4',6-Diamidino-2-phenylindole, a Fluorescent Probe for Tubulin and Microtubules*

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A new fluorophor for tubulin which has permitted the monitoring of microtubule assembly in vitro is reported. DAPI (4',6-diamidino-2-phenylindole), a fluorophor already known as a DNA intercalator, was shown to bind specifically to a unique tubulin site as a dimer ($K_{D(\text{app})} = 43 \pm 5 \, \mu M$ at 37°C) or to tubulin associated in microtubules ($K_{D(\text{app})} = 6 \pm 2 \, \mu M$ at 37°C) with the same maximum enhancement in fluorescence. When tubulin polymerization was induced with GTP, the change in DAPI affinity for tubulin resulted in an enhancement of DAPI binding and, consequently, of fluorescence intensity. DAPI, whose binding site is different from that of colchicine, vinblastine, or taxol, did not interfere greatly with microtubule polymerization. It induced a slight diminution of the critical concentration for tubulin assembly due to a decrease in the depolymerizing rate constant. Moreover, DAPI did not interfere with GTP hydrolysis correlated with tubulin polymerization, but it decreased the GTPase activity at the steady state of tubulin assembly. Even at substoichiometric levels DAPI can be used to follow the kinetics of microtubule assembly.

Tubulin is a major protein of microtubules, a key cytoskeletal constituent of eukaryotic cells, being involved in mitosis, cell division, cell shape, intracellular transport, and motility (Roberts and Hyams, 1979). The ability of cells to effect rapid changes in the assembly of microtubules appears to be of major importance for the expression of various cellular functions (Olmsted and Borisy, 1973). Until now tubulin assembly in vitro has been monitored essentially by turbidimetry (Sheinanski et al., 1973), a method which has severe limitations (Berne, 1974), while a more sensitive fluorometric method has been developed for actin polymerization using fluorescent probes covalently attached to actin (Kouyama and Mishashi, 1975; Lee al., 1981), and this fluorescein-labeled tubulin has been developed for actin polymerization using fluorescent probes covalently attached to actin (Kouyama and Mishashi, 1975; Lee al., 1981). Such attempts have been unsuccessful with tubulin. A known fluorescent probe for tubulin is 8-anilino-1-naphthalenesulfonate (Bhattacharyya and Wolff, 1975; Lee et al., 1975; Steiner, 1980). Using 8-anilino-1-naphthalenesulfonate, Steiner reported that a temperature-induced change in tubulin configuration could occur independently of its polymerization into microtubules. Microtubule proteins have been labeled with dichlorotriazinyl fluorescein (Keith et al., 1981), and this fluorescein-labeled tubulin behaved essentially like pure tubulin. No significant change in the fluorescence signal could be detected during fluorescein-tubulin assembly into microtubules.

Recently, a fluorescent DNA-intercalating dye, DAPI, was also shown to bind to negatively charged molecules such as polyphosphate and polyglutamate (Tijssen et al., 1982). Since tubulin has an unusual acidic COOH-terminal sequence (Pontaing et al., 1979) and hydrophobic autoassociation sites, ability to bind DAPI would be expected.

DAPI which was first synthesized by Dann et al. (1971) as a trypanocide has been shown to bind to double-stranded DNA (Williamson and Fennell, 1974) to form a fluorescent complex. DAPI exhibits a preference for repetitive A-T base pairs (Kania and Fanning, 1976; Hajduk, 1976). As a cytotoxic chemical probe, it allows visualization of DNA in mycoplasma and virus-infected cells (Russell et al., 1975). The specific intercalation of DAPI into DNA has been used to develop a fluorometric DNA microassay (Kapukinski and Skoczylas, 1977; Brown et al., 1981). DAPI is also widely used as a DNA probe in cytofluorometry (Coleman et al., 1981).

We demonstrate here that DAPI binds to dimeric and to polymeric tubulin with differing affinities and thus is useful in setting up a new method for fluorometric monitoring of microtubule assembly. Moreover, DAPI itself has little effect on the essential features of tubulin such as critical concentration, assembly kinetics, and GTP hydrolysis inside the microtubule. Therefore, DAPI appears as a good extrinsic fluorescence probe for tubulin.

MATERIALS AND METHODS

Chemicals—MES was purchased from Calbiochem, guanosine 5'-triphosphate trilithium salt from Boehringer Mannheim, EGTA, DAPI, and vinblastine sulfate from Sigma, colchicine from Prolabo; glicerol and all salts used in buffers were Merck analytical grade. Guanosine 5'-[\gamma-3P]triphosphate was purchased from Amersham Corp.

Taxol was purified from Taxus baccata in Dr. P. Potier's laboratory at the Institut de Chimie des Substances Naturelles and kindly given to us by Dr. Daniel Guénard.

Tubulin Purification—Microtubule proteins were purified from fresh pig brain through 3 cycles of assembly-disassembly according to Shelanski et al. (1973) and stored at -70°C as a pellet. Prior to use, pellets were thawed rapidly and resuspended in buffer A (50 mM MBS, pH 6.6, 0.25 mM Mg(CH3COO)2, 0.5 mM EGTA). Free nucleotides and residual glicerol were removed by gel filtration on Sephadex G-25 in buffer A. Microtubule protein solutions were then centrifuged at 25,000 rpm for 30 min to remove undissociated material. This tubulin preparation will be called in the text either cyclized tubulin or microtubule protein.

Tubulin was separated from the MAPs by chromatography on phosphocellulose according to Weingarten et al. (1975). The protein

1 D. Bonne and D. Pantaloni, unpublished data.
2 The abbreviations used are: DAPI, 4',6-diamidino-2-phenylindole; AMP-PNP, adenosine 5'-diphosphoribosinate; EGTA, ethylene glycol bis(\beta-aminoethyl ether)-N,N',N''-tetraacetic acid; MAPs, microtubule-associated proteins; MES, 2-(4-morpholino)-ethanesulfonic acid.
Fluorescence of Microtubule-bound DAPI

was stored at -70 °C in buffer A also containing 4 mM glycerol and 0.1 mM GTP. It will be termed pure tubulin.

Protein concentration was determined either spectrophotometrically using an extinction coefficient ε280 nm for tubulin of 1.2 mg cm⁻¹ (Detrich and Williams, 1978) or by Lowry assay (Lowry et al., 1951) with a slight correction for tubulin (David-Pleutet et al., 1977). In the case of cycled tubulin, MAP's content was estimated to be 15% of protein concentration and subtracted.

Polymerization Measurements—Tubulin polymerization was monitored by turbidimetry at 350 nm as previously described (Carlier and Pantaloni, 1978).

GTPase Activity Measurements—The GTPase activity of tubulin during polymerization was monitored by the extraction of radioactive P- labelled during hydrolysis, as described previously (Carlier and Pantaloni, 1981). AMP-PNP-Mg²⁺ (1 mM) was added to inhibit GTP hydrolysis by MAP's ATPase activity.

Electron Microscopy Analysis—Microtubule samples were negatively stained with 2% uranyl acetate on 76-mesh carbon-coated grids after 0.2% isothermal glutaraldehyde fixation following tubulin dilution to 1 μM.

Microtubule Length Distribution—Electron photomicrographs at a final 2000-2000-fold magnification were used to determine the length distribution histograms. 600-1000 microtubules were measured with a stylus digitizer connected to a Kontron V 800 computer, giving the length histogram and the mean length of the distribution.

Direct Measurement of Microtubule Weight—Microtubules could be rapidly separated from tubulin dimer solution (in less than 40 s) by centrifugation at 160,000 × g for 4 min in a Beckman Airfuge using a prewarmed rotor at 37 °C. In the determination of microtubule weight, a correction was made for the occasional formation of aggregates, by cooling at 4 °C and centrifuging the parallel samples at 20 °C for 4 min. The quantity of microtubules was then calculated from the difference between the concentrations of the supernatants of the cold and warm centrifugation. This correction for aggregate formation, although always small, was not negligible.

Colchicine-binding Assay—The binding of [H]colchicine to tubulin was determined by the adsorption of the tubulin-colchicine complex to DEAE filters (Borisy, 1972). Pure tubulin (20 μM) was preincubated during 10 min at 37 °C in buffer A containing eventually DAPI (10-100 μM) or podophyllotoxin (50–100 μM) and then incubated (45 min at 37 °C) in the presence of 50 μM [H]colchicine (20 Ci/mol) so as to reach equilibrium. At the end of incubation, aliquots were filtered on DEAE filters which were washed, then dried and the radioactivity determined.

Absorption Measurements—All absorption spectra were recorded on a Cary 219 spectrophotometer. Absorption difference spectra were determined using a set of 2-compartment quartz cuvettes (Hellma 238/QS-K75) with a 2 × 4.375-mm path length. The base-line was set with two identical cuvettes, both containing DAPI and tubulin in each compartment. Difference spectra were recorded after mixing the components of the sample cuvette.

Fluorescence Measurements—Steady-state fluorescence measurements were made with a Perkin-Elmer MPF-44A spectrofluorimeter. The instrument was operated in the ratio mode. The excitation light source was a high-pressure xenon lamp. Samples were illuminated either at 370 nm or at 390 nm in order to minimize inner filter effects. Emission can be scanned from 380 to 600 nm but was usually measured at 450 nm. Samples were placed in a temperature-controlled cuvette holder and maintained at 8 or 37 °C. Absolute quantum yields were determined according to Steiner (1980). Quinine sulfate was used as a reference. Its quantum yield in 0.1 N sulfuric acid was taken as 0.55. Concentrations of DAPI and quinine sulfate were, respectively, 10⁻⁴ M and 2.5 × 10⁻⁴ M. Integrated areas of the emission spectra were measured by weighing the paper under each peak. The quantum yield of tubulin-bound DAPI was obtained from the emission spectra of Fig. 2; the amount of bound DAPI was calculated from the Kᵣₒₜ, obtained as described under “Results.” Tubulin solutions were titrated with DAPI delivered from an Agla-Micrometer syringe; the added volume was less than 4% of the sample volume (2 ml). Fluorescence readings were corrected for dilution, for light scattering, and for the inner filter effect.

Correction for the reduction of excited light by self-absorption of the fluorophore was made according to Brand and Witholt (1967), where (F) is fluorophore molarity, ε is molar absorbance coefficient at excitation wavelength, ΔY is y₁ - y₂ = emission slit width = 0.2 cm in usual conditions, and y₁ is position of the slit relative to the cuvette.

In order to minimize the inner filter effects in titration curves of tubulin with DAPI, fluorescence was stimulated by light of wavelength 370 nm. The molar absorption coefficient for DAPI is 3100 M⁻¹ cm⁻¹ at 390 nm. Therefore, at DAPI = 50 μM, the correction factor for inner filter effects Kᵣₒₜ is only 1.19. Corrections were unnecessary for DAPI concentrations below 4 μM.

The contribution of Rayleigh light scattering due to tubulin in the absence of fluorophor was determined by measuring the apparent fluorescence emission at 450 nm with excitation at 390 nm and was found to be small (<0.5% of signal in the presence of DAPI).

The number and the affinity of binding sites of the fluorophor for the tubulin were calculated from the fluorescence enhancement of the tubulin-DAPI complex in the presence of increasing amount of DAPI. Data were usually plotted according to Scatchard (1946). Fm, the value of maximum fluorescence, was extrapolated from reciprocal binding data. DAPI titrations with tubulin were avoided as microtubule proteins have a strong tendency to aggregate and give rise to turbid solutions even at concentrations of 1 mg/ml. The loss of light due to scattering could not be rigorously measured. The ratio of binding was measured by the ratio of the corrected fluorescence of bound ligand to extrapolated maximum fluorescence. The free ligand concentration was taken as the difference between total and bound concentrations.

Data were computed on a Kontron 980 calculator with a bilinear regression program.

True fluorescence spectra were recorded with Perkin-Elmer LS-5 luminescence spectrophotometer with a temperature-controlled cuvette holder maintained at 18 or 37 °C. The maxima values were slightly different in uncorrected spectra monitored on the spectrofluorimeter currently used in the laboratory.

RESULTS

Spectroscopic Characteristics of Tubulin-bound DAPI

DAPI exhibited an electronic transition in the visible spectral region (Fig. 1) at 342 nm (εₘ = 33,000) with fluorescent light emission of maximum wavelength 470 nm (Fig. 2). Upon binding to tubulin, the spectral properties of DAPI changed significantly as seen in the difference spectra (Fig. 2). The isosbestic point at 360 nm and the two extrema at 325 and 385 nm, whose amplitude was dependent on tubulin concentration, indicated that the chromophore was red-shifted on tubulin binding (Δλₘₚₓₓ = 3 nm) and possibly rendered less accessible to aqueous solvent (Donovan, 1969). This red shift was observed and measured directly on the fluorescence excitation spectrum (Fig. 2).

![Fig. 1. Spectral data on tubulin-DAPI interaction. Simple absorption spectra: ---, DAPI, 10 μM in buffer A (λₘₚₓₓ = 342 nm); ---, DAPI, 10 μM + tubulin, 10 μM in buffer A (λₘₚₓₓ = 345 nm). Difference spectra (---) due to the association of 10 μM DAPI with pure tubulin in buffer A: I, tubulin = 2.5 μM; II, tubulin = 5 μM; III, tubulin = 10 μM.](image-url)
Fluorescence of Microtubule-bound DAPI

Determination of the Affinity of DAPI for Tubulin

Within experimental error, fluorometric titrations of 1 µM dimeric tubulin at 37 °C (Fig. 3b, curve A) and 13.6 µM polymerized tubulin at 37 °C (Fig. 3a, curve A) with DAPI were analyzed according to Scatchard (Fig. 3, insets). Tubulin was maintained in solution at 37 °C by addition of 100 µM GTP. This nucleotide was replaced by GTP when microtubules were needed. It has been shown that the nature of the nucleotide does not affect the affinity of DAPI for tubulin in solution at 8 °C (see further). As high DAPI concentrations could not be used because of inner filter effects and interaction with GTP (see further), the binding isotherms approached saturation only with microtubules (Fig. 3a, curve A). The maximum fluorescence intensities were extrapolated from reciprocal binding data.

For dimeric tubulin, it was found that \( F_{37} = 0.8 \times F_m^8 \) with \( F_m^8 = \) specific maximum fluorescence at 37 °C and \( F_m^8 = \) specific maximum fluorescence at 8 °C.

The quantum yield for free DAPI, obtained by comparison with quinine sulfate, was 0.023. It increased about 14 times upon binding to tubulin (18 °C) (Fig. 2). For tubulin assembled into microtubules at 37 °C, the maximum fluorescence intensity was also found to be very near \( F_{37} \). From the data presented in Fig. 3, the apparent dissociation constant \( K_{D(app)} \) for the reaction of tubulin with DAPI at 37 °C can be calculated as 43 ± 5 µM for free tubulin (average of 3 experiments) and 6.0 ± 2.0 µM for microtubules (average of 4 experiments). In both cases, \( n = 1.00 \pm 0.25 \) molecules of DAPI bound/ tubulin dimer. Similar values for maximum fluorescence were found whether DAPI was bound to free or to assembled tubulin, after taking into account the 20% diminution of the signal due to the temperature shift. Note that in microtubule titration some free tubulin was present and bound DAPI with lower affinity. For the sake of simplicity, this was neglected.

The addition of substoichiometric amounts of DAPI to microtubule proteins before and after assembly into microtubules was accompanied by a striking enhancement of fluorescence. Examination of DAPI fluorescence and of a mixture of DAPI with tubulin (18 °C) or with microtubules (37 °C), presented in Fig. 2, showed that free ligand (10⁻⁸ M) exhibited little fluorescence while bound ligand fluorescence was increased more than 4 times after tubulin binding (18 °C) and 9 times after microtubule binding (37 °C). As a temperature shift from 8 to 37 °C produced a 20% decrease in observed free DAPI fluorescence intensity, the enhancement of fluorescence observed after DAPI binding to microtubules maintained at 37 °C is obviously not a temperature effect. The binding time course could not be followed since the emission intensity was found to be maximal instantaneously. Similarly dissociation of ligand by dilution experiments was complete at once.

The excitation spectrum of DAPI bound either to free tubulin or to microtubules exhibited a shoulder at 298 nm which does not exist in the spectrum of DAPI. As the excitation spectrum of bound DAPI (\( \lambda_{\text{max}} = 350 \) nm) overlaps the emission spectrum of the tubulin tryptophans (\( \lambda_{\text{max}} = 339 \) nm, 4 in each tubulin monomer), an energy transfer from a tryptophan to the tubulin-bound probe may occur, indicating that the acceptor (DAPI) is quite close to the tryptophan donor. Conversely, the interaction of DAPI with tubulin also produces quenching of intrinsic tryptophan fluorescence. By exciting the protein solution at 295 nm all fluorescence emission at 339 nm is due to tryptophanyl residues. In these conditions, DAPI fluorescence is negligible. The addition of increments of DAPI (10 to 40 µM) to pure tubulin (10 µM) in buffer A produced a progressive blue shift of the emission maximum of the tryptophanyl fluorescence from 339 to 326 nm with a strong decrease in intensity (data not shown). Therefore, DAPI seems to be bound in close vicinity to tryptophanyl residues.

![Fig. 2. True fluorescence excitation and emission spectra of free tubulin- and microtubules-bound DAPI.](image)

**Fig. 2.** True fluorescence excitation and emission spectra of free tubulin- and microtubules-bound DAPI. Excitation (Ex) spectra: a = DAPI at 18 °C; b = DAPI-tubulin at 18 °C; c = DAPI-microtubules at 37 °C. Emission (Em) spectra: a = DAPI at 18 °C; b = DAPI-tubulin at 18 °C; c = DAPI-microtubules at 37 °C. Microtubule protein concentration in samples b and c was 12 µM. DAPI concentration in all samples was 1 µM. All samples were prepared in buffer A in the presence of 0.25 mM GTP. The excitation measurements were made at emission wavelength 460 nm for Ex a and 450 nm for Ex b and Ex c. The emission spectrum measurements were made at excitation wavelength 342 nm for Em a and 350 nm for Em b and Em c.

![Fig. 3. Fluorometric titrations of porcine brain microtubule protein and microtubules with DAPI.](image)

**Fig. 3.** Fluorometric titrations of porcine brain microtubule protein and microtubules with DAPI. a, polymerized microtubule proteins (13.6 µM) in the presence of 100 µM GTP in buffer A at 37 °C (curve A); microtubules (13.6 µM) with 100 µM GTP in buffer A at 8 °C (curve B); DAPI in buffer A (---). In order to avoid GTP limitation, microtubules (54 µM) were rapidly assembled and then diluted 4-fold in buffer containing 100 µM GTP. In a parallel sample, the reversibility of the assembly was checked by the amplitude of cold depolymerization. Inset, Scatchard plot of data of curve A. b, tubulin (1 µM) with 100 µM GTP in buffer A at 37 °C (curve A) and 8 °C (curve B). Inset, Scatchard plots of data from curves A and B. Fluorescence, recorded at 450 nm, was excited by light of wavelength 390 nm. The ordinates give fluorescence intensities in arbitrary units. \( F_{\text{max}} \) was extrapolated from the reciprocal representation of binding data. All fluorescence intensity measurements have been corrected for the fluorescence emission of increasing concentration of DAPI in the absence of proteins, for dilution, and for inner filter effects.
in the calculations. Moreover taxol-stabilized microtubules, whose critical concentration is extremely low (Schiff et al., 1979; Carlier and Pantolinni, 1983) bound DAPI with similar parameters. In a typical experiment done at 8 μM microtubule proteins, the DAPI-binding site in the presence of 10 μM taxol was characterized by n = 0.90, K_{D(app)} = 6.5 μM, whereas, without taxol, it was found that n = 0.86 and K_{D(app)} = 7.4 μM (data not shown). This indicated too that taxol did not interfere with the DAPI-binding site.

When tubulin was maintained in the cold (Fig. 3b, curve B), the apparent affinity of DAPI was 20 ± 2 μM (mean of 5 experiments), regardless whether GTP or GDP was present.

Fluorometric titration of pure tubulin devoid of MAPs with DAPI at 8 °C led to the same apparent affinity; the presence of MAPs apparently does not alter DAPI binding to tubulin.

When assembly of tubulin was induced by a temperature shift from 8 to 37 °C, the apparent dissociation constant of DAPI for tubulin decreased from 20 to 6 μM. Therefore, the observed enhancement of fluorescence (Fig. 2 and Fig. 3a, curves A and B) was due to an increased binding of the fluorescent probe rather than to an increase in the quantum yield, as the fluorescence, at saturating amounts of DAPI, was found constant for tubulin and microtubules.

In Vitro Visualization of Microtubules with DAPI

DAPI was used to visualize microtubules using the fluorescence microscope. Spermatozoa flagella and paramecium cilia (result not shown) appeared blue colored in the presence of 10 μM DAPI. In vitro the undiluted solutions of assembled microtubules (result not shown) appeared cloudy.

**Effect of Various Tubulin Ligands on the Fluorescence of Tubulin-bound DAPI**

The binding of [3H]colchicine (50 μM) to pure tubulin (20 μM) was measured in the presence of DAPI. Whereas a preincubation of tubulin with podophyllotoxin (50 μM) almost completely abolished tubulin-colchicine complex formation, the presence of 5 to 100 μM DAPI during preincubation did not affect the interaction of colchicine with tubulin (data not shown). Moreover, the tubulin-colchicine complex, formed by incubation of tubulin with an excess of colchicine for 30 min at 37 °C, followed by gel filtration on Sephadex G-25 to eliminate free colchicine, was found to bind DAPI with the same apparent association constant as for pure tubulin. Also, the observed fluorescence intensity of the tubulin-colchicine complex was the same, if allowance was made for the absorbance of colchicine at the excitation wavelength of DAPI (370 nm). DAPI and colchicine-binding sites appeared to be independent.

As seen in the previous paragraph, fluorometric titration of microtubules (8 μM) in the presence or absence of 10 μM taxol indicated that the apparent K_a for DAPI was not affected, showing that the taxol and DAPI sites are independent.

Fig. 4a shows the ability of Mg^{2+} ions to displace tubulin-bound DAPI without affecting the fluorescence of free DAPI. No tubulin precipitation was detected, as could be expected at low concentrations of tubulin (0.5 mg/ml). Data of Fig. 4a, when plotted according to Scatchard, showed an apparent dissociation constant of 10^{-5} M for Mg^{2+} ions involved in DAPI displacement (DAPI = 5 μM). A Hill plot of the same data exhibited a slope, n = 0.8, which indicates that only 1 magnesium ion is involved. In summary, tubulin-bound DAPI can be displaced by 1 magnesium ion with an apparent dissociation constant of 10^{-5} M. This value is higher than the mean apparent affinity 10^{-2} M of magnesium ions (n = 48 ± 5) which can associate with tubulin at 25 °C in phosphate buffer (Frigon and Timasheff, 1975).

The effect of nucleotides such as GTP and AMP-PNP on free and tubulin-bound DAPI fluorescence was investigated. An unexpected interaction of DAPI with both nucleotides was observed, although a contradictory result has been reported (Tijssen et al., 1982). In the presence of GTP or GTP-Mg^{2+}, the fluorescence of DAPI was quenched, suggesting a DAPI-GTP interaction with an apparent affinity constant of 1 mM with one binding site (Fig. 4b). The presence of GTP-Mg^{2+} also quenched the fluorescence of tubulin-bound DAPI with the same apparent affinity. This last quenching is probably due to DAPI binding to free GTP which prevented DAPI binding to tubulin. The complex AMP-PNP-Mg^{2+} was shown to enhance DAPI fluorescence in the same concentration range (1–20 mM). Low affinity DAPI interaction with nucleotides should not be inconvenient in the study of DAPI-tubulin binding where the affinity is more than 2 orders of magnitude stronger. Nevertheless, high concentrations of nucleotides (>1 mM) should be avoided in fluorescence studies with DAPI.

**Mode of Action of DAPI on Microtubules**

**Microtubule Assembly in the Presence of DAPI**—Fig. 5 shows the kinetics of assembly and disassembly of microtubule proteins (25 μM), measured by turbidity at 350 nm in buffer A containing 0.5 mM GTP with or without 60 μM DAPI. The assembly was induced by addition of GTP to the preheated (37 °C) tubulin. The initial rate of assembly was apparently not affected by DAPI, but the amplitude of the plateau was found to be 10 to 20% higher in the presence of DAPI. No lag was observed, as is usual with cycled tubulin. Microtubule disassembly, induced by cooling at 4 °C, was slowed down when 85% of tubulin molecules bound DAPI. The apparent rate of dissociation, measured by the initial slope of the disassembly kinetics was half of the control value. Nevertheless when DAPI was added in trace amounts (0.1 μM, for example) no effect was detected on either polymerization or depolymerization.

Microtubules, assembled in the presence of high concentrations of DAPI, observed at high magnification in the electron microscope appeared indistinguishable from control microtubules. The length distributions of 40 μM microtubules assembled in the presence or absence of 70 μM DAPI (34 μM buffer (Frigon and Timasheff, 1975).
We demonstrate here that DAPI, a known DNA-intercalating dye and a polyanionic polymer-binding probe, is able to interfere with the light-scattering signal.

The lowering of the critical concentration of microtubules in the presence of DAPI is in agreement with the higher affinity of the probe for polymers than for dimeric tubulin. It appears that DAPI favored the polymerization of tubulin, probably by lowering the dissociation rate constant of tubulin as seen at 4 °C (Fig. 5).

**GTpase Activity Related to Microtubule Assembly in the Presence of DAPI**—The kinetics of GTP hydrolysis during assembly by cyclized tubulin were measured under the same conditions except for the addition of AMP-PNP in order to inhibit in major part the contaminating GTPase activity of MAPs (Carlier and Pantaloni, 1981). GTP hydrolysis correlated with microtubule assembly was vinblastine-inhibitable even in the presence of DAPI. This indicates that DAPI binding did not prevent vinblastine association with tubulin or the inhibition by the antimitotic drug of microtubule formation and GTPase activity.

**Polymerization of Tubulin Detected by DAPI Fluorescence Intensity**—The kinetics of tubulin assembly at 37 °C, detected by turbidity measurement at 350 nm, was measured in a fluorimeter either by light scattering in the Rayleigh band with 370-nm excitation or by DAPI fluorescence emission at 450 nm. Zero time was on addition of GTP. Even at very low DAPI doses (0.1 μM), microtubule assembly could be easily detected by following light emission at 450 nm (Fig. 5). The kinetic data obtained by fluorescence can be analyzed in terms of 2 phases: first, a large amount of the fluorophor binds to the microtubules as soon as they are polymerized, and second, after 2 min the emitted light increases less than expected from turbidity data. This is possibly due to scarcity of free DAPI and also to some loss of emitted light due to scattering by microtubules. Polymerization of tubulin at different DAPI doses in various conditions is under study in the laboratory in order to get new insights into the mechanism of microtubule formation, particularly during the early events in assembly.

**DISCUSSION**

We demonstrate here that DAPI, a known DNA-intercalating dye and a polyanionic polymer-binding probe, is able...
to bind specifically to a unique high affinity site on tubulin, either as the dimer or associated in microtubules. The binding of DAPI to tubulin is characterized by a strong enhancement of the probe fluorescence indicating an increase in the rigidity of the DAPI molecule and/or hydrophobicity of its binding site in the protein. The inhibition of the internal rotation in the molecule of this dye has been correlated with an increase in fluorescence intensity (Kapuscinski and Skoczylas, 1978).

On the other hand, the red shift observed in the difference spectra indicates that the bound probe is less accessible to aqueous solvent. DAPI binding to depolymerized tubulin was found to be temperature dependent; the apparent dissociation constant was 20 \pm 2 \mu M at 8 °C and 43 \pm 5 \mu M at 37 °C. This result is in agreement with previous studies on the binding of another fluorophor, 1-anilino-8-naphthalenesulfonate, to tubulin which indicated that a temperature-induced change in tubulin configuration occurs independently of polymerization into microtubules (Steiner, 1980). However, DAPI binding was found to be looser upon a temperature increase from 8 to 37 °C, and this indicates that polar interactions may be involved too in DAPI-tubulin association, which could be expected from the two positively charged amidine groups in DAPI.

When tubulin at 37 °C is induced to polymerize with GTP, DAPI affinity increases 6 or 7 times (from $K_{d(app)} = 43 \pm 5 \mu M$ to $K_{d(app)} = 6 \pm 2 \mu M$) resulting in an enhancement of DAPI binding and, consequently, of fluorescence intensity. At saturating concentrations of DAPI, no change in maximum fluorescence emission was found for dimeric or assembled tubulin. At substoichiometric levels, DAPI binding to dimeric tubulin was much weaker than to the assembled tubulin. This may explain partly why DAPI gave, in this condition, the highest fluorescence change without significantly affecting tubulin polymerization.

At saturating concentrations of DAPI, the kinetics of temperature-induced assembly into microtubules was almost the same as for the control, whereas the cold-induced dissociation rate of microtubules decreased by a factor of two. This was in agreement with the lower critical concentration of microtubules measured in the presence of the probe. It seems likely that DAPI favors tubulin assembly and stabilizes microtubules by decreasing the dissociation of tubulin, i.e., the exchanges of free tubulin at the ends of the polymers. This effect may possibly explain the noticeable inhibition of steady state GTP hydrolysis. A direct effect of DAPI on GTP hydrolysis by tubulin at the very ends of microtubules cannot be rejected. However, the ability of tubulin to hydrolyze GTP during assembly into microtubules is maintained in the presence of DAPI. The rate of P, production during the prestationary phase was identical to the control, indicating that DAPI did not interfere with the molecular events following assembly and triggering of GTP hydrolysis. This should support the idea that GTP hydrolysis during the burst and at microtubule ends is functionally different. When a tubulin molecule is in contact with four neighboring molecules in the microtubule body, GTP bound to the E-site is hydrolyzed in a single turnover, and the GDP formed becomes nonexchangeable. Tubulin bound at the end of the microtubule is in contact with only two or three neighboring protomers, and the conformation change subsequent to the interaction with these three neighbors induces GTPase activity at the E-site which remains exchangeable and can bind GTP and GDP. The common part of both phenomena is GTP hydrolysis which is not affected by DAPI in the microtubule body. DAPI probably inhibits the unbound event, i.e., the turnover of nucleotide at the tubule ends. More information on the mode of action of DAPI on tubulin exchanges and relationship with GTP hydrolysis at microtubule ends will be given by a study of treadmilling in the presence of DAPI which is underway in the laboratory. Note that DAPI, like taxol, is also able to affect only the linear component of GTP hydrolysis associated with microtubule polymerization.

We propose that the DAPI-binding site on tubulin is located partly in the carboxyl-terminal region of tubulin in which the last 40 positions have 19 acidic side chains (Krauhs et al., 1981). The high content of glutamyl residues in this segment suggests that it may be responsible for binding cationic material. Displacement of bound DAPI by one Mg\(^{2+}\) ion ($K_{d(app)} = 1 \mu M$) supports this hypothesis. It is known that magnesium ions in millimolar concentrations bind preferentially to helical poly-L-glutamic acid and stabilize it (Jacobson, 1964). As the presence of MAPs did not significantly affect the apparent association constant of DAPI, their binding sites must be different from those of DAPI, even if proximal. On the other hand, DAPI was shown to be an acceptor of intrinsic energy transfer from tryptophan. Consequently, its binding site must be in close proximity to tryptophanyl residues which, in tubulin, are probably located in the hydrophobic regions of the protein (Steiner, 1980). In particular, the sequence Trp-Tyr located in 297-308 (Krauhs et al., 1981) in the COOH-terminal region of tubulin chain could possibly be involved in the DAPI-binding site.

DAPI has permitted us to follow the kinetics of polymerization in vitro and the direct visualization of microtubules in cilia and flagellae. We were not able, thus far, to visualize the microtubule network and mitotic spindles in 3T3 fibroblasts either in fixed and permeabilized or unfixed preparations. This may be due to tight binding of DAPI to DNA which hindered DAPI binding to tubulin. Another possibility could be that the binding site on tubulin is not accessible in the cell. As DAPI is able to interact strongly in vivo with DNA and with negatively charged polymers such as polyphosphate (Tijssen et al., 1982), it is not a good probe to visualize microtubules in cells.

However, DAPI exhibited all the required properties of an extrinsic fluorescence probe toward tubulin: (i) the fluorophor was tightly bound at a unique location; (ii) its fluorescence was sensitive to environmental conditions; and (iii) it did not affect the essential features of the macromolecules that were investigated (Freifelder, 1982). Moreover, it should be very useful to follow the kinetics of microtubule formation when turbidimetric measurements are not feasible: at low tubulin concentration, during the nucleation phase, etc.

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