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Ultraviolet Light Irradiated Collagen Macromolecules*

Eiji Fujimori

ABSTRACT: Collagen macromolecules undergo photopolymerization when irradiated with ultraviolet light of 2537 A. The absorption spectrum of irradiated collagen shows an increase in extinction at the longer wavelengths (290–400 mμ). The decrease of ultraviolet fluorescences due to phenylalanine and tyrosine is accompanied by the concomitant appearance of new blue fluorescences excited at 350 mμ. Although the ultraviolet fluorescences of tyrosine and phenylalanine decrease uniformly with an increase in temperature, the intensity of the blue fluorescences shows an anomalous rise in the temperature range from 30 to 40°. This temperature range corresponds to that of a helix to random-coil transition as shown in the change of viscosity, optical rotation, and far-ultraviolet absorption. This transconformation temperature is lower in irradiated collagen than native collagen. The specific optical levorotation of irradiated collagen is greater than that of native collagen.

Irradiated collagen is still able to form segment-long-spacing (SLS)-like aggregates, even though the ability to form native fibers is lost. These studies show that the blue fluorescences result from the photoproducts of phenylalanine and tyrosine. These photoproducts are probably integral parts of the new linkages. The blue fluorescences are dependent on the structural conformation of a helical rigid body which is not seriously damaged by radiation.

It is known that ultraviolet light induces some proteins to aggregate and others to decompose (McLaren, 1949; McLaren and Shugar, 1964). However, very little is known about their molecular interaction or the change of molecular dimension in these processes. Most of the proteins studied so far are globular which contain disulfide, tryptophan, tyrosine, and phenylalanine. These processes effected by ultraviolet light at 2537 A involve, as the primary chemical reaction, photolysis of disulfide and aromatic residues (McLaren and Luse, 1961; McLaren and Shugar, 1964).

The aggregation of the structural proteins, fibrinogen (Slayter and Hall, 1964) and myosin A (Kaldor *et al.*, 1964), by ultraviolet light has recently been demonstrated. These proteins contain the above-mentioned four amino acid residues. Collagen, a stiff rodlike protein with the dimension of 2800 × 15 A does not contain disulfide bonds and tryptophan, but phenyl-

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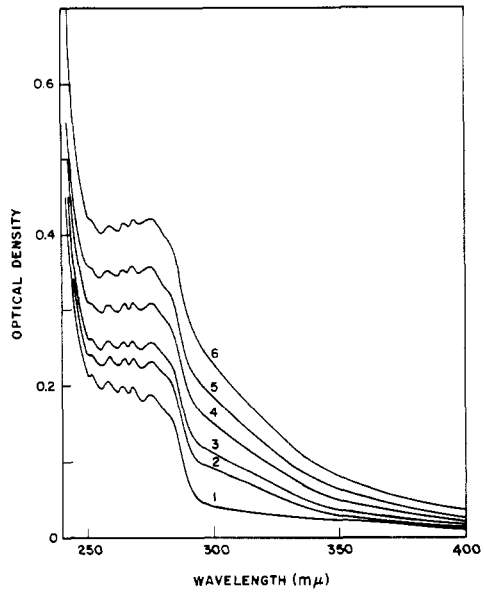

FIGURE 1: Absorption spectra of collagen irradiated with ultraviolet light: (1) before irradiation; (2) 0.5-hr; (3) 1-hr; (4) 2-hr; (5) 3-hr; and (6) 4-hr irradiation.

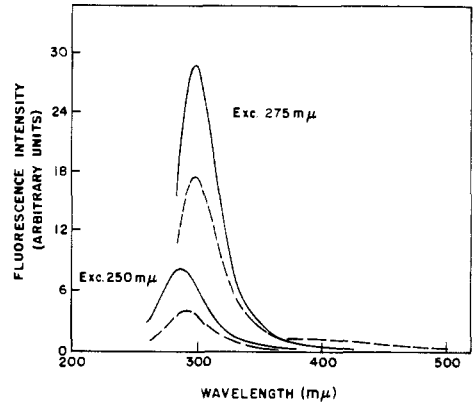

FIGURE 2: Ultraviolet fluorescence spectra of native collagen (———) and 4-hr-irradiated collagen (--------) excited at 250 and 275 mμ.

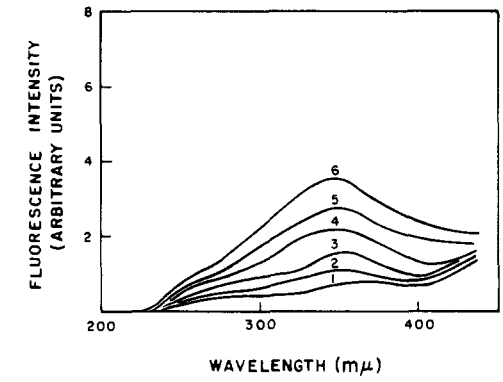

FIGURE 3: Action spectra of blue fluorescences at 450 mμ of irradiated collagen shown in Figure 1.

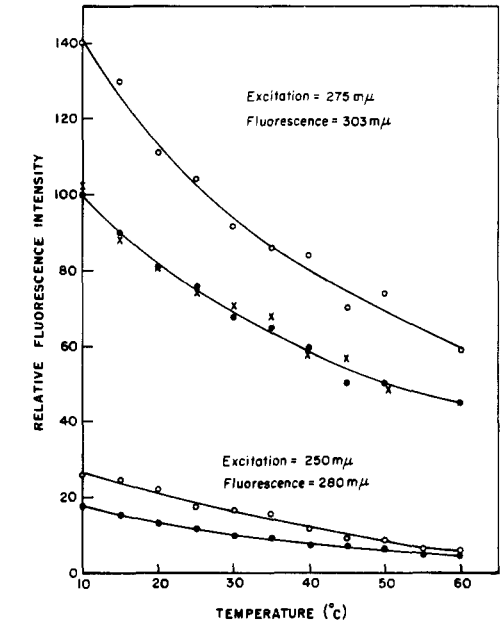

FIGURE 4: Ultraviolet fluorescence of native and irradiated collagen as a function of temperature. The fluorescence of tyrosine was excited at 275 mμ and measured at 303 mμ for native collagen (–O–O–) and 4-hr-irradiated collagen [(–●–●–), on heating from 10 to 60°, and (–×–×–), on cooling from 60 to 10°]. The fluorescence of phenylalanine was excited at 250 mμ and measured at 280 mμ for native collagen (–O–O–) and 3-hr-irradiated collagen (–●–●–).

alanine with a small amount of tyrosine. These aromatic amino acids appear to be located in the peptide chains which extend from the rigid helix body (Rubin *et al.*, 1963).

A previous paper (Fujimori, 1965) demonstrated that collagen, when irradiated with ultraviolet light at 2537 A, shows an increase in its length without appreciable change in its width. The irradiated collagen lost its ability to form native-type fibrils. It was suggested that tyrosine and phenylalanine are involved in the photochemical modification of the collagen molecule where more or less longitudinally linked molecules are formed.

This paper reports the finding of new blue fluorescences originating from the photoproducts of tyrosine and phenylalanine. These blue fluorescences are shown to be related to protein conformation. Further investigations of irradiated collagen on viscosity, far-ultraviolet absorption, optical rotation, and formation of segment-long-spacing (SLS) aggregates are correlated with its conformational change.

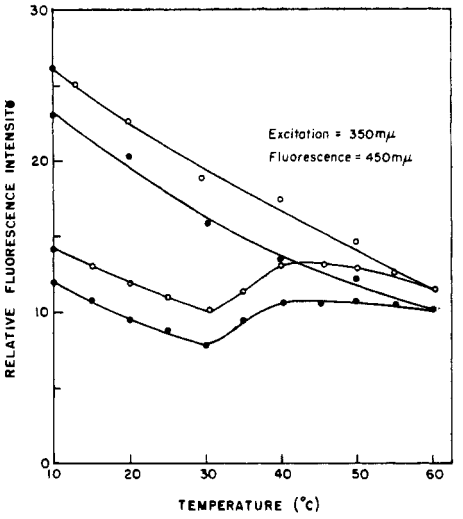

FIGURE 5: Blue fluorescence of irradiated collagen as a function of temperature. The blue fluorescence was excited at 350 mμ and measured at 450 mμ, for 4-hr-irradiated collagen (-O-O-) and 3-hr-irradiated collagen (-●-●-). In each case, a lower curve shows the change of fluorescence followed on heating from 10 to 60° and an upper curve on cooling from 60 to 10°.

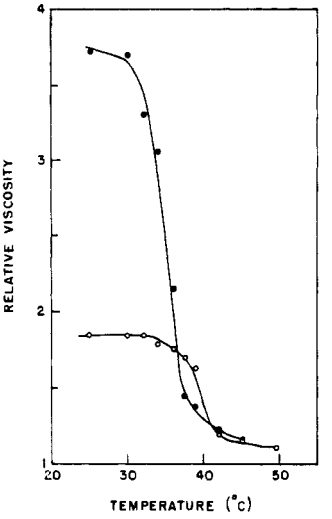

FIGURE 6: Viscosity of native collagen (-O-O-) and 4-hr-irradiated collagen (-●-●-) as a function of temperature.

Methods

An 0.05% acetic acid solution of 0.265% calf skin collagen in a shallow Petri dish was irradiated in air at a distance of 5 cm with an 8-w Minerallight SL 2537 (Ultraviolet Products, Inc.). A filter was used which transmits 35.7% of the radiation at 2537 A with no transmission below 2400 A. Aliquots were removed periodically and used for the various measurements without further dilution except for far-ultraviolet absorption measurement. The calf skin collagen was a gift from the Orthopaedic Research Laboratory, Massachusetts General Hospital, Boston, Mass.

Far-ultraviolet absorption spectra were measured with 1-mm cells in a Zeiss spectrophotometer which was flushed with nitrogen. Near-ultraviolet absorption spectra were measured on a Cary spectrophotometer, Model 14. Fluorescence spectra were determined with an Aminco-Keirs spectrophosphorimeter equipped with a RCA 1P28 photomultiplier for ultraviolet fluorescence. A RCA 1P21 photomultiplier was used for visible fluorescence. Fluorescence and action spectra are uncorrected for variation in photomultiplier sensitivity and spectral intensity of the light source (Xenon lamp), respectively. Monochromator calibration of the spectrophosphorimeter was made using a mercury lamp of pencil type. A Rudolph and Sons photoelectric spectropolarimeter 200 S was used to measure the optical rotation. Jacketed cells were employed for the optical measurements at different temperatures. Viscosity measurements were performed with a capillary viscometer.

The ability of native and irradiated collagen to form native-type fibrils and SLS-type aggregates was tested in the following ways. For native-type fibrils, solutions of native and irradiated collagen were dialyzed against 0.1 M Tris buffer (pH 7.2) overnight at 4°. SLS-type aggregates formation was tested by the addition of 1.5% adenosine triphosphate (ATP) in 0.05% acetic acid solution to the samples of native and irradiated collagen which were dialyzed against 0.05% acetic acid solution at 4°. The final concentration of ATP was 0.1%. The precipitates obtained in both treatments were mixed with 1% phosphotungstic acid in 0.15 M sodium acetate solution, and the solutions were treated with acetic acid to a pH of 6.3. The suspensions mounted were kept at room temperature for 2 min. The preparations were washed with distilled water, dried, and examined with a Siemens electron microscope.

Results

The absorption spectrum of collagen exhibits structural bands of phenylalanine at 251, 257, 263, and 267 mμ in addition to a band at 275 mμ due to the small amount of tyrosine in collagen (Figure 1, 1). The irradiation of collagen with ultraviolet light at 2537 A increased the extinction at these wavelengths, in addition to an absorption appearing at a longer wavelength region (>290 mμ) (Figure 1, 2–6). The change in absorption spectrum during irradiation of proteins in solution could generally result from photochemical reaction, from aggregation which causes light scattering, or a combination of these events. Previous studies of the photoproducts of both tyrosine and phenylalanine in solution which absorb at the longer wavelengths (290–400 mμ) (McLaren and Shugar, 1964) suggest that there is a real absorption in this region, due to the photoproducts

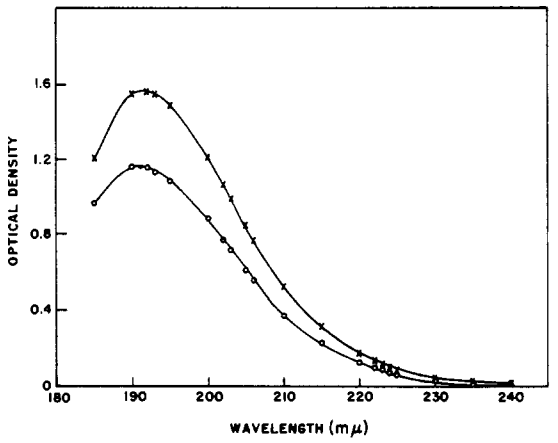

FIGURE 7: Far-ultraviolet absorption spectra of native (-O-O-) and 4-hr-irradiated collagen (-X-X-) (0.013%).

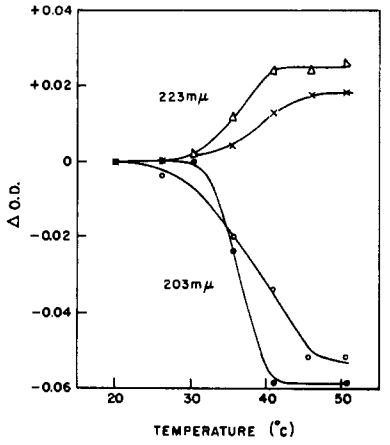

FIGURE 8: Far-ultraviolet absorption of native collagen [(-O-O-) at 203 mμ, and (-X-X-) at 223 mμ] and 4-hr-irradiated collagen [(-●-●-) at 203 mμ, and (-Δ-Δ-) at 223 mμ] (0.013%) as a function of temperature.

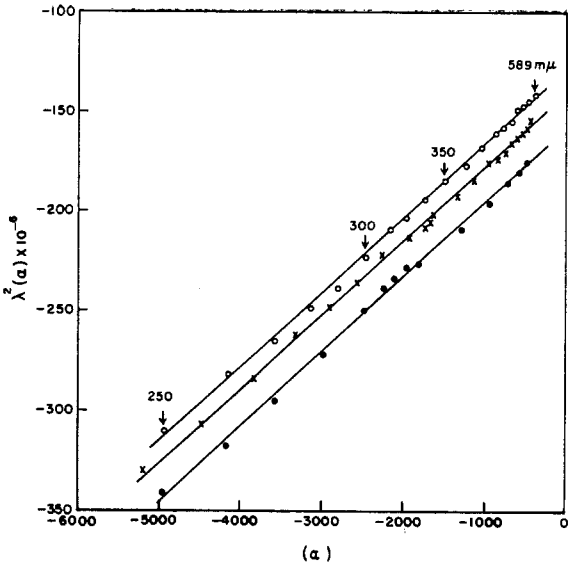

FIGURE 9: Drude plot of the optical rotatory dispersion of native and irradiated collagen at room temperature: (-O-O-) native; (-X-X-) 1-hr-irradiated; and (-●-●-) 4-hr-irradiated collagen.

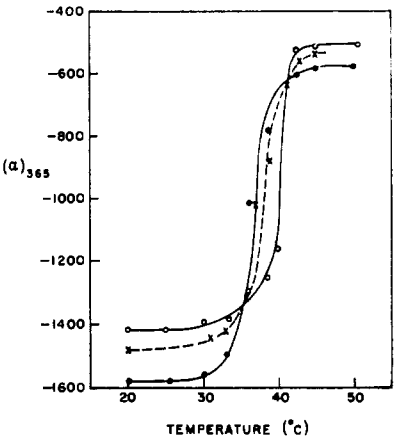

FIGURE 10: Optical rotation of native and irradiated collagen as a function of temperature: (-O-O-) native; (-X-X-) 2-hr-irradiated; and (-●-●-) 4-hr-irradiated. $[\alpha]_{365}$ is a specific optical rotation at 365 mμ.

of tyrosine and phenylalanine, in irradiated collagen.

Photochemical changes of tyrosine and phenylalanine in collagen were detected more directly from the change of their fluorescence characteristics. Tyrosine and phenylalanine of collagen fluoresce at about 300 and 280 mμ when excited with light of 275 and 250 mμ, respectively (Figure 2). This result shows that excited phenylalanyl residues in collagen are capable of fluorescing, although some of the excitation energy might possibly be transferred to tyrosine which fluoresces. As shown in Figure 2, irradiated collagen showed a decrease in the fluorescences of tyrosine and phenylalanine, indicating a photochemical change in these aromatic residues of the irradiated collagen. The observed shift of the fluorescence towards longer wavelengths when irradiated collagen is excited at 250 mμ suggests the formation of tyrosine from phenylalanine as an intermediate. The formation of tyrosine from irradiated phenylalanine solution has been reported (Matsuda *et al.*, 1954; Luse and McLaren, 1963).

Additional evidence to show that the photoproducts of tyrosine and phenylalanine in collagen actually absorb in the range from 290 to 400 mμ is provided by the observation that irradiated collagen, when excited at 350 mμ, emits blue fluorescences which can also be produced when 2537-A-irradiated solutions of tyrosine

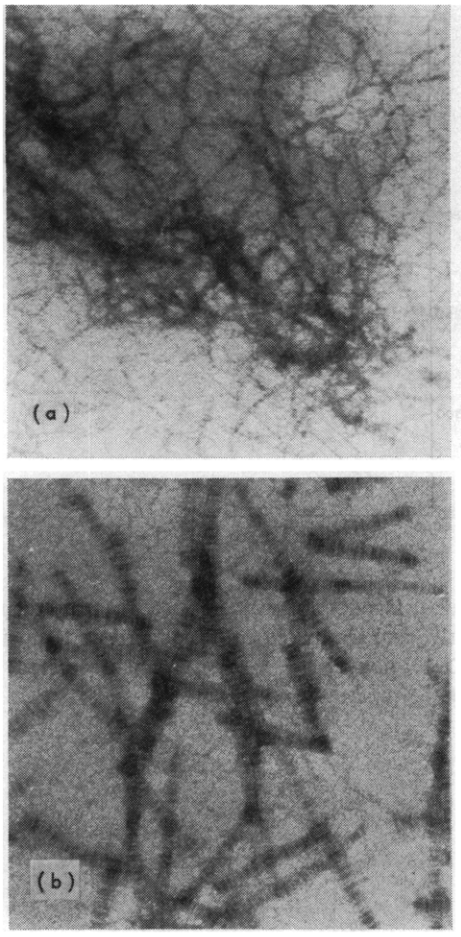

FIGURE 11: Electron micrographs of (a) 1-hr-irradiated collagen treated to form native-type fibers (×38,000) and (b) 4-hr-irradiated collagen treated to form SLS-type aggregates (×21,000).

or phenylalanine are excited at 350 mμ. The blue fluorescences at 450 mμ increased during irradiation of collagen, and this was accompanied by the decrease of the ultraviolet fluorescences from tyrosine and phenylalanine. The action spectra of the total blue fluorescences at 450 mμ of irradiated collagen show a maximum at about 350 mμ (Figure 3). These observations indicate without doubt that this maximum can be attributed to the photoproducts formed from the irradiation of the aromatic amino acids, tyrosine and phenylalanine, in collagen.

In order to establish this finding, a detailed study of the absorption, fluorescence, and action spectra of irradiated solutions of tyrosine and phenylalanine was undertaken. This confirmed that not only the increase of absorption in a longer wavelength region (290–400 mμ) but also the appearance of the blue fluorescence accompanied by the concomitant decrease of the ultraviolet fluorescences is due to the irradiation products of tyrosine and phenylalanine. The blue fluorescence from irradiated tyrosine appears at the same wavelength range as that of irradiated phenylalanine, though the former is of higher intensity than the latter. The detailed results will be published elsewhere.

Collagen undergoes conformational changes when heated, dissociating into subunits (Harrington and von Hippel, 1961). During the thermal transition from a helical structure to a random coil, no irregular change in the ultraviolet fluorescences of phenylalanine and tyrosine was observed in both native collagen and irradiated collagen as shown in Figure 4. However, the blue fluorescence at 450 mμ of irradiated collagen, when excited at 350 mμ, did show a unique feature in the thermal profile of fluorescence intensity followed on heating from 10 to 60°. Figure 5 illustrates the peculiar rise in the fluorescence intensity in the temperature range from 30 to 40°. On cooling from 60 to 10°, the fluorescence intensity increased uniformly but at an elevated value. The temperature range for the anomalous rise corresponded to that of the helix to random-coil transition. This was shown by the changes of viscosity, optical rotation, and far-ultraviolet absorption spectra as a function of temperature.

As reported in a previous paper (Fujimori, 1965), the viscosity of irradiated collagen is greater than that of native collagen. The viscosity of the irradiated collagen began to decrease at 30°, indicating a transition temperature at about 35° (Figure 6). Native collagen has a transition temperature of about 38°. This lower transformation temperature in irradiated collagen was further confirmed by far-ultraviolet absorption and optical rotation studies.

The far-ultraviolet absorption spectrum of collagen has only one peak at 190 mμ without any splitting (Gratzer *et al.*, 1963). Irradiated collagen showed a higher extinction than native collagen over the wavelength range of 185 to 240 mμ (Figure 7). On heating irradiated and native collagen, the optical density at 223 mμ increased and that at 203 mμ decreased (Figure 8). These results also demonstrate the lower transition temperature in irradiated collagen.

Collagen exhibits a large specific optical levorotation [α] (Harrington, 1958; Harrington and von Hippel, 1961). The specific optical levorotation of irradiated collagen was greater than that of native collagen. Linear plots of $\lambda^2[\alpha]$ against [α] indicate that both native and irradiated collagen obey the Drude equation with the same optical rotatory dispersion constant ($\lambda_c \sim 195$ mμ) (Figure 9). The conformation change from a helix to a random coil brings about a considerable decrease in the specific levorotation. This change of optical rotation occurred at a lower temperature for irradiated collagen (Figure 10).

The previous investigation showed that the ability of collagen to form native-type fibrils is lost with ultraviolet irradiation. Observations with the electron microscope confirmed this finding and also indicated that irradiated collagen is capable of forming SLS-like aggregates. Native collagen forms both native fibers and SLS aggregates. As shown in Figure 11(a), native fibers were no longer formed after irradiation for 1 hr. It is apparent in Figure 11(b) that SLS-like aggregates were still formed even after 4 hr of irradiation.

Discussion

The primary chemical reactions in 2537-A-irradiated proteins, containing aromatic amino acids but not disulfide bonds, involve the photolysis of these amino acids which form free radicals (Swenson *et al.*, 1963). Subsequent reactions involve photooxidations. It was suggested previously that photolysis of peptide bonds might be a significant process in the irradiation of proteins at 2537 A. Various studies have proved that the effect of irradiation at 2537 A is not manifested in the rupture of the peptide bond, but in the aromatic amino acids and disulfide bonds. Photolysis of peptide bond and histidine plays a role when the proteins are irradiated with light consisting of wavelengths below 2400 A. This region of the spectrum is absorbed appreciably by these groups (McLaren and Shugar, 1964).

The mechanism of the photooxidation of aromatic amino acids is still relatively unknown. As mentioned before, the formation of tyrosine as one of the photoproducts from phenylalanine has been reported. Dihydroxyphenylalanine (dopa) reported among the photoproducts from irradiated tyrosine and phenylalanine (Luse and McLaren, 1963) undergoes further oxidation to form a melanin-like pigment probably *via* a dopa-quinone type product. The blue fluorescent photoproduct reported here would be some intermediate which absorbs at the near-ultraviolet region. This blue fluorescence has not been reported in the investigations on the photochemistry of proteins. In the present investigation, a peculiar characteristic of this blue fluorescence in irradiated collagen is shown in its relation to protein conformation.

A number of proteins containing aromatic amino acids fluoresce in the near-ultraviolet region (Konev, 1957; Teal, 1960). In some proteins, the fluorescence of tyrosine and tryptophan changes with the conformational change of the protein structure. In the case of ribonuclease, the change in protein structure is clearly reflected in the dependence of tyrosine fluorescence intensity upon temperature (Gally and Edelman, 1962) and denaturating agents (Cowgill, 1964). Structural changes also modify the fluorescent intensity of tryptophan in γ-globulin, chymotrypsinogen (Steiner and Edelhoch, 1963), and lysozyme (Steiner, 1964). If most of the aromatic amino acids are situated in the randomly coiled portion of collagen, the regular thermal changes observed in the fluorescences of tyrosine and phenylalanine in collagen would be expected.

However, the blue fluorescences from the photochemically modified tyrosyl and phenylalanyl residues in irradiated collagen are shown to be greatly dependent on the conformation of protein structure. The blue fluorescences are quenched to a certain extent in the helix conformation. This quenching effect is removed by the thermal transition to a random coil, resulting in the increase of the blue fluorescence. It has further been shown that the temperature for this helix–coil transition becomes lower in irradiated collagen. These results point to an association between the helix conformation and new linkages which are probably responsible for the blue fluorescence. This new linkage in some way interacts with the helix portion of the molecule to lower the energy of transition. This interaction is shown by the increase of the blue fluorescence intensity with the decrease of helix content in the transition at a lower temperature. The chemical nature of this blue fluorescent center and the new linkage involved are open to future investigations.

The various properties of irradiated collagen indicate that there is no serious photodamage in the rigid helical portion of the collagen molecule. This is shown by the conformation-dependent far-ultraviolet absorption as well as by the temperature-dependent high viscosity for the irradiated collagen. Generally, the far-ultraviolet absorption spectra of α-helical proteins show two bands at 190 and 205 mμ due to exciton splitting of π–π^* band (Imahori and Tanaka, 1959; Rosenheck and Doty, 1961; Gratzer *et al.*, 1961) and one band at 225 mμ, a n–π^* transition (Schellman and Oriel, 1962). Optical densities of these wavelengths are known to depend on the conformation of the protein structure. Although only one band at 190 mμ is observed in case of collagen, the results show that the optical densities at 203 and 223 mμ are dependent upon the conformation of both native and irradiated collagen.

The results of the optical rotation studies also support the conclusion that the helical structure of irradiated collagen is nearly intact. The α-helical conformation has a negative Cotton effect at about 225 mμ (Simmons *et al.*, 1961), which arises from the n–π^* transition of the peptide chromophore. A positive Cotton effect exists at 190 mμ due to the π–π^* transition (Blout *et al.*, 1962; Holzwarth *et al.*, 1962). Collagen shows no 225-mμ Cotton effect, but a negative Cotton effect has recently been found at 195–196 mμ (Blout *et al.*, 1963). Irradiated collagen exhibits a greater specific levorotation than native collagen, although each possesses the same optical rotatory dispersion constant at 195 mμ. The loss of helical structure in thermal denaturation is reflected in a large decrease in the specific levorotation.

The above results show that the rigid helix body is rather insensitive to ultraviolet irradiation at 2537 A and tends to remain unchanged. The intact helix body would be essential for forming SLS-like aggregates. Irradiated collagen is still capable of forming these aggregates to some extent. On the other hand, the structure of the collagen molecule responsible for the formation of native-type fibers is particularly susceptible to ultraviolet light. According to the previous investigation (Rubin *et al.*, 1963), this structure of the molecule probably consists of dangling, flexible peptide chains, protruding from the rigid helix body, which are rich in aromatic residues.

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