The Effect of Ultraviolet Irradiation on Acid-Soluble Collagen

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1. A study has been made of the effect of ultraviolet irradiation on the conformational changes taking place in cooled solutions of thermally denatured acid-soluble calf-skin 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 principal effect of ultraviolet irradiation is the fission of the primary collagen chains, eventually yielding chain lengths incapable of stabilizing a helical structure. 4. The effects of ultraviolet irradiation on acid-soluble collagen may be closely correlated with similar effects on neutral salt-soluble collagen.

The formation of collagen fibrils is thought to be due to the intermolecular interaction of the telopeptides protruding from the rigid parent molecule (Boedtker & Doty, 1956; Hodge & Schmitt, 1958; Schmitt *et al.* 1964). These telopeptides are thought to be rich in both tyrosine and phenylalanine (Schmitt *et al.* 1964; Hodge, Highberger, Deffner & Schmitt, 1960; Rubin, Pfahl, Speakman, Davison & Schmitt, 1963), and possibly play a vital role in such fibril formation. Since tyrosine and phenylalanine are both photosensitive to ultraviolet irradiation (McLaren, 1949; Setlow, 1957; McLaren & Luse, 1961; Luse & McLaren, 1963), a study of the photochemistry of collagen may well shed light on the role of these aromatic amino acids in fibril formation.

The effect of ultraviolet irradiation on collagen fibres or skin would indicate that cross-linking (Bottoms & Shuster, 1963) as well as peptide-bond fission (Ramanathan, 1962; Cooper & Davidson, 1965) may result. With soluble collagens, Fujimori (1965, 1966) has shown that photopolymerization may take place. The present study is confined to the effects of ultraviolet irradiation on the conformational changes taking place in cooled solutions of thermally denatured acid-soluble calf-skin collagen. The effects may be compared with a similar study carried out on neutral salt-soluble collagen (Cooper & Davidson, 1966).

MATERIALS AND METHODS

Preparation of acid-soluble collagen. Acid-soluble collagen was prepared from the skin of a 4-week-old bull calf by the method of Piez, Eigner & Lewis (1963). Purification by phosphate reprecipitation (Gross, 1958) was carried out once.

Ultraviolet irradiation. Unless otherwise stated, collagen solutions were prepared in 0.15 M-potassium acetate buffer, pH 4.8, which is known to have the minimum effect on mutarotation (Piez & Carrillo, 1964). These samples were irradiated at 0–4° and at a distance of 46 cm. from the source as described in a previous paper (Cooper & Davidson, 1966).

Column chromatography. The method was similar to that of Piez *et al.* (1963), the same stock acetate buffer, pH 4.8 and *I* 0.5, being used. For the 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. The column (2.5 cm. × 30 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 the above authors. The effluent was continuously monitored in a 1 cm. flow cell at 230 m μ by a Beckman model DB spectrophotometer and recorder. Collagen solutions were prepared and irradiated in *I* 0.06 buffer (2 mg./ml.) and denatured at 45° for 30 min. Solutions containing 40–50 mg. of denatured collagen were run on to the column and eluted at a flow rate of 120 ml./hr. by using a Beckman Accu-Flo pump. The column was kept at 40°. With the 6 and 17 hr. irradiations, gelling resulted; on heating at 45° a small insoluble precipitate formed, which was removed before chromatography.

Ultracentrifugation. Denatured acid-soluble collagen was examined by sedimentation velocity at 35° by schlieren optics in a Spinco model E ultracentrifuge. Collagen solutions were prepared and irradiated in 0.15 N-acetic acid, after which they were dialysed against 0.15 N-acetic acid, freeze-dried and then 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 being placed in the ultracentrifuge cell. Native collagen was treated in the same way. The patterns reproduced in Fig. 3 were taken at 80 and 120 min. after a speed of 56100 rev./min. had been reached. At irradiation times exceeding 2 hr., the formation of an insoluble gel at 40° made sedimentation studies impracticable.

Viscosity. Viscosities were measured at $15 \pm 0.05^\circ$ as described previously (Cooper & Davidson, 1965).

Optical rotation. Optical rotation was measured at $15 \pm 0.07^\circ$ in a 10cm. water-jacketed tube by a Perkin-Elmer model 141 polarimeter. Readings were taken at 589, 578, 546, 436 and 365 $m\mu$.

The procedure for following helix formation was to irradiate the collagen solutions for a given period at $0-4^\circ$, heat-denature at 45° for 15min. and then cool for 45sec. under tap water. A test sample was then immediately transferred to the viscometer or polarimeter tube kept at 15° , and readings were taken at regular intervals for periods up to 48hr.

RESULTS

Chromatography on CM-cellulose. Chromatography was carried out to study the effect of ultraviolet irradiation on the sub-unit composition of acid-soluble collagen. The chromatography of unirradiated thermally denatured acid-soluble collagen (Fig. 1) resulted in the recovery of approximately 21% α_1 , 5% β_{11} , 13% β_{12} and 61% α_2 . Davidson & Cooper (1967) have shown that the large amount of the α_2 sub-unit obtained by this method may be attributed to the presence of four additional components having similar elution characteristics to the α_2 sub-unit. These four additional components designated A, B, C and D have been correlated with the dimeric subunits $\alpha_2\alpha_2$, $\alpha_1\alpha_1$, $\beta_{11}\beta_{11}$ and $\beta_{12}\beta_{12}$ respectively.

The chromatography of acid-soluble collagen irradiated for 2, 6 and 17hr. (Fig. 2 and Table 1) indicates that the β_{12} and α_2 fractions together with components A, B, C and D are most labile towards ultraviolet irradiation, resulting in the elution of degraded material in the α_1 position.

Ultracentrifugation. The ultracentrifugation of thermally denatured acid-soluble collagen resulted in the separation of the α and β sub-units with

sedimentation coefficients $S_{20,w}$ 2.6s and 3.4s respectively (Piez *et al.* 1963). The ultracentrifugation of thermally denatured acid-soluble collagen that had been irradiated for 2hr. resulted in a similar sedimentation pattern except for a considerable decrease in the β peak (Fig. 3). Chromatography has, however, shown a marked decrease in the composite α_2 fraction. Davidson & Cooper (1967) have shown that their major components A ($\alpha_2\alpha_2$) and B ($\alpha_1\alpha_1$) are eluted with the α_2 fraction by the method of Piez *et al.* (1963), but sediment with the β sub-units. The labile nature of these components A and B would explain the large decrease in the β peak obtained after ultraviolet irradiation.

Optical rotation and viscosity. In the first series of

Fig. 2. Elution patterns of approximately 50mg. of irradiated acid-soluble calf-skin collagen on CM-cellulose at 40° after denaturation at 45° for 30 min. (a) Irradiated for 2hr.; (b) irradiated for 6hr.; (c) irradiated for 17hr. The column was eluted with a linear salt gradient by 500ml. of acetate buffer, I 0.06, and 500ml. of acetate buffer, I , 0.16.

Fig. 1. Elution pattern of approximately 50mg. 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 by 500ml. of acetate buffer, I 0.06, and 500ml. of acetate buffer, I 0.16.

Table 1. Effect of ultraviolet irradiation on the sub-unit composition of acid-soluble collagen

Irradiation dose (hr. at $0-4^\circ$)	Recovery of soluble protein after irradiation (%)	Composition (%)		
		α_1	$\beta_{11} + \beta_{12}^*$	$\alpha_2^\dagger$
0	100	21	18	61
2	96	23	20	57
6	80	37	20	43
17	70	49	20	31

* Poor resolution of the β_{11} and β_{12} sub-units after irradiation made an individual assessment impossible.

† The α_2 fraction was found to be a composite of the α_2 sub-unit and components A, B, C and D (Davidson & Cooper, 1967).

Fig. 3. Sedimentation patterns of (a) thermally denatured acid-soluble collagen, (b) thermally denatured acid-soluble collagen after ultraviolet irradiation for 2 hr. The photographs were taken after 80 and 120 min. at 56100 rev./min. and 35°. The solutions contained 0.4% of protein in sodium formate buffer, pH 3.75 and I 0.15. Sedimentation is from left to right.

Fig. 5. Reduced viscosity (η_{red}) as a function of time for solutions of acid-soluble collagen (1.36 mg./ml.) irradiated with ultraviolet light at 0-4°, thermally denatured at 45° and then kept at 15°. Δ , Not irradiated; $\square$, irradiated for $\frac{1}{2}$ hr.; $\blacksquare$, irradiated for 1 hr.; $\circ$, irradiated for 3 hr.; $\bullet$, irradiated for 19 hr.

Fig. 4. Specific rotation ($[\alpha]_{365}^{15}$) as a function of time for solutions of acid-soluble collagen (1.36 mg./ml.) irradiated with ultraviolet light at 0-4°, thermally denatured at 45° and then kept at 15°. $\square$, Not irradiated; $\blacksquare$, irradiated for $\frac{1}{2}$ hr.; $\circ$, irradiated for 3 hr.; $\bullet$, irradiated for 18 hr.

Table 2. Rate of collagen-fold formation of acid-soluble collagen after ultraviolet irradiation and cooling to 15°

Solutions were irradiated at 0-4° for the specified times, and then thermally denatured at 45° for 15 min., before being cooled to 15°.

Irradiation time (hr.)	Initial rate of mutarotation at 15° ($d[\alpha]/dt)_0$ (deg./min.)		$10^{-2} \times$ Initial rate of reduced viscosity recovery at 15° ($d\eta_{red}/dt)_0$ (dl./g./min.)
	589 m μ	365 m μ	
Not irradiated	0.80	7.0	2.0
$\frac{1}{2}$	0.59	4.3	1.2
1	—	—	0.7
3	0.45	3.3	0.4
19	0.02	1.5	0.0

experiments, solutions of acid-soluble collagen (1.36 mg./ml.) were irradiated in the presence of air for periods of up to 19 hr. and denatured at 45° for 15 min., and the helix formation was followed by

Fig. 6. Specific rotation ($\bullet$) and reduced viscosity ($\circ$) as functions of irradiation time at 0-4°, for solutions of acid-soluble collagen (1.36 mg./ml.) irradiated with ultraviolet light, heat-denatured and then kept at 15° for 48 hr.

optical rotation while the solution was kept at 15° (Fig. 4). The results for a non-irradiated solution are included, showing a recovery of 80% of the helical content of the native collagen. This recovery for native acid-soluble collagen is considerably higher than the 59% recovery reported for neutral salt-soluble collagen (Cooper & Davidson, 1966), but is in agreement with other reported values (50-86%; Harrington & Hippel, 1961).

It is evident from the irradiation data (Fig. 4 and Table 2) that ultraviolet irradiation decreases the initial rate of mutarotation, i.e. ($d[\alpha]/dt)_0$, and considerably decreases 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). Further,

with irradiation periods of about 19hr., both the recovery of helical nature and the association of sub-unit chains (see below) virtually cease to take place. The specific rotation after 19hr. of irradiation followed by storage at 15° for 48hr. ($[\alpha]_D^{15} = -154^\circ$; $[\alpha]_{365}^{15} = -550^\circ$) was considerably lower than for the non-irradiated material, and approaches the mean residue rotation of collagen in the denatured form ($[\alpha]_D^{15} \approx -90^\circ$ to -120° ; $[\alpha]_{365}^{15} \approx -450^\circ$), indicating almost complete loss of the poly-L-proline II-type helix.

A parallel study was also made of the change in reduced viscosity at 15° (Fig. 5 and Table 2). Ultraviolet irradiation caused a decrease in the initial rate of viscosity recovery, $(d\eta_{red.}/dt)_0$, and the amount of chain association occurring parallels the regain in helical content.

Fig. 6 illustrates the relationship between reduced viscosity on cooling for 48hr. at 15° and the irradiation time. This relationship is thus a measure of aggregation or interchain association (Harrington & Hippel, 1961), which is directly related to irradiation damage. The main effect of irradiation would appear to be almost complete in the first 5hr. under the prescribed conditions, which indicates that the first breaks in the polypeptide chain are clearly more effective than subsequent breaks in reducing sub-unit chain association. The relationship between specific rotation and irradiation time is also illustrated in Fig. 6, representing the effect of irradiation damage on the helical content or intrachain reaction (Hippel & Wong, 1963*a,b*).

Cooper & Davidson (1966) found a similar relationship on irradiating neutral salt-soluble collagen with ultraviolet light, indicating that the first breaks in the chain are more effective than

later ones in decreasing the initial rate of mutarotation, and that the initial rate of mutarotation decreases as the chain length is diminished (Hippel & Wong, 1963*b*). It is reasonable to presume that the irradiation time is proportional to the number of breaks in the chain.

To investigate the order of reaction for the recovery of optical rotation and reduced viscosity at 15°, with and without irradiation, $\log(d[\alpha]/dt)$ and $\log(d\eta_{red.}/dt)$ were plotted against time. Fig. 7 illustrates a typical relationship. The non-linear relationship shows that the random coil-to-helix reaction is not a first-order reaction (Piez & Carrillo, 1964; Harrington & Hippel, 1961). Similar findings were noted with neutral salt-soluble collagen (Cooper & Davidson, 1966).

DISCUSSION

The effect of ultraviolet irradiation on the sub-unit composition of acid-soluble collagen has indicated the labile nature of the β_{12} and α_2 sub-units, as well as the components A, B, C and D (Davidson & Cooper, 1967). The labile nature of the β_{12} and α_2 sub-units may be expected, as the α_2 -chain has been shown to be rich in both tyrosine and phenylalanine residues (Piez *et al.* 1963). Although it has been suggested that components A, B, C and D are terminally linked dimeric sub-units (Davidson & Cooper, 1967), their labile nature may also be expected, as tyrosine is thought to play a significant role in the intermolecular linkage of such sub-units (Rubin *et al.* 1963). Cooper & Davidson (1965) have shown that both tyrosine and phenylalanine residues in soluble collagens are labile under the prescribed conditions. The relative decrease in the β -sub-unit content, indicated by ultracentrifugation, would also suggest that depolymerization of the major components A ($\alpha_2\alpha_2$) and B ($\alpha_1\alpha_1$) takes place at an early stage in the ultraviolet-irradiation reaction.

The formation of an insoluble precipitate, resulting from the excessive irradiation of acid-soluble collagen, was not encountered when neutral salt-soluble collagen was being irradiated (Cooper & Davidson, 1966). The formation of such a precipitate may be indicative of a minor cross-linking mechanism taking place.

Piez & Carrillo (1964) regarded the pyrrolidine ring content, the sub-unit content and the solvent employed as being the prime factors that control the rate and extent of mutarotation in solutions of cooled gelatins. According to Harrington & Hippel (1961), the re-formation of the collagen-type structure occurs in a three-stage mechanism. (1) The pyrrolidine-rich portions of the peptide chain undergo initial changes that nucleate the poly-L-proline II-type helix. (2) This helix structure is

Fig. 7. Plots of $\log(d\eta_{red.}/dt)$ as a function of time at 15°, for solutions of acid-soluble collagen (1.36 mg./ml.) irradiated with ultraviolet light at 0–4° and thermally denatured at 45°. ●, Not irradiated; ○, irradiated for 1 hr.; ■, irradiated for 3 hr.

propagated outwards along single gelatin chains (Flory & Weaver, 1960). (3) Lateral chain association through interchain hydrogen bonding is made possible by the helix formation of individual chains (Veis, 1964). The first stage may be followed by amino acid analyses and the second and third stages may be monitored by optical rotation and viscometry respectively.

Since the current experiments have shown that ultraviolet irradiation affects both the rate and the extent of helix formation in acid-soluble collagen, it is necessary to consider which of the above factors are affected by the irradiation. The amino acid analyses (Cooper & Davidson, 1965) show that no significant decrease in the hydroxyproline and proline content occurs after irradiation, and therefore the initial nucleation step involving the pyrrolidine ring cannot be affected by irradiation. The amino acid analyses also show that, apart from phenylalanine and tyrosine, which are present in small amounts, no great loss of amino acids occurs on irradiation. Although tyrosine and phenylalanine are thought to play a critical role in the intermolecular interaction resulting in fibril formation (Schmitt *et al.* 1964), the role played by these residues in controlling the rate and extent of mutarotation in solutions of cooled gelatins does not appear to have been considered (Piez & Carrillo, 1964; Cooper & Davidson, 1966). Although the main effect of irradiation has been shown to be the conversion of the α and β sub-units into smaller peptide chains (Cooper & Davidson, 1966), destruction of tyrosine and phenylalanine residues resulting in considerable depolymerization may well be a contributory factor. These residues may well play a critical role in the nucleation of pyrrolidine-rich portions of the peptide chain.

Therefore the effect of ultraviolet irradiation on the collagen-fold formation would appear to be the formation of shorter peptide chains, in the random-coil form, which hinder the propagation of the helical structure starting at the pyrrolidine rings and proceeding along the single-chain random coils (Hippel & Wong, 1963b). This would presumably also decrease the amount of lateral chain association between single helical chains, and any single chain folding giving helices stabilized by intramolecular association (Veis, 1964).

Since the mutarotation studies of irradiated

acid-soluble collagen and of neutral salt-soluble collagen (Cooper & Davidson, 1966) give very similar results, similar intramolecular structures for both acid-soluble and neutral salt-soluble collagens are suggested.

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