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A Circular Dichroism Study of Microtubule Protein†

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ABSTRACT: The conformation of tubulin, the 6S protein subunit of microtubules, has been studied by circular dichroism (CD) spectroscopy. The native protein at 4° and pH 6.5 contains approximately 22% α helix, 30% β structure, and 48% random coil. This protein is characterized by its ability to bind the antimitotic drug colchicine. At 37°, where colchicine binding is optimal the protein undergoes a slow conformational change resulting primarily in loss in α helix. This change increases the lability of the protein to irreversible denaturation accompanied by aggregation. The protein can be protected by the presence of various nucleotides such as GTP, GDP, GMP, GMPPCP, and the antimetabolites colchicine and vinblastine. Colchicine binding shows a sharp optimum at pH 6.5. Variations of pH away from this value lead to mild alternations in circular dichroism at 37°, much larger effects at 4°. All of the results obtained are consistent with the idea that colchicine-binding ability is a critical function of the protein conformation. A few preliminary measurements on the CD of intact sea urchin sperm tail microtubules are reported. GMPPCP and colchicine increase the rate of thermal dissociation of the tubule, as observed by changes in the CD spectra.

Microtubules, 240-Å diameter tubular structures, are widely distributed in plant and animal cells (Porter, 1966). These tubules make up the spindle fibers of the mitotic cell, the axonemal complex of cilia and flagella, and are prominent in the axons and dendrites of neurons. The precise function of the microtubule is unknown although they have been implicated in such diverse functions as axoplasmic transport, ciliary motion, morphogenesis, and form maintenance (Shelanski and Feit, 1971). While the microtubules in cilia and flagella appear to be stable over a wide range of temperature, pressure, and drug treatments, cytoplasmic microtubules are very labile. This lability is best demonstrated by the experiments of Tilney (Tilney *et al.*, 1966; Tilney and Porter, 1967) on the microtubule rich axopods of the heliozoan Actinosphaerium. In these thin processes the microtubules are depolymerized and the process retracted on exposure of the cell to low temperature (4°), high pressure (3000 psi), or the antimitotic drug colchicine. All these effects are reversible. The microtubules are stabilized by exposure to D_2O.

The subunit protein of the microtubule, tubulin or MTP, has been isolated from cilia (Renaud *et al.*, 1968), sea urchin sperm flagella (Shelanski and Taylor, 1967, 1968; Stephens, 1968), and numerous cytoplasmic sources, especially brain (Borisy and Taylor, 1967a,b; Weisenberg *et al.*, 1968). In all cases the subunit isolated was a 6S, 120,000 dimer which has approximately 2 moles of guanine nucleotide bound per dimer. The protein obtained from cytoplasmic sources and from the central pair of microtubules of the sperm flagellum

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‡ Supported by the National Institutes of Health Predoctoral Fellowship 5F01 GM46,899.

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also bound 1 mole of colchicine/dimer. Recent work has indicated that more than one species of tubulin may be present in the flagellar axoneme (Stephens, 1970; Jacobs and McVittie, 1970) and that each of these species is composed of dimers in which the monomers are nonidentical (Feit *et al.*, 1971; Bryan and Wilson, 1971).

In addition to colchicine, other metaphase-blocking antimitotic drugs interact with microtubules and microtubule protein. Some such agents as colchamide and podophyllotoxin appear to bind at or near the colchicine sites (Wilson, 1970) while others, primarily the vinca alkaloids, bind at a site separate from the colchicine site. Vinblastine, an antimitotic vinca alkaloid, causes and aggregation and precipitation of microtubule protein (Weisenberg and Timasheff, 1970; Ventilla *et al.*, 1970; Marantz *et al.*, 1969; Olmstead *et al.*, 1970). The precipitation proceeds in a stepwise fashion and the precipitate has a highly ordered paracrystalline structure (Bensch *et al.*, 1969).

In this work the circular dichroism (CD) of MTP has been measured at various temperatures, pH's, and solvent conditions. This was done in an attempt to correlate secondary structure of the protein with functional and higher order structural changes. The major structural change of interest is aggregation. The functional changes of interest are the vinblastine-induced aggregation and colchicine binding ability. In addition the protecting effects of various nucleotides on these activities were examined. Nucleotides are known to play an important role in MTP aggregation. CD measurements can determine if this role is at all correlated with secondary structure.

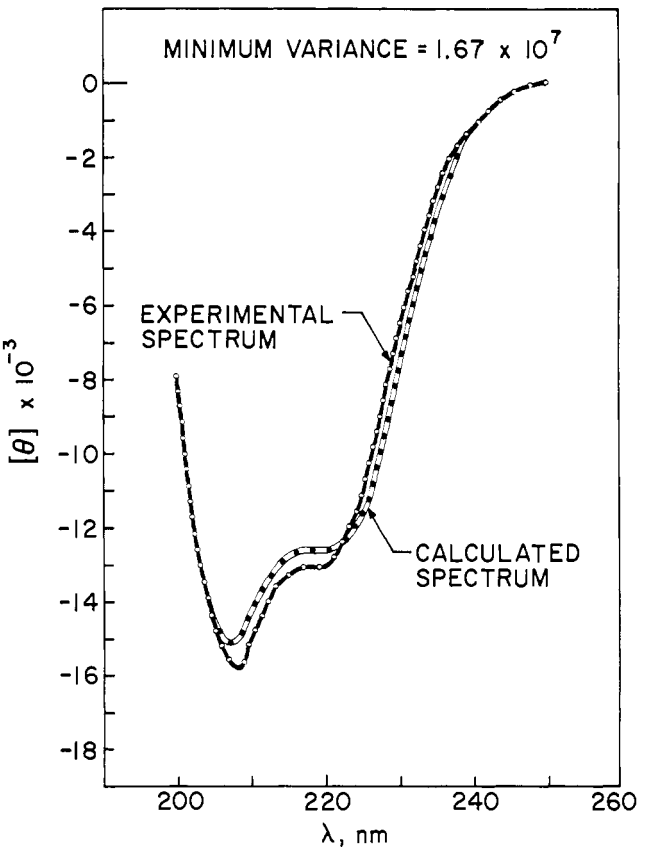

FIGURE 1: Experimental and computer calculated CD spectra of MTP at pH 6.5 at 4°.

Methods and Materials

Microtubule Protein. It was of extreme importance to prepare the protein fresh prior to CD measurements in order to minimize the effect of aggregation which inevitably occurs upon aging. This could cause scattering problems, and artifacts in measuring the optical activity. The protein was prepared from pig brains, using the procedure of Weisenberg *et al.* (1968). The purity of the preparation and the absence of aggregates was checked by measuring the sedimentation velocity in the analytical ultracentrifuge, zone sedimentation in sucrose gradients, and polyacrylamide gel electrophoresis. The colchicine binding activity was also tested. The protein used gave a single 6S peak, a single electrophoretic band and its colchicine binding was between 0.5 and 0.7 mole per dimer. The concentration of the protein was determined in order to calculate the residue ellipticity after each CD run. The method used, was that of Lowry *et al.* (1951). The routine standard was bovine serum albumin; this was calibrated against the Lowry test of lyophilized MTP which had been previously dialyzed overnight against distilled water. The protein concentration used for CD was around 0.1 mg/ml. The protein used in CD measurements was 12–24-hr old, and was clarified at 40,000 rpm for 30 min in a 50.1 rotor just prior to optical measurements. Concentration determination was carried out on each CD sample individually. The vinblastine used in our experiments was a generous gift from Ely Lilly and Co., the colchicine and the nucleotides were purchased from Sigma. Tritiated colchicine was obtained from New England Nuclear.

Sample Preparation. The protein was kept in pH 6.5 phosphate buffer, at 4° unless otherwise indicated. The buffer was 0.05 M in phosphate, 10^{-2} M in Mg^{2+}, and 10^{-3} M in GTP. The pH was varied when necessary by addition of minute drops of HCl or NaOH. The colchicine- and vinblastine-treated samples along with the nucleotide bound ones were incubated for 1 hr at 37°, and chromatographed prior to CD measurements on G-100 Sephadex columns. The above buffer minus GTP was used for elution and all CD measurements were made in this buffer unless otherwise indicated. The columns were 1 cm in diameter and 30 cm long. The chromatography was necessary because of the optical activity of the drugs and nucleotides. At concentrations of bound drug and nucleotide, assuming a 1:1 complex with MTP, their contribution to the optical activity was negligible. But in order to get maximum binding, 10^{-3} M concentrations were used initially. The optical activity was measured in a Cary 60 spectrophotopolarimeter, using the 6001 CD attachment. A 1-cm path-length cylindrical cell (Opticell, Inc.) was used with thermostated jacket, holding about 0.6-ml samples. The cell temperature was controlled with a Lauda k2/R circulating temperature bath.

Analysis of Optical Activity. The CD results are expressed in terms of $[\theta]$—the mean residue ellipticity, in units of (deg cm^2)/dmole. In all cases the mean residue weight was taken as 115 (Greenfield and Fasman, 1967, 1969). Data reduction was performed as previously described (Cantor *et al.*, 1970) except that the spectra were digitized at 1.0-nm intervals. All spectra were resolved into probable contributions from α helices, β structures, and random coil using an IBM 360-91 computer program written by Peter Kahn. The program uses as input standard curves for 100% α helix, β structure, and random coil. The data used were obtained by Pflumm and Beychok (1969). The program calculates all possible CD spectra for 1% increments of α, β, and random coil compositions. The calculated spectrum showing the least variance from the

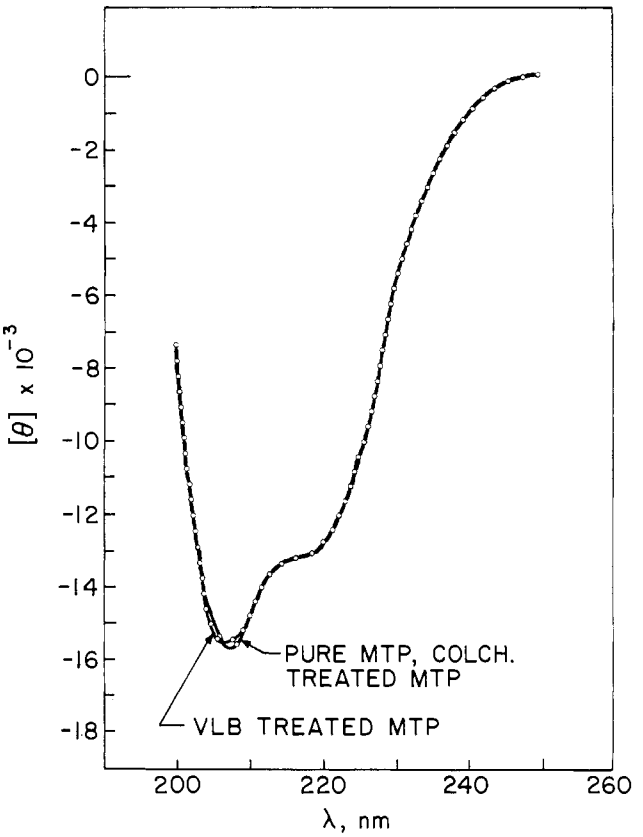

FIGURE 2: Experimental CD spectra of MTP at 4°, pH 6.5, VLB (vinblastine)-, and colchicine-treated protein. The spectra of MTP with and without colchicine are superimposable within experimental error.

experimental is defined as the best fit. For various experimental spectra the variance was generally in the order of 10^7 for an average fit, 10^8 for the worst, and 10^6 for the best one. Figure 1 illustrates an average fit between theoretically calculated and experimental spectra. The agreement is fairly good. The fits are very sensitive to the conformational parameters used. For a given experimental curve the variance changes quite sharply when the proposed conformation is perturbed from the values which give a minimum variance. The precision of the fits is about 1%. The accuracy of a conformation determined from a CD measurement is however much less reliable since so many assumptions must be entered including the accuracy of the input curves, and neglect of side chains.

Results and Discussion

CD of Isolated MTP Dimers. Figure 1 illustrates the CD spectrum of isolated MTP dimers (The term "dimers" is used to indicate a condition where the 6S form is the major form present.) under standard conditions, pH 6.5, and 4°. The protein is in its 6S form, and at the concentrations used the solution showed no visible evidence of turbidity. The measured residue ellipticity is directly proportional to the concentration over the range of 0.05–0.9 mg/ml. The observed spectrum can be matched by a linear combination of the CD of α, β, and unordered structure. The agreement with theoretically calculated spectra is illustrated in Figure 1. For the best fit one can conclude that the protein contains about 48% random coil, 22% α helix, and 30% β structure. The fact that we were able to fit the experimental results into a plausible combination of α-helical, β, and random coil conformations suggests the absence of significant artifacts arising from turbidity.

There are two earlier reports of the amount of α helix in microtubule protein. One was obtained by the optical rotatory dispersion measurements of flagellar outer fiber proteins. Applying the Moffitt–Yang equation Renaud *et al.* (1968) have calculated about 28% helix. Considering the fact that this protein is from a different source than ours, and that our measurements have shown there is strong evidence for β content, the agreement with our results is very good. The second study utilized protein prepared in a similar manner to ours. Falxa and Gill (1969) reported a zero helix content for this protein. However, they reduced the protein with ascorbic acid and alkylated the sulfhydryl groups prior to CD measurement. They also exposed the protein to extreme pH conditions. These procedures may well have caused irreversible changes which altered the helical content.

TABLE I: Estimates of the Secondary Structure of MTP as Determined by Computer Fits to CD Spectra.[a]

	Sample	Minimum Variance[b]	Turbidity[c]	r	α	β
	pH					
4°	8.7	2.9×10^7	Moderate	63	25	12
	8.3	1.8×10^7	Moderate	50	24	16
	7.4	2.5×10^7	Moderate	56	22	22
	6.5	1.6×10^7	None	48	22	30
	6.0	5.3×10^7	Slight	51	7	42
	5.25	1.3×10^8	Moderate	59	0	41
	3.55[d]	3.1×10^7	Very	80		6
	3.50[d]	1.2×10^7	Very	73		7
	pH					
37°	9.2	3.64×10^7	Moderate	52	0	48
	7.08	2.89×10^7	Moderate	48	2	50
	6.50	2.73×10^7	None	44	0	56
	6.05	5.23×10^7	Slight	47	0	53
	5.05	2.95×10^8	Moderate	47	0	53
	3.35	2.12×10^7	Moderate	55	0	45
	T°					
pH 6.5	4	1.6×10^7	None	48	22	30
	15	1.6×10^7	None	46	14	40
	25	1.8×10^7	Slight	45	7	48
	50	1.9×10^7	Moderate	43	0	57
	Alcohol					
pH 6.5, 4°	0	1.6×10^7	None	48	22	30
	20%	7.6×10^6	Moderate	41	19	40
	50%	4.7×10^7	Moderate	35	8	57
pH 6.5, 4°	Colchicine	2.8×10^7	None	46	21	38
	Vinblastine	2.6×10^7	Slight	46	23	33
	MTP	1.6×10^7	None	48	22	30

[a] One must keep in mind that there is always considerable uncertainty in helical and β-structure contents determined by CD. [b] Best-fit conformational parameters taken in 1% increments; wavelength range was 250–200 nm unless otherwise specified. [c] Determined by visual inspection. [d] Wavelength range was 250–222 nm.

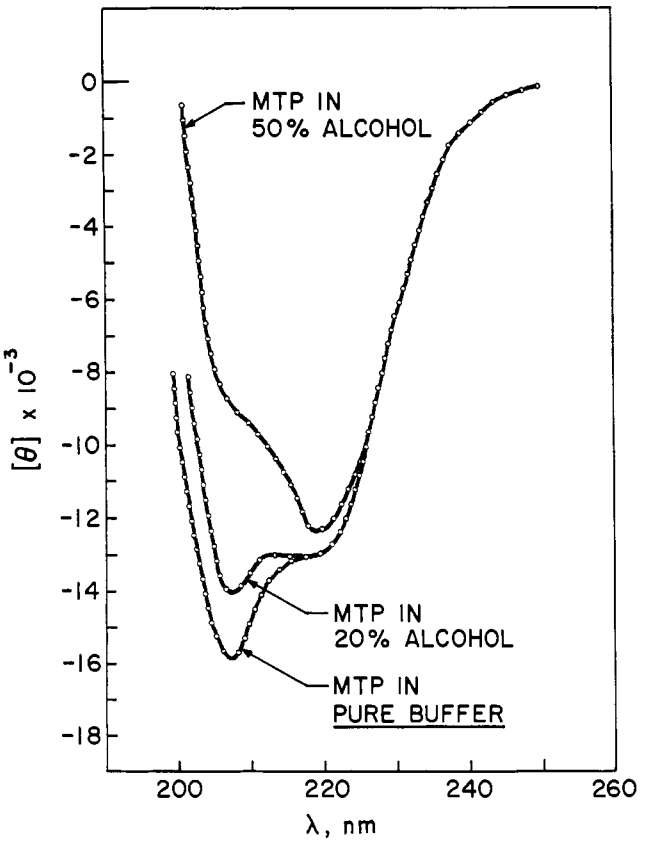

FIGURE 3: Effect of alcohol on the CD spectrum of MTP. Measurements were done at 4°, pH 6.5 in thermostated 1-cm path-length CD cells.

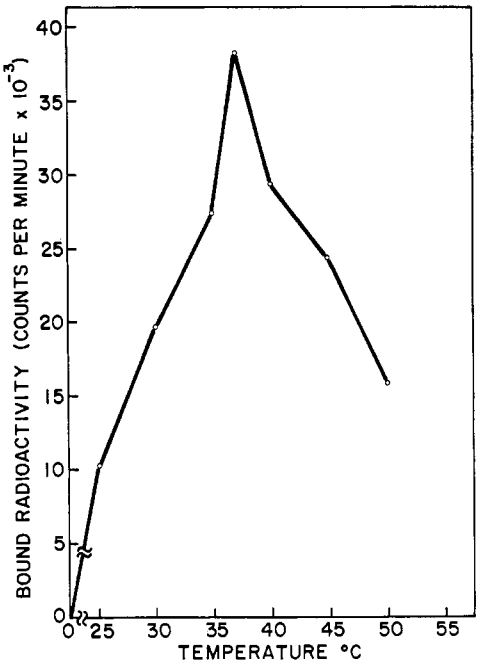

FIGURE 4: Colchicine-binding activity as a function of temperature. Activity was measured at pH 6.5.

Effects of Different Drugs on the Optical Activity. The spectrum of untreated, colchicine-treated, and vinblastine-treated protein is presented in Figure 2. An analysis of the probable secondary structures of these samples, obtained by computer fits of the CD is illustrated in Table I. These samples were measured at pH 6.5 and 4°. The drug-containing samples were incubated for 1 hr at 37°, and then chromatographed prior to CD measurements. Colchicine is still bound to MTP at 4°, during the period of time it takes for the CD measurement. It is clear that vinblastine and colchicine cause at most a small change in the CD of MTP. The only measurable difference between the vinblastine-treated and untreated protein is that the vinblastine-treated protein's spectra shows a slight blue shift. Such small shifts are often exhibited by the disaggregation of proteins (Steim and Fleischer, 1968; Urry and Ji, 1968). However, the solution has a low turbidity and aggregates are small at low protein concentrations (Weisenberg and Timasheff, 1970). The CD spectra shown in Figure 2 can still be fitted easily into theoretical combinations of the standard conformations. One can conclude that the possibility of scattering artifacts in our samples is minimal. Thus there is no marked secondary structure change induced by colchicine or vinblastine binding.

Effect of Alcohol. A number of studies have been reported on solvent perturbation of both side chain and peptide optical activity (Quadrifoglio and Urry, 1968a,b; Yang, 1967; Timasheff *et al.*, 1967). We were interested to see how MTP behaves under conditions studied for other proteins. The solvent chosen was ethanol. In the case of MTP added alcohol causes denaturation of the protein and marked turbidity. The overall CD is shown in Figure 3. As the alcohol content increases the CD intensity decreases. This decrease is exhibited markedly in the lower wavelength region. The 222-nm peak at 0 and 20% alcohol is almost identical and even for 50% alcohol the change is slight.

The effect of alcohol on the protein conformation derived from computer fits is quite remarkable. The results are summarized in Table I. The higher the alcohol content is, the greater the β conformation. This increase is accompanied by a slow decrease in the unordered structure, and a moderate diminuation of the helix content. These results are in agreement with earlier reported work on the effect of alcohol and other polar solvents on the conformation of polypeptides. Yang reported that the addition of dioxane or methanol to aqueous solutions of silk fibroin results in the appearance of β conformation (Yang, 1967). Timasheff *et al.* (1967) also reported that the change in solvent polarity affects the random coil conformation only moderately, but has a marked effect on the appearance of β structure. The extent of interconversion from random coil to β structure as reported by Quadrifoglio and Urry (1968a,b) is enhanced by the presence of organic solvents. The higher the percentage of organic solvent in the mixture is, the greater the enhancement of the β type CD curve.

Effect of Temperature. MTP is very sensitive to temperature changes. A temperature of 4° must be maintained during the isolation procedure to prevent heat denaturation. To illustrate this sensitivity when temperature is raised the colchicine binding ability was measured at different temperatures. The results are summarized in Figure 4. As one can see, the maximum binding occurs at 37°, though the protein is not stable at temperatures above 4°. Similar results have been obtained by Borisy (1966). One plausible assumption is that colchicine binding may require a specific protein conformation, different from the stable low-temperature form. This conformation might involve some unfolding of the protein to permit complex formation. Once colchicine is bound the complex stabilizes this conformation.

In order to verify this assumption CD measurements were

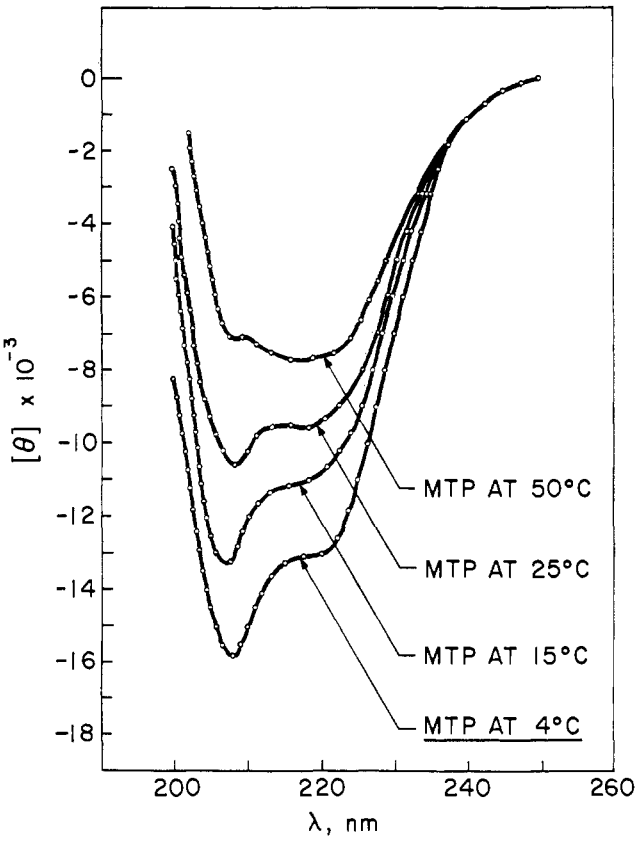

FIGURE 5: Effect of temperature on the CD of MTP at pH 6.5.

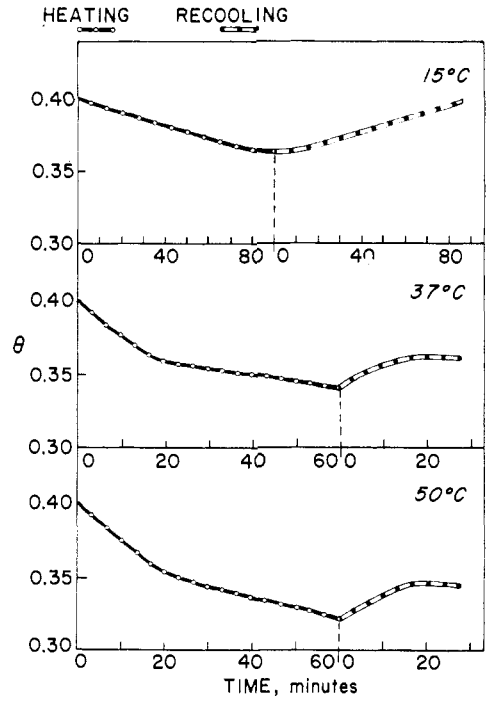

FIGURE 6: Reversibility of the heat denaturation of MTP at pH 6.4. Recooling was at 4°, heating was at temperatures as shown in the figure.

carried out at different temperatures. Samples were incubated for sufficient amount of time for changes in the CD to almost level off. The overall CD changes are illustrated in Figure 5 and the related conformational changes tabulated in Table I.

As we can see in Figure 5 the overall CD decreases with increasing temperatures, indicating a structural change occurring parallel to heating. Heating affects mostly the helical and β conformation. The amount of unordered structure is only slightly altered between 4 and 50°, while the helical content goes down from its original 22% value, measured at 4° to 0%, and the pleated-sheet conformation is almost doubled at 50°. At 4° the β conformation is 30% and at 50° it is 57%. It is interesting to note that at 37° where the colchicine binding is the highest the protein practically has no helical content, it is half random coil, half β conformation.

Figure 6 illustrates the extent of reversibility of these changes. We have recorded the time dependence of the CD at 220 nm at several temperatures, 15, 37, and 50°. Note that these samples have not been incubated long enough to allow completion of the CD change. However, in 1 hr most of the change has occurred. For comparison, see Figure 9. A 1-hr maximum incubation was used to allow most direct comparison to standard colchicine binding assays. The change at 15° is gradual, the slope of the curve is small, and the overall decrease in CD is 10%. Upon recooling the sample to 4° the optical activity goes back to its original value indicating a reversible change. At 37° the decrease in CD is less slow in the beginning, and after the first 20 min it becomes more gradual. The total change is 15% and one-third of it is reversible after recooling. The spectrum at 50° is basically similar to the 37° one, but the overall change in optical activity is higher. The total decrease is 20% and only a little more than one quarter of it is recovered after recooling.

The time required for the CD cell to equilibrate to a desired temperature is short compared to the rate of these CD changes. Thus they can be attributed to conformational changes rather than an intrinsic temperature dependence of the CD spectra. The concentrations of the samples were identical. The helical content vanishes at somewhat lower temperatures than 37°. It is possible that colchicine binding is maximum at conformations without helical content. It is interesting to note that the maximum initial rate of colchicine binding occurs at 48° (Weisenberg, 1968). In contrast the total binding capacity of the protein reaches a maximum value at 37° and then declines at higher temperature as shown in Figure 4. This suggests that at 48°, the protein conformation is the most favorable for colchicine binding, but the higher temperature causes rapid inactivation of the protein, thus the overall binding is decreased.

Effect of pH upon Protein Conformation. MTP is very sensitive to pH changes. Decrease or increase in the pH results in visible denaturation of the protein. The orginally transparent protein solution becomes opaque after adjusting the pH to very low or very high values. It is interesting to see what is happening to the protein between those extreme changes. The colchicine binding activity under various pH conditions at 37° is illustrated in Figure 7. It is maximal at pH 6.5. The binding decreases rapidly as the pH is changed above and below this region. The CD spectra in this pH range were measured at both 4 and 37°. At 37° the CD is fairly constant between pH 5.25 and 7.4. Outside this range the amount of random coil increases somewhat. The results are summarized in Table I. This is consistent with the 37° binding activity but the results are not very dramatic. At 4° the CD of the protein is much more sensitive to pH. These results are shown in Figure 8 and Table I. As the pH is dropped

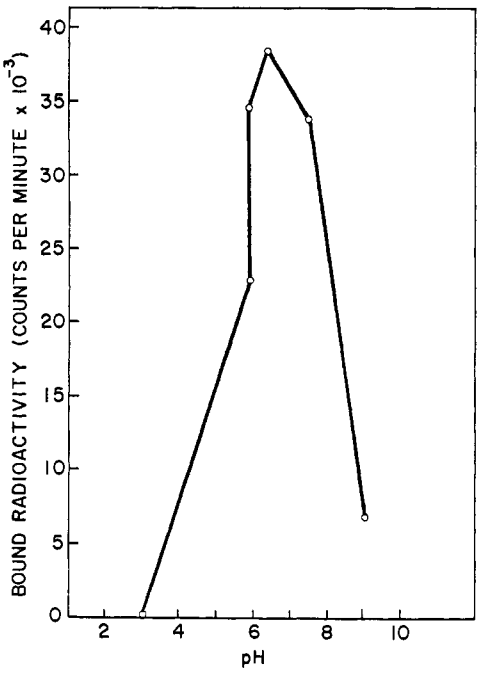

FIGURE 7: Colchicine-binding activity of MTP at 37° as a function of pH. Colchicine-binding assay was carried out with the DEAE paper method of Weisenberg *et al.* (1968).

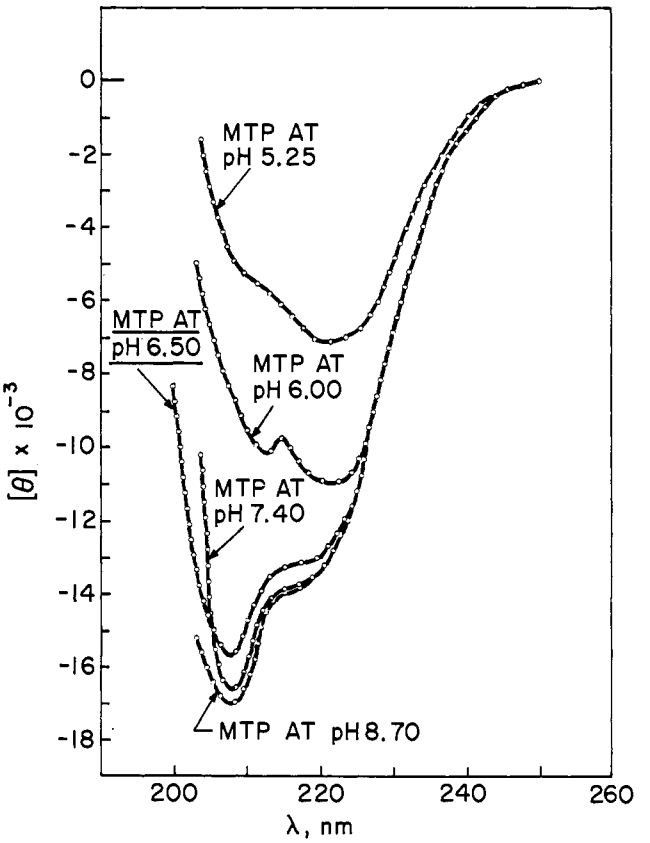

FIGURE 8: The effect of pH on the optical activity of MTP at 4°.

below 7.4 the protein undergoes a drastic change in secondary structure. The amount of random coil remains roughly constant while all α helix is apparently converted to β structure by pH 5.25. At even lower pH's the protein seems to exist in almost all random coil but we have to take these results with caution. Absorption flattening and scattering artifacts may well dominate the spectra. These can alter the apparent helical content significantly. Some evidence of the artifacts in the samples are the poor fits from the standard library of CD curves. The conformational change induced by acid at 4° is irreversible and is accompanied by aggregation and visible turbidity. One cannot correlate this directly with colchicine binding since that cannot be measured at such low temperatures.

Effect of Added Nucleotides and Antimetabolites on Colchicine Binding and Conformation. Weisenberg *et al.* (1968) and Wilson (1970) have studied the effect of added nucleotides on colchicine-binding activity. Preincubation of the protein with different amount of nucleotides such as GTP, GDP, and ATP increases the colchicine-binding activity. Recent results confirm this effect, and also demonstrate that vinblastine, vinchristine, and colchicine itself have similar effects (Wilson, 1970). We have paralleled these experiments by recording the CD of the samples in order to correlate conformational changes with the protection effect of nucleotides. Different samples of MTP at the same concentrations were incubated with GTP, GDP, GMPPCP, colchicine, and vinblastine for 1 hr at 37° and the CD at 220 nm was monitored at this temperature as a function of time. The results can be seen in Figure 9. Preincubation with all of these five substances protects against the conformational change induced by increase in temperature. The "unincubated" sample shows a 20% decrease in CD and the protected samples have a much smaller decrease, 4–6%.

A simple model is consistent with our CD results and the activity studies. Consider three forms of the protein as shown:

$$A \rightleftharpoons B \rightarrow C$$

A is the original MTP at 4°. It has minimal colchicine binding, 22% α helix, 30% β, and 48% random coil. The equilibrium between A and B is strongly influenced by temperature. Form B is favored between 37 and 48°. It has maximum colchicine binding, minimum helix, is roughly half β structure and half random coil. Form B is not nearly as sensitive to pH as form A. Form B can irreversibly convert to form C. This form shows minimum colchicine binding and no helical structure. The rate of A–B interconversion must be very slow to account for the extremely slow rate of colchicine binding. The rate of the B → C reaction is also slow and has a

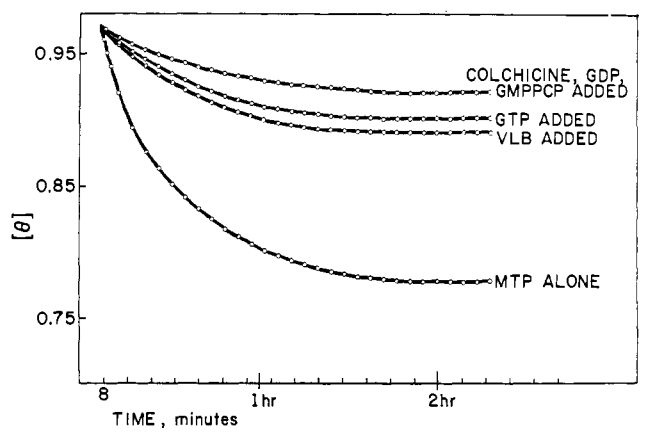

FIGURE 9: Protective effect of added nucleotides, colchicine, and vineblastine on the heat inactivation of MTP as a function of time. The samples were chromatographed prior to CD measurement and their MTP concentration was identical.

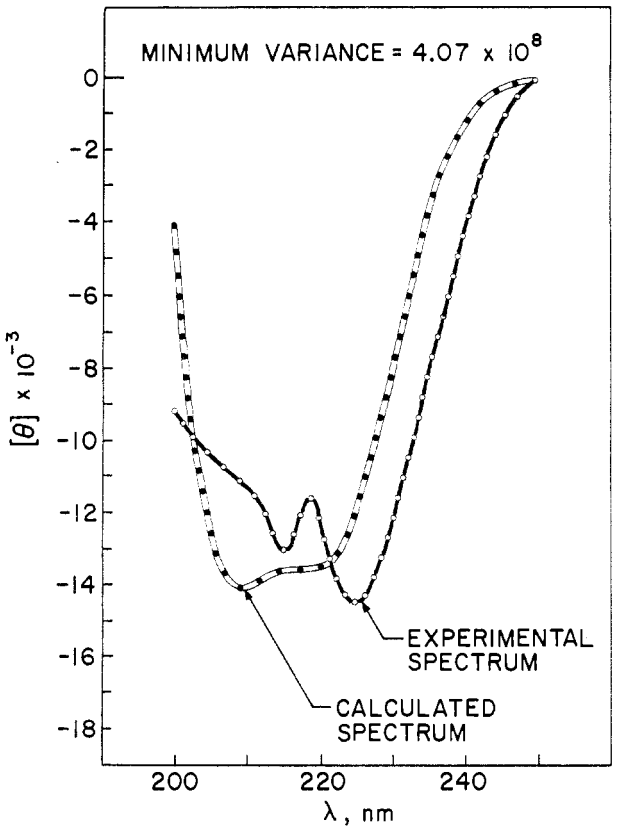

FIGURE 10: Experimental CD spectrum and computer calculated best theoretical fit of intact tubules at 4°C.

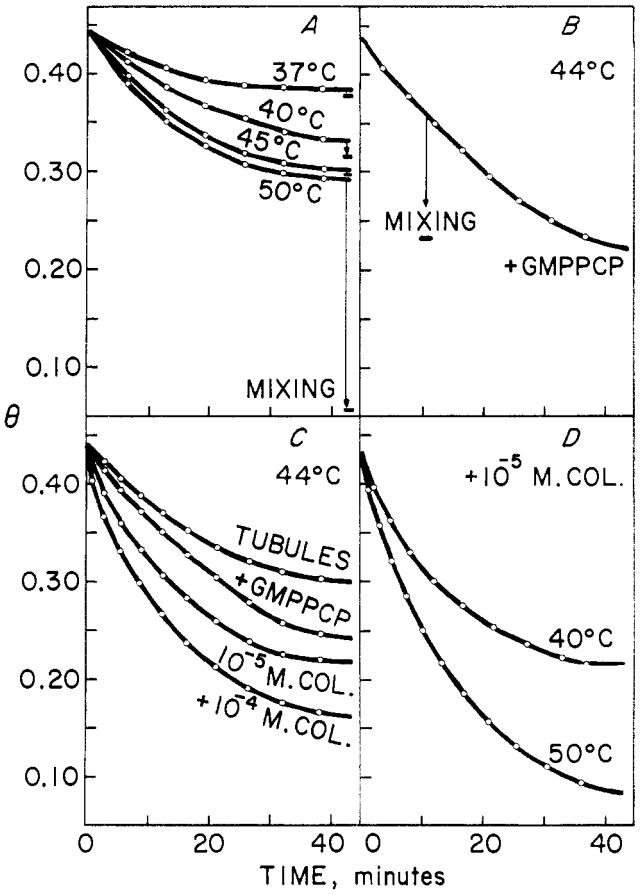

FIGURE 11: (A) CD of intact tubules as a function of time and temperature. The effect of mixing is indicated at the end of each measurement. (B) CD of intact tubules, preincubated with GMPPCP as a function of time at 44°. The same sample was tested with and without mixing. (C) The effect of possible disruptive agents on the CD of intact tubules. Samples were preincubated for 1 hr prior to measurements and optical activity was measured at 44°. (D) Disruptive effect of 10^{-5} M colchicine on tubules as a function of time and temperature.

high-activation energy. Some agents, such as nucleotides, vinblastine, and vinchristine, have a stabilizing effect on the B form and thus prevent the protein from conversion into form C which can no longer bind colchicine. Form C is a highly aggregated protein, and the effect of bound nucleotides may be to slow down this aggregation. Measurements of turbidity verify this assumption, the preincubated samples show markedly less turbidity, even after few days, than the pure protein. The application of GMPPCP is of particular interest, since samples preincubated with this GTP analog, which is incapable of hydrolysis to GDP, cannot be precipitated with vinblastine. The fact that GMPPCP has a similar protecting effect on the colchicine binding as GTP suggests that the nucleotide protection does not require hydrolysis of the triphosphate.

However, the model present above is only one of a number of possibilities. It is certainly an oversimplification and further experiments are needed to examine intermediate states in the inactivation of MTP as well as the steps in the aggregation process. Nonetheless it is gratifying that optical changes appear to parallel so closely the changes in activity.

Intact Microtubules. In order to correlate conformation of the MTP subunit with that of intact tubules a few preliminary experiments were done using purified outer doublet tubules from the sea urchin *Lythecinus pictus*. The tubules were isolated by the method of Shelanski and Taylor (1968). The intact tubules even in very dilute solutions such as 0.1–0.05 mg/ml showed some turbidity. This turbidity had a marked effect on the measured optical activity, and thus makes any unequivocal interpretation of the data difficult. As Figure 10 illustrates, it was not possible to fit the data accurately into computerized combinations of standard conformations. The minimum variance of the best fit is almost 100 times greater than the dimeric protein. The CD spectra shows a 2- to 4-nm red shift presumably as a result of turbidity.

The effect of temperature on the tubules is illustrated in Figure 11A. The CD was monitored at 220 nm as a function of time. Different samples of the tubule preparation with the same concentration were heated in the cylindrical thermostated CD cell to 37, 40, 45, and 50°. After 40-min incubation the samples were mixed in the cell (assuring a fixed cell position) and the change in optical activity was measured. There is a slow decrease in CD as a function of time, similar to the results obtained with subunits. Mixing has no effect except at 50°, where it causes nearly complete loss of optical activity. Much of the observed CD change probably results from diminution of scattering and dichroic artifacts. The extent to which the protein conformation is changing cannot be extracted from these data without much further work. The simplest plausible explanation for the loss of optical activity in samples heated to 50° could be that mixing at this high temperature causes the sudden disruption of tubule structure. The disruption of the tubule is a result of the combined effect of heat and mechanical agitation, since the sample can be heated for hours, and without disturbing it the optical activity seems to level off at a final value greater than that of

the stirred sample. The effects of added colchicine and GMPPCP were also tested. Both GMPPCP and colchicine tend to speed the heat denaturation of the sample. The higher the colchicine concentration used, the stronger the effect is (Figure 11C,D). At 50° the sample with added colchicine retains only a minimal optical activity, though pure tubule samples show considerable CD at this temperature. As we can see from Figure 11B, these effects are quite complicated. For example in the sample containing GMPPCP, mixing below 50° after 10 min seems to accelerate the decrease in CD. Both mixed and unmixed samples reach the same value after about 1 hr.

It is interesting to note that the same substances which stabilized the protein subunit against thermally induced denaturation and aggregation stimulated the denaturation of the intact tubule. These results clearly indicate the complexities of the denaturation process of intact microtubules. Considerable additional experimental data will be needed before a detailed explanation of these phenomena can be constructed.

Acknowledgment

The authors thank Dr. Peter Kahn for his valuable work in providing us with the computer program, Drs. Robert Marantz and Howard Feit for valuable discussions, and Piroska Szerdahelyi for her assistance in making the drawings. We are grateful to Dr. Irving Listowsky for allowing us to perform some of our CD measurements on his instrument.

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