The Effect of γ -Irradiation on Soluble Collagen

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1. A study was made of the effect of γ -irradiation on the sub-unit composition, as well as the conformational changes taking place in cooled solutions of thermally denatured neutral-salt-soluble and acid-soluble collagen. 2. The increase in negative rotation and viscosity at 15° for irradiated and thermally denatured collagen solutions becomes less as the irradiation dose is increased. 3. The initial effect of γ -irradiation is the depolymerization of the dimers found in both neutral-salt-soluble and acid-soluble collagen. 4. The principal effect of γ -irradiation up to 10 Mrads is the fission of peptide bonds, yielding crystalline irradiation-resistant portions of the molecule incapable of associating to the native structure. 5. The effects of γ -irradiation on both neutral-salt-soluble and acid-soluble collagen are very similar and bear a close resemblance to the effects induced by ultraviolet irradiation.

Some physicochemical effects of γ -irradiation on collagen have been reported. Bowes & Moss (1962) showed a decrease in the tensile strength and shrinkage temperature of irradiated ox hide at 5 and 50 Mrad doses. They also reported a limited destruction of acidic and basic amino acids, as well as those having a ring structure. Relatively little hydrolytic scission of peptide bonds was indicated from terminal-group analysis. Ramanathan, Mohanaradhakrishnan & Nayudamma (1965) reported similar decreases in tensile strength and shrinkage temperatures for irradiated tail-tendon collagens at 10 and 30 Mrad doses, as well as modified electron micrographs, X-ray-diffraction patterns and optical birefringence. Cassel (1959) irradiated tail-tendon collagen (5–220 Mrad doses) and found little damage to amino acid residues at doses less than 20 Mrads. Methionine, phenylalanine and threonine were shown to be most labile at higher irradiation doses, and alanine, glycine, hydroxyproline, proline and arginine were least affected. Strakhov & Shifrin (1964) reported that at 10^4 – 10^5 rads of γ -irradiation cross-linking of collagen molecules occurs, whereas at irradiation doses above 10^7 rads the destructive effect predominates.

When a solution is irradiated, the principal effect is to dissociate the solvent into free radicals, which may then attack the solute (the 'indirect effect'). Previous studies have concerned the irradiation of proteins in aqueous solution, when the 'indirect effects' of irradiation are important. The present work concerns two fractions of collagen prepared

from the skin of a newborn calf. Both fractions were freeze-dried and exposed to γ -irradiation in the absence of oxygen. After irradiation the fractions were dissolved in buffer and then thermally denatured before further physicochemical study.

MATERIALS AND METHODS

Preparation of soluble collagen. Neutral-salt-soluble and acid-soluble collagen were prepared from the skin of a newborn calf by the method of Piez, Eigner & Lewis (1963). Purification by phosphate reprecipitation (Gross, 1958) was carried out once in each case.

γ -Irradiation. Freeze-dried samples of neutral-salt-soluble and acid-soluble collagen were dried under vacuum over P_2O_5 for 24 hr., sealed under vacuum into glass tubes and irradiated at 22° from a 300–400°C ^{60}Co source. Irradiation was limited to 10 Mrads.

Column chromatography. The method was similar to that of Piez *et al.* (1963), with the same stock acetate buffer, pH 4.8 and *I* 0.50. For the initial linear gradient elution, 500 ml. of starting buffer, *I* 0.06, and 500 ml. of limiting buffer, *I* 0.16, prepared from the stock buffer, were placed in the two flasks of the linear-gradient elution system. After elution with 900 ml. of buffer from this gradient device, a change was made to a buffer of higher ionic strength, *I* 0.26. This buffer was prepared from the starting buffer by the addition of NaCl. The column (2.6 cm. $\times$ 33 cm.) was packed with CM-cellulose (lot no. 4146; BioRad Laboratories, Richmond, Calif., U.S.A.), which was regenerated between runs by the method of Piez *et al.* (1963). The column was kept at 40°. The column effluent was continuously monitored with a Beckman model 130 Spectrochrom Analyzer. The extinction of the effluent was monitored at 220, 230 and 278 m μ , and at two optical path lengths (2.5 mm. and 10 mm.). Solutions of irradiated

collagen were prepared in *I* 0.06 buffer (2mg./ml.) and denatured at 45° for 30min. Solutions containing 50–70mg. of denatured collagen were run on to the column and eluted at a flow rate of 100ml./hr. The Spectrochrom Analyzer also gave continuous recordings of the pH and conductivity of the column effluent, thus enabling a check to be kept on the consistency of the buffer gradient.

Ultracentrifugation. Denatured soluble collagen was examined by sedimentation velocity at 35° by using schlieren optics in a Spinco model E ultracentrifuge. Irradiated collagen was dissolved to give 0.4% (w/v) solutions in sodium formate buffer, pH 3.75 and *I* 0.15 (Piez *et al.* 1963). These were denatured by heating at 40° for 30 min. just before they were placed in the ultracentrifuge cell. Native collagen was treated in the same way. The patterns reproduced in Fig. 4 were taken at 80 and 120 min. after reaching a speed of 56100 rev./min.

Mutarotation at 15°. The procedure for following helix formation of irradiated collagen in 0.15M-potassium acetate buffer, pH 4.8, with viscometry and polarimetry techniques has been described previously (Davidson & Cooper, 1967a).

Amino acid analysis. Amino acid composition was determined with a Spinco Amino Acid Analyzer after hydrolysis with 6N-HCl under reflux for 24 hr.

RESULTS

Chromatography on CM-cellulose. The chromatography of non-irradiated thermally denatured acid-soluble collagen (Fig. 1) may be compared with similar findings where different methods were used (Davidson & Cooper, 1967a,b). The present study with the Spectrochrom Analyzer has resulted in improved resolution over that obtained by the method of Piez *et al.* (1963). The reasons for this are that a more exhaustive gradient involving the use of a buffer change to a higher ionic strength (*I* 0.26) was used, and that the micro-cuvette of the Spectrochrom Analyzer has a much smaller volume (0.05 ml.) compared with about 1 ml. for most flow cells with a path length of 10 mm. Component 4 probably corresponds to the α_3 -sub-unit reported by several authors (see, e.g., Piez, 1964). The large component 5 was in some cases resolved into several peaks and it is concluded that this component consists of the dimeric sub-units $\alpha_1\alpha_1$, $\alpha_2\alpha_2$, $\beta_{11}\beta_{11}$ and $\beta_{12}\beta_{12}$ described previously (Davidson & Cooper, 1967b). The resolution of the minor components 1, 2, 3, 6, 7 and 8 has not been reported in earlier findings, although components 1, 2 and 3 may be the same as the E, F and G components separated by a different chromatography procedure (Davidson & Cooper, 1967b). Since CM-cellulose is a weak cation-exchanger, the acidic nature of components 1, 2 and 3 and the basic nature of components 6, 7 and 8, together with their relatively high extinction at 278m μ (Fig. 1), due to the presence of aromatic constituents, suggest that these components are telopeptide residues. Further evidence on the nature of these components has been

Fig. 1. Elution pattern of approx. 50 mg. of acid-soluble calf-skin collagen on CM-cellulose at 40° after denaturation at 45° for 30 min. The column was eluted with a linear salt gradient with 500 ml. of *I* 0.06 acetate buffer and 500 ml. of *I* 0.16 acetate buffer followed by a change to *I* 0.26 acetate buffer.

Fig. 2. Elution patterns of approx. 50 mg. of calf-skin collagen irradiated with 1.3 Mrad γ -irradiation: —, acid-soluble collagen; ----, neutral-salt-soluble collagen. The column was eluted with a linear salt gradient with 500 ml. of *I* 0.06 acetate buffer and 500 ml. of *I* 0.16 acetate buffer followed by a change to *I* 0.26 acetate buffer.

Fig. 3. Elution patterns of approx. 70 mg. of calf-skin collagen irradiated with 10 Mrad γ -irradiation: —, acid-soluble collagen; ----, neutral-salt-soluble collagen. The column was eluted with a linear salt gradient with 500 ml. of *I* 0.06 acetate buffer and 500 ml. of *I* 0.16 acetate buffer followed by a change to *I* 0.26 acetate buffer.

reported elsewhere (Cooper & Davidson, 1968). The chromatography of non-irradiated thermally denatured neutral-salt-soluble collagen gave a similar elution pattern to that of acid-soluble collagen, except for a slightly smaller peak containing the $\alpha_1\alpha_1$, $\alpha_2\alpha_2$, $\beta_{11}\beta_{11}$ and $\beta_{12}\beta_{12}$ dimers.

The chromatography of γ -irradiated neutral-salt-soluble and acid-soluble collagen at 1.3, 2.6, 5 and 10 Mrads showed the early breakdown at low doses of irradiation of the chromatographic component containing the dimeric sub-units, resulting in the elution of degraded material just in front of the α_1 position. The chromatographic separations obtained after irradiation doses of 1.3 and 10 Mrads are shown in Figs. 2 and 3; the separations after doses of 2.6 and 5 Mrads represented intermediate stages in the degradation. The relatively high extinction of this degraded material at 278 m μ (Figs. 2 and 3) was noted in all cases.

Ultracentrifugation. The ultracentrifugation of thermally denatured neutral-salt-soluble and acid-

soluble collagen both resulted in the separation of the α - and the β -sub-units with $S_{20,w}$ 2.6 and 3.4 s respectively (Piez *et al.* 1963). The ultracentrifugation of thermally denatured neutral-salt-soluble collagen that had initially been irradiated at 5 and 10 Mrads in an anhydrous condition resulted in single heterodisperse peaks with $S_{20,w}$ 1.86 and 1.71 s respectively. Thermally denatured acid-soluble collagen irradiated at 5 and 10 Mrads resulted in similar peaks with $S_{20,w}$ 1.85 and 1.81 s respectively (Fig. 4).

Mutarotation at 15°. Solutions of irradiated and non-irradiated neutral-salt-soluble and acid-soluble collagen (1.3–1.4 mg./ml.) were denatured at 45° for 15 min. and the recovery of helix formation was monitored by optical-rotation measurements at 15°. The effects of 1.3, 2.6, 5 and 10 Mrad doses of γ -irradiation were studied in this way.

It is evident from the irradiation data (Table 1) that both the initial rates of mutarotation as well as the actual recoveries after 48 hr. for neutral-salt-soluble and acid-soluble collagen are very similar. The effects of γ -irradiation on the mutarotation of

Fig. 4. Sedimentation patterns of: (a) thermally denatured acid-soluble collagen; (b) thermally denatured acid-soluble collagen + 5 Mrads; (c) thermally denatured acid-soluble collagen + 10 Mrads; (d) thermally denatured neutral-salt-soluble collagen; (e) thermally denatured neutral-salt-soluble collagen + 5 Mrads; (f) thermally denatured neutral-salt-soluble collagen + 10 Mrads. Neutral-salt-soluble and acid-soluble collagen were irradiated from a ^{60}Co source in an anhydrous condition in the absence of oxygen, before being dissolved in sodium formate buffer and denatured at 40° for 30 min. The photographs were taken after 80 and 120 min. at 56100 rev./min. and 35°. Sedimentation is from left to right.

Table 1. Rate of collagen-fold formation of acid-soluble collagen after γ -irradiation and cooling to 15°

Solutions of irradiated collagen were thermally denatured at 45° for 15 min. before being cooled to 15°.

Irradiation dose (Mrads)	Initial rate of mutarotation ($(d[\alpha])/dt$) ₀ at 365 m μ and 15° (°/min.)		Specific rotation ($([\alpha])_{365}^{15}$) after 48 hr. at 15°	
	Neutral-salt-soluble collagen	Acid-soluble collagen	Neutral-salt-soluble collagen	Acid-soluble collagen
0	6.5	7.0	−974°	−1045°
1.3	2.8	5.4	−944	−950
2.6	2.1	4.9	−905	−927
5.0	1.2	3.1	−832	−827
10.0	0.9	1.4	−745	−783

Irradiation dose (Mrads)	10 ^{−2} × Initial rate of recovery of reduced viscosity ($(d\eta_{red.})/dt$) ₀ (dl./g. min.)		Reduced viscosity after 48 hr. at 15° (dl./g.)	
	Neutral-salt-soluble collagen	Acid-soluble collagen	Neutral-salt-soluble collagen	Acid-soluble collagen
0	2.5	2.0	5.68	6.76
1.3	1.0	0.9	3.98	4.08
2.6	0.9	0.8	3.27	3.59
5.0	0.3	0.3	1.49	1.65
10.0	0.2	0.2	0.86	1.04

Fig. 5. Specific rotation ($[\alpha]_{366}^{15}$) of γ -irradiated acid-soluble collagen as a function of time. Solutions (1.3mg./ml.) were thermally denatured at 45° and then kept at 15°. ●, Not irradiated; ■, 1.3 Mrads; ○, 2.6 Mrads; ▲, 5 Mrads; □, 10 Mrads.

Fig. 6. Reduced viscosity ($\eta_{red.}$) of γ -irradiated acid-soluble collagen as a function of time. Solutions (1.3mg./ml.) were thermally denatured at 45° and then kept at 15°. ●, Not irradiated; ■, 1.3 Mrads; ○, 2.6 Mrads; ▲, 5 Mrads; □, 10 Mrads.

acid-soluble collagen are illustrated in Fig. 5. These results show that γ -irradiation decreases the initial rate of mutarotation and the amount of helix formation in the partial re-formation on cooling of the collagen fold or some modification of this structure (Harrington & Hippel, 1961; Veis, 1964).

A parallel study was also made of the change in reduced viscosity at 15° (Table 1). Again both neutral-salt-soluble and acid-soluble collagen gave very similar results. The effects of γ -irradiation on the recovery of reduced viscosity for acid-soluble collagen are illustrated in Fig. 6. This caused a decrease in the initial rate of viscosity recovery ($\eta_{red.}/dt$)₀, and the amount of chain association occurring parallels the regain in helical content.

Fig. 7 illustrates the relationship between reduced viscosity on cooling for 48 hr. at 15° and the irradiation dose. This relationship is thus a measure of aggregation or interchain association (Harrington & Hippel, 1961) that is directly related

Fig. 7. Specific rotation (○) and reduced viscosity (●) as functions of γ -irradiation dose for irradiated acid-soluble collagen. Solutions (1.3mg./ml.) were thermally denatured at 45° and then kept at 15° for 48 hr.

Fig. 8. Plots of $\log(d\eta_{red.}/dt)$ for solutions of γ -irradiated acid-soluble collagen as a function of time at 15°. Solutions (1.3mg./ml.) were thermally denatured at 45°. ●, Not irradiated; ■, 1.3 Mrads; ○, 2.6 Mrads; ▲, 5 Mrads; □, 10 Mrads.

to irradiation damage. The main effect of irradiation on viscosity recovery appears to be almost complete at a dose of 5 Mrads, indicating that the first breaks in the polypeptide are more effective than subsequent breaks in decreasing sub-unit chain association. The almost linear relationship between specific rotation and the irradiation dose is also illustrated in Fig. 7.

To investigate the order of reaction for the recovery of optical rotation and reduced viscosity at 15°, with and without irradiation, $\log(d\eta_{red.}/dt)$ and $\log(d[\alpha]/dt)$ were plotted against time. Fig. 8 illustrates a typical relationship indicating that the coil-to-helix reaction is not a first-order reaction (Piez & Carillo, 1964; Harrington & Hippel, 1961).

Table 2. *Amino acid composition of γ -irradiated soluble collagen*

Irradiation dose ... Amino acid	Amino acid composition (g./100g. of protein)			
	Neutral-salt-soluble collagen		Acid-soluble collagen	
	5 Mrads	10 Mrads	5 Mrads	10 Mrads
Ala	8.15	7.86	8.42	8.62
Arg	7.38	7.26	7.58	7.70
Asp	6.41	6.95	6.50	6.33
Glu	9.64	10.39	9.64	9.69
Gly	18.89	18.02	19.76	20.29
His	0.59	0.75	0.57	0.56
Hyl	0.84	0.78	0.89	0.58
Hyp	11.59	10.52	11.61	11.80
Ile	1.59	1.89	1.57	1.62
Leu	2.73	3.21	2.72	2.82
Lys	3.26	3.71	3.23	3.21
Met	0.54	0.71	0.42	0.78
Phe	1.76	1.95	1.78	1.81
Pro	11.21	10.52	11.57	11.89
Ser	3.37	3.52	3.39	3.48
Thr	1.86	2.04	1.77	1.81
Tyr	0.49	0.72	0.47	0.48
Val	2.08	2.34	2.09	2.13
Total	93.16	94.05	94.82	96.44

It is apparent that the present study bears a close resemblance to previous studies with ultraviolet irradiation (Cooper & Davidson, 1966; Davidson & Cooper, 1967a).

Amino acid analysis. In Table 2 the amino acid analyses of irradiated neutral-salt-soluble and acid-soluble collagen are given. Comparison of these values with those reported for non-irradiated neutral-salt-soluble and acid-soluble collagen (Cooper & Davidson, 1965) shows that little if any destruction of amino acids occurs at the prescribed irradiation doses.

The published data on the amino acid analysis of various collagen preparations from skin (see, e.g., Tristram & Smith, 1963) show quite wide variations in the content of hydroxyproline and tyrosine, due probably to variable contamination with non-collagenous proteins. The neutral-salt-soluble collagen used in the present study did have a relatively low hydroxyproline and high tyrosine content, which indicates the presence of non-collagenous impurities. The influence of this tyrosine-containing impurity on the γ -irradiation is at present not known.

DISCUSSION

The action of γ -irradiation on both neutral-salt-soluble and acid-soluble collagen in the dry state causes significant changes in their sub-unit com-

position, even at relatively low doses. The resultant elution of these degraded components near the void volume of the chromatography column indicates either that low-molecular-weight components have been liberated by the irradiation, or that changes in the cationic character of particular components have taken place. The sedimentation studies show that degradation of the original collagen occurred on irradiation, resulting in material with a range of molecular weights generally lower than that of the α -sub-units. The absence of the β -peak indicated by ultracentrifugation of irradiated collagen suggests that depolymerization of the major $\alpha_1\alpha_1$ and $\alpha_2\alpha_2$ dimers (component 5) takes place. As the collagen was irradiated in the dry state, the indirect action of free-radical formation from any solvent cannot be implicated in the degradation observed.

It is possible that the telopeptides, which are considered to be involved in both inter- and intramolecular bonding (Drake, Davison, Bump & Schmitt, 1966), and are susceptible to proteolytic enzymes, are likewise susceptible to γ -irradiation. This could account for the ready decomposition of the polymeric forms of collagen, shown by chromatography and ultracentrifugation, even at relatively low doses of irradiation.

The chromatography and sedimentation data discussed above show that γ -irradiation doses in the range 1.3–10 Mrads bring about considerable changes in the sub-unit composition of both types of soluble collagen. Yet the mutarotation data show that even after 10 Mrads of irradiation the collagen still undergoes considerable mutarotation to give relatively high specific rotations. Thus after thermal denaturation and storage for 48 hr. at 15° non-irradiated acid-soluble collagen has specific rotations $[\alpha]_{365}^{15} - 1045^\circ$ and $[\alpha]_{D}^{15} - 324^\circ$, whereas acid-soluble collagen after 10 Mrads of irradiation has specific rotations $[\alpha]_{365}^{15} - 785^\circ$ and $[\alpha]_{D}^{15} - 251^\circ$ under the same conditions of mutarotation. The latter values are very much higher than the mean residue rotation of collagen in the denatured form ($[\alpha]_{365}^{15}$ approx. -450° , $[\alpha]_{D}^{15}$ approx. -90° to -120°), and show about 75% recovery in optical rotation when compared with non-irradiated collagen. At the lowest dose of irradiation used here, the chromatography data showed a considerable change in the sub-unit structure, but the mutarotation studies reveal that the collagen still re-forms the helical structure to a large extent, as assessed from the high specific rotation ($[\alpha]_{365}^{15} - 952^\circ$, $[\alpha]_{D}^{15} - 315^\circ$) after irradiation and mutarotation at 15° for 48 hr. This corresponds to a 95% recovery in helical content.

In contrast with the above findings, the recovery of reduced viscosity after γ -irradiation, thermal denaturation and storage at 15° for 48 hr. is relatively low. As shown by the present results,

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after 10 Mrads of irradiation the viscosity increases by only a very small amount after 48 hr. at 15°. It can therefore be concluded that even after 10 Mrads of irradiation, followed by thermal denaturation in solution, collagen still re-forms into a largely helical structure, but does not have the rod-like rigidity characteristic of native tropocollagen. This mutarotation suggests that crystalline portions of the collagen triple helix are not degraded by γ -irradiation at the prescribed irradiation doses. This is also supported by evidence from sedimentation and amino acid analyses. The viscosity data indicate that these crystalline portions are no longer capable of association to give the native molecule.

It is concluded that, for neutral-salt-soluble and acid-soluble collagen in the dry state, peptide-bond fission occurs, without significant destruction of amino acids, during γ -irradiation at doses between 1.3 and 10 Mrads. The amount of peptide-bond fission is sufficient to lower the reduced viscosity of the irradiated collagen significantly, but does not decrease the helical content to the same degree. In view of the rapid depolymerization of the dimeric sub-units (component 5 of Fig. 2), it is suggested that the initial site of action of γ -irradiation is the telopeptide residues. These contain a high proportion of aromatic amino acids (Rubin, Pfahl, Speakman, Davison & Schmitt, 1963) and are considered to be responsible for the formation of these dimers (Davidson & Cooper, 1967b) and the β -sub-units (Drake *et al.* 1966). As the irradiation dose is increased peptide-bond fission is the next stage in the reaction. In many respects the action of γ -irradiation on soluble calf-skin collagen appears to be similar to that of ultraviolet irradiation (Cooper & Davidson, 1965, 1966; Davidson & Cooper, 1967a), although no actual degradation of tyrosine or phenylalanine was observed in the present study.

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