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RADIATION CHEMISTRY OF COMPOUNDS CONTAINING THE PEPTIDE BOND

Warren M. Garrison and Boyd M. Weeks

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A few years ago we proposed that major aspects of the radiation chemistry of a wide diversity of organic nitrogeneous compounds both in the solid state and in aqueous solution could be correlated and generalized in terms of a common locus of reaction (1-4). These earlier considerations of mechanism are consistent with the viewpoint that the formation of free-radical intermediates and molecular products from compounds of the type $R_2N\text{-}CH(R)_2$ can be characterized in terms of chemical changes involving the aliphatic carbon atom adjacent to the nitrogen function. Subsequent applications of this concept to the radiation chemistry of primary amines, secondary amines, N-alkyl amides, and peptides have been made, and in a more recent report we have given detailed chemical evidence of the validity of this approach to the study of the indirect action of ionizing radiation on the variously substituted N-C bonds of protein in oxygenated solution (5). In the present paper we are concerned more specifically with the radiation-induced reactions of the peptide bond in simpler chemical compounds, both in solution and in the solid state. The N-acylamino acids provide a convenient model for such studies. The chemical and physical properties of the acetylated amino acids are fairly well defined and, in addition, there is considerable literature on analytical methods for the separation and determination of this particular class of peptide. In fact, the techniques of partition chromatography were originally developed by Martin and Synge (6) for the express purpose of analyzing protein hydrolyzates following acetylation.

Reactions in Aqueous Solution

The radiation-induced oxidation of peptides in oxygenated solution can be represented (5) in terms of the reactions

$$RCONH\text{-}CHR_2 + OH \longrightarrow RCONH\text{-}\dot{C}R_2 + H_2O \qquad (1)$$

$$RCONH\text{-}\dot{C}R_2 + O_2 + H_2O \longrightarrow RCONH_2 + RCOR + HO_2. \qquad (2)$$

The relative importance of reactions 1 and 2 in the over-all radiation chemistry of a particular peptide system will, of course, depend on the nature of the substitutions, R. However, even for aqueous protein solutions, reaction of type 1 and 2 represents the single most important path for removal of hydroxyl radicals. In the γ-radiolysis of oxygenated gelatin solutions, for example, both amide and carbonyl products are formed with $G \cong 1$ (5). It is noted that step 2 represents an over-all reaction and does not specify the nature of the intermediate involved in the reaction of $RCONH\text{-}CR_2$ with O_2 to yield the acid amide and the corresponding carbonyl product. As discussed elsewhere (5), the simplest representation of the intermediate processes of reaction 2 involves the formation of the dehydropeptide

$$RCONH\text{-}CR_2 + O_2 \longrightarrow RCON{=}CR_2 + HO_2 \qquad (3)$$

followed by

$$RCON{=}CR_2 + H_2O \longrightarrow RCONH_2 + RCOR. \qquad (4)$$

On the other hand, the peroxy radical $RCONH\text{-}C(O_2)R_2$ may be formed and undergo reactions of the type

$$RCONH\text{-}C(O_2)R_2 + H_2O \longrightarrow RCONH\text{-}C(OH)R_2 + HO_2. \qquad (5)$$

The product of 5 represents a hydrated dehydropeptide which decomposes according to

$$RCONH\text{-}C(OH)R_2 \longrightarrow RCONH_2 + RCOR. \qquad (6)$$

Recent studies of oxygenated acetylalanine and acetylglycine systems indicate that reaction 2 occurs largely through the formation of the peroxy radical. It is found that the rate of formation of pyruvic acid hydrazone, on addition of 2,4-dinitrophenylhydrazine (2,4-DNPH) to an irradiated acetylalanine solution, approximates that given by a control solution of authentic pyruvic acid. Dehydroacetylalanine, $CH_3CON{=}C(CH_3)COOH$, is relatively stable towards carbonyl reagents such as 2,4-DNPH under standard analytical conditions (7). However,

we cannot positively identify the initial product as free pyruvic acid, since the hydrated dehydropeptides $RCONH-C(OH)R_2$ also react rapidly with 2,4-DNPH (8). Current studies should resolve this ambiguity. The reaction sequence 1-2 is identical with that proposed earlier for the radiation-induced deamination of the free α-amino acids (2). The nature of the intermediate formed on reaction of $NH_2\dot{C}(R)COOH$ with O_2 via step 2 is, however, not readily subjected to experimental inquiry, since for primary amines both the dehydro intermediate (i.e., $NH{=}C(R)COOH$) and the intermediates derived from the peroxy radical represent extremely labile forms.

In the radiolysis of oxygen-free amino acid solutions, removal of $NH_2\dot{C}(R)COOH$ occurs almost exclusively through disproportionation to give $NH{=}CH(R)COOH$ which immediately hydrolyzes to ammonia and α-keto acid: dimerization of $NH_2\dot{C}(R)COOH$ also occurs but in quite low yield (2). The role of corresponding reactions in the radiation chemistry of peptides in oxygen-free solution are now under study (9). Typical data for the acetylated amino acids are described below.

Oxygen-free 0.1 M solutions of acetylglycine containing added $CH_3CONHC^{14}H_2COOH$ were irradiated in evacuated pyrex ampoules with Co^{60} γ-rays under conditions which have been previously described (2). Aliquots of the irradiated solution were analyzed directly by the method of partition chromatography adapted in this laboratory from the original method of Martin and Synge (6) and the later modifications of Marvel and Rands (10). Water adsorbed on silicic acid acts as the immobile phase; chloroform containing increasing amounts of butyl alcohol was used as the developing liquid. A second series of aliquots was hydrolyzed in 2 N hydrochloric acid for four hours at 100°C. The spectrum of nitrogen products in the hydrolyzate was then examined chromatographically on Dowex 50 (hydrogen form) after Moore and Stein (11). Hydro-

chloric acid in continuously increasing concentration (0 to 4 N) was used as the eluting agent. Analytical details including the radiometric techniques have been given (2).

Figure 1 shows a typical elution curve for a hydrolyzed fraction. Peak I contains the organic acid products which pass directly through the acid form of Dowex 50 with little or no retention. Peak II co-chromatographs with authentic aspartic acid. The major peak III is comprised of glycine liberated in the postirradiation hydrolysis of unreacted solute. Peak IV and the smaller adjacent peak correspond to the meso and racemic forms of diaminosuccinic acid. Exact correspondence between authentic diaminosuccinic acid and activity from both peaks was obtained chromatographically. Although the relative yields of these two peaks vary somewhat from one irradiation to the next, the total yield of diaminosuccinic acid is quite reproducible.

Figure 2 shows a product spectrum for unhydrolyzed acetylglycine solution as revealed by partition chromatography. Peaks III and IV have been identified as the acetylated isomeric forms of diaminosuccinic acid. The activity of peak I corresponds to free glyoxylic acid, and it is noted that the magnitude of this peak varies with the time interval between irradiation and chromatographic separation. Presumably, the labile precursor of glyoxylic acid co-chromatographs with acetylglycine or with the product peaks III and IV. Analysis for total glyoxylic acid production was made following acid hydrolysis of the irradiated solutions. In effect, this amounted to re-chromatographing peak I of Fig. 1 on silicic acid. As shown in Table I, glyoxylic acid is the major organic acid product, although formic acid is also found in low yield.

The Conway diffusion method (12) was used to determine free ammonia in the unhydrolyzed solutions. The diffusates were assayed by means of the Nessler reaction. The measured free ammonia yields were uniformly low and

did not increase appreciably above the limiting value with prolonged diffusion times. "Amide-like" ammonia was liberated by hydrolyzing the irradiated solutions *in vacuo* at 100°C in the presence of 2 N hydrochloric or acetic acid. Aliquots were taken at intervals, chilled, and assayed for free ammonia. Essentially, identical amide yields were obtained under the two conditions. Hydrolysis times required to reach the limiting values were approximately 3 hrs. and 24 hrs. for hydrochloric and acetic acids respectively.

Yields of major products formed in the γ-radiolysis of oxygen-free solutions of glycine and acetylglycine are compared in Table I. Although these data were not taken under identical conditions, they serve to illustrate the nature of the differences involved in the radiolytic response of the two systems. It is clear that a principal effect of acetylation is manifested in a pronounced increase in the yield of the diamino derivative and in a concomitant decrease in the over-all yield of the degradation reactions. Qualitatively similar results have been obtained with acetylalanine and a number of other acetylated amino acids and related compounds. By analogy with the mechanism proposed for radiolysis of oxygen-free amino acid solutions (2), we may write for the acetylated derivatives

$$2\ \mathrm{RCONH\text{-}\dot{C}(R)COOH} \longrightarrow \begin{matrix}\mathrm{RCONH\text{-}C(R)COOH}\\ \mathrm{RCONH\text{-}C(R)COOH}\end{matrix} \qquad (7)$$

$$\longrightarrow \mathrm{RCON{=}C(R)COOH} + \mathrm{RCONH\text{-}CH(R)COOH} \qquad (8)$$

$$\mathrm{RCONH\text{-}\dot{C}(R)COOH} + \mathrm{H_2O_2} \longrightarrow \mathrm{RCONH\text{-}C(OH)(R)COOH} + \mathrm{OH.} \qquad (9)$$

On this basis, formation of "amide-like" ammonia on postirradiation hydrolysis of the peptide solutions may be identified with a hydrolytic decomposition of the dehydropeptide and/or its hydrated derivative through reaction of type 4,6. Current studies of the rate of hydrolytic release of ammonia and carbonyl products indicate that both types of intermediates could be contributing. Reductive cleavage of the N-C bond via

$$H + RCONH\text{-}CH(R)COOH \longrightarrow RCONH_2 + .CH(R)COOH \quad (10)$$

$$\longrightarrow R\dot{C}O + NH_2CH(R)COOH, \quad (11)$$

does not appear to be involved to any appreciable extent in the aqueous acetylglycine system. This conclusion is based on the finding that degradation products containing the CH_2COOH radical viz glycine, acetic acid, succinic acid, and aspartic acid are found only in low yield (Table I).

The possibility that chemical change akin to the Strecker reaction (14) might be involved in the postirradiation hydrolysis of these systems has been considered in some detail.[2] Reactions of monocarbonyls with glycine, alanine, etc., are known to lead to transamination of the type $RCHO + R'CH_2NH_2 \longrightarrow R'CH_2NH_2 + RCHO$ in neutral or alkaline solution (15). However, under the conditions of acid hydrolysis such reactions are suppressed, although on prolonged heating, some evidence for transamination is obtained (16). Similar conclusions were reached in the earlier study of carbonyl products formed in the radiolysis of oxygenated peptide solutions (5). However, the diamino acids formed in oxygen-free solutions have been found to be somewhat more reactive towards monocarbonyl compounds. Hence, although the G value for carbonyl production remains constant on prolonged hydrolysis, the identity of the carbonyl product fraction is not retained. And, furthermore, at the longer hydrolysis times, some additional ammonia appears--presumably through hydrolysis of aminoaldehydes formed via transamination. Similar reactions have been observed between 1,2-dicarbonyls and monoamino acids, under the same condition. The problem here, of course, is that H reduction of acetylglycine at the acetyl group followed by dimerization of the reduced radical species would yield a 1,2-dicarbonyl derivative which may not be detected as such by our standard methods for carbonyl analysis (5). These aspects of the problem are now being investigated.

Reaction in the Solid State

From the nature of the reactions observed in our initial studies on the

radiation-induced cleavage of the N-C bond in aqueous peptide systems, we were led to the conclusion that the observed reactivity of the α-carbon locus might also be a factor in the radiation chemistry of the solid state. And, as part of these earlier studies, we found through application of the analytical methods developed for aqueous protein systems that irradiated solid pepsin and gelatin (evacuated) on dissolution in water do indeed yield high-molecular-weight compounds containing the carbonyl function (3). Subsequent hydrolysis of the products showed the constituent carbonyl compounds to include α-keto acids. Hence, we tentatively proposed that dehydrogenation followed by the hydrolysis step 4 could be involved. Meanwhile, Caputo and Dose (16) in a comparative study of solid proteins and their corresponding model hydrolyzates found that proteins, unlike the hydrolyzates, exhibited amide-like function as a major radiation chemical change. Caputo and Dose also established that the product fragments (separated electrophoretically) contained carbonyl groups, and they concluded that dehydropeptides were involved. The more recent studies of Alexander and Hamilton (17) and Bowes and Moss (18) appear to generally confirm the idea that a major chemical effect of ionizing radiation on protein in the solid state leads to degradation of the peptide chain with formation of amide ammonia and carbonyl products, both in the presence and in the absence of oxygen. However, concepts of the nature of the elementary processes involved remain largely speculative.

The initial proposal that the aliphatic carbon atom α to the NH group represents a major locus of the direct action of ionizing radiation on compounds containing the peptide bond was derived strictly from chemical evidence based on a comparison of products formed in aqueous solutions and in solid systems. Although characteristic ESR spectra of irradiated peptides had been observed, it was not until several years later that these spectra were interpreted (19,20). The ESR studies of a variety of irradiated proteins,

simple peptides, N-alkylamides, and N-acylamino acids indicate that the species $RCONH\text{-}C(R)_2$ represents the principal free radical product at room temperature (19-23).

The ESR spectra of acetylglycine and acetylalanine have been attributed to $CH_3CONH\text{-}\dot{C}HCOOH$ (doublet) and $CH_3CONH\text{-}\dot{C}(CH_3)COOH$ (tetraplet) respectively. The fact that these radical species are stable in the absence of oxygen for prolonged periods of time makes it possible to distinguish, at least in part, their separate contribution to the over-all chemistry of the irradiated solid. Three different experimental treatments are involved: (I) irradiation <u>in vacuo</u>, dissolution in oxygen-free water; (II) irradiation <u>in vacuo</u>, dissolution in oxygenated water; (III) irradiation and dissolution in the presence of oxygen. Now, it has been shown, in the case of the acetylated amino acids, that radicals of the type $RCONH\text{-}C(R)COOH$, as formed in aqueous solution, react almost exclusively in the absence of oxygen to give (acetyl) diaminosuccinic acid via step 7; whereas, in the presence of dissolved oxygen, cleavage of the N-C bond occurs through reaction akin to step 2. Yields of diaminosuccinic acid and ammonia from solid acetylglycine under the three prescribed experimental conditions are summarized in Table II. These data, obtained by direct application of the analytical methods described earlier in the section on aqueous systems, substantiate the ESR assignment of $CH_3CONH\text{-}CHCOOH$ as the long-lived radical species, and also are in accord with the mechanisms outlined here. Comparison of the diaminosuccinic acid yields under condition I and II gives $G \simeq 1.2$ as a minimum value for the yield of $CH_3CONH\text{-}CHCOOH$ in the solid state.

$$CH_3CONH\text{-}CH_2COOH \rightsquigarrow CH_3CONH\text{-}\dot{C}HCOOH + H. \qquad (12)$$

It is also seen from Table II that the loss in diaminosuccinic acid is balanced by a corresponding increase in amide ammonia on changing the experimental condition from case I to case II. This finding is in ~~a~~ quantitative agreement with the concept that reaction of $CH_3CONH\text{-}CHCOOH$ with O_2 leads to cleavage of the

N-C bond. The fact that the amide yield is increased even more if oxygen is present during irradiation (condition III) may be attributed to an interference of O_2 with radical or charge recombination and/or to reaction of O_2 with excited states which are otherwise removed in vacuo. A more complete material balance is required to distinguish the various possibilities.

The formation of amide ammonia with $G \simeq 2.0$ under the condition in which both irradiation and dissolution are made in the absence of oxygen cannot be represented simply in terms of the dehydrogenation reaction

$$CH_3CONH\text{-}CH_2COOH \rightsquigarrow CH_3CON{=}CHCOOH + H_2. \qquad (13)$$

As shown in Table II, the combined yield of glyoxylic acid plus formaldehyde corresponds to a G value of approximately 0.65. This accounts for less than one-half the amide yield. Furthermore, some glyoxylic acid and ammonia ($G \simeq 0.2$) are detected prior to hydrolysis, and this suggests the possibility that part of the carbonyl yield arises from the molecular rearrangement

$$CH_3CONH\text{-}CH_2COOH \rightsquigarrow CH_3CHO + NH{=}CHCOOH. \qquad (14)$$

Studies of the C^{14}-labeled degradation products formed in the radiolysis of $CH_3CONHCH_2C^{14}OOH$ indicate that a major fraction of the "amide" ammonia produced in excess of the carbonyl yield arises from processes involving direct cleavage of the N-C bond, for example

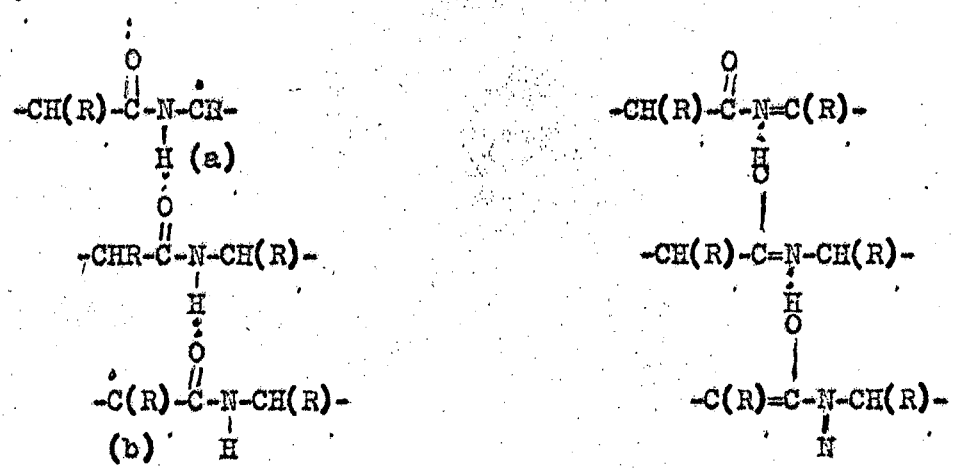
$$CH_3CONHCH_2COOH \rightsquigarrow CH_3CONH + CH_2COOH, \qquad (15)$$

followed by immediate hydrogen abstraction reactions of the products of 15 with neighboring acetylglycine molecules. Evidence has also been found for degradation of the N-C bond through intramolecular rearrangement. However, the major re-arrangement process in the acetylated amino acids is the decarboxylation reaction

$$CH_3CONHCH_2COOH \rightsquigarrow CH_3CONHCH_3 + CO_2, \qquad (16)$$

which, of course, is characteristic of organic acids in general--both in radiolysis and photolysis (24).

Finally, we should like to mention briefly some observations that indicate that the hydrogen-bond systems of solid peptides are involved in the co-valent chemistry of radiolysis. Now, the G values for production of long-lived radicals of the type -NH-CR- in the γ-radiolysis of simple peptides, N-acylamino acids, N-alkylamides, etc., are in the order of unity (20,21,23); in some instances, even higher values have been reported. On the other hand, G values for radical production in native protein are uniformly quite low; in most cases the production of a resonance center requires some 10^3 ev or more (25). Of course, part of this effect may be attributed to differences in the radiation chemical contributions of the various side chains which have not, in all cases, been separately studied in simple peptide systems. However, Blyumenfel'd and Kalmanson (25) have shown that thermal denaturation of protein prior to irradiation results in a striking increase in the yield of resonant centers per unit of absorbed energy. They find that thermal disruption of the hydrogen-bond network increases the free-radical yield by several hundred times and have interpreted this effect as evidence for electron or hole migration. We propose an alternate explanation. Consider the schematic representation of the α-helix configuration in native protein containing the radical sites (a), (b) as shown in I

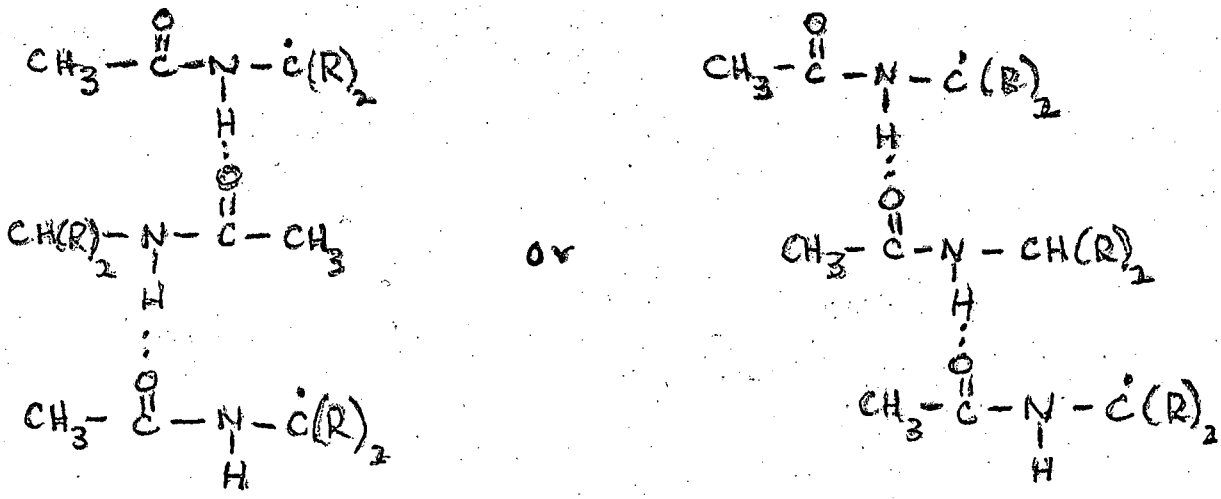


On the basis of the spur model (26) the radical sites in the solid are taken to be formed in distributions that are confined. Interaction of (a) and (b)

can occur without appreciable change in the position of atomic nuclei. Chemically, the net effect is a disproportionation reaction

$$2 \sim CONH\text{-}\dot{C}(R)\sim \longrightarrow \sim CONH\text{-}CH(R)\sim + \sim CON{=}C(R)\sim,$$

accompanied by enolization of the intervening hydrogen-bond bridge. The latter chemical change, incidentally, provides a co-valent mechanism for the localized disruption of the hydrogen-bond configuration of irradiated protein. Consider now a similar pair of radicals in a simple acylamine--either as III or IV



III or IV

The point here is that in these simple model compounds, radical H combination cannot occur simply through electron re-arrangement as is possible in the polypeptide structure, although both systems have the peptide bond in the <u>trans</u> configuration. On the other hand, it is readily shown that the amino acid anhydrides as simple peptides containing the <u>cis</u> configuration can undergo radical disproportionation and enolization. A similar formulation can be written for the nucleic acids. Chemical evidence for radiation-induced enolization of the hydrogen-bond has recently been observed in studies of the radiation synthesis of pyrazine-like derivatives from amino acid anhydrides in the solid state (27). These preliminary concepts will be treated in detail in a forthcoming publication on the role of hydrogen-bonds in the co-valent radiation chemistry of peptide systems.

SUMMARY

Radiation-induced reactions of peptides in solution and in the solid state are described. It is shown that the original proposal regarding the reactivity of the ~NH-CH(R)~ locus is in accord with the more recent chemical and physical evidence. A specific radiation-chemical role is assigned to the hydrogen-bond structure.

TABLE I

Product Yields in the γ-Radiolysis of Oxygen-free Solutions of Glycine and Acetylglycine

Product	Yield[a]	
	NH_2CH_2COOH[b]	$CH_3CONH\text{-}CH_2COOH$[c]
Ammonia	4.3	0.90
Glyoxylic Acid	2.4	0.50
Acetic Acid	1.3	~.02
Diaminosuccinic Acid	~.08	1.6
Aspartic Acid	0.25	0.13

[a] Molecules per 100 ev absorbed in water

[b] Compiled from data reported in references 2 and 13

[c] Measured after hydrolysis

TABLE II

Yields of Nitrogen-containing Products in the γ-Radiolysis of Acetylglycine, Solid[a]

Experimental Conditions	Yield[b,c]	
	Ammonia	Diaminosuccinic Acid
I	2.0	0.60
II	3.2	.12
III	4.4	<.12

[a] Dose=1 x 10^{21} ev/gm

[b] Molecules per 100 ev absorbed in solid

[c] Measured after hydrolysis

FOOTNOTES

[1] This work was performed under the auspices of the United States Atomic Energy Commission

[2] As pointed out in ref. 5, one cannot assume a priori that product groups formed on radiolysis of protein and other peptides are unreactive during postirradiation treatment. It is quite likely that part of the reported "radiation sensitivity" of the more labile amino acid residues may be attributed to "dark" reactions between these residues and the initial products. Reactions of carbonyl groups (and other unsaturated linkages) with substances such as tryptophane, histidine, and sulfur-containing moieties are well-known, and these could contribute to the observed chemical and biological properties of irradiated proteins.

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LEGENDS FOR FIGURES

Fig. 1 Typical elution curve (Dowex 50) of irradiated acetylglycine solution, hydrolyzed (Weeks, Cole, and Garrison, ref. 9).

Fig. 2 Typical elution curve (silicic acid) of irradiated acetylglycine solution, unhydrolyzed (Weeks, Cole, and Garrison, ref. 9).

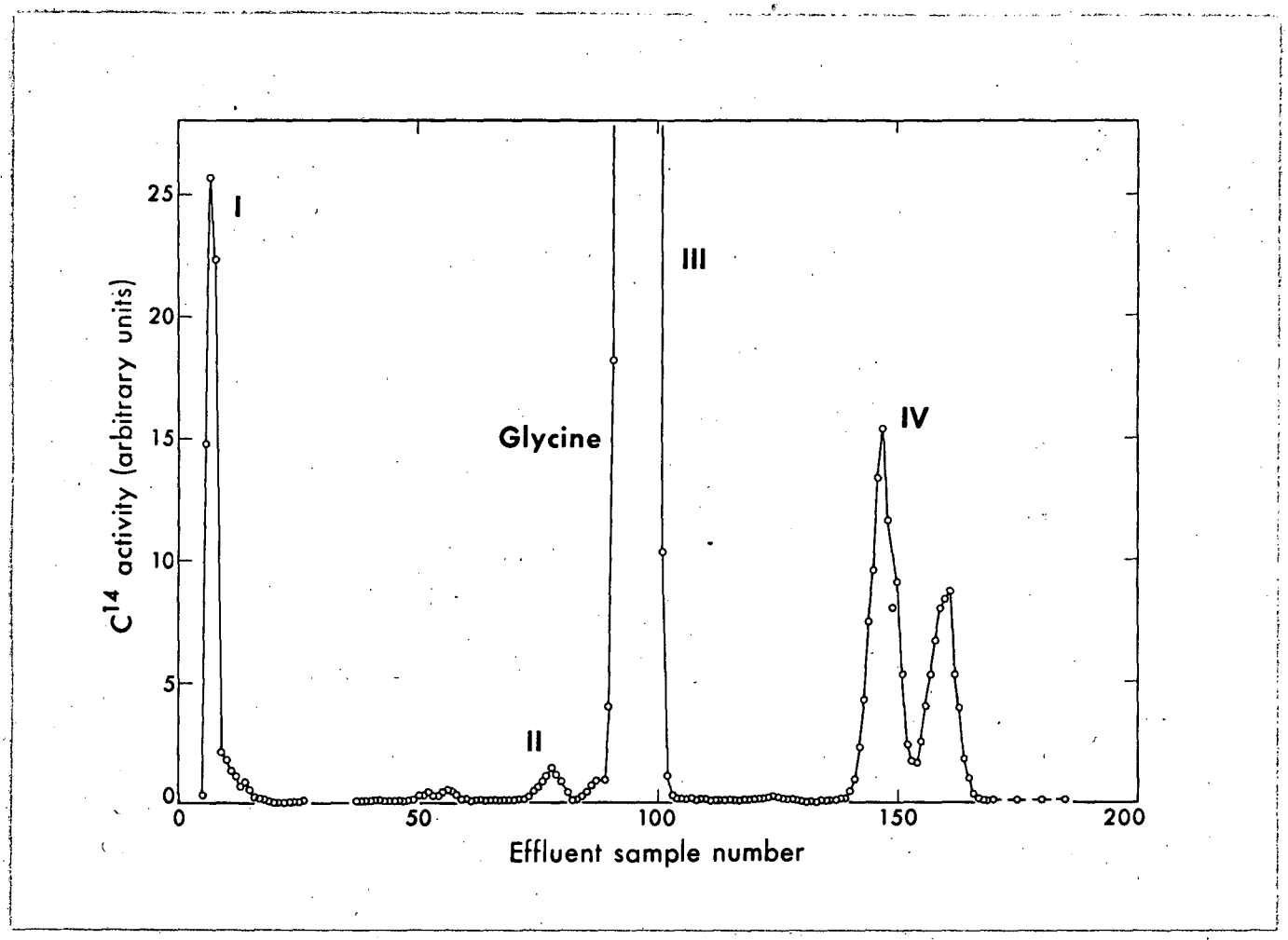

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Fig. 1

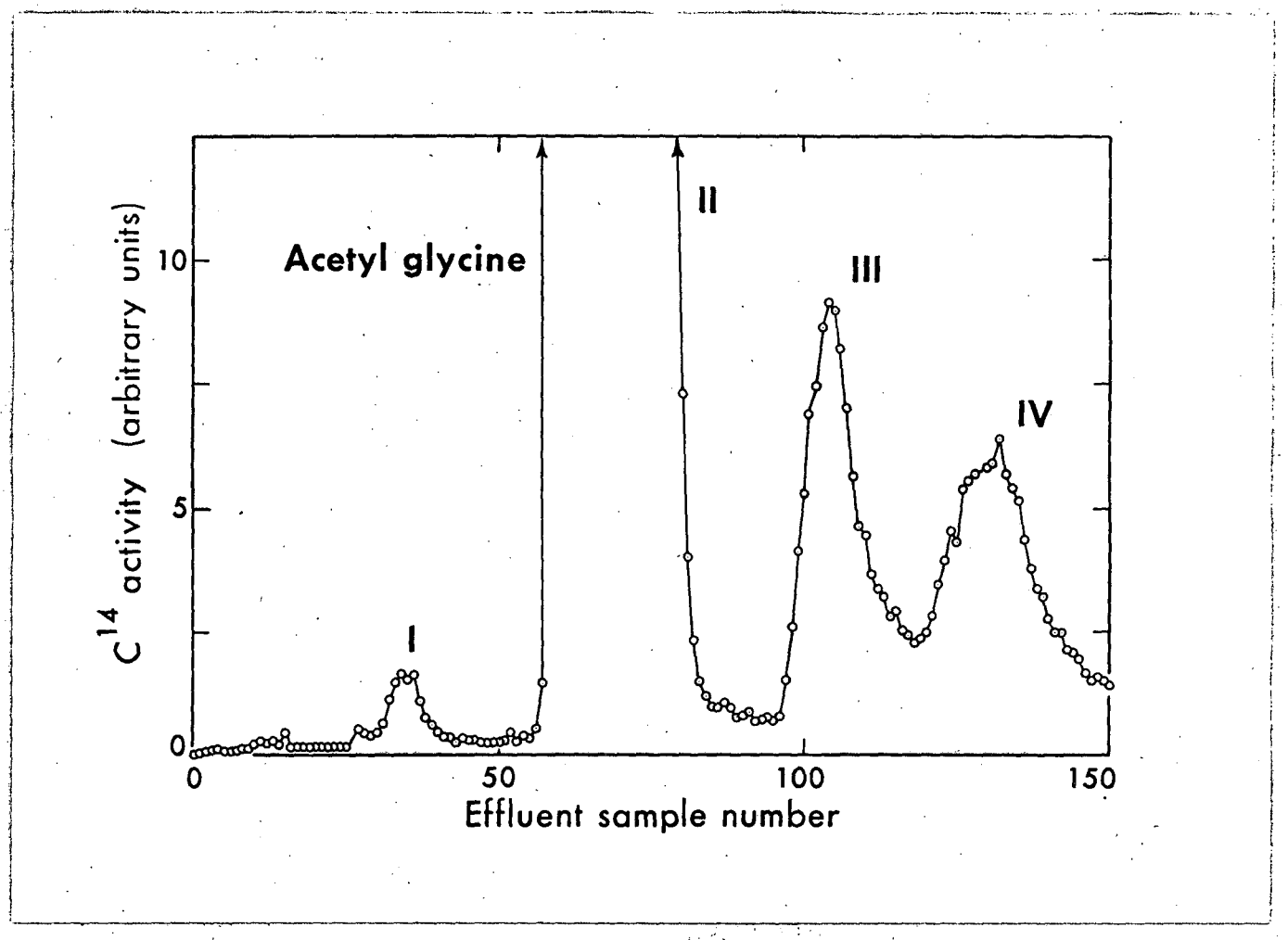

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Fig. 2