KOREAN JOURNAL OF ZOOLOGY
Vol. 13, No. 3, 75–84(1970)

Radiolysis of Oxygenated and Deoxygenated Glycylglycylglycine in Aqueous Solution and in the Solid State*

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酸素의 存在下와 無酸素下에서의 水溶液 및 固體 Glycylglycylglycine의 放射線分解

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(Received September 28, 1970)

摘 要

蛋白質의 放射線分解의 기작을 밝히는 연구의 일환으로, 특히 peptide 結合의 분해의 기작을 구명하기 위하여 glycylglycylglycine의 水溶液과 固體를 酸素의 존재하에서와 無酸素하에서 γ線을 조사하여 分解生成物을 여지크로마토그라프로 분리하였고, carbonyl 化合物과 amide를 각각 分光光度法과 微量摘定法으로 정량하였으며 放射線障害를 평가하기 위하여 赤外線 spectrum과 紫外線 spectrum을 얻어 검토하였다.

水溶液과 固體에 있어서의 peptide 結合의 분해기작은 근본적인 차이가 있는 것으로 여겨지며, 전자에서는 월등하게 분해가 많이 일어난데 반해서 후자에서는 무시할 정도에 지나지 않았다. 한편, 水溶液의 경우 酸素의 유무에 따라 현저한 영향은 보이지 않았으나 無酸素하에서는 遊離基의 再結合이 일어나는 점이 특기할만 하였다.

水溶液에 있어서의 peptide 結合의 분해기구는 Garrison 一派가 주장한 기작에 의해서 일어나는 것이 분명하여 脫水素反應에 뒤이어 加水分解反應에 의해서 amide와 carbonyl이 생성되는 것으로 보이며, 固體의 경우도 α-炭素의 부위가 방사선의 공격을 가장 많이 받는 것으로 추정되나 그 정도는 미미한 것에 지나지 않는 것으로 생각되었다.

INTRODUCTION

The reaction mechanisms for the radiolysis of the peptide bond in oxygenated and oxygen-free solutions were suggested by Garrison *et al.* (1962 a, b), who irradiated N-acylglycine, N-acylalanine and proteins for their radiolytic study, based on the yields and types of products observed.

The fundamental reaction proposed by Garrison and Weeks(1962 b) in oxygenated solutions of acetylglycine were rupture of the peptide bond under formation of amide of the N-terminal and the carbonyl derivative of the C-terminal amino acid. In oxygen-free solutions they observed similar cleavage products of peptide bond besides recombination on carbon-2, yielding diaminosuccinic acid and aspartic acid from

*This work was supported in part by a grant from the Research Foundation, Seoul National University.

irradiated acetylglycine, diaminosuccinic acid being the major product. They were concerned more specifically with radiation induced reactions of the peptide bond using convenient compound, both in solution and in the solid state. They were lead to the conclusion that the observed reactivity of α-carbon locus might also be a factor in the radiation chemistry of the solid state. And, as part of their earlier studies, they 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 (Jayko & Garrison, 1958). Subsequent hydrolysis of the products showed the constituent carbonyl compounds to include α-keto acid. Hence, they tentatively proposed that dehydrogenation followed by hydrolysis could be involved.

Hatano (1960) found formation of α-ketoisovaleric acid as well as of pyruvic acid by irradiation of alanylvaline solutions, contradicting Garrison's observation.

Liebster and Kopoldova(1960) determined the products of radiolysis of eleven dipeptides and tripeptides before and after hydrolysis of the solutions irradiated in the presence and in the absence of oxygen, showing an essential difference in the radiation chemical reactions in oxygenated and in oxygen-free solutions.

Studies of Alexander and Hamilton (1960) and Bowes and Moss (1962) appear generally to confirm the idea that a major chemical effect of ionizing radiation of 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 remains largely speculative.

The present study aimed at the elucidation of mechanisms for the gamma-degradation of both aerated and degased glycylglycylglycine in solution and in the solid state, since the results could contribute information on the radiolytic reactions of proteins.

MATERIALS AND METHODS

Glycylglycylglycine used in the irradiation experiments was chromatographically pure grade from Mann Research Laboratory.

Degasing of glycylglycylglycine in solution and in the solid state was performed in a Pyrex thimble connected with joint to a vacuum line which was equipped with a vacuum pump, oil diffusion pump, and Pirani vacuum gauge control. 5 ml each of 50mM solutions or equivalent amount of solid was taken in a thimble and placed under a vacuum of 10^{-5}mmHg, in which the thimble was chilled to -180°C by applying liquid nitrogen; then the thimble was fire sealed off.

Aqueous solutions and solid samples were irradiated in a ^{60}Co γ-source at the Atomic Energy Research Institute with a total dose of 1.84×10^{20} ev/ml, at a dose rate of 9.2×10^{18} ev/h. For the irradiation in the presence of oxygen, solutions and the solids were loosely stoppered with pin-holed aluminum foil in order that the oxygen might diffuse into the thimble during irradiation.

The decrease of the irradiated mother substance was determined by paper chromatography of the irradiated solutions and solids, elution of the spots (Giri, 1953), and colorimetric estimation. Other decomposition products of the irradiated glycylglycylglycine after hydrolysis

were isolated from the irradiated solution by means of two-dimensional chromatography with the aid of reference compounds. Hydrolysis was performed in the usual way with 6N HCl at 105°C for 4 hours.

Carbonyl compounds were determined by spectrophotometry of their alkali alcoholic 2,4-dinitrophenylhydrazones(Lappin and Clark,1951). Corresponding amide groups were determined by boric acid-hydrochloric acid procedure after hydrolysis with 3N H_2SO_4 (Conway, 1962). Amide-like ammonia was determined by subtracting the free ammonia liberated in the solution. Ammonia liberated was determined by the diffusion micromethod of Conway(1933).

The spectra of unirradiated and irradiated glycylglycylglycine solids from 2.5 to 25 microns were obtained with a Tadco Model IR-G infrared spectrophotometer equipped with sodium chloride optics. The potassium bromide was of spectro-grade and obtained from the E. Merck Company. It was used without further purification and stored in a desiccator over calcium chloride when not in use. The disks were prepared by grinding approximately 15mg of the sample in 200 mg of potassium bromide in agate mortar for 10 minutes. The resulting fine powder was placed in a sample die consisting of a stainless steel shell and two plungers. It was then connected to a vacuum pump for 10 minutes and pressed in a Carver laboratory press to a gage pressure of 450 Kg/cm^2. After pressing for 10 minutes, the disk was removed and a spectrum obtained. The instrument was calibrated using polystyrene as a standard prescribed by the manufacturer.

Ultraviolet spectra of unirradiated and irradiated solutions and solids were obtained using MPS-50L multipurpose recording spectrophotometer.

RESULTS AND DISCUSSION

Paper chromatography of irradiated solution before hydrolysis showed four ninhydrin-positive compounds as shown in Fig. 1. The largest spot was undegraded mother substance and the rest three were degradation products. In case of solid, however, no degradation product was detected. Decrease of mother substance by γ-radiolysis was determined by elution and spectrophotometry. In Fig. 2 are shown absorption spectra of eluted chromatograms showing gradual increase in rupture of peptide bond in the order of SA, LD, and LA and identical three absorption peaks at 410, 470 and 515mμ for all the samples. From this spectrum λ_{max} was determined to be 515 mμ and the quantitation was performed at this wavelength. From the absorbance G values are obtained to be 2.44, 2.36, 0.26, and 0.25 for oxygenated solution (LA), deoxygenated solution (LD), oxygenated solid(SA), and deoxygenated solid (SD), respectively.

In view of these results, it seems to be that the contribution of oxygen in peptide bond rupture is not significant since degradation of mother substance of both oxygenated and deoxygenated solutions is quite similar. This tendency is not an exception for the irradiated solids either.

Two-dimensional chromatography of LA and LD after hydrolysis revealed the presence of two spots for the former and five spots for the latter. The presence of diaminosuccinic acid was typical for LD indicating the recombination of the free radicals to occur as suggested by Garrison *et al.* (1962 b).

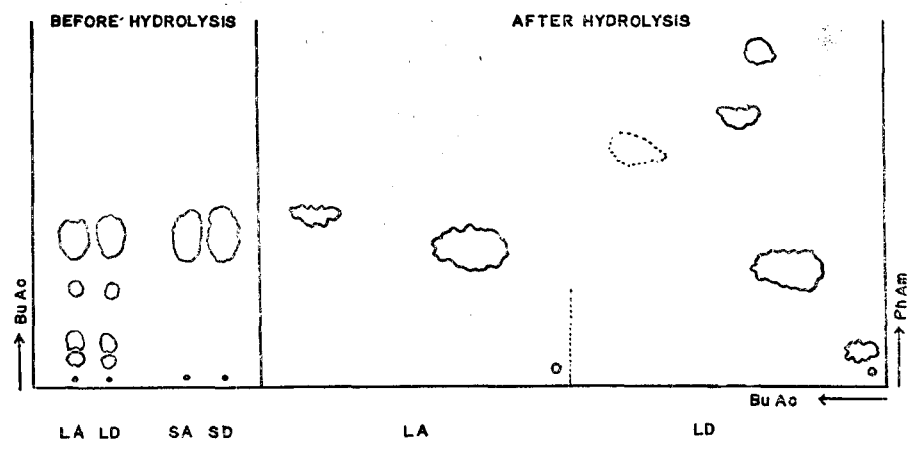

Fig. 1. One and two dimensional chromatograms of irradiated triglycine with a dose of 1.84×10^{20} ev/ml of γ-rays of ^{60}Co using three different solvent systems.

$$2NH_2CH_2CONHCH_2CONH\dot{C}HCOOH\rightarrow$$
$$NH_2CH_2CONHCH_2CONHCHCOOH$$
$$|$$
$$NH_2CH_2CONHCH_2CONHCHCOOH$$

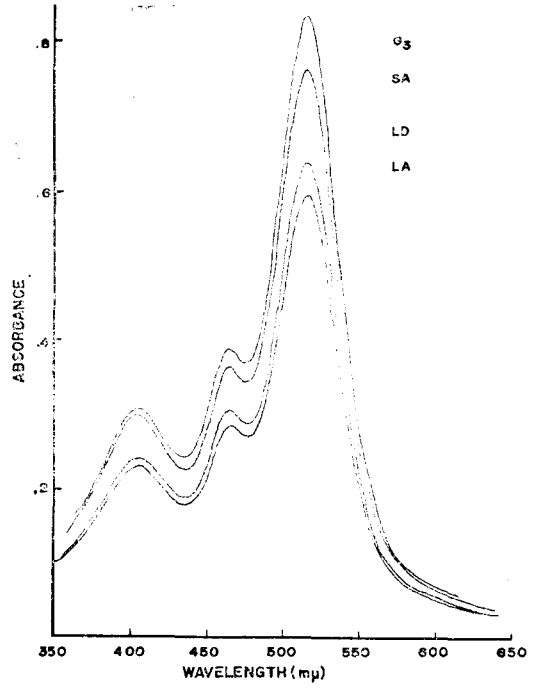

Fig. 2. Absorption spectra of eluted chromatograms of triglycine and irradiated triglycine with a dose of ^{60}Co γ-rays of 1.84×10^{20}ev/ml.

hydrolysis of the recombination products yields diaminosuccinic acid and glycine:

$$NH_2CH_2CONHCH_2CONHCHCOOH$$
$$|$$
$$NH_2CH_2CONHCH_2CONHCHCOOH$$
$$\rightarrow 4NH_2CH_2COOH + \begin{matrix}NH_2CHCOOH\\ |\\ NH_2CHCOOH\end{matrix}$$

Carbonyl formation is typical for solutions, being negligible for solids as is evident from Fig. 3. Quantitative determination was done at 470mμ employing acetone as a standard for monocarbonyl function. Total carbonyl functions expressed as G values are 0.92 and 0.70 for LA and LD, respectively, and no carbonyl function was detectable in case of solids, both of oxygenated and deoxygenated. These results indicate the possible involvement of different radiolytic mechanisms in solution and in the solid state. This finding is unconsistent with the results obtained by other workers (Jayko and Garrison, 1958), who found irradiated solid pepsin and gelatin (evacuated) on dissolution in water yield compounds containing the carbonyl function, which on hydrolysis of the products showed the constituent carbonyl compounds to

include α-keto acids.

In view of this fact, the present result on carbonyl function in solid radiolysis reached a conclusion that the carbonyl groups produced by peptide bond rupture are so minute and unstable that the detection might be impossible after dissolution in water, assessed by the fact that the corresponding amide functions were found to be produced in solid as will be shown below, though the amount produced are found to be quite low compared to those in solutions.

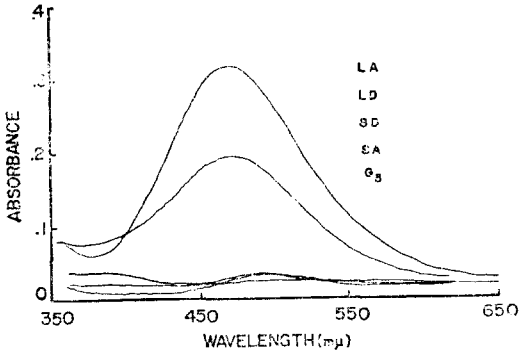

Fig. 3. Absorption spectra of alkaline alcoholic solutions of 2, 4-dinitrophehylhydrazones of triglycine and irradiated triglycine with a dose of ^{60}Co γ-rays of 1.84×10^{20}ev/ml.

Formation of amide, being a measure of peptide bond cleavage by Garrison's mechanism, occur in appreciable yield in oxygenated and deoxygenated solutions and, according to the present experiment, solid samples do also yield amide though the amount produced is quite low. Formation of amide is most likely to be affected by the presence of oxygen in solutions, while the effect of oxygen is negligible in the solid state. Formation of amide as expressed in terms of G value is 1.17, 0.46, 0.16, and 0.14 for LA, LD, SA, and SD, respectively. This finding is in good agreement with the conclusion reached by Garrison and Weeks (1962 b) that the observed reactivity of the α-carbon locus might also be a factor in the radiation chemistry of the solid state. This reasoning, however, is not consistent with the results of degradation of mother substance for good material balance.

On the basis of these results there seems to be no essential difference between oxygenated and deoxygenated samples in the mechanisms involved for the rupture of peptide bond, yielding carbonyl and amide, although the effect of oxygen was exhibited in cleavage of peptide bond. The cleavage reaction of most importance might be formulated for glycylglycylglycine as follows:

$$NH_2CH_2CONHCH_2CONHCH_2COOH+\dot{O}H\rightarrow NH_2CH_2CONHCH_2CONH\dot{C}HCOOH+H_2O$$

$$NH_2CH_2CONHCH_2CONH\dot{C}HCOOH+O_2\rightarrow NH_2CH_2CONHCH_2CON=CHCOOH+H\dot{O}_2$$

$$NH_2CH_2CONHCH_2CON=CHCOOH+H_2O\rightarrow NH_2CH_2CONHCH_2CONH_2+HCOCOOH$$

This finding is opposed by the results obtained by Liebster and Kopoldova (1964, 1966) who showed that there is practically no formation of carbonyl and amide in irradiated peptide solutions, meaning that no peptide bond cleavage occurs in oxygen-free media.

Formation of ammonia was determined for

Table 1. Quantitation of NH_3 liberated from irradiated triglycine with a dose of ^{60}Co γ-rays of 1.84×10^{20}ev/ml with the hydrochloric-barium hydroxide procedure

Sample	ml $Ba(OH)_2$	μg NH_3-N	μg NH_3-N/ml
LA	0.237±0.013	7.29	14.58
LD	0.268±0.017	6.36	12.72
SA	0.481±0.013	—	—
SD	0.481±0.018	—	—
G_3	0.480±0.011	—	—

Table 2. Quantitation of amide NH_3 of irradiated triglycine with a dose of ^{60}Co γ-rays of 1.84×10^{20} ev/ml determined as for ammonia after hydrolysis with $3N\text{-}H_2SO_4$ with boric acid-hydrochloric acid procedure

Sample	ml HCl	μg total NH_3-N/ml	μg amide NH_3-N/ml
LA	0.375±0.005	196.0	50.20
LD	0.287±0.017	146.7	19.52
SA	0.037±0.004	6.72	6.72
SD	0.036±0.006	6.16	6.16
G_3	0.025±0.003	—	—

Table 3. G values of products of irradiated triglycine with a dose of ^{60}Co γ-rays of 1.84×10^{20} ev/ml

Sample	-M	Total C=O	Amide-NH_3	NH_3
LA	2.44	0.92	1.17	3.4
LD	2.36	0.70	0.46	2.9
SA	0.26	—	0.16	*
SD	0.25	—	0.14	*

* Lead to obtain infrared spectrum.

the calculation of amide-like ammonia produced by the peptide bond rupture. It can be seen from Table 1 that formation of ammonia is found for only in solution, being generally higher in oxygenated solution. Formation of ammonia is reported by Liebster and Kopoldova (1966) to be in relation to the structure of the amino acids participating in the peptide bond and to the medium of irradiation. Also reported are ammonia formation in oxygen-free solutions represents predominantly reductive deamination of the N-terminal amino acid; in oxygenated solutions it corresponds to oxidative deamination of the N-terminal amino acid—that is, ketoacyl peptide formation plus peptide bond cleavage by the mechanism suggested by leading to formation of the aldehyde, CO_2, NH_3 and the free C-terminal amino acid (Liebster and Kopoldova, 1966). Mechanisms for the deamination in oxygenated and deoxygenated solution could not be deduced from the results obtained in the present experiment. Inability to detect ammonia in the solid state led to examination of infrared spectra to learn if any difference occurs in functional groups and in the structure of irradiated solids.

Fig. 4 shows the infrared spectra from 2.5 to 25 microns (400 to 4000cm^{-1}) of unirradiated and irradiated solids, both in oxygenated and deoxygenated. The spectra of irradiated solids were exactly the same regardless of the presence and absence of oxygen during irradiation. Also examination of these spectra reveals the alteration in position of bands, which represent various functional group absorptions such as OH, NH, C=O, C—O, C—N, C—C stretching vibrations and CH, OH, NH bending vibrations. Characteristic alterations were observed in about 6 to 7 micron regions. Unfortunately, however, the significance of these alterations was unable to be clarified. This might be attributable to the fact that irradiated solid contains mixtures of irradiation products and the interpretation of spectral alteration in mixtures, in general, is very difficult to perform. The only conclusion reached in the present experiment is that chemical structural change occurs without appreciable alteration in functional groups.

The band positions of these two spectra are compared and are shown in Fig. 5. Deamination in irradiated solid is suggested by observing the alteration of bands in 2.5 to 3 and 6 to 7 micron regions, although ammonia was not detected in the solids by means of micro-diffusion method

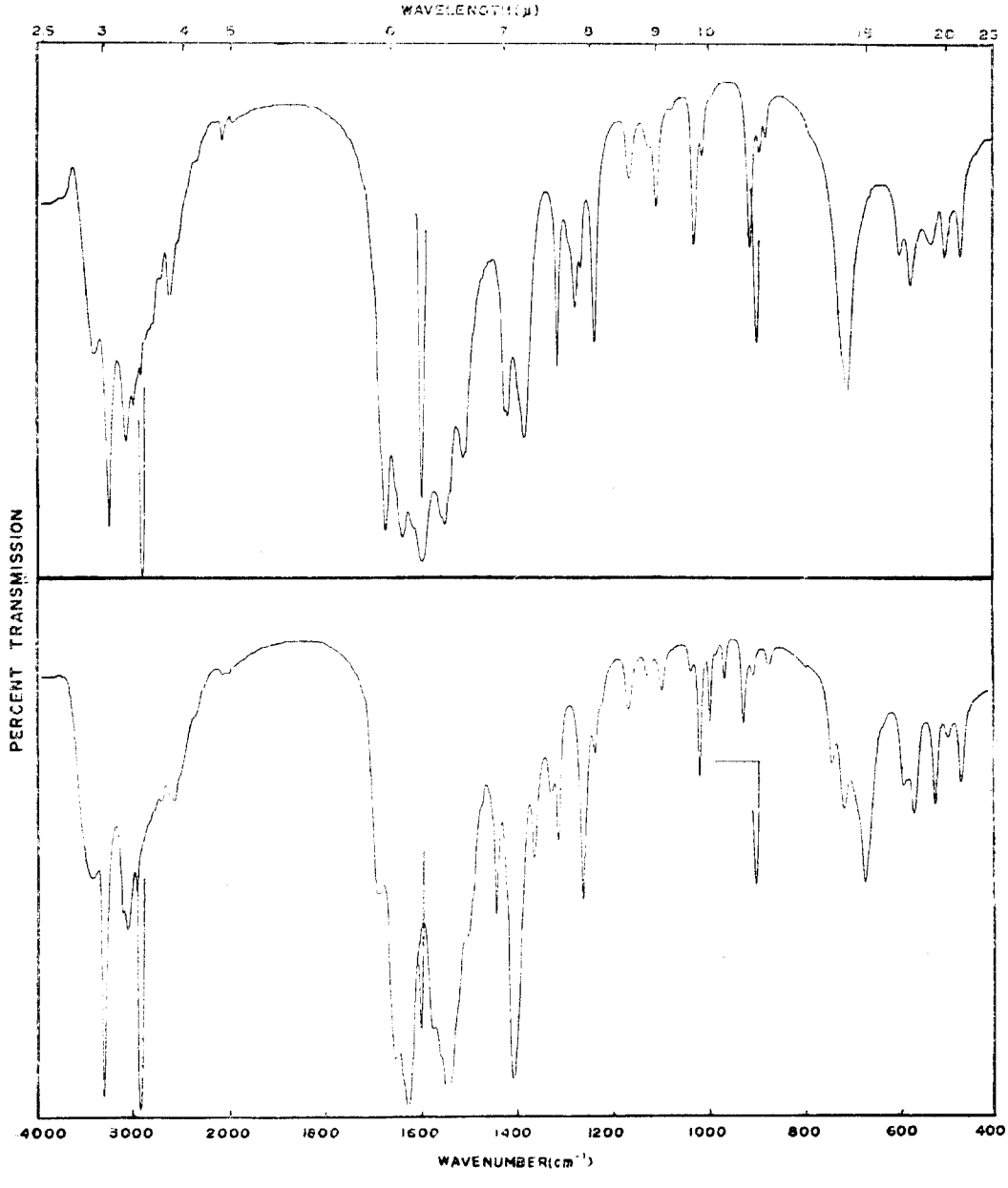

Fig. 4. Infrared spectra of unirradiated and irradiated triglycine with a dose of ^{60}Co γ-rays of 1.84×10^{20}ev/m l as potassium bromide disk.

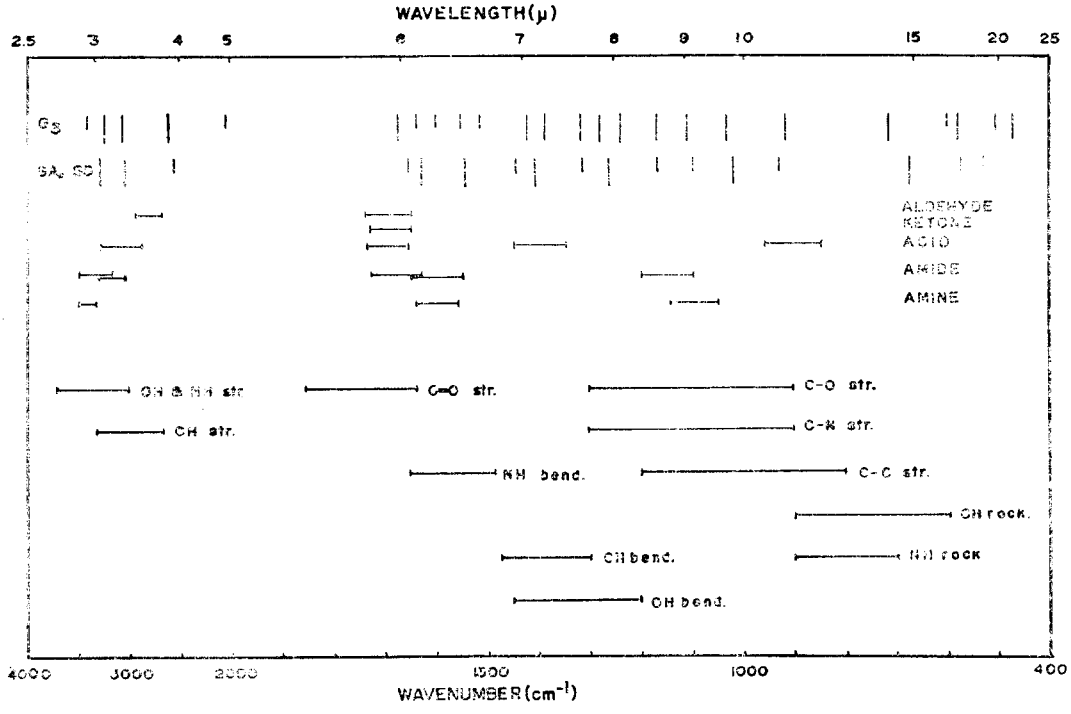

Fig. 5. Band positions of unirradiated and irradiated triglycine with a dose of ^{60}Co γ-rays of 1.84×10^{20}ev/ml from 2.5 to 25 microns.

of Conway. An interesting fact to be noted is that the change of spectrum varies with time intervals between irradiation and infrared spectrophotometry, exhibiting recovery spectral alteration of irradiated solid to the unirradiated in a month or so after irradiation. The meaning of this recovery is not interpreted in the present experiment awaiting further study.

Shown in Fig. 6 are absorption spectra in 220 to 280 mμ of unirradiated and irradiated solutions and in the solid states, both in the presence and in the absence of oxygen to observe whether any difference in ultraviolet absorbance between solution and solid exists. Molar extinction coefficients at 240mμ are determined to be 86.8, 81.0, 24.0, 23.0 and 29.0 for LA, LD, SA, SD, and G_3, respectively. Absorption coefficients for solutions are quite different from those for solids. Irradiated solids exhibit as low absorbance as unirradiated, revealing only a slight change to occur.

Setlow and Guild (1951) showed that because of the large peptide bond absorption below 230 mμ irradiation damage to peptide backbone may be observed in this spectral region. Peptide absorption maximum is reported to be 184mμ.

The spectra obtained in the present experiment, however, can not be used to determine the degree of peptide bond rupture since both remaining peptide and other degradation products are responsible for the absorption in vacuum ultraviolet region, which influences the spectra in ultraviolet region. Carbonyl and amide formation by the cleavage of peptide bond predo-

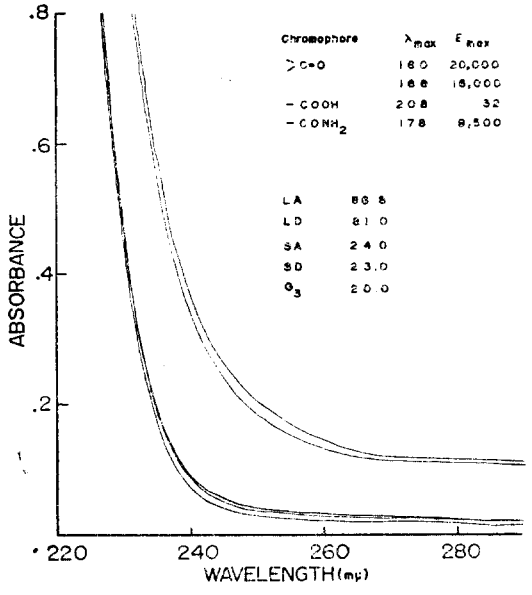

Fig. 6. Absorption spectra of triglycine and irradiated triglycine with a dose of ^{60}Co r-rays of 1.84×10^{20}ev/ml. Molar absorption coefficients at 240mμ are shown for comparison.

minate in the absorption near 160 mμ and 178 mμ (Kamlet, 1952) than that of peptide bond itself. The absorption at vacuum ultraviolet and resulting absorption in ultraviolet region bear a complex meaning. On this account the absorption by carbonyl and amide should be used instead of peptide bond absorbance for evaluation of radiation damage. It is very likely to be confirmed that the essential difference in mechanisms for degradation of oxygenated and deoxygenated solutions does not exist. And, it is unlikely that carbonyl and amide formation due to rupture of peptide bond occurs in irradiated glycylglycylglycine in the solid state in view of ultraviolet absorption, though production of amide and carbonyl in irradiated solid proteins are reported.

SUMMARY

Gamma-radiolyses of oxygenated and deoxygenated glycylglycylglycine in aqueous solution and in the solid state are observed, with special regards to peptide bond rupture for elucidation of radiolytic mechanism of proteins, by means of chromatorgraphic separation of degradation products, spectrophotometric quantitation of carbonyl compounds, micro-titration of amide formation, infrared spectrophptometry, and ultraviolet spectrophotometry for evaluation of radiation damage.

Essential difference of peptide bond rupture is observed in solution and in the solid state, being high in the former and negligible in the latter. On the other hand, the presence of and absence of oxygen in solution during irradiation are not so significant with respect to peptide bond rupture, except the recombination of free-radicals produced in deoxygenated solution.

Peptide bond rupture in solution is attributable to the mechanisms proposed by Garrison *et al.*; dehydrogenation followed by hydrolysis to yield acid amide and carbonyl function as found on the basis of radiolytic products. Peptide bond attack at α-carbon locus might be suggestive for irradiated solid but not significant in view of low degree of peptide bond rupture.

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