MICROTUBULE NUCLEOTIDE REQUIREMENTS: THE ROLE OF NUCLEOSIDE DIPHOSPHATE KINASE

A thesis submitted for the degree of
Doctor of Philosophy
in the University of London

by

Khalid Islam

Biophysics Section
Blackett Laboratory
Imperial College
University of London

November 1983

ABSTRACT

Nucleoside triphosphates are intimately involved in the structure and function of the microtubule subunit protein tubulin. Assembly-competent tubulin dimers specifically bind GTP at two sites: one of which is exchangeable (E-site) and is hydrolysed during microtubule assembly, while the other site is non-exchangeable (N-site). Nucleoside diphosphate (NDP) kinase has been proposed as the enzyme responsible for maintaining the assembly-competence of tubulin, by phosphorylating the E-site GDP *in situ* using other nucleoside triphosphates as the phosphoryl donors eg: ATP or CTP. The binding of the NDPkinase and its interactions with the microtubule proteins is therefore an important aspect in understanding the cellular regulation of microtubule assembly.

Microtubule protein isolated *in vitro* from day-old chick brain by cycles of polymerisation, contains significant amounts of NDPkinase activity. A rapid and simple procedure has been developed for the purification of this NDPkinase. The enzyme has been purified to near homogeneity, is monoisozymic, and exhibits similar physical and chemical characteristics as NDPkinases purified from various other sources.

The model of NDPkinase phosphorylating the tubulin bound E-site GDP *in situ* requires tubulin-NDPkinase

protein interaction. This has been examined in detail by gel-exclusion chromatography and by centrifugation. The model also predicts that the rate of synthesis of the E-site GTP by the NDPkinase is rate-limiting for microtubule assembly. The kinetics of microtubule assembly have been examined as a function of the GTP concentration, and the minimal GTP requirement for microtubule assembly has been determined and compared to the kinetics and minimal requirements for ATP- and CTP-induced microtubule assembly. The results indicate that the model that NDPkinase directly phosphorylates tubulin-bound GDP is incorrect.

ACKNOWLEDGMENTS

First and foremost, I would like to thank Dr. Roy Burns for his patience, helpful advice and constant encouragement which has made this work possible. I would also like to thank all the members of the Biophysics Section, in particular, Ian Coole and John Hancock for useful discussions and moral support. I am obliged to Mr. Nick Costa (Botany Department) for the use of the electron microscope and to Nick Jackson and Lou Wiggins (Physics Department) for the photography.

I am grateful to John Akins for his useful advice, frienship and moral support in the darker moments of this work and I would also like to thank Joanne (Natasha), Tahir and Aristos for lasting the distance.

Finally, special thanks to Giugi and Lucrezia for providing the badly needed extra incentive for completing this thesis.

The financial support for this work was provided by the Medical Research Council and the Cancer Research Campaign.

CONTENTS

	PAGE
CHAPTER I: GENERAL INTRODUCTION	11-50
CHAPTER II: MATERIALS AND METHODS	51-71
CHAPTER III: NUCLEOSIDE DIPHOSPHATE KINASE: PURIFICATION AND CHARACTERISATION	72-105
Introduction	73-77
Results	78-100
Discussion	101-105
CHAPTER IV: NUCLEOSIDE DIPHOSPHATE KINASE AND MICROTUBULES: PROTEIN-PROTEIN INTERACTIONS	106-139
Introduction	107-114
Results	115-134
Discussion	135-139
CHAPTER V: NUCLEOSIDE DIPHOSPHATE KINASE AND MICROTUBULES: NUCLEOTIDE REQUIREMENTS	140-170
Results	141-164
Discussion	163-170
CHAPTER VI: GENERAL DISCUSSION	171-185
REFERENCES:	186-203
LIST OF PUBLICATIONS:	204-206

LIST OF FIGURES

		PAGE
2.1	Optimal conditions for the determination of of maximal NDPkinase activity.	61
2.2	Schematic representation of coupled-enzyme reaction.	62
2.3	Chromatographic separation of nucleotides on a PEI-column.	65
2.4	Turbimetric determination of the kinetics of microtubule assembly.	69
3.1	Chromatography on Ultrogel ACA-44.	82
3.2	SDS-PAGE analysis of protein purification steps.	85
3.3	Characterisation of NDPkinase isozymes by chromatofocusing.	87
3.4	Molecular weight estimation by SDS-PAGE.	89
3.5	Bisubstrate "ping-pong" mechanism	92
3.6	SDS-PAGE analysis of NDPkinase from the soluble and particulate fractions.	95
3.7	Effect of pCMB on NDPkinase activity.	98
3.8	Effect of low ionic conditions on the elution of NDPkinase.	100
4.1	Recovery of NDPkinase activity as a function of the cycle of polymerisation.	116
4.2	Recovery of NDPkinase activity as a function of the cycle of polymerisation.	118

LIST OF FIGURES (contd.)

		PAGE
4.3	Recovery of NDPkinase and protein as a function of the initial protein concentration.	120
4.4	Recovery of NDPkinase activity as a function of the initial NDPkinase activity of 1x microtubule protein.	121
4.5	SDS-PAGE analysis of microtubule protein through cycles of polymerisation.	123
4.6	Recovery of NDPkinase and protein as a function of the initial 2x microtubule protein concentration centrifuged through a sucrose cushion.	125
4.7	Fractionation of 1x microtubule protein on a Biogel A 15m column.	129
4.8	Fractionation of charcoal-treated 2x microtubule protein on a Biogel A 1.5m column.	132
4.9	A Hummel and Dryer experiment.	134
5.1	SDS-PAGE analysis of sucrose cushion microtubule protein.	142
5.2	Electron microscopy.	146
5.3	Kinetics of microtubule assembly as a function of the GTP concentration.	147
5.4	Determination of Pseudo-first order rates at 1 mM and 20 μM GTP.	149
5.5	Eadie-Scatchard plot for GTP-binding	152

LIST OF FIGURES (contd.)

5.6	Kinetics of microtubule assembly as a function of the ATP concentration.	155
5.7	Kinetics of microtubule assembly as a function of the CTP concentration.	158
5.8	Microtubule elongation on seeds in the absence and presence of added GTP.	162
5.9	Microtubule assembly with CTP and GTP in the absence and presence of seeds	163

LIST OF TABLES

		PAGE
3.1	Distribution of chick brain NDPkinase.	79
3.2	Purification of NDPkinase from chick brain.	84
3.3	Efficiency of utilisation of triphosphates by purified monoisozymic NDPkinase.	91
3.4	Purification of chick brain NDPkinase by the Blue Dextran-Sepharose procedure.	94
4.1	Recovery of NDPkinase with microtubules under various nucleotide conditions through a sucrose cushion.	127
5.1	Efficiency of utilisation of triphosphates by NDPkinase co-purified with microtubules.	144

ABBREVIATIONS

MA buffer: microtubule assembly buffer (Chapter II)

Buffer A : see Chapter III

SDS-PAGE : Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis (Chapter II).

MES : (2⌈N-morpholino⌋ethanesulphonic acid

DTT : DL- Dithiothreitol

TEMED : N,N,N',N'- Tetramethyl-ethylenediamine

PEP : Phospho-enol-pyruvate

pCMB : p-Hydroxymercuribenzoate

GMPPCP : Guanylyl-β,γ-methylene diphosphonate

AMPPCP : Adenylyl-β-γ-methylene diphosphonate

GMPNP : Guanylyl-$^{\beta}$-γ-imidodiphosphate

dGDP : 2'-deoxyguanosine-5'-diphosphate

ddGDP : 2',3'-dideoxyguanosine-5'-diphosphate

$p(CH)_2pG$: Guanosine-5'-methylene diphosphonate

CHAPTER I

GENERAL INTRODUCTION

Microtubules were first recognised as ubiquitous cellular organelles when glutaraldehyde was introduced as a fixative, and have been observed in a wide variety of eucaryotic cells (1-3). The possibility that the microtubules were a fixation-generated artifact was quickly excluded when unfixed microtubules from lysed cells were observed by negative staining and corresponded to their counterparts in fixed tissue (4). Similarly, bundles of fixed microtubules observed by electron microscopy corresponded to birefringent fibres in living cells observed by light microscopy (5).

Most microtubules are labile and are depolymerised by the action of antimitotic drugs such as colchicine, low temperatures, high pressures and high concentrations of calcium ions. They include the microtubules of the mitotic spindle, axons and dendrites and most cytoplasmic microtubules. By contrast, the microtubules of the cilia and flagella are stable, suggesting that their functions are not linked to their assembly and disassembly properties (6-12).

This implies that the control of microtubule stability may be of functional importance and the instability of certain microtubules suggests that a dynamic equilibrium may exist between the labile microtubules and their subunits <u>in vivo</u>. Indeed, the microtubules of the mitotic spindle have been demonstrated

to exist in an equilibrium with the cytoplasmic pool of subunits *in vivo* (5). In particular microtubule disassembly is essential for the chromosome to pole movement during anaphase and the rate of disassembly regulates chromosome movement (13-14). The assembly-disassembly properties of other microtubules may be similarly linked to their function.

Control of microtubule organisation in the cytoplasm

The distribution of the cytoplasmic microtubules is an essential element in maintaining cellular asymmetry (15). In general, the distribution is both temporally and spatially controlled and the microtubules radiate out from specific structures termed microtubule organising centres or MTOC's (16). The MTOC's in the cytoplasm are frequently associated with the "centrosome" or the centriole and the electron dense pericentriolar material. For example, microtubules in the pigment granules of melanophores clearly radiate from this material (17).

Detergent-extracted cells prepared in stabilising buffer to preserve the microtubules, drug-depolymerised and then allowed to recover also show that microtubules grow from specific sites in the cell (18). The number of MTOC's per cell is controlled and this number increases at

the time of centriole replication (19-20). In addition to controlling the microtubule distribution, the MTOC's may also regulate microtubule number(19).

Cytoskeleton and Microtrabeculae

Studies on the cytoplasm suggest that far from being a "structureless soup", it is a highly ordered structure. Detergent-extracted cells show a filamentous framework which is termed the cytoskeleton (21), containing many of the components involved in cell motility such as actin filaments, intermediate filaments and microtubules (22). The similarities in the filament distribution of whole cells and the cytoskeleton has been demonstrated by immunofluorescence using antibodies to actin, myosin, tropomyosin and tubulin (23).

The spatial organisations of these filamentous systems do not appear to be independent of each other. The apparent interaction of the microtubules with the other components, coupled with the lability of many of the cytoplasmic microtubules has suggested that microtubules may play an important role in the determination of the organisation of the cytoskeleton. Most of the evidence for such an involvement has been derived from morphological and pharmacological studies, although in vitro biochemical

interactions between microtubules and neurofilaments (24), pancreatic secretory granules (25), and actin filaments (26-27) have recently been demonstrated.

Illustrative of the role of microtubules is the observation that the intermediate filament network in fibroblasts collapses following prolonged treatment with colchicine, while the removal of colchicine allows microtubules to polymerise and the intermediate filaments return to their normal distribution (28). Similarly, microtubules in the mitotic spindle influence the contractile ring of actin filaments during cell division, which is always formed perpendicular to the mitotic spindle. Indeed, mechanical displacement of the mitotic spindle causes a corresponding change in the position of the contractile ring (29).

Such interactions between microtubules and cytoskeletal components are probably effected by high molecular weight crosslinking proteins. For example MAP_2 protein, which forms the tangential side-arms of microtubules reassembled in vitro, may function to cross link microtubules and intermediate filaments. Double antibody staining reveals the co-localisation of MAP_2 with tubulin and vimentin, an intermediate filament protein, in cultured brain cells. Affinity purified anti-MAP_2 and monoclonal anti-MAP_2 also shows the presence of MAP_2 on the intermediate filaments in detergent extracted brain

cells following drug-depolymerisation of microtubules (30). Such crosslinking proteins may be an important contributor to the microtrabeculae described by Wolosewick and Porter (31).

Wolosewick and Porter (31) have examined by high voltage E-M whole cells grown on E-M grids, fixed and critical point dried. They observed that the cytoplasmic ground substance consisted of a lattice of slender strands with 3-6 nm diameter, called "microtrabeculae". The three-dimensional microtrabecular lattice was confluent with the cortices of the cytoplasm and appears to interconnect most of the well defined components of the cell such as microtubules, cisternae of the endoplasmic reticulum, polysomes and the stress fibres.

The possibility that the microtrabeculae are an artifact of the fixation/dehydration conditions appears unlikely and has been rigorously examined (31). Heuser and Kirschner (32) have used the elegant technique of rapid freezing in liquid helium, freeze-etch and rotary replication with platinum to avoid the problems of chemical fixation or dehydration. Actin, microtubules and intermediate filaments are readily distinguished by this technique and the images confirm that the three dimensional organisation of the cytoskeleton is comparable to the proposed microtrabecular lattice.

The dynamic characteristics of many of the cytoskeletal microtubules raises important questions about how the assembly-disassembly properties are regulated *in vivo*. Microtubules in cells depend upon their assembly-disassembly properties and on interactions with other components. This thesis will be primarily concerned with the assembly-disassembly of microtubules.

The structural subunit of microtubules is the protein tubulin, which was first isolated using colchicine binding assays (33) and characterised as a protein dimer of 120,000 molecular weight (34). A wide variety of isolation procedures have since been developed reflecting the source and specific properties under study. A number of accessory non-tubulin proteins, termed microtubule associated proteins or MAPs, are co-purified by certain of these procedures. Most biochemical studies use mammalian or avian brain as these are excellent sources with microtubule proteins forming as much as 40% of the soluble protein.

Microtubule substructure

X-ray diffraction studies of orientated hydrated samples of flagella and brain microtubules, show that the inner and outer diameters of these microtubules are 14 nm

and 30 nm respectively (35-36). Sections of positively stained microtubules reveal a central non-staining region of 14 nm in diameter(3). A 5-20 nm wide non-staining zone around the microtubule wall is also observed by freeze-fracture (37). Fixed and stained microtubule walls in cross-section have the appearance of a ring of beads, each bead representing an end-on-view of a longitudinal filament, known as a protofilament. "Normal" microtubules contain 13 protofilaments (38-39) including neurotubules reassembled in vitro (40). Microtubules containing 7,9,12 or 15 protofilaments can also occur but have an abnormal appearance by staining techniques (41-43).

The substructure of the protofilaments by negative staining techniques shows that they consist of longitudinal rows of globular subunits 4 nm apart, tentatively identified as the tubulin monomer, and have a basic surface lattice of 4 x 5 nm units (44). Two and three dimensional reconstruction-images confirm the 4 x 5 nm surface lattice structure for microtubules from cilia, flagella, and neurotubules (45-47). In addition an 8 nm periodicity is observed which is due to an end to end alignment of slightly dumb-bell shaped pairs of 4 nm globular units within the component protofilament, corresponding to the tubulin heterodimer (46).

Studies using tubulin sheets induced by the addition

of high concentrations of zinc ions have been used to construct two and three dimensional images (48-49), and show that alternate protofilaments are arranged in an antiparallel manner, in contrast to the assembled microtubule. Both the E-M and the X-ray data tend to support the idea that the microtubule wall is fairly deeply indented.

Microtubules reassembled in vitro from brain usually contain stoichiometric amounts of high molecular weight MAPs (see later). These accessory proteins have been visualised in sectioned pellets as fine periodic projections from the outside wall of the microtubule (50-51), while microtubules reassembled in vitro in the absence of HMW-MAPs have smooth walls (51-52). Limited proteolytic digestion of MAP_2-polymerised microtubules results in the loss of these projections (52).

The presence of MAPs alters the diffraction patterns of fixed microtubules and an additional axial spacing of 1/32 nm is observed (53). A model has been proposed (53), in which HMW-MAPs form a symmetrical superlattice, binding with a twelve tubulin dimers repeat distance along each protofilament. This model is supported by the observed biochemical stoichiometry of one MAP_2 per twelve tubulin dimers (54).

Purification of microtubule proteins:

Direct isolation of tubulin from crude brain extracts can be achieved by ammonium sulphate precipitation followed by chromatography on DEAE-anion exchanger (34). The tubulin purified by this procedure is essentially homogenous on SDS-PAGE, and although it exhibits a full range of drug binding properties, it is assembly incompetent under physiological conditions. It can though be induced to polymerise into microtubules by high, non-physiological, concentrations of magnesium ions (55-56). A further disadvantage of this isolation method is the contamination by small amounts of RNA, which can complicate studies on the properties of microtubules, although this can be removed by a modification of the procedure (57).

As many of the cellular functions of microtubules appear to depend on their ordered assembly-disassembly, a procedure for studying these events in vitro under physiological conditions is desirable. Weisenberg (58) described conditions for assembling microtubules in vitro from crude brain supernatants. He showed that microtubule assembly in vitro required the presence of GTP or ATP, low concentration of magnesium ions and the removal of calcium ions by addition of a calcium chelator. Microtubules reassembled under these conditions were morphologically similar to microtubules in vivo and exhibited similar

biochemical properties. For example, the reassembled microtubules could be depolymerised by low temperatures, by the action of colchicine and by the addition of high concentrations of calcium ions.

Two procedures have taken advantage of these observations to isolate microtubules by temperature dependent cycles of polymerisation and depolymerisation (59-60). The microtubules isolated by these procedures contain various "MAPs", which co-purify in stoichiometric amounts through successive cycles of polymerisation and depolymerisation.

The major difference between the two procedures is the presence of glycerol in the Shelanski procedure (59) and its absence in the Borisy procedure (60). There are significant differences in the relative recovery of MAPs between the two procedures (61) and the Shelanski procedure may induce artifacts (62).

The various modifications of these procedures and the use of differing sources of brain introduce an element of complexity in evaluating data on the properties of the microtubules. It must be borne in mind that contradictory results in the literature may be related to differences in the isolation procedures (63).

Tubulin:

Pure tubulin isolated by ion-exchange chromatography can participate in appropriate tubulin-tubulin interactions to form microtubules, indicating that the necessary fundamental information is carried within its primary structure. Isolated tubulin has an apparent hydrodynamic molecular weight of 110,000 and is a heterodimer composed of two non-covalently bound protein chains termed the α and β subunits (64). The nucleotide sequence of the α and β tubulins has been determined and show a high degree of homology between various species, confirming that the tubulin molecule is highly conserved throughout nature. Similarly, the α and β tubulins of chick brain show a 41% homology and sequence studies show that both have molecular weights of 50,000 daltons (65-66).

The tubulin dimer is rich in glutamate and aspartate residues and is an acidic protein with isoelectric points of 5.4 and 5.3 for the α and β tubulins respectively (67). The α and β monomers show anomalous behavior on SDS-PAGE. Both the tubulins co-migrate as proteins of molecular weight of 55,000 with the β tubulin generally exhibiting a greater electrophoretic mobility. This difference in charge and the anomalous behavior on SDS-PAGE has been successfully exploited for preparative purification of the α and β tubulins (68).

Amino acid analysis reveals ten cysteine residues for the α and eight for the β tubulin isolated from chick brain, and up to eleven cysteine residues for both the α and β calf brain tubulins (66; 69). The isolation procedure can critically affect the number of the accessible sulphydryl groups. Microtubule protein isolated by the Borisy procedure has only four accessible sulphydryl groups and oxidation of one of these sulphydryls can block assembly (70). In contrast, microtubule protein prepared by the Shelanski procedure has up to seven accessible sulphydryl groups and at least two sulphydryls must be oxidised to inhibit assembly (70-71). It has been proposed that the alteration of the redox potential of the tubulin sulphydryls in vivo, for example by glutathione, may be an important mechanism for modulating microtubule assembly (72).

Tubulin ligands:

The tubulin dimer has at least two non-covalent nucleotide binding sites. One of these sites, the N-site, is non-exchangeable and the slow turnover rate suggests that it may function as a stable structural co-factor (73). The other site, the E-site, is freely exchangeable with the medium (74). Tubulin under most conditions is considered to require GTP bound at both these sites;

the E-site is hydrolysed during microtubule assembly. There is though additional evidence for at least four possible nucleotide binding sites per tubulin dimer which will be considered later (Chapter VI).

Magnesium and calcium, both physiologically important ions, appear to have antagonistic effects with respect to microtubule assembly. Magnesium is necessary for tubulin polymerisation (58; 75), and pure tubulin can polymerise to form microtubules at high magnesium concentrations (56). At least one magnesium ion per tubulin dimer is bound to pure tubulin and during microtubule assembly one more magnesium ion is incorporated (76). Magnesium is obligatory for GTP binding and may complex with the GTP or bind directly to the tubulin (77). In contrast to magnesium, calcium ions at equivalent concentrations induce rapid microtubule disassembly, although low concentrations of calcium ions can stimulate tubulin polymerisation (60; 78-79). The calcium regulatory protein calmodulin has been proposed to sensitise microtubules to changes in the cellular calcium concentration, and consequently may regulate assembly-disassembly properties of microtubules in vivo (80).

As mentioned earlier, glycerol alters some properties of the tubulin dimer. At least five glycerol molecules can bind per tubulin dimer (62) and glycerol stabilises the binding of the guanine nucleotides to tubulin, and can

alter the nucleotide requirements.

A large variety of drugs are known to bind to the tubulin dimer, including the antimitotic drugs colchicine, podophyllotoxin, vinblastine, vincristine, maytansine, nocodazole and griseofulvin. The drug colchicine has been instrumental in the purification and characterisation of the tubulin molecule, and has been employed for quantification of tubulin in cell extracts (81). The tubulin dimer has one high affinity colchicine site and binding induces conformational changes within the dimer (82-83). Colchicine shares a part of its binding site with another alkaloid drug podophyllotoxin, which also demonstrates a similar widespread ability to bind to tubulin (84). However, unlike colchicine, podophyllotoxin binding is rapidly reversible. Both drugs are potent inhibitors of microtubule assembly.

The vinca alkaloid, vinblastine has been characterised as a poison and has at least two high affinity binding sites per tubulin dimer (85). Vinblastine induces the formation of paracrystals in cells, and has been used as a tubulin purification procedure (86). Maytansine is a competitive inhibitor of vinblastine although it induces different conformational changes (87).

Tubulin microheterogeneity:

The demonstration that microtubules are involved in a wide variety of structural and dynamic roles in the cell has prompted the "multi-tubulin" hypothesis. This hypothesis postulates the existence of different subspecies of tubulin within the same organism or in the same cell, each with a specific function (81). The spatial and temporal control of microtubule assembly and disassembly, for example the differential stabilities of the pole-pole and pole to kinetochore microtubules of the mitotic spindle, and distinct tubulins in flagellar and cytoskeletal microtubules, would lend support to this hypothesis(88-89). The demonstration that the flagellar tubulin synthesised de novo in Naegleria grubei (81) is antigenically distinct from the bulk of the cellular tubulin suggests some control at the transcription level.

The question can therefore be addressed (a) does tubulin undergo covalent post-translational modifications, and/or (b) are there multiple forms of α and β tubulins coded by different genes, which determine the assembly, stability and functions of the microtubules? The dynamics of microtubule assembly and disassembly can also be modulated by the interaction of a single species of tubulin with specific factors or the accessory non-tubulin proteins MAPs. The resolution of this question is important for understanding the possible regulatory

controls of microtubule assembly and function *in vivo*.

Tubulin, like many other proteins, appears to undergo post-translational covalent modifications. Two general approaches have been used (a) a direct chemical analysis of purified tubulin, and (b) analysis of multiple forms by SDS-PAGE or isoelectric focussing.

(a) Raybin and Flavin (90) demonstrated the specific tyrosylation of the C-terminus of the α tubulin from rat brain, which curiously was not affected by inhibitors of protein synthesis. However, the tubulin mRNA encodes for tyrosine on the C-terminal, suggesting the existence of detyrosylation-tyrosylation cycle (66). Tubulin can be phosphorylated *in vivo* to the extent of one mole per mole β-subunit (91). In addition the tubulin dimer can be glycosylated to the extent of one mole sugar per mole dimer (92) and there is also some indirect evidence for the presence of phospholipids in tubulin (93).

(b) Multiple forms of rat brain α and β tubulins can be observed with tubulin purified by cycles of polymerisation or by ion-exchange chromatography. The rat brain α tubulin can be separated into two groups α_1 and α_2 by two-dimensional gel electrophoresis and the β tubulin can be separated into a minor β_1 and a major β_2 form. However, isoelectric focussing resolves both the α_1 and the α_2 into three components each (94). Gozes and Littauer

(95) have observed an age-dependent increase in rat brain tubulin microheterogeneity by isoelectric focussing and demonstrated that it was independent of the purification procedure used. These authors further report the presence of at least five mRNA species coding for distinct tubulin forms, suggesting that the age-dependent changes may be controlled at the mRNA level (96).

The α and β tubulins isolated from chick embryo brain under denaturating conditions also display tubulin microheterogeneity when compared with respect to their molecular weights, amino-acid composition, tryptic peptide maps, amide content and isoelectric points. This observed microheterogeneity cannot be explained by the covalent post-translational modifications of the type discussed above, but may arise from the deamidation of the glutaniyl and aspariginyl residues (97). Deamidation of these residues may be a molecular clock mechanism for protein turnover (98) and can give rise to electrophoretic heterogeneity of a large number of proteins (99).

However, none of the post-translational covalent modifications have been demonstrated to affect the kinetics of microtubule assembly and disassembly. Similarly the role of the observed "structural" microheterogeneity remains unclear.

In view of the wide variety of cellular functions

performed by the microtubules and the observed microheterogeneity of the tubulin subunits, multiple tubulin assembly products might be predicted. Such polymorphism has been widely observed. The observed polymorphism may represent specific microheterogeneic forms of tubulin which may assemble to give rise to functionally distinct microtubules. The regulation, composition and role of the tubulin polymorphs may be important in the modulation of the assembly-disassembly characteristics of the microtubules and their spatial and temporal control.

Tubulin Polymorphism.

DEAE-purified tubulin spontaneously forms oligomers on addition of high concentrations of magnesium ions, and oligomer formation is augmented by the addition of colchicine or vinblastine (100-101). Frigon and Timasheff (55-56) characterised these oligomers as double-walled rings with inside and outside diameters of 27 nm and 47 nm respectively. They proposed a hydrodynamic model in which 26 6S tubulin dimers are situated around a ring with their centres at a radius of 18 nm and the long axis on the radii of the ring. The calculated sedimentation coefficient of 43S for such a model closely matches the experimentally determined sedimentation coefficient of

42S. This ring formation can be described by a concerted monomer-polymer system of one component as postulated by Gilbert and Gilbert (102).

DEAE-purified tubulin also shows the presence of sheets of tubulin under conditions which are unfavourable for microtubule assembly (103). Spirals and coils are also observed under these conditions (47). Tubulin sheets can also be induced to form when zinc ions are added (48-49).

There is however no evidence that these various structural polymorphic forms correspond to specific microhetrogeneic forms of tubulin. There is though abundant evidence that MAPs significantly alter the structural polymorphism.

Microtubule Associated Proteins:

The development of *in vitro* assembly conditions demonstrated the existence of accessory proteins, MAPs, which co-purified through successive cycles of assembly and disassembly. These MAPs can be divided into two classes: the high molecular weight (HMW) class > 200,000 and a lower molecular weight class ≈ 55,000-70,000 termed tau. The relative recoveries of these two classes of MAPs is highly dependent on the isolation

procedure employed (67; 104).

Microtubule protein can be resolved by phosphocellulose chromatography into an unadsorbed fraction, which is composed of pure tubulin dimers, and an adsorbed fraction consisting of MAPs, which depending on the microtubule isolation procedure can be mainly tau (105). This phosphocellulose purified tubulin is assembly-incompetent, but the addition of the tau fraction restores the original assembly properties. Furthermore, both the rate and the final extent of assembly of tubulin are elevated by increasing tau concentration (106). The tau proteins can be further separated into tau-I and tau-II and are highly asymmetrical proteins (10).

The HMW-MAPs can be separated from the tau and tubulin proteins by gel exclusion chromatography of depolymerised microtubules in high salt and also stimulate assembly-incompetent tubulin to assemble into microtubules (107). These HMW-MAPs have been shown to be antigenically distinct from the tau proteins (108).

There are at least six HMW-MAPs which co-polymerise with microtubules isolated by cycles of polymerisation using a wide variety of buffer conditions (Burns; personal communication), which differ in quantity depending on the buffer conditions. Several of these proteins promote microtubule assembly and the most highly characterised of

these HMW-MAPs is MAP_2, which has a molecular weight of 280,000 (109). The MAP_2 can be cleaved into two fragments by limited chymotryptic digestion of which the smaller fragment contains the assembly-promoting activity (52). Microtubules reassembled *in vitro* show a stoichiometry of one MAP_2 per twelve tubulin dimers (54).

Both tau and HMW-MAPs are cationic proteins and this property may contribute to their assembly-promoting activity. Indeed, polycationic macromolecules such as ribonuclease-A and DEAE-dextran can also facilitate microtubule assembly (110). Non-specifically binding molecules can also bind to tubulin and co-purify in constant proportions through cycles of polymerisation, if they also promote the association of tubulin (111). However, many cations and polycations induce precipitation under assembly conditions, whereas the MAPs do not induce precipitation or non-specific aggregation even at high concentrations (10).

Evidence that MAPs isolated *in vitro* are genuine structural components of cytoplasmic microtubules has come from observations that they are spatially associated with microtubules *in vivo*. Connolly et al. (112) have purified monoclonal antibodies to tau and demonstrated by indirect immunoflourescence that these antibodies bind to cytoplasmic and mitotic microtubules of mouse fibroblasts. Scherline and Schiavone (113) have made similar

observations for antibodies to HMW-MAPs demonstrating their presence in the mitotic apparatus, and Connolly et al.(108) have shown that the anti-HMW-MAPs specifically stain the cytoplasmic microtubule network.

The possibility therefore exists that the different types of tubulin and/or MAPs are required depending on the cellular process. Tubulin microheterogeneity has been discussed, but its role in the mechanism of microtubule assembly remains unknown. In contrast the MAPs have been demonstrated to modulate microtubule assembly. Perhaps, it is interesting to note that the MAPs demonstrate age-dependent changes in their composition and activity, which can directly affect the assembly-competence of tubulin at various stages of development in a number of species (114).

Tubulin polymorphism has been discussed previously and may be involved in the modulation of microtubule assembly-disassembly characteristics. However, the observed structural polymorphism does not correspond to specific microheterogeneic forms of tubulin. This polymorphism may therefore be in part due to the interaction of tubulin and the MAPs, as well as to an ability of pure tubulin to assemble in a number of distinct ways.

Modulation of Tubulin Oligomers by MAPs.

A wide variety of polymorphic species are observed in the presence of MAPs. These include microtubules, sheets, spirals, filaments, single and double rings. The ring-like structures are of particular interest as cold-dissociated microtubule protein consists primarily of rings and 6 S dimers. The dimensions, morphology and sedimentation characteristics of the rings depend on the conditions and/or methods used (61; 115-116). Double walled rings with dimensions similar to DEAE-purified tubulin rings are observed but the sedimentation co-efficient is normally around 30-36 S (61; 115).

Fractionation of microtubule protein by gel-exclusion chromatography allows the separation of the 30-36 S tubulin oligomers in the void volume from the 6 S tubulin which elutes close to the salt volume (47; 117). SDS-PAGE analysis demonstrates that the tubulin oligomers are composed of MAPs and tubulin (117).

Hydrodynamic models have been proposed in which these rings contain between 24-29 6S tubulin dimers with an average contour length of 320 nm (118-119). In addition single walled rings sedimenting at 20S are observed under certain conditions, and are thought to contain around 13 6S tubulin dimers (61; 116; 120). Spirals and coils with an average contour length similar to the 36S double rings

are also observed and may represent a segment of a protofilament (118; 121). Under certain conditions sheets of tubulin can be seen and are thought to represent open microtubules (121-122).

However, unlike the DEAE-tubulin rings the 6S-36S equilibrium does not behave as a Gilbert system. The 36S ring formation is independent of the total 6S tubulin dimer concentration (120; 123) and following gel filtration neither fraction can independently re-establish the original equilibrium (124). Furthermore, while the 36S rings can be dissociated into 6S tubulin dimers by high salt concentration, the pure 6S tubulin dimers are incapable of reforming the 36S rings. The 20S single rings, however, appear to behave as a Gilbert system (120).

Microtubule assembly is abolished or greatly inhibited upon removal of the 36S component by sedimentation or gel filtration (47; 115; 117; 125). Similarly an increase in the 20S single rings at the expense of the 36S component leads to a diminution in the rate and extent of microtubule assembly (116). The differences in the sedimentation characteristics and in the morphology of these different structures has been shown to depend to some extent on the method and conditions used. However, these differences do not represent differences in the types of tubulin isolated but in the type and amount of MAPs co-purified (52; 105).

Indeed, when HMW-MAPs are removed from the 30S oligomer by proteolytic digestion, the sedimentation coefficient increases to 39S (126). While the oligomer formation is a property of the tubulin molecule the MAPs appear to modulate the sedimentation characteristics as well as the monomer-polymer equilibrium. As the oligomer formation requires longitudinal tubulin-tubulin interactions the action of the MAPs must be to modulate these longitudinal associations.

Tubulin clearly contains all the neccessary information to self-assemble. However, microtubule protein is composed of MAPs and tubulin and the MAPs have been demonstrated to alter the tubulin assembly properties. Furthermore, the MAPs can bind to and alter the structural polymorphism exhibited by the tubulin. In addition ligands, for example the nucleotides or magnesium and calcium, which can bind to tubulin may also alter its assembly characteristics. The kinetics of microtubule assembly must therefore be analysed before the role of ligands, MAPs and tubulin polymorphism in the temporal and spatial control of microtubules can be established.

The Kinetics of Microtubule assembly in vitro

Since microtubules are rod-like structures, which scatter incident light, kinetics of microtubule polymerisation can be studied spectrophotometrically by monitoring turbidity changes (127-128). When microtubule protein, in an appropriate buffer, is rapidly warmed to polymerising temperatures a pseudo first-order increase in turbidity is normally preceded by a lag period, which is presumed to be the result of a rate-limiting nucleation step. The finite duration of the lag-phase indicates the existence of several protein associations in a sequential pathway leading to microtubule formation and the requirement of some intermediate other than the 6S tubulin dimer.

The addition of microtubule seeds, such as sheared microtubule fragments, eliminates the lag-phase and a pseudo first-order rate is observed for the elongation phase. The elongation phase can therefore be studied in the absence of the nucleation phase at a fixed number concentration of nuclei by using microtubule seeds and preparations of 6S tubulin (128).

Following the condensation-polymerisation theory of Oosawa and Kasai (129), the subunits add to or leave from the ends of polymers which undergo net growth until the concentration of the subunits $[S]$ is depleted to an

equilibrium value, the critical concentration, $[S]_c$. If the number concentration of the microtubules $[M]$ does not increase the reaction can be represented as follows:

$$M + S \underset{K_{-1}}{\overset{k_2}{\rightleftharpoons}} M$$

and the rate of change of subunits can be described as:

$$-d[S]/dt = k_2 [M] [S] - k_{-1} [M]$$

therefore at equilibrium $k_2[M] [S]_c = k_{-1} [M]$ and at eqilibrium the critical concentration $[S]_c$ is equal to k_{-1}/k_2.

Johnson and Borisy (128) analysed the initial rate of microtubule assembly <u>in vitro</u> using microtubule seeds and microtubule protein, whose capacity for self-nucleation was attenuated by high-speed centrifugation. They demonstrated that the net assembly rate is the summation of the rates of polymerisation and depolymerisation, and the rate of polymerisation was proportional to the product of the microtubule number concentration and the tubulin concentration. In addition, the depolymerisation rate was also proportional to the microtubule number concentration. They concluded that microtubule assembly occurs by a condensation-polymerisation mechanism which involves a nucleation and an elongation step. The calculated rate

constants are 4 x 10^6 M^{-1} s^{-1} and 7 s^{-1} for polymerisation and depolymerisation and a critical concentration for assembly of 0.2 mg ml^{-1}. This condensation-polymerisation mechanism is independent of the microtubule isolation method (128; 130).

Effect of MAPs on microtubule assembly kinetics

Evidence has already been presented showing that MAPs are genuine cytoskeletal components which are associated with microtubules. Further evidence that MAPs are components of microtubules derives from their specific effects on microtubule assembly. For instance, assembly-incompetent tubulin can be induced to assemble in the presence of low concentrations of MAPs (131). DEAE-purified tubulin shows a critical concentration for assembly of 1-1.3 mg ml^{-1} (56; 78), which is much higher than the critical concentration of 0.1-0.3 mg ml^{-1} observed for unfractionated microtubule protein (128). The addition of MAPs decreases the lag phase and increases the polymerisation rate with increasing concentration of MAPs (131-132).

The addition of increasing amounts of MAPs increases not only the rate but also the final extent of assembly, suggesting that there is also an effect on the elongation

phase (131). While DEAE-purified tubulin above the critical concentration can elongate microtubule seeds, suggesting that the MAPs are not absolutely required, the presence of MAPs increases the net rate of assembly. Moreover, analysis of the critical concentration of tubulin at two concentrations of MAPs shows that the increase in the net rate and final extent of assembly is solely due to a reduction in the depolymerisation rate (131). Recent studies on the phosphorylation of MAP_2 also indicates that it affects the depolymerisation rate constant (133). The assembly-promoting activity of MAP_2 has been demonstrated to reside in the 25,000 dalton tubulin binding domain of the protein (52).

Microtubule Polarity

Cytoplasmic microtubules are dynamic structures which must assemble and disassemble in a directional manner in order to affect cell shape. This structural and functional polarity in microtubule growth has important implications on models of mitosis (3; 5). X-ray diffraction studies confirm that microtubules possess a structural polarity (46), and *in vitro* growth of brain microtubule protein on microtubule seeds also demonstrates that microtubules possess an intrinsic growth polarity (134-136). Growth occurs at one end of seeds at low subunit concentration;

at high subunit concentrations assembly occurs at both ends (134). These results are consistent with the observations of Johnson and Borisy (128) that polymerisation and depolymerisation occurs at the ends of and depends on the number concentration of microtubules.

Using the technique of hook decoration (137) the polarity of the microtubules in the mitotic spindle of PtK_1 cells has been determined. The microtubules belonging to a half spindle, both kinetochore and interpolar, are orientated with the same polarity (138). Similar results have been described for the spindle of the surf clam Spisula solidissima, using axonemal dyenin to reveal the polarity (139).

Treadmilling and Head to Tail polymerisation

Antimitotic drugs block microtubule assembly in vitro at sub-stoichiometric concentrations to the free tubulin concentration. The sub-stochiometric poisoning, in the case of colchicine, occurs by the addition of the drug-tubulin complex to the microtubule ends during assembly (140). Margolis and Wilson (141) using $[^3H]$ GTP bound to tubulin found that the $[^3H]$ GDP remains blocked on the microtubule following tubulin incorporation and that the gain or loss of subunits from the microtubule was

linear with time under steady-state conditions. Pulse chase experiments also demonstrated the loss of increasing amount of label with increasing time (142). Furthermore, while podophyllotoxin blocked the steady-state assembly, it did not alter the subunit loss. On the basis of these and pulse-chase experiments they suggested that microtubule assembly and disassembly at steady-state is the summation of two different reactions which occur at the opposite ends of the microtubules *in vitro*.

A unidirectional flux of tubulin subunits from the assembly (plus) end to the disassembly (minus) end at apparent equilibrium, or treadmilling, can therefore occur. The linear loss or gain of the subunits and sub-stoichiometric poisoning of the microtubule assembly demonstrates that treadmilling occurs *in vitro* and determination of the microtubule mean length allows a rate of flux to be calculated (143). Treadmilling is a mechanism intrinsic to the tubulin molecule as pure tubulin also has the same properties (144).

Sub-stoichiometric poisoning can therefore be the result of "capping" such that further subunit addition is retarded. Bergen and Borisy (145) have studied the growth of porcine brain microtubule protein onto *Chlamydomonas* flagella, at different concentrations of subunit protein. Both the microtubule ends are dynamically active, with the plus end distal to the flagella showing a rapid net growth

rate, suggesting a head to tail polymerisation. However, the plus end shows both the higher association and disassociation rate constant and 14 associations and disassociation steps for one subunit addition at the plus end or loss at the minus end are required. However, more recent studies show that under steady-state conditions, only 4 association and disassociation steps are required and flux rates of up to 50 µm/h have been determined (146-147).

Actin filaments have also been demonstrated to treadmill *in vitro*. Wegner (148) has proposed that the hydrolysis of ATP, which occurs on polymerisation of actin, may produce a disparity in the critical concentration for assembly at the two ends of the polymer. However, more recent evidence would suggest that actin filaments do not treadmill *in vitro* under physiological conditions (149).

Models for ordered assembly-disassembly in Cells

If treadmilling occurs *in vivo* then it may have important implications for microtubule functions. Margolis and Wilson (143) have suggested that treadmilling may be used in the translocation of organelles and other substances, organisation of the locomotory apparatus and

may act as a temporal and spatial marker for morphological changes that co-ordinate with the microtubule system.

Observations of microtubule assembly *in vitro* onto microtubule seeds, suggest that the minus end is anchored to the microtubule seed and therefore "capped", while the plus end is distal to the seed and is free. Few free microtubules are observed *in vivo* in the cytoplasm and most microtubules originate from the MTOC, which is equivalent to the microtubule seed *in vitro*. Treadmilling, however, requires both the ends to be free to exchange subunits.

Kirschner (150) has proposed a model which suggests that treadmilling may be a mechanism which is used for other purposes in the cell. Treadmilling may suppress the growth of unanchored microtubules if there is a disparity in the critical concentration for assembly for the two ends of the microtubule, as demonstrated *in vitro*. For example, if the minus end is capped at the MTOC then the addition and loss of subunits can only occur at the plus end according to the critical concentration. Therefore, a microtubule with the minus end capped will be favoured to an unanchored microtubule if the critical concentration was approximately that required for the plus end but below that required for the minus end. This model would therefore account for the ordered assembly- disassembly *in vivo*, with the desired orientation, and would eliminate

any adventitious microtubule assembly. The model makes two important assumptions: (a) that the anchored microtubule is not free to exchange subunits and the MTOC "caps" the microtubule end and (b) all microtubules in the cell would be "capped" at their minus ends and all the plus ends are free to exchange subunits.

Two antimitotic drugs taxol (151) and nocodazole (152) have been shown to increase or decrease the critical concentration for assembly respectively. Cells treated with different concentrations of nocodazole show that the critical concentration for assembly is lower in the vicinity of the MTOC. Taxol treated cells however, result in the abrogation of MTOC-associated microtubules, while producing a large number of free microtubules (153).

However, polarity determinations of microtubules in the mitotic spindle show that the plus end of the kinetochore microtubules is capped (138-139). The kinetochore microtubules are also more stable to depolymerisation conditions, but have their minus end free.

De Brabander has proposed a less restrictive model for microtubule assembly in cells (154). The capping and assembly-initiating properties of the MTOC's may be separable such that anchoring may occur by lateral associations and therefore both the microtubule ends would

be free. In the case of microtubules with their ends "capped" no treadmilling would occur, but those microtubules with the minus end free would exhibit treadmilling, but would be protected from depolymerisation as a result of the lowered critical concentration in the vicinity of the MTOC.

Probable Regulatory Mechanisms

The proposed models for ordered microtubule assembly and disassembly suggest that a disparity exists in the critical concentration at the two ends of a polar microtubule. Tubulin contains all the necessary information to self-assemble, is microheterogeneic and exhibits structural polymorphism. The presence of MAPs can alter the structural polymorphism and can also affect the kinetics of tubulin polymerisation. Various physiological ligands can also bind to tubulin and may regulate the microtubule assembly characteristics. A number of mechanisms are therefore available to effect the assembly or disassembly of microtubules in the cell to exercise spatial and temporal control.

(a) The role of tubulin microheterogeneity in the regulation of microtubule assembly is unclear. None of the post-translational covalent modifications alter the

microtubule assembly characteristics.

(b) MAPs have been demonstrated to alter the critical concentration for microtubule assembly. MAP_2 has been demonstrated to affect the disassembly rate constant. In addition the post-translational phosphorylation of the MAP_2 has been demonstrated to increase the disassembly rate constant and consequently the critical concentration. Such a mechanism may be employed to cause rapid microtubule disassembly. Different MAPs can be employed for different types of microtubules and a number of MAPs are known to be phosphorylated.

(c) Calcium can cause microtubule disassembly at high concentration and may be important physiologically. Calcium regulation may be controlled <u>in vivo</u> by the calcium-dependent regulatory protein calmodulin. There is some evidence that calmodulin binds to MAPs. The calcium sequestration mechanisms may be employed to selectively disassemble specific groups of microtubules.

(d) Oligomeric species have been demonstrated to exist in the presence and absence of MAPs. The oligomers may be used as physiological storage buffers to lower the concentration of the tubulin subunits and MAPs in the cell, such that only MTOC-specified assembly can occur. On the other hand, the oligomers may be used in preference to the tubulin subunits as assembly-intermediates to promote

nucleation and rapid microtubule assembly.

(e) GTP and GDP can bind to tubulin and act as structural co-factors. The control of the nucleotide status has been suggested as a means of maintaining the growth polarity of the microtubules and the observed disparity in the critical concentration at the two ends of the microtubule. As this thesis considers the nucleotide requirements it is important to consider the possible mechanisms for regulating the tubulin nucleotide status.

Regulation of the tubulin nucleotide status

Assembly-competent tubulin under physiological conditions requires GTP bound to both the N- and E-sites. While the N-site GTP shows no turnover during microtubule assembly, the E-site GTP is hydrolysed. Following tubulin incorporation into the microtubule, the E-site GDP remains blocked on the tubulin (143 and Chapter IV).

Nucleoside triphosphates such as ATP, CTP and UTP can also promote microtubule assembly, but are reported to show no significant binding to tubulin under the assembly conditions (155-156). It has been proposed that the enzyme nucleoside diphosphate kinase (NDPkinase) utilises these triphosphates to convert the endogenous GDP to GTP which

promotes the observed microtubule assembly (155-156).

The action of nucleoside triphosphates such as ATP, CTP and UTP in promoting microtubule assembly can result by three possible mechanisms:

(a) a direct binding of these nucleoside triphosphates to tubulin at a site distinct from the E-site.

(b) utilisation of the nucleoside triphosphates and the free GDP by the NDPkinase activity and the rebinding of GTP, by exchange, to the E-site.

(c) phosphorylation of the tubulin bound GDP by the NDPkinase *in situ*, utilising the nucleoside triphosphates.

Penningroth and Kirschner (156) have proposed a model which suggests that the NDPkinase utilises the tubulin-bound GDP as the preferred substrate for phosphorylation *in vitro*. Such a model would therefore imply an important role for the NDPkinase as the enzyme responsible for maintaining the nucleotide status of the tubulin and the disparity in the critical concentration of the two ends of a polar microtubule.

In this thesis the role and requirement of the NDPkinase in microtubule assembly is examined. A procedure for the purification of the chick brain NDPkinase is developed. The purified NDPkinase is used to characterise the NDPkinase interactions with the microtubule protein.

In addition, the nucleotide requirement for microtubule assembly is determined with GTP, ATP and CTP. The requirement for the NDPkinase in ATP- and CTP-induced microtubule assembly is examined and discussed.

CHAPTER II

MATERIALS AND METHODS

Preparation of microtubule protein:

Microtubule assembly (MA) buffer

0.1 M	MES
2.5 mM	EGTA
0.5 mM	$MgCl_2$
0.1 mM	EDTA

The MA buffer was adjusted to pH 6.4 using concentrated KOH (Radiometer pH meter with a Sigma calomel electrode at room temperature) and stored at 4°C. The MA buffer was adjusted to 0.1 mM DTT before homogenisation and to 1 mM DTT for the cold-dissociation steps.

Microtubule protein was purified from day-old chick brains by cycles of assembly and disassembly, in the absence of glycerol (157-158). The chick brains were dissected and cooled to 4°C and homogenised in an equal volume (W/V) of MA buffer. The homogenate was centrifuged (20,000 x g, 30 mins) at 4°C. The decanted supernatant was adjusted to 1 mM ATP by addition of solid ATP, incubated for 10 mins at 37°C , and centrifuged (20,000 x g, 30 mins) at 30°C. The resultant microtubule pellet was homogenised in MA buffer using $^1/_{10}$ of the initial homogenisation volume, and cold-dissociated for 30 mins at 4°C. The cold-dissociated protein was then

centrifuged (65,000 x g , 30 mins) at 4°C to remove any cold-stable aggregates. The protein in the supernatant is termed "1x microtubule protein".

Further cycles of assembly and disassembly were performed by readjusting the 1x microtubule protein to 1 mM GTP followed by incubation for 20 mins at 37°C. The remainder of the steps are as described for the 1x microtubule protein except the cold-dissociation time was increased from 30 mins to 60 mins. The protein was normally stored as aliquots in liquid nitrogen and was not further purified unless specifically stated.

Sucrose Cushion:

The assembled microtubules were overlayed with a Pasteur pipette onto a 30% (W/V) sucrose solution in MA buffer. The microtubules were collected by centrifugation, through the sucrose cushion, at 100,000 x g for 30 mins. The microtubules were cold-dissociated and centrifuged as described above.

Protein Estimation:

Protein concentrations were estimated by two methods. At low protein concentrations the Bradford method (159) was employed, but wherever possible the protein concentration was determined by the modification of the Lowry method as described by Hartree (160).

The Hartree Method:

Solution A: 1 gm Potassium-Sodium tartrate and 100 gms sodium bicarbonate were dissolved in 500 mls of 1 M sodium hydroxide and diluted to 1 litre with distilled water.

Solution B: 2 gms Potassium-Sodium tartrate and 1 gm of cupric sulphate were dissolved in 90 mls of distilled water and 10 mls of 1 M sodium hydroxide.

Solution C: 1 volume of Folin-Ciocalteau reagent was dissolved in 15 volumes of distilled water and was freshly prepared.

Bovine serum albumin (BSA) at 1 mg/ml was used as the calibration standard.

Protein solutions were added in 10 x 13 mm plastic tubes and the final volume adjusted to 100 µls. Solution A was made 1:1 (V/V) with distilled water and 1.8 mls of this solution added to the tubes, which were vortexed and incubated at 50°C for 10 mins. The tubes were then cooled to room temperature and 100 µls of solution B was added vortexed and allowed to stand at room temperature for 10 mins. 3 mls of solution C was then forcefully added to the tubes which were vortexed immediately. The samples were incubated at 50°C for a further 10 mins and then cooled to room temperature. The blue colour which develops was then measured at 650_{nm} in a Beckman DU-8 spectrophotometer.

A standard calibration curve was prepared for the BSA and treated concurrently with the protein samples. The protein concentration in the samples was estimated from the calibration curve obtained for the BSA, assuming the same degree of colour development for the BSA and the protein samples.

The Bradford method:

The protein estimation makes use of the binding of Coomassie brilliant blue G-250 to proteins which causes a shift in the absorption maximum of the dye from 465_{nm} to 595_{nm} and the increase in the absorption at 595_{nm} is

determined.

Protein Reagent: The final concentrations in the reagent expressed as W/V were:

0.01% Coomassie brilliant blue G-250

4.7% Ethanol

8.5% Phosphoric acid

This solution is stable for about a month; however, it must be filtered each time before use.

Protein samples were added to plastic tubes (10 x 13 mm) and adjusted to a final volume of 50 µls. 2.5 mls of the protein reagent was added and the tube immediately vortexed. A standard calibration curve using BSA (5-50 µgs) was treated in the same manner and the absorbance at 595_{nm} was measured after five minutes in a Beckman DU-8 spectrophotometer. The sample protein concentration was estimated from the calibration curve for the BSA assuming the same degree of colour development for the standard and sample proteins.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE):

Stock solutions:

30% Acrylamide-0.8% bisacrylamide stored at 4°C.

3.0 M Tris-HCl pH 8.8

0.5 M Tris-HCl pH 6.8

0.25 M Tris-1.92 M Glycine pH 8.8

10% Sodium dodecyl sulphate (SDS)

10% Ammonium persulphate (freshly prepared)

TEMED stored at 4°C

Saturated butan-1-ol

Separation Gel:

x mls of acrylamide-bis solution to make x% gels

3.75 mls 3 M Tris-HCl, pH 8.8

0.30 mls 10% SDS

and adjusted to a final volume of 30 mls with distilled water. The radical formation was started by addition of 75 µls of 10% ammonium persulphate and the polymerisation initiated by the addition of 25 µls of TEMED. The gels were set in glass tubes (75 x 10 mm) stoppered at one end with parafilm, and the gel solution poured to a height of 60 mm. To ensure a flat surface the gel was overlayed with some saturated butan-1-ol, and the gels were allowed to

set for a minimum period of three hours at room temperature.

Stacking gel:

1.0 ml of acrylamide-bis solution
2.5 mls 0.5 M Tris-HCl pH 6.8
50 µls 10% SDS
6.4 mls of distilled water
25 µls each of ammonium persulphate and TEMED.

The butan-1-ol was removed from the surface of the separating gel and the stacking gel layered to a height of 70 mm, and overlayed with the saturated butan-1-ol. The stacking gel was allowed to set for a minimum period of two hours.

Sample buffer:

6.25 mM Tris-HCl pH 6.8
4% SDS
20% Glycerol
10% β-mercaptoethanol
a few grains of bromophenol-blue

The samples to be analysed were made to a ratio of 1:1 (V/V) with the sample buffer and boiled for two mins.

SDS-PAGE was performed essentially according to the procedure of Laemmli (161) in a gel tank comprising two chambers which were filled with the chamber buffer (a 1:10 dilution of the stock Tris-glycine buffer pH 8.3 and 0.1% SDS). The proteins were stacked at 100 volts for 10 mins. and then fractionated at a constant voltage of 75 volts. The gels were removed when the bromophenol blue front was 10 mm from the bottom edge of the tubes.

The gels were stained overnight with 1 litre of 0.025% Coomassie brilliant blue G-250- 10% acetic acid-25% propan-1-ol (162). The gels were destained with two changes of 1 litre of 10% acetic acid and scanned at 595_{nm} in a Beckman DU-8 spectrophotometer with a gel-scanning attachment.

Estimation of nucleoside diphosphate (NDP) kinase activity:

The NDPkinase activity was determined essentially according to the procedure of Bergemeyer (163)

Stock solutions:

0.1 M Tri-ethanolamine pH 7.6

15 mg/ml PEP-0.5M $MgCl_2$-2M KCl

50 mM ATP

10 mM TDP

the tri-ethanolamine was stored at 4°C, but all the other solutions were stored at -20°C.

The standard assay reaction mixture, in the final volume of 500 µls, contained :

83.3 mM Tri-ethanolamine

67.0 mM KCl

16.7 mM $MgCl_2$

1.10 mM PEP

2.20 mM ATP

0.21 mM NADH

0.70 mM TDP

and 0.24 units/ml pyruvate kinase and 7.5 units/ml lactate dehydrogenase.

The reaction was initiated by addition of 0.010-0.015 units of NDPkinase activity, and the utililisation of the NADH was monitored at 340_{nm} (E_{340}^{mM}= 6.28), using a Beckman DU-8 spectrophotometer temperature controlled at 25°C. Appropriate controls for the background activity with TDP and ATPase activity were also performed. One unit of activity is defined as that amount of enzyme which catalyses the reaction of 1 µmole ATP and 1 µmole of TDP per min at 25°C.

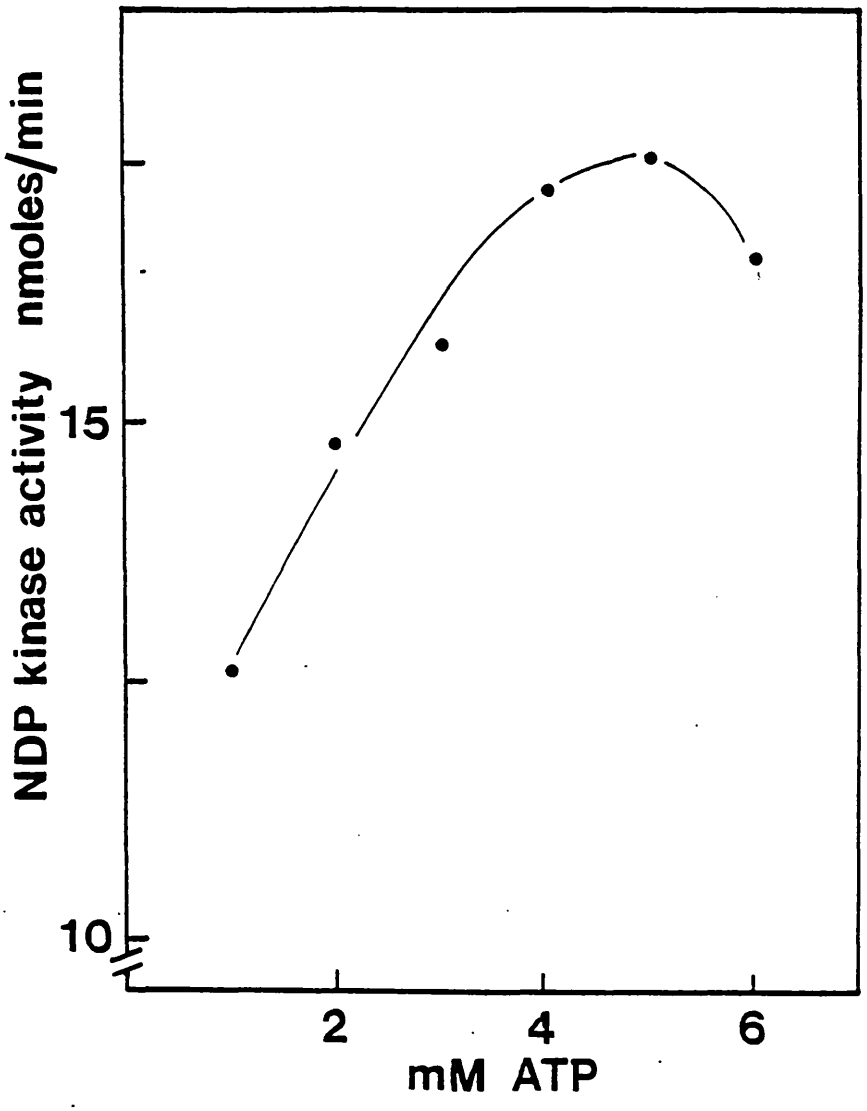


Figure 2.1. Optimal conditions for the determination of maximal NDPkinase activity.

0.014 units of NDPkinase activity, determined under the routine assay conditions, were incubated with increasing concentrations of ATP in the presence 2 mM TDP.

The conditions employed in this routine assay were found to be sub-optimal with respect to the nucleotide concentrations (Fig. 2.1). The maximal specific activity of the purified NDPkinase was therefore measured using 5 mM ATP and 2 mM TDP.

For some of the earlier experiments dGDP was used as the phosphate acceptor. However, since TDP is a poorer substrate for the pyruvate kinase, TDP was routinely used for the later experiments.

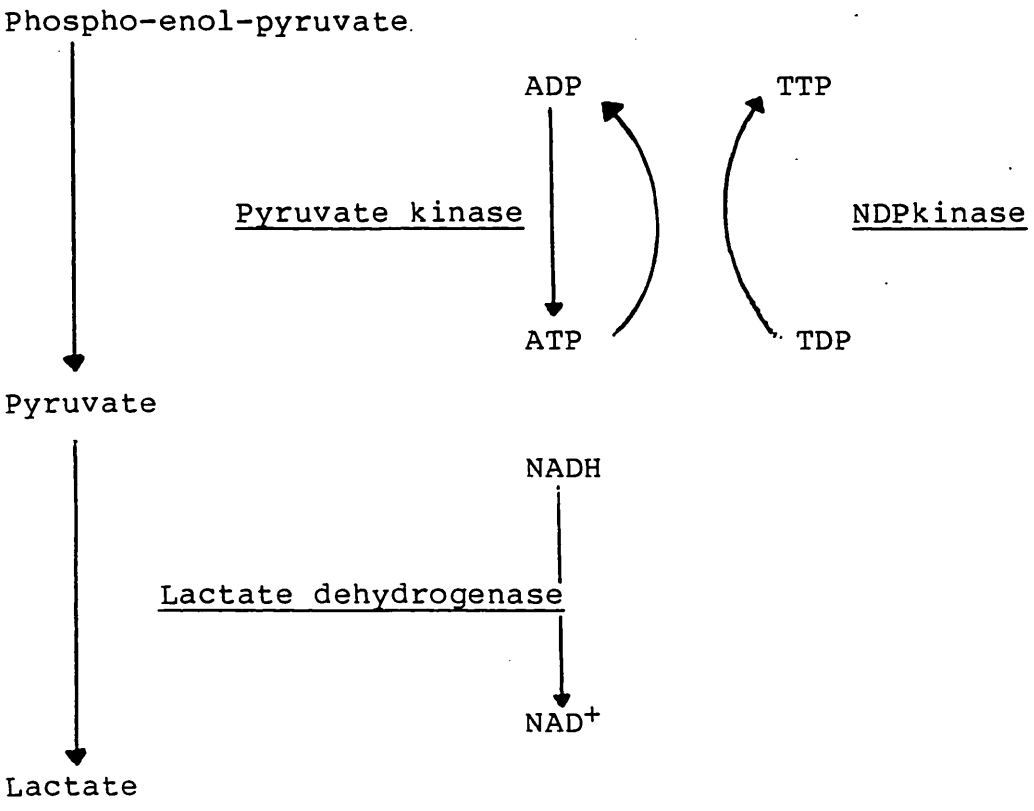


Fig. 2.2 : A schematic representation of the couple-enzyme reaction.

Characterisation of isozymes:

The isozymes were characterised by using the commercially available technique of chromatofocusing (Pharmacia Fine Chemicals, Uppsala, Sweden). The chromatofocusing column (0.9 x 28 cms) was pre-equilibrated to the start pH by washing with 15 column volumes of 25 mM ethanolamine-acetic acid (pH 9.4). The proteins were eluted at their isoelectric points by washing the column with 10 column volumes of Polybuffer-96 (pH 5.84 with HCl). The pH of the fractions were measured using a radiometer pH meter and a Sigma calomel electrode.

Analysis of nucleotides:

Total nucleotide content:

Protein was adjusted to 5% perchloric acid (V/V) and incubated at 4°C for 30 mins. The precipitated protein was removed by centrifugation (18,000 x g , 15 mins) and the nucleotide content of the supernatant determined from absorbance measurements at 257_{nm} in a Beckman DU-8 spectrophotometer. The concentration of the guanine nucleotides was determined from the extinction coefficient of GTP under identical conditions.

Separation of di- and triphosphate nucleotides:

After perchloric acid treatment and removal of proteins, the supernatant was fractionated on a PEI-cellulose (Sigma Chemical Co.) column. The column was pre-equilibrated in 20 mM KH_2PO_4 (pH 3.4) and was developed with a linear gradient of lithium chloride of 0-4 M in 20 mM KH_2PO_4. The elution profile of the di- and triphosphate form is shown (Fig. 2.3). A wide separation can be achieved by this procedure.

Column Chromatography:

Ion-exchange:

The P-11 cellulose phosphate and the diethylaminoethyl DE-52 cellulose are manufactured in the dry form (Whatman Chemical Separations Ltd.) and require precycling for conversion to the swollen gel form (Whatman Bulletin no. A1EC 201).

	First treatment	Intermediate pH	Second treatment
DE-52	0.5N HCl	4	0.5N NaOH
P-11	0.5N NaOH	8	0.5N HCl

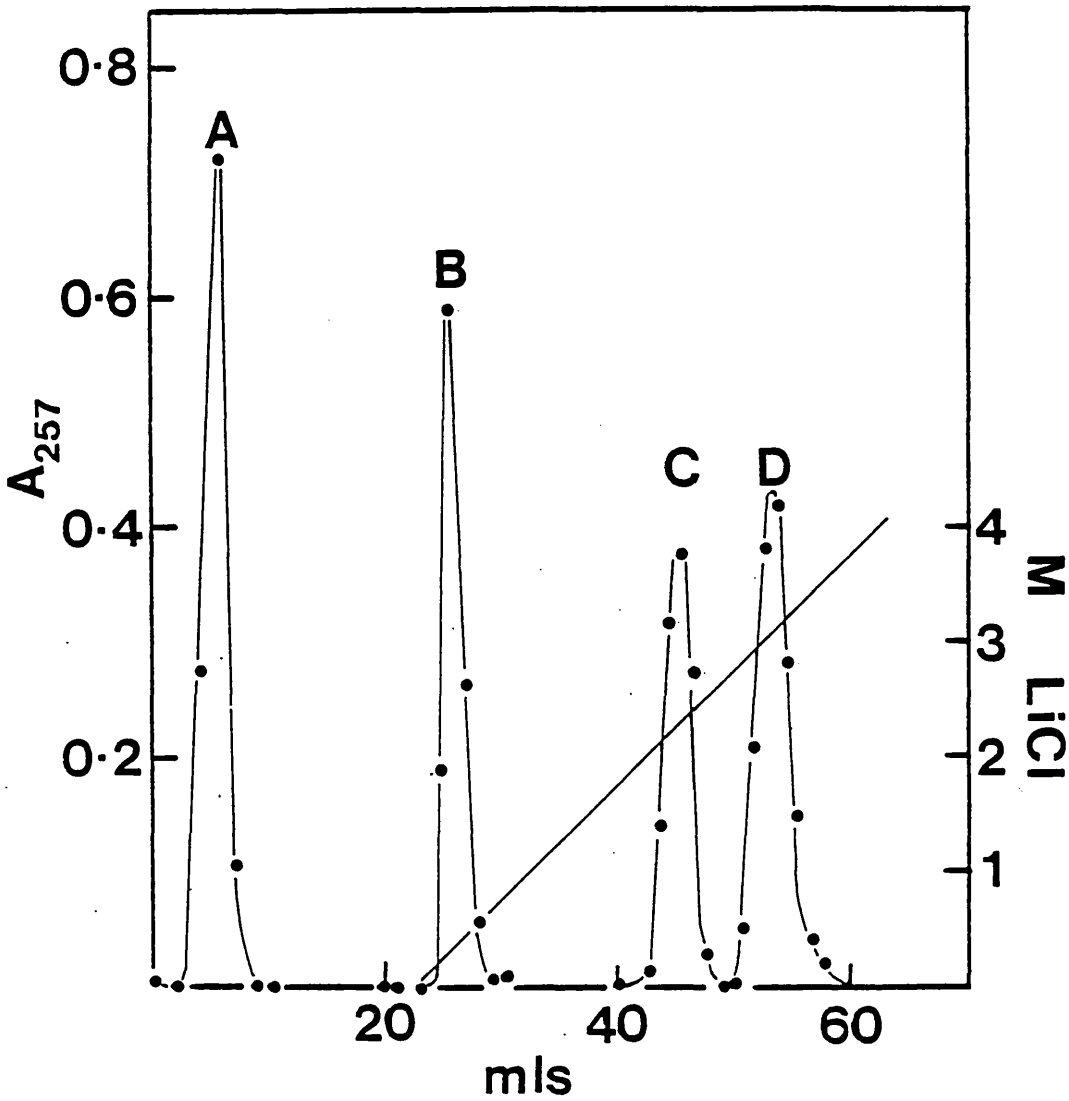


Figure 2.3. Chromatographic separation of nucleotides on a PEI-column.

A mixture of nucleotides was prepared in 0.5 mls of 20 mM KH_2PO_4, adjusted to 5% perchloric acid, and loaded onto a PEI-column (0.64 x 6 cms) pre-equilibrated with 20 mM KH_2PO_4 and washed with 20 mls of the buffer. The column was then eluted with a linear gradient of 0-4 M lithium chloride in 20 mM KH_2PO_4 (40 mls, 18 mls/h) and the absorbance of the eluted fractions at $A_{257\,nm}$ was determined. The elution profile corresponds to (A) guanosine (B) GMP (C) GDP and (D) GTP.

The ion-exchanger was stirred into 15 volumes (V/V) of the first treatment and allowed to stand at room temperature for 30 mins. This treatment converts the ion-exchanger to the fully charged form causing maximum swelling. The supernatant was decanted and the slurry washed extensively with distilled water till the intermediate pH was reached. The exchanger was then suspended into 15 volumes of the second treatment and left for 30 mins, converting the exchanger to the acid or base form. The supernatant was decanted and the slurry suspended in the equilibration buffer. After removal of fines the slurry was poured into a column and then washed with 10-15 column volumes of the equilibration buffer.

The P-11 Cellulose phosphate exchanger required a pre-treatment prior to the precycling procedure and this is discussed in Chapter III.

The hydroxylapatite exchanger was purchased in a preswollen form (LKB-Producter, Sweden) and requires no precycling. Blue dextran-sepharose was purchased from Sigma Chemical Co. and requires no precycling.

Gel-exclusion chromatography:

Sephadex G-50 was purchased in the dry form (Pharmacia Fine Chemicals) and converted to the swollen gel by suspension in 40 volumes of distilled water (W/V).

The suspension was allowed to stand overnight at 4°C and following decantation and degassing the slurry was poured into a column and equilibrated by washing with about three column volumes of buffer.

The Ultrogel ACA-44 (LKB Producter, Sweden) and the Biogel A 1.5m (Biorad Laboratories Ltd.) were purchased as preswollen gels. The gel was made 40% (V/V) with buffer and degassed prior to pouring the column. The column was then equilibrated with about three column volumes of buffer.

Charcoal Treatment:

The charcoal treatment to remove the free and partially remove the E-site nucleotides was performed essentially by the method of Penningroth and Kirschner (156). Activated charcoal (Sigma Chemical Co.) was suspended at 100 g/l in the MA buffer and 50 µls of this suspension was added per ml of microtubular protein and the charcoal was immediately removed by centrifugation (18,000 x g , 1 min). The treatment was repeated twice on the supernatant and this protein is termed "Charcoal-treated microtubule protein".

Kinetics of Tubulin Polymerisation:

As microtubules are long, rod-like structures their light scattering characteristics can be exploited to make turbimetric measurements to determine the extent and rate of microtubule assembly spectrophotometrically (127).

Cold-dissociated microtubular protein was degassed and then incubated at 37°C for 5 mins in a Beckman DU-8 Spectrophometer with a temperature-controlled cuvette compartment. On addition of the nucleoside triphosphate absorbance measurements were made at 350_{nm}. Both the maximum rate and the total absorbance change are proportional to the extent of polymerised protein. Furthermore no increase in absorbance is observed during the nucleation lag-phase, and a monotonic curve is observed with time for the elongation phase (Fig 2.4). The presence of microtubules was confirmed by electron microscopy.

Electron Microscopy (E-M):

A 0.25% formvar film was formed on a clean water surface and the E-M grids were placed onto the film, dull side down, and overlayed with ordinary writing paper. The grids and the film was pushed through the water surface

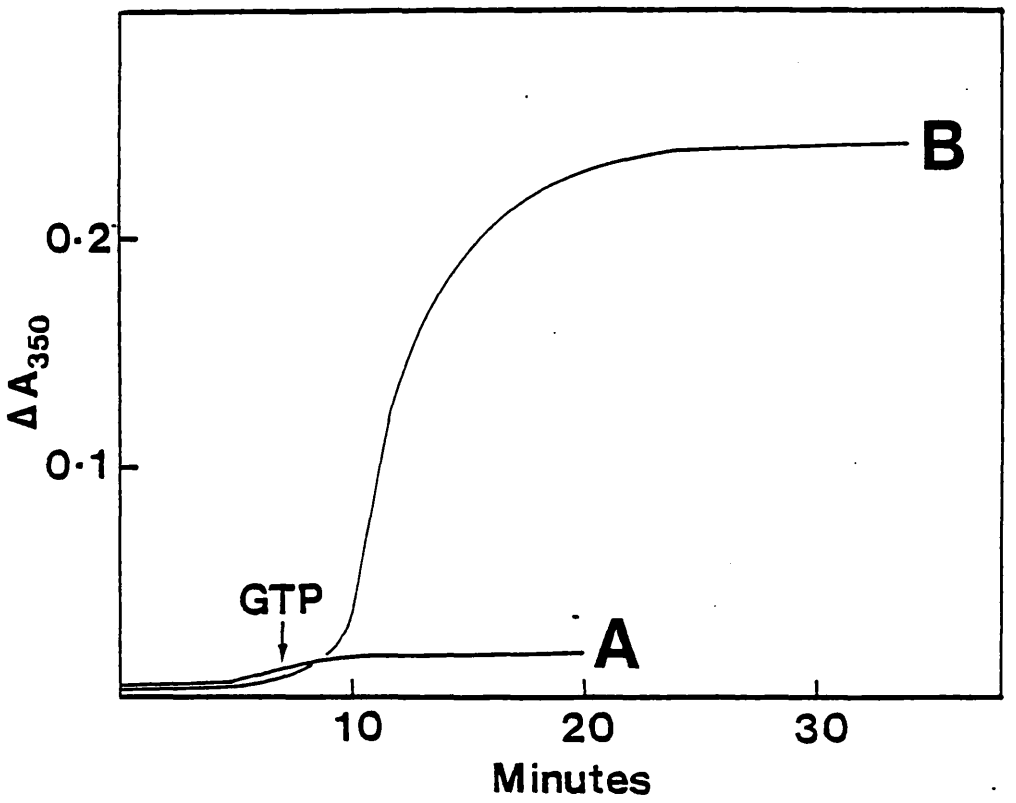


Figure 2.4. Turbimetric determination of the kinetics of microtubule assembly.

Sucrose cushion microtubule protein at 1 $mg.ml^{-1}$ was incubated at 30°C and the change in absorbance at 350_{nm} monitored in the absence (A) and the presence (B) of 1 mM GTP.

with the palm of the hand, turned around and raised through the water surface. The grids were then dried under vacuum and carbon coated.

A drop of the protein sample was applied to the carbon coated grid using a Pasteur pipette and left for 10 seconds. The sample was then rinsed with 6-10 drops of 1% uranyl acetate and the excess uranyl acetate was removed by blotting dry with filter paper. The samples were then examined in a Philips-301 electron microscope.

Microfiltration:

The microfiltration sample and receiving compartments and regenerated cellulose filters were purchased from Anachem (U.K.).

Sephadex G-50 gel slurry was prepared as described earlier and then equilibrated by washing in MA buffer. 2 mls of the gel slurry was loaded into the sample compartment and the gel was allowed to settle for 3 mins. The column was then centrifuged for 2 mins at 2500 x g and the eluate removed from the receiving compartment.

90 µls of the protein sample was carefully overlayed onto the column and immediately centrifuged at 2500 x g

for 2 mins. The eluant volume in the receiving compartment showed a constant recovery of 80-90% of the load volume. The protein concentration and the radioactivity of the eluant were determined.

CHAPTER III

NUCLEOSIDE DIPHOSPHATE KINASE: PURIFICATION AND CHARACTERISATION

Introduction:

The enzyme nucleoside diphosphate kinase (NDPkinase; EC 2.7.4.6) is ubiquitously distributed in nature. The distribution of the enzyme in rat tissues shows that it has an unusually high activity ranging from 30-68 μ moles/min/gm tissue (164). The enzyme is relatively non-specific with regard to its substrates and catalyses the transfer of the terminal phosphate group from a triphosphate to a diphosphate nucleotide by the following general mechanism:

$$N_1TP + N_2DP \rightleftharpoons N_1DP + N_2TP$$

where N_1 and N_2 are purine or pyrimidine ribo- or deoxyribonucleotides.

NDPkinase activity has been found to be associated with proteins whose biological activity is regulated by GTP, for example tubulin (155) and elongation factor 1 (165). In the context of this thesis, the interest in the chick brain NDPkinase stems from the proposals that this enzyme is responsible for catalysing the phosphorylation of tubulin-bound GDP (156). Furthermore, as discussed in chapter I, nucleotides play an important regulatory role in microtubule assembly. The nucleotide requirements for microtubule assembly and the binding of the NDPkinase with microtubules and tubulin is examined in detail in chapters

IV and V.

NDPkinase activity was first demonstrated in Yeast (166) and in pigeon breast muscle (167). The enzyme has been isolated to apparent homogeneity from Brewer's Yeast (168), human erythrocytes (169), pea seed (170), Bacillus subtilis (171), pig heart (172) and bovine brain (173), and partially purified from various sources (174). In general most of these purification procedures give a 4-20% yield of purified NDPkinase with specific activities ranging from 100-1000 µmoles/min/mg of protein and involve a large number of steps with rather poor yields.

The use of cibacron-blue F3GA dye bound to Sepharose can be employed for affinity chromatography of the NDPkinase and has been used to purify the NDPkinase simply and rapidly (172-173). However, the concentration of immobilised ligand in the gel can critically affect the binding characterstic of enzymes (172). Furthermore, cibacron-blue F3GA dye has been shown to degrade with time and has been demonstrated to inactivate or covalently modify a number of enzymes (175).

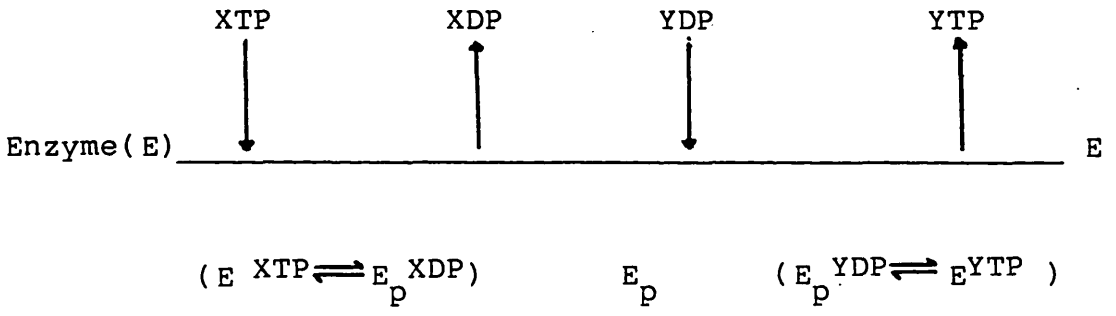
Cheng et al. (176) provided evidence for the electrophoretic heterogeneity of the human erythrocytic NDPkinases and observed six distinct peaks of enzyme activity with isoelectric points between 5.4 and 8.3 by electrofocusing and agarose electrophoresis. The

electrophoretic pattern shows no qualitative differences between individuals or races. On the basis of the molecular weights and kinetic parameters it was suggested that the isoenzymes were all distinct proteins.

Crystalline NDPkinase from Brewer's yeast has a native molecular weight of 102,000 and exhibits no electrophoretic heterogeneity. However, electrophoretic mobility as a function of pH shows a significant influence of ionic strength on the apparent isoelectric point of the enzyme, which is estimated to be ca. 8.0 at zero ionic strength (177). Palmieri et al. (178) demonstrated that it was composed of six similar subunits, each with an individual molecular weight of 17,300, which was in good agreement with the calculated molecular weight from the amino-acid composition. Similarly the crystalline bovine heart cytosolic (179) and bovine brain particulate (173) NDPkinases are composed of six similar subunits with individual molecular weights of 16,000 - 18,000.

The NDPkinase displays the classic ping-pong bisubstrate reaction and functions via a stable phosphorylated enzyme intermediate (180). The turnover number for phosphorylation is 2700/min and that for dephosphorylation is 24,000/min. The overall reaction has a turnover of 1,300/min, which strongly suggests that the phosphoenzyme is the true intermediate for the NDPkinase reaction. 1-phosphohistidine, 3-phosphohistidine, and

n-ε-phospholysine are the principal phosphorylated residues in alkaline digests of phosphorylated NDPkinase (181-183). Recently the bovine brain NDPkinase has been demonstrated to bind six phosphates confirming that the enzyme is composed of six subunits (173). The NDPkinase reaction can therefore be described in such a sequence:



Although all NDPkinases are relatively non-specific with regard to their substrates they vary greatly in their kinetic parameters with various nucleoside di- and tri-phosphates (174; 184-185). The true substrate is the Mg-nucleotide complex and all NDPkinases have an absolute requirement for divalent cations (186). The enzyme exhibits a broad pH optimum ranging from pH 6-9 (174; 185).

At high concentrations of nucleoside diphosphate the NDPkinase forms abortive complexes (187) and diphosphates may function as regulatory ligands (179). Both the di- and tri- phosphates show the classical predicted competitive inhibition patterns (169; 188). GMP can also act as a

competitive inhibitor of the ATP-dTDP reaction (169).

All NDPkinases contain essential sulphydryl groups (174; 185; 187). There are at least four hidden and four readily accessible sulphydryl groups per mole of bovine liver NDPkinase (189). The accessible sulphydryl groups can be reactivated by addition of a thiol reagent such as dithiothreitol. The presence of nucleoside di- or tri-phosphates provides some protection against inactivation of the sulphydryls, suggesting conformational changes in the enzyme. The soluble and Sepharose-bound pig heart NDPkinase while exhibiting no significant differences in their kinetic parameters, show differential sensitivity to heat-inactivation, inhibition by p-chloromercuribenzoate and urea further supporting the evidence for conformational changes in the enzyme (190).

RESULTS:

Recovery of the NDPkinase in the soluble and particulate fractions following centrifugation.

Day-old chick brains were homogenised in an equal volume of MA buffer (W/V) and centrifuged (30,000 g, 30 mins) at 4°C. The supernatant was decanted and the NDPkinase activity in this "soluble fraction" was determined. The NDPkinase activity which sediments with the "particulate fraction" was extracted by suspension in an equal volume of MA buffer containing 2% Brij-37 (a non-ionic detergent), and recovered in the supernatant following centrifugation (30,000 g, 30 mins) at 4°C. The distribution of the NDPkinase activity in the particulate and soluble fractions is shown in Table 3.1.

The chick brain NDPkinase is primarily cytosolic in common with all the NDPkinases isolated (172; 174; 185), with one notable exception of the bovine brain NDPkinase (173). The bovine brain NDPkinase has been extracted from the particulate fraction using 2% Brij-56, and purified by cibacron-blue affinity chromatography. However as discussed later the particulate and soluble enzymes may be the same protein.

TABLE 3.1 DISTRIBUTION OF CHICK BRAIN NDP KINASE.

Fraction	NDPkinase units	%
Soluble	4740	83
Particulate	982	17
Total	5722	(100)

Purification of the soluble chick brain NDPkinase:

The procedure describe the purification of the soluble NDPkinase. The conditions at each step have been optimised for the purification of the chick brain NDPkinase.

Step I. Preparation of the crude extract.

In a typical preparation (as shown in Table 3.2) 50 day-old chick brains were cooled to 4°C and homogenised in 75 mls of MA buffer containing 1 mM DTT, and centrifuged (30,000 g, 30 mins). All further steps were performed at 4°C unless otherwise stated.

Step 2. 55-80% Ammonium sulphate precipitation.

The supernatant from step 1 was adjusted to 55% ammonium sulphate by slow addition of the solid (32.6 g/100 ml), and after 10 mins the precipitated protein was removed by centrifugation (20,000 g, 10 mins). The supernatant was adjusted to 80% ammonium sulphate by addition of further solid (16.1 g/100 mls), allowed to stand for 10 mins, and centrifuged (20,000 g, 10 mins). The precipitated protein was dissolved in $1/_{10}$ of the original homogenisation volume.

Step 3. Heat treatment.

The protein from step 2 was then incubated at 60°C for 15 mins, cooled at 4°C for a further 10 mins, and then

centrifuged (20,000 g, 15 mins). The supernatant, containing the heat-stable protein, was adjusted to 80% ammonium sulphate (51.6 g/100 mls), and after precipitating for 10 mins was centrifuged (20,000 g, 15 mins).

Step 4. Chromatography on Ultrogel ACA-44.

The protein sedimented in step 3 was dissolved in a minimal volume of buffer A (same as MA buffer, but pH 6.3), and fractionated on an ACA-44 column (1.6 x 90 cms) pre-equilibrated with buffer A, and eluted with this buffer. Column fractions containing > 10 units/ml NDPkinase activity (Fig 3.1) were pooled and represent step 4.

Step 5. Phosphocellulose chromatography.

The P-11 was pretreated by washing twice in 25 volumes of 40% ethanol, prior to the precycling treatment. This pretreatment is essential to remove a yellowish coloured contaminant which inactivates the NDPkinase.

The pooled fractions from step 4 were chromatographed on a P-11 column (1.8 x 8 cms) pre-equilibrated with buffer A. The unbound protein (including about 10% of the NDPkinase activity) was eluted by extensive washing with the equilibration buffer. The column bound NDPkinase was eluted with a 60 ml linear salt gradient (0-200 mM KCl in buffer A, 35 mls/h). Column fractions containing

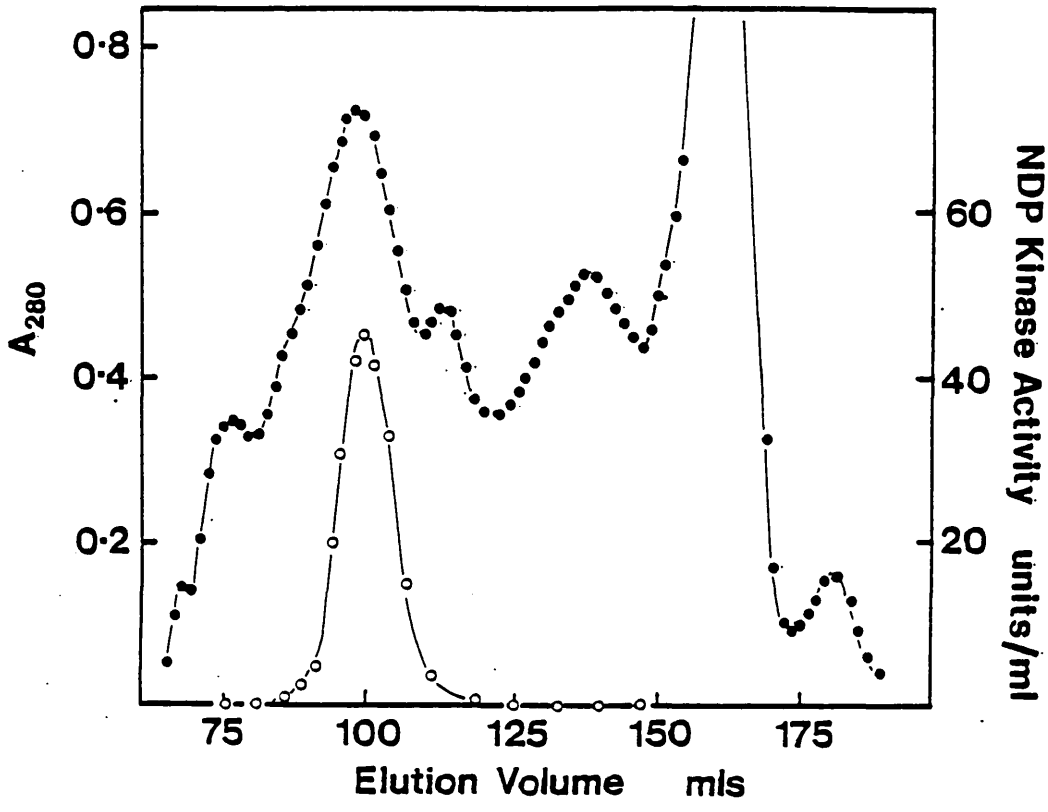


Figure 3.1. Chromatography on Ultrogel ACA-44.

This represents step 4, Table 3.2. Protein from step 3 was fractionated on an ACA-44 column (1.6 x 90 cms) pre-equilibrated in buffer A and eluted with this buffer at 10.5 mls/h. Protein concentration (●) and NDPkinase activity (o).

> 5 units/ml NDPkinase activity were pooled, and then diluted 1:3 (V/V) with deionised water.

Step 6. Hydroxylapatite chromatography.

The protein from step 5 was further fractionated on a hydroxylapatite column (0.64 x 9 cms) pre-equilibrated with 100 mM potassium phosphate pH 6.3. Unbound protein was eluted with the equilibration buffer, and the bound NDPkinase was then eluted with a linear phosphate gradient (60 mls, 100-300 mM potassium phosphate, pH 6.3, 40 mls/h). Column fractions containing > 5 units/ml NDPkinase activity were pooled.

The protein from each step was analysed for purity by SDS-PAGE (Fig. 3.2^{A-F}). This procedure leads to a 500-fold increase in the specific activity of the soluble chick brain NDPkinase and a yield of 30% from the crude extract (Table 3.2). SDS-PAGE shows the presence of a single major band and trace amounts of minor contaminants (Fig. 3.2^{F}). The purified NDPkinase has a maximal specific activity of 1500 units/mg protein. Partially purified enzyme after step 2 or 3 can be stored at 4°C for several days without any appreciable loss of activity.

TABLE 3.2. PURIFICATION OF NUCLEOSIDE DIPHOSPHATE KINASE FROM CHICK BRAIN[a].

Step	NDP Kinase Total Units[b]	Protein mgs	Specific Activity (Units/mg)	Purification Factor	Yield %
1. Crude Supernatant	1735	759	2.28	(1)	(100)
2. 55-80% Ammonium Sulphate	1260	127	9.89	4.3	73
3. Heat Treatment	1186	41.5	28.5	12.5	68
4. ACA-44	784	9.68	81.0	35.4	45
5. Phosphocellulose	615	0.63	980	430.	35
6. Hydroxylapatite	530	0.46	1150.	503.	30

[a] A typical preparation from 50 day-old chick brains.
[b] NDP kinase activity was determined in triplicate.

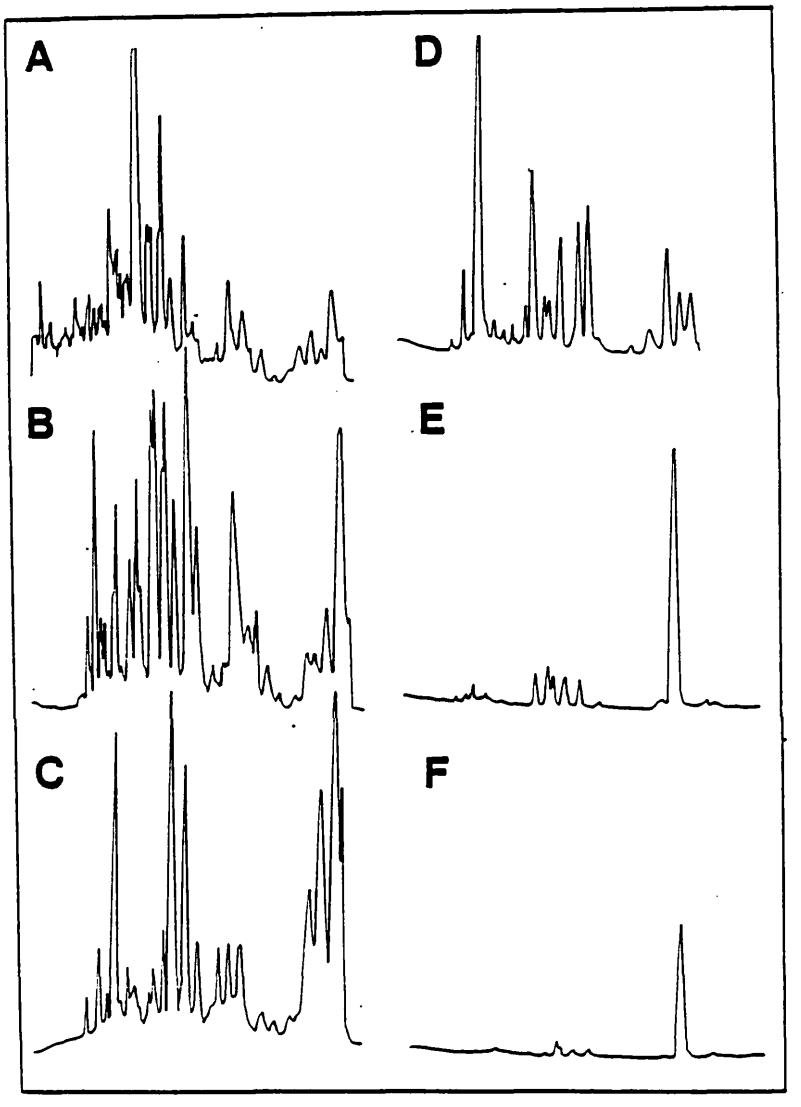


Figure 3.2. SDS-PAGE analysis of the protein purification steps.

Protein from each purification step was fractionated on 12.5% SDS-PAGE. The gels were stained with Coomassie brilliant blue, destained and scanned at $A_{595\,nm}$. (A-F) represent steps 1-6 shown in Table 3.2.

Characterisation of the NDPkinase

The purified NDPkinase has been characterised by a number of criteria and compared to NDPkinases purified by other workers from various sources. These criteria also form the framework for the subsequent analysis of the NDPkinase in microtubule assembly (Chapter IV and V).

Isozymes.

There is reasonable evidence for electrophoretic heterogenity of NDPkinases (176) and often poor resolution is obtained by isoelectric focusing (172).

The crude extract was therefore examined for the presence of NDPkinase isozymes by chromatofocusing. Only two peaks of NDPkinase activity were observed: a major peak pI 8.1, with at least 70% of the total activity, and a minor peak pI 7.7 (Fig.3.3^{A}). Similar analysis of the phosphocellulose purified NDPkinase demonstrated the presence of only a single peak of activity at pI 8.1 (Fig. 3.3^{B}). The NDPkinase purified from chick brain is monoisozymic and has a pI of 8.1, which is the predominant brain isozyme. The isoelectric point of this monoisozyme is similar to the monoisozymic NDPkinases isolated from Brewers Yeast (177-178), human erythrocytes (185), and bovine brain (173). Proteolytic breakdown (173), extent of phosphorylation (179) and ionic conditions (177) have all

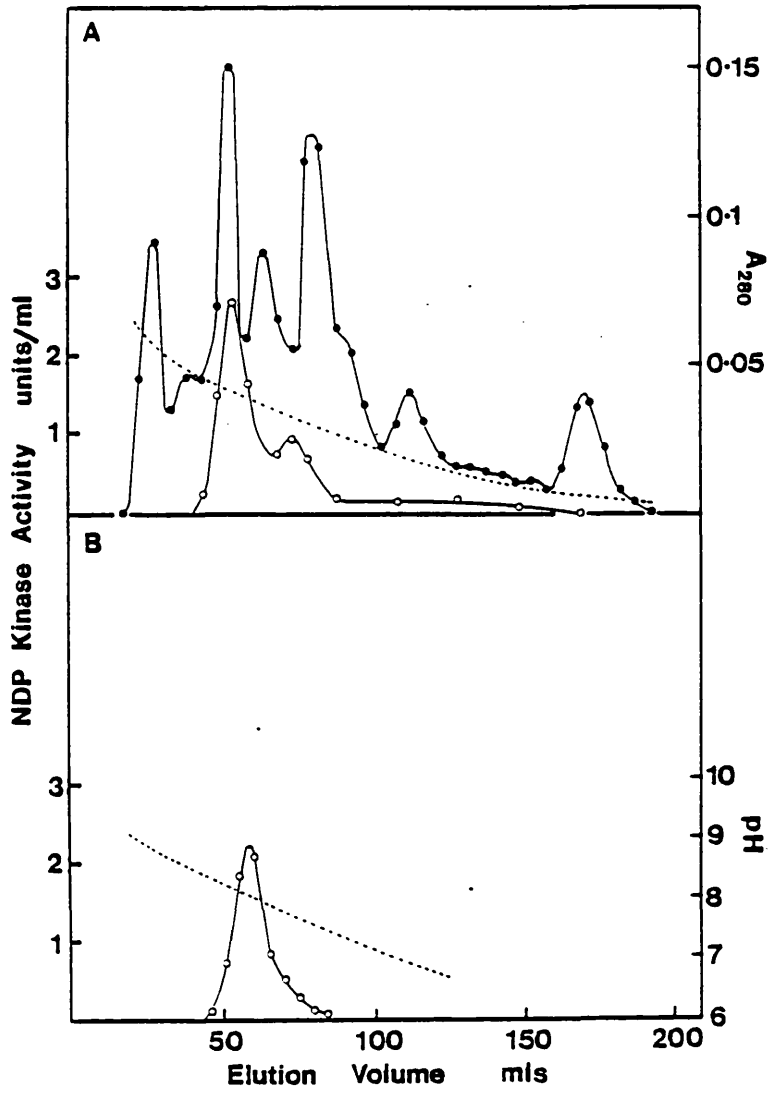


Figure 3.3. Characterisation of NDPkinase isozymes by chromatofocusing.

Proteins were fractionated on a chromatofocusing column (0.9 x 28 cms) with a pH gradient using Pharmacia polybuffer-96. Protein concentration (●), NDPkinase activity (o), and pH profile (·····) shown for Fig. 3.3^{B} only.
(A) Chick brain 20,000 x g supernantant (step 1).
(B) Purified NDPkinase (step 5).

been demonstrated to affect the isoelectric point of the NDPkinases.

Molecular weight.

The molecular weight of the NDPkinase was determined by SDS-PAGE. The purified enzyme was co-migrated on 12.5% gels with known molecular weight standards. The estimated molecular weight of the chick brain soluble NDPkinase is 16,000-18,000 daltons (Fig. 3.4). This molecular weight is in good agreement with NDPkinases purified from Brewer's yeast (178), bovine brain (173), porcine heart(172) and bovine heart (179). Furthermore, in common with these NDPkinases the chick brain enzyme behaves as a protein with an apparent molecular weight 100,000 daltons on gel-exclusion chromatography (Fig. 3.1).

Utilisation of Triphosphates.

NDPkinases have been demonstrated to exhibit broad specificity with regard to its substrates, but vary greatly in their kinetic parameters with various nucleotides (174).

The apparent efficiency of a number of nucleoside triphosphates to act as phosphate donors for phosphocellulose purified NDPkinase was examined using TDP as the phosphate acceptor. In these studies the

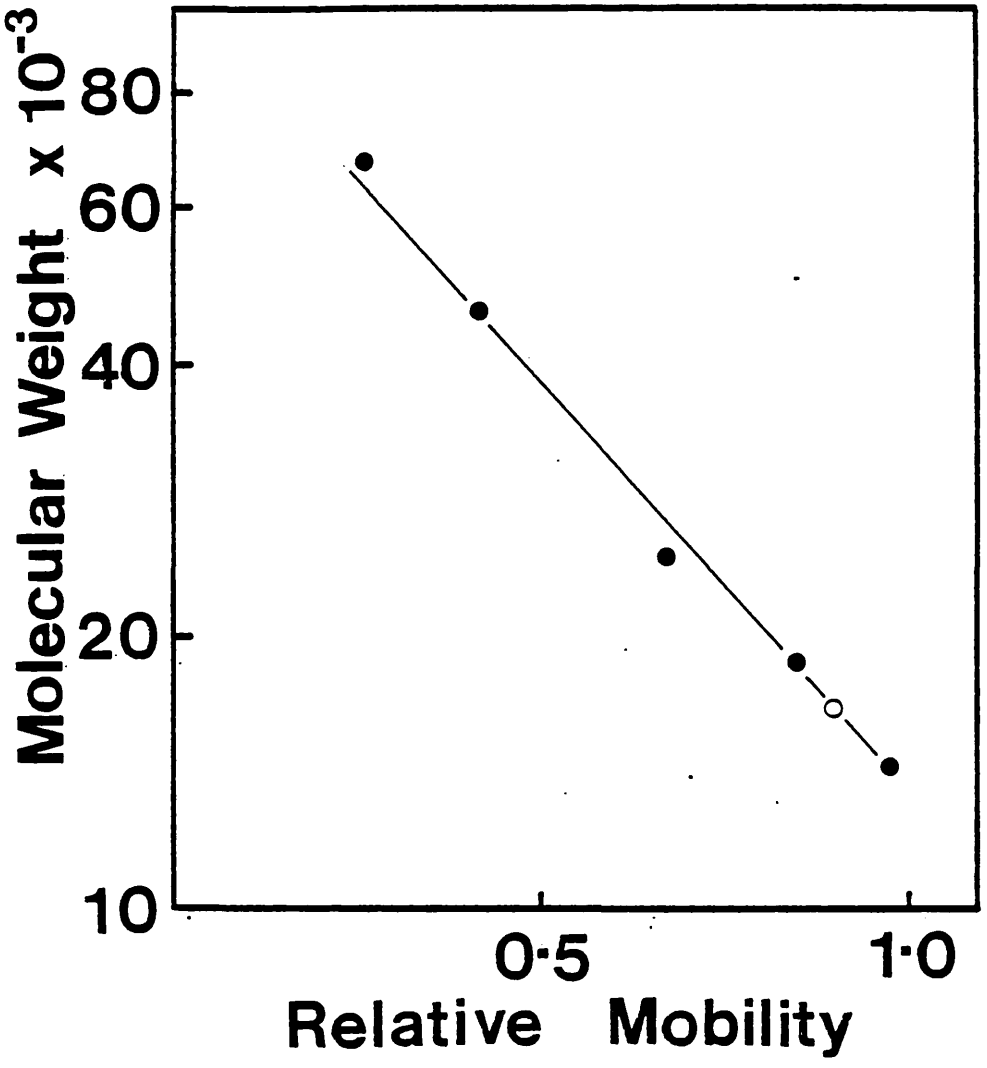


Figure 3.4. Molecular weight estimation by SDS-PAGE.

The molecular weight of the purified NDPkinase was determined by co-electrophoresis with bovine serum albumin (66,000-M_r), ovalbumin (45,000-M_r), trypsinogen (24,000-M_r), β-lactoglobulin (18,400-M_r) and lysozyme (14,300-M_r) on 12.5% acrylamide gels.

concentration of the TDP was 0.7 mM and that of the nucleotide triphosphates was 2.2 mM. The efficiency of utilisation of the nucleoside triphosphates was determined as the percentage of the ATP-TDP exchange at equivalent molar concentrations (Table 3.3). The utilisation of the purine triphosphates is much more efficient than the pyrimidine triphosphates as with the bovine brain (173) and human erythrocyte NDPkinases (185).

Initial Velocity Analysis

NDPkinases exhibit the classic "ping-pong" bisubstrate mechanism (174).

Initial velocity studies were performed with TDP as the variable substrate and ATP as the changing-fixed substrate. The reciprocal plots of data obtained from these studies yielded a family of parallel straight lines (Fig. 3.5): a result consistent with a "ping-pong" bisubstrate reaction mechanism. The corrected K_m for ATP and TDP was determined by plotting the $^1/_v$ intercepts against the reciprocal of the substrate concentration and extrapolation to the x-axis (Fig. 3.5 insert). The corrected K_m for ATP and TDP are 5×10^{-4} and 9×10^{-4} M respectively.

TABLE 3.3. EFFICIENCY OF UTILISATION OF TRIPHOSPHATES BY PURIFIED MONOISOZYMIC NDP KINASE

Nucleoside triphosphate	% ATP-TDP exchange
ATP	(100)
GTP	44
ITP	36
UTP	14
CTP	3

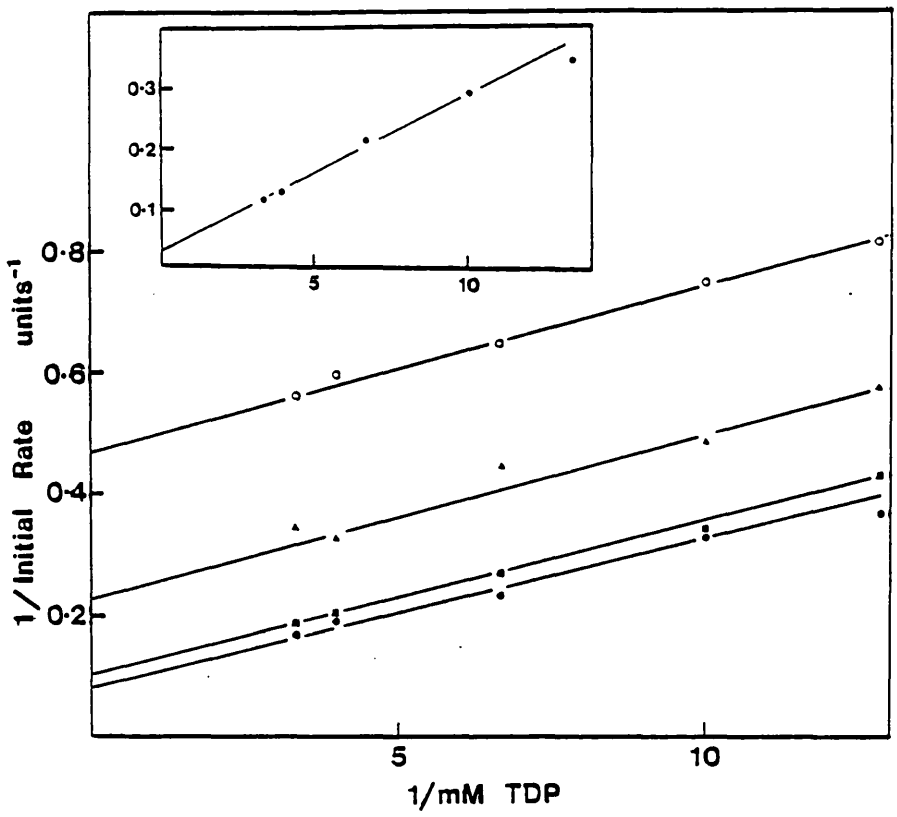


Figure 3.5. Bisubstrate "ping-pong" mechanism.

The NDPkinase activity was determined with ATP as the fixed-changing substrate at 0.05 (o), 0.10 (▲), 0.30 (■) and 0.50 (●) mM ATP and TDP as the changing substrate.

Insert: Determination of the corrected K_m for TDP.

A replot of the data with TDP as the fixed-changing substrate and ATP as the changing substrate was used to determine the 1/V intercepts($1/V_{max}$) at different TDP concentrations. The double reciprocal plot of $1/V_{max}$ versus the 1/TDP mM concentration yields the corrected $^{-1}/k_m$ at the x-intercept.

Blue Dextran-Sepharose Chromatography

Cibacron-blue F3GA dye covalently attached to dextran-Sepharose has been shown to bind the soluble NDPkinases from human erythrocytes (176) and porcine heart (172) and the particulate bovine brain NDPkinase (173). The bovine brain NDPkinase purified by blue dextran-Sepharose chromatography from the particulate fraction has been characterised as a monoisozyme (173). The isoelectric point and the kinetic parameters of the particulate bovine brain and the soluble chick brain NDPkinases are similar. It was therefore of interest to attempt the purification of the chick NDPkinase using the procedure described for the bovine brain NDPkinase (173).

As the bovine brain NDPkinase is largely found in the particulate fraction, the recovery of the chick brain NDPkinase in the soluble and particulate fractions was monitored. In contrast to the bovine brain NDPkinase the chick brain NDPkinase is mostly soluble (Table 3.4). The soluble and the Brij-extracted particulate enzymes were further fractionated on blue dextran-Sepharose chromatography. Unlike the bovine brain NDPkinase the chick brain NDPkinase only partially bound to the blue dextran-Sepharose, but no difference in the extent of binding was observed for the soluble and particulate fraction NDPkinase (Table 3.4). The blue dextran-Sepharose eluate was analysed by SDS-PAGE for purity and both the

TABLE 3.4. PURIFICATION OF NDP KINASE FROM CHICK BRAIN BY THE BLUE DEXTRAN SEPHAROSE PROCEDURE[a]

	SOLUBLE FRACTION			PARTICULATE FRACTION		
	total units	units/ mg	Yield %	total units	units/ mg	Yield %
Total units[b]	610	1.0	62	___	___	___
Brij-extract	___	___	___	380	0.72	38
Blue dextran void	344	0.8	35	208	0.40	21
Blue dextran GTP-eluate	94.5	120	9.5	66	360	6.7

[a] The method of Robinson et al. was repeated precisely as reported (173), and scaled down for the amount of NDP kinase activity present.
[b] NDP kinase activity was determined in triplicate.

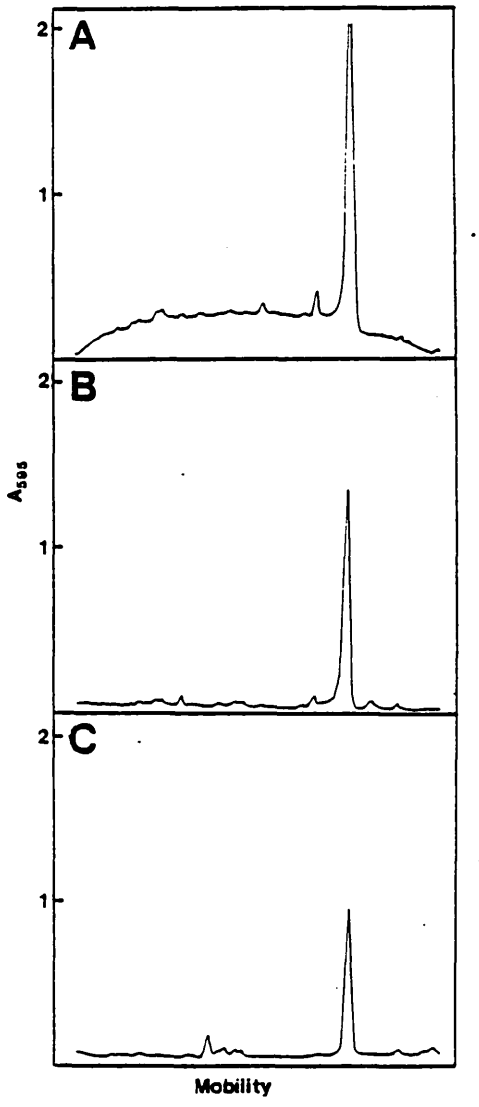


Figure 3.6. SDS-PAGE analysis of NDPkinase from the soluble and particulate fractions.

NDPkinase purified by the Blue Dextran-Sepharose procedure from (A) the soluble fraction (B) the particulate fraction and compared with (C) the purified monoisozymic NDPkinase (step 6), fractionated on 12.5% acrylamide gels. The gels were stained with Coomassie brilliant blue, destained and scanned at $A_{595\,nm}$.

particulate and cytosolic NDPkinases show only one major protein band with trace contaminants (Fig 3.6). However, both the final yield and the specific activity of the purified NDPkinases were significantly lower in comparison to the procedure described in the text (Table 3.2 and Table 3.4). Furthermore, both the particulate and soluble chick brain purified by the procedure of Robinson et al., and the enzyme purified by the procedure described in the text co-migrate as proteins of similar molecular weights (Fig. 3.6).

Inhibition studies with pCMB

All NDPkinases contain essential sulphydryl groups which can be reversibly inhibited by mercurial reagents such as pCMB (174).

Phosphocellulose purified chick brain NDPkinase (specific activity 235 units/mg) was dialysed for 5 hours against 10 mM Tris-5 mM $MgCl_2$-1 mM DTT pH 8.0 and then extensively dialysed against 10 mM Tris-5 mM $MgCl_2$ pH 8.0, to remove any traces of DTT. The enzyme (0.23 units/ml) was then incubated with increasing concentrations of pCMB (10^{-10}- 10^{-8} M) in 10 mM Tris-5 mM $MgCl_2$ pH 8.0 at 37°C for 15 min. A control sample, without pCMB, was concurrently incubated.

The NDPkinase activity remaining at the end of the incubation period was determined and expressed as a percentage of the control. The effect of increasing concentrations of pCMB on the NDPkinase activity is shown in Fig. 3.7 . The effect of the pCMB was reversible as confirmed by 80% reactivation of the enzyme following incubation with 2 mM DTT even at concentrations of 10^{-6} M pCMB. The chick brain NDPkinase appears to be highly sensitive to inactivation by pCMB, with up to 90% inactivation at 10^{-8}M pCMB, in comparison to other NDPkinases (cf. 169) which require a concentration of 10^{-4} M pCMB for similar inactivation.

In order to examine the effect of ATP or TDP on the inactivation of the NDPkinase by pCMB, the enzyme was pre-incubated for 5 min at 37°C either without added nucleotide or with 1.5 mM ATP or 1.5 mM TDP (concentrations above the k_m values), prior to incubation with 2 x 10^{-9} M pCMB. While at this concentration of pCMB only 50% of the NDPkinase remained in the absence of added nucleotide, complete activity was observed in the presence of ATP (101%) or TDP (106%). Both the triphosphate and the diphosphate afforded complete protection against inactivation by pCMB, suggesting conformational changes in the enzyme following substrate binding.

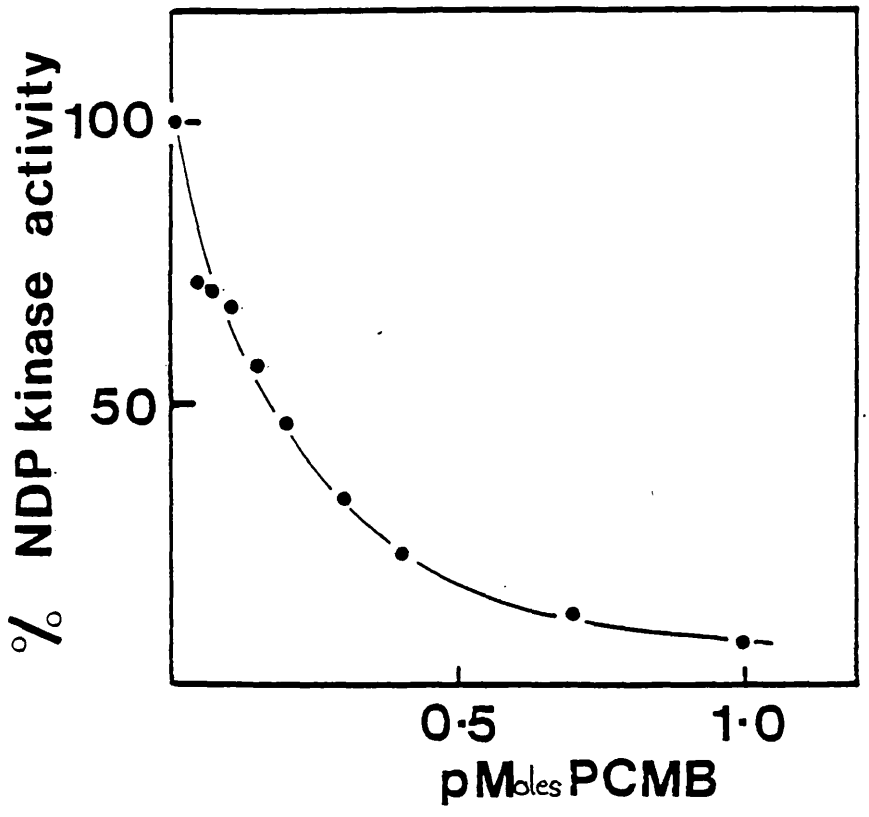


Figure 3.7. Effect of pCMB on NDPkinase activity.

0.010 units of purified monoisozymic NDPkinase was incubated with increasing concentrations of pCMB. The NDPkinase activity was determined under the routine assay conditions and is expressed as a % of the control sample (100%) incubated in the absence of pCMB.

Effect of ionic conditions on NDPkinase elution

During the development of the purification procedure for the chick brain NDPkinase it was noted that the enzyme was highly sensitive to the pH and ionic conditions and exhibited anomalous behavior on Ultrogel ACA-44 chromatography. When partially purified enzyme (Step 2 or 3) was fractionated on ACA-44 gel exclusion column at low pH and ionic conditions at least three distinct peaks of NDPkinase activity were observed (Fig. 3.8), with estimated molecular weights 35,000, 70,000, and 100,000 daltons. Inclusion of 2% Brij-37 or 20% ethylene glycol, conditions which lead to a decrease in hydrophobic interactions, did not affect this elution profile.

However, as shown in Fig. 3.1, at higher pH and ionic conditions the elution profile of the NDPkinase was dramatically altered to yield a single peak of activity with an estimated molecular weight of 100,000 daltons. Further increase in the pH of this buffer does not affect the elution profile of the NDPkinase but leads to a concommitant decrease in the specific activity of the enzyme due to co-migration of contaminating proteins. Similar anomalous behavior is observed on phosphocellulose chromatography where a slight variation in the pH results in a significant increase in the proportion of the NDPkinase eluting in the void fraction.

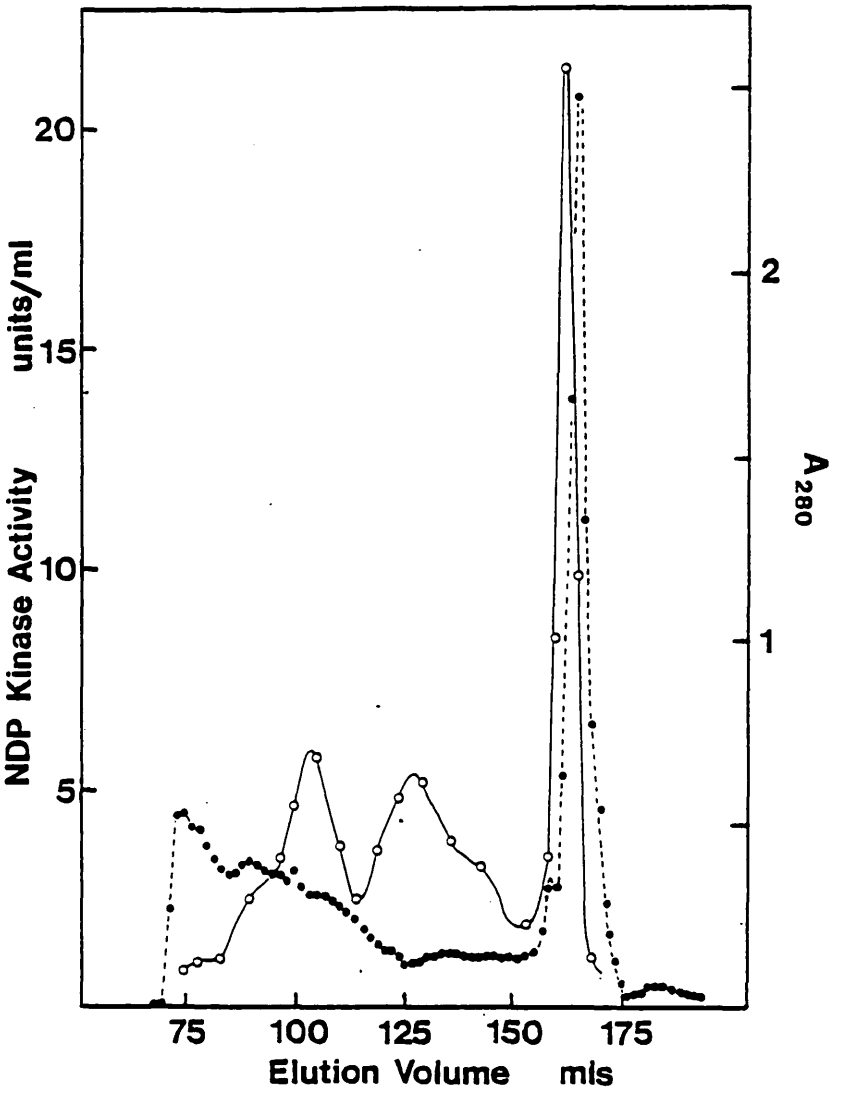


Figure 3.8. Effect of low ionic conditions on the elution of NDPkinase.

Protein was fractionated on an ACA-44 column (see Fig. 3.1) pre-equilibrated in 20 mM MES- 0.5 mM EGTA- 0.1 mM $MgCl_2$- 0.02 mM EDTA- 1.0 mM DTT (pH 6.0) and eluted with this buffer at 14.5 mls/h. Protein concentration $A_{280\,nm}$ (●) and NDPkinase activity (o).

DISCUSSION:

A procedure for the purification of the chick brain NDPkinase has been developed and the enzyme has been characterised by a number of criteria. This procedure permits the rapid purification of the soluble NDPkinase (Table 3.1), leading to a 500-fold increase in the specific activity and a final yield of 30% from the crude extract (Table 3.2). The maximal specific activity of the purified enzyme was 1500 units/mg of protein and fractionation on SDS-PAGE demonstrates the presence of a single protein band with trace contaminants (Fig. 3.2^{F}). While the specific activity and the purity of the enzyme compares favourably with homogenous NDPkinases isolated from other sources (172-174), it should be noted that a direct comparison is not possible as various workers have employed different conditions for the determination of the NDPkinase activity.

NDPkinases purified from bovine brain (172), bovine heart (179) and brewer's yeast (178), have all been demonstrated to be hexameric. In common with these NDPkinases the chick brain NDPkinase also has a native molecular weight of 100,000 daltons (Fig. 3.1) but has a molecular weight of 16,500 daltons by SDS-PAGE (Fig. 3.4), and is presumably also hexameric.

The elution profile of the chick brain NDPkinase on

Ultrogel ACA-44 chromatography shows a remarkable dependence on the pH and ionic conditions employed (Fig. 3.1 and Fig. 3.8). The sensitivity of the chick brain NDPkinase to the pH and ionic conditions has been successfully exploited in developing this purification procedure. For example, the pH and ionic conditions have been optimised to give a single peak of NDPkinase activity, thereby increasing the final yield without a corresponding increase in contamination by other proteins. In view of the anomalous behavior on phosphocellulose chromatography, it should be noted that the pH and ionic conditions are critically important in this procedure. The marked effect of the ionic conditions on the isoelectric state of the brewer's yeast enzyme has been previously noted (177).

Evidence has been presented for the existence of essential accessible sulphydryl groups for NDPkinase activity (Fig. 3.7), a property in common with other NDPkinases (174). However, the chick brain NDPkinase appears to be particularly sensitive to inactivation by pCMB. For example, 90% inhibition of enzyme activity was observed pCMB concentration ca. 10^{-8} M, while equivalent inhibition of other NDPkinases is only observed with pCMB concentration ca. 10^{-4} M (174; 185). The presence of nucleoside di- or tri-phosphate protects the enzyme against inactivation by pCMB. Similar effects of the NDPkinase substrates have been demonstrated previously and

are interpreted as evidence for conformational changes in the enzyme following substrate binding (174).

The apparent electrophoretic heterogeneity of erythrocytic NDPkinases has been demonstrated (176). Examination of the chick brain crude extract shows the presence of two isozymes by chromatofocusing (Fig. 3.3^{A}), but the purified chick brain NDPkinase is demonstrated to be monoisozymic (Fig. 3.3^{B}). However, monoisozymic NDPkinases have been shown to demonstrate heterogenity under certain conditions. Indeed, proteolytic digestion (173), phosphorylation status (179) and ionic conditions (177) have all been found to give rise to NDPkinase heterogenity. The possibility that the more acidic chick brain isozyme is the result of any of these factors has not been excluded. Furthermore, bearing in mind the complex association-disassociation properties of this enzyme, the existence of two isozymes in the crude extract should therefore be treated with caution.

Recently a homogenous monoisozymic bovine brain NDPkinase has been purified (173). A comparison with the properties of the chick brain NDPkinase which is also monoisozymic is highly interesting. Both the bovine and chick enzymes have similar isoelectric points (8.4 and 8.1 respectively). In addition, like the bovine NDPkinase, the chick enzyme is probably hexameric, and both have a subunit molecular weight of 16,500. Furthermore, both the

enzymes have similar kinetic parameters with respect to the utilisation of nucleoside triphosphates. Unfortunately, only the apparent K_m values are reported for the bovine enzyme, which displays the bisubstrate "ping-pong" mechanism, and therefore a direct comparision of the corrected K_m values for the di- and tri-phosphates is not possible.

In view of the similarities of the NDPkinases from these two species, it is interesting that the bovine enzyme was largely particulate while the chick enzyme is mainly soluble. However, the particulate nature of the bovine enzyme is not simply the result of the buffer conditions as under identical conditions the chick enzyme still remains predominantly soluble. While the procedure used for the bovine enzyme results in the purification of nearly homogenous chick enzyme, both the specific activity and the final yields are decreased (Table 3.1 and Table 3.4).

Clearly blue dextran-Sepharose chromatography cannot be employed for the purification of the chick brain NDPkinase of high specific activity. Neverthless, the method provides some interesting data for the comparison of the chick and bovine brain enzymes. While there are obvious differences in the binding properties of the chick and bovine enzymes to the blue dextran-Sepharose, this is not the result of the particulate nature of the bovine

enzyme. The chick brain enzyme isolated from the particulate and the soluble fractions exhibited no differences in binding yet both were purified to the same extent when analysed by SDS-PAGE (Fig. 3.6). The differences in the distribution of the NDPkinases purified from the two species may reflect some age-dependent difference in the nature of the proteins present, rather than any real difference in the NDPkinase.

The chick brain NDPkinase exhibits similar, if not identical, molecular weight by SDS-PAGE irrespective of the purification procedure employed (Fig. 3.6). The phosphocellulose-purified monoisozymic NDPkinase (Fig. 3.3^{B}) exhibits similar physical and chemical properties as the bovine enzyme and NDPkinases isolated from various sources. This purified monoisozymic enzyme is used to characterise the protein-protein interactions of NDPkinase and microtubule proteins (Chapter IV).

CHAPTER IV

NUCLEOSIDE DIPHOSPHATE KINASE AND MICROTUBULES: PROTEIN:PROTEIN INTERACTIONS

INTRODUCTION:

Microtubule assembly *in vitro*, under quasi-physiological conditions, requires the presence of nucleoside triphosphates (58; 60; 156; 191) and microtubule protein isolated from various sources contains associated guanine nucleotides (192-194). Since the original demonstration by Weisenberg et al.(193), it is now generally accepted that there are at least two guanine nucleotide binding sites per tubulin dimer (155; 195-196). These two sites can be further characterised by their binding properties: a readily exchangeable or *E-site* occupied by GTP or GDP (77; 197) and a non-dissociating or *N-site* containing GTP under most circumstances (156; 198).

Spiegelman et al. (73) demonstrated the non-dissociating nature of the N-site and calculated a half-life for decay of 33 hours in Chinese Hamster Ovary cells. Microtubules assembled *in vitro* through successive cycles of polymerisation and depolymerisation contain GTP at the N-site demonstrating that this GTP is not hydrolysed during polymerisation (73; 156; 198-200; 213). These results suggest that the N-site may function as a stable structural co-factor and the long half-life renders improbable a regulatory role in microtubule assembly.

While GTP promotes microtubule assembly under physiological conditions, GDP generally inhibits assembly

(59-60; 75; 156). Although GDP can block microtubule assembly or elongation, addition of GDP to pre-assembled microtubules does not result in their depolymerisation. However, the critical concentration for polymerisation, determined by dilution, in the presence of GTP or GDP is not significantly different (59; 202-203; 217).

The GTP on the E-site is hydrolysed during polymerisation, but the resultant GDP remains at this site when the tubulin is incorporated into the microtubule, and is then non-exchangeable (199). This GTPase activity during tubulin polymerisation is a characteristic of the tubulin molecule itself (200). A kinetic analysis of tubulin polymerisation and GTP hydrolysis indicates that the two processes are concommitant but are not strictly coupled (201).

Microtubule assembly can also be induced by non-hydrolysable analogues of GTP, such as GMPPCP and GMPPNP, further supporting the evidence that GTP hydrolysis is not strictly coupled to the assembly process (156; 198; 204-205). However, analogue-assembled microtubules exhibit greater stability: they are depolymerised to a lesser extent by calcium ions (204), low temperature (156), and by dilution (205). Therefore GTP may stabilise intersubunit bonds between adjacent tubulin molecules and the hydrolysis of the E-site GTP may be related to the disassembly properties of the

microtubules (196; 206).

GTP, ATP, CTP and UTP at equivalent molar concentrations generally support similar levels of polymerisation (127; 156; 218). However unlike GTP, neither ATP, nor CTP or UTP show any significant binding to assembly-competent tubulin. Furthermore, these nucleoside triphosphates do not compete for the GTP binding sites on the tubulin dimer (77; 208-210). The dissociation constant for GTP is around three orders of magnitude higher than ATP (155; 219).

Under conditions where the E-site GTP has been hydrolysed, tubulin polymerised with ATP shows a longer lag phase compared to tubulin polymerised with GTP. The lag phase is even longer with lower ATP concentrations or with more highly purified tubulin (198). Similarly UTP and CTP which induce tubulin polymerisation both show this characteristically longer lag-phase (156; 213). The lag-phase has been interpreted to be the result of a slow enzymatic reaction, namely the phosphorylation of the E-site GDP to GTP by the NDPkinase using the nucleoside triphosphates as substrates (156).

Jacobs et al. (197) suggested that ATP acts as a substrate for a transphosphorylase, NDPkinase, activity which generates GTP on the N-site, the GTP which is subsequently hydrolysed during this ATP promoted

polymerisation. It had earlier been proposed that the E-site GTP acts as a substrate for the phosphorylation of the N-site (195). However, assembly-competent tubulin contains GTP at the N-site and this GTP is not hydrolysed in the assembly process. A possible explanation for the above observations of N-site transphosphorylation can be that though the E-site nucleotide is freely exchangeable on tubulin dimers, it is only slowly exchangeable on tubulin in the oligomeric ring form (199; 211-212).

The NDPkinase can be separated from tubulin (156; 211) and from the high molecular weight and tau MAPs (211). Polymerisation of DEAE-purified tubulin, which is free of NDPkinase activity, can only be promoted by ATP on the addition of NDPkinase (200; 211). In contrast Zabrecky and Cole (219) found that ATP can induce polymerisation of DEAE-purified tubulin, provided that GTP was present but at concentrations which could not by itself induce polymerisation. They proposed that ATP binds to tubulin at a site which is distinct from the E- or N-site for GTP, and that the K_d of ATP is within an order of magnitude of the cellular ATP levels.

Penningroth and Kirschner (156) have demonstrated that UTP can generate GTP by an enzymatic reaction mediated by NDPkinase, and the UTP is not utilised by the protein kinase and myokinase activities present in their microtubule preparations. In order to determine the site

at which the NDPkinase-generated GTP is located, they incubated microtubule protein either with [^{3}H] GTP or with [^{32}P] UTP and separated the protein bound GTP by gel filtration. Under these conditions an equivalent amount of [^{3}H] GTP and [^{32}P]GTP was bound to the protein. However, as the N-site GTP is essentially non-exchangeable, repeating the binding assay with the gel filtration column equilibrated with unlabelled GTP, about 90% of both the [^{3}H] and [^{32}P] bound GTP was displaced from the tubulin. These results clearly demonstrate that the NDPkinase-generated GTP was bound to the E-site on the tubulin dimer.

To determine as to whether the NDPkinase generates GTP directly on the E-site or at a site which is physically separated from the tubulin, these authors compared the rates at which the NDPkinase generated GTP and exogenous GTP exchange onto tubulin. Increasing concentrations of [^{32}P] UTP were incubated with the microtubule protein for 2 mins at 37°C, to achieve increasing concentrations of NDPkinase generated [^{32}P] GTP, and the protein-bound nucleotide was separated by gel filtration. Similarly, microtubule protein was incubated with increasing concentrations of exogenous [^{3}H] GTP for 2 mins at 37°C and the protein-bound GTP was determined after gel filtration. The recovery of the protein-bound [^{32}P] and [^{3}H] GTP was plotted as a function of the total [^{32}P] GTP concentration determined after the 2 mins

incubation period and the [^{3}H] GTP added at zero time. By comparing the rates these authors concluded that the NDPkinase-generated GTP exchanges onto tubulin about an order of magnitude more efficiently. Although the NDPkinase could use free GDP as a substrate, these authors calculated that the affinity of the enzyme for the tubulin-bound GDP is three-fold higher, suggesting that the phosphorylation of the tubulin-bound GDP is the preferred pathway of the NDPkinase action.

However, an alternative interpretation can also describe the results presented by Penningroth and Kirschner (156). The rates of GTP and GDP binding and release are sufficiently fast relative to microtubule assembly to allow for GDP release, phosphorylation, and GTP binding to be the sole mechanism for NDPkinase action (220). The model proposed by Penningroth and Kirschner (156) would predict that low concentrations of exogenous GDP should increase the observed lag-phase for assembly, as the free GDP would compete as the alternative substrate for NDPkinase. However, analysis of the lag-phase at low exogenous GDP concentrations demonstrate a decrease in the lag-phase to a plateau value (219). Furthermore, when acetatekinase (221) or pyruvate kinase (10) is utilised as a GTP-regenerating system efficient microtubule assembly has been observed even under conditions when free GDP has been removed.

The proposed model of NDPkinase-mediated phosphorylation of the tubulin-bound GDP on the E-site has two inherent assumptions:

(a) that there must be protein-protein interactions between the NDPkinase and microtubule proteins.

(b) that the microtubule assembly effected by nucleoside triphosphates, other than GTP, is mediated by the NDPkinase.

In this chapter evidence for the protein-protein interactions is considered, and the role of the nucleoside triphosphates will be considered in chapter V.

The recovery of significant amounts of NDPkinase with microtubules assembled in vitro would be predicted if NDPkinase-tubulin protein-protein interactions exist. This has been observed (156; 211; 214-216). Similarly isolated Chlamydomonas flagella have associated NDPkinase activity which cannot be released by extensive washing, suggesting that it is an axonemal protein (216).

The NDPkinase activity migrates as a protein of M_r 100,000 daltons on gel-exclusion chromatography (211 and Chapter III). At least part of the NDPkinase activity co-migrates with the oligomeric ring fraction when cold-dissociated microtubule protein is fractionated by gel-filtration (211).

In order to examine the validity of these proposed models three approaches have been employed:

(a) The recovery of the NDPkinase activity with microtubules through cycles of polymerisation is examined.

(b) the chick brain NDPkinase has been purified and characterised with respect to NDPkinases from various sources (see Chapter III). The chick brain NDPkinase shows properties in common with other NDPkinases, and this phosphocellulose-purified monoisozymic NDPkinase has been used to examine the protein-protein interactions of the enzyme with microtubule proteins by centrifugation and gel filtration (Chapter IV)

(c) the nucleotide requirements for microtubule assembly have been re-examined (see Chapter V).

RESULTS:

NDPkinase and Microtubules

If the NDPkinase is a microtubule associated protein or "MAP" and is required for microtubule assembly, then it would be expected to be recovered with microtubules through cycles of polymerisation, in some ratio to the concentration of the free enzyme and its requirement for assembly.

Most microtubule purification procedures use reassembly buffers between pH 6.4 and pH 6.9 (6-12; 59-60) and the pH conditions may influence the NDPkinase-microtubule interaction. The recovery of the NDPkinase with assembled microtubules was therefore examined using MA buffer at pH 6.4 and pH 6.9. The specific activity of the NDPkinase recovered with the assembled microtubules decreases approximately exponentially through successive cycles of polymerisation (Fig.4.1). However, no significant differences in the recovery of the NDPkinase were observed between preparations assembled at pH 6.4 and pH 6.9. All subsequent experiments employ MA buffer at pH 6.4 routinely used for the preparation of the chick brain microtubule protein.

The purified chick brain NDPkinase has been

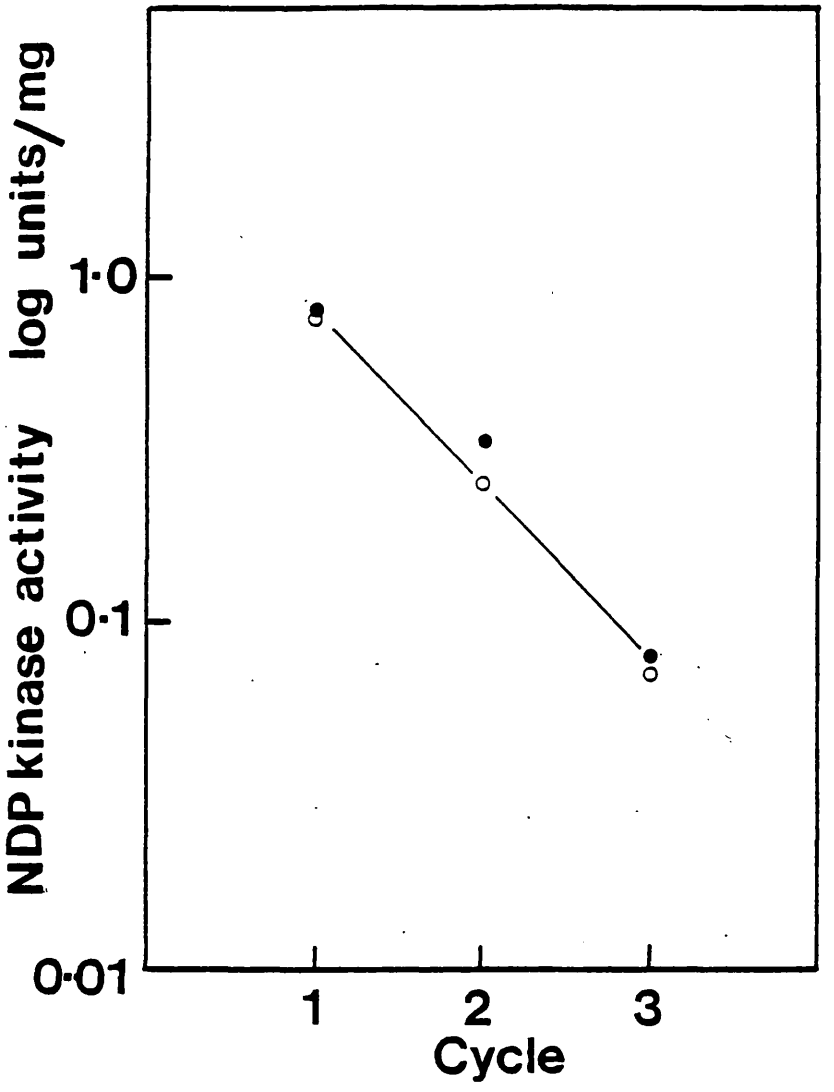


Figure 4.1. Recovery of the NDPkinase as a function of the cycle of polymerisation.

Microtubules were prepared in MA-buffer adjusted to pH 6.4 (o) or at pH 6.9 (●).

demonstrated to be non-specific with regard to its substrates, and can efficiently utilise the purine nucleoside triphosphates. GTP is required for microtubule assembly and can bind efficiently to tubulin under conditions where ATP is reported to show no significant binding (77; 156; 208-210). The nucleoside triphosphate may therefore define whether the NDPkinase is required for microtubule assembly and consequently affect the recovery of the NDPkinase with assembled microtubules. For example, while the GTP may exchange directly with the tubulin-bound GDP, ATP may utilise the NDPkinase mediated pathway to promote microtubule assembly. The recovery of the NDPkinase with microtubules assembled in the presence of ATP or GTP was examined as a function of the cycle of polymerisation. The specific activity of the microtubule protein decreases through successive cycles of polymerisation, but no significant differences in the recovery between ATP and GTP preparations were observed (Fig. 4.2).

The recovery of the NDPkinase was found to vary to some extent between preparations and this could reflect in the concentration of the microtubule protein. The recovery of the NDPkinase was examined in greater detail as a function of the initial microtubule protein concentration.

1x cold-dissociated microtubule protein was

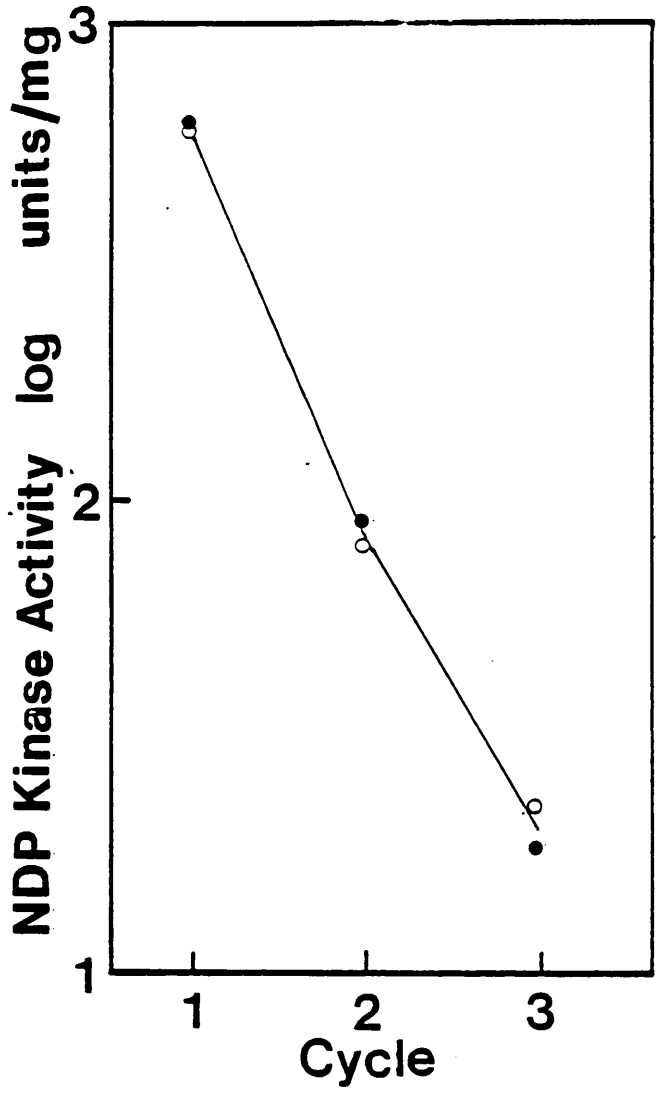


Figure 4.2. Recovery of the NDPkinase as a function of the cycle of polymerisation.

Microtubule assembly was effected by the presence of GTP (o) or ATP (●).

assembled with 1 mM GTP at increasing protein concentrations. The assembled microtubules were then pelleted, cold-dissociated and centrifuged to remove any cold-stable aggregates. An increasing amount of protein was recovered at increasing initial protein concentration (Fig. 4.3) and two components were observed with a critical concentration of 0.3 $mg.ml^{-1}$ and a second apparent critical concentration of 1.1 $mg.ml^{-1}$. The recovery of the NDPkinase also shows two components, but in contrast to the protein, the first component was extrapolated through the origin while the second component shows a critical concentration of 1.1 $mg.ml^{-1}$.

When the recovery of the NDPkinase was examined as a function of the initial NDPkinase activity, two components were observed (Fig. 4.4). At low initial activities approximately 4% of the NDPkinase activity was recovered with the microtubule protein. However, at high initial NDPkinase activities, the recovery increases to 10% of the initial activity. Two components for the NDPkinase recovery were observed as a function of the protein concentration (Fig.4.3) and two components are also observed as a function of the initial NDPkinase concentration. This suggests that there are two linear components with a critical concentration of 1 $unit.mg^{-1}$ for the second component(Fig. 4.4).

However, while the specific activity of the

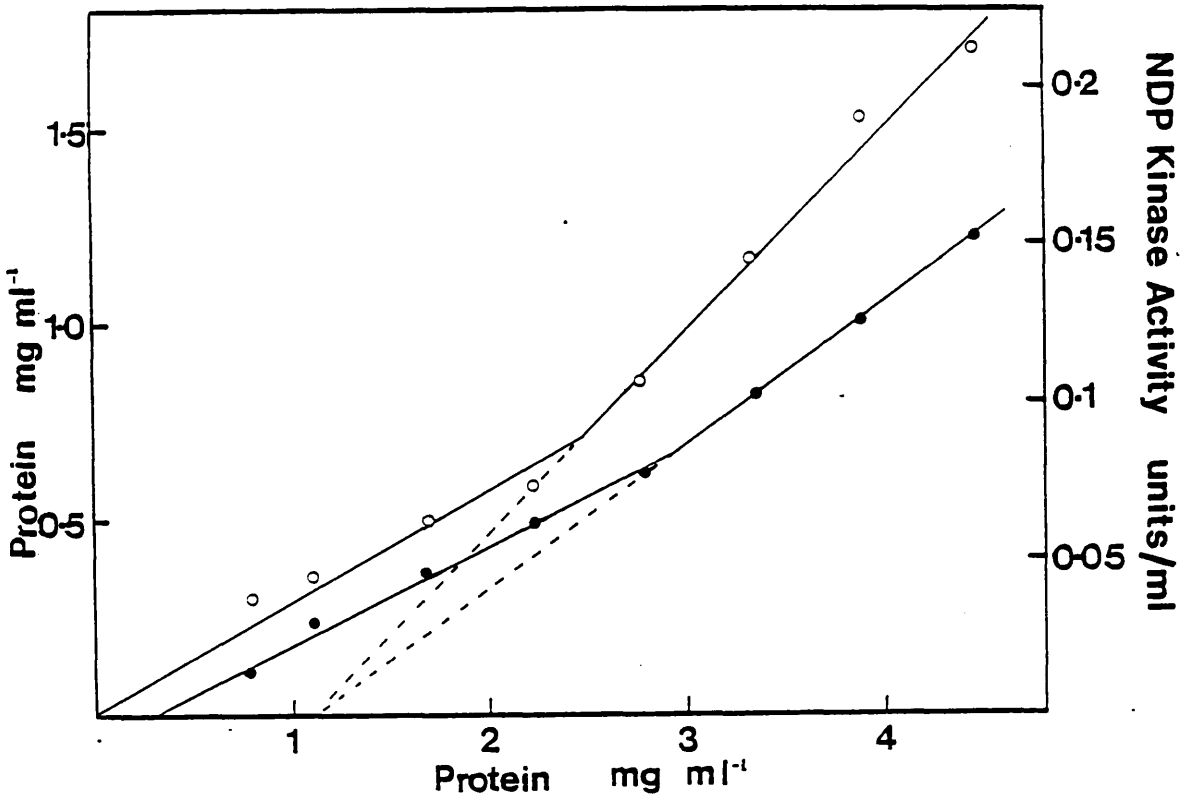


Figure 4.3. Recovery of NDPkinase (o) and protein (●) as a function of the initial protein concentration.

1x microtubule protein was assembled at increasing protein concentrations with 1 mM GTP.

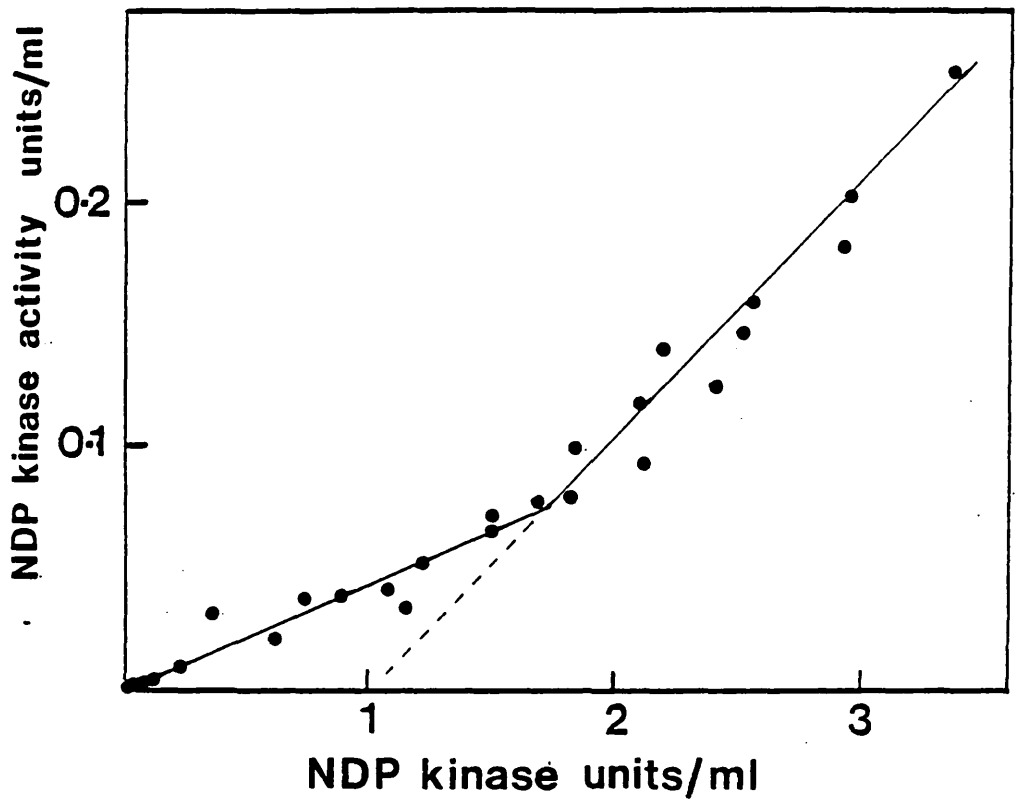


Figure 4.4. Recovery of the NDPkinase as a function of the initial NDPkinase activity of 1x microtubule protein.

Data from four different preparations of 1x microtubule protein.

microtubule protein decreases through successive cycles of assembly (Fig. 4.1 and 4.2) Penningroth and Kirschner (156) have observed a low but constant amount of NDPkinase through three cycles of polymerisation. Interestingly, Penningroth and Kirschner have washed the microtubule pellets with warm buffer and the observed differences in the specific activity may be the result of this difference in procedures. The observation of two components in the recovery of the NDPkinase may suggest the association of the enzyme with the material which may be removed by the washing procedure employed by Penningroth and Kirschner.

SDS-PAGE of successive cold-dissociated microtubule protein shows that tubulin and the MAP_2 are the major proteins present (Fig. $4.5^{A,C,E}$). By contrast the warm supernatants show the complete absence of MAP_2, but the preferential recovery of other bands (Fig. $4.5^{B,D}$). Therefore, proteins which promote microtubule assembly, for example MAP_2, are preferentially recovered with the microtubules through successive cycles of polymerisation. However, contaminating proteins which may be recovered in the 1x microtubule protein are selectively lost following further purification of the microtubules.

Therefore the recovery of the NDPkinase with assembled microtubules was further examined with more highly purified 2x microtubule protein and purified chick brain NDPkinase. In these experiments the possible

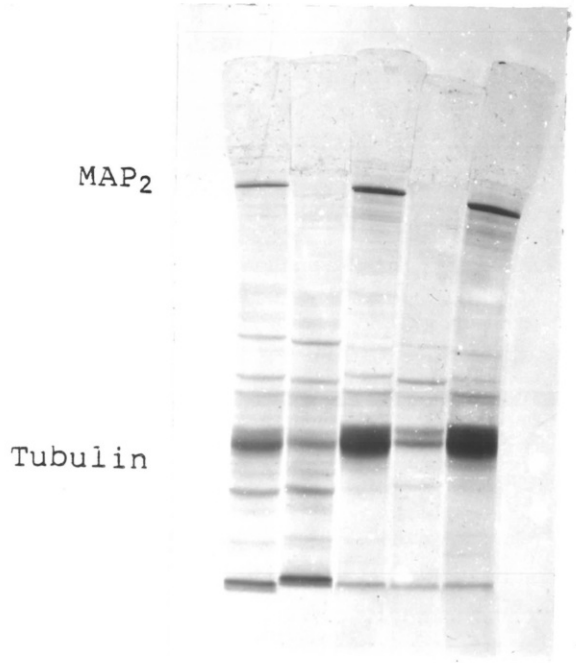


Figure 4.5. SDS-PAGE analysis of microtubule protein through cycles of polymerisation.

The protein was fractionated on 8% acrylamide gels, tubulin and MAP_2 proteins are indicated. The gels correspond to (A) 1x cold-dissociated microtubule protein (B) the warm supernatant following the second cycle of polymerisation (C) the 2x cold-dissociated microtubule protein (D) the warm supernatant from the third cycle (E) The 3x cold-dissociated microtubule protein.

entrapment of the NDPkinase was minimised by pelleting the assembled microtubules through a sucrose cushion (see Chapter II). Under these centrifugation conditions (100,000 x g, 30 mins) any oligomeric protein species or the free NDPkinase would fail to sediment with the microtubules.

2x microtubule protein with a specific activity of 0.25 units.mg^{-1} was assembled with 1 mM GTP at increasing protein concentrations in the absence and presence of 5 units of purified monoisozymic NDPkinase, and then pelleted through the sucrose cushion. The pellets were homogenised in cold MA buffer and the NDPkinase activity determined. The recovery of the microtubule protein shows a single component which extrapolates through the origin and the inclusion of purified NDPkinase does not affect the recovery of the microtubule protein (Fig. 4.6). The NDPkinase activity pelleted with the microtubule protein also exhibits a single component which can be extrapolated through the origin and has a specific activity of 0.02 units.mg^{-1}. However, the inclusion of 5 units of purified NDPkinase activity, which would be predicted to enrich the NDPkinase activity of pelleted microtubule protein, does not result in an increased amount of pelleted NDPkinase activity.

As mentioned previously the nucleotide conditions may define whether the NDPkinase is required for microtubule

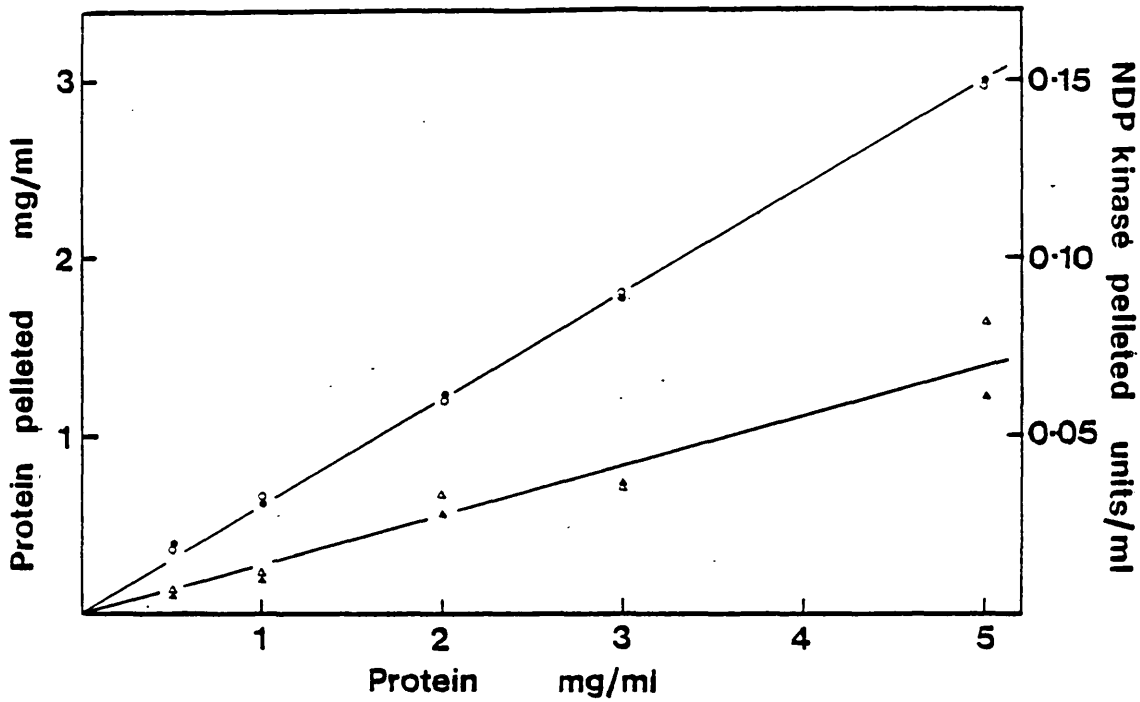


Figure 4.6. Recovery of NDPkinase (▲ ; △) and protein (o ; ●) as a function of the initial 2x microtubule protein concentration, centrifuged through a sucrose cushion.

Increasing concentrations of protein were assembled with 1 mM GTP in the absence (● ; ▲) and presence (o ; △) of 5 units of purified monoisozymic NDPkinase.

assembly and alter the recovery of the enzyme with assembled microtubules. Incubation of microtubule protein with low concentrations of GDP would increase the proportion of tubulin-bound GDP and would be predicted to increase the requirement for the NDPkinase. Similarly, if ATP utilises the NDPkinase-mediated pathway to promote microtubule assembly it may alter the NDPkinase recovery with pelleted microtubules.

The recovery of the NDPkinase with assembled microtubules was therefore examined under various nucleotide conditions. 2x microtubule protein was charcoal-treated which results in the removal of the free GDP and some of the E-site GDP (156). Analysis showed that the charcoal-treated microtubule protein contains 1.6 mole guanine nucleotide per mole tubulin. This protein was then incubated with either GTP, or ATP, or ATP and GDP, or GDP alone, and 5 units of purified monoisozymic NDPkinase. The microtubules were assembled and centrifuged through a sucrose cushion, the pellets were homogenised in cold MA buffer and the NDPkinase activity of the pellets determined (Table 4.1). No assembly and consequently no pellets were recovered with GDP alone. No significant differences in the total amount of NDPkinase activity pelleted with the assembled microtubules were observed under the various nucleotide conditions.

TABLE 4.1. RECOVERY OF THE NDP KINASE WITH MICROTUBULES THROUGH A SUCROSE CUSHION UNDER VARIOUS NUCLEOTIDE CONDITIONS.

Nucleotide conditions	Recovery of NDP kinase activity		
	total units	units. mg^{-1}	% of initial activity
100 µM GTP	0.0098	0.023	0.18
1 mM ATP	0.0088	0.041	0.16
1 mM ATP + 100 µM GDP	0.0084	0.038	0.15
100 µM GDP	-----	-----	----

NDPkinase and Tubulin Oligomers:

Two components of NDPkinase activity were observed with 1x microtubule protein (Fig. 4.4), while only a single component with a low specific activity was observed when 2x microtubule protein pelleted through a sucrose cushion was analysed (Fig. 4.6). However, electron microscopy shows the presence of rings as the predominant tubulin polymeric species apart from microtubules, which suggests that if the NDPkinase binds to tubulin, it may bind to these oligomers. Jacobs and Huitorel (211) have shown that NDPkinase activity is recovered with tubulin oligomers following separation of the oligomers from the tubulin dimers and the free enzyme.

Fractionation of microtubule protein by gel exclusion chromatography has been shown to separate the 36 S rings, composed of tubulin and MAPs, from the 6 S tubulin dimers (see ChapterI). 1x cold-dissociated microtubule protein of high NDPkinase specific activity was fractionated on a Biogel A 15m column, pre-equilibrated with MA buffer and the recovery of the protein and NDPkinase activity assayed in the fractions (Fig. 4.7). The tubulin oligomers, normally only rings were observed by electron microscopy, eluted in the void fraction and constitute approximately 60% of the total protein.

A second peak of protein which constitutes

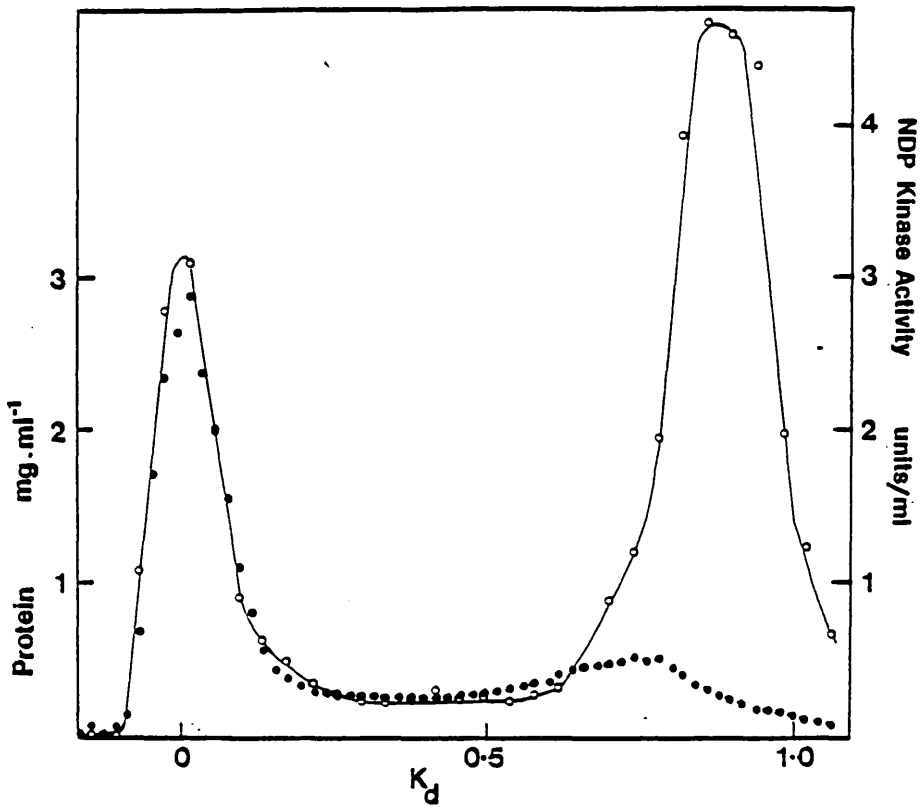


Figure 4.7. Fractionation of the 1x microtubule protein on a Biogel A 15m column (1.6 x 40 cms).

The column was pre-equilibrated in MA buffer and was eluted at 4°C. Distribution of protein (●) and NDPkinase (o).

approximately 20% of the total protein,composed primarily of tubulin, is eluted close to the salt volume. There is a significant background of protein between the two peaks which may in part reflect the equilibrium between the oligomeric and dimeric tubulin species, following the separation of the oligomer from the tubulin dimers.

Analysis of the NDPkinase activity also indicates that the enzyme is present in all the fractions between the void and salt volumes. However, in contrast to the protein distribution only 28% of the total NDPkinase activity eluted in the void fraction, while 63% of the enzyme activity eluted just after the tubulin dimer peak. This clearly demonstrates that there is no interaction between the tubulin dimer and the NDPkinase, which would be expected to elute with an apparent molecular weight of 200,000. The specific activity of the void fraction was calculated to be 1.036 units.mg^{-1} and shows that the some of the NDPkinase activity recovered with the 1x microtubule protein co-elutes with the tubulin oligomers. Furthermore, the specific activity of the protein between the void and tubulin dimer peak was calculated to be 0.945 units.mg^{-1}. This similarity in the specific activity with that of the oligomeric fraction is consistent with an interaction of the NDPkinase with the oligomeric species, which itself is in equilibrium with the tubulin dimers.

1x microtubule protein shows the presence of various

contaminating proteins which are removed following a second cycle of polymerisation (Fig. 4.4^{A-C}). To further examine the interaction of the NDPkinase with the tubulin oligomers, 2x microtubule protein with a low NDPkinase specific activity and purified monoisozymic NDPkinase was used.

The microtubule protein was charcoal-treated to remove the free nucleotides. The charcoal-treated 2 x microtubule protein was then incubated with 3.5 units of monoisozymic NDPkinase and with either 100 µM GDP, 100 µM GDP and 1 mM ATP, or with 1 mM ATP, conditions which would be predicted to result in maximal NDPkinase interaction, and fractionated on a Biogel A 1.5m column. The column fractions were assayed for protein and NDPkinase activity (Fig. 4.8^{A-C}). While 55-60% of the total protein was eluted in the void fraction, as observed for the 1x microtubule protein, only a trace amount of NDPkinase was detected. Most of the NDPkinase activity was recovered just after the tubulin dimer protein peak. The observed distribution was unaffected by the nucleotide conditions.

If the GDP bound to the E-site exchanges rapidly most of the bound GDP would be displaced from the tubulin binding site during the elution time of the protein. Consequently a Hummel and Dryer (122) type experiment was performed in the presence of GDP.

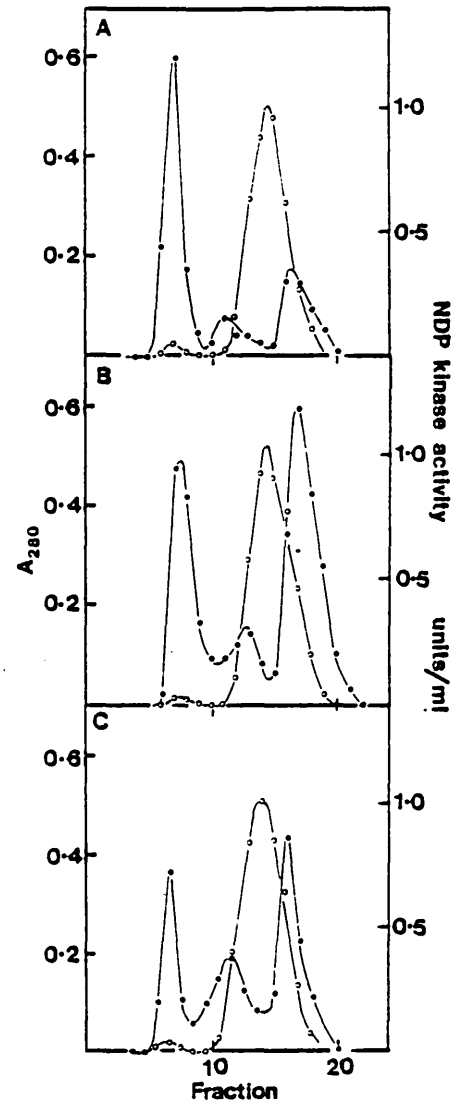


Figure 4.8. Fractionation of charcoal-treated 2x microtubule protein on a Biogel A 1.5m column (0.9 x 25 cms).

The protein was incubated with 3.5 units of purified monoisozymic NDPkinase in the presence of (A) 100 µM GDP (B) 100 µM GDP + 1 mM ATP and (C) 1 mM ATP. The column was pre-equilibrated in MA buffer and was eluted at 4°C. Distribution of protein (●) and NDPkinase (o).

Charcoal treated 2 x microtubule protein with a specific activity of 0.086 units.mg^{-1} was incubated with 0.11 unit.ml^{-1} purified monoisozymic NDPkinase activity and 100 µM GDP. The protein was then fractionated on a Biogel A 1.5m column pre-equilibrated in MA buffer containing 0.11 units.ml^{-1} NDPkinase activity and 100 µM GDP. The column was then eluted with the equilibration buffer and the fractions assayed for protein and NDPkinase activity (Fig. 4.9). On such a Hummel and Dryer type experiment a peak of activity should be followed by a trough between the void and the salt volumes.

The specific activity of the void fraction was only 0.053 units.mg^{-1}, showing no increase in the amount of NDPkinase activity co-migrating with the tubulin oligomers. No trough was observed suggesting that the NDPkinase activity on the column did not exchange with the protein.

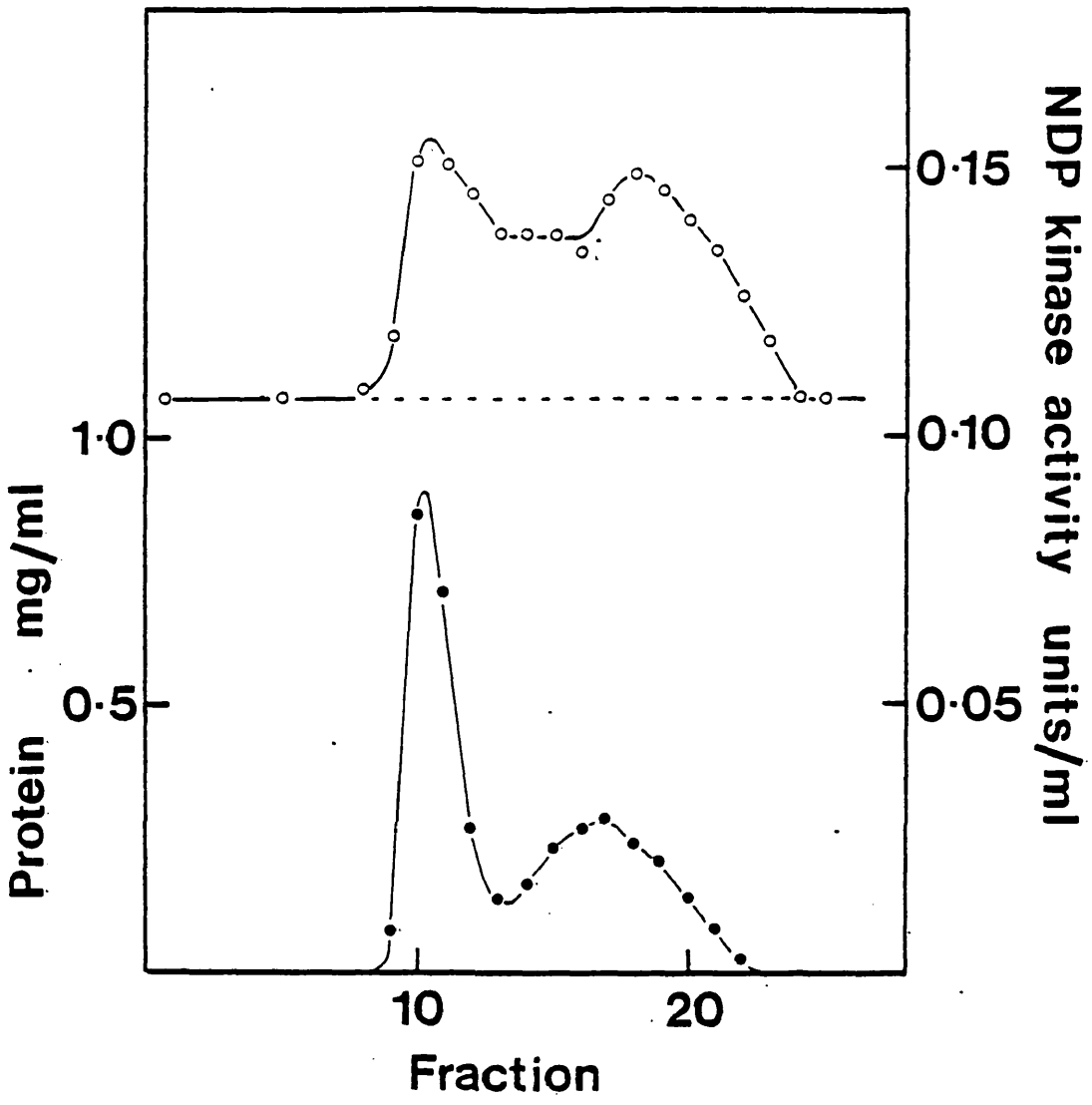


Figure 4.9. A Hummel and Dryer experiment.

Charcoal-treated 2x microtubule protein was fractionated on a Biogel A 1.5m column (0.9 x 25 cms). The column was pre-equilibrated in MA buffer containing 100 µM GDP and 0.11 units.ml^{-1} purified monoisozymic NDPkinase and eluted at 4°C. Distribution of the protein (●) and NDPkinase (○).

DISCUSSION:

Microtubules assembled by cycles of polymerisation show that the NDPkinase activity is selectively lost through successive cycles of polymerisation. The pH and the nucleoside triphosphate conditions do not significantly affect the observed recovery of the NDPkinase (Fig. 4.1 and Fig. 4.2). Nickerson and Wells (215) have also observed a similar decrease in the specific activity of the NDPkinase recovered with reassembled microtubules following cycles of polymerisation. While the observed recovery in the first cycle of polymerisation is similar to that observed by Nickerson and Wells (215), it is significantly higher than that observed by Penningroth and Kirschner (156) and Watanabe and Flavin (216). However, a direct comparison is not possible as the conditions of assay for the NDPkinase activity differ.

Two components of protein and NDPkinase activity were observed as a function of the initial protein concentration, using 1x microtubule protein (Fig. 4.3). However, while the recovery of the microtubule protein shows a critical concentration, at low microtubule protein concentrations, the recovery of the NDPkinase activity extrapolates through the origin. The NDPkinase appears to be recovered independently of the microtubules.

The recovery of the NDPkinase activity as a function of the initial enzyme activity also shows two components (Fig.4.4). However, while the first component extrapolates through the origin, the second component shows an apparent critical concentration of about 1 unit.ml^{-1}.

Under centrifugation conditions, where assembled microtubules can be separated from other protein components, for example using a sucrose cushion, only a single component of NDPkinase activity is observed and extrapolates through the origin (Fig. 4.6). However, the recovery of the low specific activity of the NDPkinase is not affected by the inclusion of purified NDPkinase. Charcoal-treated microtubule protein assembled in the presence of purified NDPkinase under a variety of nucleotide conditions also shows that the total amount of NDPkinase co-purifying with the microtubules is unaffected by the nucleotide environment (Table 4.1).

The recovery of two components as a function of the protein concentration, using 1x microtubule protein, would suggest an interaction between tubulin and the NDPkinase (Fig. 4.3). However, while microtubule protein consists of tubulin and MAPs, there are a number of additional proteins which are selectively lost following further cycles of polymerisation (Fig. 4.5^{A-F}). The observation that only a single component is observed with more highly purified microtubule protein (Fig. 4.6) strongly suggests

that the observed recovery of the NDPkinase may be the result of NDPkinase interaction with some component other than microtubules. The observed recovery of the NDPkinase with the tubulin oligomers following fractionation of 1x and 2x microtubule protein also suggests the interaction of the NDPkinase with some component other than tubulin oligomers (Fig. 4.7 and Fig. 4.8).

Nickerson and Wells (215) calculated that the observed decrease in the NDPkinase activity through successive cycles of polymerisation is less than the predicted decrease by dilution of a soluble enzyme. By contrast, Penningroth and Kirschner (156) have reported a low but constant amount of NDPkinase activity through three cycles of polymerisation. However, these authors have washed their pelleted microtubules, which may result in the loss of the NDPkinase-binding component.

If the free NDPkinase is in equilibrium with the oligomeric protein, then the Hummel and Dryer type experiment should result in a peak of NDPkinase activity in the void fraction, followed by a trough of activity further down the column. However, no increase in the specific activity of the oligomeric fraction, or a trough of activity was observed, even when the column was equilibrated with conditions which might be expected to be optimal for maximal NDPkinase interaction, suggesting no binding of the NDPkinase to the tubulin oligomers

(Fig. 4.9).

It may be argued that the Hummel and Dryer experiment would not show any NDPkinase-tubulin interaction if the proposed model of Penningroth and Kirschner (156) is correct. However, Terry and Purich (220) have demonstrated that competitive inhibition studies with free GDP do not conform to the proposed model. In addition, the NDPkinase functions via a bisubstrate "ping-pong" mechanism. Therefore the NDPkinase must be phosphorylated prior to its binding to tubulin. Yet, if the NDPkinase is phosphorylated then it would phosphorylate the tubulin-bound GDP and consequently show no further interaction with tubulin.

No NDPkinase interactions with microtubules or tubulin oligomers under conditions which result in highly purified microtubule protein were observed. However, it appears that the NDPkinase may interact with some component which partially co-purifies with microtubules. At least two reports have shown that proteins (67) and membrane vesicles and an ATPase activity (223) partially co-purify with microtubules through cycles of assembly. The recovery of these proteins and other components with the microtubules has been suggested to be the result of microtubule interactions with other components, for example neurofilaments (67) and membrane vesicles (223).

Failure to observe any specific protein-protein interactions between the NDPkinase and microtubule proteins suggests that the role of the NDPkinase is not related to phosphorylating the E-site GDP *in situ*. However, the microtubule assembly effected by the nucleoside triphosphates such as ATP or CTP may still be mediated by the NDPkinase. The nucleotide requirements for microtubule assembly are therefore examined in chapter V to determine the role of the NDPkinase in microtubule assembly.

CHAPTER V

NUCLEOSIDE DIPHOSPHATE KINASE AND MICROTUBULES: NUCLEOTIDE REQUIREMENTS

The reported requirements for nucleoside triphosphates in microtubule assembly have already been described in Chapter IV (see Introduction). In this chapter the requirements of nucleoside triphosphates GTP, ATP and CTP in promoting microtubule assembly are examined and the results are discussed with respect to the role and requirement of the NDPkinase in microtubule assembly.

RESULTS:

Sedimentation of assembled microtubules through the sucrose cushion results in the removal of free NDPkinase and the recovery of NDPkinase with the microtubules of low specific activity (see Chapter IV). For the studies on nucleotide requirements, 2x microtubule protein was routinely assembled with 1 mM GTP and centrifuged through the sucrose cushion. The pellets homogenised in cold MA buffer, cold-dissociated and centrifuged to remove any cold-stable aggregates. Fractionation of microtubule protein by SDS-PAGE showed that 80% of the total protein was composed of tubulin and 20% was composed of MAP_2 (Fig. 5.1). The co-pelleting NDPkinase has a specific activity of 0.015-0.02 units.mg^{-1}. Protein of this quality was used to examine the kinetics of microtubule assembly with GTP, ATP and CTP.

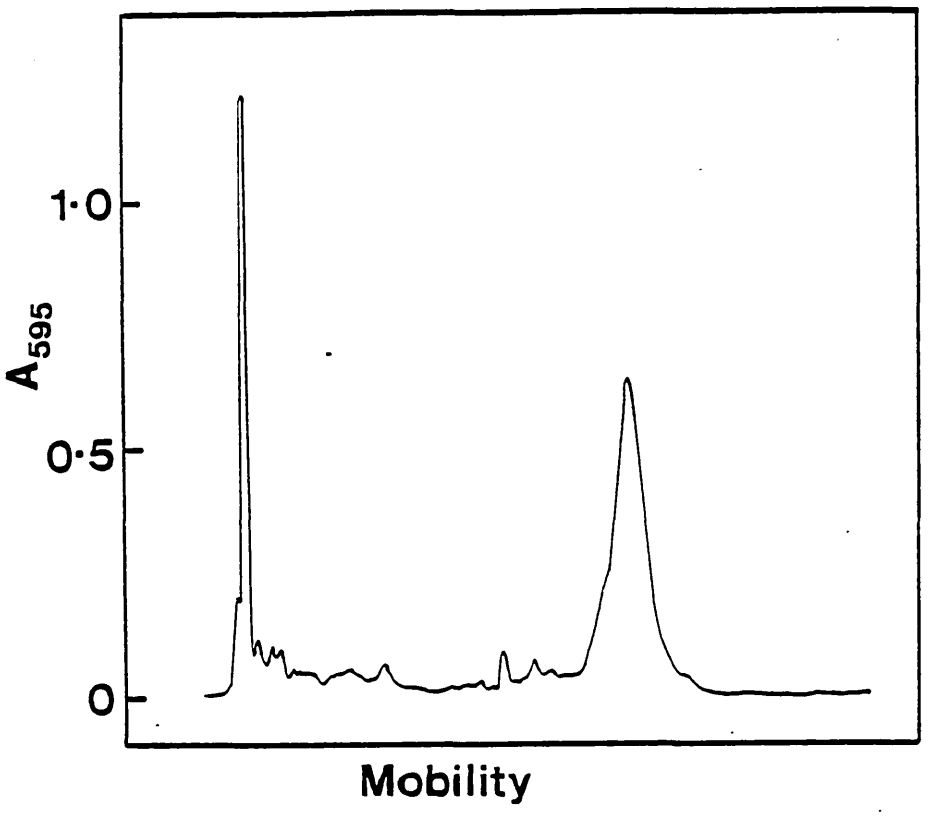


Figure 5.1. SDS-PAGE analysis of sucrose cushion microtubule protein.

The protein was fractionated on 8% acrylamide gels, stained with Coomassie brilliant blue, destained and scanned at $A_{595\,nm}$.

The possibility that the NDPkinase activity which co-pellets with the assembled microtubules is different to the purified monoisozymic NDPkinase has already been discussed (see Chapter III). However, since nucleoside triphosphates act as substrates for the NDPkinases, this co-pelleting activity must be considered in any interpretation of the role of specific nucleoside triphosphates. The efficiency of utilisation of various triphosphates by this co-pelleting NDPkinase was therefore examined, assaying the activity in the presence of 2.2 mM nucleoside triphosphates and 0.7 mM TDP. The utilisation of the triphosphates by the NDPkinase present in these microtubule protein preparations is similar to that observed with the purified monoisozymic NDPkinase (Table 3.3 and 5.1). The NDPkinase efficiently utilises the purine nucleotides but demonstrates poor reactivity with the pyrimidine nucleotides.

The nucleotide content of the sucrose cushion microtubule protein, determined by perchloric acid treatment, was normally between 1.7-2.0 mole guanine nucleotide per mole tubulin dimer. Separation of the nucleotides on a PEI-column showed that 45% of the total nucleotide exists as GDP and approximately 50% as GTP. Therefore the microtubule protein contains up to 0.8-0.9 mole GDP and 0.9-1.0 mole GTP per mole of tubulin dimer.

TABLE 5.1. EFFICIENCY OF UTILISATION OF THE TRIPHOSPHATES BY NDP KINASE CO-PURIFIED WITH MICROTUBULES.

Nucleoside Triphosphate	% ATP-TDP Exchange
ATP	(100)
GTP	42
ITP	38
UTP	6
CTP	0

Microtubule Assembly: GTP

The kinetics of microtubule assembly were examined as a function of the GTP concentration to determine the GTP requirement for tubulin polymerisation.

Microtubule protein at 1 $mg.ml^{-1}$ was degassed and following a 5 mins incubation at 30°C, polymerisation was initiated by the addition of increasing concentrations of GTP. The GTP was stored in liquid nitrogen, thawed and used immediately to minimise any GTP degradation. The polymerisation kinetics showed the characteristic lag period, the nucleation phase, which was longer at low GTP concentrations. Rapid polymerisation occured to a final plateau value after the lag period. Electron microscopy confirms the presence of microtubules at all concentrations of GTP above 4 μM GTP and suggests that the observed increase in turbidity is due to microtubule formation. The microtubules formed were morphologically "normal" and a typical example is shown in Fig. 5.2^{B}.

A plot of the final plateau value as a function of the GTP concentration shows that the final extent of assembly is virtually independent of the GTP concentration above 10 μM GTP (Fig. 5.3). There is though a minimal requirement of approximately 4 μM GTP, while high GTP concentrations result in a slight decrease in the final plateau value.

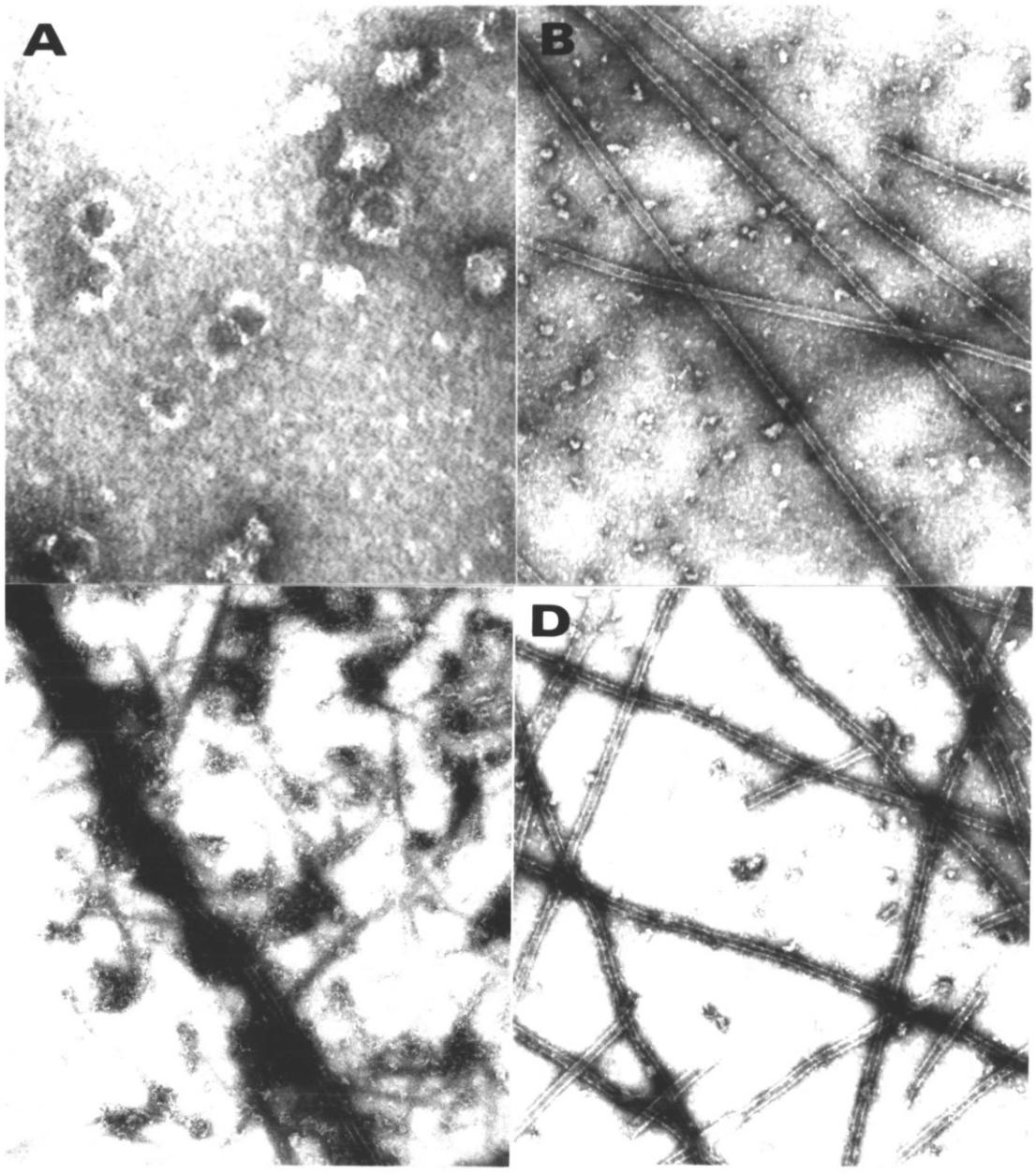


Figure 5.2. Electron microscopy.

(A) Cold-dissociated sucrose cushion microtubule protein. Magnification x 200,000. (B) Microtubules formed in the presence of 1 mM GTP. Magnification x 56,000. (C) Microtubules and filaments formed in the presence of 1 mM ATP. Magnification x 56,000 (D)Microtubules formed in the presence of 1 mM CTP. Magnification x 56,000.

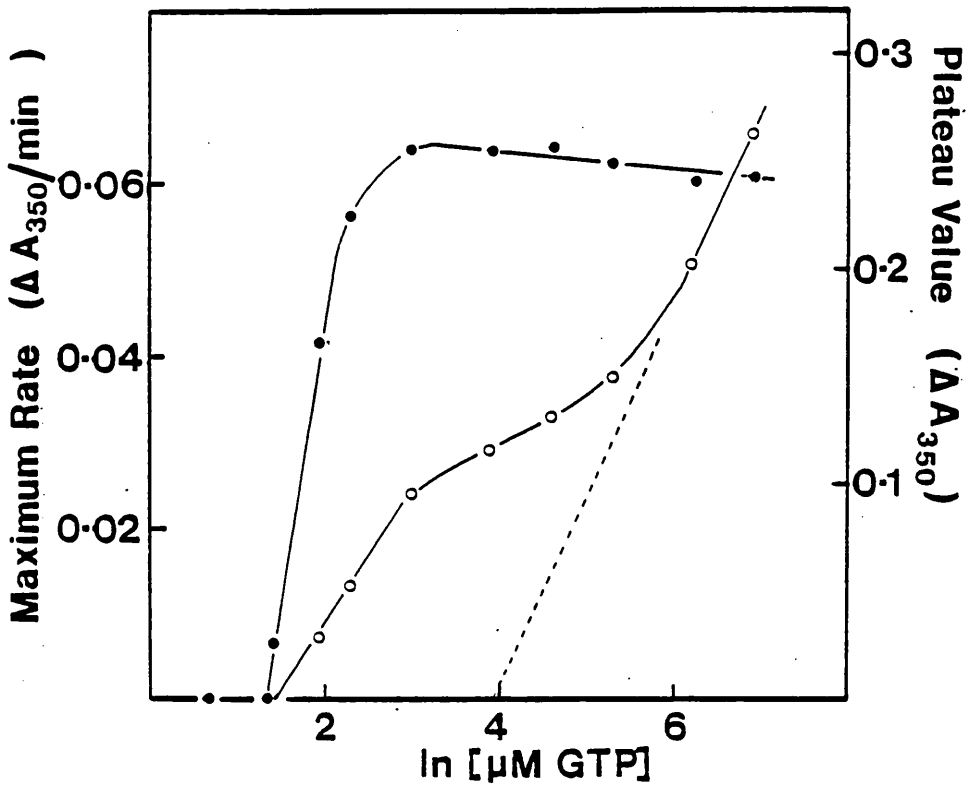


Figure 5.3. Kinetics of microtubule assembly as a function of the GTP concentration.

Sucrose cushion microtubule protein at 1 $mg.ml^{-1}$ was assembled at increasing concentrations of GTP. The maximal rate $\Delta A_{350_{nm}}.min^{-1}$ (o) and the final plateau value $\Delta A_{350_{nm}}$ (●) were determined.

The maximum rate of microtubule assembly was also determined and is defined as the fastest rate over a 60s period. A plot of the maximum rate of assembly against the ln[GTP] (Fig. 5.3) shows that this rate increases in proportion to the ln[GTP], above the minimal 4 μM GTP required for assembly, until an ill-defined plateau is attained. A second phase in which the rate of assembly increases markedly extrapolates to a GTP threshold of approximately 50 μM.

Microtubule assembly proceeds by a condensation-polymerisation mechanism (see Chapter I). A plot of $\Delta\ 350_{nm}$ vs the subunit concentration, which is expressed as $\Delta 350_{nm}$, plateau-Δ350 time, shows that the assembly is defined by pseudo first-order kinetics at 20 μM GTP (Fig. 5.4^{A}). The pseudo first-order rate constant (K_{app}) can be determined from such a plot and at 20 μM GTP is 1.25×10^{-3} sec^{-1}.

However, at 1 mM GTP the kinetics are more complex (Fig. 5.4^{A}) and appear to be described by two pseudo first-order rate constants. While at the later stages of assembly, near the plateau value, the K_{app} is identical to that observed at 20 μM GTP, at the earlier stages of assembly the K_{app} of 5.82×10^{-3} sec^{-1} is considerably greater.

The cold-dissociated microtubule protein consists of

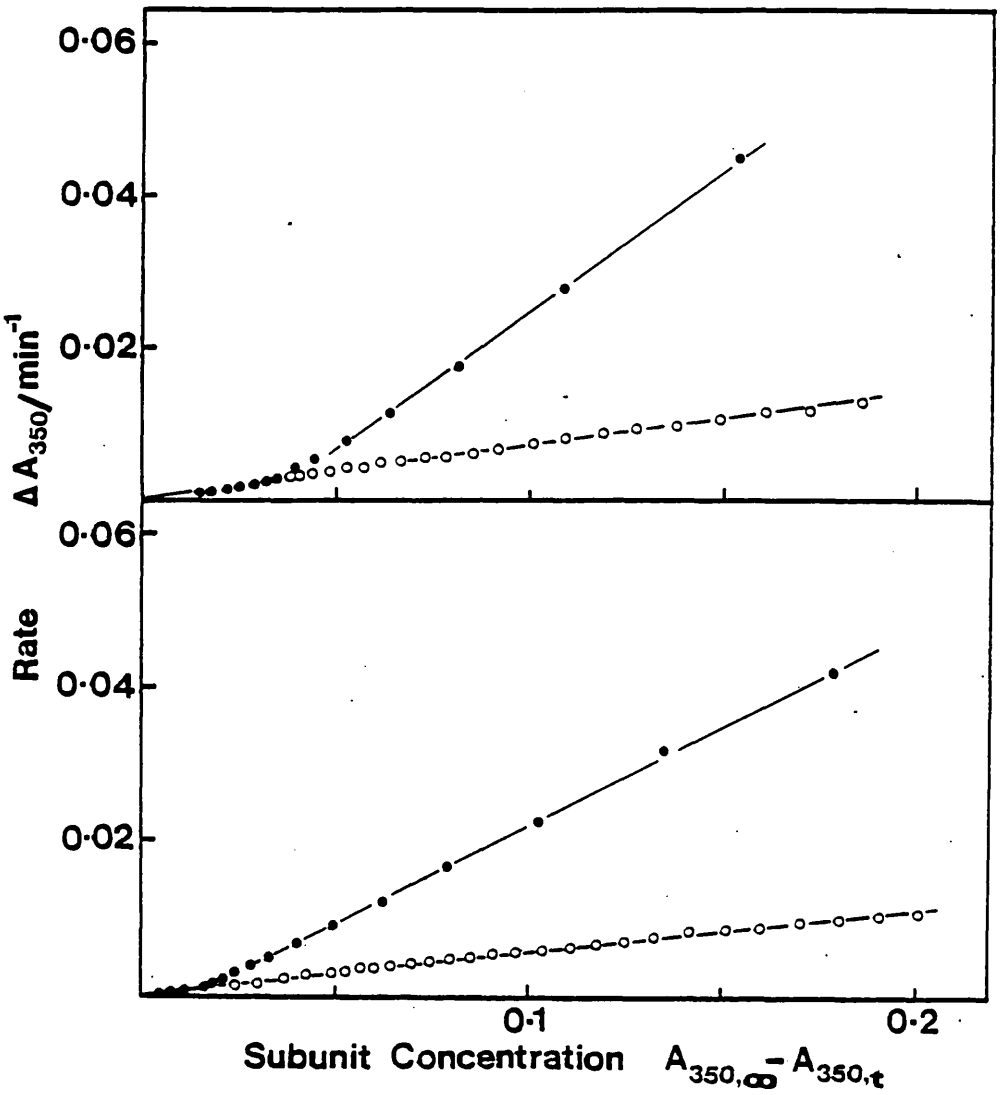


Figure 5.4. Determination of pseudo first-order rates at 1 mM GTP (●) and 20 µM GTP (O).

Sucrose cushion microtubule protein was either (A) used directly or (B) fractionated on a Biogel column pre-equilibrated in MA buffer to yield a void fraction enriched with MAP_2 and oligomeric protein. The unfractionated and fractionated microtubule protein was assembled and the final pleateau level determined. The rate of elongation was determined and is expressed as the rate $\Delta A_{350\,nm}.min^{-1}$ versus the subunit concentration $A_{350\infty} - A_{350,t}$. The slopes represent the pseudo first-order rate constants.

large numbers of tubulin oligomeric rings with estimated inside and outside diameters of 31 nm and 46 nm respectively (Fig. 5.2^{A}). These rings contain both tubulin and MAP_2. Fractionation of microtubule protein fixed with 0.1% glutaraldehyde, to prevent oligomer dissociation, shows that 53% of the total tubulin exists in the form of oligomers after correction for the MAP_2 protein content (123).

Microtubule protein fractionated on a gel exclusion column can separate the oligomers from the tubulin dimers. However, the void fraction contains both the tubulin oligomers, composed of tubulin and MAP_2, and the free MAP2 resulting in an increase of 1.4-fold in the MAP_2 concentration. The assembly of this MAP_2-enriched protein behaves as a single reaction with pseudo first-order kinetics at 20 µM GTP, while at 1 mM GTP the assembly behaves as a summation of two pseudo first-order reactions (Fig. 5.4^{B}). However, the intercept, or critical concentration, for the faster of these two reactions is lower than for the unfractionated microtubule protein. The observations of two phases as a function of the GTP concentration correspond to the assembly of tubulin dimers and the oligomeric species, with the latter only participating directly in microtubule assembly at high GTP concentrations.

The presence of oligomeric rings and 6 S tubulin

dimers means that there are two potential GTP binding species. Consequently, determination of the amount of bound GTP as a function of the GTP concentration might reveal more than one component. The technique of microfiltration has been employed to achieve rapid separation of the protein-bound GTP from the free GTP on a G-25 Sephadex column. Increasing concentrations of [^{3}H] GTP were added to microtubule protein at 1.2 mg.ml^{-1} and the protein-bound GTP was immediately separated by centrifugation.

An Eadie-Scatchard plot of bound GTP against the bound/free GTP (Fig. 5.5) deviates from linearity at high GTP concentrations indicating the presence of more than one GTP-binding species. The first component at low GTP concentrations has an apparent K_d of 5.4 µM while a second component shows an apparent K_d of 55 µM. The rate of assembly as a function of the GTP concentration showed two phases with extrapolated values of 4 µM and 50 µM GTP and the marked increase in the rate of assembly above 50 µM GTP may therefore result from the binding of GTP to the second component, thereby permitting it to participate directly in microtubule assembly.

Caplow et al. (224) have noted that the MAP_2:tubulin oligomer binds significantly less GTP than the tubulin dimers on a Hummel and Dryer type experiment using 25-50 µM GTP. It has also been demonstrated that the

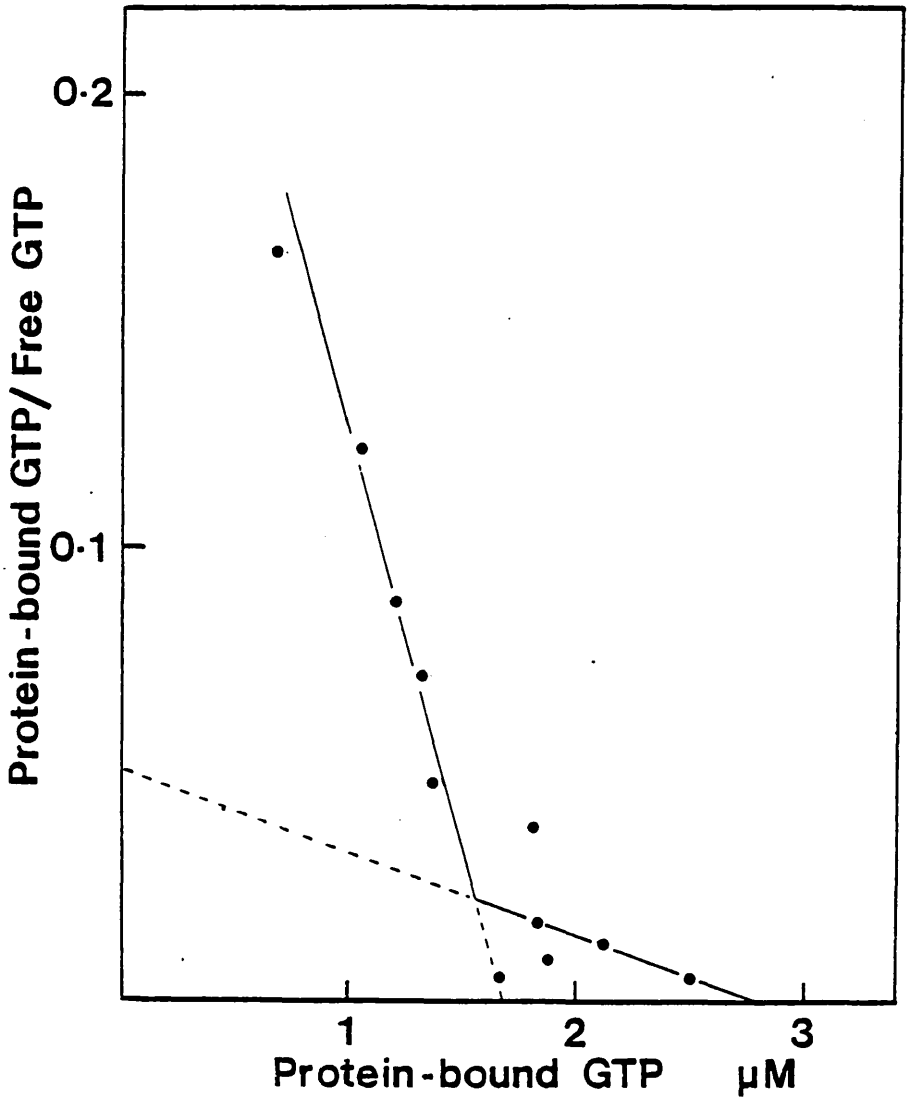


Figure 5.5. Eadie-Scatchard plot for GTP-binding.

Increasing concentrations of [^{3}H] GTP were added to sucrose cushion microtubule protein at 1 mg.ml^{-1}. The protein-bound [^{3}H] GTP was separated from the free GTP by microfiltration and the counts co-migrating with the protein determined by scintillation. 20 μls of the eluate was mixed with 5 mls of the scintillation cocktail containing 25% 2-methoxyethanol- 75% toulene-0.5% butyl-PBD.

E-site on tubulin in the ring form is only slowly exchangeable in comparison to the tubulin dimers (211-212; 225). If the two components on the Eadie-Scatchard plot represent the dimers and the tubulin oligomers then the first component derives from the GTP binding to the tubulin dimers and the second component from GTP binding to the rings.

Microtubule assembly: ATP

While GTP can exchange directly with the E-site GDP to promote microtubule assembly the polymerisation observed with ATP has been proposed to be mediated by the NDPkinase (155-156). However, the low specific activity of the NDPkinase co-pelleting with the sucrose cushion microtubule protein would predict little or no assembly with ATP.

Microtubule protein at 1 $mg.ml^{-1}$ was incubated with increasing concentrations of ATP and the kinetics of polymerisation monitored at 350_{nm}. Microtubule assembly is observed and electron microscopy confirms the presence of microtubules (Fig. 5.2^{C}) although other polymorphic forms are observed. The polymerisation kinetics exhibit a lag period which is longer than that observed with a control sample polymerised by addition of 10 µM GTP, the maximum

concentration of GTP which would be generated by the NDPkinase utilising tubulin-GDP as the substrate. The lag period was constant for all ATP concentrations above 7-15 μM ATP. The kinetics of polymerisation were similar to those observed with GTP in that a final plateau value is achieved after the lag period.

A plot of the final extent of assembly as a function of the ATP concentration (Fig. 5.6), showed a minimal requirement for ATP of approximately 4 μM: below this concentration no assembly is observed. The extent of assembly is dependent on the ATP concentration between 4-15 μM, and up to 80% assembly was observed compared to the control sample. However, increasing the ATP concentration above 15 μM, leads to a 50% decrease in the final extent of assembly for ATP concentrations of 50-500 μM.

A plot of the maximum rate of assembly as a function of the ln[ATP] shows two components (Fig. 5.6). There is a linear increase in the rate of assembly up to 20 μM ATP and then a well defined plateau is attained. Extrapolation of the first component yields a value of 4 μM ATP, the minimal concentration required to promote polymerisation. Extrapolation of the second phase yields a value of approximately 12 μM ATP. The calculated pseudo first-order rate constants (K_{app}) for 20 μM and 250 μM ATP are 6.2×10^{-3} sec^{-1} and 2.24×10^{-3} sec^{-1} respectively.

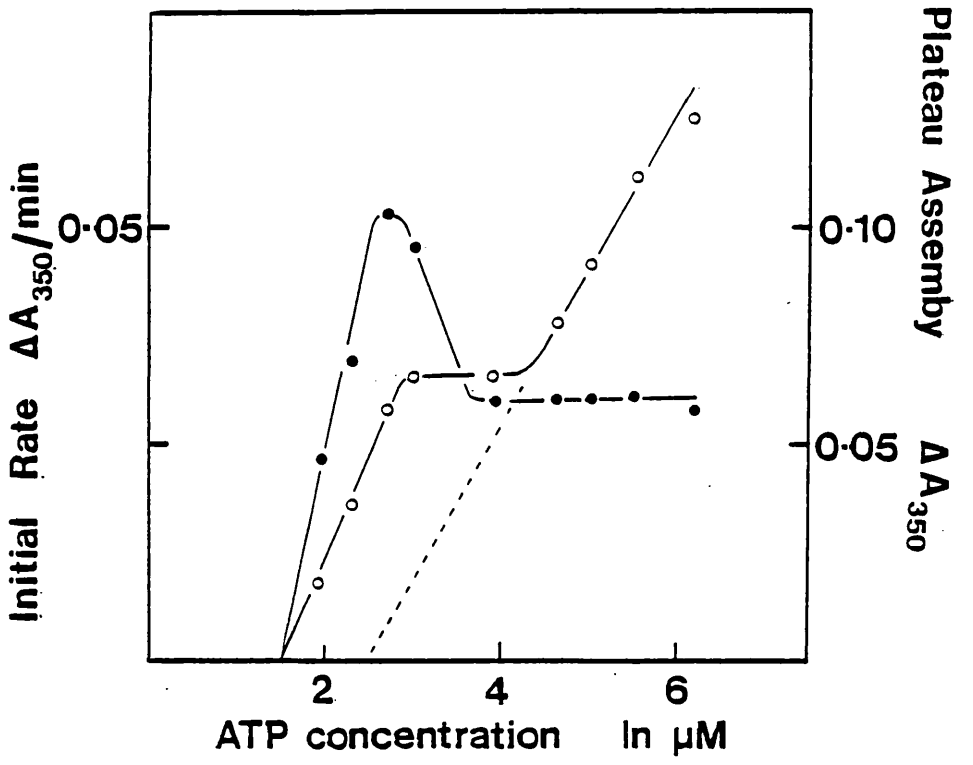


Figure 5.6. Kinetics of microtubule assembly as a function of the ATP concentration.

Sucrose cushion microtubule protein at 1 $mg.ml^{-1}$ was assembled at increasing ATP concentrations. The maximal rate $\Delta A_{350_{nm}}.min^{-1}$ (o) and the final plateau value $\Delta A_{350_{nm}}$ (●) were determined.

Microtubule assembly shows a minimal requirement of 4 µM ATP,as observed with GTP, and the rate of assembly also shows two phases. Tubulin contains stoichiometric amounts of GDP yet almost complete assembly is observed at the low concentration of 15 µM ATP. The microtubule protein contains a low amount of co-pelleting NDPkinase activity which would have to generate 4 µM GTP in the lag period and 10 µM GTP before the completion of polymerisation, if the ATP-induced assembly is mediated by the NDPkinase. The observation that the minimal nucleotide concentration for assembly is 4 µM for both GTP and ATP poses a serious challenge to the potential role of the NDPkinase. At the threshold, the assay contains about 8µM tubulin-GDP and 4 µM ATP, so that with complete conversion:

$$8\ \mu M\ GDP + 4\ \mu M\ ATP = 4\ \mu M\ GTP + 4\ \mu M\ GDP + 4\ \mu M\ ADP$$

However, utilisation of ATP by the NDPkinase is only twice as efficient as that of GTP (Table 5.1 and Table 3.3), indicating that under equilibrium conditions a significant concentration of ATP should remain. Therefore, it is unlikely that the minimal GTP concentration of 4 µM GTP required for assembly could be satisfied.

Microtubule assembly: CTP

The NDPkinase activity present in the sucrose cushion microtubule protein efficiently utilises purine nucleoside triphosphates, such as ATP, but the pyrimidine nucleoside triphosphates are poor substrates (Table 5.1). The utilisation of CTP at best is only 3% efficient compared to that of ATP at equivalent molar concentrations (Table 3.3 and Table 5.1). It would therefore be predicted that CTP should not promote microtubule assembly if the NDPkinase is required.

The kinetics of assembly were analysed under the same conditions as for GTP and ATP. A lag period was again observed which was constant for all CTP concentrations between 20-500 µM and approximately the same as for the control sample with 10 µM GTP, followed by the attainment of a final plateau value. Electron microscopy shows the presence of microtubules (Fig. 5.2^{D}).

The final extent of assembly as a function of the CTP concentration (Fig. 5.7), shows that the extent of assembly is dependent on the CTP concentration, but that complete assembly is achieved with 50-500 µM CTP. The final extent of assembly is identical to that of the control sample. There is a minimal requirement of about 12 µM CTP, below which no microtubule assembly occurs.

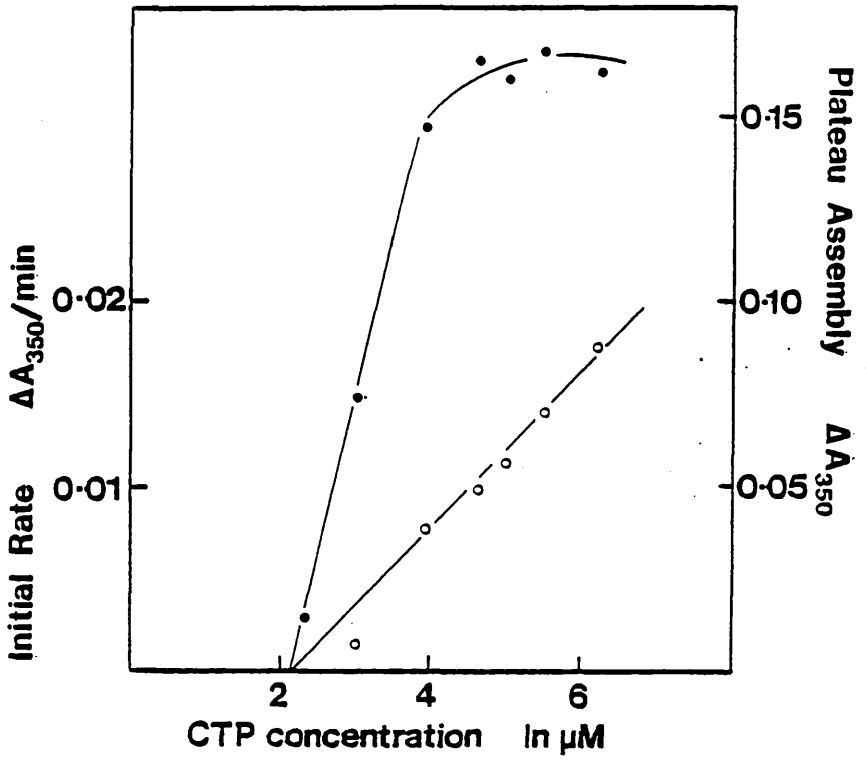


Figure 5.7. Kinetics of microtubule assembly as a function of the CTP concentration.

Sucrose cushion microtubule protein at 1 $mg.ml^{-1}$ was assembled at increasing CTP concentrations. The maximal rate $\Delta A_{350nm}.min^{-1}$ (O) and the final plateau value ΔA_{350nm} (●) were determined.

Only a single component was observed when the maximum rate was plotted as a function of ln[CTP] (Fig. 5.7). The maximum rate increases linearly and extrapolation of this component yields a value close to 12 μM CTP. The pseudo first-order rate constants(K_{app}) for 250 μM CTP is 2.0 x 10^{-3} sec^{-1}.

Nucleotide requirements: seeded preparations

Microtubule seeds, such as sheared microtubules, can eliminate the nucleation phase and the rate of elongation of microtubules can be followed, independent of any nucleation requirement (see Chapter I).

Sucrose cushion microtubule protein at 1 $mg.ml^{-1}$ was assembled with 30 μM GTP at 30°C for 60 mins. The microtubules were sheared by passaging once through a 50 μl Hamilton syringe and 10 μls of the seeds were added to initiate microtubule assembly. The addition of seeds results in the maximal addition of about 0.2 μM GTP, a concentration well below that required for microtubule assembly (Fig. 5.3).

Tubulin in the absence of added nucleotides has been reported to promote microtubule elongation (202-203; 217). It is therefore important to determine the rate of

elongation in the absence of added nucleotides, i.e. in the presence of stoichiometric amounts of GDP. Therefore, the seeded assembly of microtubule protein in the presence and absence of 10 μM GTP was examined.

Microtubule protein at 1 $mg.ml^{-1}$ was degassed and incubated for 5 mins at 30°C in the Beckman DU-8 spectrophotometer. The assembly was initiated by addition of 10 μls of the microtubule seeds and simultaneous addition of 10 μM GTP in the control sample (Fig. 5.8). While microtubule elongation can occur in the absence of added nucleotide, the addition of 10 μM GTP shows a 17-fold increase in the rate of elongation.

This elongation in the absence of added GTP means that the assembly observed with ATP and CTP could be due to the stimulation of nucleation, followed by GTP-independent elongation, provided that ATP and CTP are highly effective in inducing nucleation. In order to separate the role in nucleation and elongation, assembly of microtubule protein at $1mg.ml^{-1}$ was initiated by the addition of either 10 μM GTP or 500 μM CTP in the presence and absence of microtubule seeds. In the absence of seeds both GTP-induced assembly and CTP-induced assembly showed the characteristic lag-phase, which was totally abolished by the addition of seeds (Fig. 5.9). The observed rate of elongation immediately following the addition of seeds showed that the elongation rate with CTP is only 2-fold

slower than that observed with GTP, but is 9-fold faster than that observed in the absence of added nucleotides. CTP directly affects the rate of microtubule elongation.

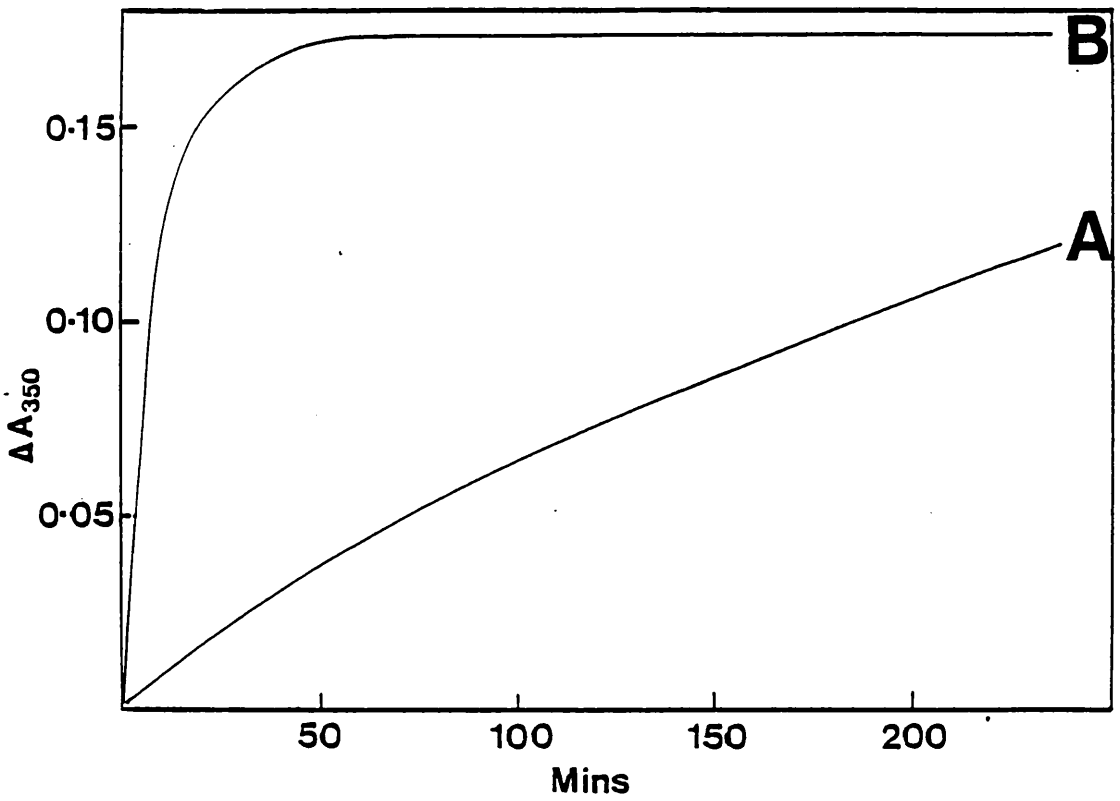


Figure 5.8. Microtubule elongation on seeds in the absence (A) and presence (B) added GTP.

Sucrose cushion microtubule protein at 1 $mg.ml^{-1}$ was assembled onto microtubule seeds in the absence and presence of 10 µM GTP.

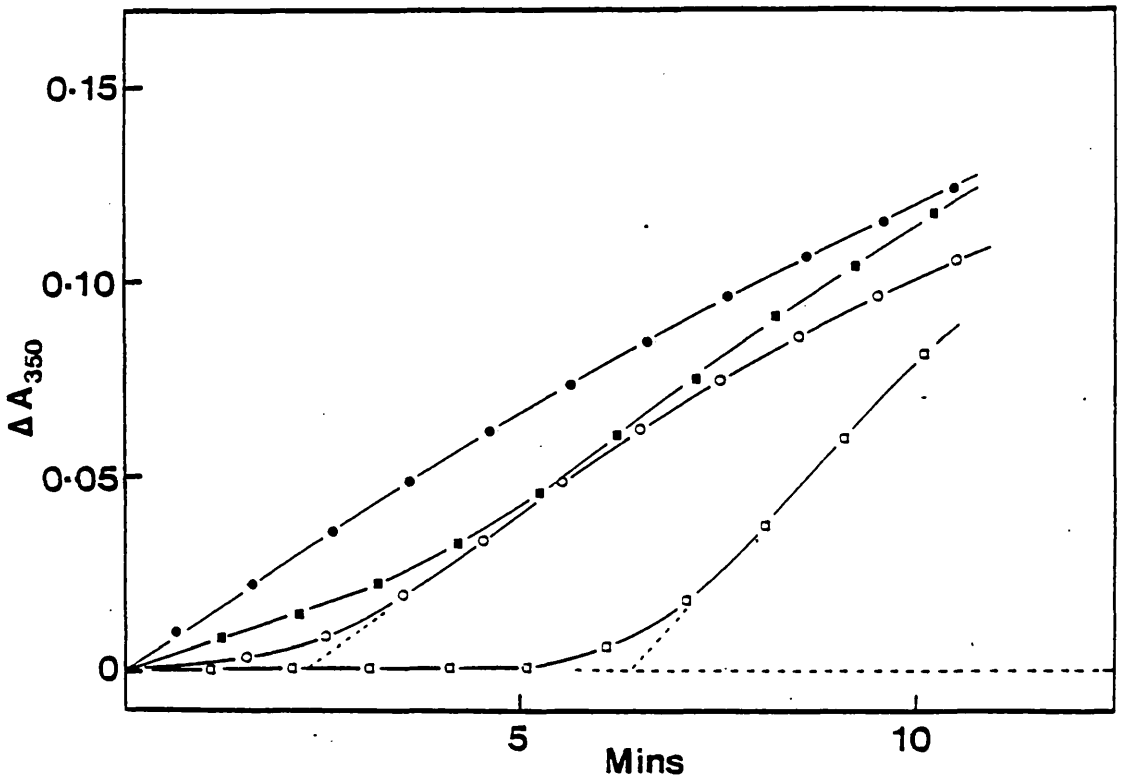


Figure 5.9. Microtubule assembly with CTP (□ ; ■) and GTP (● ; ○) in the absence (□ ; ○) and presence (■ ; ●) of seeds.

Sucrose cushion microtubule protein at 1 $mg.ml^{-1}$ was assembled in the presence of either 10 µM GTP or 500 µM CTP.

DISCUSSION

Microtubule assembly, at 1 $mg.ml^{-1}$ or about 8 µM tubulin, shows a minimal requirement of 4 µM GTP and the protein assembles to a final plateau value in the presence of approximately stoichiometric amounts of GTP (Fig. 5.3). As analysis of the nucleotide content of this microtubule protein shows the presence of stoichiometric amounts of GDP and only sub-stoichiometric amounts of GTP are required to initiate microtubule assembly.

However, GTP shows a 2.8-fold higher affinity for tubulin compared to GDP (196; 224). The minimal requirement of 4 µM GTP would therefore approximately equal the critical concentration for microtubule assembly of 1.5 µM tubulin. This suggests that all the tubulin constituting the critical concentration contains GTP on the E-site.

The cold-dissociated protein used for the assembly studies is not monodisperse: electron microscopy shows the presence of oligomeric rings (Fig. 5.2) and SDS-PAGE analysis shows that the protein is composed of tubulin and MAP_2 (Fig. 5.1). Separation of lightly-fixed microtubule protein demonstrates that 53% of the total tubulin exists in the oligomeric form while 47% is dimeric. This distribution is independent of the protein concentration (123). Consequently, any analysis of the kinetics of

microtubule assembly must accomodate this observed heterogeneity of the starting material.

Microtubule assembly at 20 μM GTP shows that the kinetics are described by a single pseudo first-order rate constant and the MAP_2-enriched protein is also described by pseudo first-order kinetics (Fig. 5^{A-B}). In contrast, microtubule assembly at 1 mM GTP is described by two pseudo first-order rate constants and the oligomeric MAP_2-enriched protein shows that the intercept for the faster reaction is lowered (Fig. 5^{A-B}).

However, the protein assembles to the same maximal extent at both 1 mM and 20 μM GTP, and the slight decrease at 1 mM GTP can be attributed to MAP_2 phosphorylation (Burns: personal communication). Clearly the oligomeric protein is utilised at both the GTP concentrations. The oligomers are in equilibrium with the tubulin dimers (see Chapter I) and any selective incorporation of the dimers into the microtubule would lead to the dissociation of the oligomers. This suggests that the assembly at low GTP concentrations is effected by the incorporation of the tubulin dimers and free MAP_2 and the oligomers participate by dissociation. In other words, the oligomers are not a direct assembly-intermediate at low GTP concentrations. By contrast, at high GTP concentrations, the kinetics are described by the summation of two pseudo first-order reactions, indicating that both the tubulin dimers and

oligomers participate directly in assembly.

At least two GTP-binding species are observed on an Eadie-Scatchard plot (Fig. 5.5) and the apparent k_d of 5.5 μM and 55 μM GTP for the dimers and the oligomers is consistent with the thresholds of 4 μM and 50 μM GTP observed for the rate of assembly as a function of the GTP concentration (Fig. 5.3). The apparent k_d of GTP-binding to the oligomers is in good agreement with that reported by Caplow and Zeeberg (224). This observation strongly supports the proposal that high GTP concentrations involve the incorporation of both the dimers and oligomers into the microtubule. However, the dimensions of the oligomeric rings prohibit direct incorporation into the microtubule, but protofilaments derived from the rings have been postulated to be directly incorporated (60; 206; 226).

The kinetics of microtubule assembly with ATP also show two phases as a function of the ATP concentration (Fig. 5.6), and a minimal requirement of 4 μM ATP. The maximal extent of assembly is observed at about 15 μM ATP, but there is a marked inhibition above this ATP concentration. This inhibition in the extent of assembly cannot be wholly attributed to phosphorylation, but must be a direct affect of ATP on polymerisation. While electron microscopy confirms the presence of microtubules even at 1 mM ATP concentration, filamentous structures are also observed (Fig. 5.2C). However, as these filaments are

observed when microtubule elongation is complete, they appear to be incapable of participating in the assembly process.

The lag period observed with ATP is much longer compared to that with GTP at equivalent molar concentrations. This longer lag period has often been referred to as the time taken by the NDPkinase to generate sufficient GTP to promote polymerisation (155-156; 198). A calculation of the total amount of GTP generated during this lag period at non-limiting concentrations of ATP can be determined from the known tubulin content (8 μM), the specific activity and amount of the NDPkinase present (0.015 units.mg^{-1}) and the length of the lag period (9 mins).

Since tubulin contains stoichiometric amounts of GDP then the total GDP content is also 8 μM. The corrected K_m for the nucleoside diphosphate (Fig. 3.5 insert), which assumes non-limiting concentrations of the donor triphosphate, can be extrapolated to determine a maximal conversion of 3.4% at 8 μM GDP. Therefore, while under the routine NDPkinase assay conditions up to 30 μM GTP.min^{-1} can be produced, only 3.4% of this conversion can be realised under the assembly conditions, or about 1 μM GTP.min^{-1}. During the lag period a maximum of 9 μM GTP can therefore be produced and this would satisfy the minimal GTP requirement.

However, while at non-limiting concentrations of ATP the minimal GTP requirement can be satisfied, a minimal requirement of only 4 μM ATP is observed for initiation of microtubule assembly (Fig. 5.6) and almost complete assembly is observed in the presence of 15 μM ATP. As the NDPkinase functions via a bisubstrate "ping-pong" mechanism (Chapter III) these low concentrations of ATP would certainly be rate-limiting. Consequently, sufficient GTP cannot be generated by the low amounts of NDPkinase activity present in these microtubule protein preparations. It therefore appears highly unlikely that the ATP-induced microtubule assembly can be mediated by the NDPkinase.

ATP is an efficient substrate for the NDPkinase compared with CTP which is an extremely poor substrate (Table 5.1 and Table 3.3). The total amount of GTP generated during the lag period of 6 mins observed with CTP, can be calculated in a similar manner, correcting for the utilisation of the CTP as a substrate by the NDPkinase (3% of that observed with ATP). In the lag period the NDPkinase can only generate a maximum of 0.2 μM GTP at non-limiting concentrations of CTP. This would be still lower at the minimal CTP concentration of 10 μM required to induce assembly.

Clearly the observation that CTP can induce microtubule assembly (Fig. 5.7) but cannot satisfy the

minimal GTP requirement strongly argues that the action of nucleoside triphosphates such as CTP and ATP on microtubule assembly is not mediated by the NDPkinase. The action of these nucleoside triphosphates must therefore be directly on the microtubule proteins. Interestingly, the observations of Penningroth and Kirschner (227) that microtubule assembly can be promoted by AMPPCP, a non-hydrolysable analogue of ATP, would further support this proposal.

Addition of microtubule seeds leads to the elimination of the lag period which represents the nucleation phase. If the lag period with CTP-induced assembly represents the time required for GTP generation by the NDPkinase then the lag period should not be eliminated by the addition of seeds. However, the addition of seeds to a control sample with 10 µM GTP, the maximal conversion that would be effected by the NDPkinase, and a sample in the presence of 500 µM CTP shows that the lag period observed in the absence of seeds is totally abolished (Fig. 5.9). Indeed, the initial rate of seeded elongation with CTP is only 2-fold slower than that observed with GTP. This seeded elongation is observed during the lag period of the CTP-sample which was not seeded, demonstrating that there is no minimal time requirement for the promotion of assembly by CTP. However, Weisenberg et al. (198) have reported a lag period for assembly in seeded preparations with ATP.

A number of laboratories have reported that tubulin in the absence of added nucleotides, such that only GDP is present, can promote microtubule elongation (202-203; 217) and the purification procedure can affect the nucleotide requirements (63). It can therefore be argued that the elongation rate observed with CTP (Fig. 5.9) represents the rate of elongation of tubulin with bound GDP. However, while tubulin in the absence of added nucleotides, and stoichiometric amounts of GDP, can promote microtubule elongation (Fig. 5.8), the rate of elongation is 9-fold slower than that observed in the presence of 500 μM CTP.

These observations that CTP, in the absence of the minimal concentration of 4 μM GTP required, can promote microtubule assembly strongly suggest that CTP, and presumably ATP, can directly effect the kinetics of assembly. The results presented and discussed in Chapter IV, which demonstrate the absence of any specific NDPkinase-tubulin interactions, and those in this Chapter suggest that there is no absolute requirement for the NDPkinase in microtubule assembly. Nucleoside triphosphates such as ATP and CTP must therefore affect microtubule assembly directly.

CHAPTER VI

GENERAL DISCUSSION

Nucleoside triphosphates are necessary for microtubule assembly *in vitro* under quasi-physiological conditions. Most reports suggest that only the guanine nucleotides GTP and GDP can bind to tubulin and the nature of the bound ligand can critically affect the kinetics of microtubule assembly (see Chapter I and IV). However, nucleoside triphosphates such as ATP, UTP and CTP, which have been reported to show no significant binding to tubulin, can also promote microtubule assembly and it has been postulated that the enzyme NDPkinase is required for this assembly-promoting activity (155-156; 196-198).

As mentioned previously (Chapter I), a wide variety of procedures exist for purifying the microtubule proteins and assembly can exhibit different requirements depending on the isolation procedure. For example, ion-exchange purified tubulin, lacking in MAPs, shows a higher critical concentration when compared to microtubule protein isolated by cycles of polymerisation and which contains MAPs. Furthermore, various procedures may isolate different MAPs, different proportions of the same MAPs, or differing co-purified enzymatic activities. The substantial differences imply that the different procedures may also result in changing the nucleotide requirements for microtubule assembly (63).

It is clearly important to report in detail the precise conditions employed for the purification

procedure and the detailed composition of the microtubule protein. The presence of "contaminating" enzymatic activities is not usually reported. The procedure employed for the purification of microtubule protein from chick brain has been fully described (157-158 ; also Chapter II). The 2x microtubule protein typically contains about 20% MAPs, which consists almost entirely of MAP_2 with trace amounts of MAP_1 and tau proteins, and tubulin (Fig. 4.5). Enzymatic activities present in these preparations include the NDPkinase, protein kinase(s) and phosphoprotein phosphatase(s). Other activities, not assayed, may be present and these may affect the specific properties of the protein. Highly purified microtubule protein can be prepared by using a sucrose cushion (Fig. 5.1).

In the context of this thesis, the question was addressed to the possible role of the NDPkinase in the regulation of the tubulin nucleotide status. The GTP bound to the N-site is thought to function as a stable structural co-factor and is not involved in a regulatory role in the dynamics of microtubule assembly and disassembly. However, the exchangeable or E-site of assembly-competent tubulin is normally occupied with GTP, which is hydrolysed following tubulin polymerisation, and the resultant GDP remains on the tubulin following its incorporation into the microtubule. This discussion will therefore use the terms tubulin.GDP or tubulin.GTP to

refer to the nucleotides bound to the exchangeable or E-site as is generally accepted in the literature.

Clearly prior to any discussion of the role of the NDPkinase it is important to outline the various functions prescribed to GTP or GDP in microtubule assembly. The kinetics of microtubule assembly can be conveniently divided into three separate phases: a nucleation phase, an elongation phase and a steady-state kinetic phase, and the role of the nucleotides may differ between the three phases.

The nucleation phase shows no change in turbidity and can be most simply defined as the formation of the shortest structure which is able to form stable lateral interactions with other tubulin molecules or protofilaments to generate a two-dimensional lattice. Recent reports using time-resolved x-ray studies to study the pre-nucleation and the nucleation events demonstrate the appearance of short protofilament fragments, which associate laterally to form a nucleating centre (226).

When microtubule protein is incubated with GTP under quasi-physiological conditions, rapid and efficient assembly of microtubules following a short lag-phase is observed. However, microtubule protein incubated with GDP under the same conditions fails to assemble (Fig. 2.4). GTP is therefore capable of nucleation while GDP is not.

Added GTP is not required for the assembly of pure tubulin to form microtubules under certain non-physiological conditions such as in the presence of high concentrations of magnesium ions or glycerol (55-56; 59). However, glycerol may induce artifacts and the observed nucleation may result from the preferential hydration of the protein under these conditions.

The lag-period or the nucleation phase is followed by rapid increase in turbidity with pseudo first-order kinetics. The lag-period can be eliminated by the addition of microtubule seeds. This pseudo first-order growth is referred to as the elongation phase which describes the addition of free subunits or fragments onto the nucleation centre until the concentration of free subunits falls to the critical concentration (see Chapter I).

No polymerisation is induced in the presence of GDP, unless microtubule seeds are added which then allow elongation to proceed (Fig. 5.8).GTP induces rapid polymerisation suggesting that tubulin.GTP not only promotes nucleation but also enhances the rate of elongation.

During microtubule assembly the tubulin.GTP is hydrolysed and the resultant tubulin.GDP becomes non-exchangeable following incorporation into the microtubule. The hydrolysis of GTP, concommitant with though not

strictly coupled to assembly, has been proposed as the mechanism which produces a disparity in the critical concentration at the two ends of a polar microtubule (see Chapter I).

Following the elongation phase a final plateau value is attained. Microtubules exist in a dynamic equilibrium with the subunits, and the kinetics of GTP-hydrolysis and pulse chase experiments suggest that microtubules treadmill (see Chapter I). The hydrolysis of GTP is thought to be the result of a conformational change in the tubulin molecule following lateral associations with other neighbouring tubulin molecules. The observed treadmilling is a consequence of the different critical concentrations at the two ends of the microtubule.

These features of the nucleotide action on microtubule assembly in vitro would suggest the existence of important control points for ordered microtubule assembly and disassembly in vivo:

(a) the level of the GTP

(b) the GTP/GDP ratio or the energy charge

(c) the activity of triphosphatases

(d) the activity of the NDPkinase

(e) the state of the N-site.

Microtubule assembly induced by ATP, CTP and UTP has been proposed to be mediated by the NDPkinase activity, by

phosphorylating the tubulin.GDP or the free GDP. Furthermore, it has been asserted that the NDPkinase preferentially phosphorylates the tubulin.GDP *in situ* (156). There are four inherent implications in this proposed model:

(a) protein.protein interactions between the NDPkinase and tubulin.

(b) the NDPkinase bound to tubulin utilises the E-site GDP and nucleoside triphosphates such as ATP, CTP and UTP as substrates.

(c) that only GTP can bind to tubulin and promote microtubule assembly.

(d) the assembly effected by the nucleoside triphosphates ATP, CTP, and UTP is mediated by the NDPkinase.

The chick brain NDPkinase has been purified to near homogeneity and migrates on SDS-PAGE as a protein of molecular weight 16,500 daltons (Chapter III). The NDPkinase is therefore clearly not intrinsic to the tubulin molecule nor to the HMW-MAPs or tau proteins. The purified NDPkinase is demonstrated to be monoisozymic, functions via a ping-pong bisubstrate mechanism and has a native molecular weight of 100,000 by gel-exclusion chromatography. The physical and chemical properties of this purified enzyme are similar to those reported for NDPkinases isolated from bovine brain and various sources (Chapter III).

Indirect evidence has been cited in support of the association of the NDPkinase with tubulin which includes the recovery of the NDPkinase through cycles of polymerisation with a constant specific activity (156), failure to fully separate tubulin on DEAE ion-exchange chromatography, ammonium sulphate precipitation, or by sucrose density centrifugation (195), co-elution on phosphocellulose chromatography (155-156) and co-elution with tubulin oligomers (211).

The evidence for NDPkinase interactions with microtubules and tubulin oligomers and dimers has been described and discussed in chapter IV. There is no evidence for specific NDPkinase:tubulin protein-protein interactions. The NDPkinase is selectively lost through successive cycles of polymerisation and its recovery is not influenced by pH or the nucleoside triphosphate conditions. Although the NDPkinase co-sediments with microtubules and co-elutes with oligomers, this apparent association appears to be related to some co-purifying contaminant.

It is therefore concluded that the NDPkinase is not a microtubule-associated protein and shows no specific interaction with tubulin oligomers and dimers.

However, there is an intrinsic problem with negative results. It therefore seems pertinent to ask whether the

NDPkinase-mediated pathway is the sole mode of action of nucleoside triphosphates such as ATP and CTP in promoting microtubule assembly. In other words, is there an absolute requirement for the NDPkinase in microtubule assembly induced with nucleoside triphosphates such as ATP and CTP and if not, how can this be reconciled with the reported absolute requirement for GTP in the assembly process?

The requirements and kinetics of microtubule assembly induced with GTP, ATP and CTP were examined. The results described and discussed in Chapter V demonstrate that there is no absolute requirement for GTP and that ATP and CTP can directly effect microtubule assembly.

Clearly while the NDPkinase can utilise triphosphates to phosphorylate diphosphates, there is no direct evidence that this enzyme is specifically involved in the regulation of the tubulin nucleotide status. Indeed the NDPkinase exhibits a wide specificity with respect to its substrates (Table 3.3 and Table 5.1) and is unlikely to function as a crucial regulatory mechanism in the cell.

If nucleoside triphosphates other than GTP can directly effect microtubule assembly <u>in vitro</u>, the question arises as to whether there is any requirement for GTP. Indeed, tubulin.GDP can elongate onto microtubule seeds although at a decreased rate compared to tubulin.GTP

(Fig. 5.8; 196; 203). Furthermore, the results presented in Chapter V also suggest that GTP is not obligatory for nucleation as this can be effected by ATP (Fig. 5.6) and CTP (Fig. 5.7). In addition, ddGDP (228) and $p(CH_2)pG$ (229), both analogues of GDP, also support polymerisation as well as various non-hydrolysable analogues of GTP (156; 202-205). Indeed, several ribose-modified analogues of GTP are much more efficient at promoting polymerisation than GTP (refs.in 228).

There is therefore no obligatory requirement for GTP to either nucleate or elongate and this raises questions about how non-GTP nucleotides effect polymerisation if the NDPkinase is not involved. Clearly the GTP and GDP analogues may simply be binding to the E-site to promote nucleation and elongation. For instance GDP promotes elongation, but at a very slow rate compared with equivalent GTP concentrations.

There is growing evidence which suggests the presence of more than one GTP-binding E-site (218; 228; 230). Hamel and Lin (218) have demonstrated that there is no relationship between the deoxy-analogues of GTP to support polymerisation and the deoxy-analogues of GDP to inhibit polymerisation. They argue that these results provide evidence for the presence of two mutually exclusive E-sites. These will be referred to as the E^1 and E^2 sites. Therefore evidence exists for the presence of at

least three guanine nucleotide binding sites: the N-site, and the E^1 and E^2 sites.

One of these E-sites has been localised on the β-tubulin using 8-N_3GTP (231-232) and the mutually exclusive nature of the E^1 and E^2 sites might indicate that both may be present on this subunit. The presence of the two guanine-specific sites (E^1 and E^2) cannot account for the promotion of assembly by ATP and CTP in the absence of adequate NDPkinase activity. This assembly would necessitate the direct binding of ATP or CTP to the tubulin subunit, yet it has been widely held that tubulin does not bind ATP.

However, Zabrecky and Cole (219; 233) have demonstrated that ATP can bind to pure tubulin and that the k_d for ATP binding is about 2×10^{-4}M. They have subsequently used the 8-N_3ATP to localise the ATP-binding site on the α-tubulin (234). This 8-N_3ATP-binding site is distinct from the N-site and the binding of 8-N_3ATP is not inhibited by GTP (234), clearly demonstrating that the ATP is not bound to either the E^1 or E^2.

There are therefore four nucleotide binding sites on the tubulin dimer: three guanine nucleotide sites (the N-site, the E^1 and E^2-sites both of which may be on the β-tubulin) and a distinct ATP binding site on the α-tubulin.

Clearly the role of nucleotides in microtubule assembly and possible cellular regulatory mechanisms requires a better understanding of the number and significance of the nucleotide sites.

The observations on the rate of assembly (Fig. 5.3) and the [^{3}H] GTP-binding (Fig. 5.5) as a function of the GTP concentration indicate the presence of two GTP-binding components. These two GTP sites appear to be related to the dimer/oligomer status of the protein rather than reflecting GTP-binding to E^1 and E^2. The observations of Hamel and Lin (218) of two mutually exclusive sites are not consistent with a correlation of the sites with the dimer/oligomer status, as the oligomers and dimers are in a dynamic equilibrium (Fig. 5.4^{A-B}). In addition, Hamel and Lin have used a "pure tubulin system", but have failed to analyse the presence of any oligomeric species.

The GTP-binding site on the rings is different from that on the dimers in that it is only slowly exchangeable (196; 199; 211-212; 224). However, a "fifth" site need not be invoked as the protein-protein interactions may modulate the binding constant of GTP to the oligomer (206). In this context, it is worth noting that Mandelkow et al. (226) have proposed that oligomeric rings are inside-out protofilaments.

The kinetics of microtubule assembly as a function of

the GTP concentration suggest that both the dimers and oligomers can directly participate as microtubule assembly-intermediates (Fig. 5.3; 60; 206; 226). However, cellular concentrations of GTP and GDP are only in the range of 10-20 μM. Most studies on the kinetics of microtubule assembly have used high concentrations of 0.1-1.0 mM GTP. The results presented in Chapter V would, however, argue strongly that only the dimers can be utilised as assembly-intermediates at cellular GTP concentrations (Fig. 5.3 and 5.4^{A-B}) and the oligomers, if they exist in vivo, act as a tubulin buffer.

However, while the cellular GTP concentration is low the ATP concentration is generally believed to be about 2 mM. Consequently, the affect of ATP on the kinetics of microtubule assembly is important. The recent demonstration that a distinct ATP-binding site is present on the α-tubulin (234) suggests that ATP is bound to tubulin in the cell. The role of ATP in microtubule assembly is crucially important but has been largely ignored as the affect of ATP was proposed to be mediated by the NDPkinase. Since ATP can directly induce assembly this raises the question of why the microtubule protein is not fully assembled in vivo.

The kinetics of assembly as a function of the ATP concentration show that ATP concentrations above 20 μM inhibit the final extent of assembly and filamentous

structures are observed by electron microscopy (Fig. 5.6 and Fig. 5.2^{C}). The formation of microtubules and short filaments has not been examined in detail, but it is possible that cellular conditions would result in the exclusive formation of these filaments by ATP.

The properties of tubulin oligomers have been widely studied and primarily single and double walled rings are found in the absence of added nucleotides and at low temperatures (see Chapter I). These oligomers do not appear to be direct assembly-intermediates at physiological GTP concentrations. The formation of oligomeric filaments at physiological temperatures and ATP concentrations is an important observation. The fact that they co-exist with microtubules strongly suggests that they may represent an assembly-incompetent oligomeric form, which may constitute the tubulin pool in vivo.

If this is the case, then the unpolymerised microtubule protein in the cell would be maintained in the filamentous form and may form part of the microtrabe culae meshwork. The release of tubulin from these filamentous structures and its incorporation into the microtubule may be controlled by GTP or by post-translational modification such as tyrosylation.

Future prospects:

At present an antibody to the NDPkinase is in preparation. An immunoabsorption column will be used to remove the contaminating NDPkinase activity from the sucrose cushion microtubule protein. The kinetics of assembly with ATP can be examined using the "NDPkinase-free" microtubule protein.

It is clearly important to determine the role of GTP and ATP in microtubule assembly at concentrations approaching those in the cell. The kinetics of microtubule assembly in the presence of GTP and ATP will be determined and the function of GTP and ATP will be examined in the presence of cellular concentrations of the nucleotides.

Finally a question will be addressed to the the presence of the two GTP-binding sites E^1 and E^2. What function could these two sites serve? It is hoped that some clues may be forthcoming from the experiments which try to replicate more accurately the in vivo conditions.

REFERENCES:

1. Slautterback, D. B. (1963) J. Cell Biol. 18, 367-388.
2. Ledbetter, M. C. and Porter, K. R. (1963) J. Cell Biol. 19, 239-250.
3. Porter, K. R. (1966) in Principles of Biomolecular Organisation (Eds. G.E.W. Woltstenholme and M. O' Connor) pp. 308-345, Ciba Foundation Symposium, J. and A. Churchill Ltd. London.
4. Behenke, O. (1965) J. Ultrastruct. Res. 13, 469-477.
5. Inoue, S. and Sato, H. (1967) J. Gen. Physiol. 50, 259-292.
6. Olmsted, J. B. and Borisy, G. G. (1973) Ann. Rev. of Biochem. 42, 507-540.
7. Wilson, L. and Bryan, J. (1974) Adv. Cell Molec. Biol. 3, 21-72.
8. Synder, J. A. and McIntosh, J. R. (1976) Ann. Rev. of Biochem. 45, 699-720.
9. Stephens, R. E. and Edds, K. T. (1976) Physiol. Rev. 56, 709-777.
10. Kirschner, M. W. (1978) Int. Rev. of Cytology 54, 1-71.
11. Dustin, P. (1978) Microtubules, Springer-Verlag, Berlin.
12. Raff, E. C. (1979) Int. Rev. of Cytology 59, 1-96.
13. Fuseler, J. W. (1975) J. Cell Biol. 64, 159-171.
14. Salmon, E. D. (1975) J. Cell Biol. 66, 114-127.
15. Solomon, F. (1981) J. Cell Biol. 90, 547-553.
16. Pickett-Heaps, J. D. (1975) Ann. N. Y. Acad. Sci.

253, 352-361.

17. Schliwa, M., Euteneuer, U., Herzog, W. and Weber, K. (1979) J. Cell Biol. 83, 623-632.

18. Spiegelman, B. M., Lopata, M. A. and Kirschner, M. W. (1979) Cell 16, 239-252.

19. Brinkley, B. R., Cox, S. M., Pepper, D. A., Wible, L., Brenner, S. L. and Pardue, R. L. (1981) J. Cell Biol. 90, 554-562.

20. Sharp, G. A., Osborn, M. and Weber, K. (1981) J. Cell Sci. 47, 1-24.

21. Brown, S., Levison, W. and Spudich, J. A. (1976) J. Supramolec. Struc. 5, 119-130.

22. Bershadsky, A. D., Gelfand, V. I., Suitkina, J. M. and Tint, I. S. (1978) Cell Biol. Int. Rep. 2, 425-432.

23. Osborn, M. and Weber, K. (1977) Cell 12, 561-571.

24. Leterrier, J-F., Liem, R. K. F. and Shelanski, M. L. (1982) J. Cell Biol. 95, 982-986.

25. Suprenant, K. and Dentler, W. A.(1982) J. Cell. Biol. 93, 164-174.

26. Satillaro, R. F., Dentler, W. L. and LeCluyse, E. L. (1981) J. Cell. Biol. 90, 467-473.

27. Griffiths, L. A. and Pollard, T. D. (1982) J. Biol. Chem. 257, 9143-9151.

28. Yamada, K. M., Spooner, B. S. and Wessells, N. K. (1970) Proc. Natl. Acad. Sci. (U.S.A.) 66, 1206-1212.

29. Rappaport, R. (1971) Int. Rev. of Cytology 31,

169-213.

30. Bloom, G. S. and Vallee, R. B. (1983) J. Cell Biol. 96, 1523-1531.

31. Wolosewick, J. J. and Porter, K. R. (1979) J. Cell Biol. 82, 114-139.

32. Heuser, J. E. and Kirschner, M. W. (1980) J. Cell Biol. 86, 212-234.

33. Borisy, G. G. and Taylor, E. W. (1967) J. Cell Biol. 34, 525-548.

34. Weisenberg, R. C., Borisy, G. G. and Taylor, E. W. (1968) Biochemistry 7, 4466-4479.

35. Cohen, C., DeRosier, D. J., Harrison, S. C., Stephens, R. E. and Thomas, J. (1975) Ann. N. Y. Acad. Sci. 253, 53-59.

36. Mandelkow, E., Thomas, J. and Cohen, C. (1977) Proc. Natl. Acad. Sci. (U.S.A.) 74, 3370-3374.

37. Stebbings, H. and Bennet, C. E. (1975) in Microtubules and Microtubule Inhibitors (eds. M. Borgers and M. DeBrabander) pp. 35-45, Elsvier-Amsterdam.

38. Ledbetter, M. C. and Porter, K. R. (1964) Science 144, 872-874.

39. Ringo, D. L. (1967) J. Ultrastruc. Res. 17, 266-277.

40. Tilney, L. G., Bryan, J., Bush, D. J., Fugiwara, K., Mooseker, M., Murphy, D., Synder, D. H. (1973) J. Cell Biol. 59, 267-275.

41. Behenke, O. (1975) Nature 257, 709-710.

42. Burton, P. R., Hinkley, R. E. and Pierson, G. B.

(1975) J. Cell. Biol. 65, 227-233.

43. Pierson, G. B., Burton, P. R. and Himes, R. H. (1978) J. Cell Biol. 76, 223-228.

44. Grimstone, A. V. and Klug, A. (1966) J. Cell Sci. 1, 351-362.

45. Chasey, D. (1972) Exp. Cell Res. 74, 471-479.

46. Amos, L. A. and Klug, A. (1974) J. Cell Sci. 14, 523-549.

47. Erickson, H. P. (1974) J. Supramolec. Struc. 2, 393-411.

48. Baker, T. S. and Amos, L. A. (1978) J. Mol. Biol. 123, 89-106.

49. Creperceau, R. H., McEwen, B. and Goldstein, S. J. (1978) Proc. Natl. Acad. Sci. (U.S.A.) 75, 5006-5010.

50. Murphy, D. B. and Borisy, G. G. (1975) Proc. Natl. Acad. Sci. (U.S.A.) 72, 2696-2700.

51. Dentler, W. L., Granett, S. and Rosenbaum, J. L. (1975) J. Cell Biol. 65, 237-241.

52. Vallee, R. B. and Borisy, G. G. (1977) J. Biol. Chem. 252, 377-382.

53. Amos, L. A. (1977) J. Cell Biol. 72, 642-654.

54. Burns, R. G. and Islam, K. (1982) Biol. of the Cell 45, 261.

55. Frigon, R. P. and Timasheff, S. N. (1975) Biochemistry 14, 4599-4566.

56. Frigon, R. P. and Timasheff, S. N. (1975) Biochemistry 14, 4567-4573.

57. Eipper, B. A. (1972) Proc. Natl. Acad. Sci (U.S.A.)

69, 2283-2287.

58. Weisenberg, R. C. (1972) Science 177, 1104-1105.

59. Shelanski, M. L., Gaskin, F. and Cantor, C.R. (1973) Proc. Natl. Acad. Sci. (U.S.A.) 70, 765-768.

60. Borisy, G. G., Marcum, J. M., Olmsted, J. B., Murphy, D. B., and Johnson, K. A. (1975) Ann. N. Y. Acad. Sci. 253, 107-132.

61. Scheele, R. B. and Borisy, G. G. (1976) Biochem. Biophys. Res. Commun. 70, 1-7.

62. Detrich, H. W., Berkowitz, S. A., Kim, H. and Williams, R. C. (1976) Biochem. Biophys. Res. Commun. 68, 961-968.

63. Lee, S-H, Kristofferson, D. and Purich, D. L. (1982) Biochem. Biophys. Res. Commun. 105, 1605-1610.

64. Detrich, H. W. and Williams, R. C. (1978) Biochemistry 17, 3900-3907.

65. Krauhs, E., Little, M., Kempf, T., Hofer-Warbineck, R., Ade, W. and Postingl, H. (1981) Proc. Natl. Acad. Sci. (U.S.A.) 78, 4156-4160.

66. Valenzuela, P., Quiroga, M., Zaldivar, J., Rutter, W. J., Kirschner, M. W. and Cleveland, D. W. (1981) Nature 289, 650-655.

67. Berkowitz, S. A., Katagiri, J., Binder, H. and Williams, R. C. (1977) Biochemistry 16, 5610-5617.

68. Luduena, R. F. and Woodward, D. O. (1973) Proc. Natl. Acad. Sci. (U.S.A.) 70, 3594-3598.

69. Luduena, R. F. (1979) in Microtubules (eds. K. Roberts and J.S. Hyams) pp. 65-116, Academic Press.

70. Nishida, E. and Kobayashi, T. (1977) J. Biochem. (Tokyo) 81, 343-347.

71. Mellon, M. G. and Rebhun, L. I. (1976) in Cell Motility, Book C (eds. R. Goldman, T.D. Pollard and J.L. Rosenbaum) pp. 1149-1163 Cold Spring Harbor Press, New York.

72. Burchill, B. R., Oliver, J. M., Pearson, C.B., Lienbach, E. D. and Berlin, R. D. (1978) J. Cell Biol. 76, 439-447.

73. Spiegelman, B. M., Penningroth, S. M. and Kirschner, M. W. (1977) Cell 12, 587-600.

74. Wilson, L., Anderson, K., Grisham, L. and Chin, D. (1975) in Microtubules and Microtubule Inhibitors (eds. M. Borgers and M. DeBrabander) pp. 103-113, Elsvier-Amsterdam.

75. Olmsted, J. B. and Borisy, G. G. (1975) Biochemistry 14, 2996-3005.

76. Lee, J. C. and Timasheff, S. N. (1977) Biochemistry 16, 1754-1764.

77. Arai, T., Ihara, Y., Arai, K. and Kaziro, Y. (1975) J. Biochem. (Tokyo) 77, 647-658.

78. Herzog, W. and Weber, K. (1977) Proc. Natl. Acad. Sci. (U.S.A.) 74, 1860-1864.

79. Cleveland, D. W., Hwo, S-Y and Kirschner, M. W. (1977) J. Mol. Biol. 116, 227-247.

80. Marcum, M., Dedman, J. R., Brinkley, B. R. and Means, A. (1978) Proc. Natl. Acad. Sci. (U.S.A.) 75, 3771-3775.

81. Fulton, C. and Simpson, P. A. (1979) in Microtubules (eds. K. Roberts and J.S. Hyams) pp. 117-174, Academic Press.

82. Heusele, C. and Carlier, M-F. (1981) Biochem. Biophys. Res. Commun. 103, 332-338.

83. Garland, D. (1977) Fed. Proc. Fed. Am. Socs. Exp. Biol. 36, 899.

84. Farrell, K. W. and Wilson, L. (1978) J. Mol. Biol. 121, 393-410.

85. Bhattacharya, B. and Wolff, J. (1976) Proc. Natl. Acad. Sci. (U.S.A.) 73, 2375-2378.

86. Ventilla, M., Cantor, C. R. and Shelanski, M. L. (1975) Arch. Biochem. Biophys. 171, 154-162.

87. Bhattacharya, B. and Wolff, J. (1977) FEBS Lett. 75, 159-162.

88. Tucker, J. B. (1979) in Microtubules (eds. K. Roberts and J.S. Hyams) pp. 315-358, Academic Press.

89. McKiethan, T. W. and Rosenbaum, J. L. (1981) J. Cell Biol. 91, 352-360.

90. Raybin, D. and Flavin, M. (1975) Biochem. Biophys. Res. Commun. 65, 1088-1095.

91. Eipper, B. A. (1974) J. Biol. Chem. 249, 1407-1416.

92. Feit, H. and Shelanski, M. L. (1975) Biochem. Biophys. Res. Commun. 66, 920-927.

93. Bryan, J. (1975) Am. Zool. 15, 649-660.

94. Marotta, C. A., Harris, J. L. and Gilbert, J. M. (1978) J. Neurochem. 30, 1431-1440.

95. Gozes, I. and Littauer, U. Z. (1978) Nature 276,

411-413.

96. Gozes, I., Baetseller, A.D. and Littauer, U. Z. (1980) Eur. J. Biochem. 103, 13-20.

97. Nelles, L. P. and Bamburg, J. R. (1978) J. Neurochem. 32, 477-489.

98. Robinson, A. R. (1974) Proc. Natl. Acad. Sci. (U.S.A.) 71, 885-888.

99. Robinson, A. R. and Rudd, C. (1974) Curr. Topics in Cell Regulation 8, 247-295.

100. Weisenberg, R. C. and Timasheff, S. N. (1970) Biochemistry 9, 4110-4116.

101. Frigon, R. P., Valenzuela, M. S. and Timasheff, S. N. (1974) Arch. Biochem. Biophys. 165, 442-443.

102. Gilbert, L. M. and Gilbert, G. A. (1973) Methods in Enzymology 27, 273-296.

103. Erickson, H. P. (1975) Ann. N. Y. Acad. Sci. 253, 60-77.

104. Scheele, R. B. and Borisy, G. G. (1979) in Microtubules (eds. K. Roberts and J.S. Hyams) pp. 175-254, Academic Press.

105. Weingarten, M. D., Lockwood, A. H., Hwo, S-Y. and Kirschner, M. W. (1975) Proc. Natl. Acad. Sci. (U.S.A.) 72, 1858-1862.

106. Witman, G. B., Cleveland, D. W., Weingarten, M. D. and Kirschner, M. W. (1976) Proc. Natl. Acad. Sci. (U.S.A.) 73, 4070-4074.

107. Keates, R. A. B. and Hall, R. H. (1975) Nature 257, 418-421.

108. Connolly, J. A., Kalnins, V. I., Cleveland, D. W. and Kirschner, M. W. (1978) J. Cell Biol. 76, 781-786.

109. Burns, R. G. and Islam, K. (1982) Eur. J. Biochem. 122, 25-29.

110. Erickson, H. P. and Voter, W. A. (1976) Proc. Natl. Acad. Sci. (U.S.A.) 73, 2813-2817.

111. Lee, J. C., Tweedy, N. and Timasheff, S. N. (1978) Biochemistry 17, 2783-2790.

112. Connolly, J. A., Kalnins, V. I., Cleveland, D. W. and Kirschner, M. W. (1977) Proc. Natl. Acad. Sci. (U.S.A.) 74, 2437-2440.

113. Sherline, P. and Schiavone, K. (1977) Science 198, 1038-1040.

114. Nunez, J., Francon, J., Lennon, A. M., Fellous, A. and Mareck, A. (1980) in Microtubules and Microtubule Inhibitors (eds. M. DeBrabander and J. DeMey) pp. 213-226, Elsvier-Amsterdam.

115. Kirschner, M. W., Williams, R. C., Weingarten, M. D. and Gerhart, J. C. (1974) Proc. Natl. Acad. Sci. (U.S.A.) 71, 1159-1163.

116. Doenges, K. H., Biedert, S. and Paweletz, N. (1976) Biochemistry 15, 2995-2999.

117. Kuriyama, R. (1975) J. Biochem. (Tokyo) 77, 23-31.

118. Kirschner, M. W., Honig, L. S. and Williams, R. C. (1975) J. Mol. Biol. 99, 263-276.

119. Scheele, R. B. and Borisy, G. G. (1978) J. Biol. Chem. 253, 2846-2851.

120. Marcum, J. M. and Borisy, G. G. (1978) J. Biol. Chem.

253, 2825-2833.

121. Erickson, H. P. (1975) Ann. N. Y. Acad. Sci. 253, 51-52.

122. McEwen, B. and Edelstein, S. J. (1979) J. Mol. Biol. 139, 123-145.

123. Burns, R. G. and Islam, K. (1983) J. Cell Biol. 97, 211a.

124. Weingarten, M. D., Sutter, M. M., Littman, D. R. and Kirschner, M. W. (1974) Biochemistry 13, 5529-5537.

125. Borisy, G. G. and Olmsted, J. B. (1978) Science 177, 1196-1197.

126. Vallee, R. B. and Borisy, G. G. (1978) J. Biol. Chem. 253, 2834-2845.

127. Gaskin, F., Cantor, C. R. and Shelanski, M. L. (1975) Ann. N. Y. Acad. Sci. 253, 133-146.

128. Johnson, K. A. and Borisy, G. G. (1977) J. Mol. Biol. 117, 1-31.

129. Oosawa, F. and Kasai, M. (1962) J. Mol. Biol. 4, 10-21.

130. Engelborghs, Y., DeMaeyer, L. C. M. and Overbergh, N. FEBS Lett. 80, 81-85.

131. Murphy, D. B., Johnson, K. A. and Borisy, G. G. (1977) J. Mol. Biol. 117, 33-52.

132. Sloboda, R. D., Dentler, W. L. and Rosenbaum, J. L. (1976) Biochemistry 15, 4497-4505.

133. Burns, R. G., Islam, K. and Chapman, R. (1983) J. Cell Biol. 97, 200a.

134. Allen, C. and Borisy, G. G. (1974) J. Mol. Biol. 90,

381-402.

135. Binder, L. I., Dentler, W. L. and Rosenbaum, J. L. (1975) Proc. Natl. Acad. Sci. (U.S.A.) 72, 1122-1126.

136. Dentler, W. L., Granett, S., Witman, G. B. and Rosenbaum, J. L. (1974) Proc. Natl. Acad. Sci. (U.S.A.) 71, 1710-1714.

137. Heidemann, S. R. and McIntosh, J. R. (1980) Nature 286, 517-517.

138. Euteneuer, U. and McIntosh, J. R. (1981) J. Cell Biol. 89, 338-345.

139. Telzer, B. R. and Haimo, L. T. (1981) J. Cell Biol. 89, 373-378.

140. Sternlicht, H. and Ringel, I. (1979) J. Biol. Chem. 254, 10540-10550.

141. Margolis, R. L. and Wilson, L. (1978) Cell 13, 1-8.

142. Margolis, R. L. and Wilson, L. (1979) Cell 18, 673-679.

143. Margolis, R. L. and Wilson, L. (1981) Nature 293, 705-711.

144. Farrell, K. W., Kassis, J. A. and Wilson, L. (1979) Biochemistry 18, 2642-2647.

145. Bergen, L. G. and Borisy, G. G. (1980) J. Cell Biol. 84, 141-150.

146. Cote, R. H. and Borisy, G. G. (1981) J. Mol. Biol. 150, 577-602.

147. Cote, R. H., Bergen, L. G. and Borisy, G. G. (1982) in Microtubules and Microtubule Inhibitors (eds. M. DeBrabander and J. DeMey) pp. 325-328,

Elsvier-Amsterdam.

148. Wegner, A. (1976) J. Mol. Biol. 108, 139-150.

149. Pollard, T. D. and Mooseker, M. S. (1981) J. Cell Biol. 88, 654-659.

150. Kirschner, M. W. (1980) J. Cell Biol. 86, 330-334.

151. Schiff, P. B., Fant, J. and Horwitz, S. B. (1979) Nature 277, 665-667.

152. Lee, J. C., Field, D. J. and Lee, L. L. Y. (1980) Biochemistry 19, 6209-6215.

153. DeBrabander, M., Geuens, G., Nuydens, R., Willeboards, R. and DeMey, J. (1981) Proc. Natl. Acad. Sci. (U.S.A.) 78, 5608-5612.

154. DeBrabander, M. (1982) Cell Biol. Int. Reports 6, 901-915.

155. Jacobs, M. (1979) in Microtubules (eds. K. Roberts and J.S. Hyams) pp. 255-278, Academic Press.

156. Penningroth, S. M. and Kirschner, M. W. (1977) J. Mol. Biol. 115, 643-673.

157. Islam, K. and Burns, R. G. (1981) FEBS Lett. 123, 181-185.

158. Burns, R. G. and Islam, K. (1981) Eur. J. Biochem. 117, 515-519.

159. Bradford, M. M. (1976) Anal. Biochem. 72, 248-254.

160. Hartree, E. F. (1972) Anal. Biochem. 48, 422-427.

161. Laemmli, U. K. (1970) Nature 227, 680-685.

162. Fairbanks, G., Steck, T. L. and Wallach, D. F. H. (1971) Biochemistry 10, 2606-2617.

163. Bergmeyer, H. U. (1974) in Methods in Enzymatic

Analysis, pp. 488-489, Academic Press.

164. Cheng, Y. C., Robinson, B. and Parks, R. E. (1973) Biochemistry 12, 5-11.

165. Wertheimer, A. M. and Kaulenas, M. S. (1977) Biochem. Biophys. Res. Commun. 78, 565-571.

166. Berg, P. and Joklik, W. K. (1953) Nature 172, 1008-1010.

167. Krebs, H. A. and Hems, R. (1953) Biochim. Biophys. Acta 12, 172-180.

168. Ratliff, R. L., Weaver, R. H., Lardy, H. A. and Kuby, S. A. (1964) J. Biol. Chem. 239, 301-309.

169. Mourad, N. and Parks, R. E. (1966) J. Biol. Chem. 241, 271-278.

170. Edlund, B. (1971) Acta Chem Scand. 25, 1370-1376.

171. Sedmak, J. and Ramaley, R. (1971) J. Biol. Chem. 241, 5365-5372.

172. Lascu, I., Duc, M. and Cristae, A. (1981) Anal. Biochem. 113, 207-211.

173. Robinson, J. B., Brems, D. N. and Stellwagen, E. (1981) J. Biol. Chem. 256, 10769-10773.

174. Parks, R. E. and Agarwal, R. P. (1973) in The Enzymes Vol. 8 (ed. P.D. Boyer) pp. 307-333, Academic Press.

175. Weber, B. H., Willeford, K., Moe, J. G. and Piszkiewicz, D. (1979) Biochem. Biophys. Res. Commun. 86, 252-258.

176. Cheng, Y. C., Agarwal, R. P. and Parks, R. E. (1971) Biochemistry 10, 2138-2143.

177. Yue, R. H., Ratliff, R. L. and Kuby, S. A. (1967)

Biochemistry 6, 2923-2932.

178. Palmieri, R., Yue, R. H., Jacobs, H. K., Maland, L., Wu, L. and Kuby, S. A. (1973) J. Biol. Chem. 248, 4486-4499.

179. Colomb, M. G., Cheruy, A. and Vignais, P. V. (1974) Biochemistry 13, 2269-2277.

180. Mourad, N. and Parks, R. E. (1965) Biochem. Biophys. Res. Commun. 19, 312-316.

181. Walinder, O., Zetterquist, O. and Engstrom, L. (1969) J. Biol. Chem. 244, 1060-1064.

182. Walinder, O., Zetterquist, O. and Engstrom, L. (1968) J. Biol. Chem. 243, 2793-2798.

183. Walinder, O. (1968) J. Biol. Chem. 243, 3947-3952.

184. Nakamura, H. and Sugino, Y. (1966) J. Biol. Chem. 241, 4917-4922.

185. Agarwal, R. P., Robinson, B. and Parks, R. E. (1978) Methods in Enzymology 51, 376-386.

186. Colomb, M. G., Laturaze, J. G. and Vignais, P. V. (1966) Biochem. Biophys. Res. Commun. 24, 909-915.

187. Goffeau, A., Pedersen, P. L. and Lehninger, A. L. (1967) J. Biol. Chem. 242, 1845-1853.

188. Roison, M. P. and Kepes, A. (1978) Biochim. Biophys. Acta 526, 418-428.

189. Pedersen, P. L. (1968) J. Biol. Chem. 243, 4305-4311.

190. Duc, M., Lascu, I., Porumb, H., Gozia, O., Schell, H. D. and Barzu, O. (1981) FEBS Lett. 127, 281-284.

191. Kobayashi, T. (1975) J. Biochem. (Tokyo) 77, 1193-1197.

192. Shelanski, M. L. and Taylor, E. W. (1968) J. Cell Biol. 38, 304-315.

193. Weisenberg, R. C., Borisy, G. G. and Taylor, E. W. (1968) Biochemistry 9, 4110-4116.

194. Bryan, J. (1972) J. Mol. Biol. 66, 157-168.

195. Berry, R. W. and Shelanski, M. L. (1972) J. Mol. Biol. 71, 71-80.

196. Carlier, M-F. (1982) Mol. and Cellular Biochem. 47, 97-113.

197. Jacobs, M., Smith, H. and Taylor, E. W. (1974) J. Mol. Biol. 89, 455-468.

198. Weisenberg, R. C., Deery, W. J. and Dickinson, P. J. (1976) in Cell Motility Vol. C (eds. R. Goldman, T.D. Pollard and J.L. Rosenbaum) pp. 1123-1131, Cold Spring Harbor Press, New York.

199. Weisenberg, R. C., Deery, W. J. and Dickinson, P. J. (1976) Biochemistry 15, 4248-4254.

200. David-Pfeuty, T., Erickson, H. P. and Pantaloni, D. (1977) Proc. Natl. Acad. Sci. (U.S.A.) 74, 5372-5376.

201. Carlier, M-F. and Pantaloni, D. (1981) Biochemistry 20, 1918-1924.

202. Carlier, M-F. and Pantaloni, D. (1978) Biochemistry 17, 1908-1915.

203. Zackroff, R. V., Weisenberg, R. C. and Deery, W. J. (1980) J. Mol. Biol. 139, 641-659.

204. Arai, T. and Kaziro, Y. (1976) Biochem. Biophys. Res. Commun. 69, 369-376.

205. Weisenberg, R. C. and Deery, W. J. (1976) Nature 263,

792.

206. Weisenberg, R. C. (1980) J. Mol. Biol. 139, 660-677.

207. Kuriyama, R. and Sakai, H. (1974) J. Biochem. (Tokyo) 75, 463-471.

208. Jacobs, M. (1975) Ann. N. Y. Acad. Sci. 253, 562-573.

209. Weisenberg, R. C. (1975) Ann. N. Y. Acad. Sci. 253, 573-576.

210. Kobayashi, T. (1974) J. Biochem. (Tokyo) 76, 201-204.

211. Jacobs, M. and Huitorel, P. (1979) Eur. J. Biochem. 99, 613-622.

212. Engelborghs, Y. and Eccleston, J. (1982) FEBS Lett. 141, 78-81.

213. Penningroth, S. M., Cleveland, D. W. and Kirschner, M. W. (1976) in Cell Motility Vol. C (eds. R. Goldman, T.D. Pollard and J.L. Rosenbaum) pp. 1233-1257, Cold Spring Harbor Press, New York.

214. Jacobs, M. and Caplow, M. (1976) Biochem. Biophys. Res. Commun. 68, 127-135.

215. Nickerson, J. A. and Wells, W. W. (1978) Biochem. Biophys. Res. Commun. 85, 820-826.

216. Watanabe, T. and Flavin, M. (1976) J. Biol. Chem. 251, 182-192.

217. Karr, T. L., Podrasky, A. E. and Purich, D. L. (1979) Proc. Natl. Acad. Sci. (U.S.A.) 76, 5475-5479.

218. Hamel, E. and Lin, C. M. (1979) Proc. Natl. Acad. Sci. (U.S.A.) 78, 3368-3372.

219. Zabrecky, J. R. and Cole, R. D. (1982) J. Biol. Chem. 257, 4633-4638.

220. Terry, B. J. and Purich, D. L. (1979) J. Biol. Chem. 254, 9469-9476.

221. McNeal, R. K., Webb, B. C. and Purich, D. L. (1977) Biochem. Biophys. Res. Commun. 74, 440-447.

222. Hummel, J. D. and Dryer, W. J. (1962) Biochim. Biophys. Acta 63, 530-532.

223. Murphy, D. B., Heibsch, R. R. and Wallis, K. T. (1983) J. Cell Biol. 96, 1298-1305.

224. Caplow, M. and Zeeberg, B. (1980) Arch. Biochem. Biophy. 203, 404-411.

225. Pantaloni, D., Carlier, M-F., Simon, C. and Batelier, G. (1981) Biochemistry 20, 4709-4716.

226. Mandelkow, E., Mandelkow, E-M. and Bordas, J. (1983) Trends in Biochemical Sciences 8, 374-377.

227. Penningroth, S. M. and Kirschner, M. W. (1978) Biochemistry 17, 734-740.

228. Hamel, E., Campo, A. A. and Lin, C. M. (1983) Biochemistry 22, 3664-3671.

229. Sandoval, I. V., Jameson, J. L., Neidel, J., McDonald, E. and Cautrecasas, P. (1978) Proc. Natl. Acad. Sci. (U.S.A.) 75, 3178-3182.

230. Jameson, L. and Caplow, M. (1980) J. Biol. Chem. 255, 2284-2292.

231. Geahlen, R. L. and Haley, B. E. (1977) Proc. Natl. Acad. Sci. (U.S.A.) 74, 4375-4377.

232. Geahlen, R. L. and Haley, B. E. (1979) J. Biol. Chem. 254, 11982-11987.

233. Zabrecky, J. R. and Cole, R. D. (1982) Nature 296,

757-759.

234. Zabrecky, J. R. and Cole, R. D. (1983) Arch. Biochem. Biophys. 225, 475-481.

LIST OF PUBLICATIONS.

Islam, K. and Burns, R. G. (1981) Multiple phosphorylation sites of microtubule associated protein(MAP_2) observed at high ATP concentrations. FEBS Lett. 123, 181-185.

Burns, R. G. and Islam, K. (1981) Nucleoside diphosphate kinase associates with rings but not with assembled microtubules. Eur. J. Biochem. 117, 515-519.

Burns, R. G. and Islam, K. (1982) Characterisation of chick brain high molecular weight, multiply phosphorylated, microtubule associated protein. Eur. J. Biochem. 122, 25-29.

Islam, K. and Burns, R.G. (1984) Purification of a soluble monoisozyme of nucleoside diphosphate kinase from chick brain: exploitation of ionic conditions. Anal. Biochem. 136, (in press)

Submitted

Burns, R. G. and Islam, K. Phosphorylation of the chick brain high molecular weight protein MAP_2 by an endogenous cAMP-independent protein kinase utilising either ATP or GTP.

Burns, R. G. and Islam, K. Determination of the chick brain microtubule associated protein MAP_2:tubulin stoichiometry and the localisation of the twelve phosphorylation and five cysteine residues.

Burns, R. G., Islam, K. and Chapman, R. Function of the twelve phosphorylation sites of the microtubule associated protein MAP_2 in regulating the critical concentration for assembly by modulating the MAP_2: tubulin interaction.

Islam, K. and Burns, R.G. Kinetics of microtubule assembly at physiological GTP concentrations.

Abstracts

Burns, R. G. and Islam, K. (1979) Purification of the transphosphorylase associated with microtubules assembled *in vitro*. *J. Cell Biol.* **83**, 353a.

Burns, R. G. and Islam, K. (1980) Nucleoside diphosphate kinase-tubulin interactions. *Cell Biol. Int. Reports* **4**, 788.

Burns, R. G. and Islam, K. (1981) Multiple phosphorylation of microtubule associated protein: evidence for four protein kinase activities. *J. Cell Biol.* **91**, 333a.

Burns, R. G. and Islam, K. (1982) Phosphorylation of microtubule associated protein MAP_2. Biol. of the Cell 45, 261.

Burns, R. G. and Islam, K. (1982) Chemical dissection of the microtubule associated protein MAP_2. J. Cell Biol. 95, 343a.

Burns, R. G. and Islam, K. (1983) Evidence that tubulin oligomers are not assembly-intermediates at physiological GTP concentrations. J. Cell Biol. 97, 211a.

Burns, R. G., Islam, K. and Chapman, R. (1983) The graduated phosphorylation of MAP_2 regulates the MAP_2:tubulin interaction. J.Cell Biol. 97, 200a.

Eur. J. Biochem. 117, 515–519 (1981)

Nucleosidediphosphate Kinase Associates with Rings but not with Assembled Microtubules

Roy G. BURNS and Khalid ISLAM

Biophysics Section, Department of Physics, Imperial College of Science and Technology, London

(Received January 12/April 21, 1981)

Microtubules reassembled *in vitro* from chick brain contain significant nucleosidediphosphate (NDP) kinase activity (EC 2.7.4.6) although the specific activity decreases with successive cycles of reassembly. However, while the recovery of microtubule protein, as a function of initial protein concentration, exhibits a critical concentration below which there is no polymerisation, the recovery of NDP kinase activity is, at low initial protein concentrations, directly proportional to the initial protein content indicating that microtubule protein and the kinase activity are independently recovered. This was confirmed by pelleting the reassembled microtubules through a sucrose cushion: the specific activity of the pelleted microtubules was reduced by approximately 90%. By contrast, when cold-dissociated microtubule protein, which is predominantly in the form of rings, is fractionated on a Biogel A 15 m column the microtubule protein and NDP kinase activity coeluted in the void volume and the specific activity remained constant throughout the ring fraction. Similarly, when microtubules were dissociated in the presence of NDP kinase the enzyme bound to the generated rings. These results suggest that NDP kinase binds preferentially to the rings compared with the microtubules, and a model is proposed to account for the presence of this enzyme in pellets of microtubule protein.

Microtubules can be purified from brain by successive cycles of assembly and disassembly (e.g. [1, 2]). During the cold-dissociation step, the microtubules break down into ring-like structures, whose dimensions, morphology, and sedimentation characteristics depend upon the conditions and/or methods used (e.g. [3, 4]), but which usually sediment at about 36 S.

Microtubule assembly requires, in general, the addition of GTP or a GTP-generating system. The tubulin dimer binds two molecules of GTP, one at a non-exchangeable site (the N site), while the other is readily exchangeable (the E site) [5, 6]. During assembly, the GTP bound to the E site is hydrolysed [7–10], while that bound to the N site remains unaltered [10]. It has been proposed that GDP bound to the E site following hydrolysis of the GTP, exchanges with free GTP before participating in further microtubule assembly, although there is some evidence that the bound GDP is charged *in situ* by nucleosidediphosphate (NDP) kinase [11]. It is however clear that NDP kinase is necessary to charge any GDP bound to the N site.

Microtubules, purified through successive cycles of assembly and disassembly, contain significant amounts of NDP kinase [10, 11, 13, 14]. Similarly, Watanabe and Flavin [12] observed NDP kinase activity in isolated *Chlamydomonas* flagella, and found that a significant amount could not be released from the detached flagella by washing, indicating that the NDP kinase is an axonemal protein. The NDP kinase of reassembled brain microtubules has been separated from tubulin [10, 11] and also from the high-molecular-weight and *tau*-like microtubule-associated proteins (MAPs) [11].

The NDP kinase specific activity has been reported to decrease through successive cycles of microtubule reassembly [10, 12, 14], demonstrating that its activity is not recovered in stoichiometric amounts. Nickerson and Wells [13] calculated that the observed decrease through successive cycles was less than that predicted by simple dilution, and concluded that the enzyme is associated, if somewhat loosely, with reassembled microtubules. By contrast, Penningroth and Kirschner [10] observed a constant, but low, specific activity through three cycles of polymerisation. In all cases, the microtubules were polymerised from brain extracts, although there were significant differences in the procedures used. In order to determine whether NDP kinase is, in fact, a microtubule-associated protein, its recovery has been monitored as a function of the initial protein concentration. The results indicate that it co-purifies with microtubules, but while it is not a microtubule-associated protein, it does associate with a tubulin oligomer.

Abbreviation. Mes, 4-morpholine ethane sulphonic acid.

Enzyme. Nucleosidediphosphate kinase or NDP kinase (EC 2.7.4.6).

MATERIALS AND METHODS

Preparation of Microtubule Protein

Day-old chick brains were homogenised in an equal (w/v) volume of cold buffer containing 0.1 M Mes, 2.5 mM EGTA, 0.5 mM $MgSO_4$, 0.1 mM EDTA, 0.1 mM dithiothreitol, pH 6.4 with KOH. After centrifugation for 30 min at 20000 × *g* at 4°C, the supernatant was adjusted to 1 mM GTP (unless otherwise stated) and incubated at 37°C for 10 min. The assembled microtubules were pelleted in the warm (30°C) by centrifugation for 30 min at 20000 × *g*. These once-reassembled microtubules were cold-dissociated for 30 min in one-tenth of the initial volume of the homogenisation buffer, and the cold-stable material removed by centrifugation for 30 min at 4°C at 65000 × *g*. In experiments which required two or three times reassembled microtubular protein, further cycles were performed by adjusting this cold-dissociated protein to 1 mM GTP, incubating at 37°C for 30 min, and centrifuging for 30 min at

30 °C at 65000 × *g*, cold-dissociating for 30 min in an appropriate volume of homogenisation buffer and centrifuging for 30 min at 65000 × *g* at 4 °C.

Measurement of NDP Kinase Activity

NDP kinase was assayed using a coupled enzyme system [15] which had been optimised for purified microtubule NDP kinase (unpublished results). The asssay mixture contained 0.1 M Mes, 5 mM $MgSO_4$, 1.1 mM phospho*enol*pyruvate, 2 mM ATP, 0.2 mM NADH, 0.4 U ml^{-1} pyruvate kinase, 8.8 U ml^{-1} lactate dehydrogenase, pH 6.9 with KOH. The reaction was started by the addition of 1 mM deoxyguanosine, and monitored by following the decrease in absorbance at 340 nm at 21 °C. The NDP kinase activity was corrected for the initial rate prior to the addition of deoxyguanosine, and for the background rate in the absence of added NDP kinase.

Protein Determination

Protein concentrations were determined using the method of Hartree [16], calibrated with bovine serum albumin.

Electron Microscopy

Samples were placed on carbon-coated grids, negatively stained with 2% uranyl acetate, and examined with a Philips 301 electron microscope at 80 kV.

Gel Electrophoresis

Proteins were fractionated by sodium dodecyl sulphate gel electrophoresis, using the method of Laemmli [17] and stained with Coomassie blue [18].

Gel Exclusion Chromatography

Microtubule protein was fractionated on columns (1.6 cm × 40 cm) of Biogel A 15 m (200–400 mesh; Bio-Rad Laboratories, Richmond, CA), preequilibrated with reassembly buffer, at a flow rate of 30 ml h^{-1}. When reassembled microtubules were fractionated, the cold-dissociated protein was adjusted to 1 mM GTP, warmed to 37 °C to facilitate polymerisation, and the assembled microtubules were sheared by gentle homogenisation in a Teflon/glass homogeniser.

Preparation of NDP Kinase

A crude preparation of NDP kinase was prepared by fractionating the warm supernatant following the second cycle of microtubule assembly on a Biogel A 1.5 m column equilibrated with reassembly buffer. NDP kinase eluted behind the tubulin dimer peak (data not presented) and had a specific activity of 25 μmol min^{-1} mg^{-1}. The preparation was extensively dialysed against reassembly buffer to remove the nucleotides and centrifuged at 65000 × *g* for 60 min to remove any precipitated protein before it was used.

RESULTS AND DISCUSSION

The specific activity of NDP kinase recovered in cold-dissociated microtubule protein decreases in successive cycles of reassembly (Fig. 1). The decrease is approximately exponential, and there is no difference between preparations reassembled in the presence of ATP compared with GTP. Under

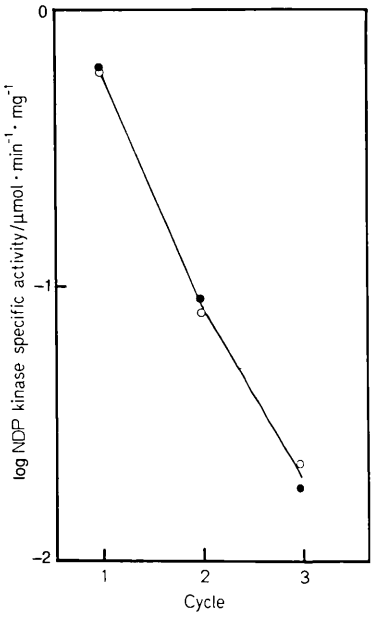


Fig. 1. *Dependence of the NDP kinase specific activity in the cold-dissociated microtubule supernatant as a function of the cycle of polymerisation.* There is no difference between preparations cycled in the presence of 1 mM ATP (●) compared with 1 mM GTP (○)

the centrifugation conditions used (30 min at 65000 × *g*), the specific activity is similar to that observed by Nickerson and Wells (1 μmol min^{-1} mg^{-1}, first cycle), but is significantly higher than that observed by Watanabe and Flavin (0.01 μmol min^{-1} mg^{-1}, first cycle) or Penningroth and Kirschner (0.07 μmol min^{-1} mg^{-1}). As it was also observed that the recovery between experiments was dependent upon the concentration of microtubular protein, this was examined in more detail.

Cold-dissociated microtubular protein, termed the initial cold-dissociated protein, from the first cycle of reassembly was diluted with homogenisation buffer, adjusted to 1 mM GTP and reassembled. Following the warm centrifugation, the reassembled microtubules were dissociated and the cold-stable material removed by centrifugation at 4 °C. An increasing amount of protein was recovered with increasing initial protein concentrations (Fig. 2), with an apparent critical concentration of 0.3 mg ml^{-1}, with a second apparent critical concentration at 1.1 mg ml^{-1} as the recovery of protein markedly increased above an initial protein concentration of 3 mg ml^{-1}. The recovery of NPD kinase activity also showed two components, but in contrast to the recovery of protein, the first component extrapolated through the origin, whereas the second component extrapolated to a critical concentration of 1.1 mg ml^{-1}.

Gel electrophoresis of successive cold-dissociated microtubule supernatants shows that tubulin is the major protein in each fraction (Fig. 3, gels A, C, and E), and that there is a marked decrease in the amount of the 80000-M_r protein (arrowed) which co-migrates with purified NDP kinase. There is the selective loss of other components, although the microtubule-associated protein MAP2 is retained as a major component through the three cycles of reassembly. By contrast, gels of the warm supernatants show the almost complete absence of MAP2 (Fig. 3, gels B and D) and the preferential recovery of other bands including NDP kinase.

Electron microscopy of once cold-dissociated microtubular protein showed that much was present as single gyre rings, and

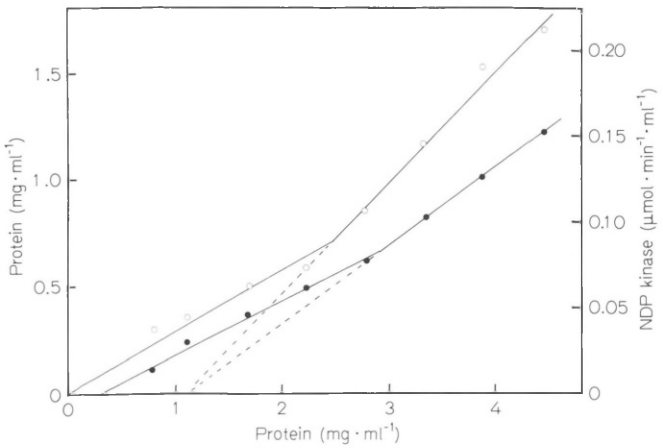


Fig. 2. *Recovery of protein (●) and NDP kinase activity (○) in the cold-dissociated microtubule supernatant as a function of the initial protein concentration.* The initial protein was once cold-dissociated microtubule protein, prepared in the presence of 1 mM GTP

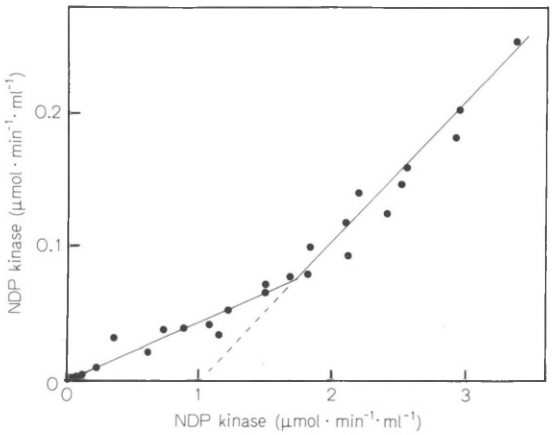


Fig. 4. *Data from four different preparations showing the recovery of NDP kinase activity in the cold-dissociated supernatant as a function of the activity of the initial protein*

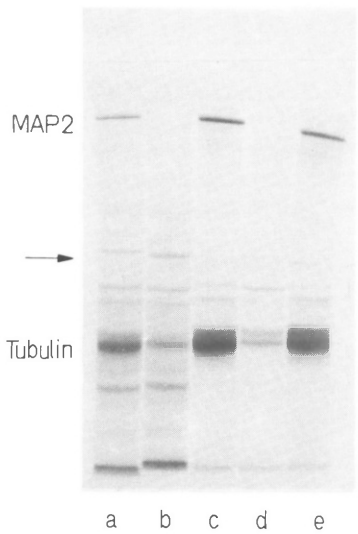


Fig. 3. *Fractionation of microtubule protein by dodecylsulphate/polyacrylamide gel electrophoresis on 8% gels.* The components corresponding to tubulin and microtubule-associated protein 2 (MAP2) are marked. The arrow indicates the 80000 M_r protein which has been shown to be NDP kinase [14]. The gels correspond to: (a) the cold-dissociated microtubule protein following one cycle of reassembly; (b) the warm supernatant following the second cycle of reassembly; (c) the twice cold-dissociated protein; (d) the third warm supernatant; and (e) the three-times cold-dissociated protein

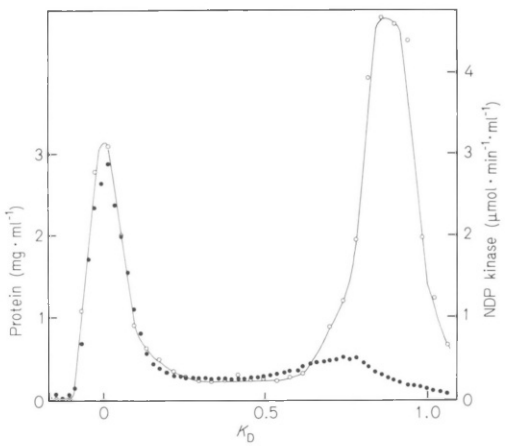


Fig. 5. *Fractionation of once cold-dissociated microtubule protein on a Biogel A 15 m column (1.6 cm × 40 cm).* The column was preequilibrated with reassembly buffer and was eluted at 4 °C. (●) Distribution of protein; (○) distribution of NDP kinase

that intact microtubules were the predominant structure after warming to 37 °C.

Data from four different preparations for the recovery of NDP kinase activity in the cold-dissociated supernatant as a function of the initial activity is shown in Fig. 4. Approximately 4% of the initial activity is recovered at low initial activities, rising to over 10% at high activities. The data from the individual experiments (e.g. Fig. 2) suggest that there are two linear components, with a critical concentration for the second phase of 1 μmol min^{-1} mg^{-1}.

The observation that at low initial protein concentrations the recovery of NDP kinase is directly proportional to the initial protein content whereas the recovery of microtubular protein exhibits a critical concentration is strong evidence that this enzyme is not bound to the microtubules, but is recovered independently. However, the observed recovery (4–10%) is more than can be accounted for by simple entrapment of NPD kinase in the microtubule pellet, as Nickerson and Wells have pointed out [13], or by sedimentation as the sedimentation coefficient of the enzyme is only 4.3 S [12].

This paradox has been examined by fractionating cold-dissociated microtubular protein, following the second cycle of polymerisation, on a Biogel A 15 m column equilibrated with reassembly buffer, and assaying the fractions for protein and NDP kinase activity (Fig. 5). Fractionation of microtubular protein by gel exclusion chromatography has been shown to separate the 36-S rings, composed of tubulin and microtubule-associated proteins, from the 6-S tubulin dimer (e.g. [19, 20]).

Approximately 60% of the total protein eluted in the void volume, and was shown by gel electrophoresis to consist of tubulin and the microtubule-associated proteins, while electron microscopy showed that the rings were the sole identifiable polymeric structure present in this fraction. No attempt has been made to determine the relative concentration of rings because of the inherent problems associated with the quantification of negative-stained preparations and because of the time-dependent dissociation of the rings (see below).

A second protein peak eluted close to the salt volume, representing less than 20% of the total protein, and which consisted primarily of tubulin. The protein content between these two peaks is significantly above background, and is due to the dissociation of the rings as they pass down the column.

Analysis of the distribution of NDP kinase indicated a similar qualitative distribution, with the enzyme present in all fractions between the void and salt volumes, although the quantitative distribution was different from that of the total protein. Approximately 28% was present in the void peak, yielding a specific activity of 1.036 ± 0.051 μmol min^{-1} mg^{-1}, while 63% was found in the salt volume, eluting slightly after most of the protein. The maximum specific activity of NDP kinase in the salt peak was 20.7 μmol min^{-1} mg^{-1}. The NDP kinase content between the void and salt peaks exactly mirrored the protein content, such that the specific activity remained constant (mean 0.945 μmol min^{-1} mg^{-1}). As the protein found in the fractions between the void and salt peaks resulted from the dissociation of rings passing down the column, the observation that the specific activity of these fractions is very similar to that of the rings eluting in the void fraction strongly suggests that the NDP kinase is bound to the rings and becomes released as the rings dissociate, and is not due to the enzyme binding to other particulate material eluting in the void volume. These results confirm the observation of Jacobs and Huitorel [11] that NDP kinase binds to a polymeric form of tubulin.

A similar finding was observed when once cold-dissociated microtubular protein was fractionated on a 5–30% sucrose gradient. Most of the rings dissociated during the course of the centrifugation (4 h at 4°C) such that there was an extensive trail from the dimeric tubulin peak. The NDP kinase activity mirrored the dissociation of the rings (unpublished observation).

The evidence therefore indicates that NDP kinase binds to rings (Fig. 5) but not to assembled microtubules (Fig. 2). Unfortunately, attempts to separate rings directly from assembled microtubules by gel exclusion chromatography have been unsuccessful, and centrifugation studies are inappropriate due to the time periods involved and the rate of dissociation of the microtubules and the rings. It is therefore only possible to offer indirect evidence to support this conclusion of differential binding.

Microtubular protein, following the first cycle of polymerisation, was assembled (see Materials and Methods), loaded onto a Biogel A 15 m column equilibrated with reassembly buffer containing 0.1 mM GTP, and fractionated at 30°C (Fig. 6). Approximately 12% of the total protein eluted in the void fractions. This apparently low value is consistent with the cumulative effects of the turnover of microtubules during the fractionation (15 min), the rate of dissociation of the rings at 30°C, and the requirement for a critical concentration for assembly, together with the possible inactivation of the microtubular proteins. By contrast, only 5.4% of the total NDP kinase activity was eluted in the void fractions, yielding a specific activity for the void protein of 0.514 μmol min^{-1} mg^{-1}. This value is significantly lower than the observed specific activity (1.0 μmol min^{-1} mg^{-1}) of the void fractions of equivalent cold-dissociated microtubular protein, i.e. rings (Fig. 5).

It should be noted that the specific activity of the void fraction when assembled microtubules are fractionated at 30°C (Fig. 6) is probably an overestimate as the column does not distinguish between intact microtubules and rings. Margolis and Wilson [21] have shown that a dynamic equilibrium exists

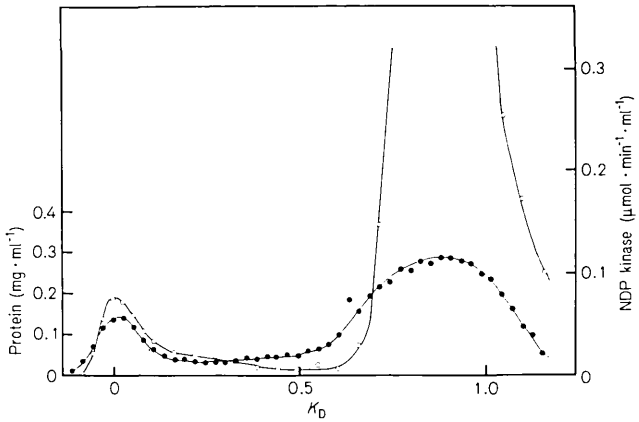


Fig. 6. *Fractionation of intact microtubules by gel exclusion chromatography on Biogel A 15 m (1.6 cm × 40 cm).* Once cold-dissociated protein was reassembled in the presence of 1 mM GTP, and the microtubules gently sheared by homogenisation. The column was preequilibrated with reassembly buffer containing 0.1 mM GTP, and was eluted at 30°C. (●) Distribution of protein; (○) distribution of NDP kinase

once maximal microtubule assembly has been attained, such that the microtubules are constantly disassembling at one end and reassembling at the other, the so-called treadmilling effect. Consequently, as the microtubules were sheared before loading onto the column, there will have been a significant increase in the rate of microtubule turnover, and hence an increased rate of ring formation. Any rings formed before the migration of the microtubular protein away from the high NDP kinase concentration in the salt volume would have been exposed to the free enzyme. Consequently, the void fraction consists of both intact microtubules and rings which may have been exposed to an environment of high NDP kinase concentration

This interpretation is supported by the striking result obtained when once cold-dissociated microtubular protein was reassembled, and the polymerised microtubules pelleted through a 50% sucrose cushion (75000 × *g* for 4 h). The specific activity of NDP kinase in the cold-dissociated pellet was only 0.0232 μmol min^{-1} mg^{-1}, compared with the usual value of 0.2–0.3 μmol min^{-1} mg^{-1} at equivalent initial protein concentrations.

It was noted that, when rings were fractionated on a Biogel A 15 m column (Fig. 5), a significant fraction of the total NDP kinase activity eluted in the salt volume and not with the rings present in the void. However, as all of the activity was pelleted with the microtubular protein, this suggests that there is an equilibrium between free NDP kinase and that bound to the rings. This was examined by fractionating the same preparation of assembled microtubules used in Fig. 6 on a Biogel A 15 m column which had been pre-equilibrated with NDP kinase (see Materials and Methods). The distribution of protein was almost identical, after correction for the protein background of NDP kinase, to that observed in the absence of added enzyme (Fig. 5). However, significantly more NDP kinase eluted in the void fraction, after correction for the background activity of 0.18 μmol min^{-1} mg^{-1}, compared with the column run in the absence of added enzyme. A comparison of the void region of the two columns is shown in Fig. 7. The specific activity of the void peak increased from 0.514 μmol min^{-1} mg^{-1} to 0.919 μmol min^{-1} mg^{-1}, an increase of over 75%. In such a Hummel-and-Dreyer-type experiment, a trough in activity should be seen in the region between the ring fraction and the salt volume. This though was not detected, partly because of the continued dissociation of the rings and the high NDP kinase

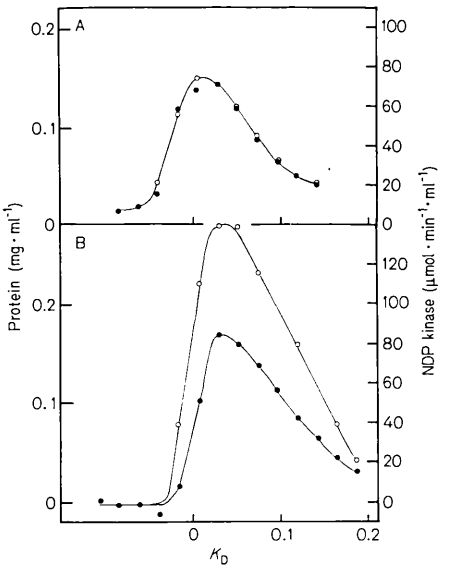


Fig. 7. *Binding of NDP kinase to microtubule proteins eluting in the void volume of Biogel A 15 m columns.* (A) An expansion of the void region of the column shown in Fig. 6. (B) The same preparation of sheared microtubules was loaded onto a parallel Biogel A 15 m column which had been preequilibrated with reassembly buffer containing 0.1 mM GTP and NDP kinase (0.180 ± 0.010 μmol min^{-1} ml^{-1}) and eluted at 30 °C. The protein and NDP kinase backgrounds have been subtracted to permit direct comparison with (A). (●) Distribution of protein; (○) distribution of NDP kinase

activity present in the salt volume (Fig. 5), and partly because the column was pre-equilibrated with a high activity of NDP kinase (0.18 μmol min^{-1} mg^{-1}) compared with the increase in activity of the void peak (0.06 μmol min^{-1} ml^{-1}). The incubation of microtubules in the presence of NDP kinase under conditions which result in the partial dissociation of the microtubules into rings therefore causes an increase in binding of NDP kinase.

The observations that NDP kinase can bind to rings (Fig. 5 and 7), that rings are the principle dissociation product of the breakdown of microtubules, and that microtubules exist in a state of dynamic equilibrium [21] suggest an explanation for the comparatively high specific activity of NDP kinase in pelleted reassembled microtubules (Fig. 1 and 2). As the assembled microtubules are pelleted through a solution containing GTP, treadmilling will continue: microtubule turnover is not fixed at the commencement of the centrifugation. The rings therefore continue to be generated throughout the centrifugation run, and many of the rings generated will be sufficiently close to the pellet that they will themselves be pelleted. As the rings, presumably free of NDP kinase, are generated they should be capable of binding the NDP kinase present in the medium (Fig. 6 and 7). There are therefore two fates for a ring loaded with NDP kinase: if it is generated close to the pellet it may become incorporated into the pellet, or alternatively it may participate in a further cycle of assembly, so-called treadmilling, with the concommitant return of the kinase to the medium.

Two alternative possibilities can be discounted: (a) that NDP kinase is pelleted independently of either the rings or the assembled microtubules as its sedimentation coefficient is only 4.3 S [12], and (b) that the NDP kinase content of the pellet represents sedimented rings which were present in the initial assembly mixture and which were not formed as a result of treadmilling, as fractionation of the warm supernatant on a Biogel A 15 m column indicates that no rings are present (unpublished observation), yet the sedimentation coefficient of the rings (36 S) is insufficient for significant pelleting to occur under the conditions used ($65000 \times g$, 30 min).

It is concluded that the observed NDP kinase activity associated with cold-dissociated microtubular protein does not result from the binding of this enzyme to assembled microtubules but to tubulin rings. This selective binding may be of considerable importance in the nucleotide regulation of microtubule assembly *in vivo*.

This work was supported by the Medical Research Council.

REFERENCES

1. Borisy, G. G. & Olmsted, J. B. (1972) *Science (Wash. DC) 177*, 1196–1197.
2. Shelanski, M. L., Gaskin, F. & Cantor, C. R. (1973) *Proc. Natl Acad. Sci. USA, 70*, 765–768.
3. Kirschner, M. W., Williams, R. C., Weingarten, M. D. & Gerhart, J. C. (1974) *Proc. Natl Acad. Sci. USA, 71*, 1159–1163.
4. Olmsted, J. B., Marcum, J. M., Johnson, K. A., Allen, C. & Borisy, G. G. (1974) *J. Supramol. Struct. 2*, 429–450.
5. Jacobs, M., Smith, E. & Taylor, E. W. (1974) *J. Mol. Biol. 89*, 455–468.
6. Arai, T., Ihara, Y., Arai, K.-I. & Kaziro, Y. (1975) *J. Biochem. (Tokyo) 77*, 647–658.
7. Arai, T. & Kaziro, Y. (1976) *Biochem. Biophys. Res. Commun. 69*, 369–376.
8. David-Pfeuty, T., Erickson, H. & Pantaloni, D. (1977) *Proc. Natl Acad. Sci. USA, 74*, 5372–5376.
9. Kobayashi, T. & Simizu T. (1976) *J. Biochem (Tokyo) 79*, 1357–1364.
10. Penningroth, S. & Kirschner, M. W. (1977) *J. Mol. Biol. 115*, 643–673.
11. Jacobs, M. & Huitorel, P. (1979) *Eur. J. Biochem. 99*, 613–622.
12. Watanabe, T. & Flavin, M. (1976) *J. Biol. Chem. 251*, 182–192.
13. Nickerson, J. A. & Wells, W. W. (1978) *Biochem. Biophys. Res. Commun. 85*, 820–826.
14. Burns, R. G. & Islam, K. (1979) *J. Cell Biol. 83*, 353a.
15. Bergmeyer, H. U. (1974) *Methods in Enzymatic Analysis*, pp. 488–489, Academic Press, New York
16. Hartree, E. F. (1972) *Anal. Biochem. 48*, 422–427.
17. Laemmli, U. K. (1970) *Nature (Lond.) 227*, 680–685.
18. Fairbanks, G., Steck, T. L. & Wallach, D. F. H. (1971) *Biochemistry, 10*, 2606–2617.
19. Erickson, H. P. (1974) *J. Supramol. Struct. 2*, 393–411.
20. Vallee, R. B. & Borisy, G. G. (1978) *J. Biol. Chem. 253*, 2834–2845.
21. Margolis, R. L. & Wilson, L. (1978) *Cell 13*, 1–8.

R. G. Burns and K. Islam, Biophysics Section, Department of Physics, Imperial College of Science and Technology, Prince Consort Road, London, SW7 2AZ