The Effect of Ultraviolet Irradiation on Collagen-Fold Formation

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1. A study has been made of the effect of ultraviolet irradiation, in the presence of air and nitrogen, on the conformational changes taking place in cooled solutions of gelatin prepared from thermally denatured neutral-salt-soluble collagen.
2. The increase in negative rotation and viscosity at 15° for irradiated and thermally denatured solutions of collagen becomes less as the irradiation time is increased.
3. The principal effect of ultraviolet irradiation is the fission of the primary collagen peptide chains, eventually yielding chain lengths incapable of stabilizing a helical structure.
4. Irradiation in both air and nitrogen results in a loss of tyrosine, histidine and phenylalanine, with more denaturation occurring in the presence of nitrogen.

Ultraviolet irradiation has been shown to modify proteins in general (McLaren, 1949; Rideal & Roberts, 1951; McLaren & Shugar, 1964) and collagen in particular (Cooper & Davidson, 1965; Nishigai, 1964). It was therefore decided to study the action of ultraviolet irradiation on the conformational changes induced in solutions of neutral-salt-soluble collagen on cooling. The conditions governing this coil-to-helix transition in cooled solutions of gelatin have been studied in detail (Harrington & Hippel, 1961; Hippel & Wong, 1962, 1963*a,b*; Piez & Carrillo, 1964; Mandelkern & Stewart, 1964; Kuhn, Engel, Zimmermann & Grassmann, 1965).

MATERIALS AND METHODS

Preparation of neutral-salt-soluble collagen. The purified neutral-salt-soluble collagen from calf skin was the same as that used in a previous publication, where the extraction and purification was described in detail (Cooper & Davidson, 1965).

Ultraviolet irradiation. Collagen solutions were prepared in 0.15M-potassium acetate buffer, pH 4.8, which is known to have the minimum effect on mutarotation (Piez & Carrillo, 1964). These solutions were irradiated with a Hanovia UVS 220A lamp, operated by a regulated power supply, at a solution temperature of 0–4° in a cold room. The lamp used is rated to give an ultraviolet intensity at 92 cm. of 420–540 $\mu\text{W}/\text{cm}^2$ of irradiation below 4000 Å. The solutions (10 ml.) were placed in fused quartz test tubes at a distance of 46 cm. from the lamp. Irradiations were either in the presence of air or oxygen-free nitrogen, the latter containing not more than 10 v.p.m. of oxygen and no oxides of nitrogen or carbon. In the latter case the nitrogen, after passing through a solution of alkaline pyrogallol and a spray trap, was then bubbled through the protein solution

for 30 min. before irradiation was commenced, and during the whole period of irradiation. The bubbling rate was about 100 ml./min. and was kept fairly low to avoid excessive frothing of the solution.

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 40 cm. water-jacketed tube by using a Bellingham and Stanley polarimeter equipped with a sodium lamp.

The procedure for following helix formation was to irradiate the collagen solution for a given period at 0–4°, heat-denature at 40° for 10 min. and then cool for 45 sec. under tap water. A test sample was then immediately transferred to the viscometer or to the polarimeter tube kept at 15°, and readings were taken at regular intervals for periods up to 48 hr.

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

Ultracentrifugation. The method was the same as that used previously (Cooper & Davidson, 1965).

Spectrophotometry. Absorption curves were measured in 10 mm. quartz cells in a Beckman model DB recording spectrophotometer.

RESULTS

Optical rotation and viscosity. In the first series of experiments, solutions of neutral-salt-soluble collagen (1.36 mg./ml.) were irradiated in the presence of air for periods of up to 19 hr., denatured at 40° for 10 min., and the helix formation was followed by optical rotation while the solution was kept at 15° (Fig. 1). The results for a non-irradiated solution after thermal denaturation are included. Here a recovery of 59% of the helical content of

Fig. 1. Specific rotation ($[\alpha]_D^{15}$) as a function of time for solutions of neutral-salt-soluble collagen (1.36 mg./ml.) irradiated with ultraviolet light at 0–4°, heat-denatured at 40° and then kept at 15°. □, Not irradiated; ○, irradiated for $\frac{1}{2}$ hr.; △, irradiated for 3 hr.; ●, irradiated for 19 hr.

Table 1. *Rate of collagen-fold formation after ultraviolet irradiation and cooling to 15°*

Solutions were irradiated at 0–4° for the times shown, or not irradiated, and then thermally denatured at 40° for 10 min., before being cooled to 15°.

Material	Initial rate of mutarotation at 15° ($d[\alpha]_D/dt)_0$ (deg./min.)	$10^{-2} \times$ Initial rate of viscosity recovery at 15° ($d\eta/dt)_0$ (dl./g. min.)
Neutral-salt-soluble collagen	0.70	2.5
Neutral-salt-soluble collagen after $\frac{1}{2}$ hr. irradiation	0.46	1.6
Neutral-salt-soluble collagen after 3 hr. irradiation	0.36	0.3
Neutral-salt-soluble collagen after 19 hr. irradiation	0	0

the native collagen was obtained, which is in agreement with reported values (50–86%; Harrington & Hippel, 1961). It is evident from these data (Fig. 1 and Table 1) 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 for about 19 hr., both the recovery of helical nature and the association of sub-unit chains (see below) virtually cease

to take place. The specific rotation is decreased to a $[\alpha]_D^{15}$ value of -89° , which corresponds to the rotation of α -helix-forming proteins and collagen in the denatured form ($[\alpha]_D \simeq -90^\circ$ to -120° ; Harrington & Hippel, 1961), indicating complete loss of the poly-L-proline II-type helix.

A parallel study was made of the change in viscosity at 15° (Fig. 2 and Table 1). The irradiation caused a decrease in the initial rate of viscosity recovery, and the amount of chain association occurring parallel with the regain in helical content.

Fig. 3 illustrates the relationship between reduced viscosity on cooling for 48 hr. at 15°, which is a measure of aggregation or inter-chain association (Harrington & Hippel, 1961), and irradiation time, which is directly related to irradiation damage. This is not linear, and the main effect of irradiation is almost complete in the first 3 hr. under the present experimental conditions. Thus the first breaks in the polypeptide chain are clearly more effective than the later ones in reducing sub-unit chain association as measured by viscosity. The relation is also presented (Fig. 3) between specific rotation, which is directly related to helical content or intra-chain reaction (Hippel & Wong, 1963*a,b*), and irradiation time. This is a linear relationship.

Hippel & Wong (1963*b*) found a similar relation between $\Delta[\alpha]_{313}$ (the difference between the specific rotation at the high- and low-temperature ends of the helix-to-coil transition) and the number of chain breaks brought about by various enzymes. They also found that the initial rate of mutarotation decreases as the chain length is diminished, and

Fig. 2. Reduced viscosity (η_{red}) as a function of time for solutions of neutral-salt-soluble collagen (1.36 mg./ml.) irradiated with ultraviolet light at 0-4°, heat-denatured at 40° and then kept at 15°. $\square$, Not irradiated; $\circ$, irradiated for $\frac{1}{2}$ hr.; $\blacktriangle$, irradiated for 1 hr.; $\triangle$, irradiated for 3 hr.; $\bullet$, irradiated for 19 hr.

Fig. 3. Specific rotation ($\circ$) and reduced viscosity ($\square$) as functions of irradiation time at 0-4°, for solutions of neutral-salt-soluble collagen (1.36 mg./ml.) irradiated with ultraviolet light, heat-denatured and then kept at 15° for 48 hr.

that the first breaks in the chain are more effective than later ones in decreasing the initial rate of mutarotation. This is also apparent from the present data (Figs. 1 and 3), if the irradiation time is taken as being proportional to the number of breaks in the chain, which is presumably a reasonable assumption in the initial stages of irradiation.

Fig. 4. Plots of $\log(d\eta_{red}/dt)$ as a function of time at 15°, for solutions of neutral-salt-soluble collagen (1.36 mg./ml.) after heat-denaturation at 40° ($\circ$), and after ultraviolet irradiation for 1 hr. followed by heat-denaturation at 40° ($\square$).

To determine the order of reaction for recovery of optical rotation and viscosity at 15°, with and without irradiation, $\log(d[\alpha]/dt)$ and $\log(d\eta_{red}/dt)$ were plotted against t . Fig. 4 illustrates a typical relationship. Except possibly for the first hour of recovery time, the period during which readings were most difficult to measure accurately as the reaction rate was high, these relationships were non-linear, showing that the re-formation reaction

in terms of helical formation and aggregation is in the main not a first-order reaction (Harrington & Hippel, 1961; Piez & Carrillo, 1964).

The accumulative effect of the irradiation, which also shows that the irradiation damage is not reversible, is illustrated in Fig. 5. In this experiment a solution of neutral-salt-soluble collagen (1.36 mg./ml.) was irradiated for 30 min., heat-denatured at 40° for 10 min. and kept at 15° for 48 hr. while the viscosity was measured at regular intervals. After

Fig. 5. Reduced viscosity (η_{red}) as a function of time for a solution of neutral-salt-soluble collagen irradiated with ultraviolet light at 0–4° for $\frac{1}{2}$ hr., heat-denatured at 40° and then kept at 15° for 48 hr. The same solution was then irradiated for a further $\frac{1}{2}$ hr., heat-denatured at 40° and then kept at 15° for 32 hr.

this the solution was immediately irradiated again for a further 30 min., heat-denatured at 40° for 10 min. and kept at 15° for 32 hr., while the viscosity was again recorded. Fig. 5 shows that after the first irradiation and 32 hr. re-formation time at 15° the reduced viscosity was 2.88 dl./g., whereas the reduced viscosity after the second irradiation and the same time for re-formation was 1.64 dl./g. For a continuous irradiation interval of 60 min. with 32 hr. re-formation time the reduced viscosity was 2.25 dl./g. (Fig. 2). The latter is not a strict comparison since the reduced viscosity had not reached equilibrium even after 48 hr. It has been reported (Hippel & Harrington, 1959; Piez & Carrillo, 1964) that viscosity can increase over a period of many days.

Experiments were then conducted to follow the differences in helix formation and aggregation brought about by ultraviolet irradiation in air and nitrogen. These data are illustrated graphically in Figs. 6 and 7, where samples of the same collagen solution were irradiated for $\frac{1}{2}$ hr. and 1 hr. in air and with pure oxygen-free nitrogen bubbling through the solution. The collagen, when irradiated in the presence of nitrogen, gave less recovery of optical rotation and viscosity at 15° than when the irradiation was done in the presence of air. Fig. 6 shows that irradiation for 1 hr. in air actually gives a greater recovery of reduced viscosity than $\frac{1}{2}$ hr. irradiation in the presence of nitrogen.

A summary is given in Table 2 of the reduced

Fig. 6. Reduced viscosity (η_{red}) as a function of time at 15°, for solutions of neutral-salt-soluble collagen (1.36 mg./ml.) irradiated in air and nitrogen with ultraviolet light, heat-denatured at 40° and then kept at 15°. ●, Irradiated for $\frac{1}{2}$ hr. in air; ○, irradiated for 1 hr. in air; ■, irradiated for $\frac{1}{2}$ hr. in nitrogen; □, irradiated for 1 hr. in nitrogen.

Fig. 7. Specific rotation ($[\alpha]_D^{15}$) as a function of time at 15°, for solutions of neutral-salt-soluble collagen (1.36 mg./ml.) irradiated with ultraviolet light for $\frac{1}{2}$ hr. in air (○) and $\frac{1}{2}$ hr. in nitrogen (□), followed by heat-denaturation at 40°.

Table 2. Summary of reduced viscosity and specific rotation values for neutral-salt-soluble collagen subjected to ultraviolet irradiation

Values were obtained after keeping solutions at 15° for 48 hr.

Treatment	Reduced viscosity (dl./g.)	Specific rotation ($[\alpha]_D$)
Control: no irradiation and no heat-denaturation	15.94	-339°
Heat-denaturation but no irradiation	4.10	-235
Irradiated for $\frac{1}{2}$ hr. in air with heat-denaturation	3.16	-230
Irradiated for 1 hr. in air with heat-denaturation	2.47	—
Irradiated for 3 hr. in air with heat-denaturation	1.30	-204
Irradiated for 10 hr. in air with heat-denaturation	0.55	-159
Irradiated for 19 hr. in air with heat-denaturation	0.28	-89
Irradiated for $\frac{1}{2}$ hr. in N ₂ with heat-denaturation	2.20	-202
Irradiated for 1 hr. in N ₂ with heat-denaturation	1.02	—

viscosity and specific rotation obtained for solutions of neutral-salt-soluble collagen kept at 15° for 48 hr., after various irradiation treatments.

Amino acid analysis. In Table 3 the amino acid analysis of neutral-salt-soluble collagen after irra-

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

Neutral-salt-soluble collagen was irradiated in nitrogen for 19 hr. before analysis.

Amino acid	Amino acid composition (g./100 g. of protein)
Ala	8.59
Arg	8.43
Asp	5.20
Glu	11.15
Gly	20.15
His	0.33
Hyl	1.15
Hyp	9.55
Ile	1.65
Leu	3.30
Lys	3.90
Met	0.77
Phe	1.07
Pro	11.52
Ser	3.48
Thr	2.06
Tyr	0.33
Val	2.45
Amide N	0.91
Total	95.08

diation in the presence of nitrogen is given. Comparison of these values with those reported (Cooper & Davidson, 1965) for neutral-salt-soluble collagen

in the native form, and after ultraviolet irradiation in air, shows that the only amino acids partially destroyed by the irradiation were histidine, phenylalanine, methionine and tyrosine. The greatest losses are apparent after irradiation in nitrogen.

Ultracentrifugation. Ultracentrifugation of native neutral-salt-soluble collagen has been shown to give two major components (S_{35} 3.48 and 4.50s), corresponding to the α - and β -components of collagen (Cooper & Davidson, 1965). After the irradiation in air one main heterogeneous component (S_{35} 2.29s) was obtained (Cooper & Davidson, 1965), whereas after irradiation in nitrogen a similar heterogeneous component with a lower sedimentation coefficient (S_{35} 1.84s) was found.

Reaction with 2,4-dinitrophenylhydrazine. Gallop (1964) has proposed a structure for the α -components of tropocollagen based on four peptide chains linked end-to-end through a hexose residue, which is adjacent to aspartic acid on one of the chains and tyrosine on the other (see Fig. 8 in Gallop, 1964). Since ultraviolet irradiation destroys about half the tyrosine in collagen this should, through steric considerations, make the hexose, if present, more readily available for reaction with the above reagents.

Accordingly, solutions of neutral-salt-soluble collagen were tested: (a) in the native form; (b) after heat-denaturation for 10 min. at 40°; (c) after irradiation for 3 and 19 hr., followed by heat denaturation. Solutions of the same initial collagen concentration (1.36 mg./ml.) were dialysed, and then an equal volume of 0.006% 2,4-dinitrophenylhydrazine in *N*-hydrochloric acid was added, the

reaction being allowed to proceed at 25° for 5 min. The spectra in the range 360–420 m μ were immediately measured. The results (Fig. 8) show that the irradiated solutions (c) gave significantly higher extinctions than the two control solutions (a and b).

DISCUSSION

The main factors that control the rate and extent of helix formation into the unique collagen-type triple helix in solutions of cooled gelatin are the pyrrolidine ring content, due to the presence of proline and hydroxyproline, the relative content of the α -, β - and γ -components, and the solvent employed (Piez & Carrillo, 1964). According to Harrington & Hippel (1961) the following three-stage mechanism occurs in the re-formation of the collagen-type structure: (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 propagates outwards from these nuclei along single gelatin chains (Flory & Weaver, 1960). (3) The formation of the unique collagen-fold along individual chains makes possible lateral chain association through inter-chain hydrogen bonding (Veis, 1964). The second step may be monitored by changes in optical rotation and the last by the increase in viscosity.

Since the current experiments have shown that ultraviolet irradiation affects the rate and extent of helix formation, it is necessary to consider which of the above factors are affected by the irradiation. The amino acid analyses 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 histidine, phenylalanine and tyrosine, which are present in small amounts, no great loss of amino acids occurs on irradiation. As reported previously (Cooper & Davidson, 1965), the main effect of irradiation is the conversion of the α - and β -components into smaller peptide chains. Therefore the effect of ultraviolet irradiation on collagen-fold formation would appear to be due to 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). Both these would result in a diminished recovery of viscosity on cooling.

Hippel & Wong (1963b) found that the degree of helical recovery taking place on cooling decreased

Fig. 8. Extinction curves for neutral-salt-soluble collagen (1.36 mg./ml.) treated with 2,4-dinitrophenylhydrazine at 25°. 1, Collagen in the native form; 2, collagen heat-denatured at 40°; 3, collagen irradiated with ultraviolet light for 3 hr. and heat-denatured at 40°; 4, collagen irradiated with ultraviolet light for 19 hr. and heat-denatured at 40°.

markedly as the molecular weight of the gelatin in solution was decreased by enzyme action. They estimate that a minimum chain length of 40–80 residues is required to generate stable elements of the collagen-type helix, and that the final helical content is independent of the method of breaking the peptide chain. The current amino acid analyses show losses of tyrosine, histidine, phenylalanine and methionine for the irradiation of collagen in air. These losses correspond to the destruction of about 11 amino acid residues/1000 residues, which is equivalent to about 11 breaks/ α -chain of mol.wt. 100 000. Hippel & Wong (1963b) have shown that no helical content is found in cooled solutions of gelatin after enzymic reaction causes about 40 breaks/polypeptide chain of mol.wt. 100 000.

As shown above the initial rates of mutarotation and viscosity recovery are decreased by irradiation, as are the final specific rotation and reduced viscosity attained after cooling at 15° for 48 hr. The initial breaks in the polypeptide chain brought about by irradiation are also more effective in decreasing these initial rates than the later ones. These results are in agreement with those found by Hippel & Wong (1963b) for chain-fission brought about by enzymes, and confirm the conclusion that the results are independent of the method of fission.

In a previous paper (Cooper & Davidson, 1965) it was concluded that the coil-to-helix transition in solutions of gelatin, prepared by the ultraviolet irradiation of neutral-salt-soluble collagen for 19 hr., followed by storing at 0°, could be one of the factors accounting for the abnormally high molecular weights obtained by gel filtration at 20°. The present results show, however, that after 19 hr. of irradiation the same gelatin preparation had a very low helical recovery on cooling to 15°.

Comparison of irradiation in air and nitrogen shows that in the latter case the amount of collagen-fold re-formation is considerably decreased for the same irradiation times. The amino acid analyses indicate a larger decrease in the histidine and phenylalanine content after irradiation in nitrogen. In addition, the sedimentation coefficient after irradiation in nitrogen is lower. These results, in conjunction with the viscosity and optical-rotation data, show that more denaturation occurs in the presence of nitrogen than in air. Generally, it appears that irradiation of proteins in the presence of nitrogen results in less denaturation when com-

pared with irradiation in air, and may in fact produce aggregation (see, e.g., Rideal & Roberts, 1951; Marsh, 1964). Therefore the present findings require further investigation to elucidate the effects of these two reaction media.

Some support for the theory (Gallop, 1964) that the α -components of collagen consist of four peptide chains linked end-to-end through hexose molecules was obtained from the increased reaction of gelatin, prepared from irradiated collagen, with 2,4-dinitrophenylhydrazine.

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