Anal. Calcd. for $C_{21}H_{23}O_4N$ (343): C, 71.4; H, 6.6; N, 4.0. Found: C, 71.8; H, 6.6; N, 4.0.

L-α-Phthalimido-β-phenylpropionaldehyde (L-VIIIa).—L-IVa, 1.69 g., was converted into L-VIIIa by the same procedure used above for the corresponding DL-compound. The crude L-VIIIa was recrystallized from ligroin, b.p. 85–100°, to give 0.73 g. (43%) of L-VIIIa, m.p. 115–117°, $[\alpha]^{25}D$ −157 ± 2° (*c* 2% in chloroform).

Anal. Calcd. for $C_{17}H_{13}O_3N$ (282): C, 73.1; H, 4.7. Found: C, 73.4. H, 5.1.

DL-α-Phthalimido-β-phenylpropionaldehyde Ethylene Glycol Acetal (DL-IXa).—The procedure of Balenović, *et al.*,[23] was employed to convert 4 g. of DL-VIIIa to DL-IXa, 3.74 g. (81%), m.p. 108–109.5° after recrystallization from hexane.

Anal. Calcd. for $C_{19}H_{17}O_4N$ (323): C, 70.6; H, 5.3; N, 4.3. Found: C, 70.4; H, 5.4; N, 4.3.

DL-α-Benzamido-β-phenylpropionaldehyde Ethylene Glycol Acetal (DL-IXd).—A solution of 1.08 g., 0.00333 mole, of DL-IXa in 40 ml. of methanol was heated for 3 hr. under refluxing conditions with 3.33 ml. of 1 *M* methanolic hydroxylamine and 3.33 ml. of 1 *M* methanolic sodium methoxide. The deep red solution was evaporated to dryness and the residue was extracted with hot ethyl acetate. The ethyl acetate extract was then allowed to react with 0.00333 mole of benzoyl chloride and 3.33 ml. of 1 *M* aqueous potassium carbonate, the ethyl acetate phase separated, dried over magnesium sulfate and evaporated to give 0.36 g. (36%) of DL-IXd, m.p. 118.5°.

Anal. Calcd. for $C_{18}H_{19}O_3N$ (297): C, 72.7; H, 6.4; N, 4.7. Found: C, 72.4; H, 6.7; N, 4.7.

The authors wish to express their indebtedness to Professors C. G. Swain and J. C. Sheehan for discussions concerning the work reported herein.

PASADENA 4, CALIFORNIA

[CONTRIBUTION FROM THE QUARTERMASTER FOOD AND CONTAINER INSTITUTE FOR THE ARMED FORCES]

Chemical Effects of Ionizing Radiation on Proteins.[1,2] I. Effect of γ-Radiation on the Amino Acid Content of Insulin

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This study was undertaken to determine the radiosensitivity of the constituent amino acids of a protein in order to provide information which might indicate the source of objectionable qualities of aroma and flavors that have been found to occur in the sterilization of food proteins by ionizing radiation. One per cent. insulin in basic (*p*H 8.5) and in acidic (*p*H 3.0) solutions was subjected to 0, 10, 20 and 40 million r.e.p. γ-radiation doses. Cystine, tyrosine, phenylalanine, proline and histidine are demonstrated to be very radio-sensitive. Leucine, valine, lysine and arginine are significantly destroyed at the high irradiation dose level. The nitrogen-terminal amino acids of insulin, glycine and phenylalanine, are shown to be deaminated. Cysteic acid is identified in the hydrolysates of the irradiated insulin. An increase in molecular size of the irradiated insulin is reported.

Introduction

A potential peacetime use of ionizing radiation is in the preservation of foods. Such use is predicated upon the solution of certain problems, one of which is the prevention or masking of disagreeable irradiation-produced odors and flavors. Although proteins have been identified as primary sources of these odors, no correlation between odors produced and amino acid content has been found.[4] Irradiation of free amino acids has resulted principally in deamination,[5] aldehyde production,[6] H_2S liberation from cysteine/cystine,[7] and hydroxylation and ring-splitting of aromatic amino acids and histidine.[8] Only two previous investigations have attempted to determine whether amino acids making up the protein structure are destroyed by irradiation of the protein. In one study,[9] fish muscle irradiated by high-voltage electrons to a dose of 5.7×10^6 r.e.p. (roentgen equivalent physical) showed no significant destruction of the "essential" amino acids. In the other,[10] irradiation of aqueous 10^{-5} *M* serum albumin solutions with X-rays (44,600 and 66,900 r.e.p.) produced 30% destruction of glycine, alanine and glutamic acid, and 21, 18, 16 and 13% destruction of lysine, threonine, tyrosine and isoleucine, respectively.

The chemical compounds responsible for the disagreeable irradiation-produced aromas and flavors are not known. A quantitative amino acid analysis of irradiated proteins of known character should indicate those amino acids in a protein which are most radio-sensitive. Further research effort could then be directed toward the characterization of the irradiation-breakdown products from those amino acids and their relation to the observed quality defects in some protein foods.

Insulin was chosen as the first protein to be investigated because of its availability in a very pure form, and its characterization as to amino acid content.[11] Since insulin does not contain cysteine, methionine or tryptophan, the necessity for hydrolysis in base and the interference of acid-hydrolysis breakdown products of tryptophan is eliminated, and cystine is left as the only sulfur-containing amino acid.

(1) Paper No. 654 in series of papers approved for publication. The views or conclusions are those of the authors and are not to be construed as necessarily reflecting the views or endorsement of the Department of Defense. The mention of commercial products does not imply they are endorsed or recommended by the Department of Defense over similar products not mentioned.

(2) Taken in part from a thesis submitted by M. P. Drake in partial fulfillment of the requirements for the M.Sc. degree. Dept. of Biochemistry, Northwestern University. Presented at Symposium on Radiation Sterilization. Agr. & Food Chem. Div., 130th Nat'l ACS Meeting, 1956.

(3) Department of Biochemistry, School of Medicine, Northwestern University.

(4) W. D. Bellamy and E. J. Lawton, *Nucleonics*, **12**, [4] 54 (1954).

(5) W. M. Dale, J. V. Davies and C. W. Gilbert, *Biochem. J.*, **45**, 93 (1949).

(6) G. Stein and J. Weiss, *J. Chem. Soc.*, 3256 (1949).

(7) S. L. Whitcher, M. Rotheram and N. C. Todd, *Nucleonics*, **11**, [8] 30 (1953).

(8) B. E. Proctor and D. S. Bhatia, *Biochem. J.*, **53**, 1 (1953).

(9) B. E. Proctor and D. S. Bhatia, *Food Tech.*, **4**, 357 (1950).

(10) E. S. G. Barron, J. Ambrose and P. Johnson, *Radiation Res.*, **2**, 145 (1955).

(11) E. J. Harfenist, THIS JOURNAL, **75**, 5528 (1953).

The dose necessary to sterilize foods has been indicated to be between 2 and 3 million r.e.p.[12] Higher levels of irradiation are necessary, however, to produce chemical changes of a magnitude large enough to be measured by the analyses available. Four dose levels were used (0, 10, 20 and 40 million r.e.p.) so that curves of amino acid survival could be drawn. An additional N_2-flushed sample was irradiated at 40 million r.e.p. for comparison of irradiation results with and without oxygen.

Experimental

Sample Preparation.—One per cent. insulin[13] solutions were used throughout this study. Aliquots (1–4 ml.) were pipetted into 13 × 100 mm. Pyrex glass test-tubes, and the tubes sealed with an oxygen-gas torch. Where N_2-flushing was desired, the end of the test-tube was drawn out to decrease the opening size, and nitrogen bubbled into the solution through a capillary tube for 20 minutes before sealing. An alkaline-pyrogallol trap was used to remove residual oxygen from the tank nitrogen, and a water trap was used to prevent dehydration of the sample solution.

For shipping and irradiation, test-tubes for each irradiation level were placed in a concentric ring near the center of a No. 2 tin can. The samples were kept frozen at all times until used, except for a 30-minute room temperature tempering period before irradiation, and during the irradiation itself. Thirty minutes had previously been determined as sufficient to thaw the samples in each can so that ice–protein concentration effects would not occur during the irradiation.

Irradiation.—The irradiation was performed on contract by personnel of the Phillips Petroleum Company who operate the Nuclear Testing Reactor at Arco, Idaho. The radiation source was γ-rays from spent reactor-fuel rods. All irradiation doses were calculated to a point in the center of each can, and are expressed in r.e.p. The r.e.p. as used here refers to a 93 erg energy absorption per gram of material.

Phenyl Isothiocyanate N-Terminal Amino Acid Method.—One-ml. sample aliquots (1.7 μ moles insulin) were used. Derivative preparation and procedure for chromatographic identification were those published by Landmann, Drake and Dillaha.[14] A spectrophotometric method[15] was used to determine that the derivatives were pure. In this method the alcohol-diluted 3-phenyl-2-thiohydantoin derivatives are read over the wave length interval of 256–275 mμ, and the ratio optical density at 260 mμ/max. optical density obtained. A derivative is considered pure if the ratio is less than 0.85.

Carboxypeptidase C-Terminal Amino Acid Method.—One hundred-μl. sample aliquots (0.17 μmole insulin) were mixed in a 200-μl. polyethylene beaker with 50 μl. (5% w./w.) of carboxypeptidase.[16] The solution was adjusted to *p*H 9 by the addition of 10 μl. of 0.01% NH_4OH, and the sample drawn into a capillary tube. After sealing, the samples were incubated at 37° for 16 hours. The enzyme was inactivated by immersion of the capillaries in boiling water for 5 minutes. The samples were emptied into a 200-μl. beaker and dried *in vacuo* over P_2O_5. For chromatographic identification, the samples were solubilized by addition of 50 μl. of very dilute HCl solution. Five-μl. aliquots were used in the analysis procedure.

Ultraviolet Absorption Spectra Analyses.—Two 200-μl. aliquots (0.34 μmole insulin) were taken from each sample, and each aliquot was diluted to 10 ml. with either 0.1 *N* NaOH or 0.1 *N* HCl. The samples were read over the wave length interval of 220–360 mμ on a Beckman spectrophotometer.

Correction formula for non-aromatic ultraviolet absorption[17]: The slope (k) of the absorption occurring in the non-aromatic absorption region of 320–360 mμ is found by the formula

$$k = \frac{\text{change in optical density}}{\text{wave length interval}} = \frac{\text{O.D. at 320 m}\mu - \text{O.D. at 360 m}\mu}{360 - 320}$$

The consumptive absorption at any wave length (λ) can then be found by substitution of k in the formula

$$\text{true absorption} = \text{optical density at wave length } \lambda - (360 - \lambda)k$$

Biological Activity Analysis.—One ml. sample aliquots were pipetted into 13 × 100 mm. test-tubes and the contents carefully lyophilized. The insulin assay was performed at The Armour Laboratories using a mouse-convulsion method.[18]

Paper Chromatography of the Amino Acids.—The method used is that published by Roland and Gross[19] with some modifications of procedure and solvent systems.

1. **Solvent Systems. a. 2-Butanol:3% NH_3 (3:1).**—This system resolves lysine, arginine, proline, tyrosine, valine, methionine, isoleucine, leucine and phenylalanine. Two hundred ml. of the solvent is placed in the bottom of each tank 16 to 24 hours prior to the addition of the chromatogram sheets in order to saturate the tank atmosphere. Two prepared chromatogram sheets are hung in each tank and equilibrated 16 hours prior to solvent development. The developing solvent is 45 ml. of a 4:1 (2-butanol:3% NH_3) mixture made up immediately before use. The use of the 4:1 mixture has been empirically determined to produce the resolution required. The chromatograms are allowed to develop for 48 hours and are then removed from the tanks and forced-air dried. A second run usually can be made with good resolution if sheets are added for the 16-hour equilibration on the same day the first sheets are removed. A loss in resolution of leucine and phenylalanine is encountered if the tank remains idle, or if more than two runs are made without cleaning the tanks and adding fresh solvent.

b. **72% Phenol.**—This system resolves cysteic acid, aspartic acid, glutamic acid, cystine, serine, glycine, threonine and alanine. Tanks are maintained "conditioned" for an indefinite period by placement of small beakers of phenol and water in the bottoms of the tanks. Ten ml. of 0.3% NH_3 is added to each tank just prior to addition of the four prepared chromatogram sheets. After 16 to 24 hours equilibration, 35 ml. of developing solvent (72% phenol) is added by hypodermic syringe and needle to each trough. The 72% phenol is made up by *thoroughly* mixing liquid phenol (88–90%) with the required amount of 0.1 *N* $NaHCO_3$ solution. The use of $NaHCO_3$ increases the resolution and decreases the background phenol-oxidation color.[20]

The sheets are allowed to develop for 32 hours, and placed in a hood for drying in a stream of air. Great difficulty is experienced in elimination of the phenol unless a continuous stream of air is maintained for 20 to 24 hours. Final elimination of the phenol is accomplished by wash-dipping the papers several times in ethyl ether.

c. **2-Butanol:1-Butanol:Glacial Acetic Acid:H_2O (9:9:2:7).**—This solvent system resolves cystine/cysteic acid (a double spot), lysine, histidine and arginine. Two hundred ml. of the solvent is placed in the bottom of the tank for conditioning and need not be changed for 2 to 3 weeks. Four prepared chromatogram sheets are placed in each tank and equilibrated 12 to 16 hours. The developing solvent is made just before use, and 50 ml. is placed in each solvent trough. The sheets are solvent-developed for 64 to 72 hours and air-dried for 12 to 16 hours.

2. **Hydrolysis Procedure (in vacuo).**[21]—After lyophilization of the samples, 2 ml. of three-times glass-redistilled 6.4 *M* HCl was added to each test-tube. Each tube was then drawn out in an oxygen-gas torch flame so that a thin tube of approximately 3 mm. diameter connected the sample part of the test-tube with the open part; the open-end of the tube was left with sufficient edge to provide support for the vacuum hose. The tube was connected through a vacuum hose

(12) R. S. Hannan, "Food Investigation Special Report #61," London, England, 1955, p. 66.

(13) H. P. Bovine Insulin, Lot No. 565, Armour & Co., Chicago, Ill.

(14) W. Landmann, M. P. Drake and J. Dillaha, THIS JOURNAL, **75**, 3638 (1953).

(15) H. Fraenkel-Conrat, J. I. Harris and A. L. Levy, "Methods of Biochem. Analysis," Vol. 2, Interscience Publishers, Inc., New York, N. Y., 1955, p. 359.

(16) Carboxypeptidase, Crystallized (5X), Feidex Biochemicals, Kankakee, Ill.

(17) T. W. Goodwin and R. A. Morton, *Biochem. J.*, **40**, 628 (1946).

(18) R. E. Thompson, *Endocrinology*, **39**, 62 (1941).

(19) J. F. Roland and A. M. Gross, *Anal. Chem.*, **26**, 502 (1954).

(20) The use of "Gilt Label" USP water-white liquid phenol (Mallinckrodt Stock No. 0024) has consistently given a low background color.

(21) W. White, personal communication, 1955.

and stopcock assembly to a water-aspirator to reduce the content of oxygen in the sample and to minimize bubbling. The assembly was then connected to a vacuum pump through a NaOH pellet–Drierite trap and a freeze trap. An alcohol–carbon dioxide freezing bath was also used to minimize bubbling, and the final vacuum was attained by freezing the solution on the bath and drawing a vacuum to 10 μ of Hg. The test-tube was sealed easily in a small gas flame after removing the assembly from the vacuum pump. Maintenance of negative pressure in the upper tube assembly indicated effective vacuum sealing of both sections.

Prior to the development of the *in vacuo* technique, the *p*H 8.5 insulin samples were hydrolyzed at atmospheric pressure.

3. **Analytical Preparations. a. Standard Amino Acid Solutions.**—Amino acid stock solutions (10% 2-propanol) were made up to contain 64 μg. of α-amino nitrogen per ml. of each amino acid used. The stock solution was diluted to provide standards at 0.08, 0.16, 0.24 and 0.32 μg. of α-amino nitrogen per 5 μl. Roland and Gross[19] applied both standards and samples in different volumes (2.5, 5 and 10 μl.). By applying both standards and samples in the same volume of solution, more quantitative separations could be obtained.

b. **Sample Solutions.**—After hydrolysis and drying, the samples were dissolved in 0.5 ml. of 10% 2-propanol solution, and a drop of 0.1 *N* HCl was added to ensure the solubility of tyrosine. The dilution of the sample was accomplished by using a 25-μl. graduated micropipet and a 50-μl. beaker. The sample was placed in an ice-bath and the aliquot drawn into the micropipet. The dilution desired was made by capillary action using an iced 10% 2-propanol solution and subsequently adjusting to the mark. Alternate ejection and filling of the pipet in the beaker thoroughly mixed the dilution solution. Four samples were routinely micropipetted to the chromatogram sheets with a 5-μl. self-filling micropipet.

4. **Color Formation.**—A freshly prepared ninhydrin solution (0.2% ninhydrin in 200 ml. of acetone plus addition of 10 ml. of H_2O and 2 ml. of glacial acetic acid) was employed for the color development. After chromatographic resolution and drying, the papers were dipped in the ninhydrin reagent and air-dried 10 minutes to evaporate the acetone. Maximum ninhydrin-color was obtained by allowing the sheets to develop for 24 hours in a 35% relative humidity chamber.[22] Petri dishes with citric acid crystals were placed on the floor of the chamber to minimize extraneous NH_3-ninhydrin background color. It was found necessary with chromatograms from the phenol solvent system to dip each sheet in the ninhydrin reagent several times for maximum color development.

An isatin reagent solution (0.1% isatin in acetone and 2% acetic acid) was used to give a greater color yield with proline. After the routine 24-hour color development, an additional five minutes in a 100° oven produced maximum color development.

5. **Quantity Measurement of Amino Acids.**—The procedure of measuring the maximum color density of the individual spots on the chromatogram sheets was used in this study. A variation from the published method[19] was the use of a slit instead of a circular aperture. This was found to give a more reproducible reading because it minimized the error which was produced by the thickness variation in the filter paper, and also because a scanning system could be employed without danger of great error being introduced if the aperture was not exactly centered on the maximum density of the spot. A Photo Volt Densitometer, Model 502, was used for the determinations. The filter used for ninhydrin and isatin color density readings was #575 which gave a peak transmission at 565 mμ (read in a Beckman DU spectrophotometer).

Four samples and four standards of varying concentrations were run on each filter paper sheet. After resolution and color development, the sheets were cut into approximately 22 mm. strips (the center being the center of the spot), the absorption density was adjusted to 0 at a blank space in the strip, and the strip was scanned by being automatically pulled through the aperture-photometer head. The maximum absorption density was tabulated for each amino acid in both samples and standards.

Roland and Gross[19] plot standard absorption density *versus* concentration curves for each amino acid on each sheet and average the sample values obtained from those curves. It was found in this Laboratory that accurate results could be obtained with far less work by averaging the values obtained for both standards and samples and plotting one curve of the absorption density *versus* concentration for each amino acid from the four sheets. The most important factor in using this simplified procedure is the necessity of exact duplication in the handling of all chromatograms in a series.

(22) E. F. Wellington, *Can. J. Chem.*, **31**, 484 (1953).

Results and Discussion

Insulin Irradiated in *p*H 8.5 Solution.—The first study attempted was paper chromatographic resolution of the protein. A marked change in the molecular weight or characteristic side-chains might be expected to result in a change in resolution, whereas a splitting of the peptide at the peptide linkages would result in the appearance of new ninhydrin-positive spots. The insulin, before irradiation, gave a single strong ninhydrin-positive spot at R_f 0.63 in a *1-butanol:glacial acetic acid:* H_2O (5:1:5) solvent system, and at R_f 0.0 in a *2-butanol:3%* NH_3 (3:1) solvent system. Chromatograms of the irradiated samples yielded no ninhydrin-positive spots. Subsequent spotting of each of the irradiated samples in higher concentration and reacting with ninhydrin without chromatographic resolution, confirmed the loss of ninhydrin reaction.

Application of the phenyl isothiocyanate method for nitrogen-terminal amino acid analysis revealed that a pure derivative was obtained only from the control sample. The spectrophotometric ratios obtained for the 0, 20 and 40 million r.e.p. samples were 0.806, 1.0 and 0.976, respectively. Chromatographic resolution of the derivatives confirmed the presence of the nitrogen-terminal amino acids, glycine and phenylalanine, in the control, and their absence in the irradiated samples. (A derivative spot was obtained at R_f 0.62 from the irradiated samples in the xylene:acetic acid:*p*H 6 phthalate buffer (10:2:5) solvent system. The spot had no counterpart in spots corresponding to known amino acid phenylthiohydantoins, and its identity was not pursued.) The radiosensitivity of the nitrogen-terminal amino acids is due most probably to an irradiation–deamination reaction similar to that occurring in free amino acids.[5]

The enzyme carboxypeptidase was used to examine the carboxyl-terminal amino acids, alanine and asparagine. Paper chromatographs of the enzyme–insulin reaction solutions revealed the presence of those two amino acids as the predominant ninhydrin-positive spots in every sample. An estimation of the relative amounts of alanine and asparagine liberated from each sample is shown in Table I.

TABLE I

AVERAGE NINHYDRIN-COLOR ABSORPTION DENSITY OF THE TWO PREDOMINANT CHROMATOGRAM SPOTS OF THOSE AMINO ACIDS SPLIT FROM IRRADIATED 1% *p*H 8.5 INSULIN BY CARBOXYPEPTIDASE

	Radiation dose (× 10^6 r.e.p.)				
	0	10	20	40	40(N_2)[a]
Alanine	0.225	0.263	0.207	0.142	0.137
Asparagine	0.080	0.073	0.054	0.040	0.040

[a] N_2-flushed before irradiation.

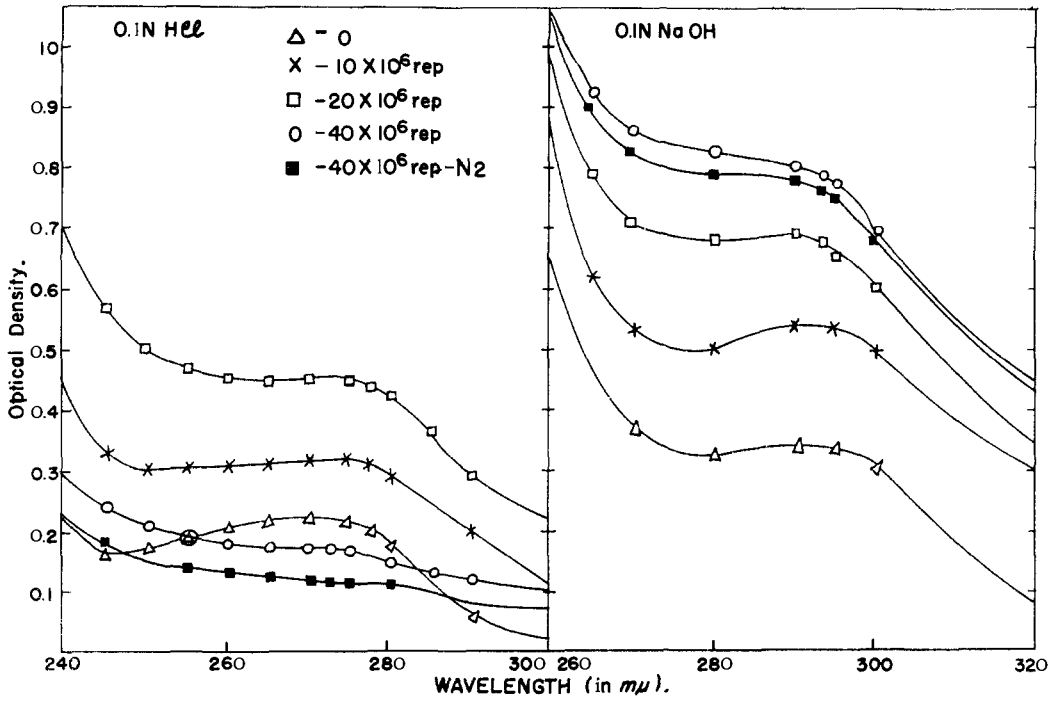

Fig. 1.—Ultraviolet absorption spectra in acid and base of aliquots (0.34 μmole insulin) from irradiated 1% *p*H 8.5 solutions. Irradiation dose levels were 0, 10, 20 and 40 million r.e.p.; the sample marked N_2 was N_2-flushed before irradiation. The spectra in acid were obtained after pepsin digestion and centrifugation; precipitates were obtained only from the 40 × 10^6 r.e.p. samples.

The low color yield for asparagine is characteristic. In general, a decrease is shown in the amounts of carboxyl-terminal amino acids split from insulin by carboxypeptidase at increasing irradiation dose. This decrease could indicate a radiosensitivity, but also could indicate that the carboxyl-terminal amino acid is not as available to enzyme action at the increased irradiation level. The appearance of glutamic and aspartic acids, serine, glycine, tyrosine, leucine and phenylalanine as trace spots on the chromatograms for every sample indicated that a trace of chymotrypsin enzyme was an impurity in the carboxypeptidase used. No isopropylfluorophosphate was available to prevent this,[23] but the predominance of the expected amino acids made it unnecessary.

The report of hydroxylation and ring-splitting occurring in irradiated solutions of aromatic amino acids was examined by use of an ultraviolet absorption analysis. Precipitation in the 20 and 40 million r.e.p. samples when diluted in acid necessitated the incubation of all acid-diluted samples with 5% (w./w.) pepsin[24] at 37° for 16 hours. The use of pepsin was successful in clarifying the 20 million r.e.p. sample, but the two 40 million r.e.p. samples had to be centrifuged to obtain clear samples for the analysis. Figure 1 shows the ultraviolet absorption of irradiated insulin in base and in acid. Note that in both acidic and basic mediums, an increase in general absorption occurred throughout the spectrum with increasing irradiation dose.

(23) F. Sanger and E. O. P. Thompson, *Biochem. J.*, **53**, 353 (1953).

(24) Pepsin Crystallized (3X) (hog mucosa), Pentex Biochemicals, Kankakee, Ill.

A literature study[25,26] indicates that when such a general absorption occurs it is sometimes due to a scattering of light by larger particles or increased molecular size (Tyndall effect). A simple formula for extrapolating the increased absorption found in regions of non-aromatic absorption (from 320–370 mμ) was used successfully for correction of ultraviolet absorption spectra.[17] Plotting of the values corrected by this procedure yields Fig. 2. The aromatic maximum is shown in acid solution to shift slightly to longer wave lengths, to increase in absolute value, and to decrease in value in relation to the adjoining minimum with increasing irradiation. The aromatic maximum is shown in basic solution to decrease in absolute value, to increase in relation to the adjoining minimum, and the minimum has shifted to shorter wave lengths with increasing irradiation. No shift of the maximum absorption in base is apparent, however, except in the 10 million r.e.p. sample.

It is most likely that the corrections applied are not exact enough to permit the fine interpretation that would appear to be necessary. The rather typical aromatic absorption curves obtained, after correction, however, strengthen the hypothesis that molecular (or particle) size increases with irradiation dose, and that the general absorption increase observed is due to the Tyndall effect. An increase in molecular size is also evident from the precipitation of the 20 and 40 million r.e.p. samples, and the

(25) G. H. Beaven and E. R. Holiday, *Advances in Protein Chem.*, **7**, 319 (1952).

(26) P. Doty and E. P. Geiduschek, "The Proteins," Vol. 1, Part A, Academic Press, Inc., New York, N. Y., 1953, p. 393.

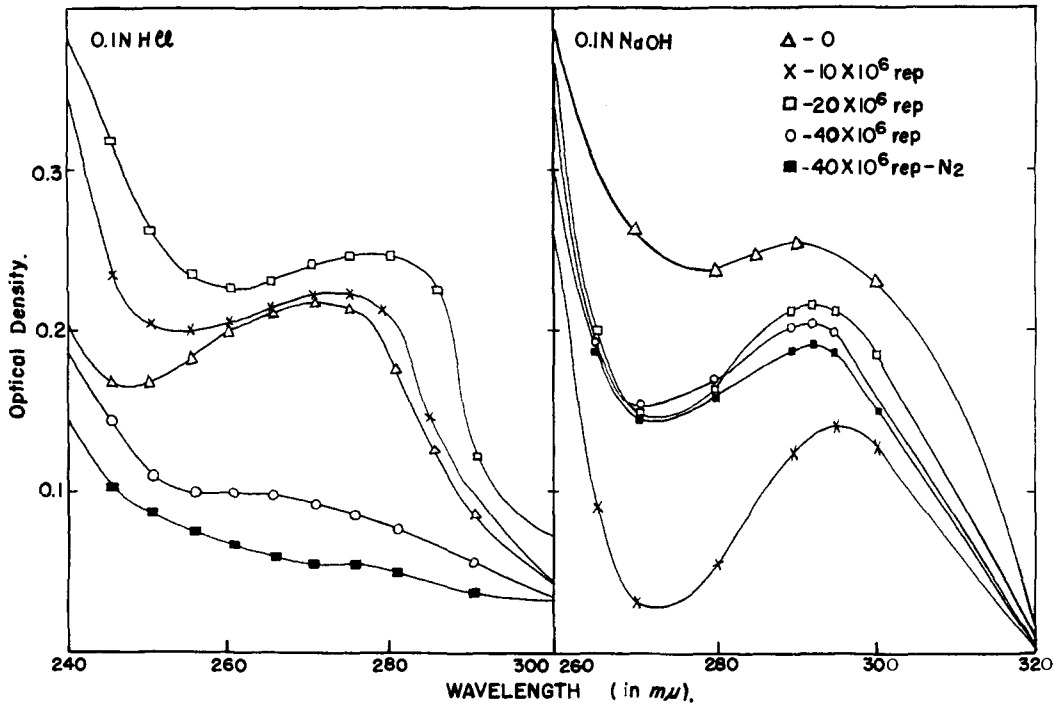

Fig. 2.—Ultraviolet absorption spectra corrected for Tyndall effect of aliquots (0.34 μmole insulin) in 0.1 *N* HCl and in 0.1 *N* NaOH from irradiated 1% insulin *p*H 8.5 solutions.

solubilizing digestion with pepsin of only the 20 million r.e.p. sample.[27]

The results of biological activity assay of the control and 10 million r.e.p. sample are indicated in Table II. The loss in biological potency upon

TABLE II

THE BIOLOGICAL ACTIVITY OF A SAMPLE OF 1% INSULIN IRRADIATED IN *p*H 8.5 SOLUTION

Samples	Activity level injected, u./mg.	Activity level found, u./mg.
0	25	22.1
10×10^6 r.e.p.	12.5	<5

irradiation of insulin is shown to be very great. The biological activity of insulin is reported to be lost when reagents attack phenolic groups, carboxyl-terminal groups, and disulfide linkages.[28] Two of the three groups necessary for activity, phenolic and disulfide, are shown below to be changed or destroyed by irradiation.

Only enough sample remained after the previous studies to run one hydrolysis period for the amino acid analyses. The hydrolysis was carried out in sealed test-tubes with 6.4 *M* HCl at 100° for 24 hours. A full residue drop of both glycine and phenylalanine, occurring at the 10 million r.e.p. dose, confirmed the destruction of these nitrogen-terminal amino acids. In general, the results of the hydrolysis were similar to those obtained in the analysis of the *p*H 3 irradiated insulin reported later.

(27) A. G. Pasynsky has similarly postulated that the increase in ultraviolet absorption is due to a Rayleigh scattering of the ultraviolet radiation by aggregated protein molecules: Conf. Acad. Sci. (USSR) on Peaceful Uses of Atomic Energy, July, 1955.

(28) C. H. Li, "The Proteins," Vol. 2, Part A, Academic Press, Inc., New York, N. Y., 1954, p. 647.

Insulin Irradiated in *p*H 3.0 Solution.—The appearance of gels in the irradiated sample tubes upon thawing of the samples prevented the taking of aliquots for hydrolysis or other tests. Additional samples were made up for irradiation at 0, 10, 20 and 40 million r.e.p. for the quantitative amino acid analysis. One ml. was placed in each test-tube and the same test-tubes were used for all the operations of irradiation, drying and hydrolysis to eliminate possible transfer errors. The samples were hydrolyzed *in vacuo* to eliminate the formation of black humin; two samples were hydrolyzed from each irradiation level, one for 20 hours and the other for 60 hours.

TABLE III

AMINO ACID RESIDUE ANALYSIS OF IRRADIATED 1% INSULIN IN *p*H 3.0 SOLUTIONS COMPARED TO LITERATURE VALUE (HARFENIST[11])

Amino acid	Irradiation dose ($\times 10^6$ r.e.p.) 0	10	20	40	Lit. value
Aspartic acid	2.9	2.9	2.6	2.6	3
Glutamic acid	6.9	6.3	6.4	6.1	7
Serine[a]	2.9	2.7	2.7	2.6	3
Glycine	4.0	3.4	3.2	2.9	4
Threonine[a]	0.93	1.0	0.98	0.96	1
Alanine	3.4	3.5	3.6	3.8	3
Cystine	2.9	1.4	1.0	0.74	3
Lysine	1.3	1.2	1.1	0.98	1
Histidine	2.1	1.8	1.6	1.2	2
Arginine	1.1	1.0	0.96	0.89	1
Tyreosine	3.3	2.6	1.9	0.98	4
Valine	5.0	4.7	4.5	3.8	5
Isoleucine	Not determined				1
Leucine	5.8	5.3	4.6	4.3	6
Phenylalanine	2.8	1.7	1.4	1.1	3
Proline	1.1	0.86	0.68	0.50	1

[a] Corrected for hydrolysis destruction (Rees[29]).

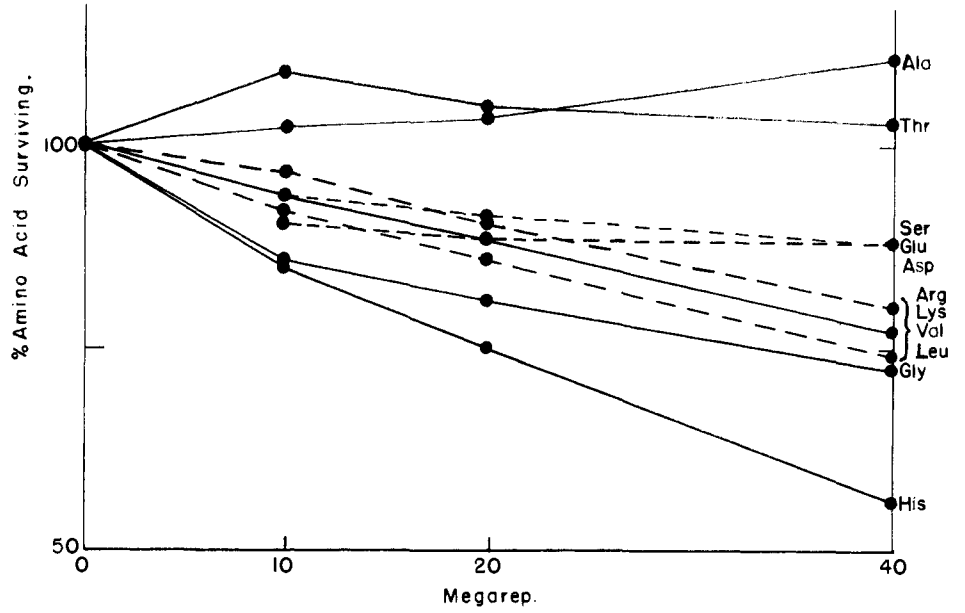

Fig. 3.—Per cent. of amino acid survival in irradiated 1% insulin in *p*H 3.0 solution as a function of irradiation dose; amino acid value in control (0 dose) used as 100%.

In Table III, the best values for each amino acid were selected from one of the two hydrolysis periods on the basis of their standard error, their known lability to acid hydrolysis (cystine, serine, tyrosine, threonine) or their stability as dipeptides to the hydrolysis (leucine, valine). Isoleucine was omitted from the analysis inadvertently, and serine and threonine have been corrected for hydrolysis destruction using the factors determined by Rees.[29]

The low residue value for tyrosine may be due to its reaction in the hydrolyzing medium with carbohydrate materials. The decrease has been constant and reproducible in the analyses of three control samples made up independently over a period of one year, and only slight improvement was obtained by use of hydrolysis *in vacuo*. The hydrolysis *in vacuo* did improve the value for cystine which gave only 2.5 residues when hydrolyzed in air. The values for alanine, arginine, and lysine are high, and the difference is greater than the standard error. No attempt has been made to further purify the material, however, and the presence of small peptide impurities may be expected. (The residue values established by Harfenist[11] were obtained from material purified by a 1000-plate countercurrent fractionation which had been demonstrated to be capable of separating insulin differing by one amide bond.)

Figures 3 and 4 show the percentage of amino acid remaining at each level of irradiation with the value in the non-irradiated sample taken as 100%. In general, a trend toward some destruction is shown for all amino acids except threonine and alanine. The slopes of the lines for alanine and threonine are significant, although the absolute value differences are not. The increase in value for these two amino acids may be due to the formation of non-amino acid ninhydrin-positive compounds which chromatograph at the threonine and alanine position. It is also possible that a by-product of the destruction of some other amino acid is one or both of these amino acids.[30]

The marked decrease in phenylalanine at 10 million r.e.p. dose correlates with the destruction of the nitrogen-terminal phenylalanine residue, but the decrease in glycine does not reach a full residue until the 40 million r.e.p. dose. This may indicate a difference in the rate of deamination from irradiation of glycine in acid solution similar to that previously observed in X-irradiation of free amino acids.[5]

The amino acids bonded in the protein molecule and shown to be radiosensitive at the lower irradiation doses (10 or 20 million r.e.p.) are cystine, tyrosine, proline, phenylalanine and histidine. It is interesting that the last four are all ring-containing. Leucine, valine, lysine and arginine show a decrease in analyses value which has become significant at the 40 million r.e.p. dose. These nine amino acids (plus possibly isoleucine which was not included in the analyses, and tryptophan, methionine and cysteine which are not present in insulin) are indicated in these studies to be possible sources for the production of objectionable qualities of aroma and flavor in the sterilization of food proteins by irradiation.

An extrapolation of the amino acid destruction curves to a 5 million r.e.p. sterilizing dose tends to confirm Proctor and Bhatia's results[9] of no significant destruction of "essential" amino acids in fish muscle. Even the most radio-labile of the "essential" amino acids common to the two studies (histidine and phenylalanine) would probably not be detected at this dose level by a microbiological assay. No real correlation of the extent of radiation

(29) M. W. Rees, *Biochem. J.*, **40**, 632 (1946).

(30) A. Scott and A. H. Livermore reported the formation of alanine from irradiation of cysteine: Symposium on Radiation Sterilization of Foods and Pharmaceuticals, 126th ACS Meeting (1954).

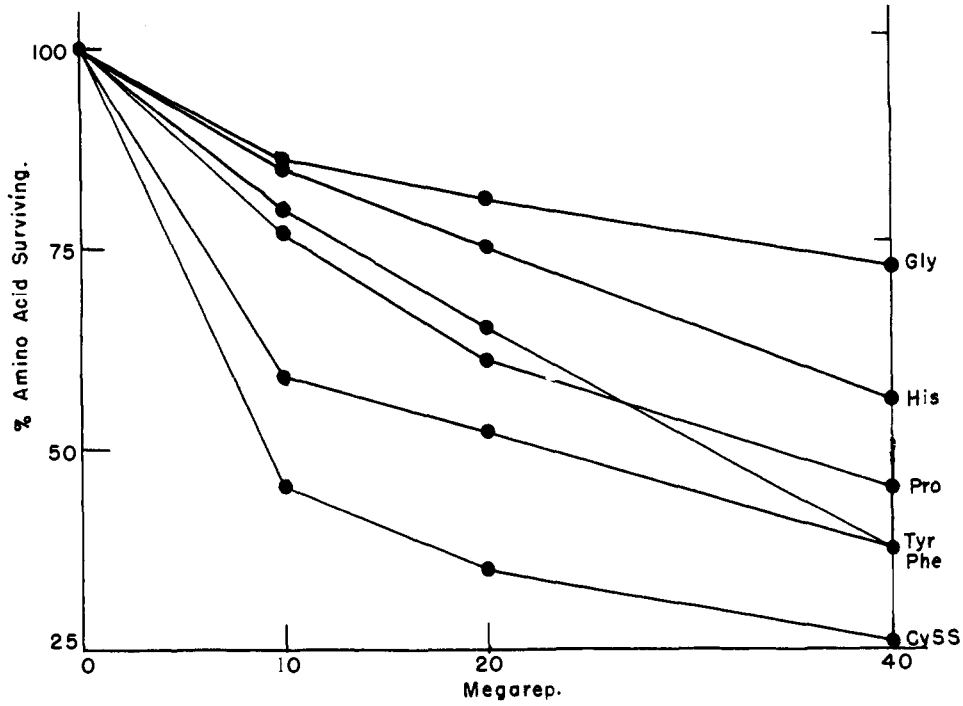

Fig. 4.—Per cent. of amino acid survival in irradiated 1% insulin in *p*H 3.0 solution as a function of irradiation dose; amino acid value in control (0 dose) used as 100%.

damage and of the particular amino acids affected, however, is apparent between this study and that of Barron on serum albumin.[10] Moreover, none of the chief variations in the two investigations (protein used, protein concentration, dose level and type of irradiation) are sufficient to account for the differences reported if either investigation is to be used as an example of the effects of irradiation on protein amino acids. Part of the differences found probably may be ascribed to reactions involving oxygen since the high electron-affinity possessed by oxygen would have its greatest effects in the very dilute solutions used by Barron. Further studies are indicated to be necessary, however.

Although its value was not determined, cysteic acid was identified by paper chromatography to be present in the hydrolysates of the irradiated insulin samples at all irradiation dose levels. The formation of sulfinic and sulfonic acids has been reported by Shapiro and Eldjarn[31] in the irradiation of cystamine.

Properties of the Insulin Gels.—The irradiated *p*H 3.0 insulin gels appeared to be fibrous and had the appearance of a wad of cotton in the test-tube. On examination with a polarizing microscope, the gel showed only weak birefringence and had the appearance of a lacy network of film and fibers. The addition of strong base was marked by a noticeable swelling of the gel, while acid caused contraction. This correlates with the finding of cysteic acid in the hydrolysates of irradiated insulin, if this behavior is analogous to the polyelectrolyte gels of Katchalsky[32] in which adjacent acid groups repel each other in a medium wherein they become ionized.

The gel could be solubilized by prolonged heating with thioglycolic acid in acid, neutral, or basic solution, while heating with a saturated solution of urea had little effect. This behavior would indicate that the rearrangement of disulfide bonds is more responsible for the polymerization than is an increase in hydrogen bonding.

One further experiment was performed which implicated oxygen and freezing as necessary for the gel formation by irradiation. One per cent. *p*H 3.0 insulin solutions (including one sample which had been highly evacuated) were irradiated with γ-rays (10, 20 and 40 million r.e.p.) at Argonne National Laboratory. This procedure permitted the examination of the samples immediately after irradiation and eliminated frozen shipment. In holding these test-tubes at room temperature, no precipitation appeared at any dose level even after two weeks of standing. Gels could be readily and irreversibly formed, however, by holding them for several hours at a freezing temperature. The evacuated test-tube sample produced a slight gelation upon freezing which solubilized on warming to room temperature.

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(31) B. Shapiro and L. Eldjarn, *Radiation Res.*, **3**, 393 (1955).

(32) A. Katchalsky, *Prog. in Biophy. and Biophys. Chem.*, **4**, 1 (1954).