The Effect of Ultraviolet Irradiation on Soluble Collagen

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(Received 4 January 1965)

1. The effect of ultraviolet irradiation on acid-soluble and neutral-salt-soluble calf-skin collagen was studied by chromatography, gel filtration, amino acid analysis and sedimentation of the sub-units, and the reaction kinetics of degradation were obtained from viscosity and optical rotation measurements. 2. It was demonstrated that, whereas the structure of neutral-salt-soluble calf-skin collagen may be represented by the formula $(\alpha_1)_2\alpha_2$, the acid-soluble extract has the formula $\alpha_1.(\alpha_2)_2$. The acid-soluble collagen is also unusual in containing a large amount of a component that could be β_{22} . 3. Ultraviolet irradiation causes the progressive degradation of the collagen molecule into smaller molecular fragments that subsequently lose their helical nature. The rate constants show that the denaturation of soluble collagens by ultraviolet irradiation is much slower, under the conditions used, than denaturation by heat or enzymes.

It has been known for a number of years (Orekhovitch & Shpikiter, 1955) that the thermal denaturation of soluble collagen yields molecular fragments, and these have recently been resolved into α - and β -components (Harrington & Hippel, 1961; Piez, Eigner & Lewis, 1963). The effect of ultraviolet light on skin (Bottoms & Shuster, 1963) has been interpreted as causing 'aging' in collagen through cross-linking within the collagen fibrils. The present investigation on the ultraviolet irradiation of separated neutral-salt-soluble and acid-soluble collagen was undertaken to study in detail the effect of this type of irradiation on the collagen molecule and its α - and β -components.

MATERIALS AND METHODS

Preparation of neutral-salt-soluble and acid-soluble collagens. The skin of a 2-month-old Jersey bull calf was taken off immediately after slaughter. This was cooled in ice, cleaned free of blood, flesh and fat, and minced to a fine pulp in the presence of ice to keep the mincer and skin cold.

Neutral-salt-soluble collagen was prepared as follows. The skin pulp was extracted three times with five times its weight of 10% (w/v) NaCl solution, each successive extraction being done at 0–4° for 96 hr. The extracts were clarified by centrifuging at 14000g and –2° through the continuous-action rotor of the MSE model 18 centrifuge. The clear extracts were saturated with solid $(\text{NH}_4)_2\text{SO}_4$, and the precipitated proteins collected by centrifugation. This precipitate was dissolved in m-NaCl and dialysed against a large volume of this solvent to remove excess of salts. The protein solution was then filtered through paper pulp and brought to pH 3–4 with acetic acid. After standing overnight the precipitated protein was collected and redissolved

in 0.5N-acetic acid. This solution was centrifuged at 32000g at –2° for 1 hr. to remove insoluble material. The neutral-salt-soluble collagen was precipitated by the addition of solid NaCl to give a final concentration of 5% (w/v). The collagen was dissolved in 0.5N-acetic acid and the solution clarified by centrifugation at 32000g at –2° for 1 hr. and dialysed against three lots of 0.02M- Na_2HPO_4 until precipitation was complete. The precipitated collagen was redissolved in 0.15N-acetic acid, dialysed against several changes of this solvent and then freeze-dried. The dry salt-free neutral-salt-soluble collagen was stored over silica gel at 4°.

The acid-soluble collagen was prepared from the skin of a 1-week-premature bull calf by extracting with 0.2M-NaCl solution as above, followed by adjusting the pH of the skin pulp to 4.0, and extracting with three lots of 0.01N-acetic acid. The acid-soluble collagen was then purified as above. Except where stated all operations were carried out in a cold room at 0–4°.

Column chromatography. The method of column chromatography at 40° was similar to that of Piez *et al.* (1963), with their stock acetate buffer, pH 4.8 and *I* 0.5. For gradient elution 500ml. of their starting buffer, *I* 0.06, and 500ml. of their limiting buffer, *I* 0.16, prepared from the stock buffer, were used. The column (2.5 cm. $\times$ 34 cm.) was packed with CM-cellulose (lot no. 11073; Schleicher and Schüll), which was regenerated between runs by the method of the above authors. The effluent fractions were collected in 10ml. portions, the fraction collector being kept at 7°, and the extinctions measured at 230m μ with 20mm. cells in a Unicam SP.700 spectrophotometer. At a later stage a Beckman model DB spectrophotometer was used to obtain continuous recordings at 230m μ of the column effluent. Neutral-salt-soluble collagen solutions were prepared in 0.15N-acetic acid (4–5mg./ml.) and denatured at 45° for 30 min., and acid-soluble collagen solutions also in 0.15N-acetic acid were denatured at the same temperature for

60 min. before chromatography. Protein recoveries of about 90% were obtained after chromatography.

Ultraviolet irradiation. Collagen solutions were prepared in 0.15 N-acetic acid and irradiated at room temperature or in a cold room with a Hanovia UVS 220A lamp operated by a regulated power supply, the solution temperatures being about 20° and 0–4° respectively. The solutions were placed in fused quartz tubes at a distance of 46 cm. from the lamp, unless otherwise specified. The lamp is rated to give an ultraviolet intensity at 92 cm. of 420–540 $\mu\text{W}/\text{cm}^2$ of radiation below 4000 Å.

Determination of viscosities. Viscosities of collagen solutions were measured in a Cannon–Fenske viscometer (size 50, B.S. 188) at $20 \pm 0.05^\circ$. Solutions for viscosity and optical rotation measurements were clarified by centrifuging at 32000g at 5° for 90 min. before irradiation. For both these measurements solutions were irradiated at 4°, but not heat-denatured, and then equilibrated at 20° before taking readings. Viscosity measurements were made on solutions containing 0.2–0.8 mg. of protein/ml. of 0.15 N-acetic acid.

Determination of optical rotations. Optical rotation was measured at $20 \pm 0.25^\circ$ in the 40 cm. tube of a Bellingham and Stanley polarimeter equipped with a sodium lamp. Measurements were made on solutions containing 0.4–0.8 mg. of protein/ml. of 0.15 N-acetic acid.

Amino acid analysis. The amino acid composition of freeze-dried irradiated and non-irradiated preparations was determined with a Spinco amino acid analyser after hydrolysis with 6 N-HCl under reflux for 24 hr.

Determination of molecular weights. Molecular weights of the collagen sub-units and residues after irradiation were estimated by the gel-filtration method (Whitaker, 1963; Andrews, 1964), with a column (52.5 cm. $\times$ 2.5 cm.) of Sephadex G-200 (lot no. T02510; Pharmacia, Uppsala, Sweden) kept at 20°. The proteins were eluted at a flow rate of 30 ml./hr. with sodium phosphate buffer, pH 5.8 and I 0.12, the elution volumes being determined by recording the extinction of the effluent at 230 m μ in the 10 mm. flow cell of a Beckman model DB spectrophotometer, equipped with a scale-expansion attachment. A calibration curve was obtained with purified preparations of the following proteins: bovine plasma albumin and bovine γ -globulin (Armour Pharmaceutical Co., Eastbourne, Sussex), egg albumin, trypsin and pepsin (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.) and undenatured neutral-salt-soluble and acid-soluble collagen (mol.wt. 345000). A graph of elution volume against log (molecular weight) gave a linear relationship over the molecular weight range 20000–350000. The method has been shown to be satisfactory for globular proteins, but there may be some doubt with the higher molecular weights of asymmetrical molecules when the column is calibrated with globular proteins (Andrews, 1964).

Ultracentrifugation. Denatured collagen and collagen fragments were examined by sedimentation velocity at 35° by employing schlieren optics in a Spinco model E ultracentrifuge. The collagen preparations after irradiation were dialysed against distilled water, 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° just before placing in the ultracentrifuge cell. Native collagens were treated in the same way. All patterns reproduced in Fig. 3 were taken 80 or 120 min. after reaching a speed of 56100 rev./min.

RESULTS

Chromatography and ultracentrifugation of ultraviolet-irradiated collagens. The initial chromatography runs were done on non-irradiated samples to study the normal content of the α - and β -components (Piez *et al.* 1963) of neutral-salt-soluble and acid-soluble collagen of calf skin and premature calf skin respectively (Figs. 1 and 2). In several repeat runs the neutral-salt-soluble collagen did not show the presence of the β_{11} -component [in the new nomenclature of Bornstein, Martin & Piez (1964) rather than the old notation of β_2], in spite of the well-defined separation of the other three components (α_1 , α_2 and β_{12}). There were indications of a

Fig. 1. Elution patterns of approx. 140 mg. of denatured neutral-salt-soluble calf-skin collagen on CM-cellulose at 40°. —, Non-irradiated; ----, ultraviolet-irradiated for 19 hr. at a distance of 46 cm. at 0–4°.

Fig. 2. Elution patterns of approx. 130 mg. of denatured acid-soluble calf-skin collagen on CM-cellulose at 40°. —, Non-irradiated; ----, ultraviolet-irradiated for 19 hr. at a distance of 46 cm. at 0–4°.

Table 1. *Approximate distribution of sub-units for ultraviolet-irradiated and non-irradiated neutral-salt-soluble and acid-soluble collagen*

For the irradiated material, the components with the same elution volumes as the α - and β -components are listed under these, but in fact are mostly degraded α - and β -components with similar elution volumes from the CM-cellulose columns (see the text). The distributions were calculated from areas of the chromatography elution curves for duplicate runs.

		Distribution of collagen sub-units (%)				
Compound	Ultraviolet irradiation	α_1	α_2	β_{12}	β_{11}	X
Neutral-salt-soluble collagen	None	47	19	34	0	—
	4 hr. at 20°	35	0	65	0	—
	6 hr. at 4°	32	14	54	0	—
	19 hr. at 4°	28	0	72	0	—
Acid-soluble collagen	None	14*	65*	10*	11*	—*
		6	27	4	5	58
	19 hr. at 4°	12*	44*	44*	—	—*
		8	30	30		32

* Composition calculated excluding the X-component.

slight inflexion at the base of the α_1 -peak, which could correspond to a small percentage of the β_{11} -component. The composition of the non-irradiated collagens is given in Table 1.

The initial ultraviolet irradiations of neutral-salt-soluble collagen were at solution temperatures of approx. 20°, when it was found that as the irradiation time was increased the amount of the α_1 - and α_2 -components decreased while a considerable increase resulted in the component corresponding in elution volume to the β_{12} -component, namely component β_{12}^* (Table 1). For the sake of clarity, and for reasons given below on the composition after irradiation, chromatographic components obtained after irradiation and corresponding in elution volumes from the CM-cellulose column to the α - and β -components are designated with an asterisk. Thus the component after irradiation having a similar elution volume to the β_{12} -component is labelled β_{12}^* . Since irradiation at ambient temperatures could introduce complicating factors, such as thermal denaturation, subsequent irradiations were done at solution temperatures of 0–4°. Under these conditions longer irradiation times were required to reach the same stage of reaction (Table 1).

After irradiation for 19 hr. at 0–4° neutral-salt-soluble collagen contained no α_2^* -component, the concentration of the α_1^* -component was practically halved and the concentration of the β_{12}^* -component was doubled (Table 1 and Fig. 1). Considerable

Table 2. *Ultracentrifugal analysis of neutral-salt-soluble and acid-soluble collagen before and after ultraviolet irradiation*

The sedimentation coefficients were measured at 56 100 rev./min. and 35°, and are uncorrected for concentration-dependence.

Sample	$S_{20,w}$ (s)
Neutral-salt-soluble collagen	2.6, 3.3
Neutral-salt-soluble collagen after irradiation for 19 hr. at 0–4°	1.7
Acid-soluble collagen	2.6, 3.4

changes also occurred in the elution pattern of the acid-soluble collagen after irradiation for 19 hr. at 0–4° (Table 1 and Fig. 2). The biggest change noted was the decrease in the content of the X-component from 58 to 32% of the whole preparation. The β_{12} -component was replaced by a heterogeneous component consisting of two peaks, with an overall increase in the concentration of the β^* -components. The amount of the α_2 -component was decreased, leaving an apparently heterogeneous component, α_2^* . From the areas of the elution curves it appears that some of the degradation products of the α_2 - and X-components are eluted as β^* -components.

Ultracentrifugal analysis (Table 2 and Fig. 3) showed that the neutral-salt-soluble collagen after thermal denaturation consisted of α - and β -components with a similar sedimentation pattern to those illustrated by Piez *et al.* (1963) and Bornstein *et al.* (1964). A small component sedimenting faster than the β -component, and presumably corresponding to the γ -component, was also evident. If the Johnston-Ogston effect (Johnston & Ogston, 1946) is taken into account, the ratio of α - to β -components (α/β ratio) from the sedimentation pattern appears to be similar to the 2:1 ratio found by chromatography. After irradiation the ultracentrifuge pattern (Fig. 3) shows one heterogeneous peak of lower sedimentation coefficient ($S_{20,w} = 1.7$ s) than those obtained for the homogeneous peaks of the non-irradiated material ($S_{20,w} = 2.6$ and 3.3 s). The pattern shows a range of sedimenting components corresponding to a wide range of molecular weights.

The sedimentation pattern for non-irradiated acid-soluble collagen ($S_{20,w} = 2.6$ and 3.4 s; Fig. 3) is similar to that for neutral-salt-soluble collagen except that the α/β ratio is much less. The X-component must sediment with the β -components, since this comprises 58% of the preparation, and β -components are the largest sedimenting fraction. From the chromatography data, if the X-component is included with the β -components then the α/β ratio [i.e. $\alpha/(\beta + X)$] is 1:2, whereas if the α/β ratio is calculated with the X-component excluded

Fig. 3. Sedimentation patterns of (a) heat-denatured neutral-salt-soluble collagen, (b) heat-denatured neutral-salt-soluble collagen after ultraviolet irradiation for 19 hr. at 0–4° and (c) heat-denatured acid-soluble collagen. The photographs were taken after 80 and 120 min. at 56 100 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.

this becomes 4:1. The former would agree approximately with the α/β ratio estimated from the sedimentation areas, with the Johnston–Ogston effect being taken into account. Gel filtration of non-irradiated X-component gave a molecular weight about 600 000, but this value could be too high as estimated by gel filtration for the reasons discussed below. These sedimentation data, particularly the relatively low sedimentation coefficients (see Altgelt, Hodge & Schmitt, 1961; Veis, Anesey & Cohen, 1962), would seem to exclude the possibility of the X-component being the γ -trimer, and therefore it could be the β_{22} -component (i.e. $\alpha_2 \cdot \alpha_2$) of Bornstein *et al.* (1964) or an aggregate of this. According to these authors the β_{22} -component is eluted from CM-cellulose after the α_2 -component, as found in the present case for the X-component.

The freeze-dried preparation of irradiated acid-soluble collagen was, for reasons being investigated, not soluble in the formate buffer, even after heating at 40°, so that no sedimentation data were obtained for this preparation.

By using the relationship of Lewis & Piez (1964)

for the concentration-dependence of the sedimentation coefficients, $S_{20,w}^0$ values of 3.1 and 4.2 s were obtained for the α - and β -components respectively, which agree with reported values (see, e.g., Altgelt *et al.* 1961).

To follow the separation of α - and β -components from the collagen molecule after ultraviolet irradiation, but without introducing the complicating factor of thermal denaturation, solutions of the soluble collagens after irradiation were placed on the chromatography column kept at 20°. Unfortunately, the high viscosity of the solutions after irradiation, particularly with acid-soluble collagen, considerably decreased the amount of material eluted from the column. Nevertheless, material corresponding in elution volume to the α - and β -components was found. In addition, a large component emerged from the column immediately on eluting. A similar component was noted by Piez *et al.* (1963) from their heat-denatured collagens, which they attributed to breakdown products of the collagens when in the relatively unstable heat-denatured form. This component was also obtained when neutral-salt-soluble collagen was irradiated for long periods and heat-denatured.

At the concentrations used for the chromatographic separations both the neutral-salt-soluble and the acid-soluble collagen gelled after irradiation for 19 hr. Since soluble collagen solutions of much higher concentrations can be prepared at this pH without setting, the gelling after irradiation indicates either the splitting of the tropocollagen molecule into sub-units (Orekhovitch & Shpikiter, 1958), or the formation of intermolecular cross-links.

Molecular weights of ultraviolet-irradiated collagens.

The sedimentation studies discussed above show that after irradiation neutral-salt-soluble collagen contains a range of low-molecular-weight products, as deduced from the low sedimentation coefficient of the heterogeneous peak, the molecular weights being lower than those of the original material. Thus ultraviolet irradiation appears to degrade the tropocollagen molecule and its sub-units into lower-molecular-weight fragments. To confirm this finding the sub-units and products of irradiation from both collagen preparations were separated by chromatography and the molecular weights determined by gel filtration.

The procedure adopted after consideration of the many possibilities was to standardize the method at 20° with globular proteins of known molecular weights. It appeared that 35–40° would have been a better temperature at which to do the determinations, since this is above the melting point of the α - and β -components (Piez & Carrillo, 1964), but this led to difficulties with the stability of the standard proteins and possible decomposition of the β_{12} -component (Lewis & Piez, 1964). The procedure

whereby the separations were done at 40°, the fractions stored at 0–4° and the molecular weights determined at 20° gave in general higher molecular weights for the α - and β -components of non-irradiated collagen than those reported in the literature (see, e.g., Lewis & Piez, 1964). This could be due to collagen fold formation, which occurs in dilute solutions of cooled gelatin (Altgelt *et al.* 1961; Hippel & Wong, 1963a,b; Piez & Carrillo, 1964), where the coil-helix transition could give incorrect results with gel filtration when the method is standardized with nearly spherical proteins (Whitaker, 1963; Andrews, 1964). A further factor that could account for the high molecular weights is aggregation (Piez & Carrillo, 1964), since evidence was obtained from protein recovery and flow rates that some precipitation occurred on the Sephadex G-200 column.

A wide range of molecular weights (10000–1 000 000) was obtained for the irradiated collagens. For the reasons given above, and the fact that the column was only standardized up to a molecular weight of 300 000, these results must be interpreted with caution, especially the higher values. Thus the sedimentation results for irradiated neutral-salt-soluble collagen that were obtained at 35°, and that were therefore not complicated by the effect of collagen fold re-formation, did not give any indication of the presence of high-molecular-weight material.

To minimize the effect of collagen fold re-formation that occurs when fractions are stored at 0–4°, the two collagen preparations were irradiated at 0–4° for 19 hr., denatured by heating at 40° and then immediately placed on the gel-filtration column at 20°. The molecular weight determinations took 8 hr. with the highest-molecular-weight components eluted after 3 hr., and therefore this procedure offered a maximum of 3 hr. at 20° for coil-helix transition. Nevertheless, both collagen preparations, even though they were irradiated, gave molecular weights in the range 300 000–600 000 for the bulk of the protein. The results of Piez & Carrillo (1964) show that even at 15° helix formation, accompanied by an increase in molecular weight, for α - and β -chains is rapid at low concentrations (1.6 mg./ml.), and would be even more rapid at the concentrations (4–5 mg./ml.) used for the molecular weight determinations in the present work.

Amino acid analysis of ultraviolet-irradiated collagens. The amino acid compositions of neutral-salt-soluble and acid-soluble collagen before and after irradiation for 19 hr. at 4° are given in Table 3. The irradiated collagens were dialysed against several changes of distilled water to remove any degraded protein before being freeze-dried for amino acid analysis. These analyses show that, though the α - and β -components are degraded into low-

Table 3. *Amino acid composition of native and ultraviolet-irradiated neutral-salt-soluble and acid-soluble collagen*

The results for neutral-salt-soluble collagen are based on a single analysis, and those for acid-soluble collagen are the averages of duplicate analyses.

Amino acid	Amino acid composition (g./100 g. of protein)			
	Non-irradiated		Irradiated with ultraviolet light for 19 hr.	
	Neutral-salt-soluble collagen	Acid-soluble collagen	Neutral-salt-soluble collagen	Acid-soluble collagen
Ala	8.32	9.13	8.95	8.33
Arg	8.20	7.35	8.54	7.66
Asp	5.06	5.61	4.37	5.70
Cys	Trace	None	Trace	None
Glu	10.88	10.06	11.50	9.85
Gly	19.46	21.22	21.08	21.48
His	0.71	0.48	0.51	0.44
Hyl	1.17	1.15	1.27	1.19
Hyp	9.60	11.15	10.00	12.75
Ile	1.70	1.33	1.70	1.05
Leu	3.38	2.87	3.43	2.05
Lys	3.89	3.40	3.99	3.74
Met	0.96	0.59	0.67	0.35
Phe	1.93	1.87	1.22	0.75
Pro	11.47	12.80	12.17	14.00
Ser	3.44	3.27	3.39	3.41
Thr	2.04	1.73	1.86	1.77
Tyr	0.65	0.51	0.34	0.14
Val	2.46	2.05	2.44	1.58
Amide N	(0.88)	(0.77)	(0.98)	(0.94)
Total	95.32	96.57	97.43	96.24
Total N	18.2	18.3	18.9	18.6

molecular-weight fragments by the ultraviolet irradiation, only limited changes occur in the amino acid composition. Thus the amounts of tyrosine and phenylalanine are decreased. Both collagen preparations, before and after irradiation, contained a small amount of 3-hydroxyproline (Piez *et al.* 1963), which appeared just before the methionine sulphoxide peak in the amino acid elution. After irradiation both preparations gave a ninhydrin-positive component appearing after leucine in very small amounts (about 0.1%).

Viscosity of ultraviolet-irradiated collagens. The fractional change in reduced viscosity for acid-soluble collagen as a function of time of irradiation is shown in Fig. 4. Similar results were obtained for neutral-salt-soluble collagen. The reaction rate plots for viscosity were obtained from the relationship between $\log(d\eta/dt)$ and t , as derived from eqn. (1) (Hippel & Harrington, 1959):

$$\ln\left(\frac{d\eta}{dt}\right) = \ln(-k\eta_0) - kt \quad (1)$$

Fig. 4. Fractional change in specific rotation and reduced viscosity as a function of irradiation time at a distance of 46 cm. and $0-4^\circ$ for acid-soluble calf-skin collagen. ○, $(\eta_t - \eta_{20})/(\eta_0 - \eta_{20})$, where η_t is the reduced viscosity at time t , and η_0 and η_{20} are the reduced viscosities at zero time and after irradiation for 20 hr. respectively. Δ, $([\alpha]_t - [\alpha]_{20})/([\alpha]_0 - [\alpha]_{20})$, where $[\alpha]_t$ is the specific rotation at time t , $[\alpha]_0$ is the specific rotation of the zero-time control, and is equal to -415° , and $[\alpha]_{20}$ is the specific rotation after irradiation for 20 hr., and is equal to -114° (0.08%, w/v).

Fig. 5. Plots of $\log(d\eta_{red.}/dt)$ as a function of irradiation time at a distance of 46 cm. at $0-4^\circ$: ○, 0.010% (w/v) acid-soluble calf-skin collagen; Δ, 0.033% (w/v) acid-soluble calf-skin collagen; ●, 0.042% (w/v) neutral-salt-soluble calf-skin collagen; Δ, 0.080% (w/v) acid-soluble calf-skin collagen.

where η is the reduced viscosity at time t , η_0 is the total change in reduced viscosity during the reaction and k is the apparent first-order rate constant. The results (Fig. 5) show that the change in viscosity

Fig. 6. Plots of $\log(d[\alpha]/dt)$ as a function of irradiation time at a distance of 46 cm. at $0-4^\circ$: ○, 0.074% (w/v) neutral-salt-soluble calf-skin collagen; ●, 0.080% (w/v) acid-soluble calf-skin collagen.

Fig. 7. First-order rate constant, k , calculated from viscosity data as a function of acid-soluble calf-skin collagen concentration. Collagen was irradiated at 46 cm. at $0-4^\circ$.

induced by irradiation is a first-order reaction, with a much greater rate constant, k , than that for optical rotation (see below and Fig. 6). The rate constants are 8.5×10^{-3} and $10.5 \times 10^{-3} \text{ min.}^{-1}$ for neutral-salt-soluble and acid-soluble collagen respectively at concentrations of 0.042 g. of protein/100 ml.

The rate constant k was found to be inversely related to concentration (Fig. 7). Therefore at the concentrations used in chromatography (0.3 g./100 ml.) the change in viscosity due to the irradiation will be extremely slow. After irradiation for 19 hr. the tropocollagen sub-units in the solutions used for chromatography should therefore retain a high helical content (see below).

Optical rotation of ultraviolet-irradiated collagens. The fractional change in the specific optical rotation for acid-soluble collagen as a function of time of irradiation is shown in Fig. 4. Similar results were

obtained for neutral-salt-soluble collagen. The reaction rate plots for optical rotation were obtained from the relationship between $\log(d[\alpha]/dt)$ and t , as derived from an eqn. (2) similar to eqn. (1):

$$\ln\left(\frac{d[\alpha]}{dt}\right) = \ln(-k[\alpha_0]) - kt \quad (2)$$

where $[\alpha]$ is the specific optical rotation. The results (Fig. 6) show that the change in optical rotation induced by irradiation follows a first-order reaction ($k = 2.6 \times 10^{-4}$ and $3.7 \times 10^{-4} \text{ min}^{-1}$ for neutral-salt-soluble and acid-soluble collagen respectively, at concentrations of 0.075 g. of protein/100 ml.).

DISCUSSION

A detailed analysis of the α - and β -components of calf-skin collagens has been reported (Engel & Beier, 1963) that shows the wide variation in the α/β ratio that is obtained. The α/β weight ratio for neutral-salt-soluble collagen has been given as approx. 1:2 (Wood, 1962; Piez *et al.* 1963), whereas in the present case a value close to 2:1 has been found. The weight ratio values cited for acid-soluble collagen are 1:2 (Doty & Nishihara, 1958) and 1:1 (Piez, Weiss & Lewis, 1960), as compared with the present value of 4:1 for premature calf skin, if the X-component is excluded, and 1:2 if it is included as a β -component. Some of the reported values cannot be correlated since different extraction methods have been used on tissues of different ages, and in some cases the neutral-salt-soluble collagen was not removed before extracting the acid-soluble collagen. Because of these factors, and in view of the interconversion of these components, it is doubtful whether an integral proportionality will always exist (Chun & Doty, 1958; Bakerman & Hersh, 1964).

No direct evidence was found for the presence of the β_{11} -component in normal neutral-salt-soluble collagen from calf skin, although its presence has been reported in rat-skin collagen (Piez *et al.* 1963; Nanto, Maatela & Kulonen, 1963).

The bulk of the evidence from the ultraviolet irradiation of proteins shows that denaturation and decomposition occur (see, e.g., Doty & Geiduschek, 1953; Luse & McLaren, 1963), with some evidence that ionizing radiations (Prusak & Sciarrone, 1962; Baily, 1963) and ultraviolet light (Kuntz, 1960, 1962; Bottoms & Shuster, 1963) can introduce covalent cross-links. It is known that ultraviolet irradiation does induce free radical formation (Inglis & Lennox, 1963; Crawshaw & Speakman, 1954) through which these cross-links could be formed. The ultraviolet irradiation of both neutral-salt-soluble and acid-soluble collagen brings about striking changes in the α - and β -component compo-

sition, as these are largely degraded, on the sedimentation evidence, to low-molecular-weight fragments. The amino acid analyses show that this degradation does not arise from considerable destruction of amino acids and must therefore result from the breaking of ultraviolet-labile peptide bonds. Studies by Schleyer (1962) showed that on alkaline degradation the α - and β -components of calf-skin collagen yielded A- and B-components with molecular weights of about 50000. The A- and B-components had similar elution volumes to those of α_1 -component and β_{12} - or β_{11} -component respectively. In the present instance the degradation products from ultraviolet irradiation had similar elution volumes to the α - and β -components, which illustrates the caution that must be exercised in interpreting the chromatography elution curves, especially if the original collagens may have been degraded.

The acid-soluble collagen from the premature calf skin has some unusual features when compared with published results. The high α_2 -component content (65% of the α - and β -components or 27% of the α -, β - and X-components) of this preparation is most striking. It is therefore postulated that some of the basic units of the tropocollagen molecule must be in the $\alpha_1 \cdot (\alpha_2)_2$ or $\alpha_1 \cdot \beta_{22}$ form to account for this. Bornstein *et al.* (1964) have concluded that the β_{22} -dimer can only be formed by intermolecular cross-linking, and that the usual extraction procedures with salt and acetic acid solutions do not solubilize these intermolecular cross-linked dimers. The present evidence, however, suggests that the β_{22} -component from premature calf skin is a result of intramolecular cross-linking. The existence of the four possible basic units, namely, $\alpha_2 \cdot \beta_{11}$, $\alpha_1 \cdot \beta_{22}$, $\alpha_2 \cdot \beta_{12}$ and $\alpha_1 \cdot \beta_{12}$, of the tropocollagen molecule could possibly account for the apparent heterogeneity reported in collagen preparations (Fessler, 1960*a,b*).

The second feature of the acid-soluble collagen preparation is the large amount of the X-component, which can be eluted from the CM-cellulose by modifying the gradient of Piez *et al.* (1963) to give I 0.26. This could correspond to the β_{22} -component or the Y-component of Bornstein *et al.* (1964) and Schleyer (1962) respectively, although a very much greater proportion was obtained in the present work. This could be attributed to the present preparation being from premature calf skin, whereas most acid-soluble collagens reported in the literature have been made by extracting more mature calf skin. By gel filtration the X-component appeared to have a molecular weight of about 600000, but the sedimentation data would indicate a lower molecular weight, by comparison with the γ -component, which has a molecular weight of 350000 with an $S_{20,w}^0$ value of 4.7 s and $S_{40,w}^0$ value of 7.2 s (Veis *et al.* 1962).

The large amount of the X-component would appear to preclude the possibility of this being the γ -component (Piez *et al.* 1963; Altgelt *et al.* 1961), since this has only been isolated in small quantities (8%) from calf skin, and the premature calf skin used in the current work should contain a minimal amount of this relatively highly cross-linked component. In addition, Bornstein *et al.* (1964) found that the γ -component was eluted from CM-cellulose on the leading edge of the β_{12} -peak, whereas the X-component was eluted after the α_2 -component. The content of the X-component in acid-soluble collagen was decreased from 58 to 32% of the whole preparation after irradiation for 19 hr. at 0–4°.

Though the gel-filtration method was not successful in giving a full picture of the immediate products from irradiating collagen, these appeared to be of relatively low molecular weight as deduced from the sedimentation data; it did show that the products readily underwent the coil-helix transition on cooling to 0° or 20°. This held for both the mixed and separated α^* - and β^* -chains. It has been shown that collagen degraded with enzymes also undergoes this transformation (Hippel & Wong, 1963b).

Though the main effect of irradiation found by column chromatography and ultracentrifugation was the degradation of the α - and β -components into low-molecular-weight fragments, viscosity and optical rotation measurements at lower concentrations showed a fall in these properties with irradiation time. It has been reported (Seifter, Gallop & Meilman, 1958; Hippel & Harrington, 1960) that when collagenase reacts with ichthyocol the viscosity changes faster with time of reaction than the optical rotation. Since viscosity is a function of the axial ratio of a molecule, whereas optical rotation is related more to spatial configuration, due either to the helical natures of the individual chains or interchain alignments (Veis & Anesey, 1958; Drake & Veis, 1964), this is interpreted as an initial scission of the collagen molecule into polypeptides with a relatively low axial ratio, having a helical configuration, followed by randomization of the polypeptide chains. The latter is probably due to thermal denaturation. Nishihara & Doty (1958a,b) found similar changes in these physical properties on ultrasonic degradation of calf-skin collagen, where viscosity and sedimentation indicated that the molecule was progressively fragmented into shorter segments.

The thermal denaturation of soluble calf-skin collagen has been interpreted as an all-or-nothing type of transition on the basis of viscosity and optical rotation changing at the same rate (Doty & Nishihara, 1958). The denaturation brought about by ultraviolet irradiation would appear to involve progressive degradation and decrease in chain length, with the subsequent loss in helical content.

This is borne out by the fact that the optical rotation changes at a lower rate than viscosity on irradiation.

The magnitude of the first-order reaction rate constants show that the denaturation of soluble collagens brought about by ultraviolet irradiation is much slower, under the conditions used, than denaturation by enzymes (Hippel & Harrington, 1959) or thermal denaturation (Doty & Nishihara, 1958). This makes the method most useful for controlled degradation studies on the collagen structure.

The amino acid analysis of both types of collagen before and after irradiation shows mainly a decrease in the tyrosine and phenylalanine contents. No decrease in hydroxyproline was detected (Bottoms & Shuster, 1963). It is reported (Inglis & Lennox, 1963; Luse & McLaren, 1963) that most amino acids are affected by ultraviolet irradiation depending on the source of light and the position of the amino acids in the polypeptide chain.

Since ultraviolet irradiation produces peroxide, the oxidation of collagen must also be considered. Gallop, Seifter & Meilman (1958) have found that oxidation of ichthyocol results in splitting of the parent polypeptide chains, leading to the production of peptides of much lower molecular weight. They conclude that hydroxyproline and proline are oxidized under these conditions. On the other hand, it has been reported (Gustavson, 1958) that milder oxidation techniques do not affect these amino acids. No evidence for loss of hydroxyproline or proline was found in the present instance, and splitting of the parent polypeptide chain was the main result.

This work was financed by the annual grants of the Livestock and Meat Industries Control Board and the Council for Scientific and Industrial Research. The authors are indebted to Mr P. Mellet of the South African Wool and Textile Research Institute for the amino acid analyses, and to Dr F. J. Joubert of the Council for Scientific and Industrial Research for the ultracentrifuge analyses.

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