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Effects of Ultraviolet Light on the in Vitro Assembly of Microtubules†

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ABSTRACT: Exposure of microtubular protein to ultraviolet light inhibits its assembly into morphologically normal microtubules. This effect appeared to result primarily from damage to the tubulin dimers. The damage consisted of a conformational change, a loss of two free sulfhydryl groups, a production of higher molecular weight cross-linked species, and the formation of aggregated amorphous material upon polymerization.

Previous studies in this laboratory have indicated that damage induced in microtubular protein by ionizing radiation is due primarily to the loss of two free sulfhydryl groups in the tubulin dimer (Zaremba & Irwin, 1981). These studies have been confirmed by Coss et al. (1981). The results suggested that the mitotic delay observed following exposure of cultured cells to ionizing radiation could be due to damage of microtubular protein. Studies in several laboratories have demonstrated that the microtubules of the mitotic spindle are formed from a pool of tubulin subunits rather than by de novo protein synthesis (Brinkley et al., 1975; Fulton & Simpson, 1979). Therefore, radiation-induced damage of tubulin could result in a delay in the formation of the mitotic spindle. In addition, since microtubules have been reported to function in cell shape, orientation of cell surface receptors, transport of neurotransmitters [for reviews see Olmsted & Borisy (1973), Roberts (1974), and Sloboda (1980)], the immune response (Snyderman & Goetzl, 1981; Malawista, 1975), hormone release (McDaniel et al., 1980; Wolff & Bhattacharyya, 1975), and regulation of adenylate cyclase (Rasenick et al., 1981) and microtubule depolymerization has been reported to initiate DNA synthesis (Crossin & Carney, 1981), such damage could have far-reaching effects in vivo.

Ultraviolet (UV) light administered at various stages of the cell cycle has also been reported to cause mitotic delay, spindle destruction, and inhibition of chromosome movement (Carlson, 1961; Smith, 1964; Inoue, 1964; Rustad et al., 1964; Brown & Zirkle, 1967; Sillers & Forer, 1981). The greatest delay in the onset of mitosis has been demonstrated to occur if cells are irradiated during S phase and is apparently due to repair of damaged DNA (Djordjevic & Tolmach, 1967; Domon & Rauth, 1968; Scaife, 1970; Bootsma & Humphrey, 1968; Han et al., 1971). However, some mitotic delay has also been shown to occur following exposure of cells to UV light during the G_2 phase of the cell cycle (Djordjevic & Tolmach, 1967; Domon & Rauth, 1968; Scaife, 1970; Bootsma & Humphrey, 1968; Carlson, 1976a,b) and therefore could be due to a mechanism other than DNA repair.

In this report we demonstrate that UV light inhibits microtubule assembly apparently by causing a loss of two sulfhydryl groups in the tubulin dimer, and therefore, a common mechanism of action could explain the effects of ionizing radiation on mitosis and UV light on spindle destruction and on mitotic delay during G_2.

Materials and Methods

Materials

Guanosine 5′-triphosphate (GTP), 2-(*N*-morpholino)-ethanesulfonic acid (MES), dimethyl sulfoxide (Me_2SO_4), and dithiodinitrobenzoic acid (DTNB) were purchased from Sigma Chemical Co. Acrylamide, methylenebis(acrylamide), ammonium peroxydisulfide, and *N,N,N′,N′*-tetramethylethylenediamine (TEMED) were from Bio-Rad Laboratories. Glycerol, 2-mercaptoethanol (BME), and [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA) were obtained from Eastman-Kodak Chemical Co. Other chemicals used were reagent grade from Baker or Fisher Chemicals. Calf brains were obtained from a local slaughterhouse within 2 h of slaughter.

Methods

Isolation of Microtubular Protein. Microtubules were isolated from calf brains by successive cycles of polymerization and depolymerization in the presence of glycerol according to the method of Shelanski et al. (1973). The microtubular protein was stored in 0.1 M MES buffer (0.1 M MES, 1 mM EGTA, and 0.5 mM $MgCl_2$, pH 6.5) containing 2 M glycerol

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at a protein concentration of 12–16 mg/mL at −70 °C. Protein determinations were performed by using the method of Lowry et al. (1951) with lysozyme (3× crystallized) as a standard. Before each experiment, the microtubular protein was depolymerized (either with or without 2 mM $CaCl_2$) on ice for 30 min and centrifuged at 17000*g* for 15 min at 4 °C to remove any aggregated material.

In Vitro Polymerization. The polymerization reaction was followed by measuring the increase in turbidity which results from microtubule formation and was monitored at 380 nm in a Cary 118 spectrophotometer equipped with a recorder. The temperature of the sample compartment was maintained at 37 °C with a circulating water bath. Protein samples (generally 1 mL) were placed in cuvettes on ice, GTP (final concentration 1 mM) was added and mixed, and the increase in turbidity at 380 nm was recorded as a function of time at 37 °C.

Electron Microscopy. Electron microscopy was performed on a Phillips EM 300 electron microscope. Whole mounts were prepared on 200-mesh copper grids coated with carbon. One drop of microtubular protein solution was placed on the grid and allowed to stand for 5 s, the grid was blotted, one drop of 1% uranyl acetate was placed on the grid and allowed to stand 5 s, and the grid was again blotted and allowed to dry.

Circular Dichroism (CD). A Jasco J-500A spectropolarimeter with a spectral range from 185 to 700 nm, calibrated with D-pantolactone (Konno et al., 1975), was used to record CD spectra of irradiated and control tubulin. The samples were diluted to approximately 1.0 mg/mL and were placed in a 1-cm cell for the near-UV region, while a concentration of approximately 0.20 mg/mL and a 0.1-cm cell was used for the far-UV region. All solutions were filtered through 0.45-μm Millipore filters prior to CD analysis. Because of the lability of PC–tubulin, the order of running the samples (i.e., control or irradiated) was reversed in every other experiment. In addition, the effect of time was examined by comparing duplicate spectra obtained 80–90 min apart. All data were obtained at 20 °C.

Fluorescence. Intrinsic fluorescence emission spectra were obtained on a Perkin-Elmer MPF-44B fluorescence spectrophotometer equipped with a DCSU-2 differential corrected spectra unit. Irradiated and control tubulin samples were diluted to approximately 80 μg/mL, and the emission spectra were integrated from 300 to 375 nm after excitation at 290 nm.

Ultraviolet Light Exposure. Irradiations were performed by using a 2.5-kW mercury–xenon lamp (Conrad-Hanovia) focused through two Schoeffel grating-type monochromators arranged in tandem. The dose rate for each experiment was obtained with a UDT-40A light meter with a UV-enhanced detector which had been previously calibrated over the wavelength range 230–635 nm. The dose rate used for all experiments was 2×10^{-4} W/cm^2. The protein samples (1–1.4 mL) was irradiated in open Petri dishes (60-mm diameter) at an approximate protein concentration of 3 mg/mL in 0.1 M MES buffer.

Whole microtubular protein was irradiated with 280-nm UV light over the dose range 0–0.25 J/cm^2 to obtain a dose–response curve. The action spectrum was obtained by irradiating whole microtubular protein with a total dose of 0.2 J/cm^2 at each wavelength tested over the range 240–302 nm. For the remaining experiments, the irradiations were performed with tubulin purified from whole microtubular protein by phosphocellulose chromatography. The tubulin was exposed to 280-nm UV radiation at a total dose of 0.2 J/cm^2.

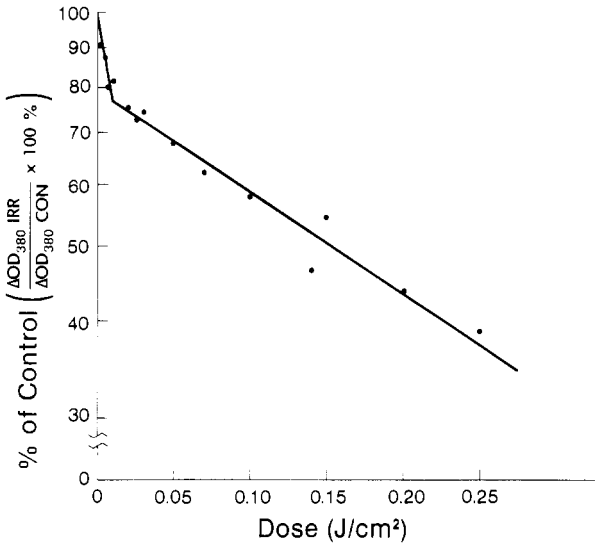

FIGURE 1: Effect of different doses of 280-nm UV radiation on the formation of microtubules. Depolymerized microtubular protein was irradiated with different total doses of 280-nm UV light, keeping the dose rate constant at 2×10^{-4} W/cm^2. The polymerization was monitored by change in absorbance at 380 nm after 30 min at 37 °C in the presence of 1 mM GTP, 0.1 M MES, 2 mM EGTA, 0.5 mM $MgCl_2$, and 1 mM $CaCl_2$, at a protein concentration of 2 mg/mL. Each dose was administered in five separate experiments, and the standard deviations did not differ from the mean by more than 5%.

Other Methods. Fractionation by phosphocellulose chromatography, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), analytical ultracentrifugation, and sulfhydryl titrations were performed as previously described (Zaremba & Irwin, 1981).

Results

Turbidity Development. Since microtubules are structures that scatter light, polymerization can be followed spectrophotometrically by monitoring the apparent increase in absorbance at 380 nm (or any suitable wavelength) that accompanies their formation. The amount of increased absorbance (or turbidity development) has been shown to be directly proportional to the mass of microtubules formed and independent of their length (Gaskin et al., 1974). The dose–response curve shown in Figure 1 was obtained by dividing the change in absorbance of the irradiated sample by the change in absorbance of the control sample at the plateau regions for each dose of 280-nm UV light. The change in absorbance and, therefore, the mass of microtubules formed was always less for the irradiated samples at every dose tested. It can also been seen that the curve obtained is biphasic, with the fast component occurring between 0 and 0.01 J/cm^2 and the slow component occurring in the 0.01–0.25 J/cm^2 range. Thus, it appears that UV light is inducing two rates of damage, one of which is evident at low doses and the other at high doses.

The action spectrum shown in Figure 2 was obtained by monitoring the change in absorbance during polymerization after exposing the microtubular protein to different wavelengths of UV light at the same total dose of 0.2 J/cm^2. It can be seen that the greatest amount of inactivation was induced by 278–280-nm UV light which corresponds to the absorbance maximum of the major component, tubulin.

The absorption spectrum, however, exhibits a broader peak (Zaremba & Irwin, 1981) than the action spectrum in the lower wavelength region which is caused by the presence of bound guanine nucleotides. This suggests that the primary site of interaction with UV light is at tyrosine or tryptophan

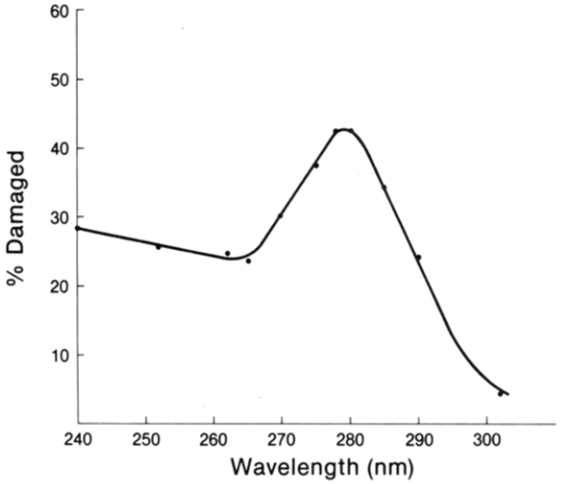

FIGURE 2: Effect of different wavelengths of UV radiation on the formation of microtubules. Depolymerized microtubular protein was irradiated with a total dose of 0.2 J/cm^2 UV radiation at each wavelength indicated by a dot. The dose rate was 2×10^{-4} W/cm^2. The polymerization was monitored as indicated in the legend to Figure 1. The change in absorbance was calculated as percent of control, and this value was subtracted from 100% to give the percent damaged. Each wavelength was tested in four separate experiments, and the standard deviations did not differ from the mean by more than 5%.

residues of the protein rather than at the bound guanine nucleotides. Furthermore, a sample containing unirradiated protein in irradiated buffer showed no reduction in the amount of turbidity produced during polymerization. Therefore, the decrease in the mass of microtubules formed following exposure to UV light appears to be a result of damage to the protein and not a consequence of the formation of degradation products in the buffer or some other artifact.

Electron Microscopy. In the foregoing turbidity development studies it was observed that the irradiated samples (280 nm, 0.2 J/cm^2) exhibited a longer lag period and a depressed initial rate along with a decreased final extent of polymerization compared with the controls. To determine the source of this abnormal kinetic pattern, aliquots were withdrawn from the irradiated and control samples at various times during the assembly process and examined by electron microscopy. Ultrastructural analysis revealed that at 1 min after the onset of polymerization, the control samples had many short microtubules (~3 μm) while the irradiated samples had only rings. At 2.5 min, the control samples exhibited numerous long microtubules (~7 μm) which increased in length up to 6 min. By contrast, the irradiated samples showed a few short microtubules (~2 μm) at 2.5 min which increased mainly in number, not length, over the same time period. At 18 min, the control samples (Figure 3A) had many microtubules that were as long or longer than the major axis of the grid area examined (>14 μm) while the irradiated samples (Figure 3B) still had only short microtubules (2–3 μm) and some aggregated material. These results demonstrate that the irradiation is primarily damaging the free 6S tubulin dimers so that they have a reduced ability to participate in the elongation phase of microtubule assembly.

Polymerization with Me_2SO_4. Since tubulin comprises approximately 75% of the microtubular protein used in the foregoing experiments, and in view of the above ultrastructural results, it was of interest to examine the effects of UV light on the tubulin dimers alone. Tubulin was obtained by phosphocellulose chromatography of whole microtubular protein. It has been shown that tubulin is capable of forming microtubules in the absence of the microtubule-associated proteins (MAP's) which apparently serve to lower the activation energy

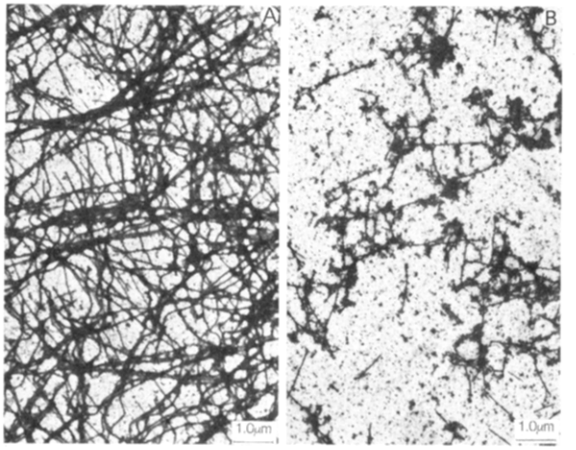

FIGURE 3: Electron micrograph of microtubules formed from (A) control microtubular protein and (B) UV-irradiated microtubular protein (0.2 J/cm^2 280-nm UV light). Microtubular protein was polymerized at 1.5 mg/mL in 0.1 M MES, 0.5 mM $MgCl_2$, 1 mM EGTA, and 1 mM GTP, pH 6.5. The samples were taken after 18 min of assembly at 37 °C.

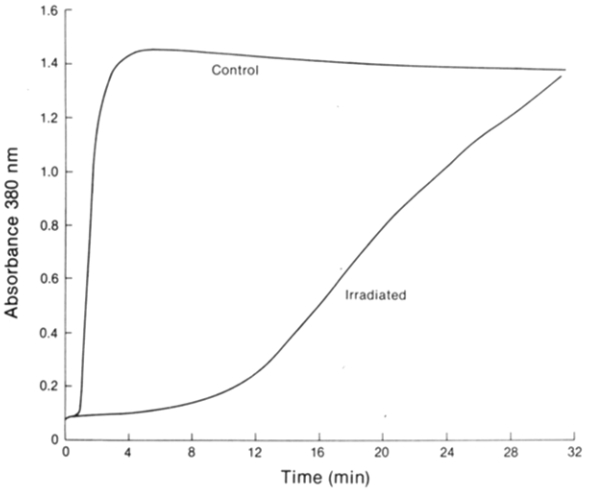

FIGURE 4: Polymerization of control and UV-irradiated tubulin in the presence of Me_2SO_4. The tubulin received a total dose of 0.2 J/cm^2 at a dose rate of 2×10^{-4} W/cm^2 at a wavelength of 280 nm. The polymerization was monitored as an increase in absorbance at 380 nm at 37 °C in the presence of 1 mM GTP, 0.2 M MES, 1 mM EGTA, 0.5 mM $MgCl_2$, and 10% Me_2SO_4 at a protein concentration of 2 mg/mL.

for the polymerization process (Murphy et al., 1977; Murphy & Borisy, 1975; Gaskin et al., 1974). One method to effect assembly of pure tubulin at a relatively low protein concentration (1–3 mg/mL) is to add Me_2SO_4 to a final concentration of 10% (Himes et al., 1976, 1977).

Figure 4 shows the polymerization kinetics of control and UV-irradiated tubulin in the presence of Me_2SO_4. It can be seen that the control sample exhibited a short (about 1 min) lag followed by an exponential increase in absorbance to a plateau level. These kinetics are typical of the normal polymerization reaction. However, the irradiated sample which received a dose of 0.2 J/cm^2 280-nm UV light exhibited quite different kinetics consisting of a gradual increase in absorbance with no sign of a plateau region. This type of kinetic behavior has been reported to result from aggregation rather than polymerization (Penningroth & Kirschner, 1978). To determine if this were indeed the case, aliquots from both control and irradiated samples were examined by electron microscopy. Figure 5A is an electron micrograph of the microtubules formed from control tubulin. Numerous microtubules of normal morphology can be seen along with some aggregated

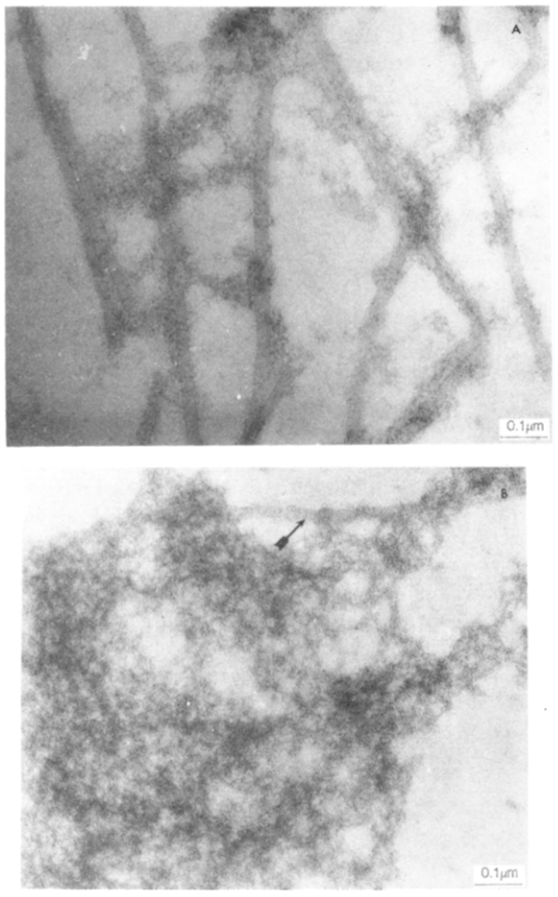

FIGURE 5: Electron micrograph of microtubules formed from tubulin polymerized as indicated in the legend of Figure 4. (A) Control tubulin; (B) UV-irradiated tubulin (note only a single microtubule is present as indicated by the arrow). The samples were obtained after 30 min at 37 °C and were stained with 1% uranyl acetate.

material. Figure 5B is an electron micrograph of microtubules formed from UV-irradiated tubulin. Perhaps the most striking aspect of this micrograph is the presence of a tremendous amount of aggregated amorphous material with only a single microtubule visible. In fact, in all of the representative aliquots examined, it was difficult to find microtubules in the irradiated samples; most contained only aggregates, with no discernible ultrastructure. Thus, it appears that the aberrant polymerization kinetics observed with the irradiated tubulin may primarily result from amorphous aggregation along with a small amount of polymerization.

Table I shows the results of a number of experiments in which both control tubulin and irradiated tubulin were polymerized in the presence of Me_2SO_4. It can be seen that the maximum rate of polymerization obtained with the irradiated sample in each experiment was only a fraction of that obtained with the control sample. Concomitant with this was an increase in the lag time for each irradiated sample compared with its control. With reference to the control samples, it can be seen that the maximum rate decreases and the lag time increases as the age of the protein increases. However, in the case of the irradiated samples, the maximum rate and the lag time remain fairly constant regardless of the age of the protein. Thus, as previously reported, the microtubular protein tends to degrade somewhat in storage so that it does not polymerize optimally, and therefore, the difference between control and irradiated samples appears to be less with older samples (Zaremba & Irwin, 1981). Nevertheless, it is clear that UV light is damaging the tubulin dimers, regardless of their age, so that both the lag time (indicative of nucleation) and the maximum rate (indicative of elongation) of the polymerization reaction are inhibited.

Table I: Effect of Ultraviolet Light on the Polymerization of Tubulin in the Presence of Me_2SO_4[a]

maximum rate[b]		% of	lag time[c]			
con	irr	con	con	irr	irr/con	age[d]
0.521	0.031	6.0	1.0	13.3	13.1	9
0.409	0.019	4.6	2.0	32.7	16.4	7
0.237	0.023	9.7	5.0	37.0	7.4	27
0.222	0.017	7.7	3.3	36.0	10.9	32
0.098	0.016	16.3	8.5	47.5	5.6	41
0.093	0.021	22.6	8.9	35.6	4.0	40

[a] Measured by turbidity development at 380 nm at 37 °C. All samples contained 1.2–2.4 mg/mL tubulin (purified on phosphocellulose) in 0.2 M MES, 0.5 mM $MgCl_2$, 1 mM EGTA, 1 mM GTP, and 10% Me_2SO_4. [b] Maximum rate was obtained at the steepest portion of the curve and was calculated as the change in absorbance at 380 nm/min divided by the protein concentration in milligrams per milliliter. [c] Number of minutes elapsed from the time samples were placed at 37 °C until the time of maximum increase in turbidity at 380 nm. [d] Age in days from the day of isolation of microtubular protein to the day of polymerization with Me_2SO_4.

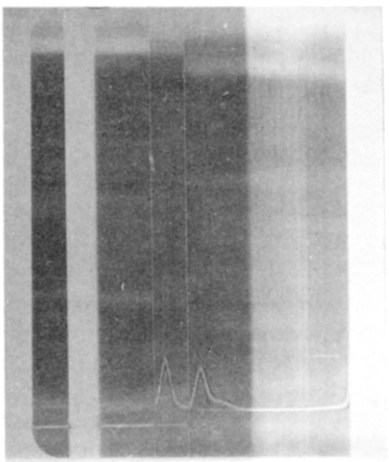

FIGURE 6: Schlieren peaks obtained during analytical ultracentrifugation of irradiated (280 nm, 0.2 J/cm^2) and control tubulin. The irradiated sample volume was 0.30 mL and is the peak on the right; the control sample volume was 0.35 mL and is the peak on the left. Both samples contained 3.4 mg/mL tubulin in 0.1 M MES, 1 mM EGTA, and 0.5 mM $MgCl_2$. The photograph was at 9 min after the rotor has reached its maximum speed of 52000 rpm. The temperature was 20 °C.

Analytical Ultracentrifugation. Photographs taken during analytical ultracentrifugation showed the presence of a faster sedimenting shoulder on the irradiated tubulin peak not present on the control peak (Figure 6). This is indicative of the formation of some higher molecular weight species with an approximate *s* value of 12 S as a result of irradiation. Furthermore, the results of several sedimentation velocity experiments indicated a reduction in the sedimentation coefficient of tubulin from 6.03 ± 0.12 to 5.72 ± 0.13 S following irradiation. These results suggest that UV light may be causing the tubulin dimer to unfold into a more open conformation (lower *s* value) and is inducing the formation of higher molecular weight species (shoulder on peak).

Gel Electrophoresis. SDS–polyacrylamide gel electrophoresis was performed on control and irradiated tubulin. The control sample contained only a small amount of material to the left of the large tubulin band corresponding to proteins of higher molecular weight. This material represented only 10% of the total protein. The irradiated sample, however,

Table II: Effect of Irradiation on CD Spectrum of Tubulin[a]

λ	C_1[b]	I_1[b]	C_2[c]	I_2[c]	I_1/C_1	I_2/C_2
287	−28.12 (±2.4)	21.62 (±2.5)	−46.15 (±4.0)	−33.31 (±5.2)	0.77	0.72
280	−16.71 (±3.1)	−6.965 (±3.3)	−39.09 (±3.3)	−26.41 (±3.8)	0.42	0.68
265	45.04 (±4.6)	46.47 (±5.4)	9.745 (±2.7)	14.29 (±1.7)	1.03	1.47
220	−13193 (±1013)	−11654 (±808)	−13054 (±1111)	−11784 (±1609)	0.88	0.90
210	−13000 (±490)	−11806 (±799)	−12017 (±1051)	−10797 (±1204)	0.91	0.90

[a] Values are expressed as $[\theta]$ in deg cm²/dmol; each value is the average of four experiments. The numbers in parentheses are the standard deviations. [b] Tubulin was prepared from 2-month-old microtubular protein. [c] Tubulin was prepared from 5–19-day-old repolymerized microtubular protein.

exhibited five additional bands or peaks in this region which amounted to about 20% of the total protein. The molecular weights of these proteins were 111 000, 138 000, 173 000, 208 000, and 268 000. These proteins may be the result of UV-induced cross-linking of tubulin dimers (or monomers and dimers) since there was a concomitant 10% decrease in the amount of tubulin present in the major peak of the irradiated sample. These results, along with those obtained by analytical ultracentrifugation, indicate that UV radiation causes the formation of higher molecular weight species by inducing cross-linking between tubulin *n*-mers. Furthermore, the tubulin dimers that are not cross-linked are also damaged by the UV light since they exhibit a lower sedimentation coefficient as well as a decreased ability to polymerize into microtubules.

CD and Fluorescence Spectra. Since irradiated tubulin exhibited a lower sedimentation coefficient than control tubulin, it was of interest to examine if this resulted from a conformational change. Examples of CD spectra obtained in the near-UV region for control and irradiated tubulin are shown in Figure 7. Although there was some variation in the amount of ellipticity obtained with the tubulin samples between each experiment, the irradiated protein always exhibited an increased ellipticity in the 260–270-nm range and a decreased ellipticity in the 280–287-nm range. The bands occurring between 260 and 270 nm are most likely due to phenylalanine residues, while the bands occurring between 280 and 287 nm and between 295 and 310 nm appear to result from tyrosine and tryptophan residues, respectively (Strickland, 1974; Detrich et al., 1981). Examples of CD spectra obtained in the far-UV region are also shown in Figure 7. It can be seen that the irradiated tubulin exhibited decreased ellipticity at 220 and 210 nm compared with that of the control; this pattern was always seen although the values obtained for $[\theta]$ showed some variation from one experiment to another.

Table II is a summary of the data obtained from all of the CD studies performed. It may be seen that the molar ellipticities obtained in the near-UV were affected by the age of the protein preparation tested. Tubulin isolated from repolymerized protein had a larger $[\theta]$ at 287 and 280 nm and a smaller $[\theta]$ at 265 nm than tubulin purified from older protein; the direction of these changes is the same as that observed following irradiation. Table II also shows that the ratio I/C obtained at each CD band is approximately the same for old and new tubulin at 287, 220, and 210 nm but not at 280 and 265 nm. This suggests some degree of correlation between targets in both old and new UV-exposed tubulin. However, the change in ellipticity at 265 nm following irradiation was greater with new tubulin than with old tubulin, while the reverse was true at 280 nm. This essentially means that new tubulin is more susceptible to the type of UV-induced damage which leads to an increase in ellipticity at 265 nm while it is less susceptible to the type of UV-induced damage which leads to a decrease in ellipticity at 280 nm (see Discussion for explanation of these observations). Clark et al.

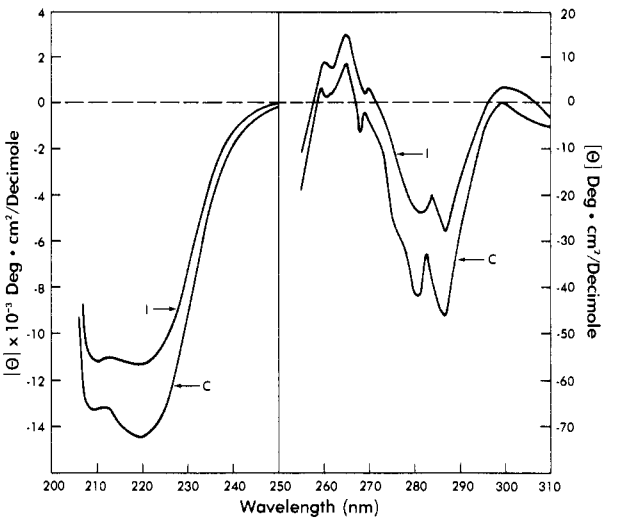

FIGURE 7: Circular dichroism spectra of irradiated (I) and control (C) tubulin in 25 mM MES, 0.5 mM $MgCl_2$, and 0.1 mM EDTA, pH 6.5, at 20 °C. Protein concentration was 0.2 mg/mL for the far-UV region (200–250 nm) and 1.3 mg/mL for the near-UV region (250–310 nm).

(1981) have also reported alterations with time at 37 °C in the near-UV CD spectrum of phosphocellulose-purified tubulin. However, these authors did not see an increased ellipticity at 265 nm or a concomitant change in the far-UV region. We observe a decrease in ellipticity at 220 and 210 nm following irradiation, but not as a result of protein aging, as shown in Figure 7 and Table II. Therefore, these results suggest that while aging of tubulin is accompanied by a conformational change as shown by the changes in the CD spectral contributions of aromatic residues in the 250–300-nm range, UV irradiation induces further conformational changes in the aromatic residues as well as in the α-helical structure.

Additional evidence for a conformational change following exposure to UV light was obtained by intrinsic fluorescence emission. These studies demonstrated that while both irradiated and control tubulin exhibited an emission peak at 330 nm following excitation at 290 nm, the integrated area was 10% less for the irradiated samples (data not shown). Therefore, these results suggest that UV radiation induces alterations in tryptophan residues in the tubulin dimer and support the results obtained with CD.

Sulfhydryl Titrations. Since certain specific sulfhydryl groups have been reported to be important for the polymerization process (Kuriyama & Sakai, 1974; Lee et al., 1981; Deinum et al., 1981), it was of interest to examine the effect of UV light on the number of free sulfhydryl groups in the tubulin dimer. These results are shown in Table III. It can be seen that the control tubulin contained approximately 25 free sulfhydryl groups per tubulin dimer, which is a good agreement with the previously reported number of 24 per dimer (Zaremba & Irwin, 1981). The UV-irradiated tubulin, how-

Table III: Number of Free Sulfhydryl Groups[a]

control	irr	con-irr
25.8	24.2	1.6
25.4	23.2	2.2
25.0	20.4	4.6
26.8	25.0	1.8
23.4	22.0	1.4
av: 25.3	av: 23.0	av: 2.3

[a] Expressed as number of free sulfhydryl groups per mole of 6S tubulin dimer (M_r 110 000) as determined by titration with DTNB.

ever, contained only an average of 23 free sulfhydryl groups per tubulin dimer, or 2 fewer than the control.[1] A blockage of two free sulfhydryl groups per tubulin dimer has been reported to greatly inhibit its ability to polymerize into microtubules (Kuriyama & Sakai, 1974; Lee et al., 1981; Deinum et al., 1981). In addition, a loss of two free sulfhydryl groups was previously shown to result from exposure of tubulin to ionizing radiation; this modification also led to a decreased ability to assemble into microtubules (Zaremba & Irwin, 1981).

The results reported here suggest that the dose-dependent decrease in polymerization observed following UV irradiation of microtubular protein is due, at least in part, to damage of the major component, tubulin. This damage is reflected by a lowered sedimentation coefficient, a decrease in the number of free sulfhydryl groups, a conformational change, and the formation of higher molecular weight cross-linked species. These changes appear to be responsible not only for the decreased ability of UV-irradiated tubulin to participate in both the nucleation and elongation phases of the polymerization process but also for the production of aggregated material instead of morphologically normal microtubules.

Discussion

The results presented in this paper demonstrate that a number of alterations in the physical properties of tubulin occur following exposure of UV light. Our analytical data suggest that several sulfhydryl groups have been lost. If this loss is accompanied by disulfide bond formation, a change in tertiary structure or conformation would reasonably be expected. The lowered sedimentation coefficient exhibited by the irradiated tubulin suggests that such a change in conformation has indeed occurred. This is supported by studies which indicate that the irradiated tubulin has a decreased intrinsic fluorescence, has a decreased amount of α-helical content, and exhibits alterations in the molar ellipticities of aromatic residues; all of these findings are suggestive of a conformational change. Furthermore, a loss of two sulfhydryl groups, a lowered s value, and an alteration in intrinsic fluorescence were previously found to occur following exposure of tubulin to ionizing radiation (Zaremba & Irwin, 1981). In this case, the tubulin also showed aberrant polymerization kinetics in the presence of Me_2SO_4 which appeared to be the result of the formation of aggregated material in addition to microtubules. It was concluded from these studies that the formation of intramolecular disulfide bonds in the tubulin dimer led to a conformational change such that the dimers associated incorrectly.

[1] The paired t test indicated that the difference in the number of sulfhydryl groups between the irradiated and control samples was not statistically significant because of the small sample size (p = 0.20). However, the nonparametric sign test for trend showed that probability of the irradiated samples having fewer sulfhydryl groups than the control samples 5 out of 5 times by chance alone was only 0.03.

Coss et al. (1981) have also reported that damage of microtubular protein by ionizing radiation resulted from oxidation of free sulfhydryl groups in the tubulin dimer. Lee et al. (1981) have shown that modification of one to two sulfhydryl groups of the tubulin with 1-fluoro-2,4-dinitrobenzene inhibits microtubule assembly and produces mainly aggregated material. Therefore, it appears that the two specific sulfhydryl groups in tubulin which are required for assembly into microtubules are also sensitive to oxidation by ionizing and ultraviolet radiation.

The higher molecular weight species observed during analytical ultracentrifugation and SDS–PAGE of irradiated tubulin do not, however, appear to result from disulfide bond formation since they are present in gels containing tubulin which had been boiled in the presence of SDS and BME. This treatment should break any disulfide bonds regardless of their position within the protein. However, only about 10% of the tubulin was converted to higher molecular weight species and, therefore, cannot account either for the 90% decrease in the maximum rate of polymerization or for the formation of the large amount of aggregated material observed in the studies with Me_2SO_4. These results must, therefore, be due to the alterations induced in the other 90% of the tubulin following UV light exposure. It may be conjectured that both UV light and ionizing radiation cause conformational changes in the tubulin dimer because of disulfide bond formation, and this alteration is functionally expressed as a decreased ability to participate in the polymerization process. Disulfide bond formation could conceivably result from the interaction of UV with aromatic residues by the following highly simplified mechanism [see, for example, Grossweiner (1976)]:

$$\phi \xrightarrow{h\nu} e_{aq} \xrightarrow{x} X\cdot$$

$$X\cdot + RSH \rightarrow XH + RS\cdot$$

$$RS\cdot + RS\cdot \rightarrow RSSR$$

It was hoped that the CD studies would provide direct evidence that disulfide bond formation had occurred since cystine exhibits a broad CD band in the 250–300-nm region (Beychok, 1965; Strickland, 1974). However, the near-UV spectra of aromatic residues can obscure the disulfide contribution. Since tubulin has been reported to contain 8 tryptophan, 35 tyrosine, and 44 phenylalanine residues (Ponstigl et al., 1981; Krauhs et al., 1981), it is admittedly difficult to discern a band arising from a single disulfide bond. However, Table II shows that a smaller change in ellipticity at 265 nm has occurred following irradiation of old tubulin compared with new tubulin. The I/C obtained at this wavelength for old tubulin is 30% less than that obtained for new tubulin. This correlates well with our previous finding of a loss in the number of free sulfhydryl groups in older tubulin which would amount to approximately 29% after 2 months in storage (Zaremba & Irwin, 1981). Therefore, it is likely that increased ellipticity observed at 265 nm following UV irradiation of new tubulin is due to disulfide bond formation and that this effect is partially obscured in old tubulin due to its loss of free sulfhydryl groups. Nevertheless, old tubulin is also damaged by UV radiation as reflected by the ellipticity changes at the other wavelengths. In fact, the change observed at 280 nm was greater with old than with new tubulin. It is possible that the conformational change accompanying the loss of free sulfhydryl groups in old tubulin has exposed more aromatic residues on the surface, therefore rendering them more susceptible to UV damage during the relatively short (15 min) exposure time. Furthermore, UV radiation induces a change in the secondary structure of tubulin as shown by the alterations in the far-UV region of the

CD spectrum which is also strongly suggestive of a covalent modification such as disulfide bond formation. However, we cannot exclude the possibility that the free sulfhydryl groups were lost by a mechanism other than disulfide bond formation.

Supporting evidence that damage to tubulin is the major cause for these results can be obtained by comparing the electron micrographs shown in Figures 3B and 5B. It can be seen that in Figure 5B mainly aggregated material was formed when irradiated tubulin was polymerized in the absence of MAP's. This result indicates that irradiation of purified tubulin damages its ability to self-associate into morphologically normal microtubules. In contrast, Figure 3B shows that mainly short microtubules and a small amount of aggregated material were formed when irradiated unfractionated microtubular protein (tubulin + MAP's) was polymerized. On the basis of early reports of microtubule nucleation, it seemed likely that these short microtubules resulted from unrolling of the 36S ring structures (Kirschner et al., 1975; Bryan, 1976). However, recent evidence (Mandelkow et al., 1980; Weisenberg, 1980; Pantaloni et al., 1981; Bordas et al., 1983) has suggested that microtubules are nucleated from tubulin oligomers smaller than rings. It appears from these studies that fragments of rings are directly incorporated in the initial or nucleation stage of microtubule assembly. In the present experiments, rings were observed in both control and irradiated samples before the onset of polymerization. Therefore, it appears that UV-exposed rings or fragments are still capable of nucleating microtubule assembly. Thus, damage induced in microtubular protein by UV light is due primarily to damage to the tubulin dimers rather than to the 36S ring structures.

These studies also indicate that UV-irradiated tubulin loses its ability to elongate preexisting microtubules as well as the ability of self-nucleate microtubule assembly. Similar results were previously reported following exposure of microtubular protein to ionizing radiation (Zaremba & Irwin, 1977). Therefore, the tubulin dimer is susceptible to damage by both ionizing radiation and UV radiation which appear to induce the formation of disulfide bonds; this modification leads to a conformational change such that the dimers have a reduced ability to participate in both the nucleation and elongation phases of microtubule polymerization.

Tubulin comprises about 75% of the microtubular protein used to obtain both the dose–response curve and the action spectrum. Therefore, it seems likely that UV damage of the tubulin dimer is responsible for the majority of these results. Since the dose–response curve has two components in the dose range tested, this suggests that the population of protein molecules contains two subgroups with respect to radiation sensitivity. However, it is not possible to determine from these results if the two subgroups represent two structurally important target sites on the tubulin dimer with significantly different UV sensitivities or if they represent one functional site on the tubulin dimer and another functional site on some other component such as the τ protein, again with a different UV sensitivity.

These results could provide an explanation of the effects of UV microbeam irradiations on the mitotic spindle. It was observed that UV irradiation of the cytoplasm [$(0.08–6) \times 10^4$ J/m^2][2] caused a disappearance of the mitotic spindle as well as a delay in progression of the cells through mitosis (Brown & Zirkle, 1967). The action spectrum for spindle destruction and mitotic delay resembled the absorption spectrum of tyrosine-containing proteins. It was, therefore, concluded that the target for UV-induced destruction of the spindle as well as for mitotic delay was a single protein species present in the cytoplasm (Brown & Zirkle, 1967). The tubulin dimer is a likely candidate for the target protein since the spindle microtubules appear to be in a dynamic equilibrium with the soluble tubulin dimers. Therefore, exposure of cultured cells to UV light could delay progression through mitosis by damaging a portion of the tubulin dimers present in the pool which would lead to an inhibition in the rate and extent of microtubule formation in the mitotic spindle. This could also upset the dynamic equilibrium in favor of depolymerization of already formed spindle tubules.

Recently, Sillers & Forer (1981) have published an action spectrum for stopping chromosome movement in crane fly spermatocytes by UV microbeam irradiation. Their results differ from ours in that 290- and 270-nm UV were more effective than 280-nm UV in stopping chromosome movement. They concluded that this effect was due to damage of both the microtubules and microfilaments comprising the spindle since actin absorbs optimally at 270 nm, while myosin subfragment 1 and microtubular protein have absorption peaks at 290 and 280 nm, respectively. The optimum UV wavelength for spindle damage may depend on its composition since Forer et al. (1980) have calculated that the amount of tubulin in the mitotic apparatus varies from 0.1% to 100% depending on the cell type.

Our results could also provide an explanation for the small amount of mitotic delay observed when cells are exposed to UV light in the G_2 phase of the cell cycle. In these studies (Djordjevic & Tolmach, 1967; Domon & Rauth, 1968; Scaife, 1970; Bootsma & Humphrey, 1968), a germicidal lamp was used for the UV exposures (2.5–100 J/m^2). It has been reported that the spectral irradiance of a germicidal lamp is approximately 1000-fold greater at 254 nm than at 280 nm (Jacobson et al., 1978). It can be seen from the action spectrum in Figure 2 that 254-nm UV light is only about 50% as effective in damaging microtubular protein as compared with 280-nm UV. In addition, absorption of 254-nm light by nucleic acids is 10–20 times higher per unit weight than that by proteins at this wavelength (Jagger, 1967). Therefore, one would not expect to observe much damage to microtubular protein in cells exposed to this wavelength. Carlson (1976a) used several wavelengths of monochromatic UV light (0.5–500 J/m^2) to produce mitotic delay in grasshopper neuroblasts in culture. He found that only cells in early prophase (equivalent to G_2 in mammalian cells) exhibited a wavelength effect, with 280-nm UV being more effective in retarding mitosis than 265- or 225-nm light. In a companion paper (Carlson, 1976b), he studied the rate of transit of cells through stages of the cell cycle following exposure to monochromatic UV light. He reported that very few cells exposed in S phase to either 265- or 280-nm UV showed appreciable delay in passing through S phase; the largest delay tended to be in early prophase (G_2). However, it should be noted that cells in pre-S and S phase were more sensitive to UV light than cells in G_2 in general. Therefore, although DNA damage and repair are a major cause for mitotic delay, it seems likely that damage to microtubular protein could also delay mitosis if the UV source utilized for the exposures emits a significant amount of light in the 280-nm region.

Because microtubules appear to be involved in many other cellular processes in addition to cell division, damage induced by UV or ionizing radiation could have dramatic effects in vivo.

[2] This dose range was calculated from Figure 3 of Brown & Zirkle (1967) which showed an exposure range of 0.4–30 ergs/cell. Since the microbeam converged to a focal spot of 8 μm in diameter, we assumed that each cell received the UV dose in a 50-μm^2 area.

Transport of neurotransmitters, secretion of hormones, cell-mediated immunity, and maintenance of cell shape are some rather important functions which could be disrupted as a result of damage to microtubular protein.

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