J. Biosci., Vol. 6, Number 4, October 1984, pp. 431–457.

Molecular biology of tubulin: Its interaction with drugs and genomic organization

B. B. BISWAS, K. SEN, G. GHOSH CHOUDHURY and B. BHATTACHARYYA

Department of Biochemistry, Bose Institute, Acharya J. C. Bose Birth Centenary Building, Calcutta 700054, India

MS received 4 August 1984

Abstract. Microtubules are ubiquitous cellular structures found in eukaryotic organisms and responsible for a variety of functions. These functions include mitosis, motility, cytoskeletal architecture, intracellular transport and secretion. The major structural component of microtubules is tubulin, a dimeric protein molecule consisting of two similar but nonidentical subunits (α and *β*) each of about molecular weight 55,000. With the introduction of radioactive colchicine for the first time it has been reported that colchicine binds specifically to tubulin. At this point microtubule research stepped up to a new era linking microtubules with other spindle poisons which are structurally diverse as well as binding at different sites on to the tubulin heterodimer. These antimicrotubular agents have already provided valuable information regarding microtubule-mediated cellular functions and its association and dissociation phenomena. Tubulins appear to be conserved proteins based on *in vitro* copolymerization and comigration on polyacrylamide gel electrophoretic properties. Further, amino acid sequences of both α and *β* subunits from a variety of sources also appear to be mostly conserved. The evolutionary conservation of tubulin genes is highly reflected at the nucleic acid level as well. The estimation of the number of genes for tubulin and their organization in a variety of organisms have opened up a new dimension to microtubule and tubulin research. The multigene family for tubulins comprising also pseudogenes is suggestive that more than one gene for each α and *β* tubulin is functional in the cell. Therefore, it has been speculated that different tubulin gene products contribute to functionally different microtubules at specific stages in cell cycle and cell growth. Heterogeneity in both α and *β* tubulins has already been established during different stages of development of the cell. Obviously, it reflects that tubulin genes are highly regulated and this regulation might be at the transcriptional and/or translational level. Whatever is the actual control mechanism it appears that cells can detect an enhanced pool of depolymerized subunits and a rapid and specific control in tubulin gene expression at the transcriptional and/or post transcriptional level does occur.

Keywords. Tubulin; microtubule; drugs; tubulin mRNA; cDNA; pseudogene; evolution.

Introduction

The microtubule system has drawn attention of several workers in the recent past for its easy availability from animal brain cells, comparatively easier way of purifying tubulins to a homogeneous preparation, its easy polymerization and depolymerization *in vitro.*

Abbreviations used: SDS, sodium dodecyl sulphate; PAGE, Polyacrylamide gel electrophoresis; M_r, molecular weight; CD, circular dichroism; CHO, Chinese hamster ovary; MBC, methyl benzimidazole-2-yl carbamate; CIPC, coumarin and isopropyl-(N-3 chlorophenyl) carbamate; APM, amiprophos-methyl.

its simple assay by colchicine binding reaction, preparation of cDNA and genome analysis. The main thesis of the present review is to answer (i) what is the extent of microheterogeneity in tubulins? (ii) how colchicine and its analogues bind with tubulin? (iii) what is the mechanism of substoichiometric drug poisoning of microtubule assembly? (iv) how the biosynthesis of tubulin is regulated? (v) how many functional and nonfunctional tubulin genes are present in the cell? (vi) what is the genetic complexity of tubulin genes? and (vii) what conservation of tubulin gene signifies?

Several reviews appeared recently on different aspects of tubulin and microtubule systems (Biswas *et al.,* 1981; Carlier, 1982; Correia and Williams, 1983; Cowan and Dudley, 1983; Hill and Kirschner, 1984) but the informations regarding the questions raised above are still fragmentary and emerging. An attempt is being made here to discuss the problems enumerated and the documented information.

Tubulin: A ubiquitous protein

Tubulin is the major constituent of microtubule structure found in all eukaryotic cells. Bryan and Wilson (1971) demonstrated that purified tubulin from chick embryo brain could be resolved electrophoretically into two closely located components on 8 M urea-polyacrylamide gels after reduction and acetylation. These two subunits were termed α and β tubulins, the β subunit having greater electrophoretic mobility. This separation results mainly from a difference of charge, since sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) of the two subunits under high ionic strength and neutral pH conditions (Weber and Osborn, 1969) shows only a single band having an apparent molecular weight M_r around 55,000. However, with SDS-PAGE of lower strength and higher pH (Laemmli, 1970), separation of α and β subunits can be achieved (Wilson and Bryan, 1974; Linck, 1976). The two subunits were found in equimolar quantities in nearly all cells studied (Dustin, 1978) and β tubulin was shown to have a M_r of 54,000–58,000 and α tubulin, a M_r of 46,000–54,000 (Feit *et al.,* 1971; Olmsted *et al.,* 1971; Raff and Kaumeyer, 1973). These two subunits are distinct, but closely related proteins. A close relation between α and β tubulins has been revealed by amino acid sequence analysis (Luduena and Woodward, 1975). The 451 amino acid residues of α tubulin from pig brain and 445 residues of β tubulin display 41 % sequence identity (Postingel *et al.,* 1983). A recent study on several taxonomically distant species (Little *et al.,* 1981) demonstrated that the β chain is more conserved than the α chain during the course of evolution. Antisera raised against tubulin also cross react across species boundaries (Osborne and Weber, 1977). This suggests that the two polypeptides have been derived from a common ancestor protein. Each has been highly conserved in the course of evolution as indicated by the similarities of tubulins from two widely separated species, chick and sea urchin. However, antiserum against β tubulin does not react with α tubulin (Piperano and Luck, 1977), indicating that the two subunits are distinctly different proteins. Further, they differ also in several biochemical properties. For example, β tubulin is phophorylated at a serine residue (Eipper, 1972) and α tubulin is tyrosinolated by tubulin tyrosine ligase (Raybin and Flavin, 1977). An enzymatic

removal, rather than the addition of tyrosin residue to α tubulin has also been reported recently (Valenzuela *et al.,* 1981; Lemischka *et al.,* 1981; Cowan *et al.,* 1983). Ultrastructural data suggest that microtubules are assembled from α*β* dimers. Infact, it has been ascertained that tubulin dimers are heterodimers of α*β,* rather than a mixture of αα and *ββ* dimers (Luduena *et al.,* 1975).

In fact, hybrid microtubules may be formed *in vitro* by copolymerization of α and *β* tubulins from different species (Snyder and Mclntosh, 1976). Inspite of the similarity among tubulins from a wide variety of species, functional variation, have been observed in species to species. Thus clear immunological differences have been detected among different classes of tubulins such as outer doublets, central pair and mitotic apparatus (Mohri, 1976; Fulton *et al.,* 1971). Tubulins from different species also differ in several properties like colchicine binding (Haber *et al.,* 1972; Hart and Sabins, 1976; Davidse and Flach, 1977), *in vitro* assembly (Farrell, 1976; Langford, 1978; Murphy and Hiebsch, 1979) and immunological behaviour (Piperano and Luck, 1977; Morgan *et al.,* 1978). Recently we have isolated (manuscript in preparation) microtubule protein from higher plant *Vigna radiata* (mung bean) by streptomycin sulphate and ammonium sulphate fractionation followed by the *in vitro* polymerization of microtubule protein by Zn (II) (Banerjee *et al.,* 1982). SDS-PAGE analysis has revealed that both of the subunits of plant tubulin are different from that of brain (figure 1). Attempts to assay colchicine binding with plant tubulins using radioactive colchicine (Weisenberg *et al.,*

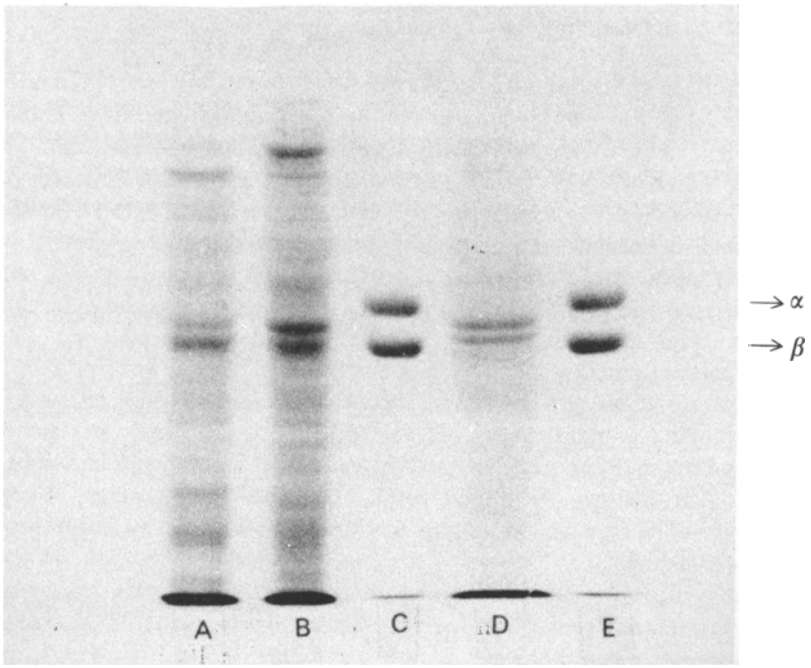

Figure 1. Purification of plant tubulin. Experimental details are as in (Sen, 1984). **(A),** Crude cytoplasmic supernatant; **(B),** ammonium sulphate fractionated protein; **(C),** purified goat brain tubulin; **(D),** purified plant tubulin; **(E)** same as in C.

1968) and by fluorometric method (Bhattacharyya and Wolff, 1974a) have not yet been successful in this laboratory.

Microheterogeneity and multiplicity of tubulins

Multiple bands of either α or *β* tubulin are obtained after gel electrophoresis in certain systems. This has been interpreted as due to microheterogeneity in tubulin (Bibring *et al.,* 1976; Kobayashi and Mohri, 1977). On SDS gels, α occasionally splits into two bands apparently differing in charge but not in size (Bibring *et al.,* 1976; Berkowitz *et al.,* 1977; Bibring and Baxandall, 1977a,b). The splitting is observed in tubulin from sea urchin mitotic spindles and in ciliary, but not flagellar outer doublets, implying a functional significance to the putative microheterogeneity (Bibring *et al.,* 1976). Purified tubulin preparations from both Ehrlich ascites tumour cells and pig brain have been found to contain a third component migrating in between the α and *β* tubulins in SDS-PAGE (Lu and Elzinga, 1977). This has been subsequently characterized as a second form of *β* tubulin, by limited proteolysis and peptide mapping (Doenges *et al.,* 1979). *Physarum* tubulin contains a subunit protein which is similar to brain *β* tubulin. However, the faster moving band on SDS gel electrophoresis in this case appearing to be altogether a different protein, after peptide mapping analysis turns out to be very similar to brain α tubulin (Clayton and Gull, 1982). This raises the question on the typical nomenclature of tubulin subunits, according to electrophoretic mobility pattern *i.e.*, fast moving constitutes the *β* subunit and the slow moving one is the α subunit.

There is substantial evidence, based on colchicine binding, which suggests that tubulin from lower eukaryotes may differ from that of higher organisms. In fact, the lower affinity of the tubulin from lower organisms and others for colchicine has been shown for *Tetrahymena* (Maekawa, 1978), *Aspergillus* (Davidse and Flach, 1977), *Physarum* (Roobol *et al.,* 1980) and higher plants (Sen, 1984). Brain cells display extensive tubulin microheterogeneity which has been found to be developmentally determined, increasing from seven isotubulins at birth to nine distinct components during early postnatal brain maturation (Little, 1979). These types of results can be obtained from the phosphorylation of the subunits which alter the charge on the molecule. This actually appears to be the case in axonemal tubulin from *Chlamydomonas,* where α splits into five components on SDS gels and *β* into two, some of these bands differing in their degree of phosphorylation (Piperano and Luck, 1976). These arguments could also explain the splitting of α on hydroxyapatite chromatography and the splitting in both α and *β* on isoelectric focussing (Kobayashi and Mohri, 1977; Lu and Elzinga, 1977; Feit *et al.,* 1977a,b; Witman *et al.,* 1972). However, multiplicity of bands on electrophoresis does not necessarily imply multiplicity of amino acid sequence.

Moreover, recently genetic analysis from different laboratories reveal that for both α and *β* tubulin there is more than one gene. Thus, Lopata *et al.* (1983) have reported four unique *β* tubulin genes in chicken. Mischke and Pardue (1983) showed the presence of a multigene family for α in *Drosophila.* Besides these a testis specific *β* tubulin is expressed in *Drosophila* (Kemphues *et al.,* 1980, 1982; Raff and Kemphues, 1983). Thus though analysis of the data in the protein level does not signify totally the presence of

microheterogeneity, the genetic analysis reveal that there is some microheterogeneity in both α and *β* tubulin. Tubulin heterogeneity in the Trypanosome, *Crithidia fasciculata* has been adequately established (Russel *et al.,* 1984). The interphase cell of *C. fasciculata* has three discrete and separable tubulin populations: the subcellular microtubule, the axonemal microtubule and the nonpolymerised cytoplasmic pool of tubulin.

Microtubule associated proteins

Tubulin purified by assembly disassembly procedure, contains some accessory proteins which copurifiy with tubulin through repeated cycles of assembly and disassembly. Two classes of accessory proteins have been reported. The first one is a set of two high M_r proteins having M_r around 300,000, were termed HMW by Borisy and coworkers (Borisy *et al.,* 1975; Murphy and Borisy, 1975) and MAP by Rosenbaum and coworkers (Dentler *et al.,* 1975). Another class of low M_r accessory proteins (M_r 55,000–70,000) has been reported by Kirschner and coworkers and were termed as τ proteins (Weingarten *et al.,* 1975). The contents of accessory proteins in a preparation depends on the conditions used in the *in vitro* assembly. All these microtubule associated proteins are often collectively termed MAPs. MAPs could be separated from tubulin and are believed to be involved in the assembly of microtubules (Snyder and McIntosh, 1976). The role of MAPs in microtubule assembly has been discussed (Scheele and Borisy, 1979). Some of the enzyme activities have been observed to be associated with isolated microtubules: adenosine triphosphatase (Gelfand *et al.,* 1978), guanosine triphosphatase (David-Pfeuty *et al.,* 1977), protein kinase (Eipper, 1974), phosphoprotein phosphatase (Jameson *et al.,* 1980) nucleoside diphosphokinase (Jacobs and Huitorel, 1979), adenylate cylase (Margolis and Wilson, 1979), 3′,5′-cyclic AMP phosphodiesterase (Watanabe *et al.,* 1976), glutamate dehydrogenase (Karr *et al.,* 1979), tyrosine hydroxylase (Borisy *et al.,* 1975), alkaline phosphatase (Prus and Wallin, 1983) and DNA polymerase (Avila, 1980).

Interaction of tubulins with drugs

Several antitumour drugs, *viz.,* colchicine and its analogues podophyllotoxin and vinca alkaloids inhibit mitosis and other cellular functions by speicifically binding to tubulin and inhibiting its assembly into microtubule. Consequently, these drugs have become valuable tools in understanding the role of microtubules in diverse cellular functions.

Colchicine and its structural analogues

Colchicine is one of the oldest drugs in the pharmacopoeia and has been named after the meadow saffron *Colchicum autumnale* (Eigsti and Dustin, 1955). It is a tropolone derivative with three rings – one trimethoxybenzene ring (*A*-ring), one saturated seven membered carbon ring (*B*-ring) with a substituted acetamido group and a tropolone ring (*C*-ring) with one oxo and one methoxy group (figure 2) (Margulis, 1975).

The binding of colchicine with tubulin is one of the primary characteristics of the

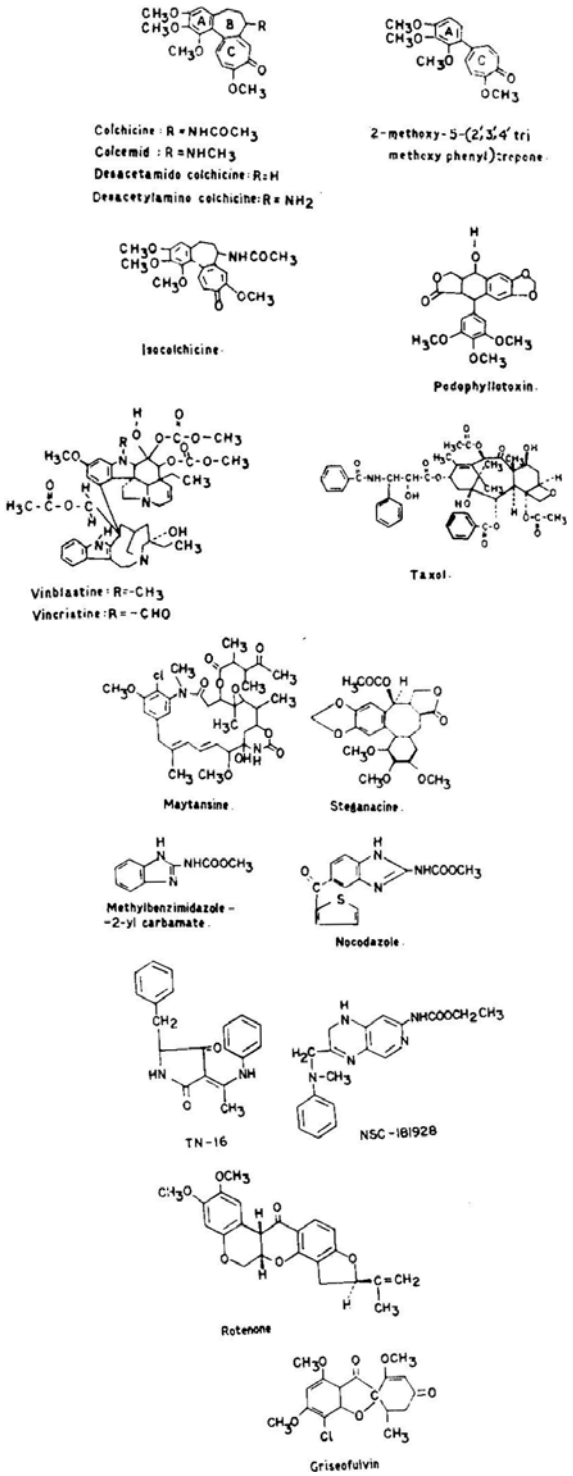

Figure 2. Chemical structures of the tubulin binding drugs.

protein and colchicine binding has been studied with tubulin prepared from many organisms and the higher plants (Flanagan and Warr, 1977; Hart and Sabins, 1973; Luduena *et al.,* 1976; Miller, 1973; Stephens, 1977; Wilson and Friedkin, 1967; Wilson and Meza, 1973). Since, the binding reaction is highly specific and the colchicine-tubulin complex is very stable, [^{3}H]-colchicine can be used as an assay for quantitative analysis of the binding reaction. To measure colchicine binding, various methods have been used which include gel filtration, filtration through DEAE cellulose filters, adsorption by charcoal (Borisy, 1972; Rappaport *et al.,* 1975; Wilson and Bryan, 1974). Bhattacharyya and Wolff (1974a) have developed another unique method based on the promotion of fluoroscence upon binding of colchicine to tubulin. This method does not require the separation of the free ligand, since unbound colchicine does not have any fluorescence. This method thus permits the measurement of kinetic and thermo-dynamic parameters under equilibrium conditions.

The tubulin dimer has one binding site for colchicine (Weisenberg *et al.,* 1968; Shelansky and Taylor, 1967; Wilson *et al.,* 1974). The dissociation constants, K_d, in animal tubulins are in the range of $3{\cdot}0–9{\cdot}1 \times 10^{-7}$ M when measured by equilibrium method (Barnes *et al.,* 1977; Borisy and Taylor, 1967b; McClure and Paulson, 1977; Owellen *et al.,* 1974; Sherline *et al.,* 1975); when measured by kinetic methods, however, the dissociation constant is an order of magnitude lower (McClure and Paulson, 1977; Sherline *et al.,* 1975; Bhattacharyya and Wolff, 1976b; Garland and Teller, 1975). The interaction between colchicine and tubulin is noncovalent and the drug is not altered chemically upon binding (Wilson and Friedkin, 1967; Borisy and Taylor, 1967a). The binding reaction is slow (Wilson and Bryan, 1974; Bhattacharyya and Wolff, 1974b) and almost irreversible in nature. Garland (1978) proposed a two step mechanism for the interaction of colchicine with tubulin: after a fast pre-equilibration step, a slow conformational change in the tubulin molecule leads to the formation of the fluorescent complex. Engelborghs's group (Lambeir and Engelborghs, 1981) by using a fluores-cence stopped flow technique, arrived at the same conclusion as made by Garland. Ventilla *et al.,* (1972) on the basis of the circular dichroic studies, have also demonstrated that colchicine binding leads to a conformational change of tubulin molecule.

The colchicine binding site on tubulin is subject to decay whose properties have been intensively studied. The decay is an $_{\text{all}}$ or none phenomenon; the binding site simply disappears with first order kinetics, without a gradual change in affinity. The half life of decay of mammalian tubulin is about 5–7 h (McClure and Paulson,1977; Sherline *et al.,* 1975; Solomon *et al.,* 1973), but solubilized sea urchin outer doublet tubulin decays with a half time of 5·2–5·6 h (Wilson and Meza, 1973). This decay is stabilized by salt, vinblastine, GTP, glycerol, sucrose, and dithiothreitol and by colchicine itself(Wilson and Meza, 1973; McClure and Paulson, 1977; Sherline *et al.,* 1975; Solomon *et al.,* 1973). The decay rate is linearly influenced by temperature and pH, being most stable at 0°C and pH 6·75 (Wilson, 1970).

Fluorescence studies using various analogues and derivatives of colchicine have revealed that atleast two moieties on the colchicine molecule are involved in the binding to tubulin (Bhattacharyya and Wolff, 1974a). One is the *A*-ring in which insertion of a bulky group causes complete loss of binding. The other moiety is the tropolone ring or *C*-ring (figure 2). It has been suggested that *C*-ring in combination with *A*-ring might be

responsible for the promotion of fluorescence. Replacement of tropolone ring by a phenyl ring in colchicine causes complete loss of binding (Zweig and Chignell, 1973). Correct positioning of the carbonyl moiety in *C*-ring appears to be necessary also. Modification in the *C*-ring (as in lumicolchicine) also leads to the complete loss of binding (Wilson, 1970). On the other hand, colchicine analogues modified at the *B*-ring moiety are known to have potent antimitotic activity which apparently rules out any major role of this moiety in the binding of colchicine to tubulin (Fitzgerald, 1976). But it has been shown from this laboratory that a minor change in the *B*-ring substituent may significantly affect the mechanism as well as the nature of binding. Thus, colcemid binds to tubulin fairly rapidly and reversibly, unlike colchicine (Banerjee and Bhattacharyya, 1979). Recently, we have shown (Ray *et al.,* 1984) that tubulin has two distinct colcemid binding sites. One site has a very high affinity while the other has low affinity. The affinity constants are respectively $1{\cdot}3 \times 10^{-5}$ M^{-1} and $0{\cdot}7 \times 10^{-5}$ M^{-1}. The activation energy of the colcemid binding to tubulin has been found to be 9·8 K_{cal}/mol, a value lower than that for colchicine (19·5 K_{cal}/mol) (Ghosh Choudhury, 1984).

Recently, from circular dichroism (CD) studies, Detrich *et al.* (1981) have shown that when colchicine binds to tubulin, the 340 nm CD band vanishes, from which they concluded that a conformational change in the colchicine molecule is taking place. Moreover, the binding of colchicine to tubulin induces a change from conformation 1 to conformation 2 which provides a potential explanation for the enhancement of colchicine fluorescence (Bhattacharyya and Wolff, 1974a; Detrich *et al.,* 1981). However, it has been recently reported from this laboratory (Ghosh Choudhury *et al.,* 1983a,b) that the two colchicine analogues desacetamidocolchicine and 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl) tropone (figure 2) can bind to tubulin with a stoichiometry of one. The affinity constants for both of these drugs are $1{\cdot}6 \times 10^{6}$ M^{-1} and $0{\cdot}58 \times 10^{6}$ M^{-1} respectively. The activation energies for binding of these drugs to tubulin have been determined (Ghosh Choudhury, 1984). The values are 6·4 K_{cal}/mol for both desacetamidocolchicine and 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl) tropone respectively. From Dreiding model building and energy calculation it has been found in this laboratory (Ghosh Choudhury, 1984) that for binding and promotion of fluorescence, both of these analogues also require a conformational change like that of colchicine. Thus the activation energies for binding of these drugs to tubulin decrease in the order:

Colchicine > Colcemid > Desacetamidocolchicine > 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl) tropone.

Moreover, 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl) tropone can bind tubulin even at 4°C (Ray *et al.,* 1981; Ghosh Choudhury, 1984). This indicates that atleast for this drug, tubulin does not require the so called time and temperature dependent conformational change (Lambeir and Engelborghs, 1981) like that needed in the case of colchicine. In the case of desacetamidocolchicine the case is nearly similar to that of 2-methoxy-5-(2′,3′,4′-trmiethoxyphenyl) tropone (activation energy = 6·4 K_{cal}/mol). So from the existing literature and from a systematic and rigorous study in our laboratory the colchicine binding reaction may be postulated as:

$$\text{Colchicine (1)} \rightleftharpoons \text{Colchicine (2)} + T \rightleftharpoons T\text{-colchicine (2)} \rightarrow T\text{-colchicine* (2)}$$

Colcemid(1) ⇌ Colcemid (2)+ T ⇌
T-colcemid (2)(?)⇌T-colcemid (2)

Desacetamidocolchicine (1) ⇌ Desacetamidocolchicine (2) + T →
T-Desacetamidocolchicine* (2)

2-methoxy-5-(2′,3′,4′–trimethoxyphenyl) tropone (1) ⇌
2-methoxy-5-(2′,3′,4′-trimethoxyphenyl) tropone (2)+T
⇌T-2-methoxy-5-(2′,3′,4′-trimethoxyphenyl) tropone*,

where 1 and 2 are the conformation 1 and conformation 2 of the drugs and asterics represents the fluorescent species of drug-tubulin complex.

On the basis of the assumption that colchicine behaves as a bifunctional ligand during binding to tubulin, Andrew and Timasheff (1982) postulated a model in which they suggested that ring *C* of colchicine (figure 2) binds first and this binding would induce in the protein a conformational change bringing the *A*-ring domain to proper position to bind the ring. But the discrepancy in this model is that there is no room for *B*-ring or *B*-ring substituent (acetamido group). But recent studies from this laboratory (Ray *et al.,* 1981; Ghosh Choudhury *et al.,* 1983a,b; Ghosh Choudhury, 1984) have revealed that there is a vast difference in activation energy and rate of binding among colchicine and its *B*-ring analogues. This finding leads to a conclusion that not only *A* and *C*-ring, but *B*-ring of colchicine also plays a major role in its binding to tubulin and determines some of the characteristics of the tubulin-colchicine interaction.

However, it has not been established which of the subunits of tubulin carries the colchicine binding site. The report by Roussett and Wolff (1980a) had indicated that the dimeric state of tubulin was not necessary for colchicine binding and that the colchicine binding site is located on either the α or *β* subunit. It has been found that lactoperoxidase, an enzyme used in the iodination of proteins, binds tubulin and dissociates α and *β* monomers as α-tubulin-lactoperoxidase and *β*-tubulin-lactoperoxidase complexes (Rousset and Wolf, 1980b). They found that the colchicine binding activity of tubulin was virtually unaltered after complete dissociation of subunits. It is known that the yeast tubulin can not bind colchicine (Haber *et al.,* 1972) and yeast tubulin has been reported to contain an altered *β* subunit (Clayton *et al.,* 1979). In this context it is note worthy that α and *β* tubulins from a protease deficient strain of *Saccharomyces cerevisiae* comigrate with brain α and *β* tubulins (Ghosh Choudhury and B. B. Biswas, unpublished observation). Colchicine resistant mutants from Chinese hamster ovary (CHO) cells have been found to possess an altered *β* subunit (Cabral *et al.,* 1980). All these information collectively indicate the presence of the colchicine binding site on the *β* subunit.

Colchicine binds only to tubulin dimers but not to intact microtubules. It has been demonstrated that the colchicine binding site of tubulin gets burried within the microtubule (Sherline *et al.,* 1975; Wilson and Meza, 1973; Wilson *et al.,* 1974). The anti-microtubular action of colchicine, therefore, believed to be mediated through an inhibition of microtubule assembly rather than a direct interaction with the micro-tubules (Wilson, 1975; Margolis and Wilson, 1977) which exist in a dynamic equilibrium with the subunit protein tubulin (Inoue and Sato, 1967). Thus, in the presence of colchicine, soluble tubulin gets complexed with the drug and becomes

inactive for microtubule formation, which in turn shifts the equilibrium and leads to the disassembly of microtubules.

Podophyllotoxin

The antimitotic drug podophyllotoxin (figure 2) is extracted from the root of the May apple *Podophyllum peltatum* (Kelly and Hartwell, 1954). In general the antimitotic activity of podophyllotoxin is qualitatively indistinguishable from that of colchicine (Pfeffer *et al.,* 1976; Wilson *et al.,* 1976; Cortese *et al.,* 1977). Although colchicine and podophyllotoxin share the same binding site on tubulin perhaps because of common trimethoxy benzene moiety (*A*-ring; figure 2) the mechanism of binding of podophyllotoxin appears to be somewhat different from that of colchicine as shown by Cortese *et al.* (1977). Thus, (i) podophyllotoxin binds very rapidly, about 10 times as fast as colchicine; (ii) podophyllotoxin binds readily at 0°C; (iii) podophyllotoxin binding is freely reversible (Wilson *et al.,* 1974; Wilson, 1975; Cortese *et al.,* 1977). The association rate constant for podophyllotoxin binding to rat brain tubulin has been found to be $3{\cdot}8 \times 10^6$ M^{-1} h^{-1}. The dissociation rate constant is $1{\cdot}9$ h^{-1}.

Vinca alkaloids

Vinblastine and vincristine (figure 2) are potent antimitotic drugs obtained from the plant (*Catharanthus roseus*) G. Don (*Vinca rosea* L.) and are used in the treatment of neoplastic diseases. Vinblastine binds to tubulin at sites distinct from colchicine and podophyllotoxin (Bryan, 1972). The vinblastine binding reaction is very different from that of colchicine; the binding is reversible, temperature independent and rapid (Wilson, 1975; Mandelbaum-Shavit *et al.,* 1976; Owellen *et al.,* 1972; Wilson *et al.,* 1975, 1978). The binding reaction is not affected by GTP, colchicine or calcium, although colchicine and sucrose stabilise the decay of the site (Owellen *et al.,* 1972; Wilson *et al.,* 1975; 1978; Bhattacharyya and Wolff, 1976a). The decay is first order with a half time of 3·5 h and is unusual in that it affects the affinity as well as the number of sites available (Wilson *et al.,* 1978; Bhattacharyya and Wolff, 1976a). Two high affinity vinblastine binding sites with about the same affinity have been reported (Lee *et al.,* 1975; Wilson *et al.,* 1975). In contrast Bhattacharyya and Wolff (1976a) found two binding sites, differing in affinity. The reported vinblastine dissociation constants for mammalian tubulin range from $1{\cdot}25 \times 10^{-7}$ M to $4{\cdot}5 \times 10^{-5}$ M (Owellen *et al.,* 1972, 1974; Lee *et al.,* 1975; Bhattacharyya and Wolff, 1975).

In addition to high affinity sites, there may be several low affinity binding sites for vinblastine, about 20–30 for chick brain tubulin (Wilson, 1975). Vinblastine induces tubulin to aggregate into a series of highly organized structures. Vinblastine (2×10^{-5} M) causes tubulin to dimerize (Lee *et al.,* 1975); at $1{\cdot}0 \times 10^{-4}$M vinblastine, larger structures are seen, including ring, spirals and double helical structure called a macrotubule, consisting of two spirals with a centre-to-centre spacing of 18-28 nm (Erickson, 1975; Fujiwara and Tilney, 1975; Maratz and Shelansky, 1970). In some preparations, closely packed arrays of macrotubules constitute a crystal, which can also be induced in some cells *in vivo* (Maratz and Shelansky, 1970; Bensch and Malawista, 1969; Bensch *et al.,* 1969; Bryan, 1971).

Griseofulvin

Griseofulvin (figure 2) is a widely used antifungal antibiotic isolated from *Penicillium griseofulvum.* It arrests mitosis and causes disorientation of microtubules *in vivo* and can prevent microtubule assembly *in vitro* (Grisham *et al.,* 1973a; Gull and Trinci, 1974). The K_i of griseofulvin for preventing assembly of recycled bovine brain microtubules is $5{\cdot}0–6{\cdot}7 \times 10^{-6}$ M (Wilson *et al.,* 1975). There are contradictions about the specific binding of this drug to tubulin. It has been reported that [^{3}H]-griseofulvin does not bind to pure tubulin (Grisham *et al.,* 1973b). On the other hand Roobol *et al.* (1977) have shown that griseofulvin binds to a fraction containing MAPs.

Taxol

Recently, it has been suggested that the antimitotic drug taxol (figure 2) an alkaloid from the plant *Taxus brevifolia* acts by a somewhat different mechanism. In contrast to other drugs mentioned, taxol acts as a promoter of microtubule assembly *in vitro* and renders microtubules resistant to depolymerization by cold (4°C) and Ca^{2+} *in vitro* and tissue culture cells (Schiff *et al.,* 1979; Schiff and Horwitz, 1980). Taxol specifically inhibits cell separation in *Trypanosoma cruzi.* However, this taxol treatment permits continued multiplication of cellular organelles, including the nucleus, kinetoplast and flagellum. This observation suggests the presence of atleast two classes of microtubules in *Trypanosoma cruzi* as determined by taxol sensititivity (Baum *et al.,* 1981). Tritium labelled taxol binds directly to microtubules *in vitro* with a stoichiometry approaching one (Parness and Horwitz, 1981). [^{3}H]-Taxol binds to the macrophage like cell line, *J* 774·2 in a specific and saturable manner. Scatchard analysis of the species binding data demonstrates a single set of higher affinity binding sites (Manfredi *et al.,* 1982). Using ultra-violet mutagen treated CHO cells, Cabral *et al.* (1981) selected taxol resistant cells. By two dimensional gel electrophoresis and peptide mapping these authors reported that the taxol resistant cells contain an altered α tubulin. This indicates that the taxol binding site may be on the α tubulin. From a study in this laboratory, some of the characteristics of the taxol induced purified tubulin polymerization *in vitro* have been elucidated (Ghosh Choudhury *et al.,* 1983b; Ghosh Choudhury, 1984). The antimitotic drug colchicine inhibits taxol induced purified tubulin polymerization *in vitro* with a K_i of $1{\cdot}5 \times 10^{-6}$ M. Moreover, like normal microtubules, colchicine binding site is burried in taxol induced polymers. Unlike normal assembly, however, taxol induced assembly is not inhibited by the colchicine analogues like desacetamidocolchicine and 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl) tropone. Rather this analogue tubulin complex can copolymerize in the presence of taxol into microtubules. Moreover, from a rigorous thermodynamic analysis it has been shown that like normal MAPs induced microtubule assembly, taxol induced purified tubulin polymerization is also an entropically driven process (Johnson and Borisy, 1979; Ghosh Choudhury *et al.,* 1983b; Ghosh Choudhury, 1984).

Other antimicrotubular drugs

Benzimidazole derivatives which were first introduced as fungicides, are another group of important microtubule poisons. These include methyl benzimidazole-2-yl carbamate

(MBC) (figure 2), mebendazole, and oncodazole or nocodazole (Davidse and Flach, 1977). These drugs exhibit antimitotic action similar to colchicine. A unique feature of MBC is that it selectively destroys the microtubules of parasitic worms (Borgers *et al.,* 1975) without any effect on those of the host. Moreover, it does not affect the *in vitro* assembly of porcine brain tubulin (Davidse and Flach, 1978). Nocodazole shows potent antimitotic activity in mammalian system and also inhibits the *in vitro* microtubule assembly (DeBrabander *et al.,* 1976). It competes with colchicine for the binding site on tubulin. It is noteworthy to mention that nocodazole has got similarity with colchicine only with its *B*-ring moiety and both of them induce a change in the conformation of tubulin molecule after binding (Lee *et al.,* 1980).

Another important antimicrotubular agent is steganacin (figure 2) which has been extracted from the wood and stems of *Steganotaenia araliaces* Hochst. It blocks Hela cell replication in mitosis. This drug is a competitive inhibitor of colchicine binding to tubulin and inhibits microtubule assembly *in vitro* (Schiff *et al.,* 1978; Schiff and Horwitz, 1981).

A potent antileukemic macrolide maytansine (figure 2), isolated from *Maytenus ovatus,* inhibits mitosis and also the *in vitro* assembly of microtubules (Remillard *et al.,* 1975). It competes with vinblastine and vincristine for their high affinity binding site on tubulin but does not lead to the formation of tubulin crystals (Bhattacharyya and Wolff, 1977).

Rotenone (figure 2) is another potent antimitotic drug which interacts reversibly with the colchicine binding site on tubulin and inhibits *in vitro* assembly of microtubules (Brinkley *et al.,* 1974; Marshall and Himes, 1978). Besides these, recently two compounds TN-16 (Arai, 1983) and antileprosy drug dopasone (Rajagopalon and Gurnani, 1983) have been reported to bind tubulin and inhibit microtubule assembly. Coumarin and isopropyl-(N-3 chlorophenyl) carbamate (CIPC) have been found to affect the mitotic division in lower eukaryotes suggesting that these have antimitotic activity (Katz *et al.,* 1982). Coumarin has effects similar to those produced by antimicrotubule agents such as colchicine, CIPC and benzimidazole derivatives (Welker, 1982). Coumarin apparently does not affect spindle microtubules. This different specificity for cytoplasmic and spindle microtubules might be used to probe the microtubule functions and its heterogeneity.

In the case of plants the herbicides, amiprophos-methyl (APM) have been found to poison specifically microtubule dynamics (Morejohn and Fosket 1984). The potent antimicrotubule action of APM has already been used to investigate the regulation of tubulin synthesis in *Chlamydomonas* (Collis and Weeks, 1978). Characteristic features of some important antimitotic drugs have been presented in table 1.

Mechanism of substoichiometric drug poisoning of microtubule assembly

Antimicrotubular drugs like colchicine, vinblastine and podophyllotoxin inhibit microtublue assembly both *in vivo* and *in vitro* in a substoichiometric manner, *i.e.* concentrations of drugs needed to inhibit the assembly is far substoichiometric to the free tubulin concentration in solution (Olmsted and Borisy, 1973; Wilson *et al.,* 1976). The drug-tubulin complex rather than the drug itself, has been found to be the

Table 1. Some kinetic and thermodynamic parameters of drug tubulin interaction.

Drug	No. of binding sites	Affinity constnat	Activation energy	References
Colchicine[a]	1	$3{\cdot}33$–$1{\cdot}09 \times 10^6\ M^{-1}$	$19{\cdot}5\ K_{cal}$/mol	Barnes *et al.* (1977), Borisy and Taylor (1967b), McClure and Paulson (1977), Owellen *et al.* (1974), Sherline *et al.* (1975), Ghosh Choudhury (1984).
Colcemid[a]	2	$0.7 \times 10^5\ M^{-1}$ $1.3 \times 10^5\ M^{-1}$	$9{\cdot}8\ K_{cal}$/mol	Ray (1980), Ghosh Choudhury (1984), Ray *et al.* (1984).
Desacetamido	1	$1.6 \times 10^6\ M^{-1}$	$6{\cdot}4\ K_{cal}$/mol	Ray *et al.* (1980), Ghosh Choudhury *et al.* (1983a), Ghosh Choudhury (1984).
2-methoxy-5-(2′,3′,4′-trimethoxyphenyl) tropone[a].	1	$0{\cdot}58 \times 10^6\ M^{-1}$	$4\ K_{cal}$/mol	Ray *et al.* (1980), Ghosh Choudhury *et al.* (1983a), Ghosh Choudhury (1984).
Podophyllotoxin[a]	1	$1{\cdot}8 \times 10^6\ M^{-1}$	$14{\cdot}7\ K_{cal}$/mol	Cortese *et al.* (1977).
Vinblastin[b]	2 and/ or more	$6{\cdot}2 \times 10^6\ M^{-1}$ $8 \times 10^4\ M^{-1}$	N.D.	Bhattacharyya and Wolff (1976a).
Vincristine[b]	1	$1{\cdot}8 \times 10^7\ M^{-1}$	N.D.	York *et al.* (1981)
Maytansine[b]	1	N.D.	N.D.	Bhattacharyya and Wolff (1977)
Steganacin[a]	1	N.D.	N.D.	Schiff *et al.* (1978), Schiff and Horwitz (1981)
Nocodazole[a]	1	N.D.	N.D.	Lee *et al.* (1980)
Methyl-benz-imidazole-2-yl carbamate[a]	1	N.D.	N.D.	Borgers *et al.* (1975), Davidse and Flach (1977).
NSC-181928[a]	1	N.D	N.D.	Hamel and Lin (1982).
Rotenone[a]	1	N.D.	N.D.	Brinkley *et al.* (1974), Marshall and Himes (1978).
TN-16[a]	1	N.D.	N.D.	Arai (1983).
Taxol	1	N.D.	N.D.	Parness and Horwitz (1981).
Dopasone	N.D.	N.D.	N.D.	Rajagopalan and Gurnani (1983).

[a,b] Indicate competitive binding site for colchicine and vinblastine respectively. N.D., Not determined.

inhibitory species (Margolis and Wilson, 1977). *In vitro* assembly is inhibited half maximally when only 2 % of the unpolymerized tubulin is complexed with the drug.

The mechanism by which colchicine exhibits substoichiometric poisoning, has been studied by several workers in recent years. Margolis and Wilson (1977) demonstrated that colchicine-tubulin dimer (CD) complex adds on to the growing end of the

microtubule and irreversibly caps that end so that further addition of dimers becomes impossible. Microtubule assembly would therefore be poisoned owing to a reduction in the number of the assembly competent ends. Alternatively, Sternlicht and Ringel (1979) demonstrated that CD complex decreases the affinity of a microtubule end for further dimer addition. In this case, the poisoning of assembly results owing to a decrease in the apparent rate of dimer addition of microtubules without any decrease in the number of the assembly competent ends.

Lambeir and Engelborghs (1980) have reported that the binding of CD to microtubule ends is reversible and the affinity of tubulin and CD for microtubule ends is of the same order of magnitude. Farrell and Wilson (1980) have re-examined the poisoning mechanism and found that the binding of CD complex to microtubule end was not irreversible. In time, some free tubulin dimer addition occurs over the CD block, ultimately resulting in recovery from that block. The recovery depends on the molar frequency of the addition of free tubulin dimers to that of CD complex. At high tubulin/CD ratios (>250) the recovery is essentially complete and copolymer formation occurs without a reduction in the number of assembly competent ends. However, at a very low ratios (< 14), frequency of addition of free tubulin dimers to that of CD complex falls below a critical ratio, and under such condition the inhibition of assembly becomes almost complete and the number of assembly competent end is reduced to zero.

Deery and Weisenberg (1981) have suggested that it is the colchicine-tubulin oligomer complex that is the actual inhibitory species. According to their model, the elongation of microtubules requires the addition of tubulin oligomers to the end of the microtubule. The presence of a single colchicine molecule to the end subunit of a linear oligomer could lead to an inhibition of the interaction of oligomers with the microtubule end. The extent of inhibition may depend upon the fraction of tubulin subunits in an oligomer that contain colchicine. Colchicine containing subunits might also inhibit the lateral co-operative interactions between the subunits of adjacent oligomers.

Tubulin mRNA during growth and development

It has been observed that tubulin synthesis can be regulated in the presence of drugs. Drugs (colchicine and nocodazole) that depolymerize microtubule decreases and those (taxol and vinblastine) favouring polymerization increase the synthesis of tubulin mRNA (Cleveland *et al.,* 1981).

Initial experiments have shown that mRNA fractions separated from the total mRNA of chick embryo brain by sucrose density gradient centrifugation could be translated *in vitro* in rabbit reticulocyte cell-free lysates containing [^{35}S]-methionine. Both actin and tubulin were detected in the product by electrophoresis (Gilmore-Herbert and Heywood, 1976). Recently, Kirschner and his associates have described the isolation of two mRNAs coding for α and β tubulins by fractionation of poly (*A*) containing RNA from embryonic chick brain, thus giving evidence for two tubulin genes (Cleveland *et al.,* 1978). These two mRNAs are very similar in M_r (650,000) as judged by mobility on denaturing gels containing methylmercury. However, as

reported by Bryan *et al.* (1978), the separate mRNAs coding for the α and *β* subunits were resolved on native gels, α migrating faster than *β,* indicating that α mRNA had more secondary structure than that of *β* mRNA. It has also been shown by Cleveland *et al.* (1978) that tubulin is synthesized in both free and membrane-associated polysomes. Peptide mapping in all cases confirmed that the *in vitro* translation products are α and *β* tubulins, which can coassemble into microtubules with added carrier microtubule proteins. Similarly, evidence for the synthesis of tubulin on membrane bound and free ribosomes from rat brain has been shown. In our laboratory it has been observed that colchicine can bind with the polysomes isolated from rat brain. Tubulin messenger RNA thus enriched from polysomes was used for cDNA synthesis and cloning (Chakraborty *et al.,* 1983; Sen *et al.,* 1984). Tubulin has also been detected among the *in vitro* translation products of poly (*A*) containing RNA from *Tetrahymena* in cell free wheat germ and rabbit reticulocyte systems. Isoelectric focusing of the products reveals heterogeneity in both α and *β* tubulins (Portier *et al.,* 1979). Similar heterogeneity has also been observed in rat brain tubulins synthesized *in vivo* or by *in vitro* translation of rat brain RNA in a wheat germ or rabbit reticulocyte cell-free system (Saborio *et al.,* 1978).

Gozes *et al.* (1980) have reported that prenatal rat brain tubulin can be resolved by isoelectric focusing into five or six components, while in mature brain nine distinct forms of tubulin were evident. Also, tubulins isolated from various regions of the brain displayed a quantitative difference in their microheterogeneity. Obviously, the question arises whether such microheterogeneity results from post-translational modification or from mulitple species of mRNA. Gozes *et al.* (1980) have shown that mature brain mRNA translated *in vitro* results in the synthesis of five tubulin forms. The mRNA could be resolved into several species coding for these distinct tubulin forms. An age dependent enhancement in the relative translation of the mRNA coding for a particular tubulin species has also been observed, this being characteristic of mature brain and not apparent among the *in vitro* translation products of prenatal mRNA. These results clearly indicate that at least some of the variations in tubulin microheterogeneity may be controlled at the mRNA level.

Although the flagellar tubulin of *Naegleria* has been shown to be similar to tubulins isolated from other sources, it is interesting to note that antibodies prepared against the outer doublet tubulin react only with the flagellar tubulin and not even with the tubulin of the amoeboid form (Kowit and Fulton, 1974). Using this specific antibody, Lai *et al.* (1979) have demonstrated that during differentiation of an amoebae to a flagellate, the flagellar tubulin was the predominant product of translation in a wheat germ cell free system as directed by the poly (*A*) containing RNA extracted from these differentiating cells. However, no flagellar tubulin or tubulin message could be detected by the translation assay or using *β* tubulin clone probe (Fulton and Lai, 1982) against RNA extracted from amoebae prior to differentiation. These facts indicate that flagellar tubulin mRNA appears only during differentiation. However, it is not settled whether such appearance of translatable mRNA arises from *de novo* synthesis of mRNA or from post transcriptional processing of a preexisting mRNA species to a translatable form.

A similar marked increase in the amount of translatable tubulin mRNA has also been observed during the regeneration of flagella by gametes of *Chlamydomonas reinhardii*

(Weeks and Collis, 1976). Of course, a major difference between the *Chlamydomonas* and *Naegleria* systems is that while the gametes of the former species contain preexisting flagella and sufficient tubulin for about 50% flagellation by length in the absence of protein synthesis (Weeks and Collis, 1976; Lafebvre *et al.,* 1978), in the latter system no flagella preexist in the amoebae nor does any flagellation occur in the absence of prior protein synthesis. However, in both systems, flagellation during regeneration or differentiation is accompanied by an increase in translatable tubulin mRNA within 15–20 min after the stimulus, reaching a maximum amount at about 1 h and then declining as regeneration or differentiation is completed (Lai *et al.,* 1979). Using cDNA probe for hybridization with RNA, it was observed that tubulin sequences in RNA increased within 8 min following deflagellation of *Chlamydomonas reinhardii,* reached maximal levels by 50 min and then began to decrease by 80 min after deflagellation. One hybridization band was detected with use of the β tubulin probe, but two RNA size classes hybridised to the α tubulin probe (Silflow and Rosenbaum, 1981). From their study, it appears that tubulin synthesis after deflagellation is regulated essentially at transcriptional level.

Gene expression and mRNA of tubulin have been studied in ciliated protozoa *Tetrahymena pyriformims* (Fliss and Suyama, 1979; Zimmerman *et al.,* 1983). The electrophoretic mobility of α tubulin subunit on SDS-PAGE differs but β tubulin subunits have the same mobility. *In vitro* synthesized tubulin was found to have exactly the same mobility as well. The pattern of fluctuation of tubulin mRNA was studied by *in vitro* translation and by hybridization with tubulin probe during cell cycle. This suggests that as the cell progresses through the cell cycle, tubulin synthesis is controlled at the mRNA level.

Tubulin gene expression has been extensively studied in *Drosophila melanogaster*. During embyronic development, in *Drosophila* β_3 tubulin subunit (β tubulin varient) is expressed transiently with concomitant increase of α tubulin in order to keep α and β tubulin ratio constant (Raff *et al.,* 1982). A testis specific β tubulin has also been identified which is used in constructing the motile sperm tail (Kemphues *et al.,* 1979). However, the products of this testis specific gene are not restricted to a single functional class. A family of four α tubulin genes have been isolated having difference in nucleotide sequence and atleast three of them have different patterns of transcription during *Drosophila* development. In addition, atleast two of the genes have more than one RNA product. The concentrations of the different RNA products from a single gene also vary independently during the development of the organism (Kalfayan and Wensink, 1981).

Hybridization of cloned cDNA probes of sea urchin, *Layterhimus pictus* to filter blots of RNA from developmental stages show that tubulin synthesis is regulated at translational level as well as by the variations in the accumulation of different tubulin transcripts. The α sequences in the egg reside primarily in RNAs that are larger (2·8 and 2·5 kb) than the mature forms of the mRNA (1·75–2·2 kb); these are converted to the smaller forms after fertilization. In contrast, β mRNAs (1·8-2·2 kb) do not undergo any obvious size reduction after fertilization (Alexandraki and Ruderman, 1981). Some authors demonstrated the existence of atleast three different β tubulin mRNA and also developmental regulation of different α and β tubulin sequences.

Autoregulatory mechanism of control of expression of α and β tubulin has been shown

by Ben Ze'ev *et al.* (1979) and Cleveland *et al.* (1981) (Cleveland and Havercroft, 1983; Cleveland and Kirschner, 1982). Ben Ze'ev *et al.* (1979) demonstrated that colchicine, which depolymerizes microtubules and raises the level of tubulin, causes a cessation of tubulin synthesis by inhibiting the formation of new tubulin mRNA of cultured fibroblast cells. Similar conclusion was drawn by Cleveland and co-workers using immunoprecipitation and chicken α and β tubulins cDNA probe to monitor the cellular response of several cell types to a wide range of antitubulin drugs. Thus, it has been postulated that in virtually all higher eukaryotic cells there is an autoregulatory mechanism which apparently acts to maintain a specific level of depolymerized tubulin subunits and responds rapidly to lower an elevated level of unpolymerized subunits and the effect is possibly at the level of transcription process. However, the synthesis of tubulin proteins in chicken fibroblasts does not respond to microtubule depolymerizing drugs in the same manner as cell lines from mammalian species, though the mRNA half life appears to be short (Cleveland *et al.,* 1981). Recently Cleveland and Havercroft (1983) have demonstrated that the rates of tubulin mRNA synthesis were essentially unchanged in isolated nuclei from CHO cells whether the cells were treated with colchicine or not. Thus, autoregulatory control of tubulin mRNA is not mediated through the regulation of transcription process *per se.* Alternatively this apparent regulation of tubulin mRNA by depolymerized tubulin might be due to inhibition of proper tubulin mRNA processing and/or transport from the nucleus. In this connection an interesting observation may be mentioned where it has been shown that nuclear matrix, nuclear envelope and cytoplasmic skeletal elements appear to be involved in maturation, transport and decay of mRNAs in general (Muller *et al.,* 1983). An interaction between the microtubules and the nuclear pore complex including the nucleoside triphosphatase might play a crucial role in the apparent autoregulatory control of tubulin mRNA synthesis.

In addition to regulation in response to unpolymerized tubulin levels, tubulin genes are differentially expressed during development. In *Drosophila melanogaster* there are four α tubulin genes, all located at different sites on the third chromosome (Kalfayan and Wensink, 1981). Each gene yields characteristic mRNA levels at different stages of development as judged by RNA blot analyses using 3′-subcloned probes (Kalfayan and Wenskin, 1982). Atleast two human β tubulin genes each specifying a distinct isotype, are expressed in Hela cells, and the 2·6 kb mRNA band appears to be a composite of atleast two comigrating β tubulin mRNAs (Hall *et al.,* 1983).

Cloning and organization of tubulin genes

Tubulin gene from a variety of species has been cloned and sequenced (Cleveland, 1983) in a very short span of time. The first report of tubulin cDNA cloning starting from partially purified chick brain mRNA came from the laboratory of Kirschner (Cleveland *et al.,* 1980). The authors adopted the most widely used strategy for cDNA cloning. Tubulin mRNA which was purified by sucrose density gradient centrifugation and confirmed by its *in vitro* translated product, was transcribed by reverse transcriptase and then by DNA polymerase I. After Sl nuclease treatment, the double stranded cDNA was tailed at the 3′-end with cytosine residue using terminal polydeoxynucleotidyl transferase.

The sizing of cDNA (1000 to 2500 base pairs) was monitored and these species were annealed to plasmid pBR 322 that was linearized at the pst-1 restriction site and tailed with guanosine residues. Transformation of *Escherichia coli* with this recombinant DNA containing putative α and *β* tubulin gene was performed according to the method of Goodman and MacDonald (1979). Selection of colonies was done by the colony hybridization technique (Grunstein and Hogness, 1975) using nick translated double stranded cDNA derived from enriched mRNA for tubulin. Hybrid plasmids containing cDNA sequences complementary to either α or *β* tubulin were further identified by hybrid selected translation and identities of both α and *β* tubulin clones have been confirmed by DNA sequencing. A similar approach was also used to prepare cDNA clone of α and *β* tubulin genes in *Chlamydomonas* (Silflow and Rosenbaum, 1981). *Drosophila* α tubulin clones were isolated by screening genomic DNA library using nick translated double stranded cDNA derived from purified mRNA specific for *Drosophila* tubulin (Kalfayan and Wensink, 1981). Tubulin genes have been cloned from human, rat, sea urchins, *Trypanosomes, Leishmania* and yeast using chicken brain cDNA as probe to screen either genomic library or cDNA library of respective species under appropriate stringent condition of hybridization to select colonies for α or *β* tubulin (Cowan *et al.,* 1981; Lemischka *et al.,* 1981; Grinzburg, *et al.,* 1981; Alexandraki and Ruderman, 1981; Thomashow *et al.,* 1983; Landfear *et al.,* 1983; Neff *et al.,* 1983). Recently, the tubulin sequence complexities and their corresponding genomic organisation have been studied in a variety of species (Cleveland 1983).

Chlamydomonas gene organisation and expression have been studied by two groups of workers using cloned α and *β* tubulin genes (Silflow and Rosenbaum, 1981; Brunke *et al.,* 1982a,b) who demonstrated the existence of atleast two α tubulin and two *β* tubulin genes in this organism. Hybridization experiments further suggest that during tubulin induction four tubulin mRNA of discrete sizes are produced (two α and two *β* tubulin specific mRNA). *Chlamydomonas* cells contain several types of microtubules, including those found in the flagella, basal body, mitotic apparatus and cytoskeleton. Eventually it should be possible to determine whether all tubulin genes are expressed in this organism and to determine the type of microtubule(s) in which each gene product is found.

Alexandraki and Ruderman (1981) have analysed the multiplicity, heterogeneity and organization of the genes encoding the α and *β* tubulins in the sea urchin, *Lytechinus pictus* by using cloned cDNA and genomic tubulin sequences. Hybrid selection performed at different stringency demonstrated the presence of several heterogeneous, closely related tubulin mRNA, suggesting the existence of heterogeneous α and *β* tubulin genes. Hybridization analysis indicated that there are atleast 9 to 13 sequences for each of the two tubulin gene families per haploid genome, α Tubulin genes and *β* tubulin genes are not found to be linked but in contrast, some genes within the same family are separated or dispersed. Exact number of functional genes of α and *β* tubulin could not be ascertained out of a large number of tubulin genes estimated but the existence of atleast three different α tubulin mRNAs which have considerable divergence in the 3′-nontranslated regions, could be demonstrated.

Recently, cDNA and genomic clones containing α and *β* tubulins have been constructed from *Trypanosome brucei* (Thomashow *et al.,* 1983; Seeback *et al.,* 1983). Both groups of workers established that in contrast with the dispersed organization of

tubulin genes in other organism, Trypanosome α and *β* tubulin genes are physically linked and clustered in tandem repeats of approximately 13–17 copies per haploid genome of alternating α and *β* tubulin sequences. This arrangement may facilitate coordinate expression of the α and *β* tubulin subunits in species where cycles of polymerization and depolymerization are major features of the cell cycle. The arrangement of developmentally regulated α and *β* tubulin genes has been studied in the parasitic protozoan, *Leishmania enriettii* by using southern blot hybridization analysis (Landfear *et al.,* 1983). The α tubulin genes occur in a tandem repeat whose monomeric unit may be represented by a 2-kilobase pst-1 fragment. Similarly, the *β* tubulin genes probably occur in a separate tandem repeat consisting of approximately 4-kilobase units unlinked to the tubulin repeats. In contrast to multiple α or *β* tubulin genes found in all the organisms so far analysed, the lower eukaryote *Saccharomyces cerevisiae,* having simple nonmotile life cycle has only one α and one *β* tubulin each in a haploid genome (Neff *et al.,* 1983). Presence of an unique tubulin gene in the yeast genome was shown by complementation of a benomyl resistant conditional-lethal mutation which carries mutation at the gene(s) specifying *β* tubulin with a cloned DNA fragment from yeast homologous to a chicken *β* tubulin cDNA and which has an essential function in yeast. In *Aspergillus nidulans,* structural gene for *β* tubulin was found to be situated at benomyl binding site and benomyl resistant mutants sometimes exhibit an altered *β*-tubulin protein (Sheir-Neiss *et al.,* 1978) and suppressor mutation of revertant of temperature sensitive ben *A* (benmyl resistant) mutant strain has been identified to be a structural gene mutation for α tubulin in *Aspergillus nidulans* (Morris *et al.,* 1979). In rats and humans there are about 15 copies, well dispersed, each of α and *β* tubulin sequences (Lemischka and Sharp, 1982; Cleveland *et al.,* 1980) and in chicken 4–5 copies each of these sequences (Cleveland *et al.,* 1980; Lopata, *et al.,* 1983), have been reported. Further, analysis of an expressed human *β* tubulin gene shows four coding regions of 57, 109, 113 and 1053 bp. The size of these exons is also similar to those in a second expressed human *β* tubulin gene (Cowan *et al.,* 1981; Lee *et al.,* 1983). This similarity in exon structure also extends to the exons of four expressed *β* tubulin genes of chicken (Lopata *et al.,* 1983). The size of the intervening sequences however, is highly variable. This suggests that sequences in exons in tubulin genes are well conserved while those in introns are not.

The tubulin gene system particularly *Chlamydomonas reinhardii* shows rapid and coordinate induction of mRNA synthesis. Sequence analyses of those clustered genes reveal a short consensus sequence of 16 bp: [*GCTC*(*G/C*) *AAGGC*(*G/T*)(*G/C*) –(*C/A*)(*C/A*)*G*] just upstream of *TATA* box (Brunke *et al.,* 1984). In general, the putative regulatory elements within a particular gene set including developmentally and homonally controlled genes (Cochet *et al.,* 1982) and tubulin gene are similar but not identical (9–24 bp) and are present more than once in the upstream of TATA box. A summary of the sequence complexities of tubulin and the genomic organization in a variety of species is given in table 2. It is abundantly clear that the tubulin genes are present in large numbers in certain species and no significant correlation as a function of evolution could be established. The tubulin genes of most species thus examined constitute dispersed multigene families (Cleveland, 1983). The analysis of tubulin multigene family in different cases has shown that many of these sequences are pseudogenes (Lee *et al.,* 1983; Lemischka and Sharp, 1982).

Table 2. Summary of tubulin gene sequences in various species.

Species		No. of subunit gene per haploid genome	No. of functional genes	Sequence organization	References
Trypanosomes	α	13–17	—	Tandemly duplicated as α/β pairs	Thomashow *et al.* (1983).
	β	13–17	—		
Leishmania	α	7–15	—	Clustered α's	Landfear *et al.* (1983).
	β	7–15	—	Clustered β's	
Yeast	α	1	1	Single gene	Neff *et al.* (1983).
	β	1	1	Single gene	
Chlamydomonas	α	2	2	Dispersed	Silflow and Rosenbaum (1981)
	β	2	2	Dispersed	Brunke *et al.* (1982b).
Sea urchin	α	15	—	Dispersed	Alexandraki and
	β	15	—	Dispersed	Ruderman (1981).
Drosophila	α	4	4	Dispersed	Sanches *et al.* (1980),
	β	4	4	Dispersed	Kalfayan and Wensin (1981, 1982), Mischke and Pardue (1982), Raff *et al.* (1982).
Chicken	α	4–5	4	Dispersed	Cleveland *et al.* (1980),
	β	4–5	4	Dispersed	Lopata *et al.* (1983).
Rat	α	15–20	2	Dispersed	Lemischka *et al.* (1981),
	β	15–20	2	Dispersed	Ginzberg *et al.* (1981), Bond and Farmer (1983).
Human	α	15–20	2	Dispersed	Hall *et al.* (1983).
	β	15–20	3	Dispersed	

Tubulin pseudogenes and evolutionary significance

As has been mentioned earlier, the human tubulin gene family contains a number of pseudogenes thereby increasing the multiplicity of genes. In the case of the human β tubulin family, of the ten genomic sequences analyzed (Wilde *et al.,* 1982; Hall *et al.,* 1983; Lee *et al.,* 1983), seven are pseudogenes and the remaining three sequences represent functional genes. These pseudogenes contain multiple deletions and/or in frame translation termination codons within the exon sequences. Two of these seven are traditional pseudogenes that contain intervening sequences. The other five are of novel type to the extent that (i) each sequence lacks intervening sequences, (ii) downstream from the *AAT AAA* consensus signal for poly (*A*) addition each carries a long coded tract of *A* residues and (iii) the entire sequence is flanked by a short direct repeat of 10–15 base pairs. It seems, therefore, that this type of pseudogenes orginates by a reverse transcription event in which a mature mRNA is copied into DNA. That the pseudogenes are, however, discovered for a number of other genes beside tubulin gene suggests that this is a common feature in eukaryotic cell. Pseudogenes have also been described in the rat α (Lemischka and Sharp, 1982) and human α tubulin gene (Lee *et al.,* 1983). It is predicted that multigene families whose expression occur in the germline cells of higher vertibrates are likely to contain members of the processed type. In that

ease similarities between the functional and processed pseudo human β tubulin gene are moderate, but an exclusive homology between 3′-untranslated regions is discernible (Hall *et al.,* 1983). Thus extensive homology between 3′-untranslated regions implies a close evolutionary relationship. In pseudogenes, mutations are found to be random since these are not subjct to selective pressure. The functional gene, however, can acquire the changes that are silent (*i.e.* no amino acid change). Therefore, changes in the third codon position represent the cumulative effects of evolutionary drift in both functional and pseudogenes. Assuming that about half of the third base differences are due to changes in human β tubulin pseudogene and a rate of divergence of 0·7 % per 10^6 years for neutral mutations (Perler *et al.,* 1980), this pseudogene may be estimated to have originated approximately $4{\cdot}4 \times 10^6$ years ago (Hall *et al.,* 1983). It is apparent that these integrated transcripts (pseudogenes) retain many of their original features for millions of years and might be recruited, in whole or in part, for the generation of new functional sequences.

Conclusion

Microtubules are present in most eukaryotic cells where they fulfill diverge functions very vital to the existence of the cell. Until recently, tubulin research has been restricted exclusively to protein isolated from mammalian brain tissue. It is becoming increasingly apparent that, despite its conserved nature, tubulin exists as a family of proteins. As the range of organisms from which tubulin has been isolated broadens, the extent of diversification of the protein becomes clearer. Eukaryotic microorganisms are found to be particularly useful in revealing the heterogeneity of tubulins. It is interesting to study as to how the cells control differential assembly and dissociation of microtubules with the existence of different isotypes of tubulin subunits. The question also arises how to identify functionally different microtubules at specific stage of cell cycle and cell growth? In fact, different isotypes of tublulins have been identified in cytoplasmic pool, in flagella and in the pellicle of certain eukaryotic microorganisms. It will definitely be interesting if heterogeneity of tubulins from the spindle apparatus can be established.

That multiple tubulin genes are expressed differentially as a function of differentiation is now established. Whether each polypeptide produced is truly functionally distinct remains to be answered. Autoregulatory mechanisms of control of expression of α and β tubulins have been well documented by the experimental results that colchicine, which depolymerizes microtubules and raises the level of free tubulin causes inhibition of tubulin synthesis by lowering tubulin mRNA production. However, this autoregulatory control of tubulin mRNA seems not to be mediated through the regulation of transcription process *per se,* instead, tubulin mRNA processing and/or transport from the nucleus might be the cause for this regulation. The question arises as to how tubulin exerts its effects on the processing and/or transport of tubulin mRNA. Considering that nuclear matrix, nuclear envelope and cytoskeletal elements might be involved in maturation, transport and decay of mRNAs in general, it would be interesting to find out any interaction of these components on the autoregulatory control of tubulin mRNA synthesis.

For what reason do the cells synthesize new tubulin for the flagella during differentiation inspite of the presence of abundant amount of tubulin? What leads to the expression of flagellar tubulin genes and what causes the disappearance of flagellar tubulin mRNA as the differentiation is completed? How, utilization of flagellar tubulin is related to its synthesis? These are the questions yet to be resolved.

Several antitumour drugs such as colchicine and its analogues, podophyllotoxin and vinca alkaloids inhibit mitosis and other cellular functions by specifically binding to tubulin and inhibiting its assembly into microtubule. The number of other chemical compounds which bind specifically with the tubulin particularly from eukaryotic microorganisms and plants has ever been increasing. However, data on the physico-chemical characteristics of the binding of these compounds except colchicine with tubulin are still limiting to propose any unifying concept for their binding with tubulin. It might turn out to be interesting to use these chemical compounds to delineate the different classes of microtubules and/or different populations of tubulins within a single microtubule.

The tubulin genes except the yeast gene are organized in multigene families. These are either tandemly arranged, clustered or well dispersed. The multigene family encoding tubulin proteins is of particular interest because, whereas numerous lines of evidence point to conservation of tubulin proteins, different species possess a broad range of tubulin like sequences. Although the coding regions of tubulin genes are rigidly conserved, the untranslated regions including the introns are not. Interestingly, the analysis of tubulin multigene families in different species has shown that many of these sequences are pseudogenes. Information on the sequences of these pseudogenes, both α and *β*, suggest that most of these were derived *via* an mRNA intermediate. The accumulation of genetic lesions and the occurrence of oligo *A* tracts in intronless pseudogenes indicate that they are functionless and therefore not subject to selective pressure. The question then arises how these pseudogenes are retained? Since the pseudogenes retain many of the features of expressed genes it is possible that the generation of new functional sequences might arise out of them. In any case, the precise relationship between genetic complexity and microtubule function has not yet been elucidated.

Acknowledgements

Thanks are due to Department, of Science and Technology, New Delhi for financial support and to Drs. Kunal Roy, Ambica C. Banerjee, Asoke Banerjee, Sukla Roychoudhury and Mr. Sankar N. Maiti for the work originated from the authors' laboratory.

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