nitrosobutane as the trapping reagent (Minegishi et al., 1980).

Cleavage of the peptide main-chain with formation of amide and fatty acid functions is a major process in the solid-state radiolysis of peptide derivatives of almost all aliphatic amino acids. Values of G(amide) ~ 3 and G(fatty acid) ~ 2 have been obtained with peptide derivatives of glycine, alanine, α-aminobutyric acid, glutamic acid, leucine, and methionine (Garrison et al., 1968). The α-carbon radical, ~CONH$\dot{C}$(R)~, is the long-lived radical species in each case as observed by direct ESR measurements of the irradiated solids (Sevilla et al., 1979a).

With peptide derivatives of aspartic and glutamic acids, the evidence is that the positive hole is located preferentially at the side-chain carboxyl group (cf reaction 106)



(109)

where the radical product of reaction 109 then abstracts H from the main-chain to form α-aminobutyric acid derivative as a major product (Ogawa et al., 1980; Sevilla et al., 1979b).

Although both methionine and cysteine undergo C-S bond cleavage on reaction with the hydrated electron, e^-_{aq}, in aqueous solution (reactions 75, 79, 80) the evidence from both product analysis (Peterson et al., 1969) and ESR (Saxebol, 1979; Sevilla et al., 1979b) studies is that such reactions are relatively unimportant in the solid state. The C=O linkage of the peptide bond appears to be the major trapping center for e^- in the radiolysis of methione and cysteine peptides as solids.

The yield of main-chain cleavage as measured in terms of amide production is appreciably lower with peptide derivatives of the aromatic amino acids, phenylalanine and tyrosine (Garrison et al., 1968). The ESR evidence is that the unsaturated side chains of phenylalanine, tyrosine, and histidine compete effectively with the peptide bond for e^- in the solid state (Sec. III.C) (Sevilla et al., 1979b).

B. Proteins

The yields of major degradation products formed in the γ-radiolysis of solid proteins, both globular and fibrous, have been summarized as follows: amide function (G ~ 2.5), keto acid plus aldehyde (G ~ 1.0),

fatty acids (G ~ 1.0) and long-lived free radicals (G ~ 5) (Friedberg, 1969; Garrison, 1981). These findings strongly support the idea that the reaction stoichiometries represented by equations 99, 100 for the polyamino acids are also of major importance in the radiolysis of solid proteins.

Extensive ESR studies of a large number of model peptides (Sevilla et al., 1979a), indicate that the dominant long-lived radicals observed in irradiated proteins (Gordy and Shields, 1958; Riesz and White, 1970) are, indeed, the α-carbon radicals P-CONHĊ(R)-P as formulated above. This is the case not only for peptide derivatives of the aliphatic amino acids - glycine, alanine, serine, threonine, lysine, sarcosine, aspartic and glutamic acids but also for methionine, histidine and phenylalanine. With the tyrosine residue, the tyrosylphenoxy radical ~ΦȮ, is produced. Combination of these long-lived radicals in irradiated proteins yields high molecular-weight aggregate products (Haskill and Hunt, 1967; Friedberg, 1969a).

Although the formation of amide and fatty acid functions in irradiated proteins via eqs. 99-105 explicitly states that cleavage of the protein main-chain does occur, this does not necessarily mean that lower molecular-weight products will be observed. The average number molecular weight of solid polyamino acids and fibrous proteins does indeed decrease on irradiation in the solid state (Hayden et al., 1966; Friedberg, 1969b). However, globular proteins show a much lower yield of molecular fragments even after reduction of intramolecular disulfide bonds (Friedberg, 1969b; Ray and Hutchinson, 1967; Stevens et al., 1967).

The reason for this difference can be related to fact that in the irradiation of protein and high molecular weight polypeptides a number of main-chain breaks plus the concomitant radical pairs would be introduced into the macromolecular structure even at the lowest practicable dosages. On the dissolution of irradiated globular proteins, radical combination within the hydrated globule would be favored by the constraints imposed by the secondary and tertiary structures. With the polyamino acids and fibrous proteins such constraints are minimal and the separation of radical fragments on dissolution would be competitive with combination (Friedberg, 1969b; Garrison, 1972).

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