REFERENCES

(1) Smith, G., *J. Assoc. Offic. Agr. Chemists*, **42**, 462 (1959).
(2) "Official and Tentative Methods of Analysis of the Association of Agricultural Chemists," 6th ed., Association of Agricultural Chemists, Washington, D. C., 1945, p. 675.
(3) Parke, T. V., Ribley, A. M., Kennedy, E. E., and Hilty, W. W., *Anal. Chem.*, **23**, 953(1951).
(4) Tinker, R. B., and McBay, A. J., *J. Am. Pharm. Assoc., Sci. Ed.*, **43**, 315(1954).
(5) Jones, M., and Thatcher, R. L., *Anal. Chem.*, **23**, 957 (1951).
(6) Winefordner, J. D., and Latz, H. W., *ibid.*, **35**, 1517 (1963).
(7) Hollis, D. P., *ibid.*, **35**, 1682(1963).
(8) Pernarowski, M., and Padval, V. A., *J. Pharm. Sci.*, **52**, 218(1963).
(9) Josephina, A. C., *Anales Fac. Farm. Bioquim. Univ. Nacl. Mayor San Marcos (Lima)*, **6**, 519(1955); through *Chem. Abstr.*, **53**, 13506*i*(1959).
(10) Bukowska, H., *Chem. Anal. (Warsaw)*, **4**, 105(1959); through *Chem. Abstr.*, **53**, 14419*g*(1959).
(11) Machek, G., *Sci. Pharm.*, **29**, 73(1961).
(12) Reilley, C. N., and Crawford, C. M., *Anal. Chem.*, **27**, 716(1955).

Effects of Ionizing Radiation on Two Gelatin Fractions III

Carbonyl Group Analyses and Electron Spin Resonance Studies

By LEONARD P. PRUSAK* and BARTLEY J. SCIARRONE

Two fractions, F-I and F-II, obtained by alcohol fractionation of commercial pigskin gelatin, and having molecular weight values of 173,000 and 86,000, respectively, showed the following relationship with respect to additional carbonyl group content resulting from low-level irradiation under a 3 Mev. Van de Graaff: 1 per cent solution > 5 per cent solution (gel) > film. Irradiated films (F-I and F-II) showed doublets with 25 gauss line separation in electron spin resonance studies. These, together with previously published data, indicate that the gelatin fractions undergo molecular weight changes through free radical mechanisms involving scission, crosslinking, and weak bond formation.

In previous papers (1, 2) the authors showed the isolation of 2 fractions, F-I and F-II, from commercial pigskin gelatin; the irradiation technique used with a 3 Mev. Van de Graaff accelerator; acid-base titration behavior; sedimentation velocity and intrinsic viscosity studies; and molecular weight determinations.

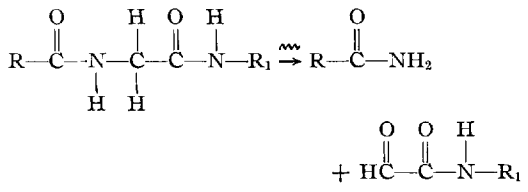
We now give further experimental data which indicate that irradiation of these gelatin fractions produces changes in structure through free radical mechanisms.

The currently accepted concept regarding radiation effects on organic materials is that chain scission is synonymous with molecular weight decrease, and crosslinking is a sign of molecular weight increase. Intermediate variations are explained on grounds that both scission and crosslinking occur. This thinking may be extended to the irradiation of complex polymers, assuming that conditions are employed which lead to these ultimate effects. It is doubtful, on the basis of experimental data available today, that gelatin is susceptible to predictable irradiation behavior.

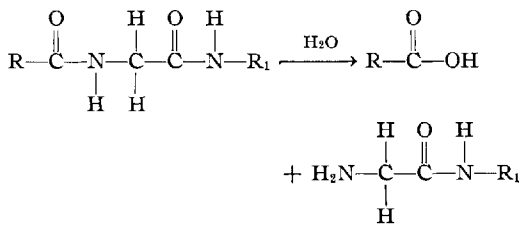
Some factors which must be considered before rupture or linkage are proposed as explanations for shifts in molecular weight of irradiated gelatin include solvent–solute interaction, absorbed irradiation dose, presence or absence of oxygen, solute concentration, nature of solvent, thermal history, aging, irradiation temperature, etc.

Since the formation of free radicals in solids and liquids exposed to irradiation has been established, explanations of molecular weight shifts in irradiated gelatin require consideration of free radical formation. This, in turn, poses the question as to indirect effects of the solvent if irradiation is carried out on a fluid system. Experimental evidence indicates that irradiation of oxygenated protein solutions, such as gelatin, results in the formation of carbonyl functions (3, 4) and that these are traceable to intermediate free radicals. Radiolytic cleavage of the peptide chain yields an amide and an additional carbonyl group:

Received March 9, 1962, from the College of Pharmacy, Rutgers—The State University, Newark, N. J.
Accepted for publication January 27, 1966.
Abstracted in part from a dissertation submitted by L. P. Prusak to the Graduate School, Rutgers—The State University, Newark, N. J., in partial fulfillment of Doctor of Philosophy degree requirements.
The assistance of Mr. W. A. Yager and Mr. S. H. Glarum, Bell Telephone Laboratories, Murray Hill, N. J., in obtaining and analyzing spin resonance data is gratefully acknowledged.
The authors thank Dr. Charles Artandi, Research Director, Ethicon, Inc., Somerville, N. J., for valuable advice during the irradiation phase of these studies.
* Present address: Johnson & Johnson, New Brunswick, N. J.



as opposed to hydrolytic fracture, which yields an acid and free amino group:



Proof of additional carbonyl group formation has been obtained (3) by isolation of a number of α-keto acids from irradiated gelatin—namely, oxalacetic, α-ketoglutaric, glyoxalic, pyruvic, and phenylpyruvic. Formation of these is believed to occur through an imino intermediate which yields the acid on hydrolysis.

Numerous details regarding the precise mechanistic sequences and interrelationships of free radical formation, chain scission, and crosslinking are yet to be resolved, but it can be said with reasonable certainty that these events do occur in irradiated gelatin. Data are presented here to show that additional carbonyl group formation affords an excellent means for correlating the degree of scission and/or crosslinking in the gelatin fractions as functions of irradiation dose and physical state (film or aqueous solution).

Since the formation of stable free radicals containing unpaired electrons in irradiated organic solids has been established, the application of electron spin resonance to the determination of the free radical quanta has assumed considerable importance. Because others (5, 6) have shown that the application of this method to proteins gives meaningful results, spin resonance measurements on irradiated gelatin films were included in the present work.

Table I.—Results of Carbonyl Analysis

	F-I μmoles Carbonyl/Gm.	F-II μmoles Carbonyl/Gm.
1% Soln., Mrad.		
0.41	23.4	11.8
0.60	39.5	13.5
1.15	53.1	25.3
2.10	66.6	47.6
5% Soln., Mrad.		
0.41	3.6	1.1
0.60	6.0	1.2
1.15	13.8	1.8
2.10	36.0	4.9
Film, Mrad.		
0.41	0.2	0.06
0.60	0.4	0.15
1.15	0.8	0.65
2.10	1.1	0.95

Table II.—Resonance Curve Values Normalized to Sweep 50, Gain 50

Peak-to-Peak Distance (Arbitrary Scale)	Sweep	Gain	Normalized Value	Dose, Mrad.
		F-I		
8.4	320	200	0.328	0.461
6.1	100	200	0.762	0.679
5.0	50	100	2.5	1.71
8.8	50	50	8.8	3.12
		F-II		
6.7	100	500	0.335	0.461
6.3	100	100	1.575	0.679
6.4	50	100	3.2	1.71
7.9	50	32	12.35	3.12

EXPERIMENTAL

Carbonyl Analysis.—Determination of carbonyl group formation was made according to the procedure of Lappin and Clark (7) using the 2,4-dinitrophenylhydrazone derivative of acetaldehyde as a reference standard. Hydrazone content was determined in terms of μm. carbonyl/Gm. of gelatin at 440 mμ with a Beckman DU spectrophotometer. Additional carbonyl group formation was obtained by difference between controls and irradiated samples. The data are given in Table I.

Electron Spin Resonance Studies.—Film strips of F-I and F-II were submitted to the scanned beam of the 3 Mev. Van de Graaff accelerator as previously described (1), and the resulting average absorbed doses were calculated immediately following irradiation. They were: 0.46, 0.67, 1.71, and 3.12 Mrads, respectively. The films were exposed to these doses at room temperature (24°C.), inserted in heat-sealed aluminum foil packages, and immediately immersed in liquid nitrogen (77°K.). Controls were included to the point of irradiation.

Spin resonance measurements, conducted on all samples on the day of irradiation, were made using a Varian 100 kc. field modulation EPR spectrometer at a microwave frequency of 9000 Mc./sec. Various degrees of attenuation of resonance intensity were used to obtain readable signals. Therefore, in order to have comparable measures of free radical population all resonance intensities, measured on an arbitrary scale, were normalized to the instrument parameters sweep 50 and gain 50. (See Table II.) A log-log plot of normalized resonance signal intensity *versus* dose is shown in Fig. 1.

RESULTS

Carbonyl Analysis.—In the case of 1 and 5% solutions of both fractions, straight line relationships were obtained from plots of dose (Mrad.) *versus*

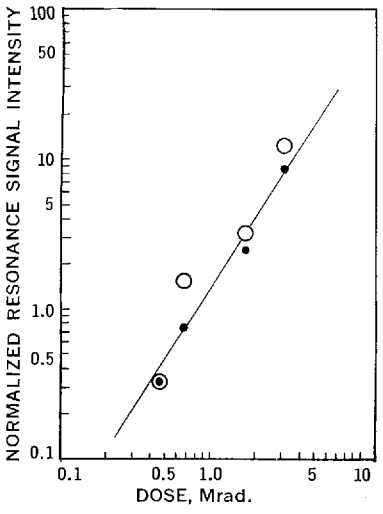


Fig. 1.—Plot of the linear relationship between EPR signal intensity, normalized to the instrument parameters sweep 50 and gain 50, and the dose impinging upon films of F-I (●) and F-II (○).

μm. carbonyl formed per Gm. of protein. Furthermore, some correlation was evident between the same concentration levels of the separate fractions. Figure 2 shows a plot of dose *versus* μm. carbonyl/Gm. of gelatin in 5% solution of both fractions. Data for obtaining the best possible straight line were computed by regression analysis. Slope values for 1% solutions of F-I (4.2×10^{-2}) and F-II (6.2×10^{-2}) were remarkably close, but the values for 5% solutions differed by a factor of 10 (*cf.* Fig. 2). Nonetheless, it can be stated that in irradiated solutions the number of carbonyl groups formed per Gm. of protein is directly proportional to the dose imparted and is concentration dependent.

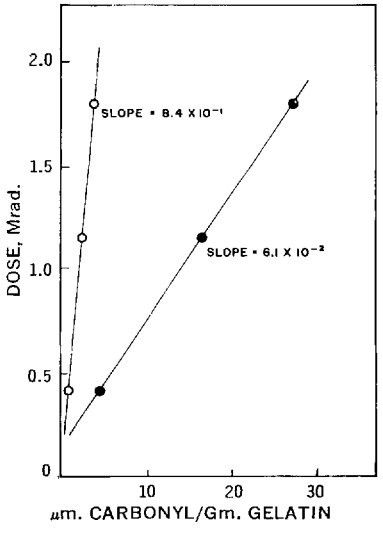


Fig. 2.—Arithmetic relationship between the dose, impinging upon a 5% solution of F-I (●) and F-II (○), and the resulting extra μm. of carbonyl groups produced/Gm. of the gelatin fraction.

Figure 3 shows a semilog plot of dose *versus* μm. carbonyl/Gm. of gelatin in irradiated films of F-I and F-II. In contrast to 1 and 5% solutions, the films showed less carbonyl formation, and proportional relationship to the logarithm of the dose. It would appear that carbonyl groups were formed at the same rate in the 2 gelatin fractions despite widely different average molecular weights.

G values, or the number of events (carbonyl groups formed) per 100 ev. (electron volts) absorbed energy, were derived from the formula:

$$G = \frac{\text{No. of changed molecules} \times 100}{E\ (\text{ev.})}$$

$$= \frac{(\mu\text{m./Gm.})\ (6.06 \times 10^{17})\ (100)}{(\text{Mrad.})\ (\text{ev./Gm./Mrad.})}$$

The expression above applies to materials irradiated in the dry state, *i.e.*, films, since the second term

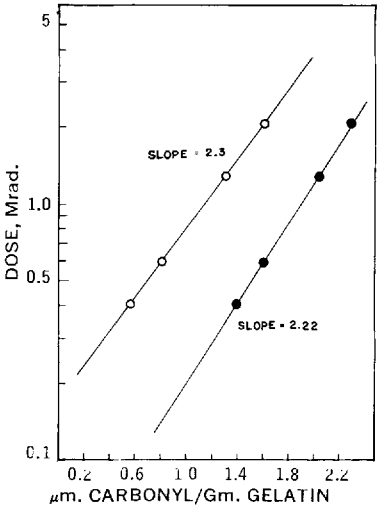


Fig. 3.—Semilog relationship between the dose, impinging upon films of F-I (●) and F-II (○), and the resulting extra μm. of carbonyl groups produced/Gm. of the gelatin fraction.

in the denominator refers to grams. In the case of 1% solutions, the second term was appropriately adjusted to enable calculation of energy absorption (*E*) on the basis of 100 ml. (1 Gm. of protein in 100-ml. vol.). Similarly, with the 5% solutions, another adjustment was made in the second term to permit calculation of energy absorption of a 20-ml. vol. Thus, the second expression in the denominator is, respectively, for

film: 6.37×10^{19} ev./Gm./Mrad.
1% solution: 6.37×10^{21} ev./100 ml./Mrad.
5% solution: $6.37 \times 10^{20} \times 2$ ev./20 ml./Mrad.

The *G* (carbonyl) values for both fractions are given in Table III.

Results for all samples were somewhat lower than the $G = 1.2$ reported by Garrison (3) for gelatin solutions (5 mg./ml.) irradiated with ^{60}Co γ-rays at a dose rate of about 2×10^{17} ev./ml./min. This difference in values can be accounted for by observing that Garrison's rate is equivalent to about 0.2

TABLE III.—EXPERIMENTALLY DETERMINED CARBONYL FORMATION *G* VALUES

Mrad.	F-I	F-II
	1% Soln., G Values	
0.41	0.54	0.27
0.60	0.63	0.21
1.15	0.44	0.21
2.10	0.30	0.22
	5% Soln., G Values	
0.41	0.42	0.12
0.60	0.47	0.10
1.15	0.57	0.08
2.10	0.82	0.11
	Film, G Values	
0.41	0.46	0.14
0.60	0.63	0.24
1.15	0.66	0.54
2.10	0.50	0.43

Mrad./hr. (2×10^{17} ev./ml./min. = 1.2×10^{22} ev./L./hr. Since 1 Mrad./hr. = 6.37×10^{22} ev./L./hr., 1.2/6.37 = 0.188, or 0.2 Mrad./hr.). In the 3 Mev. Van de Graaff, on the other hand, all dose rates were imparted on a "per second" basis. Thus, in the range of doses used in the present experiments (0.4–2.0 Mrad.), the dose rates differed by factors ranging from 7,200 to 36,000 (greater). Since the efficiency of free radical formation is reduced at higher dose rates through recombinations caused by high local concentration, the higher dose rate is responsible for the lower *G* (carbonyl) values.

Of collateral interest in connection with the *G* (carbonyl) value calculations, are the previously referred to Wiederhorn experiments (2), which showed that in parent gelatin (from which F-I and F-II were fractionated) the molecular weight between crosslinks increased as the irradiation dose increased. This implies the formation of thermostable covalent bonds, a requirement for copolymerization. F-I and F-II, on the other hand, did not respond to the crosslinking test at any irradiation level (at least through the crosslinking mechanism), hence failed to polymerize despite carbonyl formation. This, in turn, would suggest that either the number of carbonyl groups formed was inadequate (Table III), or that a crosslinking factor present in the parent source was lacking in the purified fractions, or both.

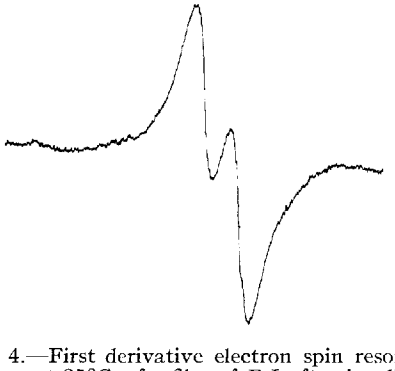


Fig. 4.—First derivative electron spin resonance spectrum at 25°C. of a film of F-I after irradiation with accelerated electrons giving a total dose of 3.12 Mrad. Instrument parameters used were sweep = 50, gain = 50.

Electron Spin Resonance Studies.—Figures 4 and 5 are first derivative resonance curves of F-I and F-II irradiated at 3.12 Mrad., and Figs. 6 and 7 show resonance curves on controls, all observed at room temperature. Figures 4 and 5 also illustrate the typical doublet of 25 gauss line separation obtained for all irradiated samples.

One of the samples, F-I, irradiated at 3.12 Mrad. at 25°C. was observed at 77°K. Total erasure of the doublet occurred when the gain was reduced to 125 and the sweep held at 50. By increasing the gain to 160 and reducing the power by 15 decibels, the same sample (at 77°K.) gave a doublet of 25 gauss line separation, indicating that at low temperature the signal was readily saturated. Shouldering beyond the points of inflection was evident, indicating possible dipolar broadening.

Shifting the sweep and gain to 32 and 200, respectively, retaining the sample at 77°K., and reducing the power by 20 decibels gave a doublet of 20 gauss line separation and retained evidence of shouldering. The sample was then removed from the nitrogen atmosphere and raised to room temperature (25°C.). At sweep 32, gain 500, −15 decibels, the pattern approached that originally obtained at room temperature with a doublet of 25 gauss. Retention of the doublets despite shifts in resonance temperature conditions (following irradiation at room tempera-

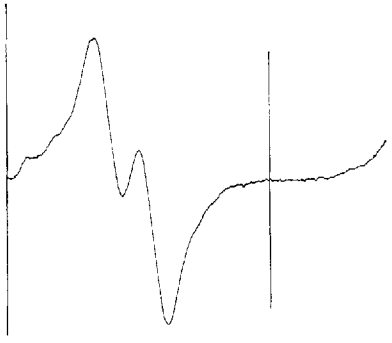


Fig. 5.—First derivative electron spin resonance spectrum at 25°C. of a film of F-II after irradiation with accelerated electrons giving a total dose of 3.12 Mrad. Instrument parameters used were sweep = 50, gain = 32.

ture) from 25°C. to 77°K. to 25°C. shows (5) that free radicals produced at room temperature remain in the same chemical form upon cooling and subsequent warming, and that changes in room temperature patterns following lowering of the temperature to 77°K. are reversible. The increase in signal strength at low temperature is primarily due to $1/T$ dependence of the magnetic susceptibility.

By normalizing the resonance curves to sweep 50 and gain 50 constant values (Table II), and plotting the resulting data as a function of dose (Mrad.) on a log-log scale, linear trends were obtained (Fig. 1) for F-I and F-II. Deviations from the straight line plot can be attributed to variations in sample weight and positions of the films in the cavity.

From Table III one can readily determine the relative increases in free radical formation for F-I and F-II when subjected to identical increases in irradiation dose, keeping the initial free radical and

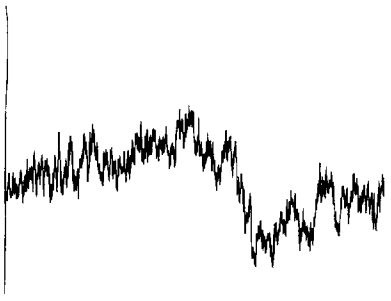


Fig. 6.—First derivative electron spin resonance spectrum at 25°C. of a control (unirradiated) film of F-I. Instrument parameters used were sweep = 500, gain = 800.

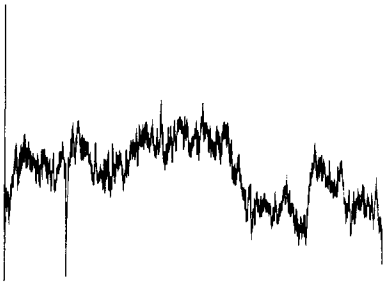


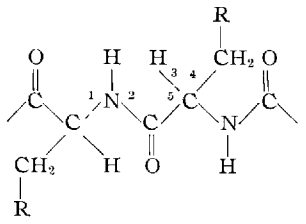
Fig. 7.—First derivative electron spin resonance spectrum at 25°C. of a control (unirradiated) film of F-II. Instrument parameters used were sweep = 500, gain = 800.

dosage norms as bases for comparison. A 32% increase in radiation from 0.461 to 0.679 Mrad. elicited a 56% increase in free radicals in F-I and a 78% increase in F-II. A further increase of 60% in radiation produced 69% more free radicals in F-I, but only a 50% increase in F-II, the latter indicating a downward trend in free radical formation despite a 2.5-fold increase in energy over the previous run. Finally, a further 45% increase in irradiation produced 71% more free radicals in F-I and a 75% increase in F-II. In both films, therefore, the higher the dose absorbed, the greater the excess of unpaired electrons. Contrary to the expectation that radical concentration, in terms of gain strength, should be directly proportional to the absorbed radiation, the data show that radical concentration is proportional to the three-halves power of the radiation dose.

DISCUSSION

Assuming that the predominating amino acid components in gelatin are alanine, glycine, proline, and hydroxyproline with no representation of —S—S— linkages in the absence of cystine (8), the most probable site of radiation damage would appear to be the α-carbon in the polypeptide back bone.

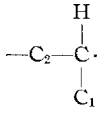
Viewing the protein configuration as:



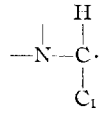
fracture of the C—N bond at 1 would require 25 gauss for the proton, with a possible structure for —CH_2, and a 10 gauss triplet at the nitrogen. Fracture at 2 would require a 25 gauss proton, a 10 gauss triplet at the nitrogen, and a single line for the carbon with a double bonded oxygen. Should fracture occur at 3, the proton would split off, with possible evidence of a 5 gauss triplet at the nitrogen and a —CH_2 triplet. Fracture at 4 would require a 25 gauss triplet to account for the —CH_2—R group, as well as a 25 gauss doublet (carbon) and a 5 gauss triplet (nitrogen). This could occur, for example, in the event of recombination of ·CH_2—R. Finally, fracture at 5 would give a singlet, a 25 gauss doublet, a 5 gauss triplet, and possibly evidence for CH_2.

It would appear that the observed spectra are characteristic of the

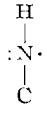
free radical. Formation of



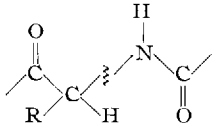
would give a doublet of 25 gauss, but protons on C_1 and C_2 might lead to additional structure, if they were noncoplanar with the carbons. Formation of



also would give a 25 gauss doublet, and a possible triplet, with 5 gauss separation due to the nitrogen. The latter splitting could be broadened by nitrogen quadrupolar interactions. If

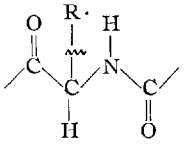


formation occurred, a 25 gauss doublet (H) and a 10 gauss triplet (N) splitting would be evident if quadrupolar broadening did not occur. Of the 2 remaining possibilities



appears to be the less likely since it would involve twice the nitrogen splitting. It seems reasonable to

conclude that formation of the following free radical structure:



is feasible since the data are indicative of spin being on an atom which is bonded to only one hydrogen.

Proof is not available here for postulating with absolute certainty at which of these positions free radical formation occurs. However, it is interesting to note Gordy's (6) conclusions, based on more extensive experimental data, that the peptide free radical resulting from radiation damage to proteins forms at a glycine residue.

It should be observed that the spin-proton interactions did not elicit any qualitative differences in resonance between the 2 protein fractions studied, despite a 2:1 ratio in molecular weight values. Doublets were conspicuously persistent at all irradiation levels, although one fraction showed a successively lesser tendency to increase in molecular weight as the dose was increased, and the other increased in molecular weight as higher doses were applied.

CONCLUSIONS

Low temperature alcohol fractionation of acid-treated pigskin gelatin yielded 2 distinct fractions having different average molecular weights and characteristic acid/base binding properties (1). Dilute solution intrinsic viscosity studies on these fractions (2) showed higher values for the higher molecular weight (173,000) fraction, F-I, than the lower (86,000) fraction, F-II, as expected. The similarity in acid/base binding capacity in the 2 fractions may be best explained on the ground that F-II was constituted of shorter fragments of chains similar to those prevailing in the larger fragments. The virtually superimposable acid-base titration curves over the entire titration range support the proposition that the same N-terminal and C-terminal groups existed in the high molecular weight, F-I, and low molecular weight, F-II, controls.

Of additional importance, particularly with respect to films, is the total absence of water, hence the lack of OH· and H· radicals, to cause alterations in protein structure through secondary effects. Further evidence that more concentrated gelatin is less susceptible to radiation damage is provided in Table I, which shows lower values for carbonyl production in 5% solutions and films of F-I and F-II, than corresponding 1% solutions.

It is *not unreasonable* to suggest that fracture of electrovalent bonds in the gelatin structure occurs on irradiation and that these breaks are responsible for molecular weight reduction. Indeed, evidence that free radicals were formed in films of F-I and F-II contributes to the validity of this proposal, especially when considered in the light of Fig. 3. These data indicate that as the radiation dose is increased, the rate of free radical formation rises. More importantly, at least when considered as a film, the damaged gelatin appears to retain the unpaired electron structure. Thus, retention of high numbers of free radicals reflects minimal recombination, hence absence of crosslinking.

Since the intrinsic viscosity studies and the molecular weights calculated from these data were based on experiments with 1% solutions (2), evidence of disproportionate retention of stable free radicals in films cannot be extrapolated to interpretation of changes in molecular weights predicated on solution experiments. Nonetheless, it seems permissible to reason that free radicals are formed in solutions (as they are in films), but the differences in radiation environment lead to dissimilar results. Irradiated films tend to retain free radicals because the compactness of the molecule locks the radicals into their created positions. Ultimately, after absorption of moisture, the molecular structure becomes loosened, and the radicals are able to shift. Irradiated solutions, on the other hand, do not retain free radicals in abundance because the concurrent high energy impact on solute and solvent creates, in addition to solute free radicals, unpaired electrons in the solvent structure. These are, in the case of water, OH· and H·. The formation of stable free radicals and a structurally less compact solute configuration facilitates (*a*) inactivation by abstraction of hydrogen from another molecule and (*b*) recombination and coupling. Assuming that each of these reactions occurs to a variable degree in irradiated gelatin solutions, it could be suggested for F-I that (*b*) predominates at low radiation levels, causing an increase in molecular weight. As the irradiation levels become greater in intensity, the ability of the free radicals to recombine and couple is progressively decreased and (*a*) controls.

Gelatin in solution answers the description of a polyelectrolyte (9), its isoelectric behavior being dependent upon which groups, the carboxyl or the amine, predominate in the gross structure. Its principal amino acid components are glycine, proline, and hydroxyproline, with lesser amounts of lysine, hydroxylysine, histidine, and arginine. Cystine and tryptophan are lacking.

The acid-base titration curves previously reported (1) tend to confirm the differences in behavior between the high and the low molecular weight fractions. At 1% concentration, irradiation did not appear to affect either the acid or the base functions of either fraction until the dosage was increased to the 2 and 3 Mrad. levels. At that point the basic groups were reduced in number, with a slight increase in bound carboxyls. The midportions of the 1% curves were unaffected in F-II, but F-I showed a decrease in bound imidazole groups at the low radiation level, with a gradual approach to the control value as the energy was increased.

In 5% concentrations, neither F-I nor F-II imidazole groups were affected. However, both fractions suffered carboxyl and amino depletion with the greatest damage imparted to the higher molecular weight protein. Apparently as the concentration of the protein is increased, radiation effects become more pronounced and tend to disrupt drastically the charge organization without affecting the isoelectric point. Irradiation of F-I and F-II in the solid state, on the other hand, caused little deviation in charge.

Carbonyl analyses in terms of μm./Gm. follow an arithmetic progression in approximate proportion to

the absorbed dose (Table I). Conversion of these data to G (carbonyl) values shows somewhat more carbonyl groups in F-I than in F-II per 100 ev. absorbed dose. By taking averages of the G values and drawing comparisons between F-I and F-II within the respective physical states irradiated, it will be seen that in the 1% solutions the averaged G values differed by a factor of 2; in 5% solution the difference increased to a factor of 6; but in films the difference factor fell to about 1.5. Within each group, on the other hand, there appeared to be reasonable consistency in G values and a noticeable lack of deviation, irrespective of variation in water content.

The anomalies may be due, at least in part, to the fact that irradiation was conducted in the absence of low temperature controls. Thus, at room temperature some free radicals tend to recombine to form carbonyl groups. Others enter into crosslinking reactions. The rates of recombination will vary depending on solute concentration, temperature variations, dose imparted, and other factors. In films, the conditions are such that the free radicals are not only deprived of free movement due to the relatively fixed configuration of the molecule, but also of participation in secondary reactions with solvent free radicals.

Notwithstanding this shortcoming, the relative pattern of carbonyl production on a weight basis assigns the lowest quantity to films and the highest to 1% solutions, the 5% solutions being intermediate. The relationship offers at least a partial basis for concluding that a greater number of free radicals are formed in irradiated dilute solutions of proteins than in those more concentrated because of solute–solvent interaction during the ionization event. Although G values in all cases remained below unity, there is no reason to minimize the conclusion that these data are in substantial agreement with Garrison's $G = 1.2$ (3) for carbonyl production in gelatin solutions influenced by ^{60}Co γ-rays. As explained in detail earlier, the lower values obtained in our experiments are attributable to the higher dose rate imparted, which functionally reflects the efficiency of free radical production.

Decarboxylation and deamination occurred in irradiated gelatin solutions in proportion to absorbed dose. Dilute solutions of the low molecular weight fraction were not severely damaged by low energy radiation, but the higher molecular weight fraction appeared to be injured at the imidazole portion. Neither the high nor the low molecular weight fraction was altered in charge groups when irradiated in the dry state, but free radicals were formed in both when irradiation was imparted at room temperature. No alteration in free radical structure occurred when irradiated gelatin films were brought to 77°K. nor when they were subsequently returned to room temperature.

Variations in solvent–solute ratios of gelatin solutions reflected differences in the number of new carbonyl groups formed on a weight basis. Higher carbonyl values per unit weight of protein were obtained in dilute than in concentrated solutions, confirming the secondary effects of water in altering the solute structure on irradiation. Relatively lower G (carbonyl) values were obtained in F-II than in F-I.

It seems permissible to conclude that the end effects of low level irradiation emanating from an electron beam upon fractions of gelatin obtained from the same source are predictable only to the extent that free radical and carbonyl group formation occur. The degree of radiation damage is proportional to the absorbed dose. The presence or absence of water in the system being irradiated also determines the extent of denaturation as reflected in carbonyl group formation. No direct evidence is available that aggregation plays the dominating role in molecular weight increase as a result of irradiation. In view of the extensive free radical formation and the strong indications of solvent–solute interaction during the irradiation event, it is a fair inference that increases in molecular weight in irradiated dilute gelatin solutions can be attributed to crosslinking through covalent and weak hydrogen bonds.

REFERENCES

(1) Prusak, L. P., and Sciarrone, B. J., *J. Pharm. Sci.*, **51**, 1046(1962).

(2) *Ibid.*, **52**, 546(1963).

(3) Garrison, W. M., Weeks, B. M., Jayko, M. E., Bennett, W., Cole, S., and Hughes, G., U.S.A.E.C. Contract No. W-7405-eng-48, Biology and Medicine Semiannual Report, April through September, 1958; dated October 21, 1958, Univ. Cal. Rad. Lab.—8513.

(4) Bennett, W., and Garrison, W. M., *Nature*, **183**, 889 (1959).

(5) Patten, F., and Gordy, W., *Proc. Natl. Acad. Sci., (U. S.)*, **46**, (No. 8) 1137(1960).

(6) Gordy, W., and Shields, H., *ibid.*, **46**, (No. 8) 1124 (1960).

(7) Lappin, G. R., and Clark, L. C., *Anal. Chem.*, **23**, 541 (1951).

(8) Ward, A. G., and Saunders, P. R., in "Rheology, Theory and Applications," vol. II, Eirich, F. R., ed., Academic Press Inc., New York, N. Y., 1958, Chap. 8, p. 319.

(9) Williams, J. W., Saunders, W. M., and Cicirelli, J. S., *J. Phys. Chem.*, **58**, 774(1954).