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INDIRECT ACTION OF IONIZING RADIATION ON SOME ORGANIC COMPOUNDS CONTAINING THE C-N BOND

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ABSTRACT

Observations on the indirect action of ionizing radiation on compounds containing primary amine, secondary amine, N-alkylamide, and peptide-bond configurations are correlated in terms of related mechanisms involving the C-N linkage as the locus of chemical change. The radiation chemistry of aqueous solutions of various amino acids and proteins is considered in detail from this viewpoint. Effects of oxygen and other solutes are discussed. Particular reference is given to: (a) studies of the radiation-induced oxidation of protein in oxygenated solution, (b) studies of radical-radical reaction in oxygen-free protein solutions containing a simple C^{14}-labeled organic solute such as $CH_3C^{14}OOH$.

INTRODUCTION

Of the various classes of organic compounds containing the C-N bond, certainly the α-amino acids and derivatives--largely because of their biological import--have received the greatest attention in radiation chemistry. This paper develops certain specific aspects of mechanism in the radiolysis of aqueous amino acids, both free and combined as native protein. The treatment is based on various literature sources and on information derived from investigations at this laboratory.

The latter have involved (a) detailed radiation chemical studies of particular chemical bonds and groups in the simpler compounds and (b) parallel studies of corresponding processes as they occur in increasingly complex systems. One of the specific objectives of this research has been the development of experimental approaches that will provide detailed and precise information on the loci of radiation-induced reaction in protein systems.

It has often been noted that the radiation chemistry of even the simpler aquo-organic systems may be complicated considerably by the fact that a multiplicity of products can be formed. Further, these products may be encountered in small amount and at low concentrations. Fortunately, the

use of C^{14}-labeled materials in conjunction with appropriate radiometric and chromatographic techniques can greatly facilitate the experimental study of these systems. In large part, the considerations presented herein have been derived through various applications of such methods.

THE INITIAL REACTION

Studies of radiation-induced reaction in aqueous solution are generally consistent with the theory that the earliest detectable products of water decomposition include H atoms, OH radicals, H_2, and H_2O_2. The initial chemical reaction is conveniently described in terms of the notation[1, 2]

$$H_2O \rightsquigarrow H,\ OH,\ H_2,\ H_2O_2.$$

The 100-ev yields of the initial products are denoted by $G_w(H)$, $G_w(OH)$, $G_w(H_2)$, $G_w(H_2O_2)$; the yields of final products are denoted G(product).

GENERAL ASPECTS OF INDIRECT ACTION

The following brief summary of the intermediate processes of indirect action will serve to establish the viewpoint of subsequent discussion.

In oxygen-free solutions, the removal of H and OH in reactions with organic solute may involve simply the hydrogen-abstraction steps

$$RH + H \longrightarrow R + H_2,$$

$$RH + OH \longrightarrow R + H_2O.$$

However, with certain solutes other (competing) processes such as displacement,

$$RH + H \longrightarrow R'H + R'',$$

and addition,

$$RH + H \longrightarrow R',$$

can be of importance in the removal of both H and OH. Regardless of other possible intermediate steps which often complicate the picture, ultimate removal of the organic free-radical intermediates involves either disproportionation,

$$2R \longrightarrow RH + R'H,$$

and (or) combination,

$$2R \longrightarrow R\text{-}R,$$

depending on the nature of the solute. It is to be noted that products of radical combination in some systems may be relatively unimportant in determining over-all product stoichiometry and yet may be of considerable

interest when considered in relation to the question of reaction mechanism. Often, information on the nature of the free-radical intermediates can be deduced directly from the chemical structure of high-molecular-weight products formed in radical-combination processes.

In oxygenated solution, reactions of H atoms with solute can be effectively blocked by the competing process

$$H + O_2 \longrightarrow HO_2,$$

whereas reaction between OH and organic solute is unaffected. The chemistry of the oxygenated system, then, is determined primarily by the properties of intermediates formed via

$$R + O_2 \longrightarrow RO_2.$$

For certain systems this reaction may simply be written

$$R + O_2 \longrightarrow R'H + HO_2.$$

In such cases the ultimate radical-removal step involves only

$$2HO_2 \longrightarrow H_2O_2 + O_2.$$

Generally, however, removal steps involving both HO_2 and RO_2 must be invoked.

Some specific applications of these considerations to the radiation chemistry of nitrogenous compounds are described in the following sections.

OXYGEN-FREE SOLUTIONS

The major effects of indirect action on α-amino acids of the general type $RCH(NH_2)COOH$ result in the formation of NH_3, $RCHOCOOH$, RCH_2COOH and H_2 as principal products. Smaller amounts of RCHO and CO_2 have also been found.[3-8]

Quantitative data are most complete for the particular case of glycine. Radiolysis studies have been made with x-rays, γ rays, β rays, and cyclotron-produced helium ions. The radiation chemistry of the oxygen-free solution has been shown[8] to be in accord with the mechanism

$$H_2O \rightsquigarrow H,\ OH,\ H_2,\ H_2O_2,$$

$$H + NH_2CH_2COOH \longrightarrow H_2 + NH_2CHCOOH$$

$$\longrightarrow NH_3 + CH_2COOH,$$

$$OH + NH_2CH_2COOH \longrightarrow H_2O + NH_2CHCOOH,$$

$$CH_2COOH + NH_2CH_2COOH \longrightarrow NH_2CHCOOH + CH_3COOH,$$

$$H_2O_2 + NH_2CHCOOH \longrightarrow H_2O + OH + NHCHCOOH,$$

$$2NH_2CHCOOH \longrightarrow NHCHCOOH + NH_2CH_2COOH,$$

$$H_2O + NHCHCOOH \longrightarrow NH_3 + CHOCOOH.$$

Substantiating evidence for the formation of the proposed intermediates CH_2COOH and $NH_2CHCOOH$ came from detailed chromatographic studies of the high-molecular-weight product fraction.[8] The entire spectrum of nitrogen-containing products was examined by a procedure which was essentially a modification of the method of Moore and Stein.[9] The irradiated glycine solution containing $NH_2C^{14}H_2COOH$ or $NH_2CH_2C^{14}OOH$ was added to a Dowex-50 column (hydrogen form) and the radiolysis products plus parent glycine were eluted by hydrochloric acid in progressively increasing concentration (0 to 4 N). Effluent samples were dried to yield the hydrochloride salts, and product peaks were determined by assaying for C^{14} activity and (or) by titrating the hydrochloride salt with 0.01 N sodium hydroxide. Figure 1 shows a typical elution curve for oxygen-free 0.25 M glycine solution irradiated with 30-Mev helium ions. A qualitatively similar curve was obtained with Co^{60} γ rays. An interesting feature of these chromatographs is the relatively large number of products peaks observed at a radiation dose corresponding to a removal of less than one percent of the parent solute. Two of these activity peaks from their approximate positions were suspected of being aspartic acid and diaminosuccinic acid respectively. An exact correspondence between the C^{14}-labeled product and authentic carrier in titratable amounts was subsequently observed in both cases. These elution curves are reproduced in Fig. 2. The nonnitrogenous organic-acid fraction which passed through the ion-exchange column with little or no fractionation or retention (peak 1, Fig. 1) was subsequently separated by partition chromatography on a silicic acid column through application of methods adopted from the procedure of Marvel and Rands.[10] Activity peaks were found in the regions corresponding to acetic, glyoxylic, and succinic acids. Subsequent co-chromatographs of each fraction with the appropriate authentic carrier confirmed these identifications. The simplest explanation for the formation of succinic acid, aspartic acid, and diaminosuccinic acid is that small fractions of the CH_2COOH and $NH_2CHCOOH$ radicals undergo the competing combination reactions

$$2CH_2COOH \longrightarrow \text{succinic acid},$$

$$NH_2CHCOOH + CH_2COOH \longrightarrow \text{aspartic acid},$$

$$2NH_2CHCOOH \longrightarrow \text{diaminosuccinic acid}.$$

It is noted that the formation of succinic acid via dimerization of CH_2COOH is a principal reaction in the radiolysis of oxygen-free acetic acid solutions.[11]

The above considerations suggested the possibility that loci involved in the indirect action of radiation on protein molecules could be ascertained through study of radical-radical reactions in protein solutions containing a second organic solute. For example, in the radiolysis of oxygen-free solutions containing protein molecules RH and a simpler organic solute R'H, there is a given probability of radical combination of the type $R + R' \rightarrow R - R'$. If the nature of the radical R' is known from independent studies, information on the configuration of the protein radical R could be obtained through identification of products formed in conventional hydrolysis

of the modified protein R - R'. It is obvious that the use of C^{14}-labeled solutes, R'H, would greatly facilitate such studies. We have at this laboratory undertaken studies of the radiation chemistry of a number of protein systems in which the second solute, R'H, is C^{14}-labeled acetic acid, ($C^{14}H_3COOH$ or $CH_3C^{14}OOH$). This solute species as mentioned earlier yields the radical CH_2COOH on H and (or) OH attack. Reactions of CH_2COOH with radicals derived from pepsin, gelatin, and β-lactoglobulin have been studied.[12, 13]

The oxygen-free solutions containing acetic acid and protein at concentrations of 10^{-2} M and 10^{-4} M to 10^{-5} M respectively were irradiated with 40-Mev helium ions from the Crocker Laboratory cyclotron. Irradiation techniques have been described in earlier publications.[11] The irradiated solutions were distilled to dryness in vacuo to recover C^{14}-labeled acetic acid. The residue was redissolved in 1 N hydrochloric acid and dialyzed for 3 to 4 hours in cellophane against running water in the cold. The C^{14} activity decreased rapidly to a limiting value which did not change appreciably after about 2 hours. (There was negligible retention of C^{14} activity in duplicate manipulations of unirradiated protein - $C^{14}H_3COOH$ solutions). The dialyzed material was made 4 N in hydrochloric acid, evacuated, and hydrolyzed for 24 to 36 hours at a temperature of about 90°C. The hydrolyzate was taken to dryness in vacuo and chromatographed on Dowex-50 as described above. Elution curves obtained with gelatin, pepsin, and β-lactoglobulin are reproduced in Figs. 3, 4, 5 respectively. Each chromatograph shows a series of distinct C^{14}-activity peaks. The fraction designated peak A in each figure was found to co-chromatograph with authentic aspartic acid. A typical curve is shown in Fig. 6. An aliquot of fraction A from pepsin was treated with nitrous acid to obtain the corresponding hydroxy acid. The material was then chromatographed on the silicic acid column with 35% butanol (v/v) in chloroform. Correspondence between C^{14} activity and authentic malic acid titer was observed. The simplest explanation for the formation of C^{14}-labeled aspartic acid under the conditions of this study is that $CH_2C^{14}OOH$ radicals combine with protein radicals containing the -NH-CH-CO- configuration. The fact that a series of C^{14}-labeled nitrogen-containing products is produced in the radiolysis of protein-$CH_3C^{14}OOH$ solutions suggests that the formation of aspartic acid represents but one path of the general radical-radical reaction which can be written

$$\text{-NH-C(R)-CO-} + CH_2COOH \longrightarrow \text{-NH-}\overset{\overset{\displaystyle CH_2COOH}{|}}{C}\text{(R)-CO-}$$

where R represents any of the amino acid residues. An extensive study of the relative contributions of these various possible protein free radicals is being made. In this regard it is noted that peak A, as would be expected, is the major C^{14}-labeled product from gelatin, since this protein contains a large proportion of glycine residues. However, the fraction of alanine residues is also relatively high. In attempting to measure the relative contributions of -NH-CH-CO- and -NH-C(CH_3)-CO- in the formation of observed products we were surprised to find that aspartic acid and methyl aspartic acid are not completely resolved on the ion-exchange column, at least under the conditions employed in this study. Analysis of such unresolved amino-acid pairs is now being made after conversion to the corresponding hydroxy acids by nitrous acid treatment. Partition chromatography on the silicic

acid column[10] is then employed to effect a separation.

OXYGENATED SOLUTIONS

The indirect action of radiation on amino acids in oxygenated solution can be represented to a first approximation by the net over-all reaction:[7]

$$RCH(NH_2)COOH + O_2 + H_2O \rightsquigarrow RCOCOOH(RCHO + CO_2) + NH_3 + H_2O_2$$

The following detailed reaction scheme[8] has been derived primarily from studies of oxygenated glycine solutions:

$$H_2O \rightsquigarrow H, OH, H_2, H_2O_2,$$

$$H + O_2 \longrightarrow HO_2,$$

$$OH + NH_2CH_2COOH \longrightarrow NH_2CHCOOH + H_2O,$$

$$O_2 + NH_2CHCOOH \longrightarrow NHCHCOOH + HO_2,$$

$$H_2O + NHCHCOOH \longrightarrow NH_3 + CHOCOOH,$$

$$2HO_2 \longrightarrow H_2O_2 + O_2.$$

It is noted that reaction of $NH_2CHCOOH$ with O_2 to give the imino intermediate NHCHCOOH merely represents the simplest path for removal of $NH_2CHCOOH$ radicals consistent with the fact that ammonia and glyoxylic acid are formed in approximately equal yields in oxygen-saturated solution.[7] Formation of an unstable peroxide intermediate via

$$O_2 + NH_2CHCOOH \longrightarrow NH_2CH(O_2)COOH,$$

$$HO_2 + NH_2CH(O_2)COOH \longrightarrow NH_2CH(O_2H)COOH + O_2$$

followed by

$$H_2O + NH_2CH(O_2H)COOH \longrightarrow NH_3 + CHOCOOH + H_2O_2,$$

would of course lead to the same stoichiometry.

Cleavage of the C-N bond by the indirect action of radiation is not confined to the primary amino configuration. Secondary amines, for example, undergo radiation-induced reaction in oxygenated solution to give primary amine and aldehyde,[14]

$$RNHCH_2R' + O_2 + H_2O \longrightarrow RNH_2 + R'CHO + H_2O_2,$$

and one of the reactions involved in the radiolysis of aqueous solutions of protein under appropriate conditions apparently leads to cleavage of the peptide chain to form the carbonyl and amide functions:

$$-CO-NH-CH(R)- + O_2 + H_2O \rightsquigarrow -CONH_2 + RCO- + H_2O_2.$$

Evidence for the latter radiation-induced oxidation has been derived largely from studies of carbonyl production in oxygenated solutions of pepsin and gelatin.[15, 16] The carbonyl function is found to be associated with high-molecular-weight (nondialyzable) products which on conventional acid hydrolysis yield a series of α-keto acids. Carbonyl compounds in addition to the simple α-keto acids are also observed.

Because of the probable importance of these reactions in radiation biological phenomena, we have undertaken extensive studies of the production of > C=O bonds in the radiolysis of oxygenated protein systems.

Irradiations were made with Co^{60} γ rays at a dose rate of ~ 2×10^{17} ev/ml/min. The solutions were exposed under one atmosphere of oxygen in sealed Pyrex tubes mounted in a motor-driven reel situated at standard distance from the source.

Immediately after irradiation each of the protein solutions (5 mg/ml) was treated with platinum black to destroy hydrogen peroxide and then treated with 2, 4-dinitrophenylhydrazine-hydrochloric acid solution to form the hydrazone derivatives. (Control runs established that protein oxidation is not induced by the addition of platinum black to these systems.) The solution was dialyzed rapidly in cellophane against running water in the cold to remove excess reagent and possible reaction products of low molecular weight. An appropriate aliquot of the dialyzed protein was then added to methanol–potassium hydroxide solution for spectrophotometric analysis after the method of Lappin and Clark.[17] A typical absorption curve (filled circles) for irradiated pepsin solution treated in this manner is shown in Fig. 7. Similar curves were obtained with gelatin. The absorption spectrum of a representative monocarbonyl derivative (acetaldehyde 2, 4-dinitrophenylhydrazone) is shown for purposes of comparison. The upper curve (open circles) shows the absorption spectrum of 2, 4-dinitrophenylhydrazine reagent under identical conditions. The absorption curve of irradiated pepsin treated with 2, 4-dinitrophenylhydrazine is seen to be characteristic of the $> C=N-NH-C_6H_4(NO_2)_2$ chromophore. Lappin and Clark have shown for monocarbonyl 2, 4-dinitrophenylhydrazones that the position of the absorption maximum (as well as the value E_{max}) is nearly independent of the structure of the carbonyl compound. If the acetaldehyde derivative is used as a standard, an initial yield of 1.2 carbonyl groups is obtained per 100 ev absorbed in the pepsin solution. Pepsin in unirradiated controls showed negligible retention of 2, 4-dinitrophenylhydrazine. Unirradiated gelatin solutions, on the other hand, always showed a small retention.

To eliminate various uncertainties involved in the above determination, a method was developed to measure G(>C=O) directly in terms of -G(2, 4-dinitrophenylhydrazine). The protein solutions were treated with 2, 4-dinitrophenylhydrazine-hydrochloric acid, and after reaction was complete, any excess reagent was removed for assay by successive extractions with equal volumes of ether. The time required for stoichiometric reaction was determined in separate kinetic studies. Figure 8 shows the optical densities (in methanol-potassium hydroxide) of control and irradiated gelatin solutions as a function of the initial concentration of 2, 4-dinitrophenylhydrazine. The data of Fig. 8 give -G(2, 4-dinitrophenylhydrazine) = 1.5. This value is

essentially the same as that obtained by the method in which acetaldehyde 2, 4-dinitrophenylhydrazone is used as a reference standard. Figure 8 also shows that there is some interaction between unirradiated protein and 2, 4-dinitrophenylhydrazine reagent.

Part of the carbonyl function measured by the above techniques has been found to be associated with α-keto acids liberated on hydrolysis of the irradiated and dialyzed protein. In these studies the irradiated protein solutions were hydrolyzed in 4 N HCl (in vacuo) for 24 hours prior to treatment with 2, 4-dinitrophenylhydrazine reagent. (We found it necessary to add the hydrazine reagent after hydrolysis, since hydrazone derivatives are generally rather rapidly destroyed by heating in mineral acids.) The hydrazone derivatives were extracted with chloroform and examined chromatographically.[18] It was found that the irradiated samples yield a series of α-keto acids. The relative yields of these products roughly reflect the amino acid composition of the initial protein. It is of interest to note also that control runs with unirradiated pepsin showed the presence of measurable amounts of pyruvic acid. This apparently arises from the decomposition of serine during acid hydrolysis. Pyruvic acid was observed only in trace amounts in gelatin hydrolyzates, presumably because of the lower serine content of the latter. Most of the keto acid identification studies were therefore made on the irradiated gelatin system. The following keto acid products have been identified: oxaloacetic, α-ketoglutaric, glyoxylic, pyruvic, and phenylpyruvic. Identification was based on the fact that in each case the 2, 4-dinitrophenylhydrazone derivatives could not be distinguished chromatographically from the corresponding authentic material. Typical chromatographs of the α-keto acid hydrazones recovered from an irradiated gelatin solution and an identical control solution are shown in Fig. 9. As a second independent method of identification, each of the separated keto acid hydrazones was converted into the corresponding amino acid by catalytic hydrogenation. Chromatographic characterization of the amino acid in each case confirmed the original product identification. We also considered the possibility that transmutation reactions may occur during hydrolysis once a particular keto acid has been released. If such a reaction did occur it would, of course, introduce an uncertainty in the identification of the initial carbonyl products. The results of a series of control runs, however, vitiated this possibility. (See also reference 19). Carbonyl products in addition to those associated with the α-keto acid fraction have also been observed. Studies of these other fractions are in progress.

Substantiating evidence for the proposed radiation-induced oxidation of the peptide bond has also been obtained from studies of the amide content of reaction products formed by γ-ray-induced oxidation of aqueous gelatin.[20] Commercially available lime-process gelatin was found to be particularly suitable for this study since most of the amide groups of the glutamine and asparagine residues of the parent collagen are removed through hydrolysis during the manufacturing process. Unmodified proteins, in general, contain amide groups in sufficient number to mask the radiation-induced reaction at the lower radiation dosages necessarily invoked in studies of initial reaction products. Analyses were made for "free" ammonia and for amide groups, both before and after irradiation. Analytical methods essentially were those developed by Vickery. The data indicate that the formation of amide groups ($G \approx 1$) corresponds to one of the principal reactions involved in the radiolysis of aqueous solutions of gelatin. The G value for this product group is very

close to that obtained for total carbonyl formation under equivalent irradiation conditions. Although the simplest explanation for this correspondence would seem to involve the postulated reaction we cannot conclude at the present time that other undefined reactions do not contribute to the amide yield as measured. Ammonia formation ($G \approx 0.3$) may be attributed to a radiation-induced oxidation of terminal and (or) side-chain amino groups.

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LEGENDS FOR FIGURES

Fig. 1. Typical elution curve of nitrogen-containing products formed in the helium-ion irradiation of 0.25 M glycine solution containing $NH_2C^{14}H_2COOH$. (*) Diaminosuccinic acid is eluted in two fractions. (From Weeks and Garrison.[8])

Fig. 2. Co-elution curves of authentic aspartic and diaminosuccinic acids with C^{14}-labeled products from glycine. (From Weeks and Garrison.[8])

Fig. 3. Typical elution curve of C^{14}-labeled compounds obtained from irradiated gelatin-$CH_3C^{14}OOH$ solutions after dialysis and hydrolysis. (From Weeks, Cole, and Garrison.[13])

Fig. 4. Typical elution curve of C^{14}-labeled compounds obtained from irradiated pepsin-$CH_3C^{14}OOH$ solutions after dialysis and hydrolysis. (From Weeks, Cole, and Garrison, UCRL-8265, April, 1958.)

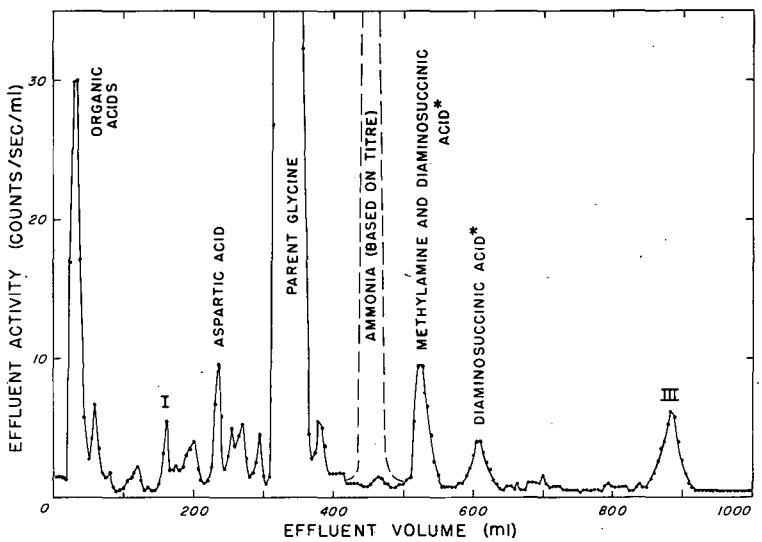
Fig. 5. Typical elution curve of C^{14}-labeled compounds obtained from irradiated β-lactoglobulin-$CH_3C^{14}OOH$ solutions after dialysis and hydrolysis. (From Weeks, Cole, and Garrison.[13])

Fig. 6. Co-elution curve of authentic aspartic acid with C^{14} activity from peak A of Fig. 4. (From Weeks, Cole, and Garrison, UCRL-8265, April, 1958.)

Fig. 7. Absorption spectra of hydrazone derivatives. (From Jayko and Garrison, UCRL-8265, April, 1958.)

Fig. 8. Effect of irradiation on the reaction of 2, 4-dinitrophenylhydrazine with gelatin. (From Jayko and Garrison.[16])

Fig. 9. Chromatogram of 2, 4-dinitrophenylhydrazones of α-keto acids from control and irradiated gelatin solutions. (From Jayko and Garrison.[16])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ASPARTIC ACID
PARENT GLYCINE
AMMONIA (BASED ON TITRE)
METHYLAMINE AND DIAMINOSUCCINIC ACID*
DIAMINOSUCCINIC ACID*
III

Fig. 1.

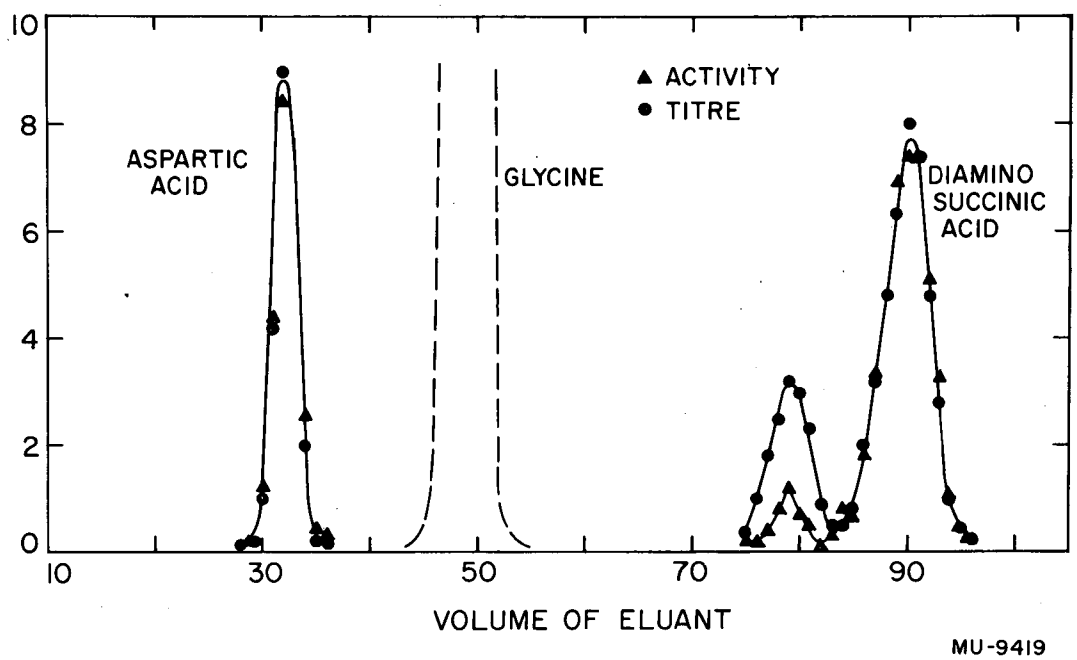
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Fig. 2.

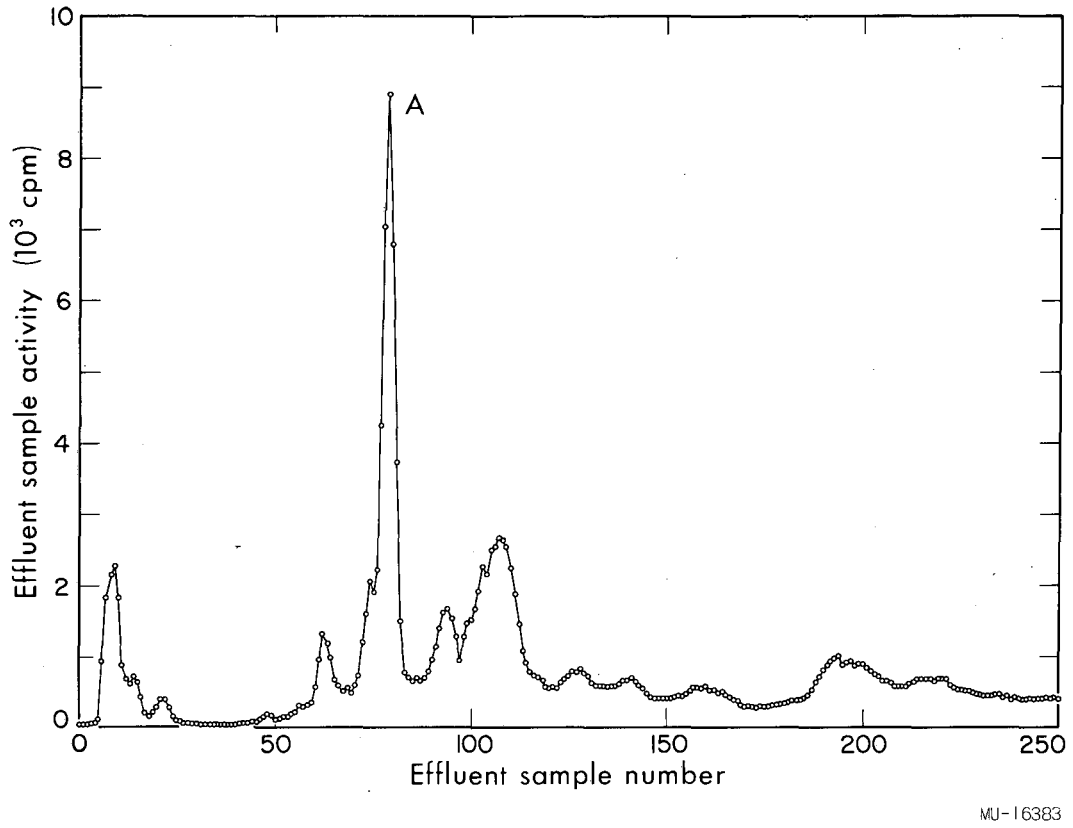
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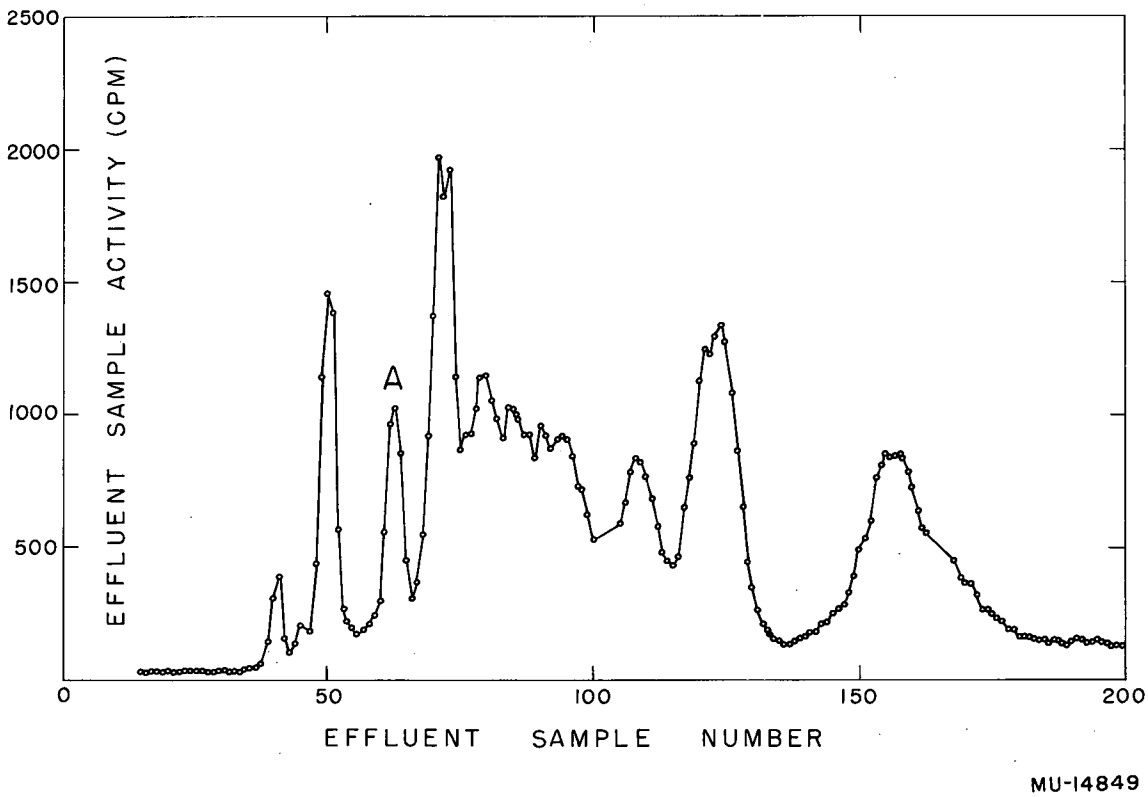
Fig. 3.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200
EFFLUENT SAMPLE NUMBER
MU-14849

Fig. 4.

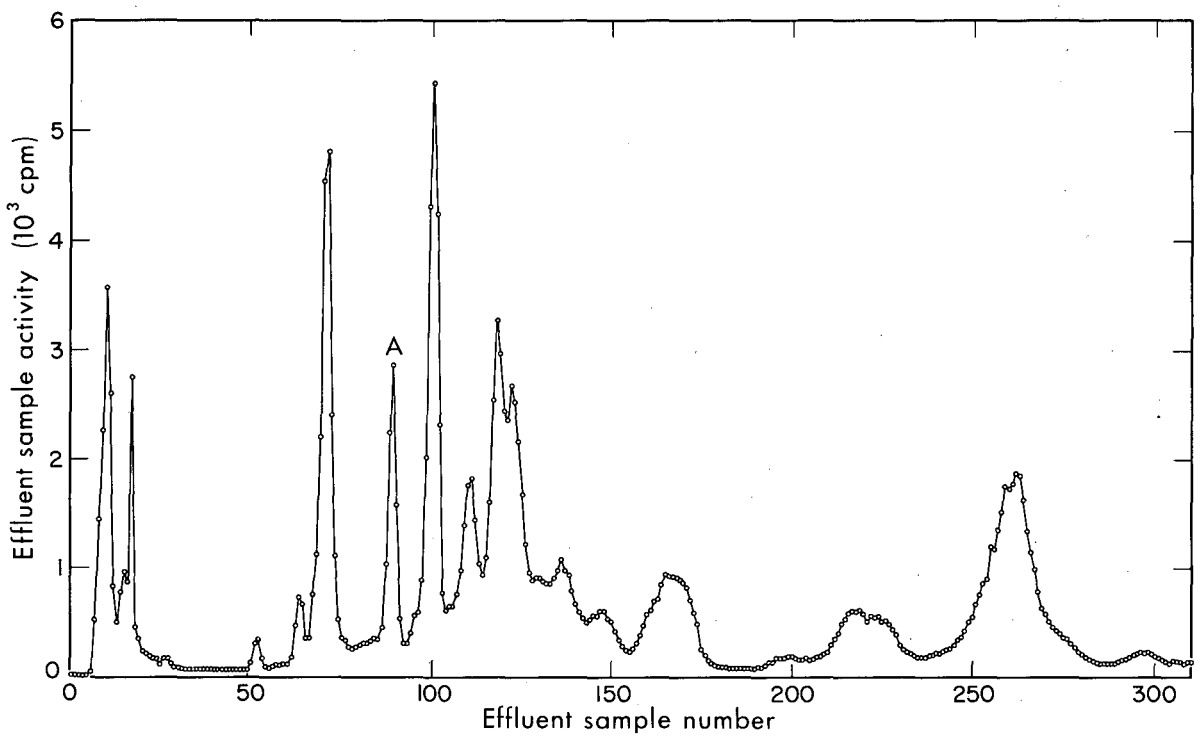
Effluent sample activity (10^3 cpm)
Effluent sample number
A
0
1
2
3
4
5
6
0
50
100
150
200
250
300
MU-16384

Fig. 5.

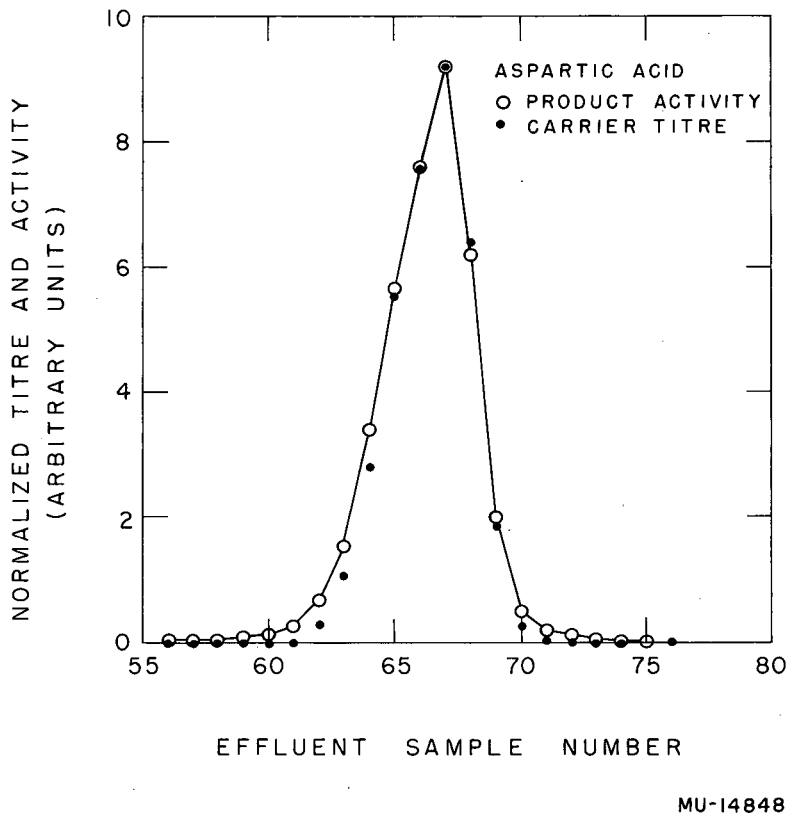
ASPARTIC ACID
O PRODUCT ACTIVITY
• CARRIER TITRE
NORMALIZED TITRE AND ACTIVITY
(ARBITRARY UNITS)
0
2
4
6
8
10
55
60
65
70
75
80
EFFLUENT SAMPLE NUMBER
MU-14848

Fig. 6.

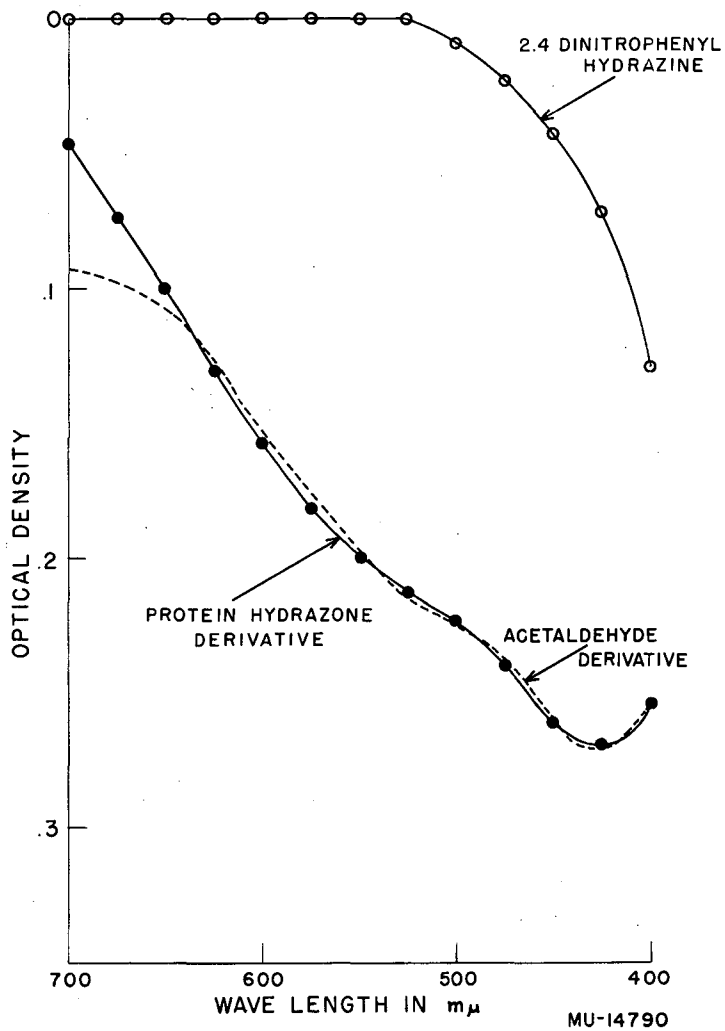
0
.1
.2
.3
OPTICAL DENSITY
700
600
500
400
WAVE LENGTH IN mμ
2.4 DINITROPHENYL HYDRAZINE
PROTEIN HYDRAZONE DERIVATIVE
ACETALDEHYDE DERIVATIVE
MU-14790

Fig. 7.

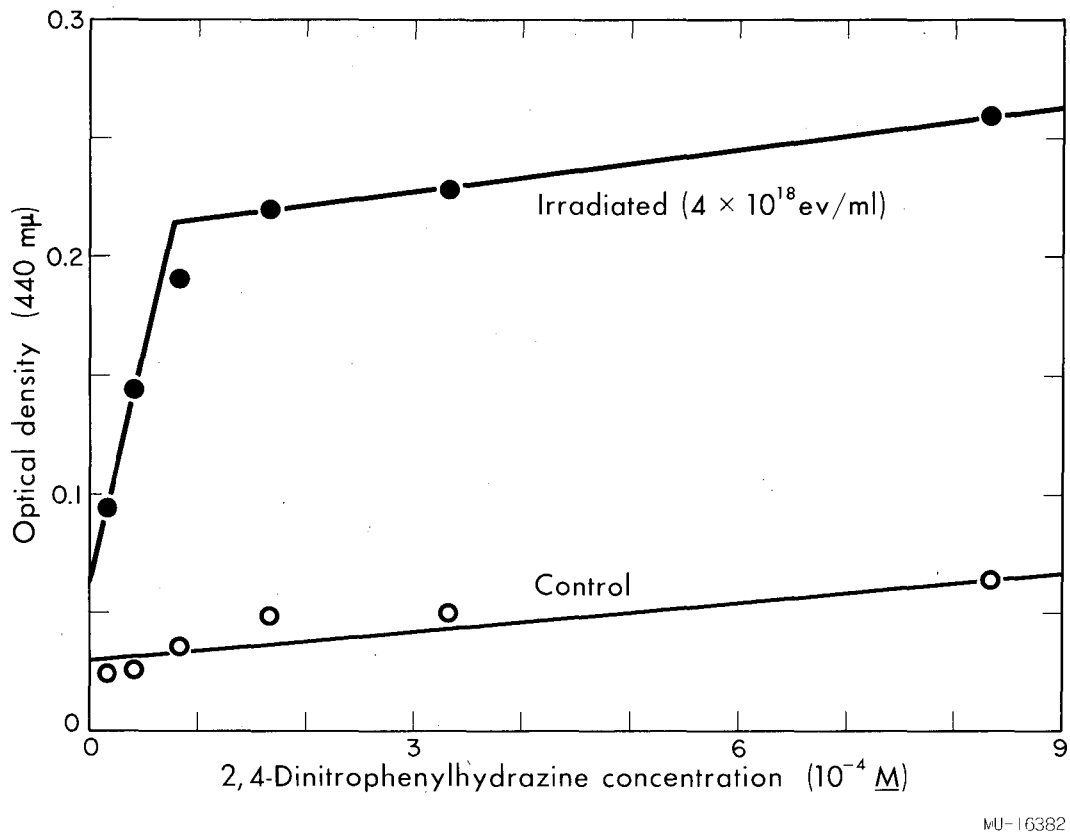
0.3
0.2
0.1
0
Optical density (440 mμ)
Irradiated (4 × 10^18 ev/ml)
Control
0
3
6
9
2, 4-Dinitrophenylhydrazine concentration (10^-4 M)
MU-16382

Fig. 8.

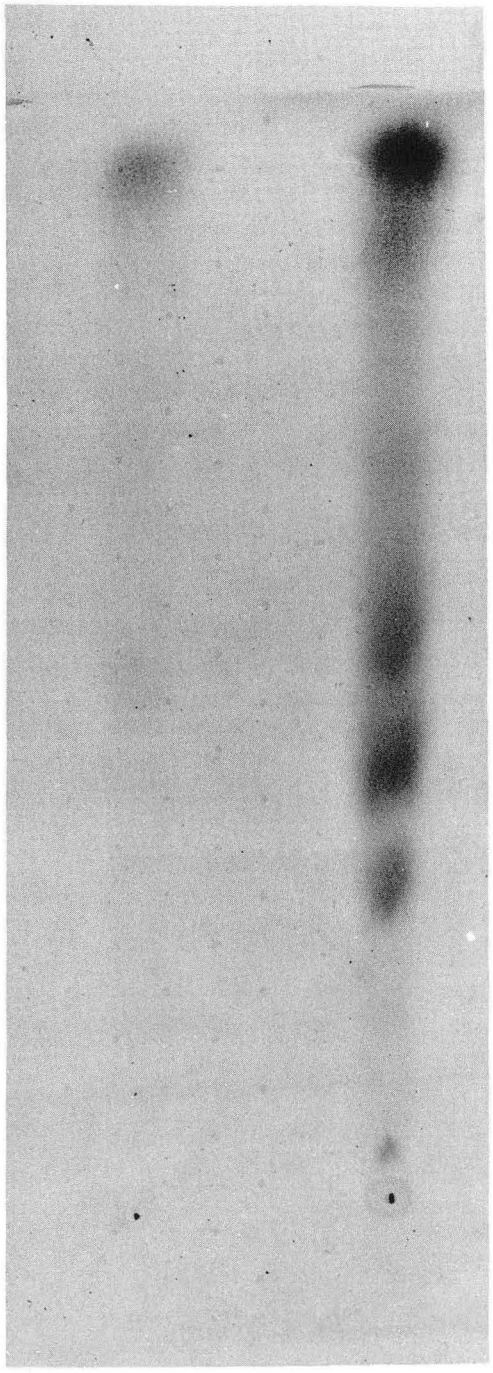

Fig. 9.

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