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MECHANISMS OF RADIATION-CHEMICAL REACTION IN BIOCHEMICAL SYSTEMS

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ABSTRACT

Current concepts related to mechanism in the radiolysis of aqueous amino acids and peptides are reviewed. Emphasis is given to recent studies of the peptide linkage as a locus of indirect action. It is shown that radiolytic reactions of protein, both in oxygenated and in oxygen-free solutions, lead under appropriate conditions to cleavage of the polypeptide chain. Reaction in oxygenated solution corresponds to a radiation-induced step that gives rise to amide and carbonyl terminal functions at the locus of cleavage. In oxygen-free solutions, cleavage occurs predominantly through postirradiation hydrolysis of acyl imino linkages (-CO-N=C(R) -) to give essentially the same final products. Observed differences in the rates of the postirradiation liberation of ammonia for the two cases support the mechanisms proposed.

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By way of introduction to this session on "The Effects of Radiation on Molecules in Cellular Solution," I propose to review some of the current concepts related to the mechanism of radiation-induced reaction in aquo-organic systems--particularly systems of biological interest. I shall discuss a number of specific reactions that have been found important in the radiolysis of aqueous solutions of amino acids, peptides, and related compounds. And finally, I will attempt to correlate various of these observations with recently reported data on the indirect action of radiation on protein.

Some of the material to be presented has not yet appeared in the open literature; I am indebted to Dr Boyd M. Weeks, Dr. Michael E. Jayko, Dr. Mathilde Kland-English, and Miss Winifred Bennett of the Radiation Chemistry Group at Crocker Laboratory, for the opportunity of referring to this work at this time.

I should like to start out by discussing briefly the initial radiation-induced reaction in aqueous solution, i.e., the radiation decomposition of water. Now, many radiolysis studies of aqueous solutions have been found to be generally consistent with the theory that the earliest detectable products of water include H atoms, OH radicals, H_2, and H_2O_2. The molecular products H_2 and H_2O_2 are formed in tracks or "spurs" by primary combination of H and OH, which otherwise escape by diffusion into the bulk of the solution. The net initial reaction has been conveniently described in terms of the notation

$$H_2O \rightsquigarrow H,\ OH,\ H_2,\ H_2O_2, \qquad (1)$$

and the 100-ev yields (G values) of these initial products are denoted G(H), G(OH), etc. Yields of final products are represented by G(Product). There is a detailed and rigorous treatment of this reaction, in the recent review by Allen and Schwarz.[1]

Let us consider now the indirect action of radiation on some organic molecules of biological interest. Of the various classes of compounds in this category, certainly the amino acids and derivatives have received the greatest attention. The early studies of indirect action of x-rays on aqueous amino acids by Dale[2] and by Weiss[3] established that ammonia is a major product--both in vacuo, and in oxygenated solution. Later work, principally by Maxwell

*Based on transcript of a paper given at the Second UCLA Conference on Radiobiology, September 1959.

and co-workers,[4,5] gave the major stoichiometric relationships between ammonia and various organic decomposition products from glycine and alanine. Most amino acids, with the exception of cystine, undergo deamination (or the equivalent) as a characteristic radiation-induced reaction, although certainly the relative importance of deamination in the over-all radiation chemistry of any particular amino acid may depend on the nature of the side chain R. Until recently no detailed mechanism for radiation-induced deamination in these systems has been formulated. The following treatment,[6] based largely on experimental data obtained with glycine and alanine, has been found to provide a useful working hypothesis in the interpretation of the radiation- induced deamination of most amino acids.

In the radiolysis of oxygen-free glycine solutions the major products are ammonia, glyoxylic acid, acetic acid, and hydrogen. Correspondingly, the major organic products from alanine are pyruvic and propionic acids, etc. In formulating the intermediate processes we refer first to the yields of the initial products derived from water. To a good approximation, we may assume, for x- and γ-ray irradiations, $G(H) \approx G(OH) \approx 3$, $G(H_2) \approx G(H_2O_2) \approx 0.4$. Now, if all available H atoms were removed by hydrogen abstraction,

$$H + NH_2CH(R)COOH \rightarrow H_2 + NH_2\dot{C}(R)COOH, \qquad (2)$$

then the observed product yield $G(H_2)$ should approach 3.4. Actually, the value for 1 M glycine is considerably less than this, viz. $G(H_2) \approx 2$, and it is apparent that reaction in addition to (2), is involved. On the basis of various considerations, it appears that the competing process involves reaction of the type

$$H + NH_2CH(R)COOH \rightarrow NH_3 + \dot{R}CHCOOH, \qquad (2a)$$

followed by

$$\dot{R}CHCOOH + NH_2CH(R)COOH \rightarrow RCH_2COOH + NH_2\dot{C}(R)COOH. \qquad (3)$$

It can also be shown (a) that the most likely path for OH removal is probably

$$OH + NH_2CH(R)COOH \rightarrow H_2O + NH_2\dot{C}(R)COOH; \qquad (4)$$

and (b) that the intermediate radical species $NH_2\dot{C}(R)COOH$ formed in steps (2), (3), and (4) are ultimately removed by disproportionation

$$2\ NH_2\dot{C}(R)COOH \rightarrow NH{=}C(R)COOH + NH_2CH(R)COOH. \qquad (5)$$

The imino acid produced in Reaction (5) then hydrolyzes immediately to give α-keto acids and ammonia,

$$NH{=}C(R)COOH + H_2O \rightarrow NH_3 + RCOCOOH. \qquad (6)$$

Although the main stoichiometric relations can be interpreted both qualitatively and quantitatively in terms of the above mechanism, we have found that small amounts of higher-molecular-weight products are also formed. Among these from glycine are succinic acid, aspartic acid, and diaminosuccinic acid. The simplest explanation for the formation of these products is that small fractions of the $\dot{C}H_2COOH$ and $NH_2\dot{C}HCOOH$ radical intermediates derived from glycine undergo competing combination reactions.

In oxygenated solutions the net over-all reaction can be represented to a first approximation by the equation

$$NH_2CH(R)COOH + O_2 + H_2O \longrightarrow NH_3 + RCOCOOH + H_2O_2. \quad (7)$$

Carbon dioxide and the corresponding aldehyde RCHO are also formed in low yield. The main features of the over-all reaction are consistent with a mechanism in which (a) H atoms are removed by

$$H + O_2 \longrightarrow HO_2, \quad (8)$$

$$2\ HO_2 \longrightarrow H_2O_2 + O_2, \quad (9)$$

and (b) amino acid oxidation occurs via Step (4) followed by

$$NH_2C(R)COOH + O_2 + H_2O \longrightarrow NH_3 + RCOCOOH + HO_2. \quad (10)$$

The justification for Eq. (10) as written is simply that it leads to the correct over-all product stoichiometry. In actuality, it no doubt represents a more complex reaction sequence. For example, an organic hydroperoxide could be involved,

$$NH_2C(R)COOH + O_2 \longrightarrow NH_2\overset{O_2}{\overset{|}{C}}(R)COOH, \quad (11)$$

$$NH_2\overset{O_2}{\overset{|}{C}}(R)COOH + HO_2 \longrightarrow NH_2\overset{OOH}{\overset{|}{C}}(R)COOH + O_2, \quad (12)$$

$$NH_2\overset{OOH}{\overset{|}{C}}(R)COOH + H_2O \longrightarrow NH_3 + RCOCOOH + H_2O_2, \quad (13)$$

or conceivably the imino intermediate postulated for the oxygen-free case could be formed,

$$NH_2C(R)COOH + O_2 \longrightarrow NH{=}C(R)COOH + HO_2, \quad (14)$$

and then hydrolyzed via (6). However, since there appears to be no appreciable postirradiation deamination in amino acid solutions, we cannot readily distinguish between these possibilities. We can say, though, that if hydroperoxide substitution at the α carbon does occur, the resultant organic hydroperoxide is essentially as labile as the α-amino acid.

Now, if cleavage of the N-C bond by indirect action occurred only at the primary amino configuration, I suspect that the reaction would have limited significance in radiation biology. However, radiation-chemical changes analogous to those observed with the amino acids (and with primary amines in general) also occur with secondary amines,[7] and it is to these reactions that I should like to direct our attention now. Obviously the secondary amines of interest to us here are acyl amines--i.e., peptides--and among the simplest chemical models that can be employed in the study of radiation--induced reactions of the peptide bond are the amino acid anhydrides (diketopiperazines). Now, if an oxygenated solution of glycine anhydride, for example, is assayed for "free" ammonia immediately after irradiation, the $G(NH_3)$ value obtained is only 10% or so of the value given by glycine under identical conditions. However, if the irradiated glycine-anhydride solution is subjected to acid hydrolysis, then the total yield of liberated ammonia approaches that obtained with the free amino acid. A qualitatively similar postirradiation effect is obtained with solutions that have been irradiated under vacuum. There is, though, an important difference between the two cases in that for the same hydrolysis conditions the rate of ammonia liberation in the oxygen-free reaction is much the slower. For example, a glycine anhydride solution that has been γ-irradiated under oxygen gives, on hydrolysis in 1 N hydrochloric acid at 90°C, a limiting $G(NH_3)$ value of about 5 within a few minutes, whereas in the oxygen-free case the yield of ammonia increases almost linearly with time up to at least 18 hours, at which point $G(NH_3)$ is about 2.5. Although the amino acid anhydrides in some ways represent a special case because of their cyclic structure, we have obtained qualitatively similar results with representative linear peptide models, and in interpreting these effects, we have proposed that H and OH in oxygen-free solution react with peptides at the α-carbon via steps analogous to (2) and (4) to give radicals of the type RCONHC(R)COOR. These are then removed by disproportionation to yield the acyl imino derivatives as initial products *in vacuo*,

$$2\ \mathrm{RCONH\dot{C}(R)COOR} \rightarrow \mathrm{RCON{=}C(R)COOR} + \mathrm{RCONHCH(R)COOR}. \qquad (15)$$

Acyl imino compounds as a class (unlike the imino acids NH=C(R)COOH) are moderately stable in water but are slowly hydrolyzed in acid or base,[8]

$$\mathrm{RCON{=}C(R)COOR} + H_2O \longrightarrow \mathrm{RCONH_2} + \mathrm{RCOCOOR}, \qquad (16)$$

$$\mathrm{RCONH_2} + H_2O + H_2O \longrightarrow \mathrm{RCOOH} + NH_3, \qquad (17)$$

and we accordingly attribute the postirradiation effect to reactions of type (16), (17). Hence, on the basis of this representation, (a) cleavage of the peptide bond would not be considered an initial effect of indirect action *in vacuo*, (b) postirradiation hydrolysis would liberate not only ammonia but also carbonyl products. Experimentally, this is precisely what is found.

The results obtained with oxygenated systems, on the other hand, are consistent with the idea that cleavage of the peptide bond occurs as a radiation-induced step. The postirradiated data (together with other types of evidence) indicate that (a) the cleavage step is analogous to Reaction (10) of the mechanism formulated for amino acid systems, i.e.,

$$RCONHC(R)COOR + O_2 + H_2O \rightarrow RCONH_2 + RCOCOOR + HO_2, \quad (18)$$

and (b) the postirradiation reaction corresponds simply to a hydrolysis of the amide product. In oxygenated peptide solutions, then, the carbonyl derivatives are initial irradiation products, and the postirradiation hydrolysis step affects only the ammonia values. The possibility that the postirradiation step involves the decomposition of an organic hydroperoxide has been examined. We have determined organic peroxides in a number of irradiated peptide and protein solutions just prior to the hydrolysis treatment, and find that peroxide intermediates can account for only a small fraction of the liberated ammonia. This is not to suggest, however, that organic peroxides cannot be involved as labile intermediates in the formation of the amide products. Step (18) represents only an over-all reaction which may actually proceed via processes analogous to those given earlier in Eqs. (11) to (13).

The fact that the peptide linkage is the principal reaction site in "simple" chemical systems does not necessarily imply, of course, that similar reactions are of equal importance in the radiolysis of aqueous protein. Certainly, reactive groups of the various amino acid residues present in protein would be expected to provide "built-in" chemical protection against reaction at the peptide chain. Furthermore, such protection could conceivably be enhanced by various steric factors introduced by secondary and tertiary bonding. Nevertheless, the fact remains that the peptide linkage represents the single most recurrent configuration, and on the basis of our studies of small-molecule reactions, we postulated some time ago[7] (a) that main-chain cleavage could be of importance in the radiolysis of aqueous protein solutions, and (b) that high-molecular-weight products containing amide and carbonyl functions might be expected. We have developed a number of chemical techniques for investigating radiation-induced reactions of protein under various experimental conditions, and I should like now to outline very briefly some of our observations.[9, 10]

That high-molecular-weight carbonyl compounds are formed as initial irradiation products in oxygenated protein solutions can be easily demonstrated by use of the carbonyl reagent 2,4-dinitrophenylhydrazine (2,4-DNPH). The protein solutions are treated with 2,4-DNPH and are then dialyzed to separate excess reagent and any products of low molecular weight. Control runs with unirradiated solutions have shown for most of the proteins studied only a negligible retention of 2,4-DNPH reagent. In any case, the absorption spectra of the hydrazone products (in methanol--potassium hydroxide solution, after Lappin and Clark)[11] are characteristic of the $>C=N-NH-C_6H_4(NO_2)_2$ chromophore and are readily distinguished from the reagent spectrum. Quantitative applications of this colorimetric procedure give (initial) G values of about 1.2 to 1.5 for total carbonyl production in γ-irradiated solutions of pepsin and gelatin. A modified procedure in which excess reagent is extracted with ether and then assayed to give G(>CO) in terms of -G(2,4-DNPH) gives esentially identical results. Although most of our studies to date have been with protein of low sulfhydryl content, measurable reaction has also been observed with typical "sulfhydryl" proteins. For example, the initial G(>CO) value for alcohol dehydrogenase under identical conditions is about 0.5.

Of course, the above measurements provide no specific information on the positions of the carbonyl bonds. In one of the methods we have employed

to obtain data of this kind, the irradiated solutions are acid-hydrolyzed to elementary compounds before treatment with 2, 4-DNPH reagent. The hydrazone derivatives are then extracted with chloroform and examined chromatographically. All the proteins studied give a series of α-keto acids as the principal component of the total hydrazone fraction. The relative yields of the individual keto acids roughly reflect the amino acid composition of the initial protein. It should be noted that control runs of the unirradiated proteins always show some pyruvic acid. Since this arises[12] from the decomposition of serine during the hydrolysis step, most of our keto-acid studies have been made with gelatin because of its low serine content. Oxaloacetic, α-ketoglutaric, glyoxylic, pyruvic, and phenylpyruvic acids have been identified.

Measurements of ammonia and amide production in oxygenated protein solutions are also generally consistent with the mechanism outlined. Here again we found gelatin (lime-processed) to be particularly suitable for study because of the low amide content of this derived protein. The analytical methods employed are based on the work of Vickery.[13] Analyses for free ammonia and for amide groups both before and after irradiation are quite straightforward. An aliquot of each solution is made slightly alkaline and distilled at room temperature in vacuo into a receiver at low temperature. A second aliquot is made 1 N in hydrochloric acid and hydrolyzed to liberate ammonia from amide linkages. As with irradiated solution of simple peptides, the rate of ammonia liberation from irradiated gelatin solutions (in terms of hydrolysis time) appears to be typical of the amide group. Here again hydrogen peroxide and organic peroxides were found not to be involved in the postirradiation reaction. Initial G values for formation of free ammonia and acid amide groups in the oxygenated gelatin systems are about 0.3 and 1.3 respectively. It is likely that the free ammonia arises in part from terminal and (or) side-chain amino groups.

Also of interest here is the question whether or not the various side-chain linkages on the protein molecule are preferrentially attacked before the peptide chain becomes involved. Dose-yield data for amide and carbonyl production indicate that the peptide bond in the gelatin system, at least, must be considered as one of the loci of initial reaction. For example, amide production is found to be directly proportional to dose over the range 1×10^{18} to 1×10^{19} ev/ml (in a 1% solution) and extrapolates essentially to zero at zero dose. The α-keto acid production shows the same linear dose dependency. In other words, the side-chain and main-chain reactions occur in parallel, not consecutively.

All these observations on oxygenated solutions, then, are in essential agreement with the formulated mechanism in which the amide and carbonyl functions are formed directly in a radiation-induced cleavage of the polypeptide chain.

Although information on oxygen-free solutions is not complete, data obtained so far support the idea proposed earlier--namely, that cleavage of the polypeptide chain in this case does not occur as an initial consequence of indirect action. For example, treatment of pepsin and gelatin solutions with

2,4-DNPH immediately after irradiation gives total G(>CO) values of less than 0.2, as compared to 1.5 for the oxygenated case. If, however, the irradiated solutions are hydrolyzed and then analyzed, the α-keto acid and ammonia yields approach those found in corresponding studies of the oxygenated systems. These results fit in very nicely with the proposal that the acyl imino derivative is formed as one of the products of indirect action on the polypeptide chain *in vacuo*. It is quite possible, of course, that these acyl imino intermediates

$$-CH(R)-CO-N=C(R_1)-CO-NH \qquad \text{I}$$

are in the isomeric form

$$-CH(R)-CO-NH-\overset{\overset{CH(R_2)}{\|}}{C}-CO-NH, \qquad \text{II}$$

although the final hydrolysis products of both dehydropeptide forms would be the same. It is also worthy of note that various authentic dehydropeptides have been reported to be hydrolyzed enzymatically by tissue extracts.[14] The possibility that *dehydropeptidases* are involved in radiation biological phenomena does not seem to have been investigated.

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