Effects of ultraviolet radiation on the type-I collagen protein triple helical structure: A method for measuring structural changes through optical activity

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A detailed study of the effects of ultraviolet radiation on type-I collagen has been conducted. We have confirmed that exposure to ultraviolet radiation lowers the denaturation temperature of type-I collagen and that the triple helical state is destroyed provided that the radiation dose exceeds a threshold level, which is defined as the incident radiation dose that raises the sample temperature above the (lower) denaturation temperature. For incident radiation doses below threshold, the collagen molecule remains in a triple helical state. Denaturation is determined by changes in the optical activity of the collagen solution. Furthermore, a new instrument has been developed and tested to measure the optical rotatory dispersion properties of chiral molecules. The advantage of this instrument is that it enables a real-time measurement of the optical activity of chiral macromolecules while exposing samples to ultraviolet radiation and requiring no special sample preparation techniques. Using a differential measurement scheme, system errors have been minimized.

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I. INTRODUCTION

The effects of ultraviolet (UV) radiation on the collagen molecule are not completely understood at the present time; certain characteristics of the denaturation process have been researched in detail, but a complete model does not exist. Earlier ultraviolet radiation experiments on collagen samples failed to quantify in a satisfactory manner the intensities and wavelengths of the radiation. Existing data are limited and in certain instances appear to be contradictory. Some experiments report measurable effects of UV radiation on the denaturation processes and/or cross-linking properties, while others claim that no observable effects exist. Hayashi *et al.* [1] found that exposing collagen solutions to the UV light in circular dichroism spectrometers damaged the molecule and affected its denaturation profile. Na [2] found that there were no measurable affects on the denaturation profile of collagen when exposed to the ultraviolet light of a spectrophotometer. More recently, Miles *et al.* [3] have suggested that exposure of collagen to UV light cleaves the molecule at random points, while preserving the triple-helical structure, resulting in a lower denaturation temperature. Fujimori [4] reported on cross-linking in collagen by exposing samples to ultraviolet light; it was determined that cross-linking was strongly dependent on the solution and oxygen concentration. Fujimori [5–7] reported further that UV light cleaves the collagen molecule without destroying the triple-helical structure. The studies of Kato *et al.* [8] and Miyata *et al.* [9] analyzed the degradation of collagen when exposed to ultraviolet light. In the work of Davidson and Cooper [10–12], it was found that the effect of ultraviolet light on collagen resulted in the formation of shorter peptide chains in a random-coil formation rather than a helical formation. Kaminska *et al.* [13] concluded that the levorotation of collagen gradually decreased under exposure to ultraviolet light, indicative of a change in the triple-helical structure of the collagen molecule. Yova *et al.* [14] found that irradiation of collagen by 337-nm radiation changed the fluorescence spectrum of collagen and was similar to the fluorescence spectrum of denatured collagen. Also there exist experiments which point toward a multistep denaturation process which is irreversible and strongly dependent on the solution. Other experiments indicate reversible processes [15,16].

A better understanding of the effects of ultraviolet radiation on collagen is important since this is the most abundant protein in biological tissue. These data are particularly significant since UV photoactive dyes are being explored as a means of altering or enhancing collagen's structural and biological properties [17,18]. Since changes in optical rotatory dispersion are a function of the triple-helical concentration, our experiments have been able to measure changes in the collagen helical content. As is well known, collagen denaturation results in a complete loss of molecular triple-helical content. Our data support the evidence that the collagen triple-helical structure is destroyed under specific conditions. In our work, type I collagen samples were exposed to broadband and narrowed bandwidths of ultraviolet radiation while monitoring incident ultraviolet intensity, sample temperature, and duration of exposure. The data indicate that certain doses and wavelengths of ultraviolet radiation damage the collagen

molecule but do not completely destroy the triple-helical structure unless the radiation dose exceeds a threshold level. This threshold level was determined experimentally and is defined as the incident radiation dose needed to raise the sample temperature above the (lower) denaturation temperature which caused a change in optical activity in the collagen sample equal to that caused by thermal denaturation.

The resolution and stability of current instrumentation limits detailed real time studies of macromolecular denaturation through optical activity. In this experimental work, we also present a description of a novel instrument, based on interferometry, developed to study real time denaturation events through optical activity.

II. OPTICAL ACTIVITY IN MACROMOLECULES

Optical activity is defined as a property in which a substance will absorb incident (optical) radiation and/or change its polarization state. Such optically active molecules are known as chiral. These molecules are also asymmetric and hence possess a handedness. In protein molecules (i.e., biopolymers or macromolecules), optical activity is related to the secondary structure of the molecule. Helical proteins, such as collagen, consist of long flexible chains which comprise their secondary structure. The collagen molecule has a characteristic triple-helical structure that resembles a rope-like structure which results in its optical activity [19]. The denatured state of collagen is characterized by the unraveling of the triple helix; the denaturation process is referred to as a helix to (random) coil transition. This process causes a change in its optical activity.

The optical rotatory dispersion phase measurement system (ORDPMS) is the instrument developed to conduct these experiments. It has been designed to give a real time measurement of optical rotatory dispersion and is currently being used to study changes in biopolymer structure.

Optical rotatory dispersion (ORD) occurs when a material exhibits a difference in its index of refraction for right-handed (n_r) and left-handed (n_l) circularly polarized light. Circular dichroism (CD) is the observed elliptical polarization of right-handed and left-handed circularly polarized light produced by an optically active medium [19]. An optically active material will preferentially absorb either left-handed or right-handed circularly polarized light.

A. Electromagnetic theory

The general expression for the electric field of a propagating electromagnetic wave is given by

$$\vec{E}(\vec{z},t)=(\hat{\varepsilon}_1\vec{E}_1+i\hat{\varepsilon}_2\vec{E}_2)\exp(i\vec{k}\cdot\vec{z}-i\omega t). \tag{1}$$

This may be rewritten to describe the electric fields of left-handed and right-handed circularly polarized electromagnetic waves (same amplitude),

$$\vec{E}_r=\vec{E}_o(\hat{i}\cos\theta_r+\hat{j}\sin\theta_r), \tag{2}$$

$$\vec{E}_l=\vec{E}_o(\hat{i}\cos\theta_l+\hat{j}\sin\theta_l), \tag{3}$$

with

$$\theta=2\pi\omega\left[t-\left(\frac{\eta z}{c}\right)\right]. \tag{4}$$

Here η describes a complex index of refraction. For left-handed and right-handed circularly polarized electromagnetic waves, the complex index of refraction is

$$\eta_r=n_r+ik_r, \tag{5}$$

$$\eta_l=n_l+ik_l. \tag{6}$$

The difference in the *real* part of the indices of refraction is related to optical rotatory dispersion R_{ORD} while the difference in the *imaginary* part of the indices of refraction is related to circular dichroism (absorption), R_{CD} according to

$$R_{\mathrm{ORD}}\equiv\frac{\pi z(n_l-n_r)}{\lambda}, \tag{7}$$

$$R_{\mathrm{CD}}\equiv\frac{\pi z(k_l-k_r)}{\lambda}. \tag{8}$$

B. Theory of optical activity

From a group-theoretical treatment, optical activity occurs only for asymmetric molecules and arises from the nonorthogonality of molecular electric and magnetic dipole moments [19]. The physics is as follows. Helical molecules promote electron mobility along a helical path, while the magnetic dipole transition moment causes electrons to move in a circular path wherein the electric dipole transition moment causes linear electron motion. The result is a coupling between electric and magnetic dipole transitions giving rise to optical activity. Hence, the helix is an ideal structure for supporting optical activity.

Using semiclassical theory, it was postulated that Maxwell's equations were incomplete in describing the interaction of polarized radiation with optically active media. Applying the standard form of these equations for a nonconducting medium, solutions are of the form

$$\vec{D}=\varepsilon\vec{E}, \tag{9}$$

$$\vec{B}=\varkappa\vec{H}. \tag{10}$$

Here ε and $\varkappa$ are the electric and magnetic susceptibilities, respectively. The *Ansätze* for corrections to the electric and magnetic fields in chiral media are given by [19,21]

$$\vec{D}=\varepsilon\vec{E}-4\pi N\frac{\beta}{c}\frac{\partial\vec{H}}{\partial t}, \tag{11}$$

$$\vec{B}=\varkappa\vec{H}-4\pi N\frac{\beta}{c}\frac{\partial\vec{E}}{\partial t}. \tag{12}$$

The additional terms account for the interaction of the electric and magnetic fields with matter in an optically active

medium. Through a further quantum-mechanical treatment one may show that the observed rotation (α) is a sum over molecular transition states:

$$\alpha = \frac{\pi d(n_l - n_r)}{\lambda_0} = \frac{16\pi^3 Nz}{\lambda_0^2 c}\beta, \tag{13}$$

$$\beta = \frac{c}{3h}\sum_b \frac{\mathrm{Im}[\langle a|\vec{\mu}|b\rangle \cdot \langle b|\vec{m}|a\rangle]}{\nu_{ba}^2 - \nu^2}. \tag{14}$$

Here ν is the frequency of the (measuring) radiation, ν_{ba} the frequency of radiation associated with the transition from the a ground state to the b excited state; $\langle a|$ is the wave function of the a state and $\langle b|$ is the wave function of the b state. Also, $\vec{\mu}$ is the electric dipole moment, $\vec{m}$ is the magnetic dipole moment, N is the number of electrons, c is the speed of light in vacuum, d is the sample path length, β is the optical rotatory parameter as defined above, and Dirac notation is employed.

C. The collagen molecule

The terms ''polymer'' and ''macromolecule'' designate high-molecular weight materials of either synthetic or natural origin. In the natural origin case (e.g., a protein), the polymer is classified as a biopolymer. In order to be considered a polymer, a molecule must consist of a great number of repeating units. Biopolymers possess a hierarchy of their structure: primary, secondary, tertiary, and sometimes quaternary. The primary structure is the sequence of repeating units in the chain. The secondary and tertiary structures are the short-scale and long-scale (respectively) order in the monomer position. The secondary structure usually contains on the order of 10 monomer units of the chain. The main secondary structures are the α, β, and triple helix. The ''untwisting'' of the helix is referred to as the triple-helix to (random) coil transition. For the collagen molecule, the helix to (random) coil transition is also referred to as denaturation. Triple-helix denaturation is accompanied by absorption of a large amount of heat used to break the hydrogen bonds that stabilize the triple helix [20,22].

Collagen has a triple-helix structure which consists of three polypeptide chains (α helices) coiled into a left-handed helix, of which two are $\alpha_1(I)$ chains and one is the $\alpha_2(I)$ chain. The differences in the $\alpha_1(I)$ chain and the $\alpha_2(I)$ chain are in the amino acid sequence. The three chains are wrapped around each other to form a right-handed triple helix [20,22–25]. The triple helix has a structural repeating distance of approximately 8.6 nm; its overall length is approximately 300 nm. The molecular weight is approximately 3×10^5 grams/mol, and a mean residue weight (the molecular weight of the polymer divided by the number of monomers, i.e., weight per unit length) of approximately 100 grams/mol.

Each of the three α helices has a primary sequence of amino acids in a repeating Gly-X-Y sequence, where Gly is the glycine amino acid; X and Y are frequently the proline and hydroxyproline amino acids, respectively [15,25,26]. Usually 33% of the amino acids which comprise the collagen molecule are found to be the glycine amino acid. Of the remainder, 15–30 % are the proline and hydroxyproline amino acids. The main stabilizing forces in the collagen triple helix are due to extensive hydrogen bonding [15,23,27,28].

D. Thermal denaturation experiments

There have been a number of researchers which have studied the optical rotation and rotatory dispersion of collagen. It has been demonstrated that the levorotation of collagen (its optical activity) diminished upon denaturation [15,24]. Specific values of the optical rotation were calculated for various types of collagen under specific conditions. The most common method of denaturing collagen is through heating. Heating type-I bovine collagen above a critical temperature T_d (the denaturation temperature) will induce denaturation fairly rapidly and completely destroy the triple-helical structure. The critical temperature for pepsin-treated type-I bovine collagen is approximately 38 °C; the duration of heating at the critical temperature for a complete helix to coil transition is approximately 5–10 min [15]. This transition is irreversible in our samples, and can be monitored by measuring the change in the UV absorption spectrum over various temperature setpoints [24].

The denaturation temperature is also defined as the point where the increase in absorption begins to plateau (while monitoring at a fixed wavelength). Figure 1 shows absorption data at 227 nm for different sample temperatures; the samples were diluted to a concentration of 0.315 mg/ml in a 0.012N HCl solution. Samples were heated, while in the spectrophotometer, in a 10-mm (path length) quartz cuvette. The denaturation temperature is seen to be approximately 38 °C. Absorbance measurements indicated a $+0.23$ A (±0.02 A) increase in absorbance measured at 227 nm wavelength [24,29,30].

E. Measurement of optical rotation

The measured optical rotation depends on the number of molecules encountered by the electromagnetic wave and is a function of the concentration of the optically active compound and path length. The standard used in measuring optical activity is the specific rotation $[\alpha]_\lambda$ [15,24,28],

$$[\alpha]_\lambda = \frac{\alpha}{\rho d}, \tag{15}$$

where α is the observed rotation, ρ is the concentration, and d is the path length.

The units of measurement in calculating the specific rotation are g/ml for concentration and decimeters (dm) for path length. In terms of the indices of refraction, the observed rotation is

$$\alpha = \frac{\pi d}{\lambda_0}(n_l - n_r). \tag{16}$$

Here n_l is the index of refraction for left-handed circularly polarized radiation, n_r is the index of refraction for

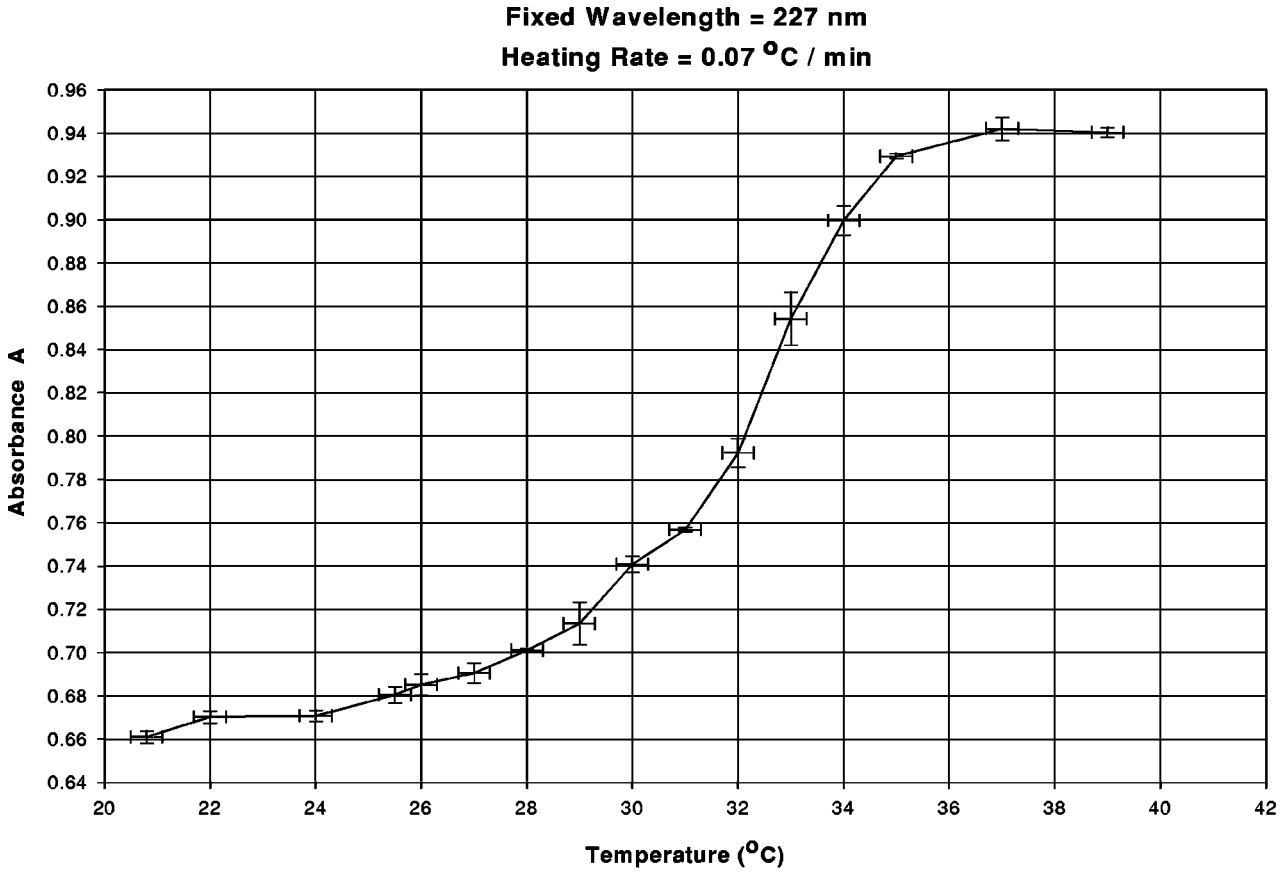

FIG. 1. Absorbance measurements (in a 10-mm cuvette) of pepsin-treated type-I bovine collagen at 227 nm. Samples were diluted to a concentration of 0.315 mg/ml in a 0.012*N* HCl solution. Measurements made for a heating rate of 0.07 °C/min. The graph shows that the transition temperature is approximately 33 °C and the denaturation approximately 38 °C.

right-handed circularly polarized radiation, d is the sample path length, and λ_o is the wavelength of radiation measured in vacuum.

Combining Eqs. (15) and (16), and converting the path length (d) and concentration (ρ) to MKS units, yields

$$[\alpha]_\lambda = \frac{10\pi}{\lambda_0 \rho}(n_l - n_r). \tag{17}$$

Equations (15)–(17) yield

$$\alpha = ([\alpha]_\lambda)\rho d. \tag{18}$$

Moffitt and Yang [31] developed a two-term equation to predict the specific rotation of optically active molecules, given by

$$[\alpha]_\lambda = \frac{100(n^2+2)}{3M}\left[\frac{a_0\lambda^2}{\lambda^2-\lambda_0^2} + \frac{b_0\lambda_0^4}{(\lambda^2-\lambda_0^2)^2}\right]. \tag{19}$$

Here, λ is the wavelength of the measuring radiation (light), and a_o, b_o, and λ_o are constants derived through curve fitting, and are referenced at the specific wavelength λ_o. These constants are reflective of the solvent, structural properties of the triple-helical molecule and wavelength. M is the mean residue weight (the molecular weight of the polymer divided by the number of monomers in units of grams per mole) and n is the refractive index of the medium. The constant a_o is strongly dependent on the solvent while b_o is relatively solvent-independent. The magnitude of the constant b_o reflects the rotational strength of the transition near λ_o, which is related to the screw sense of the helical molecule [19].

An estimate for the amount of rotation was calculated from Eqs. (18) and (19) and compared to our measured values for the optical rotatory dispersion by denaturing collagen samples through heating. The samples studied in our experiments were pepsin-treated type-I bovine collagen with a concentration of 3.15 mg/ml in a 0.012*N* HCl solution. Optical rotation was measured in our instrument using a He-Ne laser (633 nm wavelength) and samples were contained in a 40-mm path length cuvette. A mean residue weight (M) of 100 g/mol was used for collagen and a value of 1.4 for the index of refraction of the 0.012*N* HCl solvent.

Specific data for pepsin-treated type-I bovine collagen were not available. Table I contains the data used to estimate the amount of change in optical rotation from the collagen samples upon denaturation [24]. As an estimate for the amount of expected rotation, a value of -0.6 deg was calculated for the optical rotation of the native state and a value of -0.1 deg for the denatured state. Hence, the change in optical rotation for the triple-helix to random-coil transition

TABLE I. Values of the constants for the Moffitt and Yang equations (for the native and denatured states) used in calculating the specific and observed rotation for the 632.8-nm wavelength.

Conditions	a_o (deg)	b_o (deg)	λ_o (nm)	$[\alpha]_{633}$ (deg mg/g dm)	α_{633} (deg)
Native (triple-helix)	−2840	+250	202	−414	−0.6
Denatured (random-coil)	−550	−20	214	−93	−0.12

of the collagen solution was estimated at $+0.55$ deg. For a 40-cm path length cuvette, this gives a normalized value of $+0.13$ deg/cm. This value is in good agreement with our thermal experiments, which yielded a $+0.15°$/cm rotation upon denaturation.

III. APPARATUS

A new instrument has been developed and tested to conduct our experiments. This instrument has the ability to monitor changes in optical rotatory dispersion in real time, and for long-term experiments, while exposing the sample to ultraviolet radiation. Furthermore, special sample preparation techniques are not required, unlike other instruments which are limited by difficult sample preparation techniques and long-term measurement stability. We developed and characterized the ORDPMS and determined that it has outstanding resolution and stability needed to perform these measurements.

A schematic of the ORDPMS is given in Fig. 2. A fundamental component of the ORDPMS is the Zeeman laser. This single mode He-Ne laser has its center frequency split by an axial magnetic field which is generated by a permanent magnet surrounding the laser tube. A coil wound around the magnet generates an additional magnetic field and is controlled by a phase-locked loop. This stabilizes the Zeeman frequency to within 100 Hz of drift. The center frequency is stabilized by a piezoelectric transducer which adjusts the cavity length to center the two frequencies on the gain curve.

The beam is Zeeman split into right-handed and left-handed circularly polarized beams (r state and l state). The intensities are used in a feedback loop to stabilize the center frequency. Heterodyning these polarized beams generates a 1.73 MHz beat frequency. The magnetic field used to control and generate the Zeeman splitting is axial. A detailed description of this new instrument has been given elsewhere [32,33].

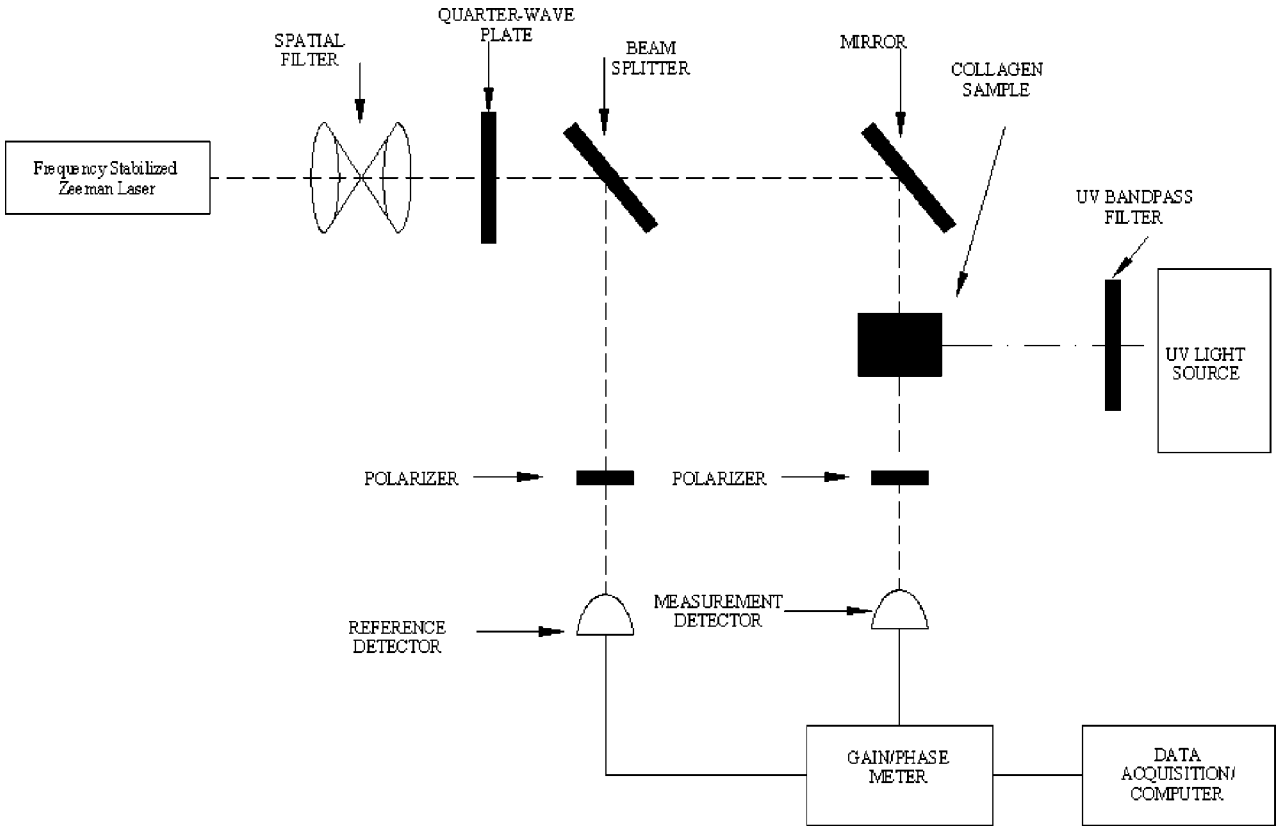

FIG. 2. Diagram of the experimental apparatus for the Optical Rotary Dispersion Phase Measurement System.

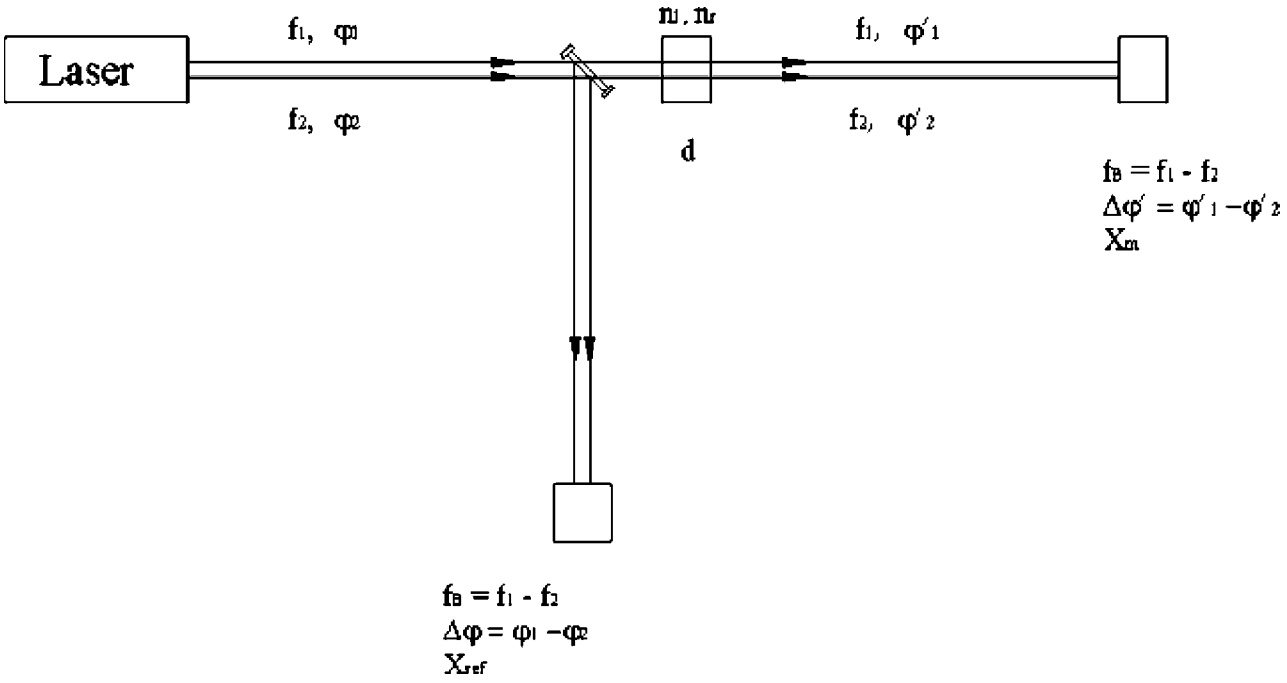

FIG. 3. Optical rotary dispersion phase measurement system: frequencies, index of refraction, and phase properties.

A. Phase rotation analysis

Since the Zeeman laser output consists of two beams of equal amplitude and frequency shifted with respect to each other, the total electric field exiting the laser is

$$\vec{E}(\vec{z},t)=\vec{E}_r+\vec{E}_l. \tag{20}$$

Substituting the expressions from Eqs. (2) and (3) yields

$$\vec{E}(\vec{z},t)=2E_0\cos\left(\frac{\theta_r+\theta_l}{2}\right)\times\left[\hat{i}\cos\left(\frac{\theta_r-\theta_l}{2}\right)-\hat{j}\sin\left(\frac{\theta_r-\theta_l}{2}\right)\right]. \tag{21}$$

The frequencies and indices of refraction for the left-handed and right-handed circularly polarized electric fields (respectively) are

$$\theta_l=2\pi\omega\left[t-\left(\frac{\eta_l z}{c}\right)\right], \tag{22}$$

$$\theta_r=2\pi\omega\left[t-\left(\frac{\eta_r z}{c}\right)\right]. \tag{23}$$

The relation between the observed phase shift and the optical rotatory dispersion may be derived as the difference between the reference and measurement intensities. Figure 3 shows the frequency, index of refraction, and phase properties in the ORDPMS from where the phase difference Φ is defined as

$$\Phi=\Delta\varphi'-\Delta\varphi, \tag{24}$$

$$\Phi=(k_l-k_r)x_{\mathrm{ref}}-(k_l\Lambda_l-k_r\Lambda_r). \tag{25}$$

Here $\Lambda_r=n_r x_m$ and $\Lambda_l=n_l x_m$ are the optical path lengths (for right-handed and left-handed circular polarizations, respectively) measured from the laser to the measurement detector, x_{ref} is the distance from the laser to the reference detector, and d is the sample thickness, where we have defined the following quantities:

$$x_\Delta=x_{ref}-x_m,$$

$$n_\Delta=n_l-n_r,$$

$$k_c=(k_l+k_r)/2.$$

Substitution into Eq. (24) gives

$$\Phi=(k_l-k_r)x_\Delta+(k_c n_\Delta d). \tag{26}$$

With the definitions

$$k=2\pi f/c,$$

$$f_B=f_l-f_r,$$

Eq. (25) may be recast as

$$\Phi=\frac{2\pi}{c}(f_B x_\Delta-f_c n_\Delta d). \tag{27}$$

The above equation gives the phase rotation measured in the ORDPMS. In our experiments, changes in the optical activity of the collagen solution were monitored while exposing samples to ultraviolet radiation. Changes in optical activity are indicative of changes of state in collagen upon denaturation (i.e., a loss of optical activity due to a triple-helix to coil transition). The phase change maybe quantified

as the change of the difference in index of refraction (i.e., Δn_Δ). Hence, a relative phase shift based on Eq. (27) gives

$$\Delta\Phi = \frac{2\pi}{c}[f_c(\Delta n_\Delta)d] = \frac{2\pi(\Delta n_\Delta)d}{\lambda_o}$$
$$= \Delta\alpha = (\Delta[\alpha]_\lambda)\rho d \equiv \Delta R_{\mathrm{ORD}}. \quad (28)$$

From Eq. (28), we see that a relative phase-shift measurement is equivalent to a measure of the change in optical rotatory dispersion of the collagen solution and is a measure of change in triple-helical content.

IV. EXPERIMENTAL PROCEDURES

The purpose of these experiments was to yield a measurement of the change in optical rotatory dispersion of type-I collagen. The optical rotatory dispersion of this macromolecule is a consequence of its chirality. Changes in its optical rotatory properties are a result of a loss of its chirality and are ultimately related to structural changes in its triple-helical structure. Thus, measuring the effects of ultraviolet radiation on the optical rotatory properties of type-I collagen provides additional information on triple-helix destabilization (denaturation) criteria of this macromolecule. The objective was to expose type-I collagen to ultraviolet radiation (broadband and specific bandwidths) and measure the incident ultraviolet radiation intensity and temperature while monitoring for changes in optical rotation. In addition, absorbance spectra, pre- and post-exposure, were measured for additional analysis since collagen denaturation has a known absorbance change.

A. Sample preparation

Samples consisted of type-I bovine collagen in a 0.012*N* HCl solution and were pepsin-treated. Pepsin treatment of samples removed the telopeptides at the ends of the triple-helical structure. This ensured that samples would consist of non–cross-linked triple helices and that denaturation would result in complete destruction of the triple helix with only random coils remaining in solution. Collagen concentration was 3.15 mg/ml, pH of 3.5; samples were from the same lot and were purchased from BD Scientific.

Absorption spectrum measurements were performed on a Perkin-Elmer Lambda EZ210 spectrophotometer. Samples were contained in 1-mm quartz cuvettes; these were standard spectrophotometer cuvettes purchased from Starna Cells, with a cell path length of 1 mm. A 1-mm quartz cuvette containing a 0.012*N* HCl solution was placed in the reference beam of the spectrophotometer. By using 1-mm cuvettes, samples did not need to be diluted, and concentration was reduced to an effective value of 0.315 mg/ml. A portion of each sample was removed before and after irradiation and differences in the absorption spectrum were analyzed.

B. System characterization

It was made certain that sample (cuvette and collagen solution) final temperature was approximately the same as the initial temperature for all experiments conducted in the ORPMS. This was done to ensure that any measured phase-rotation changes were not due to thermal stress birefringence in the quartz cuvettes. The 40-mm quartz cuvettes were annealed in order to relieve any stress birefringence.

Additional experiments were conducted to verify that heating and/or exposing the cuvettes to ultraviolet light did not cause a phase-rotation change due to stress birefringence. A similar set of experiments was also conducted to verify that the solvent (0.012*N* HCl) was not optically active and that exposing it to ultraviolet radiation had no effect on its absorption spectrum.

The results of these preliminary experiments confirmed that UV irradiation had no effect on the optical rotation of the cuvettes containing 0.012*N* HCl solution, nor on changes in the solution absorbance. Hence, we were certain that any measured phase change of the collagen samples would be due to a change of the triple-helical structure of the collagen molecule, since collagen was the only optically active substance in the solution.

C. Ultraviolet irradiation experiments

Samples were exposed to ultraviolet radiation from an EFOS Novacure high-pressure (100 W) mercury arc lamp. A total spectral output up to 7000 mW/cm^2 in the 250–550 nm region was attainable. The output beam was directed through a UV grade light guide to the sample surface. A 1.5-ml sample volume (3.15 mg/ml type-I bovine collagen) was placed in an open quartz cuvette [1 cm(W) $\times$4 cm(L)$\times$3 cm(H)] with the sample surface approximately 6 cm from the tip of the light guide. The intensity of the output beam was uniform to approximately 70% over the sample surface area. Samples were placed in the ORDPMS at ambient temperatures (T_i) and reached a maximum temperature (T_m) during irradiation. After a period of time and delivered dose, the UV light was turned off and the sample cooled to a final temperature (T_f). Cooling times were approximately 30 min. Optical rotation changes were monitored by placing the collagen samples in the ORDPMS and exposing them to the full output spectrum and selective bandwidths of the ultraviolet radiation. Bandpass filters (10-nm full width at half maximum) were used to select out various wavelengths, while the incident intensity, duration, and radiation dosage were varied and monitored for each wavelength.

For each sample run, a small portion of sample was removed pre- and post-exposure to ultraviolet radiation to perform absorbance measurements and calculate differences in absorbance. Portions removed from samples were contained in 1-mm quartz cuvettes. A 1-mm quartz cuvette containing a 0.012*N* HCl solution was placed in the reference beam of the spectrophotometer [32,33].

V. DISCUSSION

A limited portion of data from ultraviolet radiation effects on pepsin treated type-I bovine collagen is compiled in Table II and Figs. 4 and 5. The optical phase rotation ($\Delta\phi_{632.8}$) is

TABLE II. A limited portion of data from ultraviolet radiation effects on pepsin-treated type-I bovine collagen. The optical phase rotation ($\Delta\phi_{632.8}$) is measured by the ORDPMS at the wavelength of 632.8 nm. The difference in absorbance (ΔA_{227}) pre- and post-UV irradiation is measured at 227 nm. Also given are the sample initial (T_i), maximum (T_m), and final (T_f) temperatures for each experimental run. The incident intensity (I), duration of exposure, and dose are also given. In addition, the error associated in calculating the absorbance change (ΔA_{err}) is listed.

λ (nm)	Incident intensity (mW/cm^2)	Exposure time (min)	Dose (J/cm^2)	T_i (°C)	T_m (°C)	T_f (°C)	$\Delta\phi_{632.8}$ (deg/cm)	ΔA_{227} (A)	ΔA_{err} ($\pm A$)
250–400	775	40	1860	21	34	23	+0.15	0.256	0.005
	850	35	1785	23	38	23	+0.15	0.197	0.005
	415	38	946	18	32	19	0.00	0.053	0.005
	300	30	540	18	31	20	0.00	0.023	0.005
260.4	0.28	55	0.9	22.2	24.0	23.1	0.00	0.011	0.003
	0.18	72	0.8	22.3	23.2	22.7	0.00	0.009	0.003
	0.15	90	0.8	22.6	23.5	22.8	0.00	0.006	0.002
278.4	0.8	65	3.1	22.8	23.6	23.0	0.00	0.005	0.003
	1.0	38	2.3	22.2	24.8	23.8	0.00	0.009	0.003
	0.9	39	2.1	22.1	22.9	22.3	0.00	0.004	0.002
300	14.1	68	57.6	22.6	23.7	22.8	0.00	0.005	0.001
	14.5	50	43.5	22.2	23.8	23.0	0.00	0.007	0.001
	13.3	49	38.9	22.0	23.8	23.0	0.00	0.005	0.001
319.7	8.0	78	37.4	18.2	19.0	18.2	0.00	0.010	0.003
	3.2	50	9.6	24.3	25.0	24.6	0.00	0.010	0.003
	1.6	60	5.8	24.0	24.6	24.3	0.00	0.010	0.003
341.3	1.5	59	6.2	21.9	23.2	22.7	0.00	0.008	0.003
	0.5	61	1.8	21.8	23.3	22.8	0.00	0.005	0.003
	0.3	64	1.1	22.5	24.1	23.3	0.00	0.008	0.002
360.6	9.3	87	48.5	23.5	25.2	24.0	0.00	0.007	0.001
	20.2	40	48.5	21.3	23.0	22.2	0.00	0.008	0.005
	21.0	36	45.6	23.1	24.4	23.3	0.00	0.007	0.002
379.5	19.9	56	66.7	22.6	23.8	22.9	0.00	0.006	0.001
	13.3	60	47.4	23.1	24.5	23.5	0.00	0.018	0.010
	6.6	55	21.8	20.7	22.5	21.7	0.00	0.006	0.001

measured by the ORDPMS at the wavelength of 632.8 nm. The difference in absorbance (ΔA_{227}) pre- and post-UV irradiation is measured at 227 nm. Also given are the sample initial (T_i), maximum (T_m), and final (T_f) temperatures for each experimental run. The incident intensity (I), duration of exposure, and dose are also given. In addition, the error associated in calculating the absorbance change (ΔA_{err}) is listed. The measurement error ($\Delta\phi_{\text{err}}$) for optical phase rotation was ± 0.025 deg/cm. For the UV experiments, all optical phase rotation data presented are for a sample path length of 40 mm, a concentration of 3.15 mg/ml in a 0.012N HCl solution, and a sample volume of approximately 2 ml. Data shown for the optical (phase) rotation have been normalized for a 4-cm path length. For the corresponding absorbance measurements, all data presented are for a sample path length of 1 mm and a concentration of 3.15 mg/ml in a 0.012N HCl solution.

The first part of Table II shows the data for broadband irradiation. The optical (phase) rotation ($\Delta\phi_{632.8}$) data show a $+0.15$ deg/cm phase change, which indicates complete destruction of the triple helical state. The phase change is in agreement with thermal denaturation experiments. It is interesting to note that a phase rotation was observed only for doses above a certain threshold dose. It is interpreted that for doses below threshold value, the collagen molecule remains in a triple-helical state since there is no loss of optical activity. Interestingly, the data for absorbance change (of denatured samples) increase with delivered dose and range from $+0.197$ to $+0.256$ A. This absorbance change for the triple-helix to coil transition is larger than the values measured by thermal denaturation.

Figure 4 shows the optical (phase) rotation and absorbance change versus dose. It is evident that the incident dose must be sufficient to first damage the collagen molecule and then denature it, resulting in a phase change. Also, it was discovered (and in agreement with recent literature [3]) that the collagen denaturation temperature is lowered from 38 °C to approximately 34 °C. Therefore, the delivered dose must provide enough energy to also raise the sample temperature above this (lower) denaturation temperature. The difference in absorbance has a significant increase also at the threshold level. This is characteristic of the increase in absorbance in the collagen triple-helix to coil transition. The threshold level was determined to be approximately 1400 J/cm^2

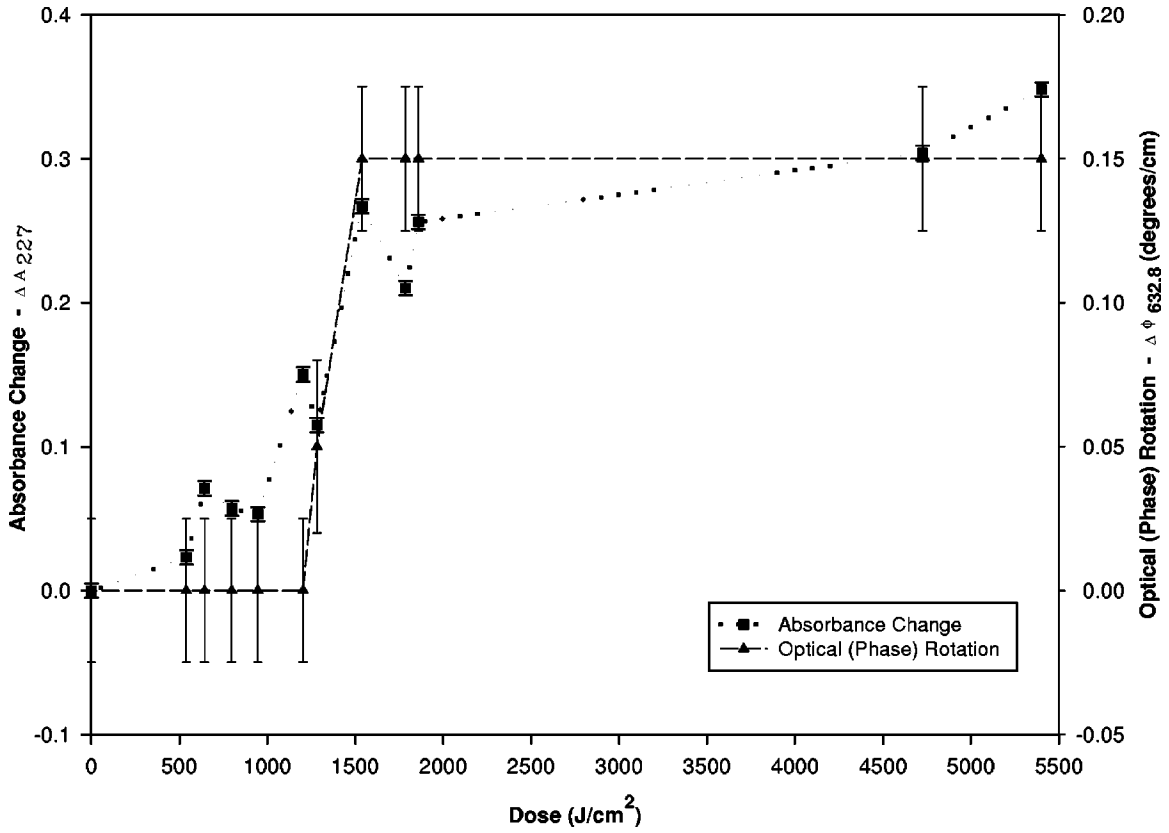

FIG. 4. Optical (phase) rotation as a function of dose for broadband ultraviolet radiation exposure of pepsin-treated type-I bovine collagen. The threshold dose is shown. Also shown is the change in absorbance (measured at 227 nm) as a function of dose.

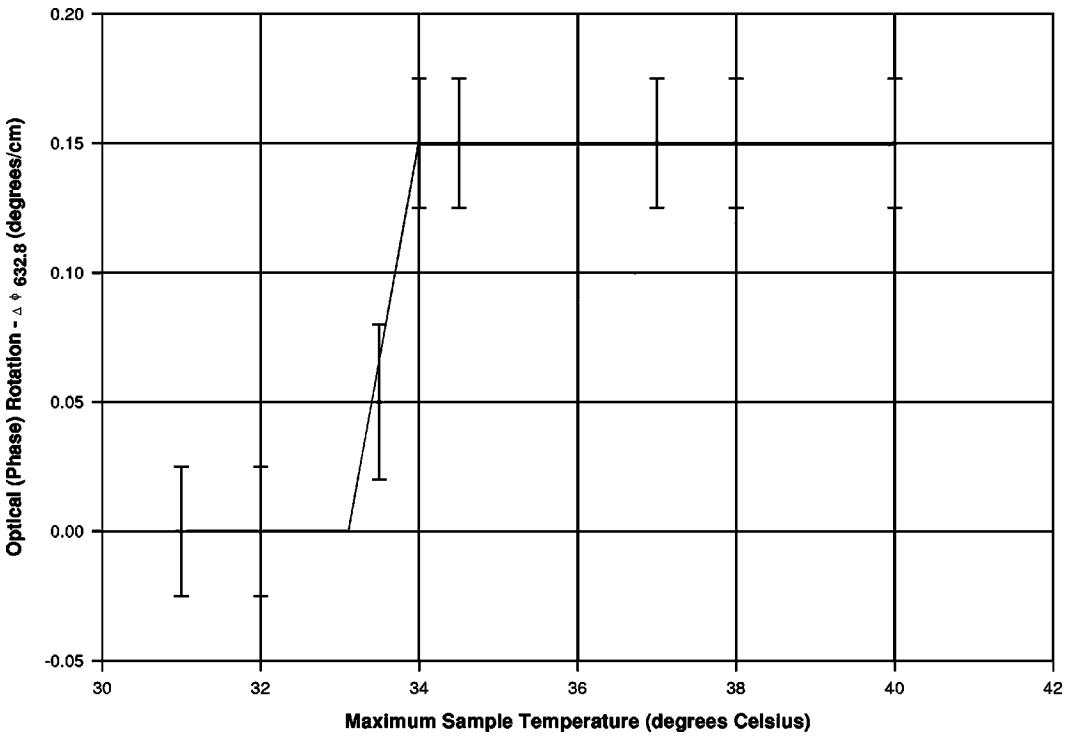

FIG. 5. Shown is the optical phase rotation versus maximum sample temperature reached during broadband ultraviolet irradiation. The data show that for runs where T_m was less than or equal to 33.5 °C no phase change was measured, while for runs where T_m was greater than or equal to 34 °C a phase change of $+0.15$ deg/cm was measured by the ORDPMS.

(± 200 J/cm^2). The threshold dose was defined as the minimum dose needed to change the optical (phase) rotation by an amount equal to that caused by thermal denaturation. The threshold dose is therefore the incident radiation dose, which damages the collagen molecule and raises the sample temperature above the (lower) denaturation temperature. For incident radiation doses below threshold, the collagen molecule remains in a triple helical state.

The broadband irradiation data also show that exposure to ultraviolet radiation damages the collagen molecule and lowers its denaturation temperature. Figure 5 is a plot of measured phase change versus the maximum sample temperature reached during an exposure. The data show that for runs where T_m was less than or equal to 33.5 °C, no phase change was measured. However, for runs where T_m was greater than or equal to 34 °C, a phase change of $+0.15$ deg/cm was measured by the ORDPMS. Hence it was determined that the type-I collagen denaturation temperature was lowered to approximately 34 °C.

Since denaturation (and hence optical phase rotation) is shown to be dependent on dose, it is reasonable to assume that the mechanism for denaturation is thermal. However, a lower denaturation temperature is indicative of a quantum-mechanical effect since the bonding structure in the molecule must be different from its initial state. More specifically, the molecule must have fewer bonds. For incident radiation doses below threshold, no phase rotation was measured by our instrument and it is inferred that the collagen molecule remains in a triple helical state. However, this state is not believed to be the native state but rather a modified triple-helix. This is based on the data that show a change in absorbance for samples that had a 0-deg phase rotation. Again, this supports our hypothesis of a quantum-mechanical damage mechanism of the interaction of the molecule with UV light, because for the various doses below threshold, the variance in absorbance change was not very large. It is interesting to note that unlike the thermal denaturation experiments, the absorbance change with UV light exposure shows a dependence on dose for the denatured samples. In the thermal denaturation experiments there was no significant increase in absorbance once the sample was denatured.

For the selected bandpass filters, the data show no observed phase rotation, which indicates the existence of a triple helical state. The spectral power output of the UV source was substantially lower in the experiments conducted at the narrowed linewidths. Nevertheless, small differences in the absorption spectrum are observable for the 260.4- and 278.4-nm UV filters. The absorbance shifts are believed to be due to cleavage effects of ultraviolet radiation on the collagen molecule. Due to limited spectrophotometer resolution, absorption changes for the other ultraviolet regions are not resolvable from system error.

In summary, we conclude that for collagen exposed to broadband ultraviolet radiation, the triple-helix to coil transition occurs only for doses above the threshold value. Otherwise, the collagen molecule remains in a triple-helical state, not necessarily its native state due to a measured change in absorbance. In addition, a significant decrease in the denaturation temperature, as compared to the thermal denaturation experiments, is indicated by the data. The damage mechanism for lowering the denaturation temperature is believed to be quantum mechanical while for the actual denaturation process the mechanism remains thermal. The UV light interacts with the collagen molecule, cleaving it at specific points along its backbone, hence lowering the denaturation temperature. Cleavage must occur at specific sites along the triple helix in order for UV damage to be a quantum-mechanical mechanism.

For all of the narrowed bandwidths (260.4, 278.4, 300, 319.7, 341.3, 360.6, and 379.5 nm), an optical (phase) rotation was not measured when samples were exposed to varying doses. This leads to a conclusion that the type-I collagen molecule structure remained in some triple-helical state. Also, for each group of experiments, the maximum sample temperature never exceeded 26°C. This again supports the thermal mechanism needed for collagen denaturation. For the 260.4-, 278.4-, and 300-nm wavelength regions, the data indicate a small change in absorbance, and are interpreted to mean that the damage to the collagen molecule may have resulted at these specific wavelengths most likely due to cleavage along the triple helix. However, additional studies need to be done to draw any conclusions. At present, we are investigating the use of amino acid analysis in order to determine the amino acid concentrations before and after ultraviolet irradiation. These results will be reported in a future study.

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