Effects of Ionizing Radiation on the Polymerization of Microtubules in Vitro†

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ABSTRACT: Exposure of phosphocellulose-purified 6S tubulin to ionizing radiation results in a reduction or loss in its ability to participate in polymerization. Evidence is presented which correlates this loss in ability to polymerize with a reduction in the number of titratable sulfhydryl groups and a lowered affinity for guanosine triphosphate.

A number of theories have been proposed to explain the mitotic delay which occurs following exposure of cultured cells to ionizing radiation (Oleinick & Rustad 1976; Okada, 1970a); however, none satisfactorily accounts for all of the observed results. Many of the studies which were performed to elucidate the target site of the damage concentrated on measurement of the rates of DNA, RNA, or protein synthesis following irradiation (Bacchetti & Sinclair, 1970, 1971; Doida & Okada, 1969; Tomasovic & Dewey, 1978). Although it is likely that radiation is affecting these processes, it is not clear if any of these effects are directly responsible for the mitotic delay observed.

Several investigators have suggested that radiation is inhibiting the synthesis of one of the proteins comprising the mitotic spindle (Walters & Petersen, 1968; Rustad et al., 1975; Dubravsky et al., 1976; Dubravsky & Withers, 1976, 1978). However, Noland et al. (1974) reported that exposure of Chinese hamster ovary cells to X-radiation had no apparent effect on the synthesis of microtubular protein. Inoue and co-workers (Inoue & Ritter, 1978; Inoue et al., 1975) have demonstrated that microtubule formation does not require de novo protein synthesis but rather appears to involve a dynamic equilibrium between the microtubule and a pool of its subunits. In addition, studies in several laboratories using fluorescent tubulin antibody have shown that the extensive array of cytoplasmic microtubules normally present in interphase cells disappears prior to the formation of the mitotic spindle (Brinkley et al., 1975; Fujiwara & Pollard, 1978). Thus, it appears that the spindle could be formed from the increased pool of subunits produced by this depolymerization of the cytoplasmic microtubules. Inoue (1964) has also demonstrated that the spindle microtubules, like those present in the cytoplasmic network, are in a dynamic equilibrium with their subunits. Therefore, it is possible that radiation damage of the microtubular protein present in this pool could affect the dynamic equilibrium and result in depolymerization of the spindle tubules, thus leading to a delay in the onset of mitosis.

Previous studies in our laboratory (Zaremba & Irwin, 1977) using microtubular protein isolated from calf brains by successive cycles of polymerization–depolymerization demonstrated that exposure of the depolymerized protein to γ irradiation resulted in a dose-dependent decrease in the extent of polymerization. Furthermore, it appeared that the tubulin dimer was more sensitive to irradiation in the free (6S) form than in the 36S oligomer where it is associated with the other microtubular components (τ, protein kinase, and high molecular weight proteins). The irradiation had no apparent effect on either protein kinase activity or [^{14}C]colchicine binding ability, and therefore the radiation was not causing a generalized molecular degradation of the microtubular protein.

In this report, we have further characterized the nature of the radiation damage of the free 6S tubulin dimer. Our results show that there is a decrease in the number of free sulfhydryl groups following the exposure of tubulin to γ irradiation; this appears to cause a conformational change which leads to a decreased ability to participate in both the nucleation and elongation events of microtubule polymerization.

Materials and Methods

Materials

Guanosine 5′-triphosphate (GTP), 2-(*N*-morpholino)-ethanesulfonic acid (MES), dimethyl sulfoxide (Me_2SO), and dithiobis(nitrobenzoic acid) (DTNB) were purchased from Sigma Chemical Co. Acrylamide, methylenebis(acrylamide), ammonium peroxydisulfide, *N,N,N′,N′*-tetramethylethylenediamine (TEMED), glycerol, and 2-mercaptoethanol (BME) were obtained from Eastman. [^{14}C]GTP (specific activity 331 or 355 mCi/mmol) was purchased from New England Nuclear. Other chemicals used were reagent grade from Baker or Fisher. 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 $MgCl_2$) containing 2 M glycerol at a protein concentration of 12–16 mg/mL at −20 °C. For some experiments the protein was isolated in the absence of glycerol by using the same basic procedure except that all of the centrifugations were performed by using a Sorvall SS-34 rotor, and the microtubules were depolymerized on ice in the presence of 2 mM calcium chloride. In this case the protein was stored in 0.1 M MES buffer at a protein concentration of 6–9 mg/mL in liquid nitrogen. Protein determinations were performed by the method of Lowry et al. (1951) by using either bovine serum albumin (BSA) or lysozyme (3× crystallized) as a standard. Before each experiment the microtubular protein was depo-

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lymerized (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 in a Gilford 2400 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) and EGTA (final concentration 2 mM) were added and mixed, and the increase in turbidity at 380 nm was recorded as a function of time at 37 °C.

Fractionation by Phosphocellulose Chromatography. Phosphocellulose (Whatman P-11) was precycled by successive washes with 50% ethanol, 0.25 M NaOH, and 0.25 M HCl. It was finally suspended and stored in MES–EDTA buffer: 0.025 M MES, 0.5 mM $MgCl_2$, 0.1 mM EDTA, and 1 mM BME.

Tubulin was separated from the accessory proteins on 10-mL phosphocellulose columns by the method of Weingarten et al. (1975). Tubulin is not retained on phosphocellulose and is thus recovered in the buffer wash (MES–EDTA buffer). The fractions containing tubulin were combined and brought to 1 mM GTP, 0.1 M MES, and 2 mM EGTA and concentrated by Amicon ultrafiltration, generally to 3 mg/mL.

Gel Electrophoresis. The purity of microtubular protein fractions was monitored by polyacrylamide gel electrophoresis by using a discontinuous gel system in the presence of $NaDodSO_4$ according to the method of Weber & Osborn (1975). Protein samples were generally diluted to 1 mg/mL with 10% $NaDodSO_4$ and 1% BME and were denatured by boiling for 3 min. After being cooled, the samples were brought to 10% glycerol and bromophenol blue was added as a tracking dye. The final concentration of protein applied to each gel was 40–80 μg, depending on the experiment. Tris–glycine–$NaDodSO_4$, pH 8.3, was used as the running buffer. The gels were stained overnight in 0.025% Coomassie blue R-250 in 30% 2-propanol–10% acetic acid. The stain was gradually removed by successive treatments with 10% 2-propanol–10% acetic acid followed by 10% acetic acid over a period of 2 days as described by Fairbanks et al. (1971).

^{60}Co γ Irradiation. Irradiations were performed with a ^{60}Co therapy unit on air-equilibrated solutions at 25 °C. Samples (1 mL) were irradiated from below in small (5 mL) Pyrex beakers open to the atmosphere and held in an apparatus designed to maintain constant geometry and distance from the source. No evaporation occurred during the period of irradiation, which typically was no longer than 30 min. The dose rate was measured by thermoluminescent disometry. TLD chips (LiF) were placed at several positions around the inside and outside surfaces of the beaker, and chips surrounded by a single layer of parafilm were placed in the solution. After irradiation, the chips were read in a Harshaw No. 3000 TLD system. Since ^{60}Co decays at the rate of 1% per month, the dose rate varied from 2381 to 1800 ± 60 rd/min over the course of these studies.

GTP Removal. Tubulin prepared by phosphocellulose chromatography of whole microtubular protein contains GTP (or GDP) both bound to the E site and free in solution (Zeeberg et al., 1977; Penningroth & Kirschner, 1978). For studies on the binding of GTP to tubulin, the exchangeable and free nucleotides must be removed. Centrifugation through Sephadex G-25 followed by charcoal treatment was used for this purpose.

Disposable 5-mL syringes were prepared containing 5 mL of Sephadex G-25 in MCE buffer (0.1 M MES, 0.5 mM $MgCl_2$, 1 mM EGTA, and 2 mM $CaCl_2$). The syringes were centrifuged at 120*g* for 10 min to remove excess buffer. Aliquots (1–1.2 mL) of 6S tubulin were layered onto the G-25 and centrifuged at 120*g* for 5 min. The excluded volume containing tubulin was transferred to 5-mL centrifuge tubes and 50 μL of a charcoal solution was added and mixed by Vortex. The charcoal solution was prepared by suspending Norit A charcoal in MCE buffer followed by repeated centrifugation and washing. The final suspension contained approximately 200 mg/mL charcoal, so that 10 mg was added to the tubulin in the 50-μL aliquot. The majority of the charcoal was removed by centrifugation at 27000*g* for 15 min. The supernatant containing tubulin was freed of the remaining charcoal by filtration through a 0.22-μm Millipore filter. Tubulin prepared in this manner was used in the binding studies following addition of EGTA to chelate the excess Ca^{2+}.

GTP Binding. The binding of GTP to tubulin was measured by rate of dialysis according to the method of Colowick & Womack (1969). The flow dialysis cell had lower and upper chamber capacities of 2 and 1 mL, respectively. The bottom chamber was filled with buffer and connected to a peristaltic pump. The top chamber contained 0.73 mL of tubulin at about 1.5 mg/mL. To start the binding reaction 20 μL of [^{14}C]GTP (specific activity 331 or 355 mCi/mmol) was added to the top chamber while buffer was pumped through the bottom chamber at a flow rate of 2 mL/min. One-minute fractions (2 mL) were collected for the duration of the experiment which was 60 min. At four 10-min intervals, 3–9 μL aliquots of unlabeled GTP (1.958×10^{-4} M) were added to the top chamber. After 50 min had elapsed, a 20-μL aliquot of a more concentrated GTP solution (4.40×10^{-2}) was added to release essentially all of the bound [^{14}C]GTP. Aliquots (1 mL) of each fraction were counted in a liquid scintillation counter using a xylene-based cocktail (ACS from Amersham-Searle).

Analytical Ultracentrifugation. Analytical ultracentrifugation was performed in a Beckman Model E ultracentrifuge equipped with Schlieren optics in order to determine if the sedimentation coefficient of tubulin was altered by irradiation. The control and irradiated samples were centrifuged at the same time by using a double sector cell. Since a different volume of each was used (0.42 mL of irradiated, 0.35 mL of control), the Schlieren peaks were offset. After the rotor had reached its maximum speed (53 500 rpm), pictures were taken at 4-min intervals over a period of 76 min. The photographic plates were developed and peak positions measured on a Nikon microcomparater.

Sulfhydryl Titrations. Reaction with DTNB was used in order to determine differences in the number of free sulfhydryl groups between irradiated and control tubulin. Samples containing 0.3–0.5 mg/mL tubulin in 0.1 M MES buffer (pH 6.4) were diluted in half with 0.1 M Tris (pH 8.7) in order to bring the pH up to 8.0–8.2. A blank containing MES buffer plus Tris was always run along with the samples. One milliliter of each sample was pipetted into cuvettes and the base lines were set at 412 nm in a Gilford recording spectrophotometer. An aliquot (10 μL) of DTNB (4 mg/mL) was added to each and mixed, and the increase in absorbance at 412 nm was monitored during the reaction. Samples were run in the native state as well as in the denaturated state, in the presence of 7 M urea.

Electron Microscopy. Electron microscopy was performed by using a Phillips EM 300 electron microscope. Whole

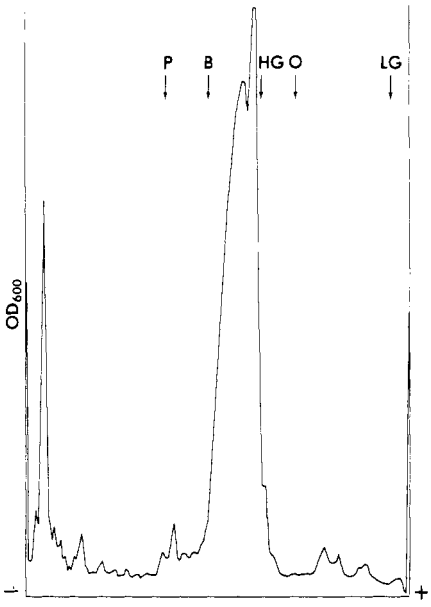

FIGURE 1: Densitometer scan of $NaDodSO_4$–polyacrylamide gel of whole microtubular protein. The amount of protein applied to gel was 71 μg. Arrows indicate the relative mobilities of protein standards: P, phosphorlase a, M_r 92 500; B, BSA, M_r 68 000; HC, heavy-chain γ-globulin, M_r 50 000; O, ovalbumin, M_r 43 000; LC, light-chain γ-globulin, M_r 23 500.

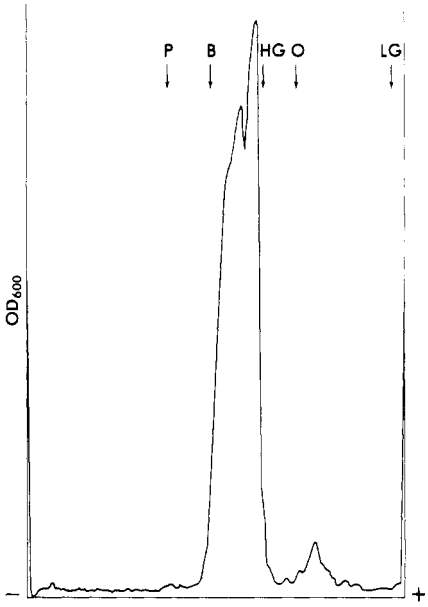

FIGURE 2: Densitometer scan of $NaDodSO_4$–polyacrylamide gel of 6S tubulin isolated by phosphocellulose chromatography. The amount of protein applied to the gel was 61 μg. Protein standards are as indicated in the legend to Figure 1.

mounts were prepared on 200 mesh copper grids coated with Formvar-carbon. One drop of the microtubular protein solution was placed on the grid and allowed to stand for 30 s, the grid was blotted, one drop of 1% uranyl acetate was placed on the grid and allowed to stand 20–30 s, and the grid was again blotted and allowed to dry. By use of this technique, microtubules were negatively stained.

UV Absorption Spectra. A Beckman Acta V double-beam recording spectrophotometer was used to obtain UV spectra of irradiated and control tubulin. The scans were obtained by using the appropriate buffer in the reference cell and generally covered the range from 350 to 220 nm.

Fluorescence. A recording spectrofluorometer was used to obtain emission spectra of irradiated and control tubulin. The excitation wavelength was 280 nm and the emission was recorded over the range 300–475 nm. Prior to measurement, all samples were diluted to an OD^{280} of 0.1 to avoid inner filter effects.

Results

Purity of Microtubular Protein. The purity of microtubular protein preparation was routinely monitored by polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$. Figure 1 shows a typical pattern obtained with whole microtubular protein purified by three cycles of polymerization–depolymerization in the presence of glycerol. The bands near the top of the gel corrspond to the high molecular weight (HMW) proteins and the large double band in the center corresponds to the α and β subunits of the tubulin dimer. The band migrating ahead of the tubulin subunits has tentatively been identified as actin, which has been shown to copurify with microtubular protein. The identity of the band migrating behind tubulin (about 92 000 daltons) is not known. Figure 2 shows a typical gel scan of 6S tubulin purified by phosphocellulose chromatography. While there are a few very minor impurities present, it can be seen that this preparation is entirely free of the HMW proteins and is approximately 95% pure 6S tubulin.

UV and Fluorescence Spectra. Since our previous results had demonstrated that the free 6S tubulin dimer was more sensitive to radiation than the 36S aggregate, all of the studies presented here were performed by using purified 6S dimer in order to determine the nature of the damage at the molecular level. After irradiation with 50 000 rd of cobalt-60 γ rays at a protein concentration of 3 mg/mL, the control and irradiated 6S samples were centrifuged through Sephadex G-25 and treated with charcoal as indicated under Methods in order to remove nucleotides exchangably bound to tubulin and free in solution. The 6S samples were then diluted with MES buffer and the UV absorption and fluorescence emission spectra were determined. Typical results are shown in Figure 3. The maximum of the absorption spectrum was at 278 nm for both irradiated and control tubulin while the average absorption per milligram of protein per milliliter was 1.92 for the irradiated and 1.87 for the control. The fluorescence emission maximum (excited at 280 nm) was at 355 nm for both irradiated and control tubulin; however, the peak intensity of the irradiated sample was an average of 16% greater than that of the control after correction for differences in protein concentration. Therefore, the irradiated tubulin exhibited both a greater absorbance and a greater emission than the control tubulin.

Analytical Ultracentrifugation. Following fractionation by phosphocellulose and irradiation (50 000 rd) at 3 mg/mL,the control and irradiated tubulin samples were placed in a double-sector cell and centrifuged as indicated under Methods. The Schlieren peaks obtained at one time point during the centrifugation are shown in Figure 4. It can be seen that with either sample only one peak is observed, suggesting that irradiation is not causing cross-linking of dimers or other changes which would result in the production of more than one sedimenting species. Analysis of the data indicated that the control tubulin had a sedimentation coefficient of 6.07 S while the irradiated tubulin had an *s* value of 5.77 S.

GTP Binding. Because of the rapidity with which results could be obtained, the rate of dialysis method of Colowick & Womack (1969) was used to determine if GTP binding at the exchangable site was affected by exposure of the tubulin to radiation. Following irradiation with 50 000 rd, the control and irradiated tubulin were freed of excess unbound and reversibly bound (E-Site) GTP by centrifugation through G-25

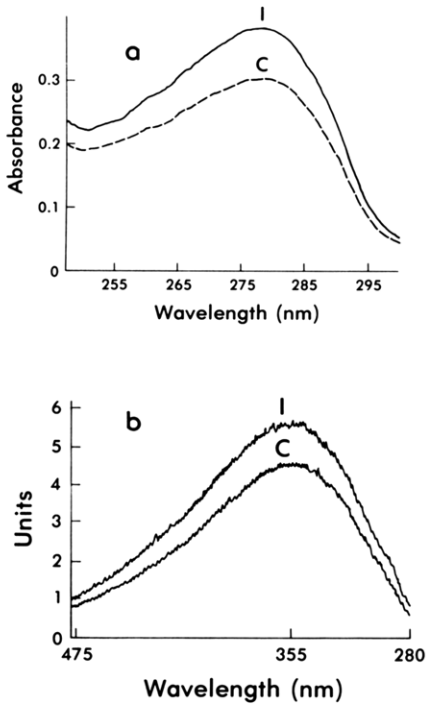

FIGURE 3: Ultraviolet absorption and emission spectra. (a) Ultraviolet absorption spectra of control (C) and irradiated (I) 6S tubulin. The sample compartment contained 1 mL of control tubulin (0.15 mg/mL) or 1 mL of irradiated tubulin (0.18 mg/mL) in 0.1 M MES buffer. The reference compartment contained 0.1 M MES buffer. (b) Fluorescence emission spectra of control (C) and irradiated (I) 6S tubulin during exicitation at 280 nm. The protein concentrations and buffer conditions were the same as in (a).

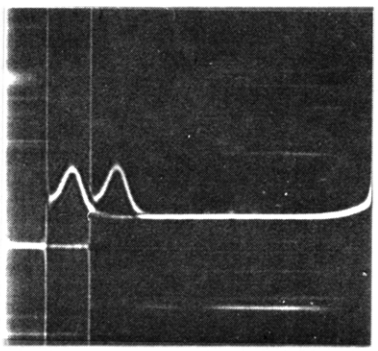

FIGURE 4: Schlieren peaks obtained during analytical ultracentrifugation of irradiated and control 6S tubulin. The irradiated sample volume was 0.42 mL and is the peak on the left; the control sample volume was 0.35 mL and is the peak on the right. Both samples were 3 mg/mL in 0.1 M MES buffer. The photograph was taken at 8 min after the rotor had reached its maximum speed of 53 500 rpm. The lens angle was 75°.

and charcoal treatment. The samples were then placed in the upper chamber of the flow dialysis cell. A typical experiment using [^{14}C]GTP and aliquots of unlabeled GTP both in the presence and absence of tubulin is shown in Figure 5. In the absence of protein, the counts per minute increase to a plateau (steady state) value and no effect is observed with the additions of unlabeled GTP. This experiment done in the absence of protein serves two purposes: (1) it establishes the steady-state plateau value for 100% free labeled GTP; (2) it demonstrates that the rate of dialysis of the labeled GTP is unaffected by further addition of unlabeled GTP. In the presence of tubulin, the initial plateau obtained after the addition of labeled GTP was much lower than that obtained in the absence of tubulin, thus indicating that most of the labeled GTP was protein

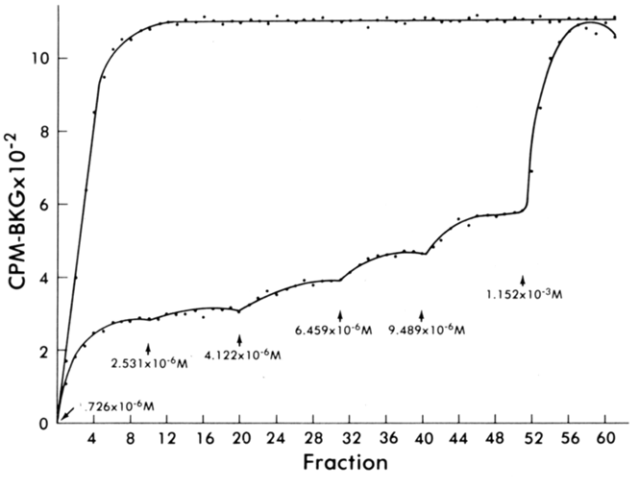

FIGURE 5: Measurement of GTP binding at varying GTP concentrations by rate of dialysis. The top curve shows the time course of the dialysis rate measurement for buffer alone, while the bottom curve shows the time course for 6S tubulin in the buffer. The buffer was 0.1 M MES buffer containing an additional 2 mM EGTA, 2 mM $CaCl_2$, and 0.24 M glycerol. The tubulin concentration was 1.336 × 10^{-5} M. The specific activity of the [^{14}C]GTP added at the beginning was 331 mCi/mmol. Aliquots of unlabeled GTP were added to give the total concentrations indicated under the arrows. The temperature was 22 °C.

Table I: GTP Binding to Irradiated and Control 6S Tubulin following Nucleotide Removal by G-25 Centrifugation and Charcoal

temp (°C)	sample	10^6K_d	n
22	Irr	4.8	0.86
22	Con	2.3	0.46
4	Irr	1.8	0.29
4	Con	1.6	0.31

bound. After each addition of unlabeled GTP, a new higher plateau value was reached, indicating that the labeled GTP was being displaced from the protein by the unlabeled GTP. The final aliquot added contained a large excess (100×) of unlabeled GTP. As can be seen from the figure, the addition of this aliquot caused a large increase in the plateau value observed and the final plateau value was very similar to that obtained in the absence of tubulin, thus demonstrating that the final addition displaced essentially all of the labeled GTP from the protein. The values obtained at each plateau together with the concentration of nucleotide added at each level were used to make a Scatchard-type plot. For the experiment shown in Figure 5, the values obtained on this plot were 2.3 × 10^{-6} M for the dissociation constant and 0.46 for the number of exchangably bound GTP molecules per molecule of tubulin dimer (n). Since the number of exchangable GTP binding sites has been reported to be 1/mol of tubulin, this latter value is rather low. In similar experiments the n value was found to vary from 0.26 to 0.86/mol of tubulin. The reason for this variation was found to be the inability to remove all of the GTP (or GDP) bound at the E site prior to the binding studies. Indeed, Penningroth & Kirschner (1978) have also reported that regardless of the treatment used to remove nucleotides from 6S tubulin, an average 0.4 mol of GTP (or GDP) per mol of tubulin remains bound at the E site. Table I shows the GTP binding results obtained at two different temperatures for irradiated and control 6S tubulin. When the binding experiment was performed on the same day, the K_d obtained with the irradiated tubulin was greater at either temperature than that obtained with the control tubulin. The Scatchard-type plot for the experiment at 22 °C is shown in Figure 6.

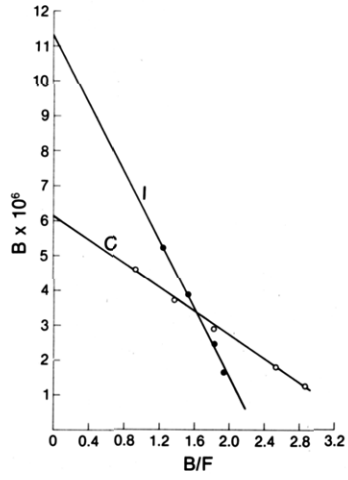

FIGURE 6: Scatchard-type plot of GTP binding to irradiated (closed circles) and control (open circles) 6S tubulin. The values were obtained by rate of dialysis measurements. The conditions were the same as indicated in the legend to Figure 5.

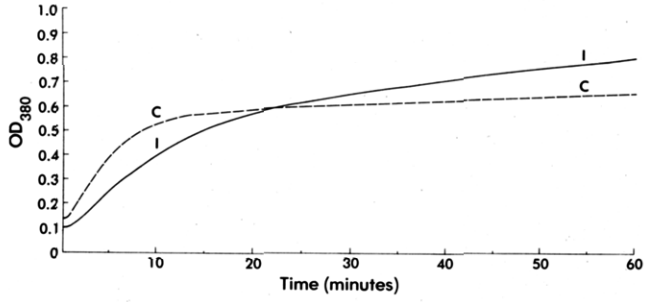

FIGURE 7: Turbidity development at 380 nm during polymerization of control (C) and irradiated (I) 6S tubulin in the presence of Me_2SO. The concentrations of control and irradiated samples were 1.614×10^{-5} M and 1.607×10^{-5} M, respectively. Both samples were in 0.2 M MES buffer, pH 6.4, containing 0.5 mM $MgCl_2$, 2 mM EGTA, 0.1 mM EDTA, 1 mM GTP, and 10% Me_2SO. The sample compartment was maintained at 37 °C.

No conclusions can be drawn from the *n* values because of the variation due to the problem of GTP removal.

Polymerization with Me_2SO. Himes et al. (1977) have reported that pure 6S tubulin will form microtubules if the protein concentration is high enough (8–10 mg/mL or greater), or at a concentration of 1 mg/mL if 10% Me_2SO and 0.2 M buffer are included in the reaction mixture. Since phosphocellulose-purified 6S tubulin contains no tubulin oligomers, Me_2SO-induced polymerization must proceed directly via association of tubulin dimers regardless of the particular mechanism involved. Therefore, turbidity assays for polymerization were performed on irradiated and control 6S tubulin in the presence of Me_2SO in order to determine the effect of irradiation on self-association events of polymerization. The results from a typical experiment in which the irradiated sample received a dose of 50 000 rd are shown in Figure 7.

Following the lag, the initial rate of polymerization was greatly depressed in the irradiated sample. It can also be seen that the total increase in turbidity was greater for the irradiated sample than for the control. However, the turbidity of the irradiated tubulin did not level off to a plateau as in the case of the control tubulin, but rather continued to increase gradually with no sign of eventually reaching a plateau. Such a gradual increase in turbidity has been reported to be indicative of aggregation rather than polymerization (Penningroth & Kirschner, 1978). Therefore, at the end of the experiment, aliquots were removed from both the control and irradiated samples and were examined by electron microscopy. Typical

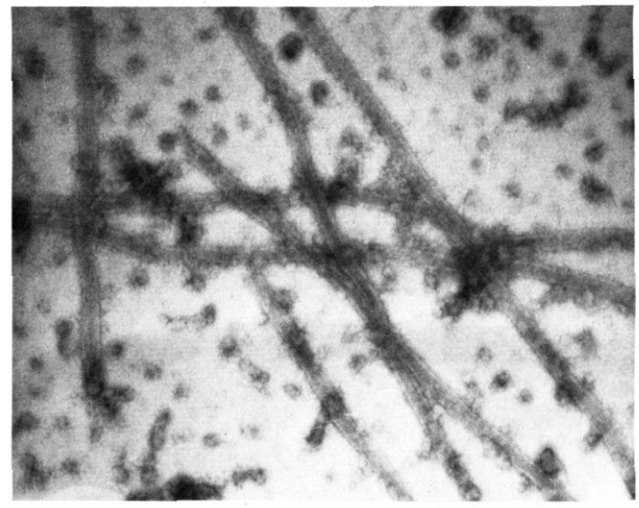

FIGURE 8: Electron micrograph of microtubules polymerized from control 6S tubulin and stained with uranyl acetate. The protein was polymerized at 2 mg/mL and was then diluted 1:10 with buffer. The buffer conditions were the same as indicated in the legend of Figure 7. Magnification 65000×.

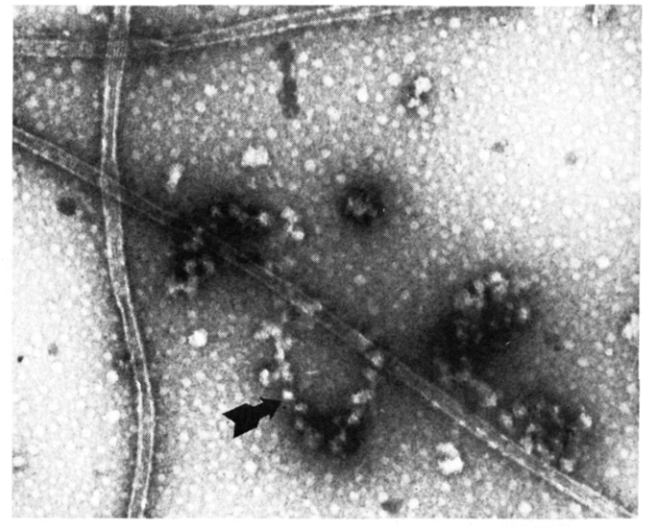

FIGURE 9: Electron micrograph of microtubules polymerized from irradiated 6S tubulin and stained with uranyl acetate. See Figures 7 and 8 for conditions. Magnification 65600×.

electron micrographs are shown in Figures 8 and 9. It can be seen that the microtubules polymerized from either control or irradiated tubulin in the presence of Me_2SO were of normal morphology. However, fewer microtubules were formed from the irradiated tubulin when compared with the control tubulin at the same concentration. This was true for every grid examined following these experiments. Furthermore, the grids prepared from the irradiated samples always contained a rather large number of elongated aggregates which could perhaps account for the residual turbidity development compared with the control samples.

In order to confirm that the greater turbidity developed by the irradiated tubulin was due to aggregation rather than polymerization, a polymerization–depolymerization experiment was performed (data not shown). Both control and irradiated samples were allowed to polymerize for 40 min at 37 °C in the presence of Me_2SO. The temperature of the sample compartment was then changed to 2 °C and the depolymerization was followed until a lower plateau was reached at 150 min. The maximum turbidity developed during polymerization for the irradiated sample was 22% greater than that developed

Table II: Effect of Irradiation on the Initial Rate of Polymerization of Tubulin in the Presence of Me_2SO

no.	sample	10^{-3} × rate/M[b]	% of control
	A. No BME[a]		
1	Irr	2.46	57.9
	Con	4.25	
2	Irr	1.57	61.8
	Con	2.54	
3	Irr	3.12	51.1
	Con	6.10	
4	Irr	2.08	44.8
	Con	4.64	
	B. With BME[a]		
1	Irr	1.93	78.1
	Con	2.47	
2	Irr	3.83	71.2
	Con	5.38	
3	Irr	3.23	58.2
	Con	5.55	

[a] Tubulin was prepared by phosphocellulose chromatography in the presence or absence of BME. [b] Obtained at the steepest portion of the curve and calculated as the change in absorbance at 380 nm per min divided by the tubulin concentration in moles per liter.

Table III: Effect of γ Irradiation on the Initial Rate of Polymerization of 6S Tubulin[a]

dose (rd)	initial rate of polymerization[b]
25 000	90
30 000	84
40 000	70
50 000	54

[a] The conditions of polymerization were as indicated in Figure 6. [b] Expressed as percent of control, based on the change in absorbance at 380 nm per minute divided by the tubulin concentration in moles per liter.

for the control. The turbidity remaining following depolymerization in the irradiated sample was 27% greater than that remaining for the control. Therefore, there is good agreement between the amount of increased turbidity during polymerization and the amount of turbidity remaining following depolymerization in the irradiated tubulin compared with the control tubulin. These results along with the electronmicroscopic analysis strongly suggest that irradiation causes an increased amount of aggregation of tubulin dimers rather than polymerization into microtubules.

The most striking effect of the irradiation was on the initial rate of polymerization as shown in Table II. In the absence of BME, the initial rate of polymerization of the irradiated tubulin was, on the average, 54% of the rate of the control tubulin. In some experiments, 1 mM BME was included in the buffer and the polymerization with Me_2SO was performed in the same manner. These results are also shown in Table II. In this case, the initial rate of polymerization of the irradiated tubulin was on the average 69.2% of the rate of the control tubulin. Thus, both in the presence and absence of BME, irradiation caused a decrease in the initial rate of polymerization, but in the presence of BME this reduction in the rate was approximately 15% less than in the absence of BME. These results suggested that the irradiation might be affecting the free sulfhydryl or disulfide groups of tubulin. Furthermore, the effect of irradiation on the initial rate of polymerization was dose dependent, as shown in Table III. It can be seen that, in the absence of BME, the initial rate of polymerization of the irradiated tubulin was 90% of the rate of the control tubulin at a dose of 25 000 rd, compared with 54% at a dose of 50 000 rd. Johnson & Borisy (1977) have reported that the initial rate of polymerization is dependent on the 6S tubulin concentration and also, since the concentration of microtubules is determined during the lag, on the nucleation events. Our previous results had demonstrated that irradiation of microtubular protein caused an average 24% reduction in the final extent of polymerization when both 36S and 6S components were present during the irradiation. This is most likely a reflection of radiation damage to the elongation reaction (addition of 6S tubulin to microtubule ends) since the oligomeric component was unaffected by irradiation. The present results with 6S tubulin alone show that irradiation causes an average 46% reduction in the initial rate of polymerization (Table II, no BME). Since the initial rate is a function of both nucleation and elongation reactions and since the irradiation affects the initial rate to a greater degree than it affects the elongation reaction, these results suggest that irradiation-induced damage of 6S tubulin inhibits its ability to participate in both the initiation and elongation events of microtubule polymerization.

Number of Sulfhydryl Groups following Irradiation. The results reported in the previous section had suggested that the presence of BME reduced the effect of irradiation on the initial rate of polymerization. Studies in other laboratories (Stephens et al., 1966; Mellon & Rebhun, 1976a,b) had indicated that sulfhydryl groups play a role in the polymerization process both in vivo and in vitro. In addition, irradiation has been reported to cause the inactivation of a number of enzymes by the oxidation or destruction of sulfhydryl groups (Okada, 1970b). Therefore, it was of interest to examine the effect of irradiation on the number of free sulfhydryl groups present in 6S tubulin.

Previous reports from several laboratories had indicated that tubulin contains 7–11 free sulfhydryl groups per 55 000 molecular weight monomer (Eipper, 1974; Kuriyama & Sakai, 1974; Mellon & Rebhun, 1976a,b; Lee et al., 1973) and one intrachain dilsulfide bridge (Lee et al., 1973). However, in two of these reports (Eipper, 1974; Lee et al., 1973), the protein was isolated by chromatography on DEAE-cellulose or DEAE-Sephadex which does not produce pure 6S tubulin (Sandoval & Cuatrecasas, 1976). In other reports (Kuriyama & Sakai, 1974; Mellon & Rebhun, 1976b), while the protein was isolated by a polymerization–depolymerization method similar to that used in this laboratory, no attempt was made to further fractionate the system. Since the protein obtained in this manner contains a variable amount of the oligomeric component depending on the protein concentration (Weingarten et al., 1974), these authors also did not use pure 6S tubulin.

Mellon & Rebhun (1976a,b) have reported that protein isolated in the presence of glycerol contains seven sulfhydryl groups per 55 000 molecular weight tubulin monomer while protein isolated in the absence of glycerol contains only four sulfhydryl groups per monomer. We therefore used microtubular protein prepared in the presence and absence of glycerol in these irradiation studies. In either case, 6S tubulin was obtained by phosphocellulose chromatography (in the absence of BME) and the number of sulfhydryl groups was determined with DTNB as described under Methods. Table IV shows the results obtained for control and irradiated 6S tubulin prepared in the presence and absence of glycerol. It can be seen that while there is a variation in the absolute number of free sulfhydryl groups obtained in all cases (the reasons for this will be discussed later), there was always a reduction in this number following irradiation. In the absence

Table IV: Effect of Irradiation on the Number of Free Sulfhydryl Groups in Tubulin

no.	conditions[a]	irradiated[b]	control[b]	Con − Irr
	A. Tubulin Prepared in the Absence of Glycerol			
1	native	6.9	8.6	1.7
2	native	8.2	9.5	1.3
3	native	5.9	7.3	1.4
4	native	4.7	7.5	2.8
5	denatured	4.6	7.1	2.5
6	native		9.2	
		av 6.06	av 8.2	av 1.94[c]
	B. Tubulin Prepared in the Presence of Glycerol			
1	native	11.9	12.4	0.5
2	native	9.1	10.0	1.1
3	native	11.0	13.6	2.6
4	denatured	11.1	11.5	0.4
5	denatured	12.2	13.5	1.3
6	native		10.6	
7	native		12.8	
		av 11.06	av 12.06	av 1.18[c]

[a] Denatured samples were in the presence of 7 M urea. [b] Results are expressed as moles of sulfhydryl per 55 000 dalton tubulin monomer. [c] This is the average difference between control and irradiated tubulin. See text for details of calculation.

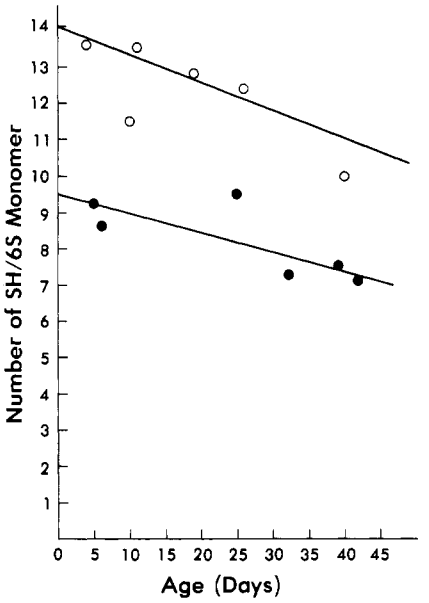

FIGURE 10: Effect of storage on the number of free sulfhydryl groups in 6S tubulin. The number of sulfhydryl groups per monomer is for control tubulin, and was taken from Table IV. The age was calculated from the day of isolation of protein from calf brains. (Open circles) Protein isolated and stored in the presence of glycerol; (closed circles) protein isolated and stored in the absence of glycerol.

of glycerol, the average difference in the number of sulfhydryl groups between control and irradiated tubulin was 1.94, while the average difference obtained in the presence of glycerol was 1.18. These numbers were calculated by subtracting the value obtained for the irradiated sample from the value obtained for the control sample in the same experiment and then averaging the differences obtained from all experiments. In the absence of glycerol, the average number of sulfhydryl groups obtained was 8.2 for the control and 6.06 for the irradiated tubulin; in the presence of glycerol the average number obtained was 12.06 for the control and 11.06 for the irradiated tubulin. Therefore, regardless of the method of calculation used, the results show that irradiation appears to reduce the number of free sulfhydryl groups by 2 per tubulin monomer in the absence of glycerol and by 1 per tubulin monomer in the presence of glycerol. Furthermore, no significant difference in the number of sulfhydryl groups was observed between native and denatured (7 M urea) control tubulin, in agreement with other reports (Eipper, 1974; Mellon & Rebhun, 1976a,b). This was also true for the irradiated tubulin and suggests that the reduced number obtained following irradiation was not due to the sequestering of free sulfhydryl groups in an inaccessible or unfavorable environment for reaction with DTNB. It should be noted that the values obtained were based on the number of sulfhydryl groups per 55000 molecular weight tubulin monomer. Since tubulin exists as a dimer in solution (M_r 110000), the irradiation has reduced the number of sulfhydryl groups by four per dimer in the absence of glycerol and by two per dimer in the presence of glycerol. Therefore, these results strongly suggest that irradiation is causing the formation of one or two disulfide bonds in the tubulin dimer depending on the presence or absence of glycerol.

As noted before, the absolute number of sulfhydryl groups obtained in all cases varied from one experiment to another. The results obtained for the control tubulin were plotted against the number of days the microtubular protein was stored following its isolation. The results are shown in Figure 10. While there is some scattering of points, a clear trend can be seen: there is a decrease in the number of free sulfhydryl groups with increasing time of storage for both glycerol and glycerol-free tubulin. If the line obtained is extrapolated back to zero days in storage, it would appear that tubulin prepared in the presence of glycerol should have 14 sulfhydryl groups per monomer, and 9–10 sulfhydryl groups when prepared in the absence of glycerol. Although these values seem high in comparison to those previously cited, a recent study (Ikeda & Steiner, 1978) has demonstrated the presence of 12 sulfhydryl groups per monomer when the protein was isolated under nitrogen. However, these authors made no attempt to fractionate the whole microtubular protein obtained.

Discussion

Sulfhydryl groups have been implicated for many years as having a role in microtubule function. Several early reports of agents which disrupted mitotic spindles, such as BME, 3,4-dithiahexane-1,6-diol, and *p*-chloromercuribenzoate (Stephens et al., 1966; Zimmerman, 1960; Mazia, 1956), led to the postulation that both free sulfhydryl groups and disulfide bonds were important for maintenance of the structure of the mitotic spindle. Recently several reports have appeared in which the relationship between sulfhydryl groups and microtubular protein structure and function were investigated (Kuriyama & Sakai, 1974; Wallin et al., 1977; Mellon & Rebhun, 1976a). Although these studies have not revealed any clear-cut or specific mechanistic role for sulfhydryl groups in microtubule polymerization, they have shown that chemical modification of sulfhydryl groups results in loss of function and, in some cases, alteration of quaternary structure. All of these studies have employed whole microtubular protein which in the depolymerized state exists as a mixture of the 6S tubulin dimer and the 30–36S tubulin oligomer. However, in no instance was there any attempt to determine whether preferential modification of one or the other components had occurred. Interpretation of these results has also been complicated by the large number of reactive sulfhydryl groups present in all tubulins resulting in the inability to correlate alteration of structure or function with the modification of specific sulfhydryl groups.

In this paper we have presented evidence which demonstrates that modification of a single pair of sulfhydryl groups located on the 6S tubulin dimer isolated in the presence of

glycerol results in reduction of its ability to be incorporated into a polymerizing microtubule and also alters its GTP binding ability. In addition, evidence from ultraviolet absorption and intrinsic fluorescence emission spectra as well as from analytical ultracentrifugation indicate that modification of the pair of sulfhydryls is accompanied by a perturbation of the proteins' conformation.

The experiments involving polymerization in Me_2SO are very useful for examining the ability of pure 6S tubulin to associate into microtubules. Thus, the unusual polymerization behavior observed with irradiated 6S tubulin is an indication that the irradiation is modifying a substantial portion of the free 6S tubulin. Our results indicate that the radiation is modifying two sulfhydryl groups per tubulin dimer and that this modification results in the abnormal polymerization behavior observed in Me_2SO. In an effort to test this hypothesis, we performed an experiment (unpublished experiments) in which 6S tubulin, purified from phosphocellulose, was reacted with pyrenemaleimide (pm), a sulfhydryl-specific reagent, and then the Me_2SO polymerization behavior of this modified tubulin was examined. The pm-modified tubulin exhibited the same aberrant polymerization kinetics in the presence of Me_2SO as that observed following irradiation of unmodified tubulin. The altered kinetics consisted of a decreased initial rate of polymerization, a more or less linear increase in turbidity with little sign of a plateau region, and a final turbidity greater than the control which was indicative of aggregation along with polymerization. This behavior was only observed with 6S material which was uniformly modified by the reaction of two or more sulfhydryl groups per dimer with the pm.

In an attempt to further characterize the effects of irradiation on 6S tubulin, we also examined the ability of irradiated material to bind GTP. Our results indicate that the binding constant for GTP on irradiated tubulin is approximately 2-fold larger than the binding constant for GTP on control 6S at 22 °C. It should be noted, however, that during the course of the GTP binding studies we encountered some difficulty in removing the endogenously bound GTP or GDP prior to performing a binding experiment. Thus, the stoichiometry of GTP binding was not correct. The results indicate that on the average we only observe the binding of 0.4 mol of GTP per tubulin dimer, whereas values reported in the literature (Jacobs et al., 1974; Levi et al., 1974; Weisenberg et al., 1976) all indicate that approximately 1 mol of GTP is exchangeably bound per free 6S dimer. Analysis of the amount of endogenous guanine nucleotide present in both the irradiated and control samples used for the GTP binding experiment revealed the presence of approximately 0.4–0.5 mol of exchangeable guanine nucleotide per tubulin dimer. Thus, the 0.4 mol of GTP per mol of dimer observed in the binding added to the 0.5 mol endogenously bound would give a value close to the observed value of 1 per dimer reported for the E site or exchangeable nucleotide. Since the technique we employed for the GTP binding studies measured only exchangeable GTP, this implies that the residual nucleotide which was not removed by our prior treatment was not in a form capable of exchanging with free nucleotide from the medium. Although Penningroth & Kirschner (1977, 1978) have also reported problems in removing nucleotide from 6S tubulin, it is not at all apparent why this should be the case. The endogenous material in our samples did not appear to be covalently bound since it was released upon perchloric acid precipitation.

The above studies of GTP binding also provide evidence that radiation is altering a fairly large fraction of the population of free 6S molecules. The Scatchard plots of the binding data obtained with irradiated 6S material are linear, indicating that they can be approximated by a single set of binding sites characterized by a single dissociation constant. These results undoubtedly represent the average behavior of all those 6S molecules capable of binding GTP and do not necessarily imply that all sites are homogeneous. However, it would be difficult to rationalize these results with the idea that radiation is only altering a very small fraction of total population of 6S molecules. In this latter case one might expect either a biphasic binding curve characterized by two distinct sets of binding sites or dissociation constants or a binding curve which was identical with the control. Our results, however, indicate that at least 50% of the 6S molecules present are capable of binding GTP and this binding can best be characterized with the dissociation constant we observed which is 2-fold greater than that for the control at 22 °C.

We thus conclude on the basis of the studies presented in this paper that irradiation of free 6S tubulin causes the loss of two sulfhydryl groups which induces a conformational change such that the affinity for GTP is reduced. This conformational change is functionally expressed as a loss or greatly decreased ability of the tubulin dimer to participate in the polymerization process.

It is interesting that tubulin isolated in the absence of glycerol contains approximately 40% fewer free sulfhydryl groups than that isolated in the presence of glycerol and still retains the ability to polymerize. It is not clear, however, why the irradiation causes the loss of four sulfhydryl groups in tubulin isolated without glycerol and only two sulfhydryl groups in tubulin isolated with glycerol. One possibility is that since glycerol has been reported to bind to tubulin (Detrich et al., 1976) it could protect certain groups from radiation damage. It was not possible to determine if the loss of two additional sulfhydryl groups would further inhibit the polymerization process since the low protein yield obtained in the absence of glycerol precluded its fractionation into 6S tubulin in sufficient quantity for polymerization studies.

Preliminary experiments have indicated that the loss of the free sulfhydryl groups following irradiation of tubulin is due to the formation of disulfide bridges. These experiments demonstrated that if the tubulin was incubated with BME in the presence of 7 M urea, there was no difference in the number of free sulfhydryl groups between irradiated and control tubulin as determined by titration with DTNB following the removal of the BME. This conclusion also seems reasonable because the radiation induces the loss of an even number of sulfhydryl groups in tubulin regardless of whether it was isolated with or without glycerol. In addition, the initial rate of polymerization was greater following irradiation if BME was included in the buffer during the fractionation procedure. Although BME is undoubtedly acting as a radical scavenger (Okada, 1970b), it also seems possible that its presence could prevent or reverse the formation of disulfide bonds due to the radiation. The disulfide bridges appear to be intra- rather than intermolecular in nature, since the Schlieren patterns obtained during analytical ultracentrifugation showed only one sedimenting species following irradiation. In addition, preliminary experiments have shown that there is no difference in the number or position of the bands obtained on gels between irradiated and control tubulin samples which were prepared for electrophoresis in the absence of BME. Therefore, our results suggest that irradiation preferentially damages the free 6S tubulin dimer by inducing the formation of disulfide bridges; this causes a conformational

change, a reduction in the affinity for GTP, and an inhibition of the polymerization process.

The large irregular aggregates observed by electron microscopy following irradiation of tubulin and polymerization with Me_2SO are also of interest. Although some aggregates were observed with the control tubulin which was prepared and polymerized under the same conditions, they were much fewer in number and were not elongated as were those associated with the irradiated samples. It is probable that these structures are responsible for the more or less linear increase in turbidity with no plateau region which is observed during the polymerization of irradiated tubulin. It also seems reasonable that the aggregates are responsible for the greater total turbidity developed during polymerization and remaining after depolymerization of the irradiated sample. These structures have some characteristics which resemble the 18S oligomers of tubulin which are present in some microtubular protein preparations (Marcum & Borisy, 1978; Vallee & Borisy, 1978). An increase in the amount of 18S oligomers was observed as the ionic strength or the pH of the buffer was increased. Under the conditions which favor the formation of the 18S species, the rate and extent of polymerization were significantly reduced (Olmsted & Borisy, 1975). Therefore, it appears that this oligomer of tubulin may not be a normal intermediate in the polymerization process but perhaps is an abnormal product whose formation prevents the proper association of tubulin dimers necessary for microtubule production. Since our results demonstrate that irradiation causes a decrease in the rate and extent of polymerization as well as the formation of elongated aggregates, it is possible that the aggregates and the 18S oligomer are produced by disulfide bond formation. This is supported by the observation that the 18S oligomer is favored at higher pH since the rate of autoxidation of sulfhydryl groups has been shown to increase with increasing pH (Jocelyn, 1972; Friedman, 1973). In addition, we have observed that with increasing time of storage of microtubular protein, there is a decrease in the amount of polymerization, an increase in aggregation, and a decrease in the number of free sulfhydryl groups. All of these findings taken together strongly suggest that disulfide bond formation, which can be induced by irradiation, storage, or higher pH levels, leads to the incorrect association of tubulin dimers into an aggregated form.

Our results also could provide an explanation for the radiation-induced mitotic delay which has been shown to occur when cells are irradiated during the G2 phase of the cell cycle. It is during this phase that the extensive array of cytoplasmic microtubules depolymerize and the mitotic spindle is formed apparently from the increased pool of soluble microtubular protein. Since the spindle microtubules appear to be in a dynamic equilibrium with the soluble tubulin dimers, exposure of cultured cells to ionizing radiation during G2 could delay 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.

Recovery from mitotic delay might result from de novo synthesis of tubulin, thus increasing the viable pool of microtubule precursors. However, Noland et al. (1974) have reported that there is no increase in the synthesis of tubulin following X-irradiation. Alternatively, recovery from mitotic delay could be a result of cellular mechanisms leading to repair of the damaged tubulin dimers. For example, glutathione (GSH) and oxidized glutathione (GSSG) have been implicated in the in vivo control of microtubule polymerization and depolymerization (Mazia, 1956, 1959). In vitro, GSSG has been shown to inhibit microtubule formation (Mellon & Rebhun, 1976a). Therefore, it seems possible that in vivo the disulfide bonds formed in the tubulin dimer as a result of irradiation could be reversed by GSH, which may be a physiological regulator of microtubule assembly.

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Resonance Raman Spectroscopy of Cytochrome Oxidase Using Soret Excitation: Selective Enhancement, Indicator Bands, and Structural Significance for Cytochromes *a* and a_3[†]

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ABSTRACT: Resonance Raman studies of oxidized and reduced cytochrome oxidase and liganded derivatives of the oxidized enzyme have been performed by using direct-Soret excitation at 413.1 and 406.7 nm, as well as near-Soret excitation (457.9 nm) and α-band excitation (604.6 nm). The Soret results clearly show selective enhancement of Raman modes of the hemes of cytochromes *a* and a_3, depending upon the excitation wavelength chosen. For the preparations employed in this study, photoreduction of cytochrome oxidase in the laser beam was not a significant problem. Resonance Raman frequencies sensitive to oxidation state and spin state or core expansion of the *a* and a_3 hemes are identified and correlated with those previously identified for other heme proteins. An unusual low-frequency (<500 cm^{-1}) spectrum is observed for oxidized high-spin cytochrome a_3, which may be due to axial nonheme structures in this cytochrome.

Resonance Raman spectroscopy (RRS)[1] has proven to be an extremely informative probe for the structures and electronic states of hemes, both in their heme protein environments (Spiro, 1975) and as protein-free metalloporphyrins (Spiro & Burke, 1976; Spaulding et al., 1975; Kitagawa et al., 1975). Information available from heme protein RRS includes oxidation state (Spiro & Strekas, 1974), ligation state (Spiro et al., 1979), and spin state (Spiro & Strekas, 1974) of the iron atom, macrocyclic "hole size" associated with the equatorial nitrogen donors of the heme (Spaulding et al., 1975; Spiro et al., 1979), and perhaps effects of protein tertiary and quaternary structure (Shellnut et al., 1979). Clearly, such information on cytochrome *c*/dioxygen oxidoreductase (cytochrome oxidase) would be invaluable in elucidating the heme structures involved in the stable forms of the enzyme and in identifying the heme species associated with the various transients in the electron-transfer sequence of reacting cytochrome oxidase.

Cytochrome oxidase has, however, proved more resistant to analysis by RRS than other heme proteins. Although cytochrome oxidase was one of the first proteins to have been examined by RRS (Nafie et al., 1973) and has been the subject of several RRS studies since (Salmeen et al., 1973, 1978; Adar & Yonetani, 1978; Babcock & Salmeen, 1979; Bocian et al., 1979; Ondrias & Babcock, 1980), the general phenomenology of cytochrome oxidase RRS is far from established, the catalogue of RR spectra of its various oxidation states and derivatives is quite incomplete, and entirely satisfactory interpretations of even the data which exist are lacking. There are several reasons for this situation. Primarily, preparations of cytochrome oxidase which are suitable for RRS study are even

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[1] Abbreviations used: RRS, resonance Raman spectroscopy; RR, resonance Raman; HRP, horseradish peroxidase; Hepes, *N*-2-(hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid.

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