Effects of Ionizing Radiation on Two Gelatin Fractions I

Material Preparation, Dosimetry, and Acid-Base Behavior

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Two fractions, F-I and F-II, were isolated from commercial pigskin gelatin by low-temperature alcohol precipitation. Both fractions, in the form of 1% and 5% solutions and as films, were exposed to variable electron beam irradiation emanating from a 3 Mev Van de Graaff accelerator to give absorbed-dose values of from about 0.1 to about 3 Megarads. Radiation dose was determined by means of Ce(IV)-Ce(III) and polyvinylchloride dosimetry. Acid-base titration curves on the commercial gelatin sample, control fractions F-I and F-II, and irradiated F-I and F-II, as 1% and 5% solutions and as films, all showed differences in —COOH and —NH_2 content.

ASIDE FROM its application to sterilization techniques, the development of ionizing radiation as a research tool has facilitated a more profound understanding of the structure of complex proteins and polymers. Radiation caused by charged and uncharged particles and electromagnetic waves has been widely used by chemists to determine structural vulnerability to high-energy impingement through bond rupture, free radical formation, crosslinking, decarboxylation, and deamination.

Interaction of high-energy radiation with proteins is known to produce a variety of chemical and physical effects, depending on the system. Gelatin was chosen as the test material to study these effects. The wide pharmaceutical importance of gelatin is evidenced by its uninterrupted inclusion in the United States Pharmacopeia since the eighth decennial revision in 1905.

Despite intensive investigation in numerous laboratories, a full explanation of the peculiar behavior of gelatin under varying environment is still unavailable. Interpretative difficulties arise because the material known as "Gelatin" is a heterodisperse system, some fractions having molecular weights as high as 400,000. The distribution of smaller protein molecules depends upon the source and treatment of its precursor, collagen, as well as its subsequent refinement. In general, therefore, more meaningful experimental results are obtainable only after appropriate fractionation, especially when dealing with commercial gelatin.

It is not known to what extent hydrolysis of the protein may occur during separation, such as treatment with alcohol, nor is it known how radiation affects the isoelectric point of the protein. This necessitates the characterization of each fraction by the determination of the milliequivalents of acid and base bound per gram of protein as a function of pH. Hence, these determinations are also useful in comparing the behavior of gelatin fractions following exposure to high-energy radiation at identical levels. This is particularly true if any one or combinations of the following four modes of interaction are operative during irradiation: (*a*) end-group hydrolysis, (*b*) polymerization, (*c*) scission, and (*d*) crosslinking phenomena.

It was intended to establish by this investigation experimental procedures to study the effects of the exposure of gelatin to high-energy radiation. Since we chose to work with commercial pigskin gelatin, a modification of a known fractionation procedure was used to reduce heterogeneity. The modified procedure gave a relatively large molecular weight fraction and a smaller molecular weight fraction both having greater sensitivity and viscosity characteristics (1). There appeared to be no justification to include, in work of this scope, a study of radiation effects on each of several gelatin fractions.

The dosimetry was established using both cericcerous and polyvinylchloride techniques The radiation doses were limited to those producing visually nondiscernible denaturation of the gelatin since acid-base titrations or other physical measurements on solutions containing precipitated protein would not yield readily interpretable results.

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Of particular interest was the effect of ionizing radiation on gelatin in the presence and absence of water. For this reason, measurements of the two fractions were attempted on diluted solutions (1%), gels (5%), and solid state films.

EXPERIMENTAL

Material Preparation.—The method of Hultin, *et al.* (1), was modified as follows: Two hundred grams of purified pigskin gelatin (Eastman Kodak Lot No. 5247) were soaked in 4500 ml. distilled water overnight at 25°. The temperature was then increased to 42° whereupon the gelatin immediately dissolved. A sufficient volume of ethanol was then added to bring the volume to 52% v/v, and the mixture was allowed to stand for 24 hours at room temperature. Alcohol was again added to bring the volume to 61%. After 24 hours standing at room temperature, the mixture was halved in volume. The alcohol content of the first half was increased to 72%. After 24 hours, the temperature of the mixture was reduced to −2° and the alcohol content increased to 76%. The mixture was allowed to stand for 72 hours. Ammonium hydroxide was added dropwise to an apparent pH 10, whereupon a precipitate formed which was allowed to settle overnight to permit decantation of the supernate the following morning, leaving Fraction I (F-I).

The second half of the separated volume was allowed to age 4 days at room temperature. The alcohol concentration was then increased to 78%, the temperature reduced to −2°, and ammonium hydroxide added to an apparent pH of 10. A white precipitate formed which was allowed to settle overnight. The supernate was decanted, leaving Fraction II (F-II).

Both fractions (F-I and F-II) were dried under vacuum at room temperature, dialyzed against water and aliquotes lyophilized. Freeze-drying *in vacuo* of F-I produced a dense, hard, white mass which was reduced to granular form. F-II, also white, was characteristically different in texture, less dense, and softer than F-I. Both fractions were stored over Drierite. Yields were 33.2% and 22.4% of F-I and F-II, respectively.

Dosimetry.—Initial experiments on solid-gelatin films and aqueous systems contained in glass were conducted at relatively high (2.5–25 Megarads) ranges on a 6-Mev linear accelerator. All samples suffered degradation, as evidenced by gas evolution and various states of separation from turbidity to heavy precipitation. According to Weiss (2), the radial dimension of glass vessels is critical because of secondary electrons emanating from the vessel wall. As an alternative, polyethylene containers were tried and found to be more satisfactory than glass but precluded irradiating oxygen-free solutions.

Successful irradiation was achieved by exposing the aqueous gelatin systems in 4.5 in. x 6.5 in. heat-sealed polyethylene containers, 3.5 mils average thickness, under the scanned beam of the 3-Mev Van de Graaff accelerator for smaller dosages. Preliminary experimentation on 1% solutions and 5% gels of F-I and F-II indicated upper tolerances of energy at about 2.5–3.0 Megarads for F-I and about 2.0 Megarads for F-II to avoid visible denaturation. When these dose limits for F-I and F-II were exceeded, both the 1% solutions and the 5% gels showed evidence of cloudiness. The 5% gel also became extremely viscous. The degree of apparent denaturation appeared to be proportional to the excessive dose. Therefore, in order to remain below the irradiation dose limits, the Van de Graaff input microamperage was arbitrarily set at 50, 150, 450, and 1000 μ amps. at constant belt speed of 70 in./-min. to give four dose levels with the maximum at about 3 Megarads. (See Table I.)

TABLE I.—COMPARISON OF PVC AND CERIC DOSIMETRY

	Dose (Mrads.) per Individual Run		Average Dose, Mrads.	
	PVC	*Ceric*	*PVC*	*Ceric*
50 μ amps.	0.1	0.1704		
	0.1	0.1588		
	0.1	0.1745	0.12	0.16
	0.1	0.1110		
	0.2	0.2080		
150 μ amps.	0.3	0.4364		
	0.4	0.4709		
	0.3	0.3984	0.38	0.45
	0.4	0.4440		
	0.5	0.5337		
450 μ amps.	1.0	1.2710		
	1.0	1.1890		
	1.0	1.3570	1.10	1.31
	1.2	1.3240		
	1.3	1.4400		
10^3 μ amps.	2.8	2.803		
	2.1	2.558		
	2.5	3.020	2.60	2.87
	2.8	2.949		
	2.9	3.020		

Exposure to the electron beam was carried out by placing one sealed polyethylene unit containing 20 ml. of a 1% solution, a second unit containing 20 ml. of a 5% solution and two film strips of a given fraction on 9 in. x 15 in. aluminum trays provided with $^3/_4$ in. wood backing to minimize back scattering. All physical measurements were made on freshly irradiated gelatin solutions and films.

In addition to the gelatin solutions and films to be irradiated, each tray included a polyethylene unit containing 20 ml. of freshly prepared 0.015 *M* ceric ammonium sulfate in 0.8 *N* sulfuric acid (in triple distilled water) for precise dosimetry calculations and one rigid polyvinylchloride (PVC) slide for additional dosage approximation. Weiss (2) found that ceric dosimetry is very reliable when the total absorbed dose is in the order of 6 million rep. Subsequently Harlan, *et al.* (3), established the reliability of ceric dosimetry for high level irradiation measurements up to 10^8 rads. In our hands the ceric system was accurate and reproducible within the dose range applied in the experiments.

Immediately prior to irradiation a 0.005 *M* standard ferrous ammonium sulfate solution was prepared. This was used to standardize the nonirradiated ceric ammonium sulfate and to back-titrate the irradiated volumes, using 1,10-phenanthroline as the end-point indicator. Irradiation runs were completed on the same day and titrations were made immediately following exposure to minimize error due to air-induced oxidation. From the original concentration data and the back-titration, the number of micro-

moles of Ce(IV) reduced (or Ce(III) produced) was calculated. The total absorbed dose, E_t in units of *ev/L.*, was determined from ceric dosimetry through the following (3, 4)

$$E_t = (6.02 \times 10^{17}) \times (\mu \text{ moles Ce(IV) reduced}) \times \left[\frac{1000}{\text{Vol. used}}\right] \times \left[\frac{100}{2.5}\right]$$

and converted to Megarads using the relationship

$$6.37 \times 10^{22} \text{ ev/L.} = 1 \text{ Megarad.}$$

Rigid polyvinylchloride (PVC) dosimetry, as described by Artandi, *et al.* (5), was used concurrently with the ceric indicator. Although the sensitivity of PVC at low levels does not approach that of the ceric-cerous system, its application in the present work as an approximation for absorbed energy proved highly desirable. Table I shows PVC and ceric determinations on a series of five irradiation runs. These data were analyzed and the best fitting equation was calculated to be

$$Y = 0.9259X - 0.0575, 0.11 \leq X \leq 3.02$$

where, Y = PVC reading and X = ceric reading. The above equation is valid only over the stated range of X. The degree of fit, or how well the Y values depended on the X values, was 98.5%, which is considered good.

Acid-Base Behavior.—All titrations were conducted under a nitrogen atmosphere on 10-ml. solutions which were 1% with respect to the gelatin concentration. Several drops of octyl alcohol were added as an antifoaming agent. Titrant volumes of 0.1 *N* sodium hydroxide and 0.1 *N* hydrochloric acid were measured with a model SB2 syringe microburet. The pH values were measured with a Beckman model G pH meter employing a glass and saturated calomel electrode system.

Titrations were performed on the commercial gelatin, irradiated and unirradiated water controls, and fractions F-I and F-II, the latter two as (*a*) controls, (*b*) irradiated 1% solutions, (*c*) irradiated 5% gels, and (*d*) irradiated films. Titration volumes were corrected by subtracting the appropriate water controls. The titration curves were obtained by plotting the observed pH (abscissa) against the milliequivalents of acid or base bound per gram of protein. Gelatin concentrations for this purpose were obtained by dry weight.

In order to provide comparison among the three forms of each fraction, as well as between the two fractions, four-level irradiation was conducted on all forms of both fractions simultaneously. The average dose imparted to each of four separate passes under the electron beam was calculated to be: (*a*) 0.37, (*b*) 0.84, (*c*) 2.00, and (*d*) 3.00 Megarads, respectively.

RESULTS AND DISCUSSION

Unirradiated F-I and F-II titration curves, although nearly identical and characterized by equilibrium pH 7.0, represented a distinct shift from their parent source which showed a pH of 4.8 at equilibrium. (See Fig. 1.) Irradiated F-I and F-II titration curves showed nearly complete superimposability for a given form at various irradiation levels. Thus F-I, irradiated as the 1% solution,

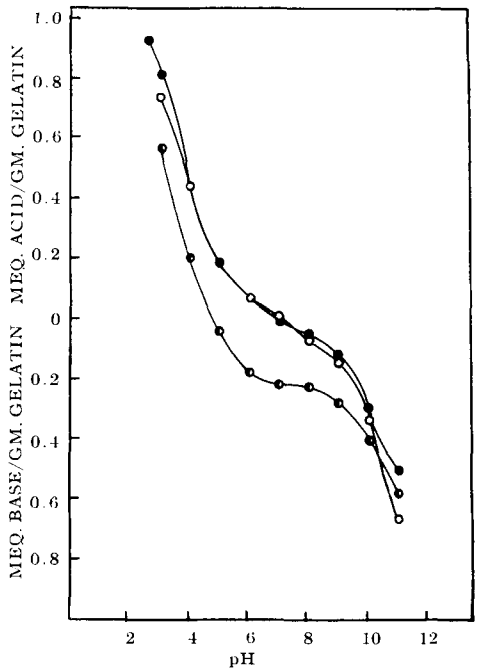

Fig. 1.—Titration curves of controls in the form of unirradiated F-I (○), F-II (●), and commercial gelatin (◐).

showed four nearly identical curves at 0.37; 0.84; 2.00; and 3.00 Mrads. The same pattern prevailed in the remaining five forms and was especially pronounced in the case of irradiated films, with slight departures at the extreme ends of the curves.

Comparison of F-I and F-II at any given radiation level with respect to a particular form, *e.g.*, 1% solution, showed differences in structure. Figure 2 is illustrative. The 1% solutions irradiated at 0.37 Mrad. appeared to be similar at either end of the titration range, but in the region from pH 4.0 to 10.0 more acid than base volumes were required to bind F-I than F-II. Values fell at pH 5.5 and pH 7.0, respectively. At the 0.847 Mrad. level, solutions of both fractions at the same concentration showed similar patterns in the extreme acid and base regions. Deviation in milliequivalents of acid/base bound per gram of protein began at pH 3.0, increased gradually to a maximum at pH 6.0–8.0, then returned slowly to near similarity. Although the equilibrium pH of F-I did not shift (5.5) despite the twofold increase in absorbed dose, a slight increase appeared in F-II (pH 7.2).

By increasing the irradiation dose to 2.0 Mrads., titration curves on 1% solutions of F-I and F-II continued to show the inversely proportional relationship between dose and milliequivalents of acid/base bound in the midportion of the curve. When the irradiation dose was further increased to 3.0 Mrads., curves of F-I and F-II approached superimposability, with only a small difference in milliequivalent values in the midportion.

Plots of F-I and F-II 5% solutions differed from their 1% counterparts along the entire titration curve. A consistently gradual approach toward pH 7.0 in the four cases studied from either extreme was apparent. Figure 3 is illustrative of this series. More milliequivalents of acid were required to bind

1 Gm. of F-I than F-II. This differential was gradually reduced until pH 7.0 where no difference existed and the curves crossed. F-II showed progressively greater milliequivalent requirements and increasing departure from F-I as the extreme alkaline end of the curve was approached.

Films of F-I and F-II, at all four irradiation levels studied, showed remarkably consistent similarity in titration curve behavior. Figure 4 shows a representative curve of a film of F-I irradiated at 0.37 Mrad. in this series. Slight variations (*ca.* 0.02 milliequivalents) among the various plots in isolated sections of the curves were not considered critical, all eight plots being substantially similar within limits of experimental error. Equilibrium values for films of F-I and F-II at all four irradiation doses consistently fell at pH 7.0.

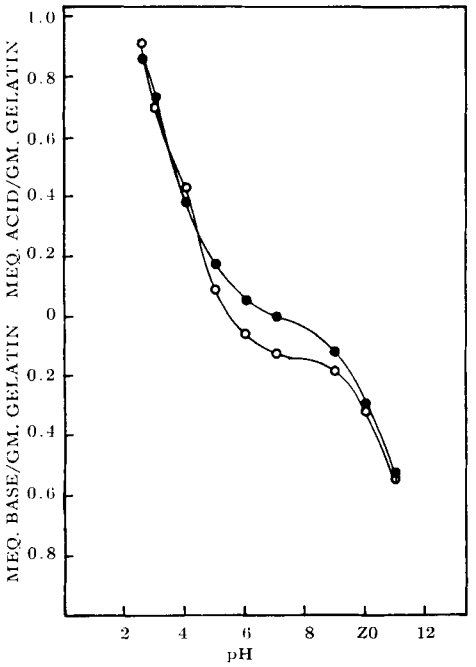

Fig. 2.—Titration curves of F-I (○) and F-II (●) after 1% dispersions of each were subjected to irradiation dose 0.37 of Mrads.

CONCLUSIONS

Treatment of commercial gelatin (pH 4.9) with alcohol under low-temperature conditions and adjustment of pH to a value on the alkaline side appears to disrupt the charge on the parent gelatin molecule, reducing its acid/base binding capacity and shifting the pH to neutrality. In changing from one form to another, there is a loss of amide nitrogen in the form of ammonia, resulting in an increase in the number of free carboxyl groups and base-fixing capacity (6). Further, the shift in position of the titration curve can be attributed to the liberation of carboxyl groups concurrent with release of amine ammonia. The shift is the same regardless of molecular weight

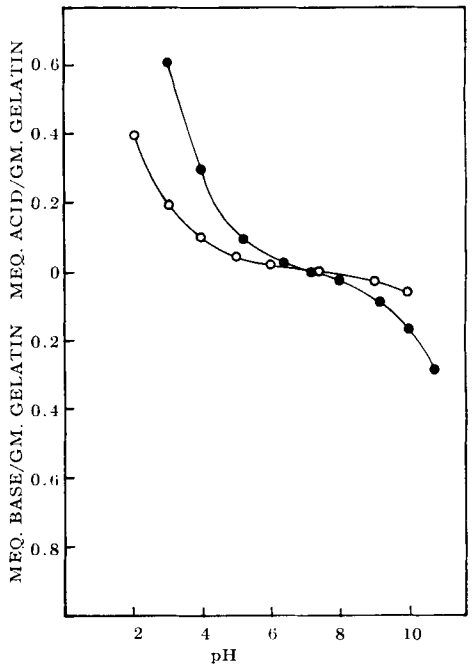

Fig. 3.—Titration curves of F-I (○) and F-II (●) after 5% dispersions of each were subjected to irradiation dose of 20 Mrads.

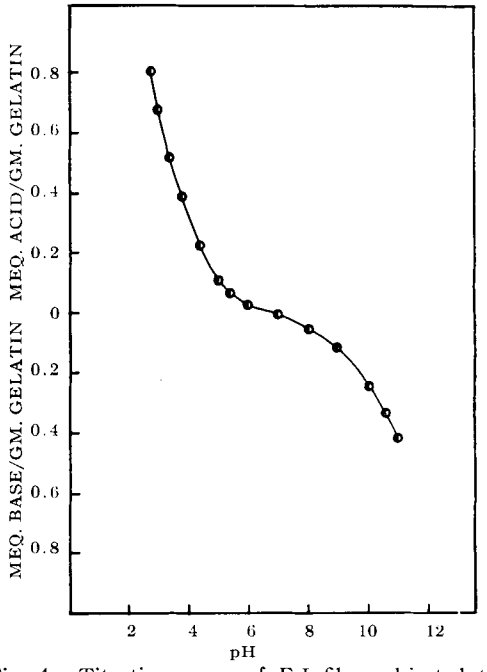

Fig. 4.—Titration curve of F-I film subjected to irradiation dose of 0.37 Mrads.

characteristics of the fractionated components.

Irradiation at similar levels of the two gelatin fractions as 1% solutions does not materially affect either their acid-binding or base-binding capacity at the distal ends of the titration curves.

In the region of neutrality, and 2 pH units on either side of it, a substantial variance occurs at the lowest irradiation level which gradually lessens as the dose is increased. To this portion of the curve are attributable the imidazole groups of the protein. It is evident that the low molecular weight fraction (F-II) requires acid/-base milliequivalents of constant value in this region whereas the high molecular weight fraction initially (0.37 Megarads) binds 0.12 milliequivalents of base; this requirement gradually diminishes as the dose of ionizing radiation is increased.

In 1:20 solute:solvent ratio during the ionization process, a given gelatin fraction shows entirely different charge structure from that when irradiation occurs at 1:100 ratio. The decrease in volume requirements of acid and base indicates a reduced availability of titratable amino and carboxyl groups.

In subsequent reports we shall present the results of further studies showing experimental evidence supporting our belief that the two fractions not only differed from each other but behaved differently under changing irradiation stress.

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Kinetics of Steroid Effects on Ca^{47} Dynamics in Dogs with the Analog Computer I

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Intravenous administration of the short-lived isotope Ca^{47} in dogs was followed by the measurement of Ca^{47} in blood, feces, and urine during a control phase, a steroid regimen (2 mg./Kg./day), and a final recovery, post-steroid phase. Methods and procedures were developed to analyze the data by a combination of digital and analog computer techniques. In all dogs and in all phases the specific rate constants of a polyexponential fit for the distribution of Ca^{47} in all hypothetical compartments under all the experimental conditions studied were similar. The cumulative fecal and urinary Ca^{47} eliminations were fitted to a first order curve as a function of time by the analog computer. The most striking effect of steroid therapy in the young dog used as his own control is the increase in first order Ca^{47} elimination within the first 200 hours to $1\frac{1}{2}$ to 2 times the control values. After cessation of steroid therapy, the per cent of the total intravenous dose eliminated decreased and approached the amount of the control period within these 200 hours. The residual Ca^{47} is tied up in the bone and is slowly released at an apparently linear rate over longer periods of time. The rate of release between 200 and 700 hours is twice as great with steroid regimen as without for both control and recovery phases.

RADIOCALCIUM and other bone-seeking isotopes have been used in kinetic analyses of changes in blood and excretion of injected radioisotope (1–4). The radioisotope, Ca^{47}, has recently become available and presents certain advantages over previous radiocalcium studies in that it is a gamma emitter of short half-life of 4.56 days and an animal can be used as his own control in short-term sequential studies of the effect of a therapeutic agent or experimental condition on the kinetics of radiocalcium distribution, metabolism, and excretion.

The advantages of this isotope have permitted us to initiate an investigation of the osteoporotic effects of adrenal steroid regimen and its potential reversibility. Preliminary studies have demonstrated that this phenomenon is common to all the adrenal steroids in varying degree (5, 6) but one was chosen for the detailed study presented in this paper.

This paper presents a detailed quantitative analysis of our first studies on the effects of adrenal steroids on the distribution and kinetics of intravenously administered Ca^{47}. The digital- and analog-computer techniques described herein greatly facilitated the quantification of the huge masses of data it was necessary to obtain in such studies.

Special emphasis was also placed on the initial disappearance curves of radiocalcium from the blood, studies of which have not received too much attention previously (7).

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