STUDIES ON THE PHOSPHORYLATION OF BRAIN MICROTUBULAR COMPONENTS

by

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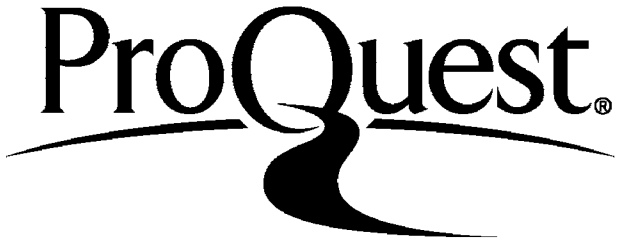

Abstract

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Microtubular protein isolated from brains of adult rats or 1 to 3-day old chicks, previously injected with ^{32}P, contained considerable amounts of serine-bound ^{32}P measured as alkali-labile protein bound ^{32}P. Between 40 and 80% of the acid-stable tubulin-bound ^{32}P was extractable with lipid solvents and was shown to be associated with several major phospholipid classes.

Experiments in which tubulin purified by polymerisation was incubated with [γ-^{32}P] ATP revealed that tubulin itself acted as a substrate for a microtubule-associated protein kinase under *in vitro* polymerisation conditions.

Further investigations concerning the biochemical properties of brain microtubulin fractions prepared in the presence or absence of glycerol revealed that glycerol brought about a re-distribution of total protein and of phospholipid-and protein-bound ^{32}P.

In a final series of experiments, the interactions between myo-inositol and purified brain tubulin were investigated. It was found that myo-inositol binds to tubulin (0.5 - 1.0 moles of myo-inositol/mole of tubulin dimer) and that myo-inositol affected the stability of *in vitro* polymerised microtubules.

The data presented in this work provide new biochemical evidence for interactions between microtubules and membranes, the functional implications of which are discussed.

Acknowledgements

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I wish to extend my appreciation to my parents for their encouragement and patience, and I hope that from this work they will at least draw some comfort.

Abbreviations

ATP	Adenosine5'-triphosphate
cAMP	Cyclic adenosine5'-monophosphate
ATPase	Adenosine triphosphatase
GTP	Guanosine5'-triphosphate
TRIS	[2-amino-2-(hydroxymethyl)propane-1,3-diol]
MES	2-(N-morpholino)ethanesulphonic acid
PIPES	Piperazine-NN'-bis-2-ethanesulphonic acid
EGTA	1,2-di(2-aminoethoxy)ethane-N,N,N',N'-tetra-acetic acid
TCA	Trichloroacetic acid
MTP	Microtubular protein

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Chapter 1 Introduction

1.1 Historical

Already in the very early days of cell biology, some of the properties of the cell, especially those attributed to the cytoplasm, demanded for their explanation the assumption of a general organisation or architecture of the cytoplasm. The facts that cells are elastic, that cells can assume, maintain and control various shapes, that Brownian particle movement is absent in the majority of cell types, could only be explained by the existence of what is today known as a cytoskeleton. Studies with the light microscope provided the first evidence for the presence of a structuring mesh of filamentous elements in the cytoplasm. Early efforts to resolve the internal structure of nerve fibres revealed the presence of longitudinal fibres of different diameters, which could not really be resolved with by light microscopy. Moreover, the finding that the then used cytological techniques of fixation and staining could produce artefactual coagulation in homogenous protein solutions made it very difficult for cytologists to believe in the validity of the patterns observed under the light microscope in stained specimens. However, later observations made with polarising optics on unfixed cells did indicate the presence of some sort of rod-like structures in the cytoplasm. In particular, the birefringence of muscle, nerve, sperm and mitotic spindles indicated the presence of long arrays of ordered molecular structures. The use of polarising optics to study whole living cells was, in a way, the

precipitating factor which convinced cytologists of the validity of the existence *in situ* of fibrous cytoskeletal elements. It is now generally recognised that microtubules are a major component of the cytoskeletons of eukaryotic cells.

1.2 General characteristics of microtubules

At first electron microscopy could only resolve the internal structure of cilia and flagella and of centrioles and basal bodies.

In basal bodies stable arrays of fused microtubules (pairs and triplets) showed up as the main fibrous components present in the cellular elements.

Microtubules as such were first noted by Fawcett and Porter (1954) as elements of the '9 + 2' fibers in cilia. Each cilium contains nine pairs of 'outer microtubule doublets' near the perifery, which are linked to each other through intradoublet links, made of the protein nexin, and to a spring-like proteinaceous sheath surrounding a 'central pair' of microtubules via spoke-like elements. The outer pairs of microtubules are referred to as A and B subfibres, and these are connected through the sharing of subunits, common to the wall of each tubule. The complete A subfibre, to which a spoke is attached, also contains a pair of side arms which have now been shown to be composed of an ATP-ase which is referred to as dynein (Satir, 1974). The '9 + 2' arrangement of microtubules is illustrated in fig. 1.1 showing a schematic drawing of the cross-section of a cilium.

A simplified version of this pattern is observed

Fig. 1.1

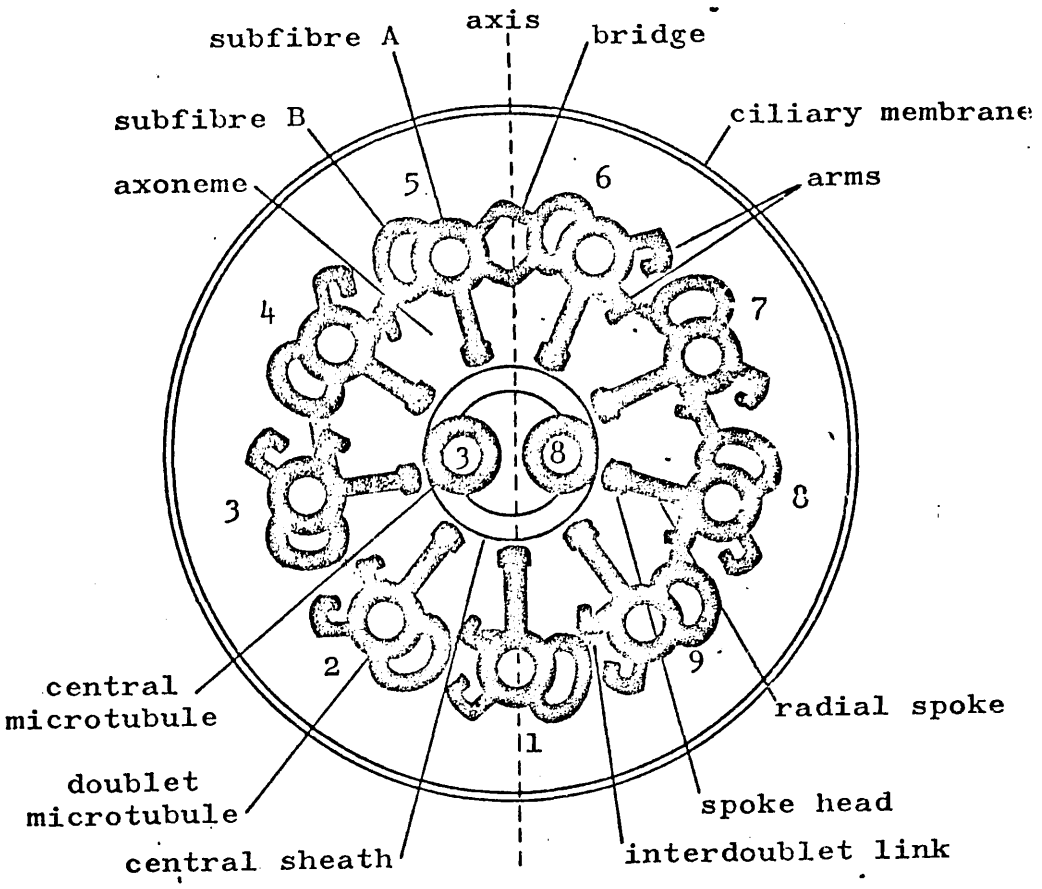
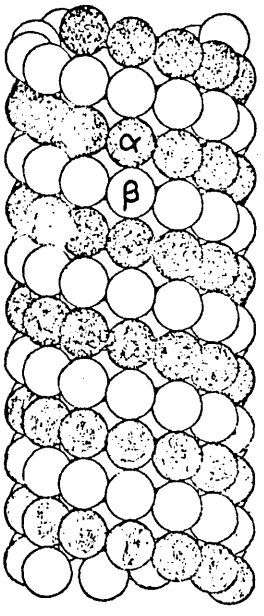


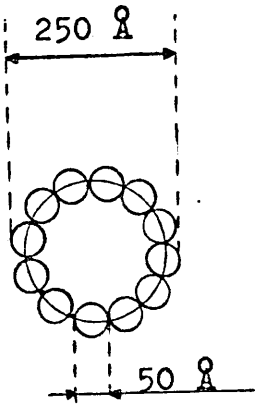
Fig. 1.2

A



Microtubule surface lattice

B



Crossection through a microtubule

in a number of sensory receptor cells where microtubules are arranged in a '9 + 0' pattern. This has led to the view that sensory receptors may be modified cilia. In one type of receptor cell, the mechanoreceptor of the cockroach leg, singlet microtubules are present in very large numbers, virtually filling the cytoplasm. In this case, only occasional inter-tubular links can be detected and the main functional linkages appear to occur between individual microtubules and the plasma membrane (Moran et al, 1971).

Glutaraldehyde appears to act as a cross-linking agent, stabilising singlet microtubules in the cytoplasm of various cells and those forming the mitotic interphase spindle. Microtubules in the axonal and dendritic processes of neurons were first referred to as neuro-tubules to distinguish them from the thinner and more stable filaments seen in axons called neurofilaments (ca 10nm in diameter). The lability and changing intracellular distribution of cytoplasmic microtubules and spindle microtubules seen in all eukariotic cells examined led to their description as 'handy portable organelles'. It was later shown by Behnke and Forer (1967) that, in fact, cytoplasmic microtubules vary in their lability in different cell types and even within a single cell type.

In the case of the mitotic spindle no strict pairing between individual microtubules is apparent, though there exists some evidence that some of the microtubules constituting the mitotic spindle are held together, temporarily at least, through inter-tubular bridges

(Hepler et al, 1970).

Electron microscopic examination of cytoplasmic microtubules in a wide variety of eukaryotic cells has shown that individual microtubules appear as straight, hollow, cylindrical structures about 250 Å in diameter, composed of 13 linearly arranged protofilaments (see fig. 1.2). Each protofilament is composed of a linear array of globular subunits, about 50 Å in diameter.

Beginning with the observations of Palay (1956) and with the application of glutaraldehyde as fixing agent microtubules have been found in most nerve fibers of all vertebrates and invertebrates examined. In developing vertebrate neurites and in mature vertebrate dendrites and unmyelinated axons, it is the microtubule, also called the neurotubule, which is the predominant linear component. In these cases the neurofilament, the remaining linear component, is usually sparsely and unevenly distributed within the neurite cytoplasm. Neurofilaments are, on the other hand, the main filamentous structure seen in the giant axons of squid and Myxicola.

Axonal microtubules appear to begin from the cell body and until recently they were thought to terminate before entering the synaptic terminals (Gray and Guillery, 1966); Wuerker and Kirkpatrick, 1972). However, Gray (1975) showed that microtubules traverse the presynaptic cytoplasm and focus on the terminal membrane. Although there is little evidence for structural links between neurotubules with each other or with other cellular elements, it was recently shown, using lanthanum staining techniques, that the surface of neurotubules in various

invertebrate nerve cords seems to be covered with amorphous filamentous extensions which frequently appear to act as intertubular links (Burton and Fernandez, 1973). In addition, an earlier study (Kirkpatrick et al, 1970), in which intact microtubules were isolated from brain homogenates showed that these microtubules have a fuzzy, filamentous coating.

Early studies on the mechanism of action of colchicine as an antimitotic agent by Inoue (1953) and Taylor (1968), showed that this alkaloid appeared to specifically interact with a protein component of the mitotic spindle, i.e. with a component of spindle microtubules. This work led to the use of radioactively labelled colchicine as a specific ligand to detect the presence of microtubular protein, defined as 'colchicine binding protein' in crude tissue extracts from non-dividing cells. In this way it was found that brain was a particularly rich source of 'colchicine binding protein'. Using colchicine binding to monitor the presence of the protein, procedures were devised to isolate what was later identified as the subunit protein of microtubules (Weisenberg et al, 1968; Shelanski and Taylor, 1968).

It was shown that microtubules could be dissociated into a relatively stable 6S protein particle, which appears to be the basic chemical subunit (Borisy and Taylor, 1967). This 6S unit has a molecular weight (MW of 110000)and is, apparently, a dimer of two polypeptide chains with a MW of about 55000 each. It has now been shown that microtubules are composed of two slightly different monomers of MW of approximately 55000, designated as α- and ß-tubulin (Bryan and Wilson, 1971) and it has been

suggested that the 6S tubulin unit is an α- ß heterodimer. It is now postulated that each of the above mentioned morphological subunits of 50 Å diameter may be identified as either an α- or ß- tubulin. The chemically defined 6S tubulin, consisting of the α- and ß- tubulin peptides, is referred to as the 6S dimer, and it is thought that the axis of the 6S dimer is oriented parallel to the axis of the microtubule.

Microtubule subunit protein (6S unit) isolated from many sources has been observed to be associated with guanine nucleotides (Shelanski and Taylor, 1968; Weisenberg et al, 1968; Bryan, 1972), and it was proposed that these nucleotides stabilised the native configuration of the protein (Weisenberg et al, 1968). Definition of the binding properties of the protein indicated that up to 2 moles of guanine nucleotide was bound per tubulin dimer, of which one mole was readily exchangeable and the other was tightly bound (Berry and Shelanski, 1972; Levi et al, 1974; Jacobs, 1975).

The 6S protein unit of tubulin can be resolved into two separate polypeptides, by electrophoresis, on SDS-urea polyacrylamide gels. The faster migrating polypeptide is referred to as the ß-subunit and the more slowly migrating one as the α-subunit. The vast majority of studies indicate a 1:1 mole ratio of the two tubulin 'monomers' from rather diverse sources. The presence of one binding site each for colchicine, tightly bound GTP and exchangeable GTP would favour a hetero-dimer concept since a homo-dimer might be expected to provide two identical sites for at least one of these molecules. More direct support for this alleged heterogeneity of

the 6S tubulin unit comes from the work of Luduena et al (1974), who employed diimidate crosslinking agents to chemically unite dimerizing species in solutions of brain 6S tubulin. Analysis of the products by gel electrophoresis indicated that the reaction mainly yielded cross-linked dimers consisting of the two dissimilar tubulin monomers.

The α- and ß- chains of both chick embryo brain and sea urchin outer-fibre tubulin have been sequenced through the first 25 NH_2-terminal amino acids by Luduena and Woodward (1973). The two subunits were found to be clearly distinct from one another, but eleven positions were identical and differences in nine other positions could result from one base-pair change, presumably implying evolution from a single ancestral protein. When homologous polypeptide chains were compared in these two species, only the ß-chains differed and this difference was in one position only. With such a high degree of sequence homology, it is really not too surprising that all the tubulin dimers studied so far have yielded α- and ß- chains with essentially identical characteristic electrophoretic properties. Specific post translational modifications of tubulin α- and ß- chains could serve as a means for bringing about differences in microtubule stability or function without the requirement for multiple gene products.

1.3 Polymerisation of microtubulin

On the basis of their morphological distribution and assumed functions, microtubules fall in two general classes: a) the microtubules of motile organelles and

b) the cytoplasmic microtubules. Studies by Tilney and Gibbins (1968 and 1969) revealed the cold-, colchicine- and hydrostatic pressure- lability of cytoplasmic but not ciliary microtubules. Because of the apparently more dynamic nature of cytoplasmic microtubules they became the system for studying the processes of microtubule assembly and disassembly.

The very nature of the mitotic apparatus requires a temporal and spatial microtubule equilibrium, so the mitotic apparatus was a more or less obvious choice for studying microtubule assembly/disassembly *in vivo*. Certain other systems maintain cytoplasmic microtubules predominantly in the polymer form, e.g. neuronal microtubules, but they too are susceptible to manipulation by cold, hydrostatic pressure or colchicine. The availability and ease of preparation of neuronal tubulin made it the preferred system for studying the assembly/disassembly processes *in vitro*.

1.3.1 In vivo polymerisation

Spindle fibers generally possess a positive birefringence. Using a sensitive polarising microscope Inoue (1953 and 1964) took a series of time-lapse motion pictures showing the birefringence changes during mitosis in several types of cell. Inoue also observed that spindle fibre birefringence is abolished in a matter of seconds by treatment with low temperature. After return to normal temperature, the birefringence recovered in the course of a few minutes, after which the mitotic spindle was fully functional again. This reversible low temperature-induced disintegration of the mitotic spindle could be repeated up to ten times in the same cell.

Colchicine, Colcemid and other drugs can abolish

birefringence of the mitotic spindle of Pectinaria oocytes, but when the cells are washed with normal sea water the birefringence of the spindle recovers in as little as ten minutes (Malawista and Sato, 1966). High hydrostatic pressure is known to destroy the spindle organisation reversibly (Margland, 1966). The reformation of the spindle fibers after direct mechanical disruption by micromanipulation was described by Nicklas (1965).

Inoue also showed that mitotic spindles, arrested in meiotic metaphase and attached by one pole to the cell membrane, were found to shorten as they lost birefringence and concomitantly the chromosomes were translated towards the stationary, membrane-attached, end of the spindle microtubules. These early observations served as the basis for numerous studies on the labile and dynamic nature of spindle microtubules and also gave rise to the idea that the physiologically controlled depolymerisation of spindle microtubules could move chromosomes.

Inoue observed that spindle fiber birefringence increased with increasing temperature and appeared to approach an asymptotic limit (Ao). Using this upper limit as a measure of the maximum amount of available spindle fiber material and taking the observed birefringence at intermediate temperatures (b) as a measure of ordered polymeric material, i.e. microtubules, in equilibrium with disordered monomer, i.e. 6S tubulin dimer, (Ao - b), Inone (1960) postulated a simple equilibrium, $K = b/Ao - b$. A log plot of this assumed equilibrium constant against reciprocal temperature (Van't Hoff plot) produced a straight line, the slope

of which yielded an enthalpy change of +28 Kcal. From this value and a near-zero free energy change he calculated an entropy change of about + 100 eu.

Similar results were obtained by Sato and Bryan (1968) who used Pisaster spindles isolated at appropriate times after a temperature shift and also found first order polymerisation and depolymerisation kinetics, in agreement with the scheme proposed by Inoue. All these studies yield thermodynamic parameters consistent with an entropy-driven polymerisation mechanism. The large increase in entropy upon polymerisation was interpretated by Inoue as being due to hydrophobic bonding between the 'monomers' of the mitotic spindle microtubules. Thus many moles of protein-associated (ordered) water would be displaced (disordered) as polymerisation proceeded. This concept was further supported by the observation that D_2O, thought to compete for protein-bound water, markedly promoted spindle microtubule polymerisation (Carolan et al, 1966).

Although growth of spindle microtubules should involve the addition of material to existing polymers, a situation which could be kinetically more complex than the simple first order monomer - polymer equilibrium assumed by Inoue, direct kinetic measurements do not support any higher order mechanisms.

The use of birefringence as a direct measure of oriented, polymerised tubulin has long been a point of controversy, but it has now been shown that the tubulin content of the isolated mitotic apparatus is directly proportional to the birefringence and the number and distribution of microtubules correlates quite well with

the magnitude of birefringence (Stephens, 1972; Sato et al, 1975). Assuming that no significant shape changes occur in spindles over the range of measurement and considering that electron microscopic evidence indicates that microtubules are essentially parallel within the spindle, birefringence may be used directly as a measure of oriented polymer, at least to a very good approximation.

1.3.2 In vitro polymerisation

In the late 60's and early 70's, sufficient information was available on the isolation and characterisation of tubulin to enable 'microtubulists' to begin the search for the proper conditions for microtubule polymerisation in vitro. This problem was tackled in two different ways. Borisy, who previously had succeeded in purifying and characterising brain microtubulin, attempted to polymerise purified brain tubulin in the presence of GTP, which had earlier been shown to be bound to tubulin and to stabilise its colchicine binding properties. They succeeded only partially in polymerising brain tubulin into non-tubular filamentous (and occasionally beaded) aggregates which were temperature and GTP dependent (Borisy et al, 1972). However, the first to succeed in reconstituting true microtubules was Weisenberg (1972), who found that crude rat brain extracts would form microtubules when warmed in the presence of GTP.

Borisy and Olmsted (1972), using porcine brain extracts and conditions similar to those employed by Weisenberg (1972), found that tubulin would not polymerise in high-speed supernatants but would readily do so in low-speed extracts. Sedimentation and electron microscopic examination of the low-speed extracts revealed

the presence of 30S particles which were identified as 290 Å -diameter disc-like structures. The amount of these 'rings' would decrease with warming as tubules were formed or increase if existing microtubules were depolymerised by cold or other means. The conclusion was reached that the disks might be incorporated into the forming microtubules and that they may, in some way, act as nucleating centres to initiate microtubule polymerisation. The basic parameters for microtubule re-assembly in brain extracts were studied viscometrically (Olmsted and Borisy, 1974), or by a sensitive sedimentation assay (Borisy et al, 1975). They found the process to be endothermic, proceeding optimally at 37°C and with a pH optimum of 6.7 - 6.9. The process of polymerisation was also dependent on nucleotides, with a clear preference for GTP. Moderate ion concentrations inhibited polymerisation, with inhibitory effects occurring at concentrations above 0.15 M for Na^+ and K^+, 10 mM for Mg^{2+} and 1 mM for Ca^{2+}. The minimal protein concentration (total protein) at which polymerisation in brain extracts could occur was about 4 mg/ml. Further development of the polymerisation procedure culminated in a purification scheme involving cycles of temperature-dependent assembly/disassembly.

Shelanski et al (1973) found that 4 M glycerol or 1 M sucrose present in the assembly buffer markedly enhanced the polymerisation of tubulin and the microtubules obtained were much more stable to low temperature or colchicine treatment. Removal of glycerol or sucrose resensitized the microtubules to cold and colchicine. Two cycles of assembly in glycerol at 37°C and disassembly

in the absence of glycerol yielded over 90% pure tubulin. Irrespectively of the purification method employed (in the presence or absence of glycerol) microtubular preparations always contained a number of minor protein components and in particular some high molecular weight proteins (MW ca. 300000 - 350000), which comprised up to 15% of the total protein. Differences found between microtubular protein fractions prepared in the presence or absence of glycerol will be described and discussed fully in Chapter 6.

The following major developments were introductory to a detailed study of the mechanism of microtubule assembly and disassembly in vitro: a) the establishing of ionic and buffer requirements (Olmsted and Borisy, 1973; Lee et al, 1974; Olmsted and Borisy, 1975); b) development of procedures for isolation of assembly-competent tubulin (Shelanski et al, 1973; Borisy et al, 1974); c) characterisation of the protein species present under depolymerisation conditions or during the early stages of polymerisation and at equilibrium (Kirschner and Williams 1974; Erickson, 1974; Weisenberg, 1974; Johnson and Borisy, 1975).

Analysis of the sedimentation velocity of tubulin species in mixtures at equilibrium revealed the presence of two principal components: a rapidly sedimenting species ($\geq$ 30OS) corresponding to microtubule polymer and a slowly sedimenting species corresponding to the 6S tubulin heterodimer. Since the only detectable species remaining in solution after sedimentation of the polymer was the 6S tubulin, it was tentatively identified as the monomer unit with respect to the polymerisation reaction.

Experiments in which the distribution of protein in monomer (6S) and polymer fractions after approaching the final equilibrium state from different directions substantiated this assumption. Determination of the distribution of protein into monomer and polymer fractions as a function of total protein concentration revealed a critical concentration below which no polymer was formed, whereas above this value the amount of polymer formed was proportional to the total protein concentration. It was also found that the initiation of polymer formation begins with an abrupt transition step, at total protein concentrations above the critical. In addition, the concentration of monomer in equilibrium with polymer was found to be independent of the total protein concentration, when the total protein concentration was above the critical (for formation of polymer). Experiments in which the microtubules were sheared showed that the monomer concentration was independent of the length and number concentration of microtubules. Therefore, this type of polymerisation reaction can be referred to as a condensation polymerisation mechanism having a monomer concentration which is independent of the mass and distribution of material in the condensed phase. By this mechanism, elongation occurs by the consecutive addition of monomer units to the end of the microtubule following a nucleation step, required to form a short polymer.

Equilibrium state experiments, showing that the equilibrium position is independent of the number concentration of microtubules do not provide any information regarding the kinetics of the transitional

processes, i.e. polymerisation and depolymerisation. In order to examine if the rate of these processes depends on the number concentration of microtubules equilibrium perturbation experiments were performed (Johnson and Borisy 1975; Gaskin et al, 1975). In such experiments, turbidity was used to follow the kinetics of assembly and disassembly of microtubules. Johnson and Borisy (1974) showed that turbidity was proportional to the polymer concentration and was insensitive to small changes in length (1 - 4 μm) and therefore can be used as a good quantitiative assay for microtubule polymer (as determined by quantitative electron microscopy).

The number concentration of microtubules in identical samples, equilibrated at 37°C, was changed by shearing (no turbidity change after shearing) and the equilibrium state of the samples was perturbed by rapidly changing the temperature to 11.5°C. Johnson and Borisy (1975) found that after a threefold increase in the number concentration of microtubules the rate of approach to the new equilibrium was increased twofold.

On the basis of the kinetic data from equilibrium perturbation studies, Johnson and Borisy proposed a mechanism for polymerisation as follows: The elongation reaction is represented by the addition of a 6S tubulin dimer (S) to the end of a microtubule consisting of n subunits (M_n). Thus the polymerisation reaction ($M_n + S \rightarrow M_{n+1}$) is a second order association reaction and the depolymerisation reaction ($M_{n+1} \rightarrow M_n + S$) is a first order dissociation reaction. At equilibrium ($M_n + S \underset{k-}{\overset{k+}{\rightleftharpoons}} M_{n+1}$) the rates of the forward and back reactions, k+ and k- respectively, are independent of

length, hence the concentrations of microtubule ends in the equilibrium expression ($K_e = [M_{n+1}] / [M_n][S]$) cancel, leaving an equilibrium constant dependent only on the subunit concentration ($K_e = 1/[S]$). Such a mechanism is consistent with the kinetic data obtained from experiments performed at equilibrium and equilibrium perturbation experiments.

Gaskin et al (1974), presented evidence for a critical concentration and interpreted their results, based on turbidity measurements, in terms of a similar condensation polymerisation mechanism as the one suggested by Johnson and Borisy (1975), discussed above. However, the experimental data presented by Gaskin et al (1974, 1975) suggests that the rate of depolymerisation is independent of the microtubule number concentration. The reason for this disparity in results has not been cleared so far.

Investigations of the kinetics of pressure-induced depolymerisation by Salmon (1975) also yielded results which are consistent with a nucleated condensation polymerisation mechanism. However, under such a mechanism spontaneous nucleation is kinetically unfavourable. It is thought that the 30S discs, originally described by Borisy and Olmsted (1972) may participate in the initiation step. According to Borisy et al (1975) under conditions favouring polymerisation, the subunits of the 340Å discs (30S discs) undergo a rearrangement resulting in the generation of a tubule segment 250 Å in diameter and elongation, occurs primarily by means of the addition of subunits (6S) to the nascent tubule fragments.

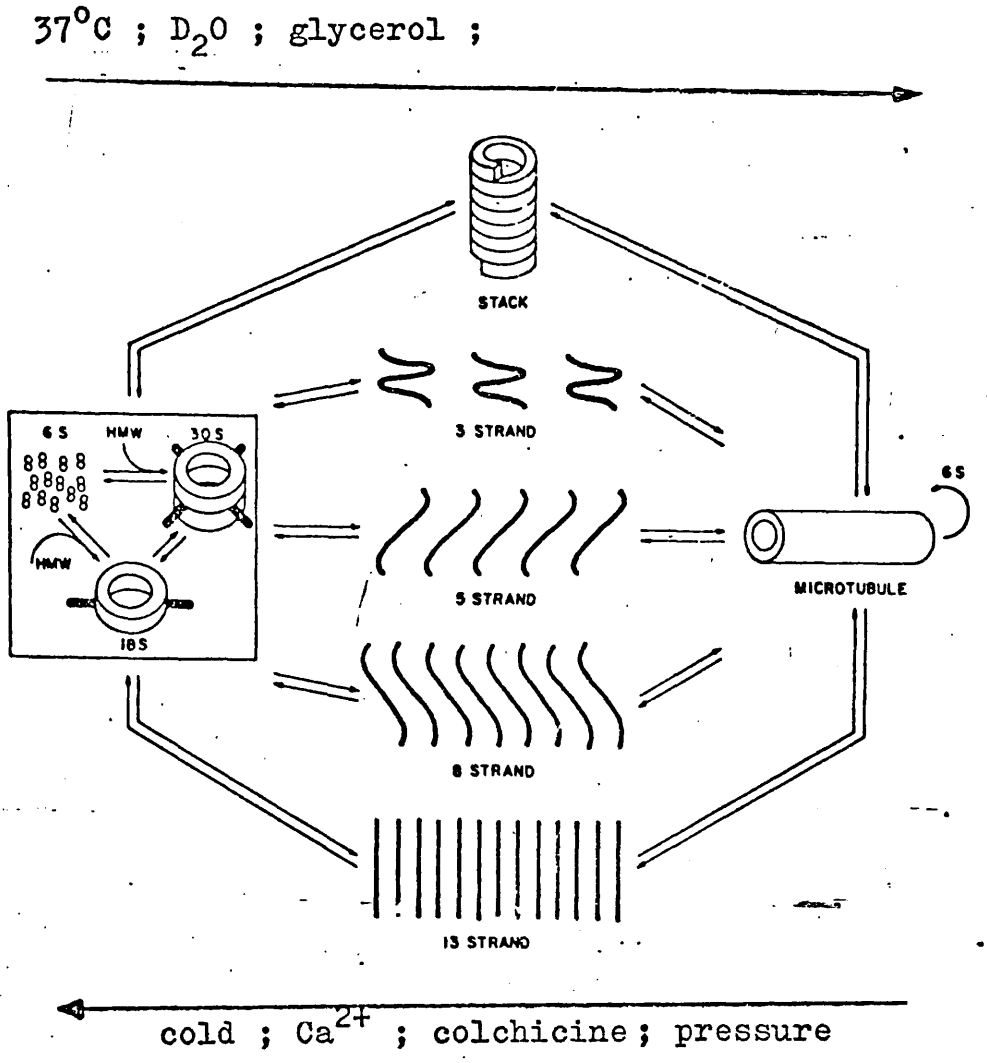
Electron-microscopic examination of the tubulin

forms present during the early stages of polymerisation revealed the presence of ribbons or sheets of up to 12 protofilaments (Erickson, 1974). These sheets seemed to grow, during polymerisation, by the addition of subunits and protofilaments. More recently, Erickson (1975) showed that tubulin obtained by depolymerisation of microtubules at 0°C exists as both 6S dimers and curved or ring shaped filaments. These two forms (dimeric and polymeric) were separated chromatographically and their properties studied in relation to the polymerisation process. The ring forms rapidly reassembled into microtubules by uncoiling into protofilaments, which in turn associated laterally to form the tubule wall. The 6S alone did not form microtubules but readily incorporated into tubules on addition of some ring 'seeds'. Similar observations were made by Kirschner et al (1974, 1975).

One fundamental difference between the Kirschner and Erickson models of assembly and the model proposed by Borisy and co-workers is that assembly, in the schemes of the former researchers, is not characterised by distinct phases of nucleation and elongation. It is difficult to reconcile the results of Johnson (1975) suggesting that elongation of microtubules occurs by the addition of 6S subunits to the growing ends of microtubules with the Kirschner and Erickson proposals where the polymerising unit is primarily the uncoiled oligomer (see fig. 1.3).

Finally, the possibility that nucleation of assembly may not involve any one of the oligomeric structures mentioned above should also be considered. Indeed, it

Fig. 1.3



Schematic representation of proposed *in vitro* polymerization mechanisms for brain tubulin. (Modified from Borisy, 1976).

has been demonstrated that proteins co-purifying with tubulin are required not only for oligomer formation but for microtubule assembly as well (Murphy and Borisy, 1975; Weingarten et al, 1975). It is possible that these accessory factors and tubulin subunits may interact directly to form a short segment of the microtubule lattice which then serves as a nucleating centre.

1.3.3 Concluding Considerations

It is important to compare the *in vitro* polymerisation process with the formation of microtubules *in vivo* in order to assess the physiological implications of the *in vitro* studies. In terms of ionic strength and temperature dependence, drug sensitivity, morphology and kinetics, microtubule assembly *in vitro* is similar to assembly *in vivo* and could provide a useful system for analysing the control of microtubule formation *in in vivo* conditions. Although the first order reaction kinetics suggested for *in vivo* polymerisation cannot be reconciled easily with the second order reaction scheme for assembly *in vitro*, the extensive similarities in the properties of microtubule assembly *in vitro* and *in vivo* argue for a common mechanism under both sets of conditions.

The in vitro polymerisation mechanisms outlined above (1.3.2) provide the possibility of suggesting mechanisms for the *in vivo* control of microtubule assembly. Because spontaneous nucleation is kinetically unfavourable, a cell could determine the spatial localisation of microtubule formation by controlled placement of nucleation centres. Centrioles, basal bodies, kinetochores and microtubule nucleating centres

have been suggested as possibilities for the spatial control of microtubule formation *in vivo*. Evidence for *in vitro* assembly of microtubules onto isolated flagellar fragments (Allen and Borisy, 1974) and basal bodies (Snell et al, 1974) provided additional support for the above suggestion. The claim for controlled nucleation relies on the kinetic argument that polymerisation will proceed faster, and energetically more economically, onto pre-existing nuclei than it would happen at random through spontaneous nucleation. Temporal control of *in vivo* polymerisation could be exercised by controlling the availability of free, polymerisable monomer, divalent ions, GTP or accessory factors (polypeptides etc.), whose function *in vivo* however, has not been experimentally demonstrated.

1.4 Phosphorylation of microtubular protein

Cyclic AMP was reported to stimulate the phosphorylation of purified tubulin (Goodman et al, 1970). It was suggested that purified microtubular protein fractions contain an intrinsic protein kinase activity (Lagnado et al, 1971). Soifer et al (1972) suggested that this kinase activity is intrinsic to tubulin itself. This was contested initially by Piras and Piras (1974) who managed to separate a protein kinase activity associated with microtubules by gel permeation chromatography. Eipper (1974), Rappaport et al (1975), Shigekawa and Olsen (1975) and Sandoval and Cuatrecasas (1976) showed convincingly that the microtubule associated kinase activity can be chromatographically separated from tubulin, i.e. it is not an intrinsic

activity to tubulin itself..

The sensitivity of the microtubule associated kinase activity to cAMP stimulation has long been a point of controversy. Sandoval and Cuatrecasas (1976) suggested that originally the kinase activity always is cAMP sensitive, and the observations of cAMP independent kinase activity are due to an artefactual desensitisation occurring during the purification procedure employed (for further details and discussion, see Chapter 4).

Piras and Piras (1974) and Eipper (1974) reported that only the ß-chain of the tubulin subunit dimer serves as a substrate for the kinase co-purifying with microtubulin in contrast to the results of Lagnado et al (1975), who showed that both the α - and ß- polypeptides become phosphorylated.

Sloboda et al (1975) stressed the much higher degree of phosphorylation of tubulin-associated HMW proteins, which might be a reflection of the differential roles of HMW proteins and tubulin phosphorylation.

In keeping with the above observations, it was reported that purified brain tubulin contains between 0.5 - 1.0 mole of protein-bound phosphate per mole of tubulin dimer (Reddington and Lagnado, 1973; Eipper, 1974; Lagnado et al, 1975). Further, Kirschner et al (1975) showed that tubulin derived from the oligomeric 36S structures contains about 0.8 moles of protein-bound phosphate per mole of tubulin dimer, whereas the 6S tubulin is hardly phosphorylated.

The direct role, if any, of the phosphorylation of tubulin or its associated proteins in microtubule

polymerisation, *in vitro* or *in vivo*, is presently unclear. However, Reddington, Tan and Lagnado (1976) suggested that: "... the ability of microtubular protein to act as an effective substrate for its associated protein kinase activity *in vitro* may be correlated with its state of aggregation, though not necessarily with the polymerisation process *per se*.". This suggestion was, later in the same year, further substantiated by the observations of Shigekawa and Olsen (1976), who showed that the phosphorylation state of tubulin affects tubulin aggregation state (6S $\rightleftharpoons$ 36S). It should also be considered that phosphorylation of tubulin or tubulin-associated HMW proteins may play a role in the determination of the rate of turnover of tubulin or in the distribution of tubulin among different cellular pools according to functional requirements.

1.5 Microtubules and membranes

The first indications for some form of interaction between membranes and microtubules were provided by the work of Inoue. The microtubules of the mitotic apparatus were shown to be almost invariably arising from or interacting with membranes, plasma and unclear, or membrane derived organelles, e.g. centrioles and kinetochores (for details and references see Chapter 6). In later studies Lieberman (1971) observed neuronal microtubules in very close proximity to the membranes of the smooth endoplasmic reticulum. However, since it was always possible to distinguish between membranes and microtubules, Lieberman concluded that the interaction between membranes and microtubules most probably through

an intermediate structure (accessory polypeptides?). Smith (1971) showed clusters of synaptic vesicles closely associated with microtubules, but it was Gray (1975) who first described microtubules, with associated vesicles, traversing the presynaptic cytoplasm and converging on the terminal membrane.

Studies on the effects of microtubule-disruptive drugs, eg colchicine and Colcemid, on cell membranes in cultured cells, revealed that treatment with these drugs resulted in a marked increase in surface activity (Vasiliev et al, 1970). It was concluded that microtubules somehow stabilised the non-active state of the cell surface, perhaps acting as a submembraneous framework. The investigations of Berlin et al (1972) showed that colchicine, Colcemid and vinblastine inhibited the self agglutination of polymorphonuclear leukocytes by the lectin concanavalin A. These workers concluded that microtubules were major determinants of the topography of lectin-binding sites. For example, Ukena and Berlin (1972) observed that colchicine disrupted the topological and functional separation of two un-related membrane-transport systems which implied the involvement of microtubules in the control of some physiological functions of the cell membrane. Yahara and Edelman (1975) reported that concanavalin A-induced inhibition of "capping" could be effectively reversed by antimitotic drugs, once again implying the participation of the microtubular system in determining plasma membrane topology.

However, the work of Furcht and Scott (1975), who showed that antimitotic drugs can have a primary effect

directly on the plasma membrane, increasing its fluidity, in addition to the typical disruption of microtubules., calls for caution when interpreting the results of experiments in which antimititoc drugs were used to disrupt the microtubular system of intact cells.

An interesting suggestion was made by Hoffstein et al, (1976) when it was observed that after treatment with concanavalin A human polymorphonuclear leukocytes contained markedly enhanced numbers of microtubules and many microtubules were seen in association with internalised plasma membrane bearing concanavalin A binding sites. They concluded that small regional condensation of membrane lipids, induced by concanavalin A binding to membrane-surface receptors, may be essential to the induction of a variety of cytoplasmic events, including microtubule assembly.

Furthermore, there is also an increasing amount of biochemical evidence showing that a significant proportion of the tubulin present in nervous tissue is tightly bound to membrane fractions which are primarily derived from isolated nerve-ending particles (Lagnado et al, 1977; Feit et al, 1971; Lagnado and Lyons, 1977; Walters and Matus, 1975). Actually, tubulin has been specifically identified as the major protein component of the subsynaptic dense material (Walters and Matus, 1975; Matus and Walters, 1975).

More recently, Sherline et al, (1977) showed that in vitro assembled microtubules can bind to pituitary secretory granules and isolated granule membranes. The authors interpret their data as suggesting a role for microtubules in the intracellular movement of granules

which is most probably brought about by crossbridges linking the tubule to the granular membrane surface.

1.6 Microtubule function

The widespread occurrence of microtubules in cells and organisms indicates that they are involved in various functions, which are outlined below.

1.6.1 Maintenance of cell shape

Numerous studies on different systems have shown that disruption of cytoplasmic microtubules by either low temperature, pressure or drugs results in a reversible loss of cell shape (e.g. see Tilney, 1968). The extent to which microtubules participate in determining the shape of anisotropic cell is still questionable. Different mechanism have been proposed to explain the participation of microtubules in cell-shape changes. Inoue and Sato (1967)proposed that in addition to being linked to chromosome movement, the growth of microtubules may also be involved in the development of anisometric cell form during cell division.

The destruction of microtubules in the axopods of Actinosphaerium, seen after lowering of temperature (Tilney and Porter, 1967), or after the addition of colchicine (Tilney et al, 1966), or on application of high hydrostatic pressure (Tilney, 1968), resulted in retraction of the axopods. D_2O, which has been shown to stabilise microtubules, prevented the pressure-and cold-induced retraction of axopods. The addition of colchicine to growing nerve cells in tissue cultures stopped the elongation of neurites without affecting the growth cone (Seeds et al, 1970; Yamada et al, 1970). Colchicine and

vinblastine also prevented the elongation of developing lens tissue (Arnold, 1966).

Although microtubules appear to maintain the cell-shape, their role in its production is still ill-defined. In many systems, microtubules exist side by side with microfilaments (see, e.g. Spooner, 1975), and there is reason to believe that there exists a cooperative interaction between these two filamentous systems.

1.6.2 Intracellular transport

A microtubule role in intracellular transport has been suggested by many authors. Theories have ranged from ones suggesting motive force production through a direct interaction between microtubules and exported particles (Roth et al, 1970; Schmitt, 1968), to others postulating the presence of an energy transducing enzyme (dynein) attached to the surface of the microtubules and interacting with the transported particles (Murphy, 1975; Smith, 1977), to a more passive role of the microtubules where they act as tracks on which other proteins may develop force (Ochs, 1972).

The possible involvement of microtubules in axoplasmic transport is of particular interest and has stimulated a vast amount of research. The initial evidence for the involvement of microtubules in axonal transport comes from studies which showed that colchicine (Karlsson and Sjostrand, 1969; James et al, 1970) blocks axonal transport. More direct evidence was presented by Lasek and Hoffman (1966). These authors studied the elements of the fast, intermediate and slow axonal transport by polyacrylamide gel analysis. They showed that the fast component provides the presynaptic

terminals with proteins primarily involved in synaptic function and does so in a matter of hours after synthesis of the exportable components. Labelling experiments showed that tubulin itself was a major component of the slowly transported material and almost exclusive to the material exported at intermediate rates. This observation by itself appears to exclude the possibility that microtubule growth can be responsible for the fast component of axonal transport. The same authors (Hoffman and Lasek, 1975) proposed that the movement of exported particles and the cytoskeleton itself is based upon actin-myosin-microtubule interactions.

The exact function of the three different classes of filaments, i.e. microtubules, neurofilaments and microfilaments, found in nerve cell processes has, so far, not been elucidated. All of the models proposed to explain the translocation of material in nerve cells in essence envision the transported components interacting with either microtubules, directly or via force generating bridges, or with membrane (or microtubule) associated filaments. Even if microtubules do not participate in the production of a motive force they may be aligning those proteins that do, as well as performing a cytoskeletal function.

1.6.3 Motility

The role of microtubules in the movement of cilia and sperm-tail flagella is readily apparent. It has been shown that microtubules in cilia slide along one another by means of their dynein side-arms which contain ATPase activity. The hydrolysis of ATP by the dynein has been proposed to provide the energy required for movement

(Satir, 1974). It has also been shown that the disruption of cytoplasmic microtubules by colchicine led to a loss of oriented movement and extension of psendopodia in cultured fibroblasts, which further supports the view that microtubules are involved in motility (Goldman, 1971).

1.6.4 Sensory transduction

The association of cilia with sensory receptors such as olfactory cells (Reese, 1965), vertebrates rods and cones (Sjostrand, 1953), and insects receptors (Gray and Pumphrey, 1958), gave rise to the suggestion that microtubules are involved in sensory transduction. Friedman (1971) observed a close 'functional' association of microtubules with the plasmalemma in a sensory organ of the cricket. More direct evidence comes from the work of Moran and Varela (1971) who showed that the application of colchicine or vinblastine to the mechanoreceptor of cockroach leg resulted in the disruption of microtubules and a failure to evoke action potentials in these receptors.

1.6.5 Secretion

Colchicine has been found to inhibit the release of insulin from ß cells (Lacey et al, 1968), the secretion of thyroxin and iodine from the thyroid gland (Williams and Wilff, 1970), and the release of catecholamines from adrenal medulla (Poisner and Bernstein, 1971). These observations have been taken as evidence for the involvement of microtubules in secretory processes.

Evidence for the inhibition of synaptic transmission by colchicine (Perisic and Cuenod, 1972; Robert and Cuenod, 1969), has been interpreted as due to the

disruptive effects of colchitine on axonal transport.

In some cases a 'stimulatory' effect of colchicine on secretory cells has been observed (Edwards and Howell, 1973). This was interpreted by Stephens and Edds (1976) as negating evidence for the participation of microtubules in secretory processes. However, there is the alternative interpretation that the observed 'stimulation' was due to the loss of metabolic order resulting in the premature secretion of a particular substance.

It should be borne in mind that just as some of the postulated force-production roles for microtubules in motility, the inferences regarding the participation of microtubules in various cell phenomena drawn solely from the effects of antimitotic drugs may be premature.

Chapter 2 General Methods

2.1. Introduction

General methods employed throughout the course of this work are described in this chapter. Specific methods used for a particular purpose are described separately in the appropriate chapter.

2.1.1. Chemicals

All standart chemicals used were of the highest purity available. All radioactive chemicals were obtained from the Radiochemical Centre, Amersham, Bucks., U.K. Unlabelled nucleotides were purchased from Boehringer Corporation Ltd. Other reagents used will be specified in the text.

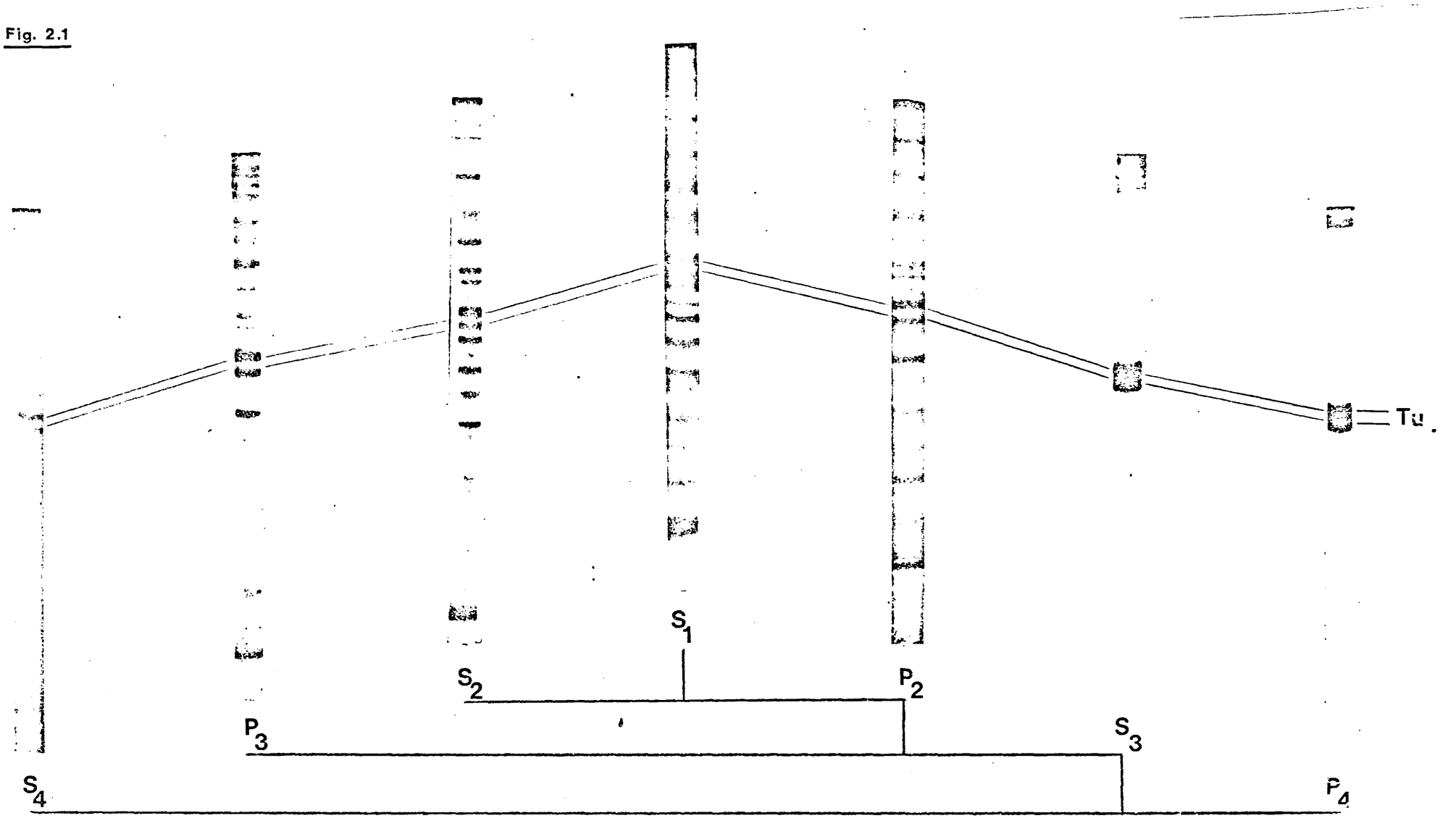
2.2. Purification of tubulin

Tubulin was routinely purified by cycles of temperature-dependent assembly/disassembly *in vitro* from crude brain supernatants. For most of the experiments to be described tubulin was purified from rat or chick brains, as specified for the individual experiments.

2.2.1. Polymerisation in the presence of glycerol

The method described by Shelanski *et al* (1973) was adopted. Freshly excised rat or chick brians were homogenised with 1.5 vol. of ice-cold reassembly buffer (0.1M, MES, 1mM E6TA, 0.5 mM $MgCl_2$, 1mM GTP; pH 6.9) in a glass homogeniser fitted with a motor driven Teflon pestle (clearance 0.08 - 0.13 mm) by 10 up-and-down strokes at 2000 rpm. The homogenate was centrifuged at 10^5xg for 1h at 4^oC and the resulting supernatant (S_1) was mixed with an equal volume of reassembly buffer containing 8M glycerol. After incubating the mixture at 37^o for 30 min, to allow for polymerisation of

Fig. 2.1

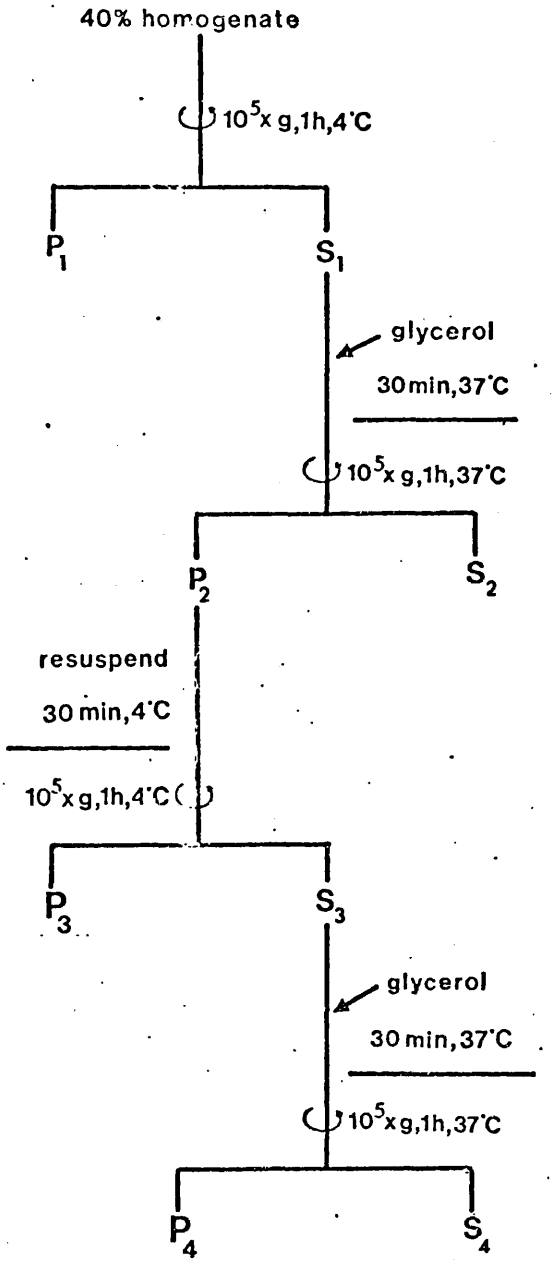


Electrophoretic patterns of MTP fractions obtained during purification of brain tubulin in the presence of glycerol.

tubulin, the microtubules were sedimented at 10^5 xg for 1h at room temperature. The pellet containing the microtubules (P_2) was resuspended in a volume of ice-cold reassembly buffer equivalent to 1/4 of the volume of S_1 and gently homogenised (3-4 strokes by hand) in a glass-Teflon homogeniser. The suspension was kept on ice for 30min, to allow for depolymerisation of the microtubules, and then centrifuged at 10^5 xg for 1h at 4^0C. The resulting pellet (P_3) contained some microtubule fragments, but mainly non-tubular aggregates, as seen by electron microscopy(J.R.Lagnado, unpublished observations); more than 80% of the protein was tubulin (see fig. 2.1; for further details and discussion see 6.2.). The supernatant (S_3) contained the depolymerisation products of microtubules (see Kirshner et al 1974) and was over 80% tubulin. This tubulin fraction, S_3,will be refered to as 'microtubular protein' (MTP) prepared by one cycle of polymerisation. To further purify this tubulin preparation S_3 was mixed with an equal volume of glycerol (8M) - containing reassembly buffer, polymerised and the microtubules were sedimented under the conditions described above (see centrifugation scheme, fig. 2.2.)

The protein yield (per gram of wet tissue) and distribution between the individual fractions (see table 6.1.) is in good agreement with those reported by Shelanski et al (1973). However, a high molecular weight (HMW) protein complex, consisting of 3-5 minor bands was consistenly observed in all fractions, which was not reported by Shelanski et al (1973).

Fig. 2.2 Scheme for purification of microtubular proteins by one cycle of assembly/disassembly in the presence of glycerol

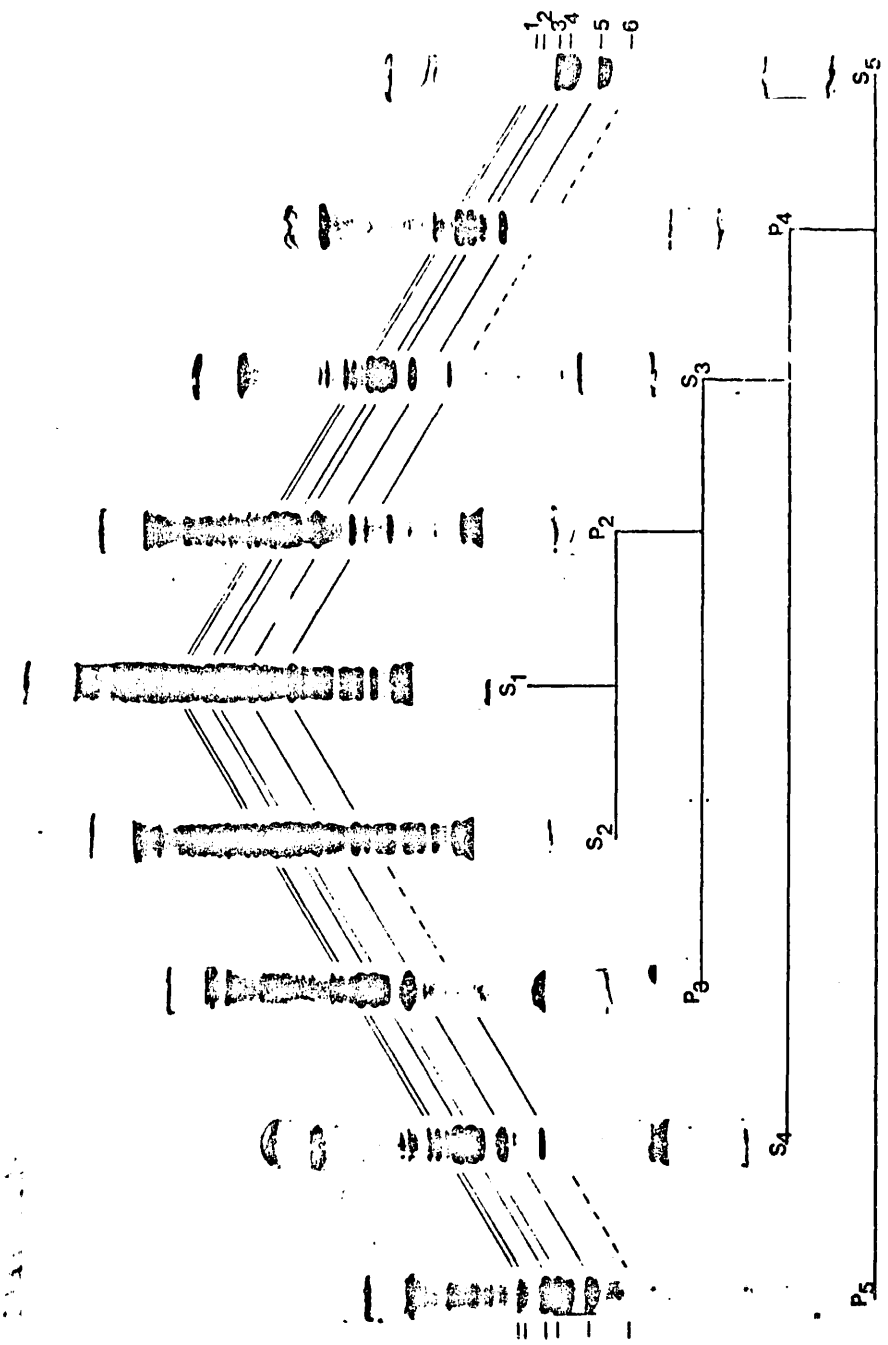


Pellet and supernatant fractions are designated as P and S respectively.

2.2.2. Polymerisation in the absence of glycerol

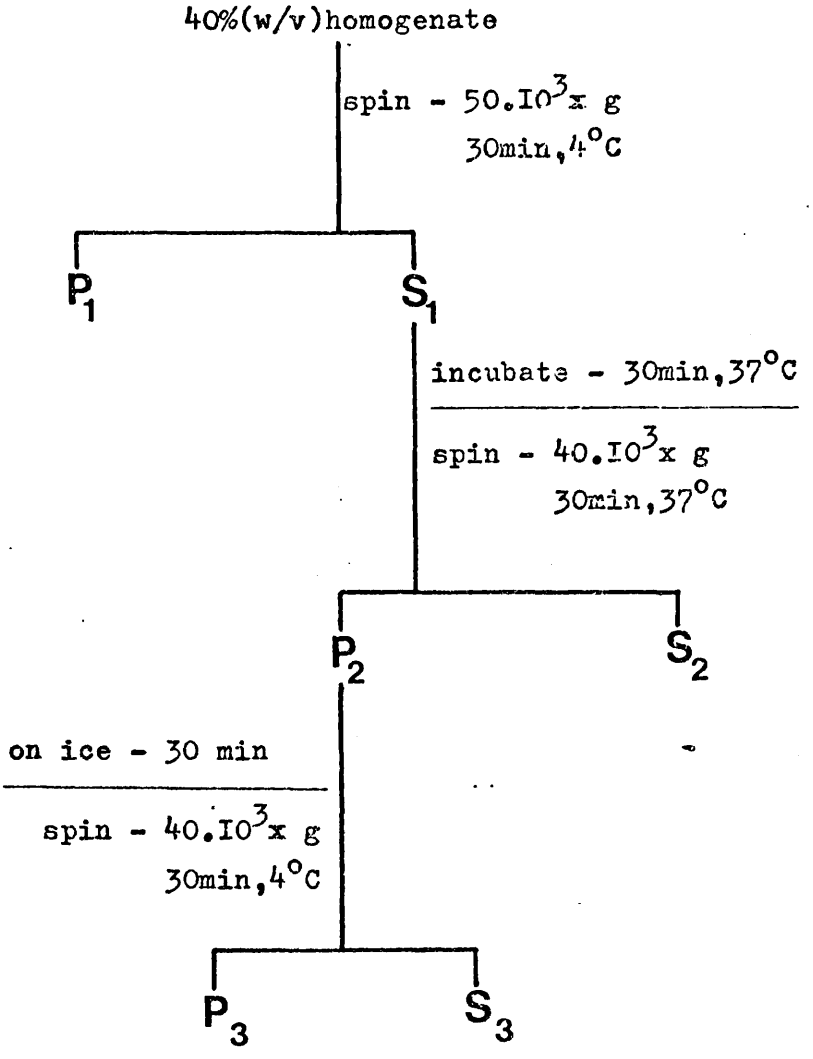
This method was adapted from the procedure described by Borisy et al (1975). For convenience, the reassembly buffer described in 2.2.1. was used instead of the buffer system described by the above authors. Brain tissue was homogenised as described in 2.2.1. and the homogenate was centrifuged at 50^3xg for 30 min. at 4°C. The supernatant (S_1) was incubated at 37°C for 30 min, to allow the formation of microtubules, which were then sedimented at 40^3xg for 30 min. Because of the lability (sensitivity to temperature changes, Shelanshi et al, 1973) of microtubules in the absence of glycerol, the centrifugation tubes and the centrifuge rotor were preincubated at 37°C and the centrifugation was performed at 34-37°C. The microtubular pellet (P_2) was resuspended in a volume of ice-cold, glycerol-free, reassembly buffer equal to 0,17 of the volume of S_1 and gently homogenised (3-4 strokes by hand) in a glass-Teflon homogeniser. After keeping this suspension on ice for 30 min, to depolymerise the microtubules, it was centrifuged for 30 min at 40^3xg at 4°C. This yielded a supernatant (S_3) containing the depolymerisation products of microtubules (see Borisy et al, 1975) and a pellet (P_3) roughly equivalent to the corresponding fraction obtained by the glycerol polymerisation method (see 2.2.1., for details and discussion see 6.2.1). Over 75% of the protein in both fractions was tubulin (see fig. 2.3.). A further cycle of polymerisation-sedimentation-depolymerisation-sedimentation, fig. 2.4.) yielded a tubulin preparation (S_5) of even greater purity.

Although the initial yield of protein (fraction P_2) is in good agreement with the results of Borisy et al (1975) the final yield of tubulin was, in all cases, considerably less than expected on the basis of Borisy's (1975) results.



Electrophoretic patterns of MTP fractions obtained during purification of brain tubulin by polymerization in the absence of glycerol; protein bands are designated as follows : 1 = H_1; 2 = H_2; 3 = α-tubulin; 4 = β-tubulin; 5 = L_1; 6 = L_2. For molecular weights of proteins 1 - 6 see 2.7.4.

Fig. 2.4 Scheme for purification of microtubular proteins by one cycle of assembly/disassembly in the absence of glycerol



Pellet and supernatant fractions are designated as P and S respectively.

It should also be noted that the purity achieved by Borisy et al after the first cycle of polymerisation could only be achieved after the second polymerisation cycle (see fig. 2.3.). Some of the proteins copurifying with tubulin, bands H_1, H_2, L_1 and L_2 in fig. 2.3. (see also 2.7.4.) remained in certain cases even after two cycles of polymerisation (for further details and discussion see Chapter 6) These apparent discrepancies could be due to the modified buffer system employed, i.e. the inclusion of Mg^{2+} ions (0,5 mM) and substitution of MES instead of PIPES as buffer (see 2.2.1. and Borisy et al 1975), but this was not further investigated.

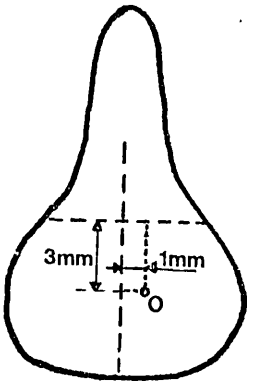
2.3. Injection of animals with ^{32}P

The experimental animals, rats or chicks, were injected intracranially, aiming at the IV-th cranial ventricle with 50- 400 µCi of $^{32}P_1$ in a maximal volume of 50 µl of solution, according to the requirements of the experiment.

2.3.1. Injection of rats

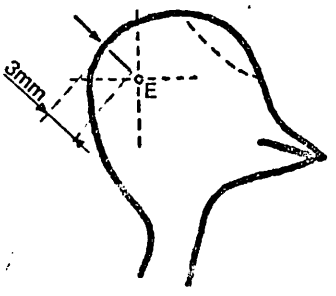
The skin on the skull of rats, anaesthetised with diethyl ether, was cut with a scalpel and pulled away to expose the sutures. A hole was drilled in the skull with a hypodermic needle (location of hole shown in fig. 2.5.). The ^{32}Pi- containing solution was injected through a 25 mm 5/10 (GILETTE, 061) needle, which was inserted in a small diameter (0.7 mm) plastic tube so that only 4 mm of the needle was exposed. This limited penetration to 3 mm (1 mm skull bone), so that the tip of the needle reached the IV-th cranial ventricle. Preliminary injections with dye showed that 85% of the injections were successful, i.e. the

Fig. 2.5



Schematic representation of the area of a rat's skull exposed on cutting and pulling away the skin. Sutures are represented with dashed lines. 'O' denotes the opening through which the radioactive solution is injected into the brain.

Fig. 2.6



Schematic representation of a chick's head. 'E' denotes ear opening. Arrow shows location and angle of injection.

dye was deposited in the IV-th cranial ventricle. After injection the skin of the skull was closed with surgical clips.

2.3.2. Injection of chicks

1-3 day-old chicks were lightly anaesthetised with diethyl ether and injected through a 25 mm 5/10 (GILETTE, 061) needle, inserted through a small diameter (0,7 mm) plastic tube, so that only 3 mm of the needle were exposed. The needle was inserted directly in the middle of a hemisphere of the chick's brain, located by touch, approximately 3-4 mm from the ear opening (fig. 2.6.) and the required amount of ^{32}P injected. Injections with dye showed that in more than 75% of the cases the dye was deposited in the immediate vicinity of the IV-th cranial ventricle or directly into it.

2.4. Gel permeation chromatography

Samples of freshly prepared depolymerised microtubule fractions, containing 1-15 mg of protein (3mg protein/ml. of reassembly buffer) were chromatographed on 1.8 x 30 cm. columns of Sepharose 6B (Pharmacia) or 1x20 cm columns of Sephadex G100 (Pharmacia), previously equilibrated, with at least 4 bed volumes, and then eluted with glycerol-free reassembly buffer minus GTP.

Chromatographic separations were carried out in a refrigerated LKB 7000 Ultrorack fraction collector maintained at 4°C. Eluates were monitored on a Uvicord LKB 4700A recording apparatus and the protein content and radioactivity were determined for each fraction (2 ml). The protein recovery from the columns was usually over 80%. Flow rates were controlled with a peristaltic pump, connected to the effluent tubing, and were 10 ml/h and 12 ml/h for Sepharose 6B

and Sephadex G100 columns respectively.

2.5. Protein estimation

2.5.1. Protein estimation by the method of Lowry

Protein was estimated according to the method of Lowry et al (1951) using the following solutions:

A/ 2% Na_2CO_3 in 0.1N NaOH (20 g Na_2CO_3, anhyd. 4 g NaOH, made up to 1 l with H_2O)

B/ 0.5% $CuSO_4.5H_2O$ in 1% Na-citrate.

C/ 50 parts of A freshly mixed with 1 part of B .

D/ Folin-Ciolcalteau Reagent (as supplied by BDH) was diluted 1:2,3 with H_2O dist.

E/ Standart protein solutions were prepared from a stock solution containing 10 mg/100 ml of bovine serum albumin.

Samples containing up to 100 ug of protein in a total volume of 0.8 ml were mixed with 4 ml of reagent C and allowed to stand for 20 min at room temperature. This mixture was treated with 0.4 ml of reagent D, ensuring a rapid mixing (within 10 sec of addition of D). The blue colour was allowed to develop for at least 30 min before measuring the absorbance at 680 nm in a MSE-Spectro-plus spectrophotometer against a blank without added protein. It is known that glycerol brings about increased colour intensity if present in the reaction mixture. It was therefore necessary to include appropriate blanks when the protein content of glycerol-containing fractions was estimated.

2.5.2. Protein estimation by spectrophotometry

The protein content of supernatants and fractions from gel permeation chromatography was estimated spectrophotometrically by the method of Warburg and Christian (1941)

on a MSE-Spectro-plus spectrophotometer. The deviation from the mean values of protein estimated for the same fractions by the method of Lowry was not more than 3%.

2.6. Determination of protein bound ^{32}P

The ^{32}P present in MTP fractions purified from *in vivo* labelled brain tissue exists mainly in the following forms:

1. Free ^{32}Pi.
2. Bound to nucleotides
3. Bound to phospholids
4. Bound to nucleic acids
5. Bound to protein

To investigate the nature of the protein-bound ^{32}P all other forms of ^{32}P must be removed from the investigated MTP fractions. The following procedures were adopted from the analytical procedures described by Weller (1977) (see also Rodnight et al, 1975).

2.6.1. Removal of free, nucleotide-, phospholipid- and nucleic acid-bound ^{32}P

The protein in the investigated fractions was denatured by the addition of 100% TCA (w/v) to the samples to a final concentration of 10%, at $4^{\circ}C$. After leaving the samples on ice for 20 min, to allow quantitative precipitation of the protein, the denatured protein was pelleted and washed two times with 5% TCA (up to 5 mg of protein/ml of acid). This treatment removes all trapped phosphate and nucleotides and presumably the acid-labile histidine- and lysine-bound ^{32}P as well (see below). Prior to extraction of phospholipids the residual acid from the pellet was removed by a diethyl ether wash, to avoid losses due to the solubility of protein

in acidified phospholipid solvents. This treatment resulted in the removal of about 40 and 80%, for pellet and supernatant samples respectively, of the total ^{32}P present in the native samples.

The dry pellets were extracted two times with chloroform-methanol (2:1, by vol.) at room temperature to remove phospholipids. This extraction does not remove phosphoinositides quantitatively , but they do not interfere with the estimation of protein-bound ^{32}P (see below).

The ^{32}P remaining in the denatured and delipidised protein pellet may be found in nucleic acids (hot acid labile) and as one of two forms of protein-bound ^{32}P: a) as phosphoserine or phosphothreonine, which are stable in acid but labile to alkali ; b) as acyl-phosphate (phosphoaspartate) which is specifically hydrolysed by hydroxylamine, but which is also labile to hot acid or alkali.

2.6.2. Determination of alkali labile ^{32}P

Protein boundphosphoserine or phosphothreonime may be determined as'alkali labile phosphate'. While the presence of nucleic acids does not interfere with the determination of alkali-labile phosphate the treatment described below will release any acyl-phosphate remaining after the cold acid washes. Acyl-phosphate could thus contribute to the phosphate defined as alkali-labile if it was not specifically excluded (see below).

The denatured and delipidised protein pellet is suspended in 1N NaOH (up to 10 mg. of protein/ml. of 1 N NaOH) and incubated at 100°C for 12 min. At the end of the incubation 1.6 ml of 1.4 M perchloric acid was added to the sample which was then cooled on ice and 0.6 ml of silicotungstate

reagent (mix 5.7 g. of sodium silicate and 79.4 g. of sodium tungstate in 900 ml of 3% sulphuric acid, reflux for 5 h, filter and make up to 1 l with H_2O dist.) added dropwise with constant stirring. After removing the precipitate by centrifugation an aliquot of the supernatant (2 ml) is transfered to a clear tube and treated with 0.3 ml of 5% ammonium molybdate. The phosphomolybdate complex is then extracted into 2.5 ml of isobutanol-benzene (1:1, by vol) and a representative sample taken for radioactive counting.

2.6.3. Determination of acyl-^{32}P

The denatured and delipidised protein sample was suspended in 0.1.M acetate buffer (pH 5.4) and incubated for 30 min at 37°C in the presence of 0.8 M hydroxylamine (freshly prepared by the addition of 2 parts of 8M NaOH to 5 parts of 4M hydroxylamine - HCl). The protein was precipitated by the addition of 0.5 vol of 30% perchloric acid at 0°C and used for determination of alkali-labile ^{32}P. The supernatant was used to estimate acyl-^{32}P.

2.6.4. Results

When the nature of ^{32}P phosphate associated with MTP purified by two cycles of polymerisation, then denatured and delipidised, was analysed as described above, it was found that:

1. If the pellet was used directly for determination of alkali labile ^{32}P, over 90% of the recovered ^{32}P behaved as alkali labile-phosphate. The pellet obtained after NaOH treatment contained about 6% of the recovered ^{32}P, presumably bound to nucleic acids.

2. If prior to NaOH hydrolysis the protein was treated with TCA, 10% of the recovered ^{32}P was extracted (acyl plus nucleic acid-P) and over 85% behaved as alkali-labile ^{32}P.

3. If prior to NaOH hydrolysis the pellet was treated with hydroxylamine, 5% of the recovered ^{32}P was extracted (acyl-P), 85% was alkali labile and 6% remained in the pellet obtained after NaOH treatment.

These results suggest that at least 85% of the protein-bound ^{32}P is bound to serine and/or threonine residues, 5% behaves as acyl-phosphate , and 6% as nucleic acid-P. Because of the almost negligible amounts of protein-bound ^{32}P, other than alkali-labile P, the hot acid or hydroxylamine treatments were left out in routine analyses and alkali-labile phosphate was assumed to represent serine- or threonine- derived ^{32}P.

The nature of protein-bound ^{32}P-phosphate was almost identical for all MTP fractions investigated, using the operational criteria described above.

2.7. Polyacrylamide gel electrophoresis

The purity and molecular weight of proteins in purified MTP fractions was determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl (lauryl) sulphate (SDS; Fig.) used to dissociate proteins into their subunit polypeptides. Under these conditions it is possible to calculate the apparent molecular weights of the individual polypeptides from their mobilities on basis of a standart curve obtained by plotting the logarithm of molecular weights versus the mobility for a series of monomeric proteins of known molecular weight (see Weber and Osborn, 1969).

Electrophoresis was carried out on 10% acrylamide gels employing the procedure described below.

Bryan and Wilson (1971) found that polyacrylamide gel electrophoresis of reduced, carboxymethylated and alkylated preparations of chick brain tubulin in an alkaline buffer system (TRIS-glycine) resulted in the splitting of tubulin into two closely migrating components which were designated as α and ß subunits according to IUPAC conventions (Webb, 1964). It was recently shown that purified tubulin can be separated into the same two components when electrophoresis is carried out in the presence of 2-8 M urea, employing various buffer systems (Geit et al, 1971; Wilson and Bryan, 1971; Fine, 1971; Lagnado et al, 1972). Under these conditions it is not necessary to reduce carboxymethylate and alkylate the protein to affect its resolution into α and ß monomers. In the present work the two subunits of tubulin were separated in a discontinuous TRIS-glycine buffered system modified from Laemmli (1970) in the presence of 4M urea, according to the method described below.

2.7.1. Preparation of gels

A stock of acrylamide solution containing 20 g of acrylamide (BDH, specially purified for electrophoresis) and 0.15 g of NN' methylene bisacrylamide (BDH) dissolved in 100ml of H_2O dist. was used for preparation of 10% gels.
To prepare 10 gels in 10 cm long siliconised glass tubes (internal diameter 5 mm), 10 ml. of gel buffer (0.75 M TRIS-HCl, pH 8.8, 0.2% SDS (w/v), 8M urea) was mixed with 9 ml. of stock acrylamide solution and the mixture was de-aerated for 10 min. After adding 2 ml of freshly prepared 1.5% (w/v)

aqueous ammonium persulphate, the polymerisation was started by the addition of 30-60 ul of NNN'N'-tetramethylethylenediamine (TEMED, Koch-Light).

The mixture was immediately poured into the glass tubes, fixed vertically, stoppered at one end with Parafilm, and a drop of water was added to each tube to improve the surface of the meniscus of the forming gel. Gelling occurred within 15-20 min and the gels could be used within one hour of preparation.

2.7.2. Preparation of protein samples and electrophoresis

Protein samples were mixed with an equal volume of sample incubation buffer (0.025 M TRIS-HCl, pH 6.8, 0.8% SDS (W/V), 2% ß-mercaptoethanol, 2M urea) such that the ratio of SDS to protein on a weight basis was kept between 2-4 and incubated at 100^{0}C for 3 min. After cooling a drop of glycerol - bromophenol blue (saturated aqueous solution) mixture (1:1, by vol) was added to the sample and aliquots containing 5-150 ug of protein, according to the purity of the sample, were loaded on the gels. The compartments of the electrophoresis bath were filled with electrophoresis buffer (0.025 M TRIS - 0.192 M glycine, pH 8.3, 0.1% SDS (w/v)) and electrophoresis was performed at 2.5 m A/gel supplied from a Shandon- Unoplan power pack. Usually electrophoresis lasted until the tracking dye (Bromophenol Blue,BDH) reached the bottom of the gels (app. 3-5 hours).

2.7.3. Fixing, staining and destaining

Proteins in the gel were fixed and stained for 1 hour in 10% (w/v) sulphosalicylic acid (SSA) containing a solution of Coomasie Brilliant blue stain (o.25% Coomasie

Brilliant blue (w/v) in 50% methanol glacial acetic acid 1:0.98 by vol.), prepared by mixing 13.3 parts of 10% SSA with 1 part of dye solution. Background staining of the gel was removed with several changes of a destaining solution containing 7% acetic acid, glacial, in 5% methanol.

2.7.4. Determination of molecular weights of polypeptides

Measuring the length of the gel before fixing, the distance migrated by the dye before fixing and the length of the gel and distance migrated by each protein band after destaining the mobility of each protein was estimated on the basis of the following equation:

$$\text{Mobility} = \frac{\text{Length before fixing}}{\text{Length after staining}} \times \frac{\text{Distance migrated by protein}}{\text{Distance migrated by dye}}$$

Plotting the logarithm of the molecular weight against the mobility of standart proteins, a linear standart curve was obtained, from which the molecular weight of the unknown protein could be calculated. From the standart curve, shown in fig. 2.7., the molecular weight of α and β tubulin subunits was calculated to be 54,000 and 57,000 respectively and the molecular weights of the H_1H_2, L_1 and L_2 bands (see 2.2.2. and fig. 2.3.) were calculated to be as follows:

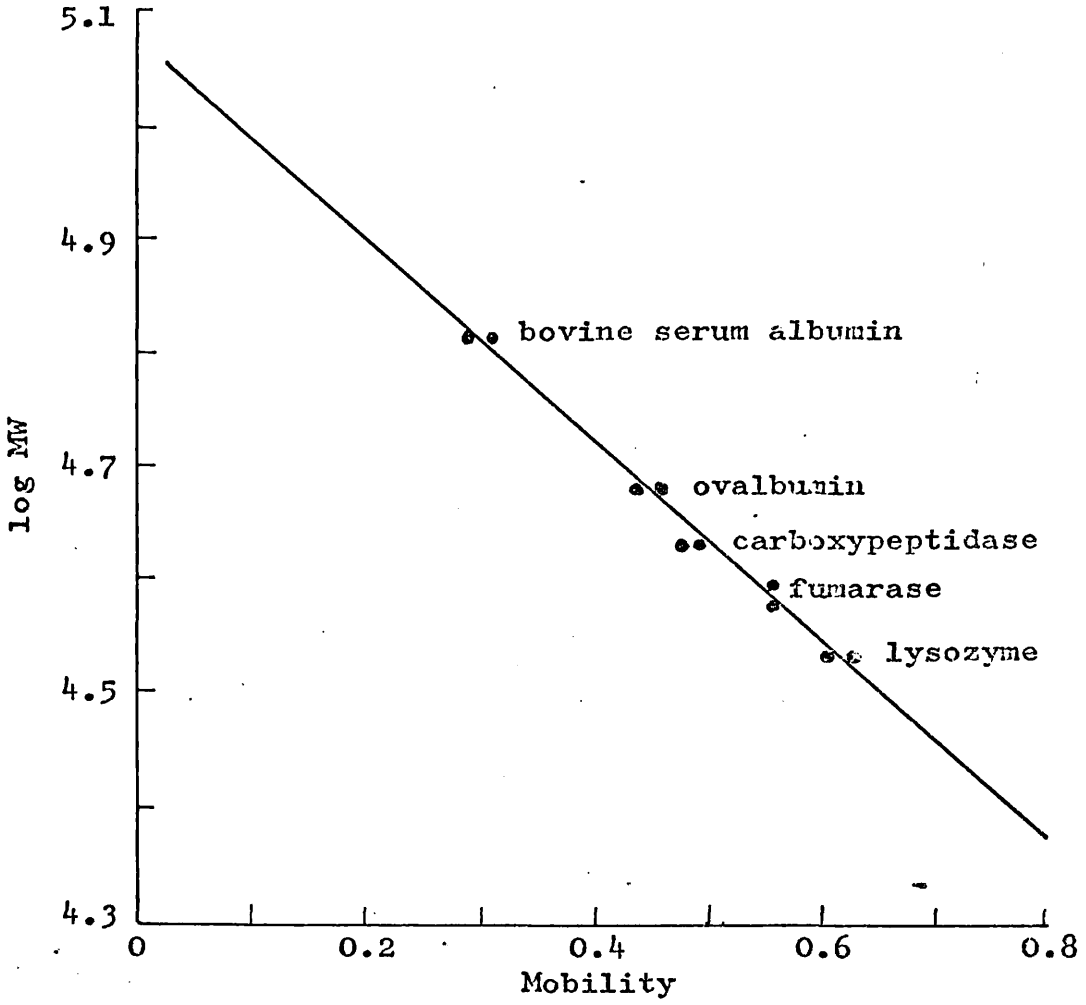
H_1 - 72,000

H_2 - 64,000

L_1 - 46,000

L_2 - 33,000

Fig. 2.7 Standart curve for determination of molecular weights



Protein	Molecular weight
Bovine serum albumin	65 000
Ovalbumin	45 000
Carboxypeptidase	43 000
Fumarase	38 000
Lysozyme	15 000

2.7.5. Preparation of polycrylamide gels for radioautography

Gels were sliced in a home-made slicing apparatus similar to that orginally described by Fairbanks et al (1965). The gel slices were dried on Whatman No. 3 paper under steam heating and reduced pressure. Drying was performed in the apparatus shown in fig. 2.8. The gel slices were arranged on a transparent polythene sheet resting on a larger sheet of silicone rubber (TC 156, ESCO Rubber Ltd., London). They were then covered with Whatman No. 3 paper, wetted with H_2O, covered by a porous polythene sheet (50 n size; Gallenkamp and Co. Ltd., U.K.) and a silicone rubber sheet with an outlet for evacuation, located at the centre of the sheet. The silicone rubber sheets were sealed by metal clips and the whole device was placed on a steaming water bath, at the same time pressure was reduced by connecting the outlet on the top rubber sheet to a vacuum pump. The drying process takes about one hour. On drying the gels are fixed on the Whatman No.3 paper and are ready for radioautography (see fig. 2.9). For radioautography Ilford Red Seal 25 FW x-ray film was used. The time of exposure of the film varied according to the amount of radioactivity loaded on the gels.

2.7.6. Densitometric scanning of gels and radioautographs

This was performed on a Vitatron densitometer using a yellow U-12 filter. The recorder was fitted with a mechanical integrator the trace of which was used to calculate the area under the individual peaks (see fig. 2.10).

Fig. 2.8 Gel - drying assembly for preparation of fixed and stained polyacrylamide gel slices for radioautography

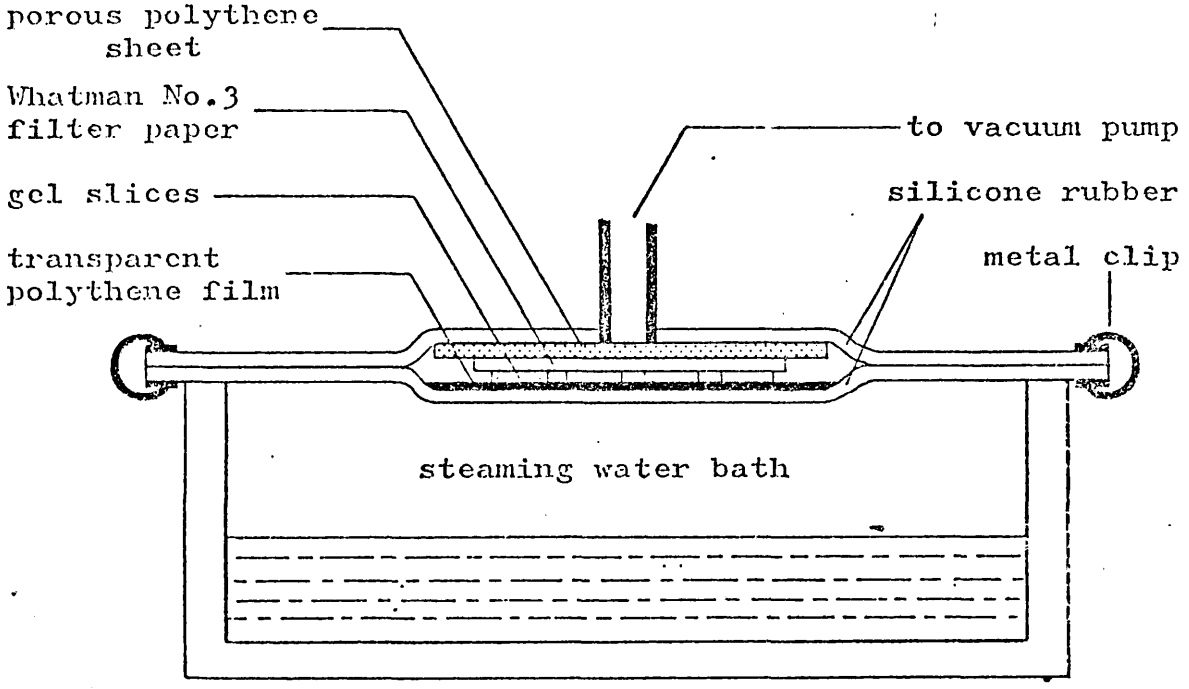
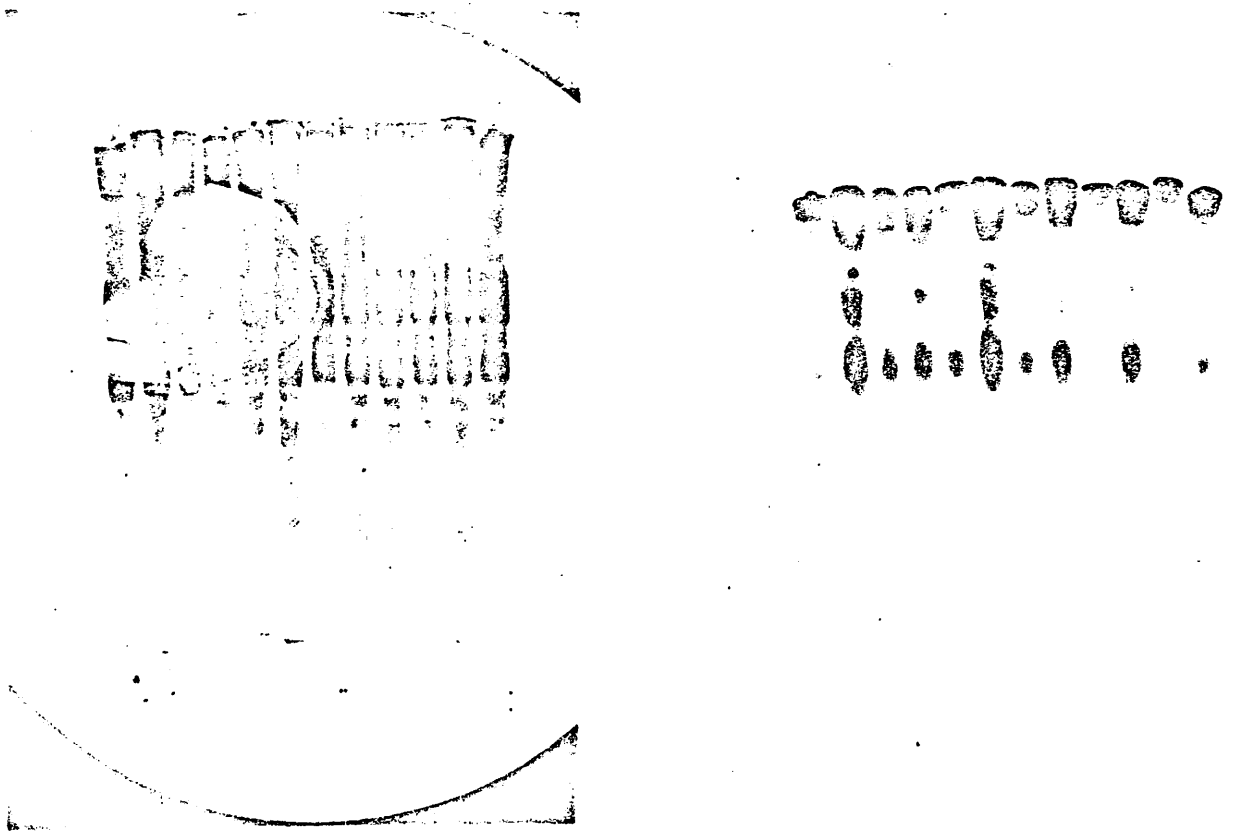


Fig. 2.9 Radioautography of sliced and dried polyacrylamide gels

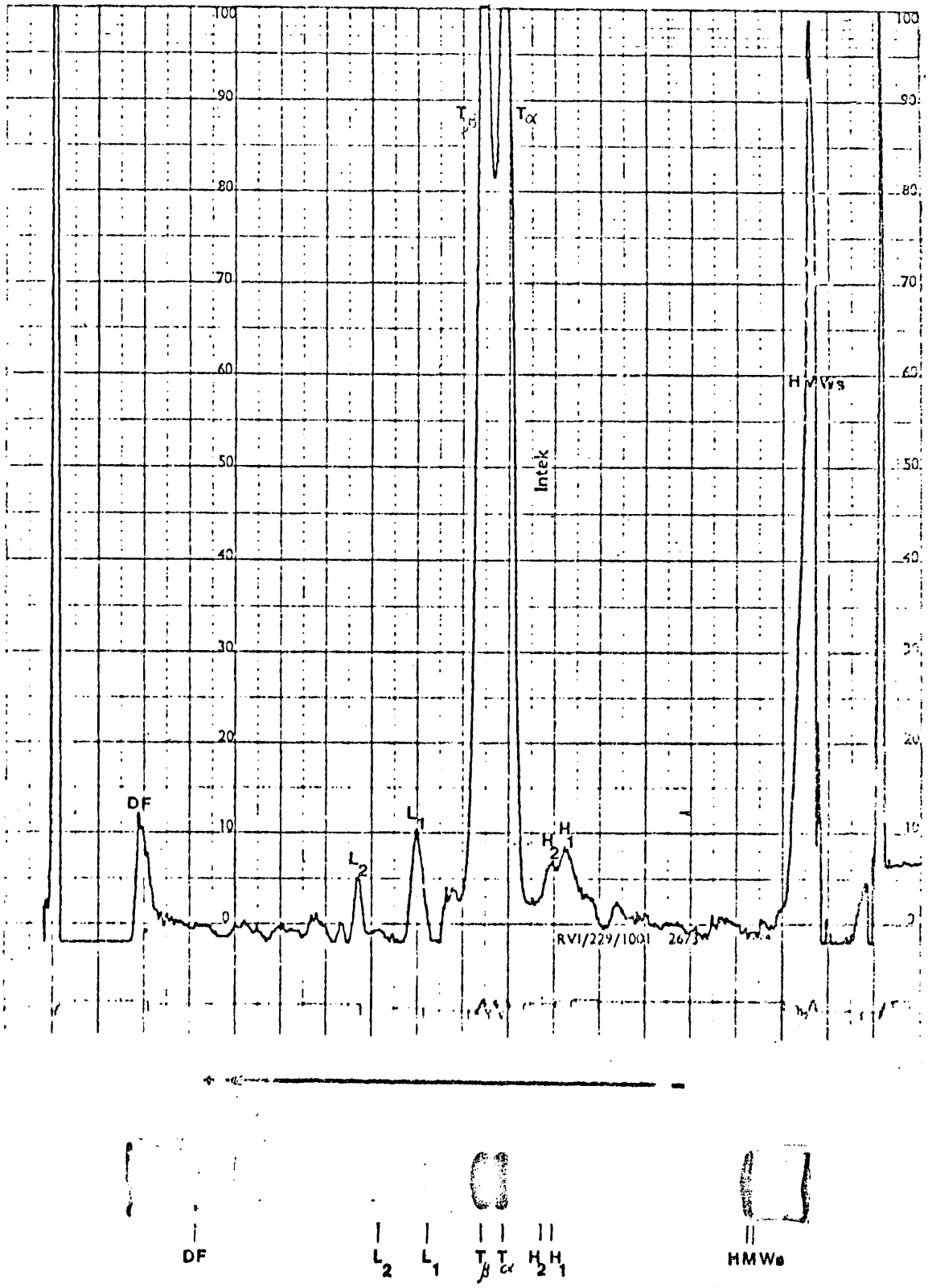


(A) (B)

(A) shows the sliced polyacrylamide gels fixed on a disc of Whatman No. 3 paper.

(B) shows the developed radioautograph of the gels shown in (A).

Fig. 2.10 Densitometric analysis of fixed and stained polyacrylamide gels



DF = dye front ; HMW - high molecular weight proteins ;

2.8. Radioactive counting

Radioactive samples were counted in 5 or 10 ml of Bray's scintillant (Bray, 1960), according to the quenching effect of the radioactive solution, and counted in a Tri-Carb Packard 3375 Scintillation spectrophotometer.

The efficiency of counting was determined by measuring the counts per minute (cpm) of a specific volume of radioactive solution of known disintegrations per minute (dpm):

$$\text{Efficiency} = \frac{\text{cpm} \times 100}{\text{dpm}}$$

The efficiencies for (^{3}H) and (^{32}P) were about 35% and 65%, respectively.

The scintillant used was essentially that of Bray (1960) except that methanol was replaced by ethoxyethanol (BDH) and the amount of the primary solute 2,5 - diphenyloxazole (PPO, Fisons) and of the secondary solutes 1,4-di-2-R (5-phenyloxazolyl)-benzene (POPOP, BDM) and naphtalene (BDH) were somewhat increased to improve counting efficiency.

2.9. Expression of data

The results of experiments in which radioactive isotopes were used were commonly expressed in terms of Specific Activity (SA), as cpm/mg of protein or, in certain cases, as moles of ligand bound per mole of protein. In some experiments, where comparison between different fractions was made, the results were calculated in terms of relative specific activities (RSA), taking the RSA of the orginal fraction as 1, according to the formula:

$$RSA = \frac{\%\ \text{cpm recovered in fraction}}{\%\ \text{protein recovered in fraction}}$$

The results presented are based on mean values of triplicate determination(variation within 5-10%) derived from at least three separate experiments.

Chapter 3 In vivo phosphorylation of microtubular protein

3.1 Introduction

Previous work has shown that MTP can be phosphorylated in vitro, in brain slices and in purified MTP preparations (see Lagnado et al, 1975). Further experiments also showed that purified MTP contains 0.5 - 1.0 moles of protein-bound P per mole of tubulin dimer (MW 110000) (see Reddington et al, 1974, Kirschner et al, 1975).

The aim of the experiments described in this chapter is to provide further evidence that brain microtubules are phosphorylated in vivo and to determine more precisely the nature of the phosphorylated components labelled in vivo that were derived from reassembled microtubules.

3.2 Materials and Methods

3.2.1 Preparation and nomenclature of in vivo labelled MTP fractions

Adult rats (4 to 6 weeks) or 1 to 3-day-old chicks were injected intracranially with 400 - 600 μCi ^{32}P per animal, as described in 2.3.1, and killed 2 h after the injection. MTP was isolated by one cycle of polymerisation, in the presence of glycerol, from either the initial high-speed supernatant (40% homogenate in glycerol free reassembly buffer, spun at 100000 x g, 1 h 4°C), designated as S, or from the ammonium sulphate fraction of S, (same expt. precipitating between 0-50% saturation), designated as $S_{0.5\text{-ppt}}$. The $S_{0.5\text{-ppt}}$ was re-dissolved in glycerol-free reassembly buffer to give a protein concentration similar to that of S_1 (ca. 8 mg

protein/ml). MTP fractions obtained on polymerisation of the re-dissolved $S_{0.5\text{-ppt}}$ were designated as $P_{2\ 0.5}$ (microtubular pellet) and $S_{2\ 0.5}$ for the supernatant.

3.2.2 Fractionation of purified MTP

The following procedures were designated to separate the aggregated forms of MTP ('36S' fraction) from the tubulin dimers ('6S' fraction) obtained on depolymerisation of microtubules prepared as described in 2.2.1 by gel permeation chromatography or by differential centrifugation according to the procedures of Erickson (1974) and Weingarten et al (1974). In addition, NaCl was used (see Weingarten et al, 1974) to dissociate the aggregated forms of tubulin ('36S' tubulin) in order to separate tubulin dimers and HMW-enriched fractions derived from the '36S'structures.

3.2.2.1 Experiments type 1 and 2

1 x polymerised microtubules were disassembled in the cold; the solutions obtained after centrifuging for 60 minutes at 10^5g (MTP, 5-12 mg protein/ml) were fractionated at 4°C on Sepharose 6B essentially as described by Erickson (1974) except that in Expt. type 2, MTP samples were treated with 0.75 M and eluted with buffers containing 0.75 M NaCl.

3.2.2.2 Experiments type 3

2 x polymerised microtubules were disassembled for 30 minutes at 4°C in MES-Ca^{2+} buffer (supplemented with 1mM $CaCl_2$) before chromatography on Sepharose 6B equilibrated in the cold with reassembly buffer, supple-

mented with 2.5 mM Ca^{2+}, essentially as described by Weingarten et al(1974) In 3B, concentrated peak I ('36S') material (of 3A) containing 0.75 M NaCl ('36S + NaCl') was separated on Sepharose 6B in the presence of 0.75 M NaCl and analysed as described in text.

3.2.2.3 Experiments type 4

2 x polymerised microtubule preparations were isolated as in Expt. 3, except that the final disassembly step was carried out at 15°C in the presence of MES-Ca^{2+} buffer supplemented with 1 mM GTP and 1 mM $CaCl_2$ (MT; 10.5 mg protein/ml). The pellet fraction (4A, '36S') obtained after centrifuging MTP for 190 minutes x 10^5g (at 8-10°C) was re-suspended in the cold in disassembly buffer containing 0.75 M NaCl ('36S + NaCl'; 4.7 mg protein/ml) and centrifuged as indicated above. The resulting pellet contained appreciable amounts of tubulin (denatured aggregates?) in addition to HMW ('HMW + T'), as revealed by SDS-gel electrophoresis.

3.2.3 Analysis of ^{32}P distribution on fixed and stained polyacrylamide gels

3.2.3.1 Slicing of gels for counting of individual [illegible] bands

Fixed and stained gels were placed on strips of Whatman No 3 paper in a shallow glass dish and covered with freshly prepared 2% agar (Oxoid).
After the agar gelled, the strips, with the gels fixed on them, were transferred to a McIlvain tissue chopper adjusted to cut 1mm slices. To avoid adhesion of individual slices to the blade, it was slightly wetted with a surfactant (e.g. Triton), and the slicing was performed

at low speed. The individual slices were removed with forceps and placed in counting vials containing 5ml of Brays scintillant.

3.2.3.2 Electrophoretic elution of protein from gels

The corresponding protein bands from 6 - 8 gels were cut out and transferred to glass tubes, the bottom opening of which was blocked with a plastic filter (porous polythene, size 50 u, Gallenkamp & Co Ltd, UK). The tubes were then inserted in tightly fitting dialysis bags and filled up with electrophoresis buffer (see 2.7.2), taking care to remove all air bubbles. After placing the tubes in an electrophoresis bath, containing electrophoresis buffer, the proteins were eluted into the dialysis bags at 3m A/tube in approximately 2.5 hours. The excess buffer from the tubes was removed with a Pasteur pipette and the protein was recovered in 0.5 - 1 ml of buffer from the dialysis bag.

3.3 Results

Reassembled microtubules isolated from rat and chick brain labelled *in vivo* with $^{32}P_i$ contained appreciable amounts of acid-insoluble radioactivity (bound ^{32}P). Up to 80% of the bound cpm in disassembled microtubular preparations from chick and rat brain was extractable with chloroform-methanol (2:1, by vol., see tables 3.1 and 3.2). Over 80% of the acid-insoluble ^{32}P remaining after extraction with lipid solvents was present as alkali-labile P, derived most probably from protein-bound serine residues (see 2.6); this will be referred to as protein-bound P. Less than 5% of the bound counts remained in

Table 3.1 The distribution of bound ^{32}P in subfractions of *in vivo* labelled brain microtubulin

Fraction	Protein	Bound ^{32}P		*Distribution of ^{32}P	
		Protein	Lipid	Protein	Lipid
	mg	cpm/ug prot		%	
S_1	82.5	15.4	5.9	74	26
P_2	8.4	17.3	6.5	72	28
$S_{0.5\text{-ppt}}$	32.4	24.2	20.5	54	46
$P_{2\text{-}0.5}$	1.5	36.2	28.3	56	44

* Calculated as % from bound ^{32}Pcpm recovered as alkali labile and phospholipid ^{32}P.

Table 3.2 The incorporation *in vivo* of ^{32}P into the protein and lipid fractions of microtubules isolated from rat brain

Expt Type	Fraction analysed	Protein recovered	^{32}P incorporated protein	^{32}P incorporated lipid	Distribution of ^{32}P protein	Distribution of ^{32}P lipid
			cpm/ug protein (%)*		%**	
1	MT		10.8	27.4	34	66
	'36S' (peak I)	23	4.6 (34)	39.3 (77)	19	81
	'6S' (peak II)	77	3.4 (66)	4.4 (23)	29	71
2	MT + NaCl		12.3	37.6	29	71
	'HMW' (peak I)	11	28.4 (42)	31.7 (20)	49	51
	'36S + 6S' (peak II)	89	4.7 (58)	14.9 (80)	27	73
3A	MT		38.5	47.5	45	55
	'36S' (peak I)	20	12.6 (14)	128.0 (87)	13	87
	'6S' (peak II)	80	8.6 (86)	4.7 (13)	78	22

* Values in brackets = % of recovered cpm in fraction.
** cpm recovered for protein + lipid in individual fraction = 100%.

Table 3.2 (continued)

Expt Type	Fraction analysed	% Protein recovered	^{32}P incorporated protein	^{32}P incorporated lipid	Distribution of ^{32}P protein	Distribution of ^{32}P lipid
			cpm/ug protein (%)*		%**	
3B	'36S + NaCl			as for '36S' in Part A		
	'HMW + T' (peak 1a)	n.d.	n.d.(4)	n.d.(8)	21	79
	'HMW + T' (peak 1b)	n.d.	n.d.(3)	n.d.(11)		
	'36S - T' (peak Ic)	(60)§	27.7 (93)	27.5 (93)	44	56
4A	MT		6.0	28.7	17	83
	'6S' (supnt.)	10	2.9 (8)	7.1 (3)	29	71
	'36S'(pellet)	90	3.6 (92)	25.0 (97)	13	87
4B	'36S + NaCl'			as for '36S' in Part A		
	'6S' (supnt.)	36	2.9 (60)	6.2 (22)	38	62
	'HMW + T' (pellet)	64	3.7 (34)	12.8 (78)	8	92

* Values in brackets = % of recovered cpm in fraction.
**cpm recovered for protein + lipid in individual fraction = 100%.
§ Estimated as a percentage of the protein applied to the column. In most experiments, 70 - 85% of the protein applied was recovered in combined eluates.

the alkali-stable fraction (see 2.6.3).

The data shown in table 3.1 reveal considerable enrichment in ^{32}P-phospholipids in microtubules reassembled from ammonium sulphate precipitated material. However, the yield of polymerisable protein prepared under these conditions was considerably less (1 - 2%) than that from the unfractionated supernatant fraction, from which about 10% of the protein was recovered in the microtubular pellet.

An obvious limitation to the experiments described so far is that it was not possible to distinguish between tubulin derived from the 36S aggregates, corresponding to the 400 nm ring-like structures seen by electron microscopy, and the 6S form of tubulin (α; ß dimer), which are both present in cold-or calcium-depolymerised microtubules isolated from <u>in vitro</u> reassembled brain microtubules (Olmsted et al, 1974; Kirschner et al, 1974).

It was recently shown that these two components, which were initially characterised on the basis of their sedimentation properties (Kirschner et al, 1974; Weingarten et al, 1974), can be separated by chromatography on Sepharose 6B (Erickson 1974; Kirschner et al, 1974; Weingarten et al 1974), to give a first peak, eluted in the void volume, which consisted mainly of 36S 'ring' structures that are readily polymerised at 37°C in the presence of GTP, and a second peak, containing essentially pure tubulin dimer, which polymerises less readily (Erickson, 1974), or not at all (Kirschner et al, 1974). Although tubulin was the major protein component found in both peaks (as seen by SDS-gel electrophoresis), the 36S component was shown to be enriched in a number of minor components, the main

one corresponding to the HMW protein complex (Erickson, 1974). In addition, it was shown that the 36S component could be reversibly disaggregated into the 6S component in the presence of high salt concentrations (Kirschner et al, 1974; Weingarten et al, 1974).

Exploratory experiments were carried out to investigate the extent of incorporation of ^{32}P into the 36S and 6S components of chick brain microtubules in vivo. In addition, it was attempted to separate the minor HMW components, which were found to be nearly exclusively associated with the 36S fraction, in order to facilitate characterisation of the ^{32}P incorporated in this fraction. The results of these experiments which are shown in table 3.2 and in figs.3.1 and 3.2, can be summarised and interpretated as follows.

When cold-or Ca^{2+}-depolymerised microtubules were fractionated by chromatography on Sepharose 6B (table 3.2, Expts 1 and 3A), most of the protein-bound ^{32}P recovered was found in the peak fraction containing 6S tubulin, while most of the phospholipid-^{32}P recovered was associated with the fraction enriched in the 36S 'ring' fraction (peak 1) (see also data for Expt 4, table 3.2). However, only about 2[illegible] the protein was recovered in the 36S fraction (see also Fig. 3.1A), even though the concentration of protein in samples before separation (MT) was such that over 60% of the protein was expected to be present as 36S aggregates (Yagihara et al, 1973). This suggests that in both experiments a substantial proportion of the 36S material was disaggregated through dilution during chromatography and/or that only a more

stable form of the 36S component can remain intact under these conditions, as was originally suggested by Erickson (1974). Thus, the tubulin present in the 6S (peak II) fraction is probably composed of a mixture of protein originally present as 6S tubulin and of tubulin derived through disaggregation from 36S structures.

The high specific activity in the phospholipid fraction associated with the 36S component could reflect the preferential association of metabolically active phospholipids with a more stable form of 36S tubulin, or, alternatively, the presence of HMW material, which indeed appears to be exclusively associated with peak 1 material (see fig. 3.1A). The relatively high specific activity of phospholipids associated with the 36S component derived from Ca^{2+} -depolymerised material (cf. Expt. 3A and 1) was generally confirmed (Expt. 4, see also 4.3).

The possible significance of the labelling seen in the HMW material that co-chromatographed with 36S tubulin was further investigated in experiments in which the 36S component was disaggregated with NaCl (Weingarten et al, 1974) before chromatography.

In Expt. 3B, the 36S fraction was concentrated by vacuum dialysis in the presence of NaCl and re-chromatographed on Sepharose in the presence of NaCl (Fig. 3.1B). Under these conditions, the material present in the first two peaks of radioactivity eluted (fractions 1a, 1b) gave identical protein patterns after electrophoresis in SDS-gels HMW was present together with some tubulin. A further broad and apparently complex peak of radioactivity (1c) was eluted in a region coinciding roughly with the elution volume of the 6S component: pooled fractions from

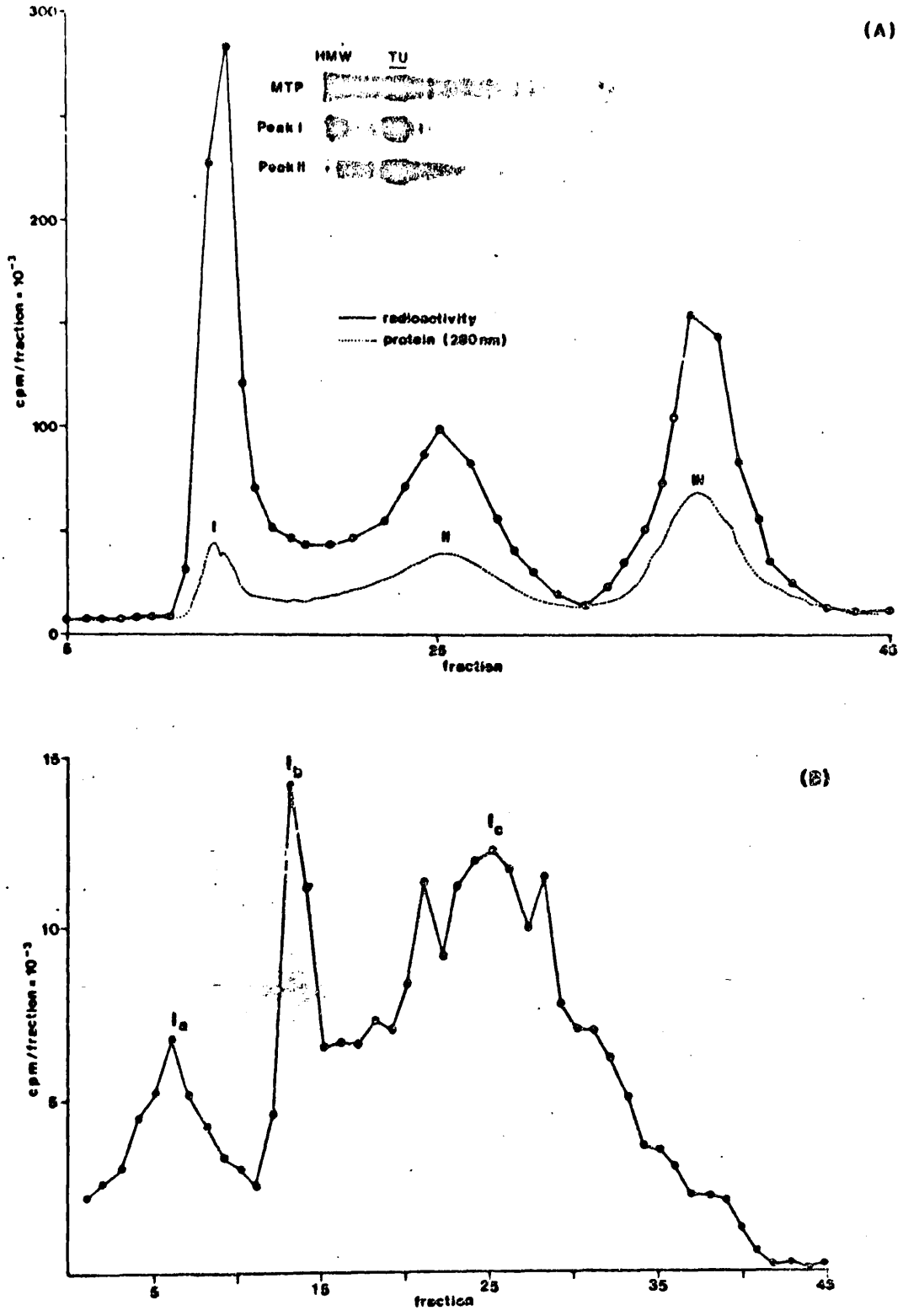
Legend to fig. 3.1

(A) In vivo labelled MTP was prepared by one cycle of polymerisation, fraction S_3, and fractionated on Sepharose 6B as described earlier. See also 3.2.2.1 and table 3.2.

(B) Peak I of (A) was concentrated and made 0.75M with NaCl by vacuum dialysis. The concentrated '36S + 6S' material was chromatographed on Sepharose 6B in the presence of 0.75M NaCl. For details see 3.2.2.2 and table 3.2.

The inset in (A) shows S-PAGE patterns for samples of unfractionated MTP and of material pooled from peak I (fractions 13-15) and peak II (fractions 23-27). HMW=high molecular weight protein(s); Tu = tubulin. Note absence of HMW in peak II material.

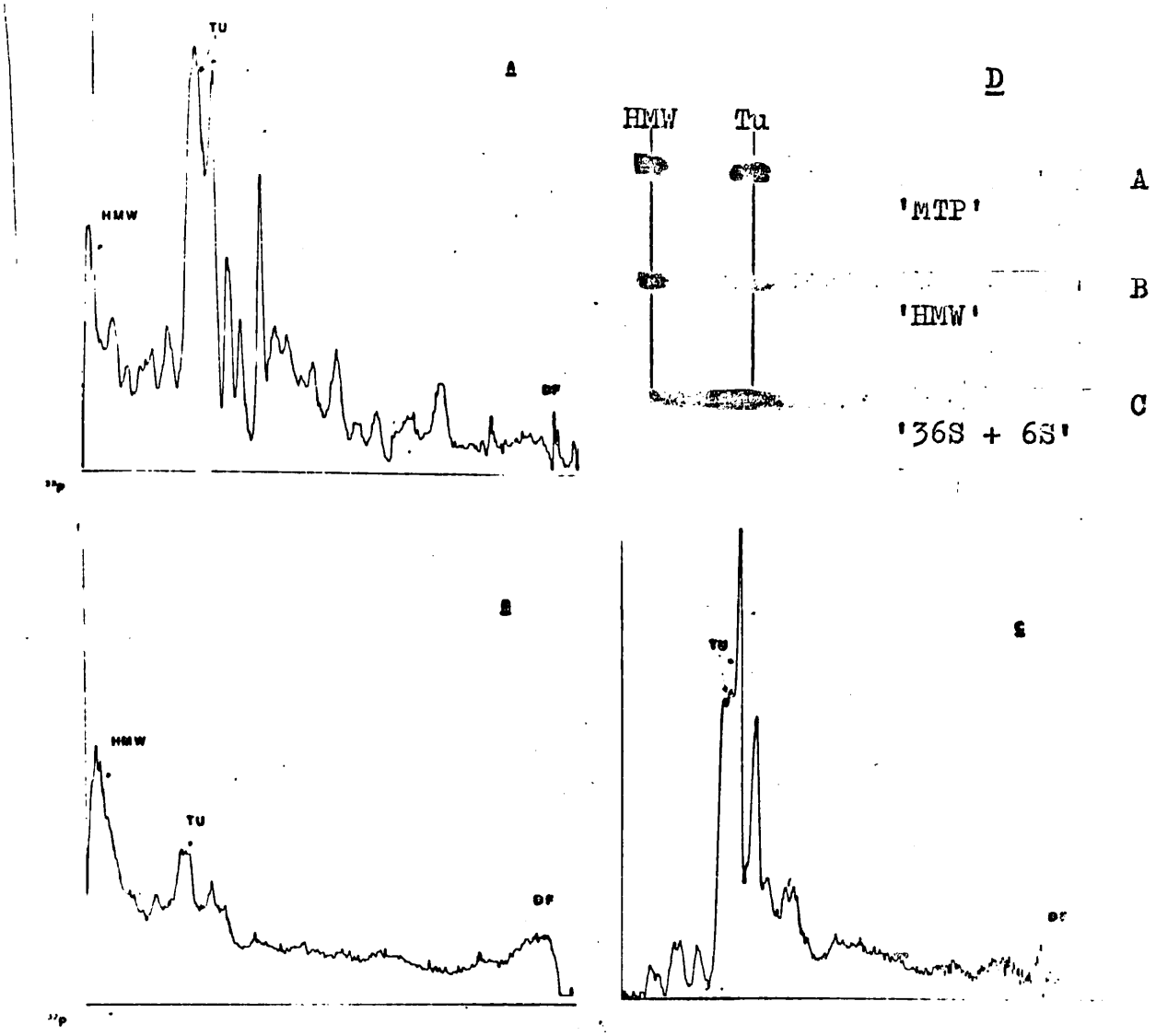
Fig. 3.1 Chromatography on Sepharose 6B of in vivo labelled MTP from chick brain



this region, which accounted for most of the recovered protein (Table 3.2, Expt. 3B), were almost exclusively composed of tubulin derived, presumably, from the 36S tubulin aggregates separated in Expt. 3A (see also Fig 3.1A). Thus, a partial separation of HMW from 36S tubulin was achieved. It is evident from the data shown in Table 3.2 (Expt. 3A) that most of the bound ^{32}P recovered as protein-bound P or as phospholipid P was present in the main tubulin fraction (peak 1c). Since, however, too little material was recovered in peak fractions containing HMW (1a, 1b, see Fig 3.1B) to permit reliable estimations of protein by the Folin-Lowry method, it was not possible to determine specific radioactivities for ^{32}P in these fractions. Nonetheless, it is tentatively concluded on the basis of the data giving the percentages of radioactivity recovered that the fractions enriched in HMW are also relatively enriched in ^{32}P-phospholipid (see discussion of Expt. 3A above). The distribution of ^{32}P labelled phospholipids in microtubular subfractions is specifically dealt with in Chapter 4.

In Expt. 2 (table 3.2, Fig 3.2), the possibility of separating HMW from both forms of tubulin was investigated by chromatography of cold-depolymerised microtubules which had been treated with 0.75 M NaCl to dissociate 36S tubulin aggregates, prior to chromatography on columns of Sepharose 6B which were equilibrated and eluted in the presence of 0.75 M NaCl. It was anticipated that this procedure would result in all the tubulin being eluted as 6S tubulin, after elution of the HMW fraction in the void volume, as had been the case when it was present together with the undissociated 36S component (see Fig 3.1A).

Fig. 3.2 S-PAGE analysis of *in vivo* labelled chick brain MTP



The densitometric scans shown in A, B and C were obtained from polyacrylamide gels of MTP fractions prepared as follows: A unfractionated MTP, fraction S_3; B and C show the HMW-enriched fraction and the '36S + 6S' fraction , respectively, obtained after treatment of S_3 with 0.75M NaCl and subsequent chromatography on Sepharose 6B in the presence of 0.75M NaCl. For details see 3.2.2.1 and table 3.2. D shows radioautographs of the same (as in A, B and C) gels. Note absence of radioactivity in the HMW-region of '36S +6S' radioautograph.

This was found to occur, as shown in the SDS-gel electrophoresis patterns of ^{32}P-labelled material which behaved similarly during elution to the 36S and 6S components: the first peak eluted now contained mainly HMW, while most of the protein present in peak II was identified as tubulin, as shown in Fig. 3.2B and 3.2C respectively. Moreover, it is evident from the radio-autographs shown (Fig. 3.2) that virtually all the label detected after electrophoresis of peak I material was associated with several HMW components near the origin, while the label found in peak II material co-migrated almost exclusively with the tubulin fraction. The data given in Table 3.2 (Expt. 2) show that under these conditions the HMW fraction was considerably enriched in bound ^{32}P, especially in respect of protein-bound P.

Finally, the results of an experiment in which the 6S and 36S components of tubulin were separated by high-speed centrifugation (Weingarten et al, 1974) (Expt. 4A, Table 3.2) showed that most of the protein was recovered in the pellet fraction which was expected to contain tubulin, present mainly as 36S aggregates. This fraction also contained most of the bound ^{32}P and was particularly enriched in ^{32}P-phospholipids. The pellet fraction, which contained tubulin and HMW in about the same proportion as in the unfractionated material (MT), was re-suspended in buffer containing 0.75 M NaCl and re-centrifuged at high speed. It was anticipated that in this way HMW components, together with some non-specific tubulin aggregates, might be recovered in the pellet, while tubulin derived from dissociated 36S aggregates would remain in solution.

This was confirmed by SDS-gel electrophoresis, though an appreciable amount of tubulin was also found in the pellet fraction. The data shown in Table 3.2 (Expt. 4) indicate once again that the HMW-enriched pellet fraction accounted for the bulk of ^{32}P-phospholipid (see also Expts. 2 and 3B). On the other hand, the supernatant was considerably enriched in ^{32}P protein, despite the absence of any HMW component (cf Expt. 2). One feature worth noting about this experiment is that the concentration of protein remained high enough throughout to maintain the 36S $\rightleftharpoons$ 6S equilibrium in favour of the 36S species (Kirschner et al, 1974; Weingarten et al, 1974); this is in contrast to the situation which obtains during the separation of 6S and 36S components on Sepharose 6B, when dilution of the sample during chromatography tends to favour disaggregation of the 36S species (see above).

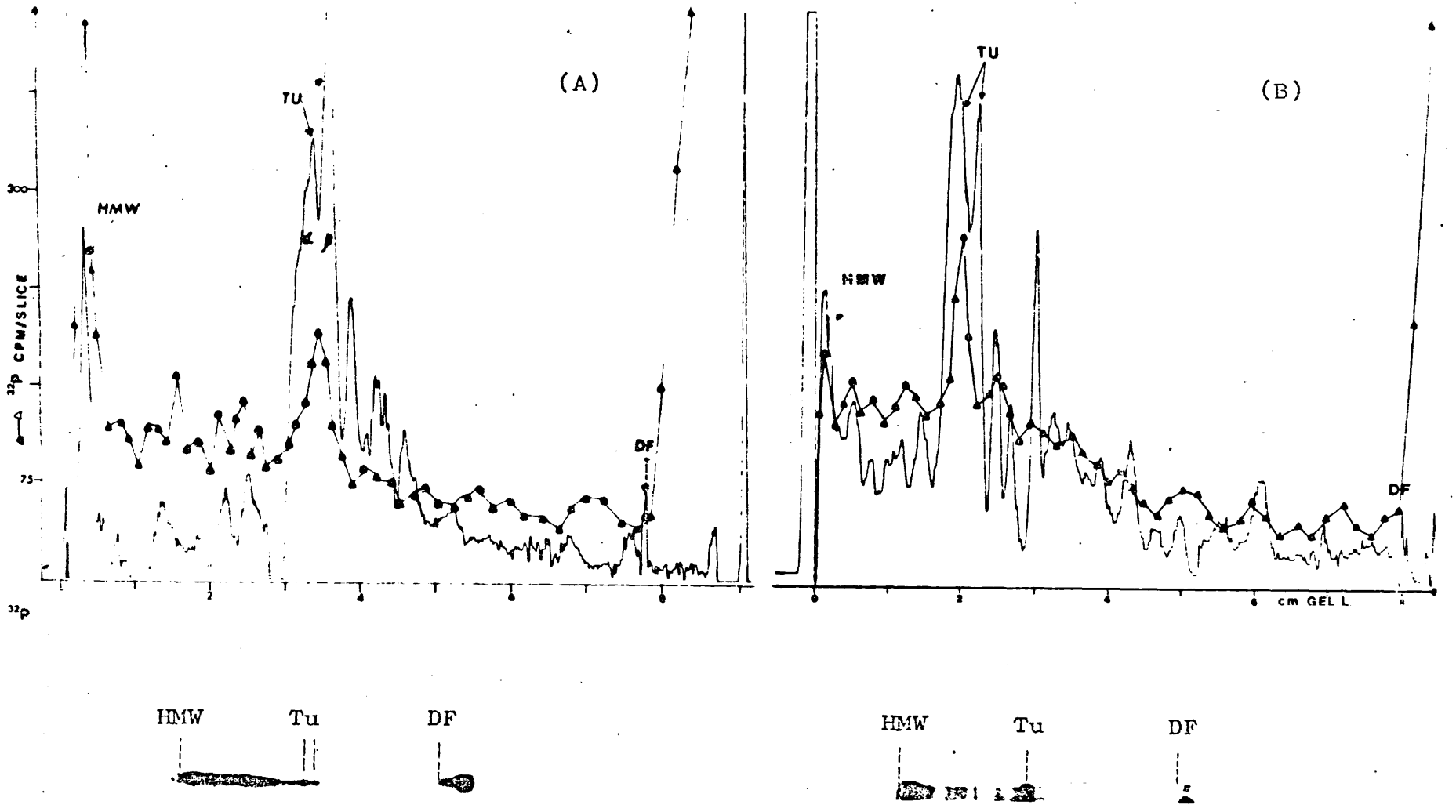
It was previously observed that a significant proportion of the radioactivity present in the microtubular fraction of ^{32}P-labelled slices from guinea pig cerebral cortex was associated with minor high-MW components ('HMW') seen after electrophoresis in SDS-polyacrylamide gels (Lagnado et al, 1975). A similar distribution of ^{32}P has now been found for <u>in vivo</u> labelled microtubular preparations (1 x polymerised) derived from rat and chick brain (Fig. 3.3).

In these preparations, a number of additional minor components migrating between tubulin (TU) and HMW fractions were also labelled; and in chick brain some labelling was associated with a minor stained component migrating ahead of the ß-tubulin band (see Fig.3.3B).

Legend to fig. 3.3

MTP was prepared by one cycle of polymerisation fraction S_3, and samples were analysed by S-PAGE. After scanning (densitometric trace shown with continuous line) the fixed and stained gels were sliced in 1mm slices and the radioactivity of each slice determined separately, as described earlier. The radioautographs shown are of gels of the same fractions (from same experiment) run in parallel. HMW = high molecular weight proteins; Tu = tubulin; DF = dye front. Smearing on radioautographs is due to overexposure of gels that were overloaded to detect minor ^{32}P-labelled components.

Fig. 3.3 Distribution of protein and ^{32}P in <u>in vivo</u> labelled brain MTP, obtained from rat (A) and chick (B)



In addition, a diffuse region of radioactivity of varying intensity was consistently observed to migrate ahead of the dye front (Lagnado et al, 1975 - submitted for publication; Lagnado et al, 1975), a region which does not stain for protein but contains an excess of SDS.

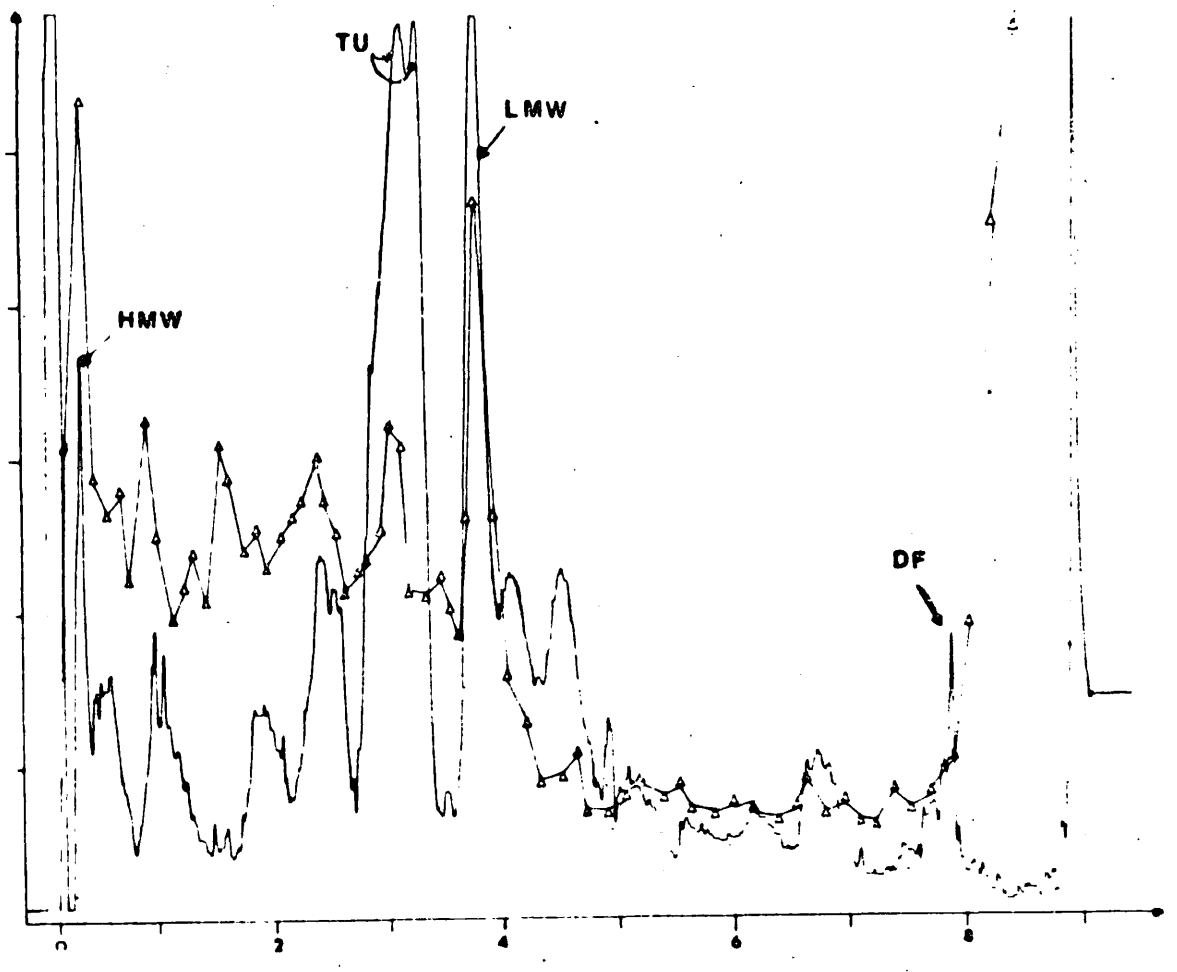
In an effort to assess the nature of the ^{32}P present in the main labelled fractions (ie, in HMW, α+β-tubulin, and 'SDS front'), these were eluted electrophoretically from the appropriate slices pooled from 6 - 7 gels (run in parallel) after fixation and staining of proteins with Coomassie Blue; the eluates were treated with ice-cold 10% TCA (in the presence of carrier albumen) and then as indicated in 2.6. It was consistently found that most of the ^{32}P associated with the HMW and tubulin fractions was recovered as protein-bound (alkali-labile), while that associated with the SDS front, which accounted for about ten times as much ^{32}P as was eluted from the other fractions, was mainly recovered as phospholipid P and acid-soluble P.

When <u>in vivo</u> labelled microtubules were polymerised from a 50% ammonium sulphate precipitate of the initial high-speed supernatant fraction (S_1) of brain, an additional, highly labelled protein component, MW ca. 48000, co-polymerised with microtubular protein, as illustrated for rat brain ('LMW', Fig. 3.4). This was not seen in microtubular preparations precipitated with ammonium sulphate after reassembly directly from the initial supernatant. Slower-migrating components that are concentrated in microtubular samples polymerised from the $S_{0.5}$-ppt. nearly obscured the labelling of tubulin

Legend to fig. 3.4

MTP was prepared by polymerisation of 50% ammonium sulphate precipitate from high-speed rat brain supernatant, fraction S_1 (see 3.2.2.1). LMW= prominent labelled low molecular weight protein band appearing only in this type of MTP preparations. Nomenclature as in fig. 3.3, see also related text for further details.

Fig. 3.4 Distribution of protein and ^{32}P in *in vivo* labelled brain MTP prepared by polymerisation from ammonium sulphate precipitate of high-speed rat brain supernatant



detected by radioautography (see Fig. 3.4). However, the estimated specific activity of the 'LMW' fraction, as determined after elution (see above), was found to be similar to that of the tubulin fraction.

The significance of this observation is not apparent although it is interesting that in vivo labelled microtubular protein from guinea pig brain is also enriched in a similar faster-migrating ^{32}P-protein when it is precipitated directly from the supernatant fraction with high concentrations of Vinblastine or Vincristine (10^{-4} M) (Lagnado, unpublished observations).

3.4 Conclusions

The evidence presented above indicates that brain microtubules contain protein-bound P and are associated with a fraction of phospholipids, both of which exhibit considerable metabolic activity *in vivo*. This confirms and extends earlier evidence based on studies of microtubule phosphorylation *in vitro* and in tissue slice experiments (Lagnado,et al, 1975; Daleo et al, 1974; Piras and Piras,1974). A more precise interpretation of these findings must obviously await further information concerning the P contents of the various components present in microtubules isolated by reassembly *in vitro*, from which labelling due to turnover of bound P and to net phosphorylation might be distinguished. Nevertheless, it was consistently observed that the 36S component of depolymerised microtubules,which consists largely of tubulin polymers stabilised into the ring-like structures, was preferentially labelled *in vivo* under

various experimental conditions as compared to the 6S tubulin component. The relative enrichment of the 36S component in metabolically active phospholipid suggest that 36S structures may be associated *in situ*, with cell membrane fractions at sites of microtubule-membrane interactions.

The relatively high incorporation of ^{32}P into phosphoserine residues (as defined in 2.6.2) of the 36S component may indicate that any regulatory factors controlling the conversion of 6S tubulin to the 36S form, from which microtubules are apparently polymerised might be acting, in part at least, as regulators of enzymes concerned with the turnover of protein-bound P. In this connection, the observation that a highly labelled minor protein (HMW), originally seen by SDS-gel electrophoresis, can be separated from salt-dissociated preparations of the 36S component by chromatography, may be of interest in the light of recent work (Weingarten et al, 1975) showing that a tightly-bound, heat-stable protein ('tau factor') required in microtubule assembly can be dissociated from 36S tubulin preparations in the presence of salt.

4.1 Introduction

The evidence presented in chapter 3 shows that tubulin can be phosphorylated *in vivo*. Lagnado et al (1972), Soifer et al (1972), Lagnado et al (1975) and Reddington et al (1976) also showed that tubulin prepared chromatographically or by cycles of temperature dependent polymerisation can act as substrate for a cyclic AMP-sensitive protein kinase which co-purifies with microtubular proteins. The above authors assayed MTP phosphorylation under conditions where the assembly of microtubules is unlikely, although some form of tubulin aggregation can occur. The experiments to be presented in this chapter were designed to investigate the *in vitro* phosphorylation of MTP under conditions favouring the formation of microtubules. Thus assays were carried out using freshly prepared MTP preparations in the presence of sufficient protein and nucleotides to ensure the formation of microtubules (see 1.3 and Gaskin et al, 1975). These experiments were not aimed at relating phosphorylation with microtubule assembly, but rather at assaying the phosphorylation reaction where the protein is more likely to preserve its native configuration, as judged by its ability to reassemble in microtubules.

4.2 Materials and Methods

4.2.1 Preparation of MTP

MTP was prepared from one day old chick or 5 - 7 week old rat brain by one cycle of assembly/disassembly, fraction (S_3), as described in 2.2.1.

The depolymerisation of the first MTP-pellet, P_2, was performed in the presence or absence of added GTP

(1mM final conc.) as specified for the individual sets of experiments. In both instances the yield of protein in the S_3 fraction was the same indicating that the state and/or type of protein obtained on depolymerisation, in the presence or absence of GTP, is very similar. Polyacrylamide gel electrophoresis in the presence of SDS (S-PAGE; see 2.7) showed the presence of two main types of protein in the S_1 fraction, namely the HMW complex consisting of three distinct protein bands of very similar mobility and tubulin. Densiometric analysis of gels stained with Coomasie blue showed that 85% of the protein in the S_3 fraction is tubulin and 15% - HMW proteins (apparent MW 300000 by S-PAGE).

4.2.2 Protein kinase assays

These were carried out at 37°C in MT-reassembly buffer in the absence or presence of GTP (1mM final conc.) and at a protein concentration of 0.5mg/ml. Where indicated 10^{-5}M cAMP was included in the reaction mixture. After 5 minutes of preincubation of the samples at 37°C the reaction was started with the addition of a mixture of ATP (100 µM final conc.) and [γ-^{32}P] ATP, 1 or 2.2 x 10^6 cpm / 0.2ml of reaction mixture, specific activity 2.7 Ci/mmol.

4.2.3 Filter disc assay of protein-bound ^{32}P

Samples of reaction mixture, 0.1ml, containing 50 µg of protein, were transferred to conical centrifuge tubes containing 1ml of ice-cold 10% TCA (w/v) and 300 µg carrier protein (bovine serum albumin) and left on ice for 15 minutes. The precipitate was transferred to glass fibre discs (Whatman GF), which were placed on a vacuum filtration assembly. Quantitative transfer of the protein precipitate from the centrifuge tubes to the filter discs was achieved

by washing the tubes two times with 10ml of ice cold 5% TCA, which was then used to wash the filter discs on the vacuum filtration device. (Randomly selected centrifuge tubes, washed in this way, were then washed with 5ml of Bray's scintillation liquid and in no case did the wash contain more than background counts). The filter discs were then washed with 20ml of diethyl ether - ethanol (1:1) and finally dried with 20ml of diethyl ether. The dry discs were transferred to counting vials containing 5ml of Bray's scintillant and counted. Blanks containing heat-denatured protein (5 minutes at 90°C) were incubated and processed in the same way and the appropriate corrections made for the test samples.

4.2.4 Preparation of samples for electrophoresis

Protein samples were prepared in two different ways:

1. Samples of the reaction mixture containing 60 µg of protein were transferred to tubes containing 25 µl of sample incubation buffer (see 2.7.2) and incubated for 3 minutes at 90°C.

2. Samples of the reaction mixture containing 80 µg of protein were transferred to conical tubes containing 1ml of ice-cold 10% TCA and 80 µg of carrier protein and left on ice for 15 minutes. The precipitate was pelleted and then washed twice with 2 ml of 5% TCA. The acid-washed pellet was resuspended in acidified chloroform-methanol (400 : 200 : 1.5 conc. HCl, by vol), and left for 15 minutes at room temperature. After sedimentation the pellet was dried with 1 ml of diethyl ether and dissolved in 80 µl of sample incubation buffer. The radioactivity recovered in this solution is defined as protein-bound ^{32}P.

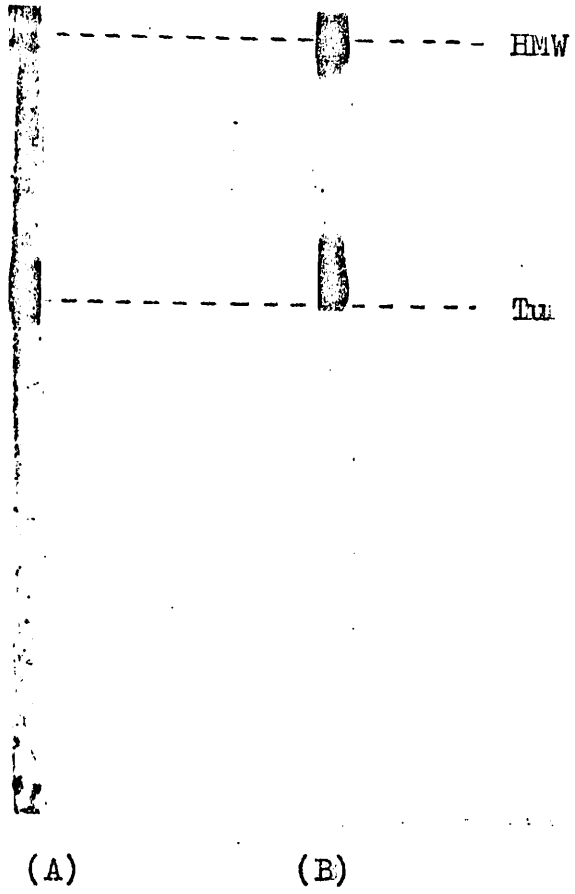
Aliquots were used to determine values of protein-bound ^{32}P in individual samples. Values are expressed either as cpm/mg protein, or as moles of bound ^{32}P per mole of protein, on the basis of the measured specific activity of the $[\gamma - ^{32}P]$ ATP in the incubation mixture. Since the same results were obtained using either method, acid precipitated samples were routinely used in the experiments described in this chapter.

4.3 Results

Radioautography of polyacrylamide gels of *in vitro* phosphorylated MTP (fraction S_3) reveals two main regions of radioactivity corresponding to the protein bands of the HMW-complex and tubulin (fig. 4.1). There are a number of minor protein bands which become labelled too, the most prominent one of an apparent molecular weight of approx. 70000. However, the total radioactivity associated with any one of those minor components is less than that found with the HMW or tubulin bands.

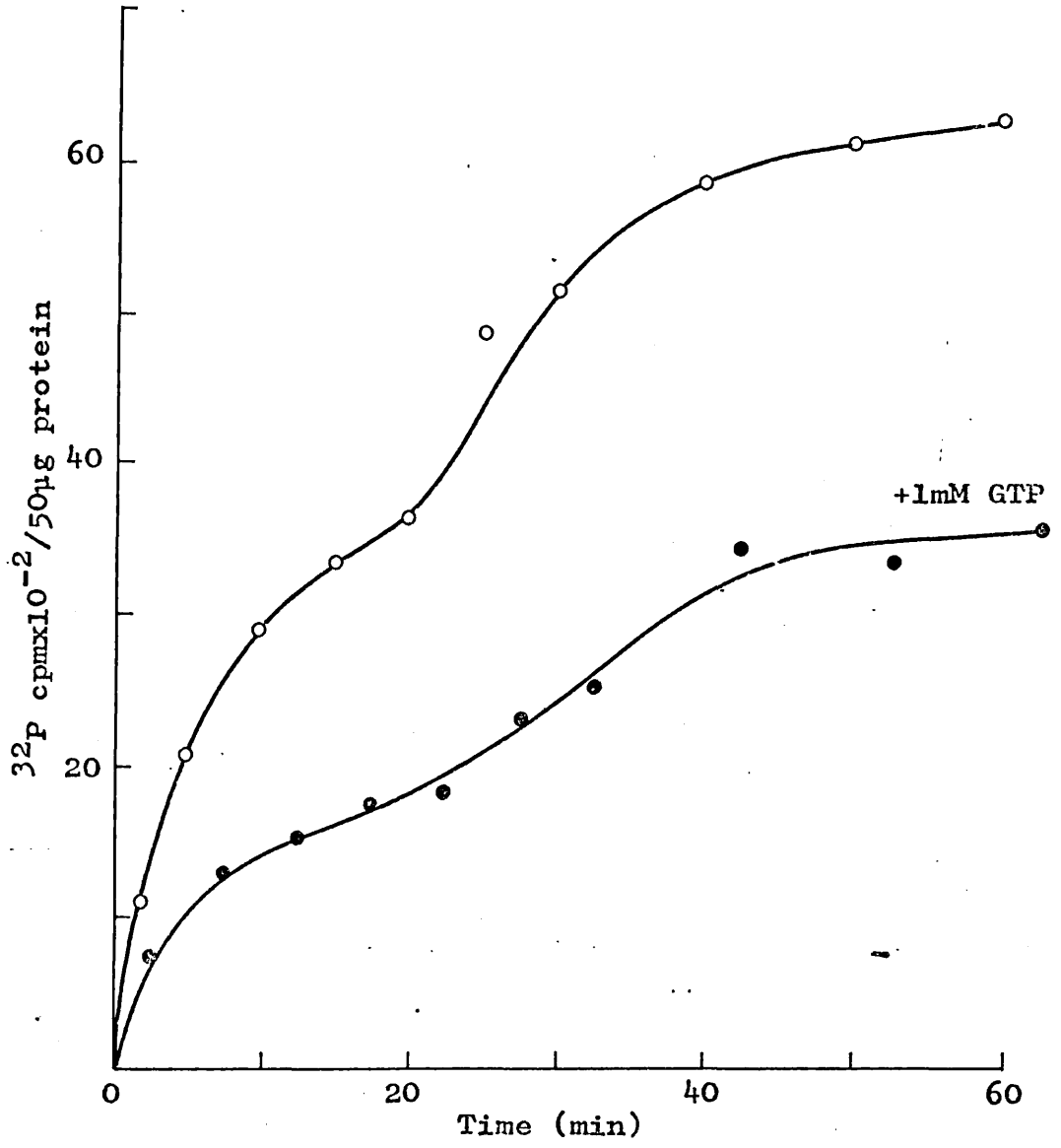
The time course of protein phosphorylation in the purified MTP fraction, S_3, was investigated by the filter disc assay described above. The curves obtained in the presence or absence of GTP were very similar, except that less labelling was seen in the presence of added GTP. This suggests that GTP competed for ATP as phosphate-donor in the phosphorylation reaction, in accordance with previous observations. (Piras and Piras, 1974.) The data illustrated in fig. 4.2 indicate that the phosphorylation reaction is bi-phasic, a rapid and linear incorporation of ^{32}P occurring during the first 5 - 10 minutes, being followed by a second phase of increased incorporation of label, as protein-bound

Fig. 4.1 In vitro phosphorylation of brain MTP purified by polymerisation



MTP, fraction S_3, was prepared by one cycle of polymerisation from rat brain. A sample of purified MTP was incubated for 20min with [γ - ^{32}P] ATP (1×10^6 cpm/0.2ml reaction mixture) in glycerol free reassembly buffer (0.5mg protein/ml of reaction mixture). The reaction was stopped by the addition of ice-cold TCA. The protein was washed further with 5% TCA, delipidised and dissilved in sample incubation buffer as described in 4.2.4, procedure 2. (A) shows the protein pattern of the sliced and dried polyacrylamide gel of the in vitro phosphorylated MTP which was used for radioautography; (B) shows the radioautograph of the gel shown in (A).

Fig. 4.2 Time-course of *in vitro* phosphorylation of brain MTP purified by polymerisation



Brain MTP prepared by one cycle of polymerisation was incubated with $[\gamma - {}^{32}P]$ATP (1×10^6 cpm/ml of reaction mixture) under *in vitro* polymerisation conditions. The extent of protein phosphorylation was estimated by the filter disc assay. For details of phosphorylation assay and filter disc assay see 4.2.2 and 4.2.3 respectively.

^{32}P, after 10 - 25 minutes. A final plateau was reached by about 50 minutes.

The observed biphasic character of the time-course for incorporation of ^{32}P into MTP was a highly reproducible phenomenon. One possible explanation of the biphasic nature of the curve is that it represents the sequential labelling of two distinct protein substrates, or even two distinct (serine ?) residues on the same protein substrate. This was investigated in parallel experiments in which the distribution of bound ^{32}P into MTP polypeptides separated by S-PAGE was examined. Experiments were performed in which the total protein-bound ^{32}P was estimated directly by the filter disc assay, at different times of incubation. The distribution of radioactivity in the same samples was investigated by estimating the radioactivity for the individual polypeptides separated on SDS-polyacrylamide gels. In this procedure the main labelled bands, detected by radioautography of the stained and dried gels, were cut out and counted (see table 4.1) or the relative distribution of radioactivity was estimated by densitometric analysis of the radioautographs.

The results shown in figure 4.3 clearly indicate that the two main phosphorylated components in MTP are tubulin and the minor proteins of the HMW-complex. A further labelled component, of approx. 70000 MW, was consistently observed. It is evident from this data that labelling in the tubulin fraction increased steadily between 5 - 50 minutes of incubation, whereas near maximal labelling of the HMW-complex was attained during the first 5 minutes of incubation. Indeed, at very short times of incubation, tubulin labelling was hardly detectable by visual inspection of

Table 4.1 Effects of incubation time on the phosphorylation of tubulin and HMW fractions of rat brain MTP (fraction S_3)

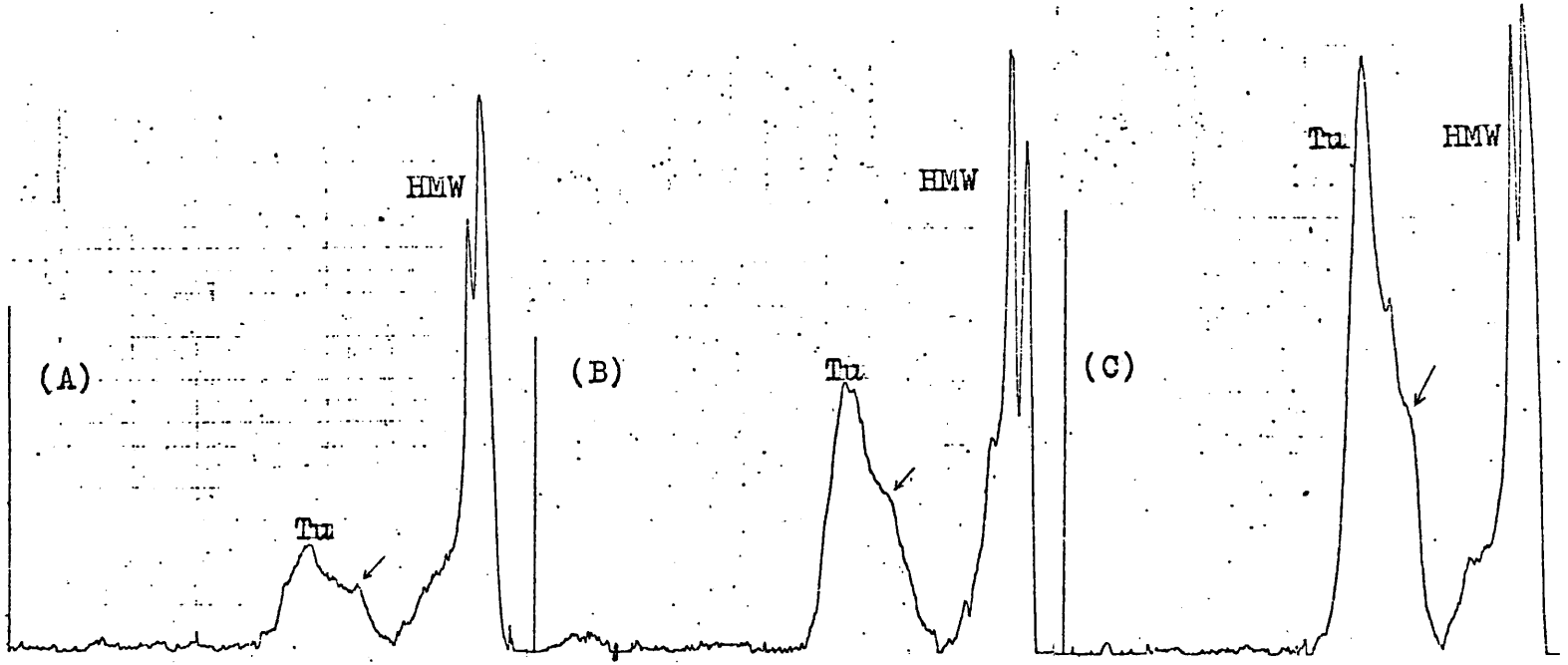
Incubation time	Protein bound ^{32}P		% increase $\left\{\frac{\text{cpm 30 mins}}{\text{cpm 5 mins}} \times 100\right\}$	
	HMW	Tubulin	HMW	Tubulin
mins	cpm/fraction		%	
5	2011	884		
			1	18
30	2042	1029		

See text for experimental details.

Legend to fig. 4.3

MTP, purified from rat brain by polymerisation, was incubated with [γ - ^{32}P] ATP under *in vitro* polymerisation conditions. Protein samples were withdrawn at 5, 20 and 50min of incubation, (A), (B) and (C) respectively, and prepared for electrophoresis as described in 4.2.4, procedure 2. The figure shows densitometric scans of radioautographs of the sliced and dried gels of the corresponding samples. Arrows point at shoulder due to a labelled protein component of an apparent MW of 70 000.

Fig. 4.3 Time course of in vitro phosphorylation of brain MTP purified by polymerisation



radioautographs. These results clearly show that the MTP preparations used contain two main classes of phosphorylatable protein substrates, the differential labelling of which, as a function of time, could account for the observed biphasic labelling curves seen in fig. 4.2.

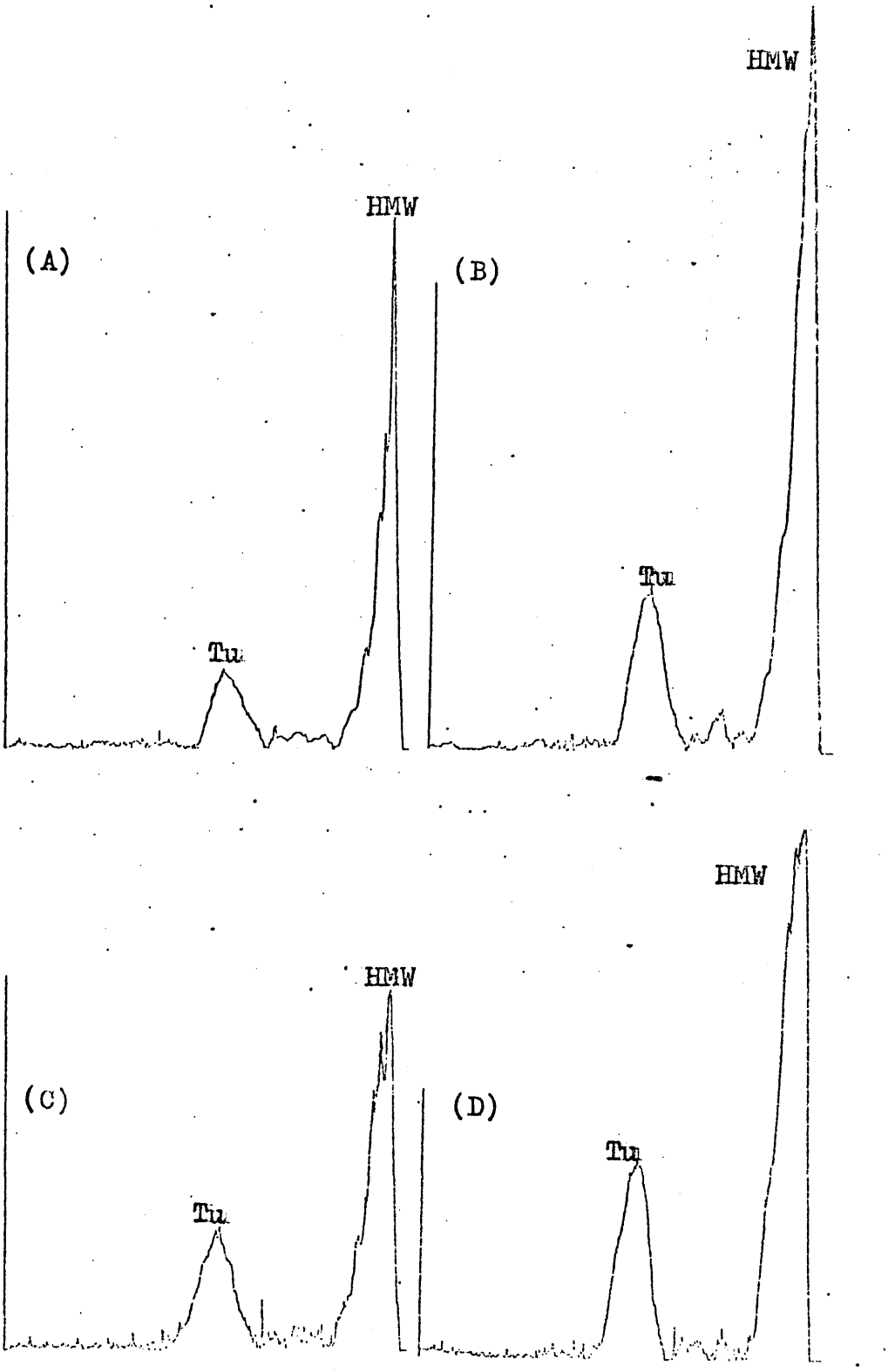
To further characterise the kinase activity of purified MTP fractions, the effects of cAMP on the phosphorylation of MTP from rat brain were investigated. The addition of 10^{-5} M cAMP to the reaction mixture at 4 ^{o}C, prior to preincubation of the samples (5 minutes, 37^{o}C), resulted in a 100% increase of the total amount of protein bound ^{32}P at 5 minutes and a 53% increase at 30 minutes of incubation (see also fig.4.4 and table 4.4). This suggests that at least part of the kinase activity found in purified MTP fractions is cAMP sensitive.

Magnesium ions, required for the transfer of the γ-phosphate of ATP, are invariably included in phosphorylation systems. Rodnight et al (1975) have shown that concentrations of Mg^{2+} of between 0.5 and 1mM are sufficient to saturate ATP at concentrations of up to 1mM. It has also been shown that Mg^{2+} at concentrations higher than 1mM does not increase, but tends to inhibit certain types of cAMP sensitive kinase activity. (Weller and Rodnight, 1973). On the other hand, Mg^{2+} in concentrations higher than 5mM can affect the aggregation state of MTP (Borisy et al, 1976), which in itself could bring about changes in the phosphorylation kinetics of MTP. Moreover there are numerous examples where, for no apparent reason, the activity of the MTP associated kinase is assayed at Mg^{2+} concentrations of up to 20mM (Sloboda et al,1975;

Legend to fig. 4.4

MTP, prepared from rat brain by one cycle of polymerisation, was incubated with [γ - ^{32}P] ATP under *in vitro* polymerising conditions in the absence, (A) and (C), or presence (B) and (D), of 10 μM cAMP. Protein samples were withdrawn at 5min, (A) and (B), and at 30min, (C) and (D), of incubation and prepared for electrophoresis after denaturation with TCA and delipidisation, as described in 4.2.4, procedure 2. The figure shows densitometric scans of radioautographs of sliced and dried polyacrylamide gels of the corresponding samples.

Fig. 4.4 Effects of cAMP (10μM) on the *in vitro* phosphorylation of MTP purified by polymerisation



Reddington et al, 1976).

These considerations gave rise to a set of experiments in which the effect of different concentrations of Mg^{2+} on the phosphorylation of purified MTP was investigated.

The results in Table 4.2 clearly show that the total amount of protein-bound ^{32}P was increased to a similar extent after 5 and 30 minute incubations in the presence of Mg^{2+} concentrations above 0.5 mM. Estimation of the amount of label associated with the major phosphorylated protein species (see fig. 4.1), by direct count of gel slices containing the corresponding protein bands, shows that the degree of phosphorylation of both HMW and tubulin was increased at the higher Mg^{2+} concentrations tested. It is also evident from the data shown in table 4.3 that:

a) the increase of HMW-associated label is considerably higher than that seen for tubulin, and

b) the increase of ^{32}P incorporation into the HMW proteins was similar at 5 and 30 minutes, whereas the increased labelling of tubulin is much higher at 30 than at 5 minutes. Both of these observations are in agreement with the suggestion that the phosphorylation, <u>in vitro</u>, of HMW-proteins is more or less complete at 5 minutes, but tubulin becomes significantly phosphorylated at a lower rate, between the fifth and thirtieth minute of the phosphorylation reaction.

The reasons for this apparent 'activation' of the kinase by higher Mg^{2+} are not clear. It is possible that Mg^{2+} ions, at sufficient concentrations, can promote the dissociation of a regulatory subunit from the protein kinase complex. This could be brought about by some form of direct interaction between Mg^{2+} and the intact kinase complex. If this was the case, it could be expected that the sensitivity

Table 4.2 Effects of the concentration of Mg^{2+} ions on the phosphorylation of MTP (fraction S_3) from rat brain

Incubation time	$MgCl_2$ concentration	Protein bound ^{32}P	% increase $\left(\frac{\text{cpm 30 mins}}{\text{cpm 5 mins}} \times 100\right)$
mins	mM	cpm x 10^{-2} per mg prot	%
5	15	32.97	72.3
5	5	32.1	67.7
5	0.5	19.14	0
30	15	37.74	70.7
30	5	33.22	63.2
30	0.5	20.35	0

Table 4.3 Effects of concentration of Mg^{2+} ions on the phosphorylation of tubulin and HMW fraction of rat brain MTP (fraction S_3)

Incubation time	$MgCl_2$ concentration	moles of bound ^{32}P/mole prot		% increase $\left(\frac{\text{moles bd } ^{32}P \text{ 30 min}}{\text{moles bd } ^{32}P \text{ 5 min}} \times 100\right)$	
		HMW	Tubulin	HMW	Tubulin
mins	mM			%	
5	15	2.18	0.032	142	29
5	5	1.63	0.037	81	42
5	0.5	0.9	0.026	0	0
30	15	2.2	0.06	141	100
30	5	1.64	0.045	80	50
30	0.5	0.91	0.03	0	0

of the kinase to cAMP stimulation should decrease at higher Mg concentrations. Subsequent experiments provided some evidence supporting this interpretation of the effects of Mg ions, thus the data in table 4.4 show that cAMP mainly stimulates the phosphorylation of HMW fractions, the extent of stimulation being inversely related to the concentration of Mg^{2+}. In contrast, the phosphorylation of tubulin was only moderately stimulated by cAMP and in addition, Mg ions had no effect on the degree of stimulation observed. It was noted earlier that cAMP stimulation of phosphorylation seen with unfractionated MTP was higher at shorter incubation times (see text above) and the data in table 4.4 indicate that this effect is specifically related to the phosphorylation of the HMW fraction of MTP (see also fig. 4.4).

The results in Table 4.4 and the proposed explanation are in agreement with the observations that the kinase co-purifying with MTP becomes cAMP insensitive if the procedures by which MTP is purified include treatment with high salt concentrations, eg ammonium sulphate precipitation and fractionation on ion-exchange resins, as summarised by Sandoval and Cuatrecasas (1976). The same authors showed that the kinase activity co-purifying with MTP prepared by a number of very different procedures is invariably inhibited by a protein kinase modulator, which has high specificity for cAMP-sensitive kinases (Walsh et al, 1971), suggesting that the apparent insensivity of MTP-associated kinases to cAMP is an artefact of the particular purification procedure employed.

Table 4.4 Effects of Mg^{2+} ions on the sensitivity of the phosphorylation of tubulin and HMW fractions from rat brain MTP (fraction S_3) in the presence of 10uM cAMP

Incubation time	$MgCl_3$ concentration	* % increase	
		HMW	Tubulin
mins	mM	%	
5	15	37.6	30.2
5	5	132.1	25.6
5	0.5	160.0	20.0
30	15	40.7	18.0
30	5	48.0	20.0
30	0.5	91.0	22.0

$$* \text{ \% increase} = \frac{\text{moles bd } {}^{32}P\text{/mole protein in the presence of 1 gm M cAMP}}{\text{moles bd } {}^{32}P\text{/mole protein in the absence of cAMP}} \times 100$$

Comparing the change in total protein-bound ^{32}P of the MTP fraction, as a function of time in the presence and absence of $10^{-5}M$ cAMP and at different Mg^{2+} concentrations, it can be seen that at the extent of labelling in the presence of cAMP is decreased (see also Leterrier et al, 1974) while an increase in labelling is observed with time, in the absence of added cyclic nuclectide. It is also apparent that the decreased labelling observed is greater at lower Mg^{2+} concentrations (Table 4.5 and fig. 4.5). Examination of the HMW and tubulin bands from polyacrylamide gels shows that a decrease of the protein bound ^{32}P is observed only in the ^{32}P bound to HMW. In the absence of cAMP the amount of total protein bound phosphate increases with time by about 5% at all Mg^{2+} concentrations and this is due mainly to an increase in the amount of label associated with tubulin (Table 4.5).

This observation suggests that the amino acid residues which are phosphorylated in the HMW proteins are different from those phosphorylated in tubulin, having different stability under the conditions employed. Such an interpretation is supported by the fact that measurable quantities of P can be released from MTP prepared by two polymerisation cycles by hydroxylamine treatment (see 2.6).

Chromatographically purified tubulin lacking HMW has been shown to contain only serine-bound phosphate (Eipper, 1974). The manner in which protein-bound ^{32}P is defined in the present experiments does not distinguish between the different forms of protein-bound phosphate.

Table 4.5 Effects of incubation time on the phosphorylation of total MTP and on the tubulin and HMW fractions of the same preparation (fraction S_3 from rat brain) in the presence and absence of cAMP (10 u M) and at different Mg^{2+} ions concentrations

* cAMP (10 uM)	$Mg\ Cl_2$ concentration	** % increase of bd. ^{32}P		
		tot. MTP	HMW	Tubulin
	mM		%	
+	15	- 18	0	69
+	5	- 32	- 30	19
+	0.5	- 41	- 35	19
-	15	5	0	87
-	5	4	0	22
-	0.5	6	0	15

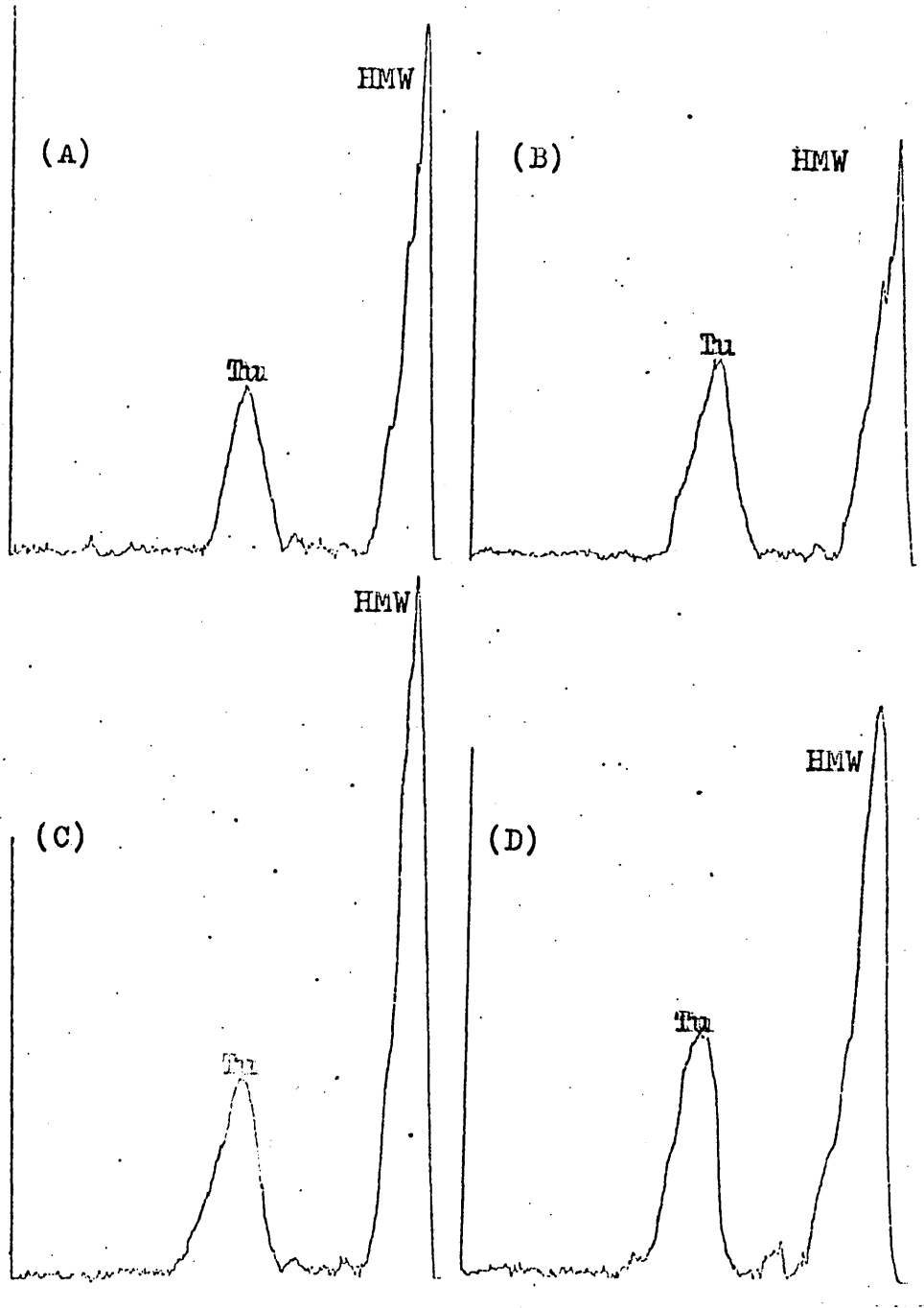
* + or - denotes presence or absence of cAMP

** % increase = $\frac{\text{moles bd. } ^{32}P/\text{mole prot. 30 min}}{\text{moles bd. } ^{32}P/\text{mole prot. 5 min}} \times 100$

Legend to fig. 4.5

MTP, prepared from rat brain by one cycle of polymerisation, was incubated with [γ - ^{32}P] ATP under *in vitro* polymerisation conditions in the absence, (A) and (B), and presence, (C) and (D), of 10 μM cAMP. Protein samples were withdrawn at 5min, (A) and(C), and at 30min, (B) and (D),of incubation and prepared for electrophoresis as described in 4.2.4, procedure 2. The figure shows densitometric scans of radioautographs of sliced and dried polyacrylamide gels of the corresponding samples. (Note that area, hence amount of radioactivity, of HMW peaks in (A) and (B) is the same, although absolute height of peaks is different.)

Fig. 4.5 Change of phosphorylation of brain MTP with time under *in vitro* polymerisation conditions in the absence and presence of 10 uM cAMP



It is also possible that the proteins, or a protein, of the HMW-complex possess some intrinsic enzymic activity, acting as an intermediate in the transfer of phosphate from the ATP to tubulin, thus accounting for the decrease of protein bound ^{32}P associated with HMW, concomitantly with the increase of tubulin bound ^{32}P.

4.4 Discussion

The experimental evidence presented above is further proof for the presence of an intrinsic cAMP-sensitive kinase activity in MTP fractions prepared by cycles of assembly/disassembly. This kinase possesses the potential to phosphorylate proteins present in the MTP fraction. The main substrates for this activity are the proteins of the HMW-complex and tubulin.

It has been estimated that HMW protein(s) contain 2 moles of bound phosphate (*). Under conditions favouring polymerisation of microtubulin (0.5 mM $MgCl_2$) up to 2 moles of ^{32}P can be transferred to the HMW proteins in the presence of 10^{-5} M cAMP (1 mol in its absence, see also Sloboda et al, 1975) which suggests that all of the HMW-bound phosphate is exchangeable. In contrast, only about 0.03 moles of ^{32}P can be transferred to tubulin. MTP purified by cycles of assembly/disassembly has been shown to contain up to 1 mole of protein bound phosphate per mole of tubulin dimer. It can be calculated that only 3% of the total tubulin population can be phosphorylated, thus suggesting

*Shigekawa and Olsen (1976)

heterogeneity of the tubulin population, with respect to phosphorylation, and a very stable MTP-phosphate bonding.

The above considerations point to the conclusion that the HMW proteins are the more dynamic component of the HMW-tubulin system. The phosphorylation of HMW proteins is thereby very likely to be related to different functional aspects of the microtubular system - interactions of microtubules with intracellular components, organelles, particles and generally membranes. The phosphorylation of tubulin itself is more likely to play a role in determining the availability of the Tu monomer for incorporation into microtubules and control of its turnover rate or distribution between different cytoplasmic pools.

The fact that phosphorylation of HMW is more sensitive to cAMP stimulation than tubulin phosphorylation (fig. 4), by almost one order of magnitude, points again to a more dynamic role for the HMW complex and suggests that HMWs are the sites of control of microtubule function in vivo (Olmsted, 1976; Piras and Piras, 1974). The sensitivity of tubulin phosphorylation to cAMP may be the expression of a control mechanism able to mobilise stored monomer for incorporation into microtubules, when required by the cell, and/or control the turnover of tubulin subunits (Olmsted, 1976; Piras and Piras, 1974).

Recently Sloboda et al (1975) investigated the phosphorylation of MTP under conditions similar to the ones employed in the experiments described in this

chapter, and obtained very similar results to the ones described in this chapter. In contrast, Leterrier et al (1974) failed to observe phosphorylation of tubulin *in vitro* under the same experimental conditions.

The significance of the observed phosphorylation of MTP during *in vivo* and *in vitro* labelling experiments will be discussed more fully in the final chapter.

Chapter 5 Identification of in vivo labelled phospholipids associated with brain MTP and related studies

5.1 Introduction

The results in Chapter 3 show that a substantial portion of the acid stable ^{32}P found in purified MTP fractions, of in vivo labelled brain, can be extracted with lipid solvents, suggestive of the presence of labelled phospholipids associated with MTP.

The aim of the experiments presented in this chapter is to identify the labelled phospholipids co-purifying with MTP; the relative distribution of radioactivity in the individual phospholipid classes; and to determine whether the labelled phospholipids are associated with specific components from brain microtubules.

5.2 Materials and Methods

5.2.1 Lipid extractions

The procedure employed was adopted from the method described by Yagihara et al (1973). The protein in the examined fractions was precipitated by the addition of 100% TCA (w/v), to give a final concentration of 10%. After leaving the samples on ice for 15 minutes, to ensure quantitative precipitation of the protein, the precipitate was sedimented and then washed two times with 5% TCA (up to 4mg prot./ml of 5% TCA). It was essential to re-suspend the protein pellets very well in the 5% TCA washes which ensured an easy and thorough re-suspension of the pellets in the lipid solvents. Breaking up the pellet with a glass rod was preferred to sonication to avoid losses of protein adhering to the

sides of the tube on sonication. After draining the acid from the second 5% TCA wash the protein pellets were re-suspended with a glass rod, in 2 ml of chloroform-methanol-conc. HCl (C-M-conc. HCl, 300:300:1.5, by vol.) and left at room temperature for 15 minutes. The protein residue from the first extraction was re-suspended in 2 ml of chloroform-methanol-conc HCl (C-M-conc. HCl, 400:200:1.5, by vol.) and again left for 15 minutes at room temperature. The two lipid extracts were combined (crude lipid extract) and the delipidized protein was dried with 1 ml of diethyl ether and stored for further analysis.

To remove the non-lipid contaminants, 1 ml of chloroform and 3 ml of 0.1 N HCl were added to the crude lipid extract to form a biphasic system. (Folch et al, 1957). On separation of the phases by centrifugation, a layer of white precipitate formed on the boundary between the aqueous and lipid phases. The aqueous phase was discarded and the lipid phase was transferred carefully to a clean tube with a Pasteur pipette, leaving the inter-phase material in the original tube.

The lipid containing lower phase was washed two times with 3 ml (per wash) of its 'synthetic upper phase'. The washed lipid extract (purified lipid extract) was stored at -20°C under N_2. Prior to separation on thin layer chromatography (TLC) plates the solvent was evaporated under a stream of N_2, and the lipid residue was dissolved in 100 - 150 µl of chloroform-methanol (2:1, by vol.) and loaded on the TLC plates.

5.2.2 Extraction of the interphase phospholipid

The interphase material presumably contains lipoproteins and a small amount of protein, known to be soluble to some extent in lipid solvents, but precipitating when the lipid solvent is saturated with water (Ansell and Hawthorne, 1969). Palmer (1977) has shown that addition of water to a lipid solvent containing a small amount of protein can bring about the absorption of phosphatidylinositides onto both basic and acidic proteins. On saturation of the lipid solvent with water, this protein-lipid complex precipitates giving rise to the residue appearing at the interphase between the two solutions. The protein-lipid complex is broken up when the residue is dried and the lipid can be extracted with lipid solvents.

To extract the phospholipids from the interphase material, it was washed with 2 ml of diethyl-ether, to remove remnants from the lipid washes, dried and then boiled sequentially with 0.4 ml of acetone and ethanol, removing the excess solution under a stream of nitrogen. The pellet was then dispersed in 2 ml of chloroform-methanol-conc. HCl (200:100:0.15, by vol.) and left for 15 minutes at room temperature, to extract the phospholipids. Usually, this extract was combined with the purified lipid extract and stored at -20°C under N_2.

5.2.3 Separation of phospholipid mixtures

5.2.3.1 Two dimensional thin layer chromatography (2D TLC)

2D - TLC was performed as described by Pumphrey (1969).

Glass plates, 0.4 x 20 x 20 cms, washed with acetone, were arranged on an inflatable mounting board (Shandon). The spreader was adjusted to give a layer thickness of 0.3 mm. 40g of Silica gel H (Merck, Darmstadt, West Germany), without binder, were stirred in 90 ml of water, to give a uniform slurry and transferred to the applicator. The Silica gel covered plates were put in an oven for 1 hour at 120°C. Immediately before use, the plates were re-heated at 120°C for 1 hour. The phospholipid samples were loaded with a 5 µl automatic pipette (Oxford) and the plates were developed in two dimensions in the solvent system described by Yagihara et al (1973), chloroform-methanol- 7 M ammonia (12:7:1, by vol.) in the first dimension and chloroform-methanol-glacial acetic acid-water (80:40:7.4:1.2, by vol.) in the second dimension. Prior to developing in the second dimension, the plates were blown dry until all the solvent from the first dimension was separated (approx. 15 minutes).

5.2.3.2. Chromatography on formaldehyde-treated paper

In cases where only the amount of ^{32}P associated with phospha[illegible]tides was of interest, the lipid extracts were subjected to one-dimensional descending paper chromatography as described by Michell (1973). The purified phospholipid mixtures were loaded on sheets of Whatman No 1 paper, treated with HCHO and developed in a system containing N-butanol-glacial acetic acid - water (4:1:5, by vol.) for approximately 12 hours. Phospholipid standards were run in parallel on the same chromatogram (see fig. 5.6).

5.2.4 Detection of lipid spots

5.2.4.1 Charring with $H_2 SO_4$

The TLC plates were dried after chromatography and sprayed with 5% $H_2 SO_4$. On heating at 120°C for 15 - 20 minutes, the phospholipids appeared as black spots. Occasionally phosphatidylcholine (PC) and phosphatidic acid (PA) could only be seen as fluorescent spots under ultra violet light.

5.2.4.2 Detection of amino-phospholipids

Amino-phospholipids were detected with the ninhydrin stain described by Marinetti (1964). The TLC plates were sprayed with a 0.25% (w/v) solution of ninhydrin in a mixture of acetone-lutidine (9:1, by vol.). Amino-lipids appeared as purple spots.

5.2.4.3 Detection with I_2 vapours

When the phospholipids were needed for further analysis, the lipid spots were localised with I_2 vapours. The developed and dried chromatograms were exposed to iodine vapours until well defined yellow spots appeared on the chromatogram. After marking the spots with pencil, the I_2 was blown off the chromatogram.

5.2.4.4 Radioautography of chromatograms

Occasionally, chromatograms containing ^{32}P labelled phospholipids were radioautographed, as described by Kates (1977) and the radioautograph was used to map the radioactive spots on the chromatogram.

In all cases, after localisation and marking of the

phospholipid spots by one of the above methods, the chromatograms were photocopied on a Xerox-type copier for a permanent record.

5.2.4.5 Elution of phospholipids from TLC plates

This was performed by a scaled down version of the acid Bligh-Dyer procedure (Bligh and Dyer, 1959) as described by Kates (1972). The silica gel from individual phospholipid spots was aspirated in 15 ml conical tubes containing 3.8 ml of acid Bligh-Dyer solvent (chloroform-methanol - 0.2 N HCl aq., 1:2:0.8, by vol.). After centrifugation, the silica gel was washed once more with the same amount of this solvent, and the two extracts were combined. 2ml each of chloroform and water were added to form a two-phase system. After thorough mixing the phases were separated by centrifugation; the upper aqueous phase was discarded and the lower, lipid containing phase was transferred to a clean tube and immediately neutralised with a slight excess of 0.2 N methanolic ammonia, to minimise acid degradation of the phospholipids. The lipid solution was stored at -20°C under N_2.

5.2.5 Counting of radioactivity

The phospholipid spots from paper chromatograms were cut out and counted directly in 10 ml of Bray's scintillant. The spots from TLC plates were aspirated in glass counting vials containing 10 ml of Bray's scintillant. The amount of silica gel aspirated from the individual spots was very similar and in most cases no corrections for the quenching effect of silica gel was needed.

5.2.6 Preparation of in vivo ^{32}P labelled MTP fractions

One to two-day old chicks were injected intracranially (see 2.3) with 300 μ Ci ^{32}P/animal and killed two or four hours after the injection, or with 50 μ Ci ^{32}P per animal and killed 20 hours later. MTP was prepared from the labelled brains by polymerisation in the presence or absence of glycerol, as described in 2.2.2.

5.3 Results

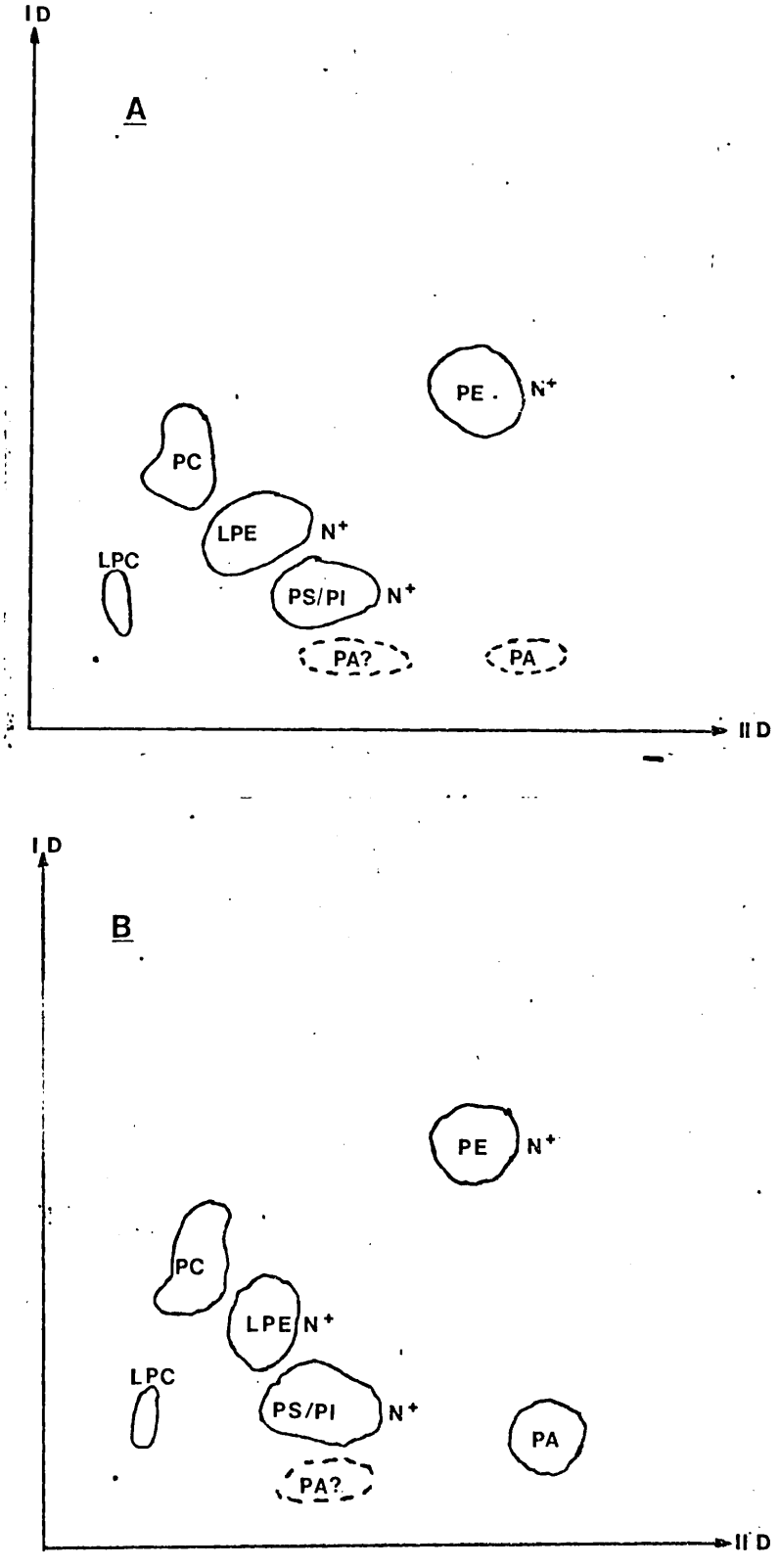
The phospholipids separated by two dimensional TLC were identified on the basis of standard samples developed under the same conditions, individually or as mixtures, and on Pumphrey's (1969) results (fig. 5.1). To confirm the identity of the phospholipids, as established by 2D-TLC, the lipids from the major spots (spots 1 - 6, fig. 5.2) were extracted in the presence of the appropriate standard lipid (30 μg lipid -P), according to the tentative identification on 2D-TLC chromatograms, and subjected to mild alkaline hydrolysis in order to identify their water-soluble products, according to the method of Dawson (1962).

To the dry lipid residue 0.2 ml of chloroform, 0.3 ml of methanol and 0.5 ml of 0.2N methanolic NaOH were added in succession. After vortexing, the mixture was left at room temperature for 15 minutes. 0.2 ml of methanol, 0.8 ml of chloroform and 0.9 ml of water were added quickly and the mixture was vortexed and centrifuged for a short time. The upper methanol-water phase was transferred quickly and as completely as possible to another tube containing about 1 g of wet Dowex 50 (H^+) resin, pre-treated with 1 N HCl, and vortexed vigourously until the supernatant was neutral or slightly acidic. The supernatant was removed and washed twice with 0.5 ml of methanol-water (10:9, by vol.) with which the ion-exchange resin has been washed in advance. The combined methanol-water phase was neutralised with a few drops of methanolic ammonia (1.5N). The solution was then dried *in vacuo* over dessicant.

Legend to fig. 5.1

A shows a two-dimensional thin layer chromatogram of a mixture of standart phospholipids. In the first dimension (I D) the chromatogram was developed in cloroform-methanol-7M ammonia (12:7:1, by vol.) and in the second dimension (II D) in chloroform-methanol-glacial acetic acid-water (80:40:7.4:1.2, by vol.) as described in 5.2.3.1. The lipid spots were localised with I_2 vapours and after evaporating the I_2 from the plate the amino-phospholipids (N^+) were detected with the ninhydrin reagent described in 5.2.4.2. PE-phosphatidylcholine; LPC-lysophosphatidylcholine; PE-phosphatidylethanolamine; LPE-lysophosphatidylethanolamine; PA-phosphatidic acid; PS-phosphatidylserine; PI-phosphatidyl-inositol. PA would move in both positions shown.

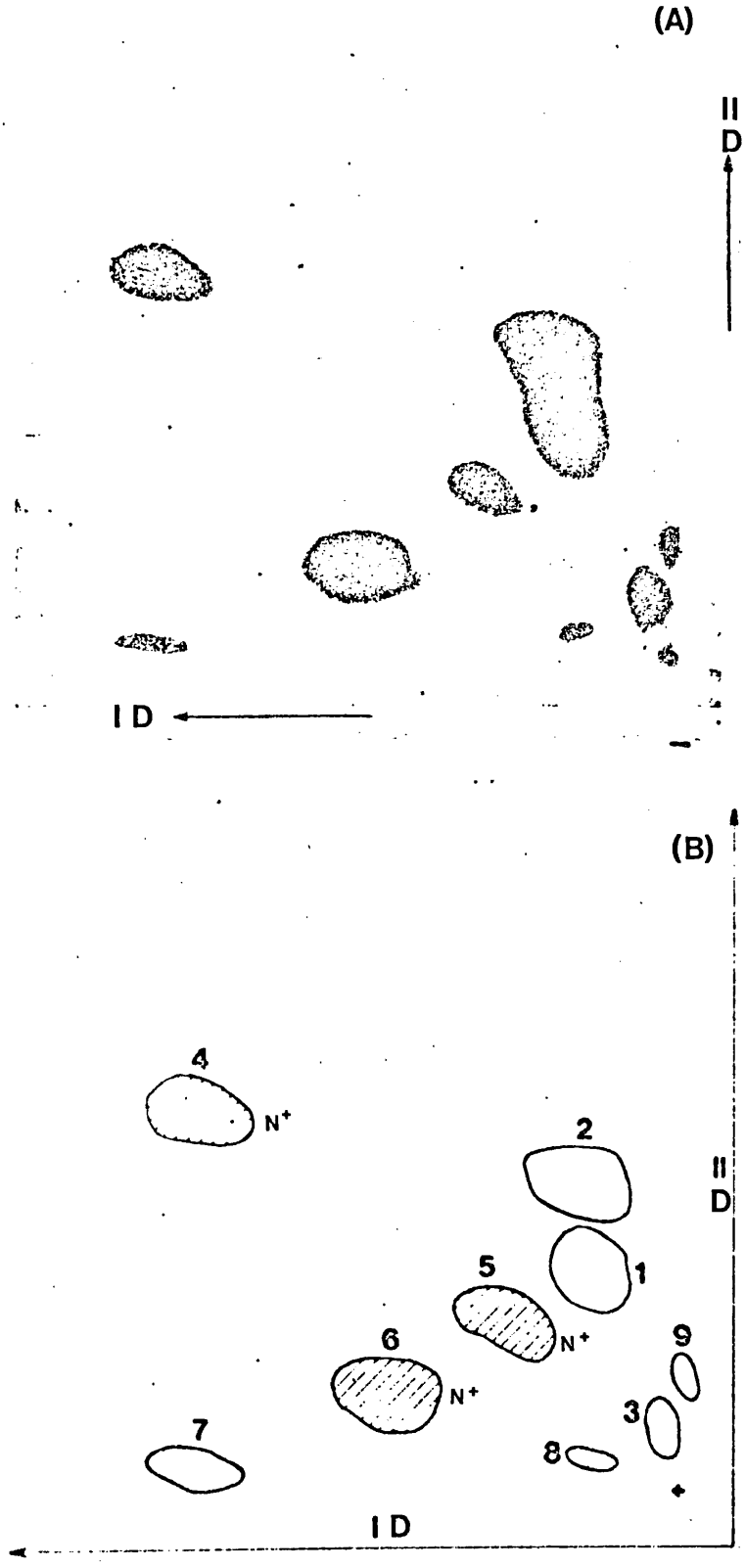
Fig. 5.1 Identification of phospholipids by two-dimensional thin layer chromatography



Legend to fig. 5.2

(A) shows the radioautograph of a two-dimensional thin layer chromatogram of phospholipids extracted from *in vivo* labelled (400 µCi/animal; 2h) MTP (fraction P_3). For conditions of chromatography see fig. 5.1. The radioactive areas on the TLC-plate were 'mapped' from its radioautograph. Localisation of the lipid spots with I_2 vapour showed that the radioactive spots coincided with the phospholipid spots. (B) shows a sketch of the TLC-plate after localisation of the phospholipid spots. To obtain further proof for the identity of the phospholipids the individual spots were eluted from the chromatogram, shown in this figure, and hydrolysed under mild alkaline conditions. The water soluble products were chromatographed on paper to determine their identity (see fig. 5.3 and legend to the same figure). For details see related text in 5.3. For N^+ see legend to fig. 5.3.

Fig. 5.2 Identification of phospholipids from in vivo labelled MTP



The water-soluble hydrolysis products were identified by one-dimensional ascending chromatography on Whatman No 1 paper. The solvent system used for the paper chromatography was phenol (saturated with water) - ethanol - glacial acetic acid (50:5:6, by vol), as described by Dawson (1962). After the solvent had ascended about 10 inches (approximately 18 hours), the chromatogram was dried and the spots localised by the sulphosalycilic acid-ferric chloride procedure for detection of phosphates (Vorbeck and Marinetti, 1965). The phosphates appeared as white spots on a mauve background. The chromatogram was then radioautographed to localise the ^{32}P-labelled hydrolysis products.

In all cases, except for PS, the radioactive spots coincided with the products of the appropriate carrier lipid. In the case of PS the radioactivity did not coincide with the glycerylphosphorylserine (GPS) spot but appeared in a spot with a R_F value of 0.17, which is different from that of GPS, but very similar to the R_F value of glycerylphosphorylinositol (GPI), see fig. 5.3. This was the first indication for the presence of PI in the phospholipids extracted from purified MTP.

To check more specifically for any labelled PI which might be present in the PS spot seen on the 2D-TLC chromatograph, a one-dimensional TLC system, currently developed in the laboratories of I.N.Hawthorne, was employed.

The TLC plates are prepared as described in 5.2.3.1, but instead of pure distilled H_2O, a 1% aqueous solution of ammonium sulphate was used to prepare the gel slurry. The solvent system used is chloroform-methanol-glacial acetic acid - water (50:30:7:3, by vol.), and the chromatogram is developed at $4^{\circ}C$.

The PS spot from two-dimensional thin layer chromatograms was eluted by the acid Bligh-Dyer procedure, as described above, and the dry residue dissolved in chloroform-methanol (2:1, by vol.), and loaded on an ammonium sulphate containing TLC plate. Marker samples of PS, PI and a mixture of both were loaded onto the same

Legend to fig. 5.3

Phospholipids extracted from in vivo labelled (400 µCi/animal; 2h) chick brain MTP (fraction P_3) were separated by 2D-TLC, (shown in fig. 5.2). After elution from the thin-layer chromatogram, in the presence of carrier phospholipids chosen according to the tentative identification of the individual lipid spots (see fig. 5.1), the individual phospholipids were hydrolysed under mild alkaline conditions and the water soluble products were identified by their R_f values measured from a paper chromatogram, which is shown in this figure. Paper chromatography was performed according to Dawson (1962) in phenol (satur. aq.)-ethanol-glacial acetic acid (50:5:6, by vol.). After developing the chromatogram was radioautographed to localise the regions of radioactivity (black regions in the figure) and then stained with the sulphosalycilic acid-ferric chloride stain for detection of phosphates (Vorbeck and Marinetti, 1965). After localising the water soluble hydrolysis products (enriched with black lines in the figure) the chromatogram and its radioautograph were superimposed. The composite picture obtained is shown in this figure. Note splitting of the PC spot, numbers 1 and 2 in fig. 5.2. 1 and 2 = PC; 3 = LPC; 4 = PE; 5 = LPE; 6 = PS/PI. Spots number 7,8 and 9 were not eluted from 2D TLC shown in fig. 5.2.

Fig. 5.3 Identification of the water soluble hydrolysis products of phospholipids extracted from *in vivo* labelled brain MTP

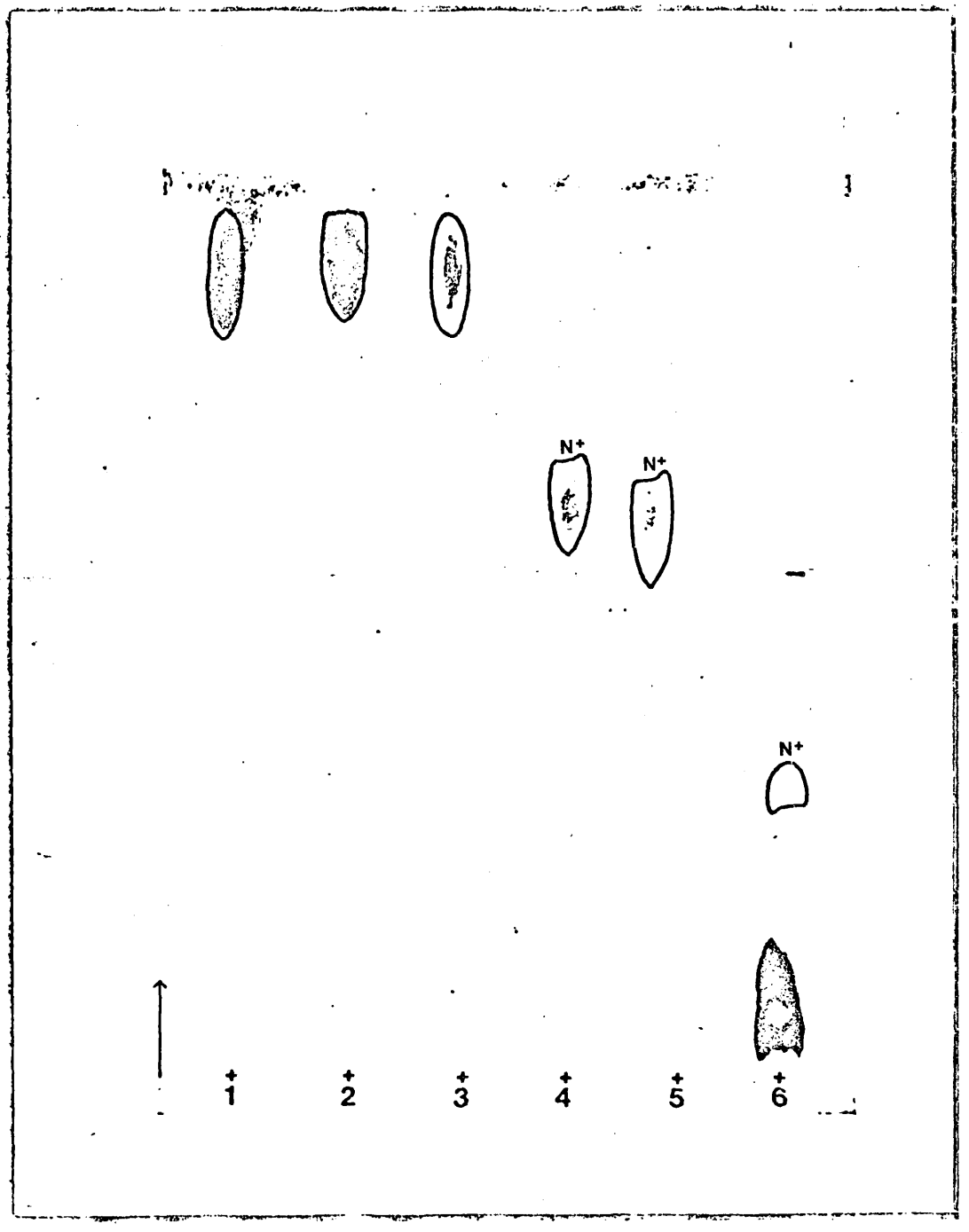
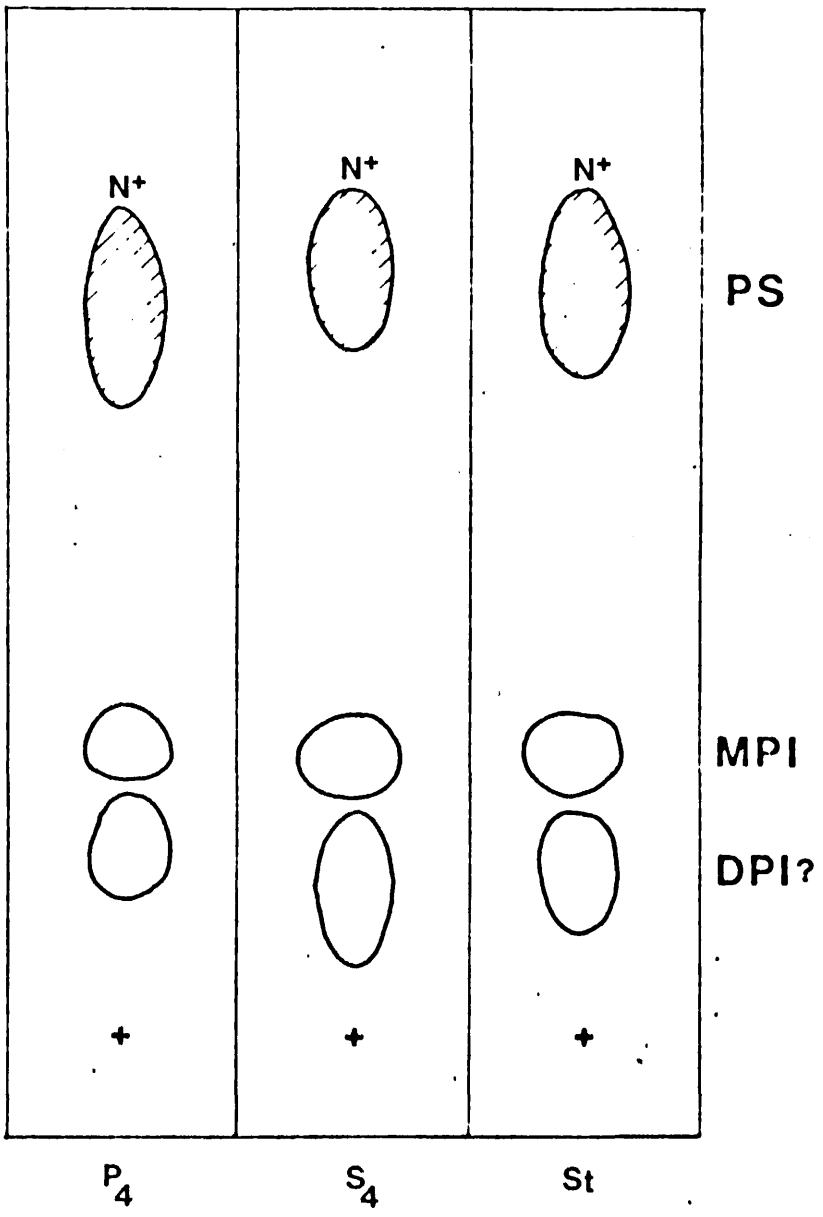


plate. After the chromatogram was developed and dried, the phospholipid spots were localised with I_2 vapours. After the iodine was removed, the plate was sprayed with ninhydrin reagent (Marinetti, 1964). The PS spots gave a positive ninhydrin reaction. It must be noted that the radioactivity in the PI region was localised in two spots, suggesting the presence of polyphosphoinositides (see fig. 5.4). Diphosphatidylinositol (DPI) was also found in the lipid extracts from interphase material (see figure in 5.5.2, Appendix to this Chapter.). However, labelling of DPI was only found after two hour ^{32}P injection, but not after 20 hour labelling injection.

Although the main phospholipids present in the lipid extracts of purified MTP fractions were PC, PS, LPC, PE, LPE (in order of relative intensity of staining with I_2 vapours), a substantial portion of the radioactivity was found in the inositide species. Under all conditions of labelling and preparation of the MTP fractions, the specific radioactivity of PI is expected to be at least two to three times higher than that of the most highly labelled phospholipid - PC, assuming that the phosphoinositide fraction contributes no more than 10% of the total phospholipid-P as is reported for various types of brain extracts (see, eg, Lunt and Pickard, 1975). In no case could the phosphoinositides be localised on TLC or paper chromatograms by any of the staining procedures employed. Phosphoinositides were localised either by radioautography or addition of lipid standards to the purified lipid extract, prior to chromatography.

The results in table 5.1 show that the distribution

Fig. 5.4 Separation of phospholipids eluted from the PS/PI spot of two dimensional thin layer chromatograms



The phospholipid from the PS/PI spot from 2D TLC plates on which the phospholipid extracts from in vivo labelled (400 µCi/animal; 2h) chick brain MTP fractions, P_4 and S_4, was eluted as described in 5.2.4.5.The eluted phospholipid was loaded on ammonium sulphate containing plates and developed at 4°C with chloroform-methanol-glacial acetic acid-water (50:30:7:3, by vol.). The lipid spots were localised with I_2 vapours. After removal of I_2 the chromatogram was sprayed with the ninhydrin reagent described in 5.2.4.2. Only one spot (from each sample) migrating similarly to the PS standart gave positive reaction . The other two spots moved similarly to the PI and DPI standarts. St-mixture of PS, PI and DPI standarts.

Table 5.1 %Distribution of radioactivity between individual phospholipid spots from chromatograms of purified phospholipid extracts obtained from *in vivo* labelled brain MTP fractions

Experiment type	Fraction (MTP)	Labelling time(h)	Glycerol	Method of separation	PE	LPE	PC	LPC	SM	PA	PS	PI
I	P_3	2	-	TLC	12.9	6.5	40.0	3.5	0.4	1.3	-	35.0
	S_3				12.6	6.7	38.9	4.3	0.2	0.9	-	36.3
II	P_3	2	+	TLC	15.3	4.0	37.3	3.0	1.8	3.1	-	35.5
	P_4				14.2	3.6	44.4	-	-	-	-	37.7
	S_4				9.1	5.4	38.8	-	-	-	-	43.5
III	P_3	2	+	HCHO		-	-	70.0		-	-	30.0
	P_4			treated		-	-	62.0		-	-	38.0
	S_4			paper		-	-	59.7		-	-	41.3
IV	P_3	4	+	TLC	11.0	5.0	52.8	2.4	0.5	0.8	-	27.4
	P_4				11.0	4.8	55.7	2.7	0.7	0.5	-	25.0
	S_4				11.4	4.3	52.2	4.0	0.5	0.4	-	28.0
V	P_3	4	-	TLC	11.8	5.9	53.0	2.7	0.5	0.3	-	26.0
	P_4				10.6	5.0	60.9	3.7	0.9	0.7	-	23.0
	S_4				10.1	5.6	48.1	7.0	1.0	2.0	-	28.0
VI	P_3	20	+	TLC	16.7	1.2	67.6	3.0	1.7	1.0	2.2	6.4
	S_3				16.7	1.0	65.4	4.2	2.7	1.0	2.4	7.5
VII	P_3	20	-	TLC	17.4	10.8	54.4	2.4	1.8	0.5	3.3	9.4
	S_3				16.8	12.4	53.7	0.2	1.8	0.6	3.9	10.5

of ^{32}P label between the individual phospholipids is specific for each labelling time, which reflects the change in the specific activity of each phospholipid class as a function of time. It is also apparent that MTP fractions prepared by two cycles of polymerisation, P_4 and S_4, contain a higher amount of labelled inositides as compared to MTP fractions prepared by one polymerisation cycle, P_3 and S_3. This can be explained by the loss of phospholipids other than phosphoinositides and suggests a tighter retention of inositides on MTP than the rest of the MTP associated phospholipids (see table 5.1, Expts. type I and II and fig. 5.5.).

In subsequent experiments, MTP purified by one cycle of polymerisation, in the absence of glycerol fraction S_3, from the brains of animals labelled for 20 hours, was chromatographed on a column of Sepharose 6B (as described in 2.4). The bulk of the phospholipid was found in the void volume (peak I) and the rest of the phospholipid co-eluted with the second protein peak (peak II) (Table 5.2). The material eluting between the two peaks contained about 5% of the recovered protein and negligible amounts of phospholipid -^{32}P. If fraction S_3, prepared as above (no glycerol, 20 hour labelling) was made 0.75 M with NaCl and eluted on the same column with buffer containing 0.75 M NaCl, a re-distribution of protein and protein-bound ^{32}P occurred, but no change in the elution pattern of the phospholipids was observed (Table 5.2). Under both conditions, with or without 0.75 M NaCl, the second peak contained two times more PI -^{32}P than the first peak. This result suggests that

Legend to fig. 5.5

The phospholipids extracted from *in vivo* labelled (400 µCi/animal; 2h) chick brain MTP, fractions P_3, S_4 and P_4, were chromatographed employing the 2D-TLC system described in 5.2.3.1. The figure shows radioautographs of the chromatograms. (See also table 5.1, experiments type II and III.)

Fig. 5.5 Distribution of radioactivity between the phospholipids extracted from *in vivo* labelled MTP fractions.

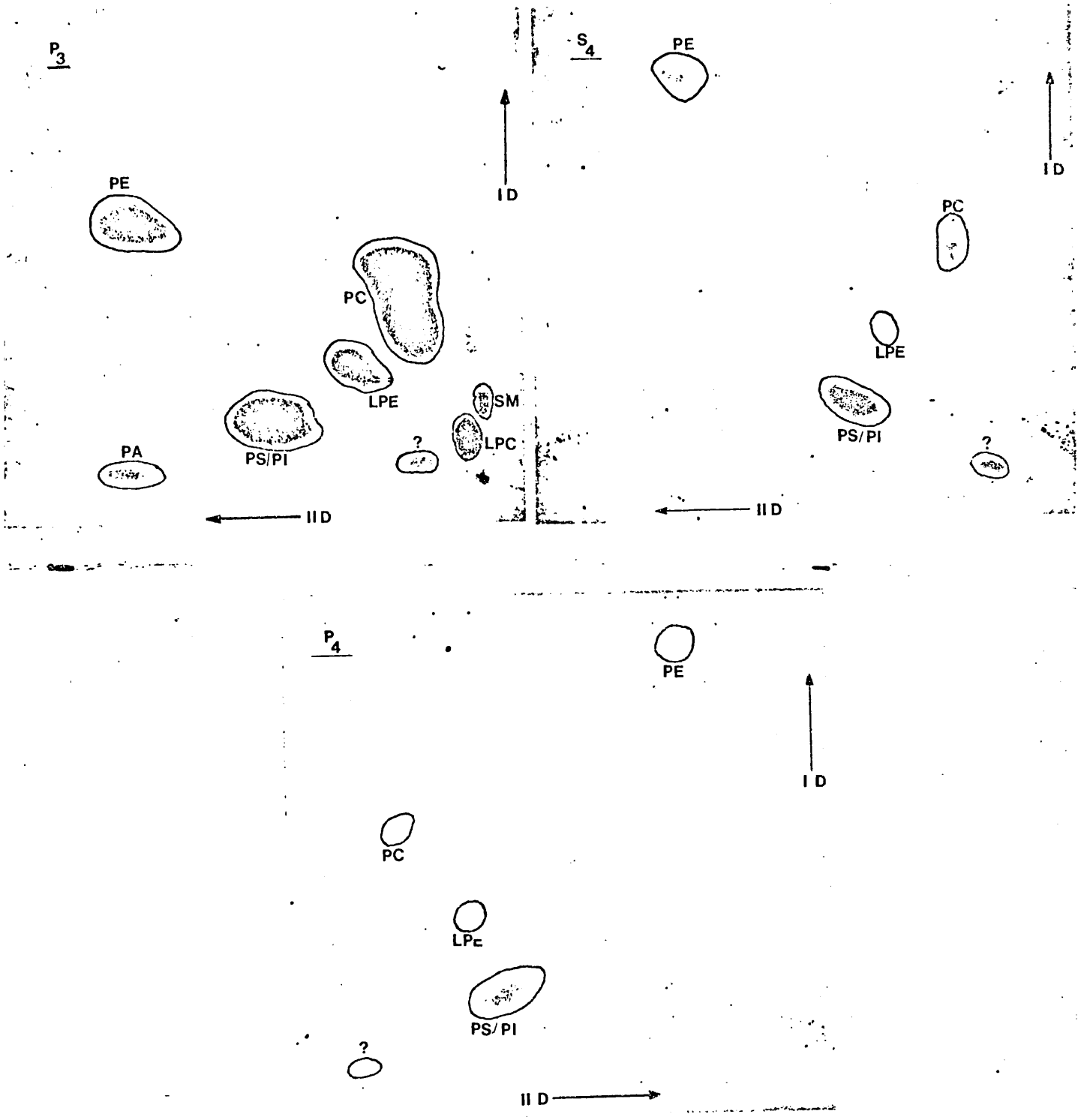


Table 5.2 Distribution of acid stable ^{32}P in ring (36S) and dimer (6S) fractions obtained by chromatography on Sepharose 6B from in vivo labelled MTP (fraction S_3)

Fraction (MTP)	Chromatographic protein peak	Protein content	% of recovered acid stable ^{32}P cpm		***% P1 ^{-32}P
			*Protein bound	**Phospho-lipid ^{-32}P	
		mg	%		%
S_3	I	4.5	81.6	91.3	10.5
	II	5.5	18.4	8.7	21.3
S_3 + 0.75 M NaCl	I	1.4	29.0	92.0	11.0
	II	8.1	71.0	8.0	21.2

* % ^{32}P cpm in acid-washed, delipidized protein pellet.

** % ^{32}P cpm in purified phospholipid extracts.

*** % P1-^{32}P from the total cpm recovered from chromatograms of purified phospholipid extracts.

a) the phospholipids associate preferentially with the 30S tubulin structures eluting in peak I and

b) that PI -^{32}P appears to associate with the 6S dimer fraction (peak II) to a greater extent than the rest of the labelled phospholipids. The fact that after dissociation of the 30S tubulin structures with NaCl to 6S dimer the bulk of the lipid still elutes in peak I, which under this condition (0.75 M NaCl) is enriched in HMW proteins and contains a residual amount of tubulin, points to the conclusion that it is the HMW proteins which preferentially associate with phospholipids and may act as a binding link between phospholipid structures, e.g. membranes and microtubules. (see final Chapter).

The objective of a further series of experiments was to test whether MTP could act as an acceptor system for phospholipids in membrane fractions which could occur purely artefactually during preparation of the protein or as a result of an exchange reaction involving phospholipid exchange proteins. Labelled microsomal membranes were prepared from the brains of two-day old chicks injected with 50 μ Ci ^{32}P each and killed 20 hours after the injection. Microsomal membranes (fraction Mic_{20}) were prepared by a modified version of the procedure of Van Leeuwen et al (1976). Prior to use, the Mic_{20} pellet was re-suspended in half the original volume used for homogenisation of the tissue of glycerol-free reassembly buffer, and sedimented at 30000 x g for 40 minutes, at 4°C. An initial high-speed supernatant fraction of two-day old chick brain (S_1, see 2.2.1) was used as a source

of 'cold' polymerisable MTP. This soluble fraction would also presumably contain phospholipid exchange proteins (Miller and Dawson, 1972).

The washed Mic_{20} pellet was re-suspended in the 'cold' S_1 supernatant and the suspension was divided in two equal parts. One part was incubated at 37°C for 30 minutes with occasional shaking. At the end of the incubation, the suspension was kept on ice for 30 minutes to allow for depolymerisation of the microtubules. Meanwhile, the other half of the suspension was kept on ice. Both suspensions were then centrifuged at 100000 x g for 1 hour at 4°C to sediment the Mic_{20} membranes. The resulting supernatants were used for preparation of MTP by one cycle of polymerisation as described in 2.2.1. The MTP fractions obtained from the suspension which had been incubated at 37°C were designated as P_3W and S_3W (W for warm), and the 'control' fractions prepared from the suspension kept on ice were designated as P_3C and S_3C (C for cold). These fractions correspond to the P_3 and S_3 fractions obtained during purification of MTP in the presence of glycerol (see 2.2.1).

From the results in table 5.3 it can be calculated that up to 0.1% and 0.01% of the total phospholipid ^{32}P of the Mic_{20} was 'transferred' in the P_3 and S_3 (W and C) MTP fractions respectively, but hardly any protein-bound ^{32}P, suggesting a selective interaction between MTP and phospholipids. The results in table 5.4 show that the whole range of phospholipids, usually found in the corresponding <u>in vivo</u> labelled MTP fractions, was 'transferred' to the cold microtubules (see also table 5.1).

Table 5.3 (Legend)

Two lots 50 mg of *in vivo* labelled Mic_{20} protein was re-suspended in two lots (3.5 ml) of high speed brain supernatant (S_1). One suspension was incubated at 37°C for 30 minutes and then on ice for 30 minutes, while the other suspension was kept on P_3C and S_3C MTP fractions were prepared as described in related text.

Table 5.3 Total protein bound ^{32}P and phospholipid ^{-32}P in MTP fractions purified from high-speed brain supernatants (S_1) incubated with *in vivo* ^{32}P labelled Mic_{20} membranes

Fraction	Protein content	*SA alkali labile ^{32}P	** SA phospho-lipid ^{32}P
	mg		
Mic_{20}	50	259.7	109.3
P_3W	2.2	0.13	15.3
S_3W	1.8	0.09	2.0
P_3C	1.9	0.11	19.0
S_3C	2.5	0.1	1.0

* SA (specific activity) alkali labile ^{32}P = alkali labile ^{32}P x 10^{-3}/mg protein in fraction.

** SA (specific activity) phospholipid ^{32}P = ^{32}P x 10^{-3} recovered in purified phospholipid extract/mg protein in fraction.

Table 5.4 % Distribution of radioactivity between individual phospholipid spots from 2D - TLC chromatograms of purified phospholipid extracts obtained from MTP fractions prepared from high-speed brain supernatants (S_1) incubated with *in vivo* labelled Mic_{20} membranes

Fraction	PE	LPE	PC	LPC	SM	PA	PS	PI
Mic_{20}	16.7	1.2	67.6	3.0	1.7	1.0	2.2	6.7
P_3W	14.6	10.4	60.9	2.8	1.0	1.8	1.8	6.7
S_3W	10.4	8.9	59.8	2.8	1.4	1.8	1.4	13.5
P_3C	15.4	12.9	52.7	2.2	2.1	2.3	3.1	9.1
S_3C	15.0	9.6	49.0	2.8	2.5	3.5	3.6	14.0

For preparation of MTP fractions in this table, see legend to Table 5.3 and related text.

It is, however, unlikely that this 'transfer' of phospholipids was brought about by the activity of phospholipid exchange proteins found in high-speed supernatant from brain, because of their apparent specificity for PC and PI and moreover because of the apparent 'transfer' which occurred in fractions kept on ice (see table 5.4). It is more likely that this 'transfer' is the result of a selective association between MTP and a particular phospholipid structure (for further discussion of this suggestion, see Chapter 6).

5.4 Discussion

A considerable amount of evidence for the presence of phospholipids in purified ^{32}P-labelled MTP fractions has been accumulated in the last four years. In 1974 Eipper reported the presence of phospholipids in brain tubulin purified by chromatography on DEAE-cellulose, (Eipper, 1974). In the same year, Daleo et al reported the presence of phospholipids in brain tubulin purified by vinblastine precipitation and by polymerisation, although they were unable to detect any labelled PI in extracts from MTP incubated with $[\gamma - {}^{32}P]$ ATP. It has also been shown that tubulin prepared by ammonium sulphate precipitation followed by polymerisation and tubulin fractions prepared by gel permeation chromatography on Sepharose 6B from tubulin purified by polymerisation contain phospholipids (see Chapter 4). These findings strongly suggest that MTP, or a class of MTP, if it is not a lipoprotein in itself, can associate with phospholipids.

The origin of the MTP associated phospholipids

is not clear, and it can still be argued that the observed association occurs artefactually during the initial stages of MTP purification, eg homogenisation of the tissue, sonication in the presence of membranes, etc.

The nature of the phospholipid - MTP interactions is also not clear. However, the preferential co-purification of phospholipids with HMW-containing tubulin fractions (peak I, table 5.3; see also related text) may suggest an intermediary role, as a link between membranes and microtubules, for the HMW proteins. In addition, the fact that a highly dynamic membrane component, the phosphoinositides, are found in the MTP - associated phospholipids may reflect the relation of microtubules to various membrane based phenomena, as will be discussed in the final Chapter.

5.5 Appendix

5.5.1 Distribution of radioactivity in fractions obtained during purification of crude lipid extracts

Generally, between 57 and 80%, for pellet and supernatant fractions respectively, of the ^{32}P cpm present in the 'crude lipid extract' (see 5.2.1) were recovered in the purified lipid extract (see table 1), overleaf.

Table 1

Distribution of ^{32}P cpm in fractions obtained during purification of crude lipid extracts

FRACTION	% of recovered ^{32}P cpm in fraction	
	Pellets	Supernatants
	%	
1st upper phase	12.5	28.6
2nd upper phase	2.3	3.8
3rd upper phase	1.1	2.1
Interphase	4.1	7.2
Purified lipid extract	80.0	58.3

The results in this table were obtained on purification of the crude lipid extracts of P_3 (pellet) and S_3 (supernatant), prepared as described in 2.2.1 from the brains of two-day old chicks injected with 300 μ Ci ^{32}P per animal and killed 2 hours after the injection.

5.5.2 Interphase phospholipids and proteins

Usually about 10 - 15% of the protein in the original MTP fraction was recovered in the interphase pellet of this fraction, obtained during the purification of its crude lipid extract. The protein was over 70%

tubulin (see fig. 1).

When the phospholipids extracted from the interphase were separated by HCHO-treated paper chromatography it was found that over 50% of the ^{32}P cpm recovered from the chromatogram were associated with phosphoinositides (see table 2 and fig. 2).

Table 2

% distribution of ^{32}P cpm on HCHO-treated paper chromatograms of phospholipids extracted from the interphase material of MTP fractions.

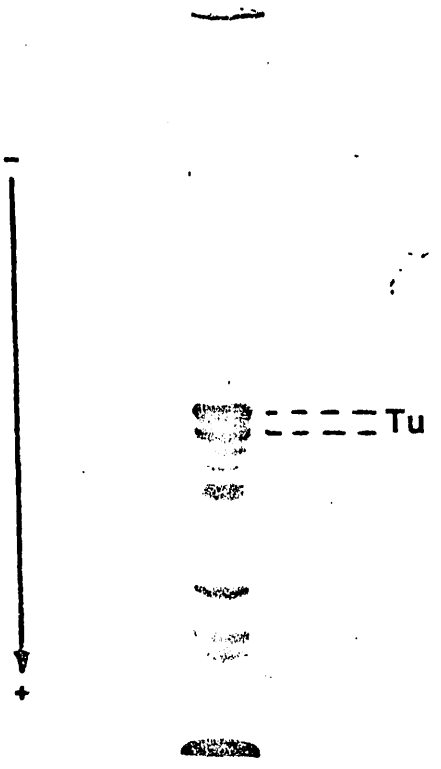
FRACTION	PI	DPI	*remaining
		%	
P_3	60.3	4.0	25.7
P_4	48.5	2.3	49.2
S_4	50.2	2.8	47.0

*remaining = phospholipids migrating ahead of phosphoinositides.

MTP fractions were prepared from two-day old chicks' brains labelled with 300 µ Ci ^{32}P per animal for 2 hours.

Appendix to Chapter 5 / Fig. 1

S-PAGE analysis of interphase protein



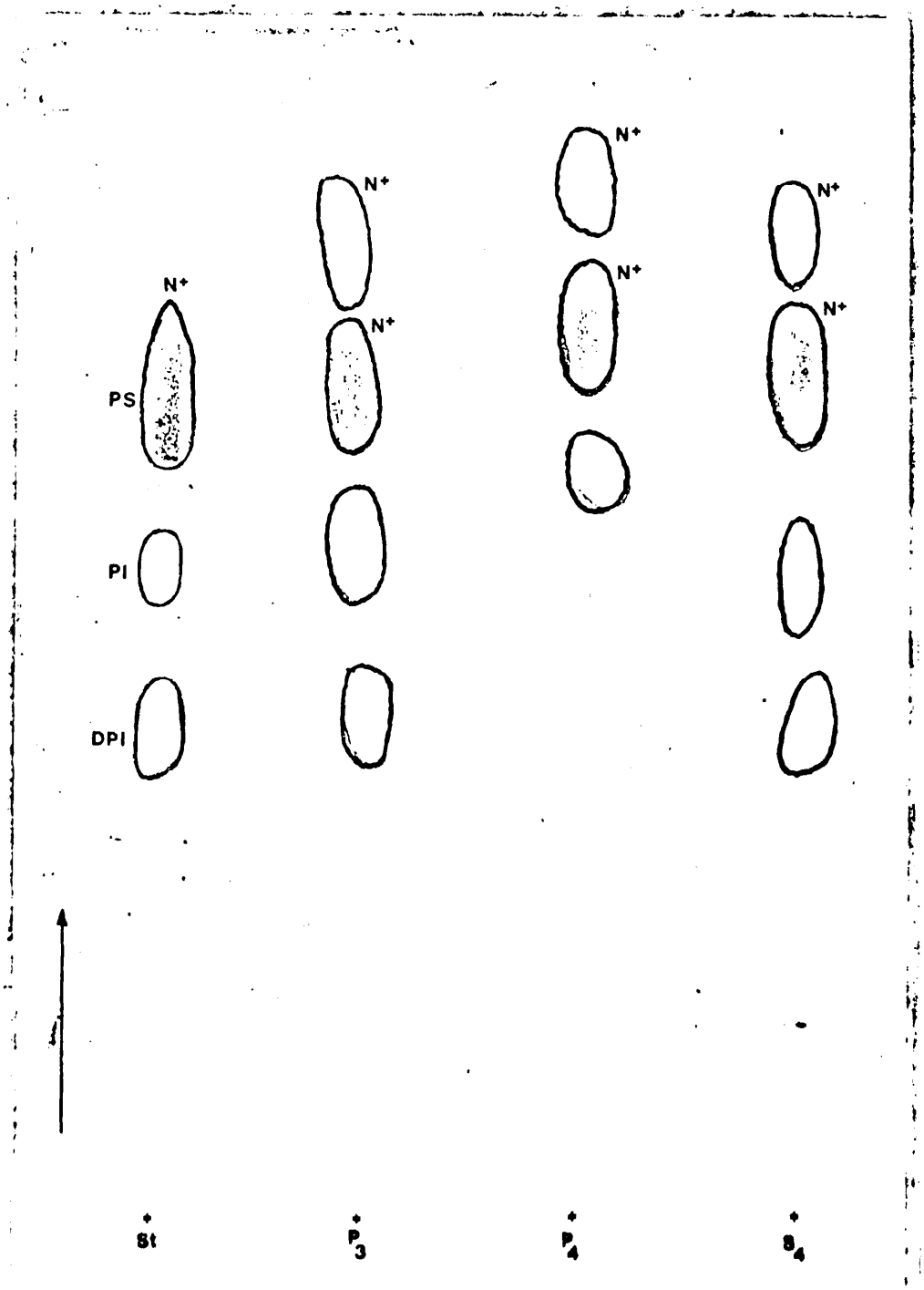
The delipidised interphase pellet (see 5.2.2) was analysed by S-PAGE according to the method described in 2.7. Tu = tubulin.

Legend to Fig. 2 / Appendix to Chapter 5

Interphase phospholipids were extracted from the interphase material obtained during purification of crude lipid extracts of *in vivo* labelled (400 µCi/animal; 2h) chick brain MTP, fractions P_3, S_4 and P_4. The interphase lipid was chromatographed on paper as described in 5.2.3.2. A mixture of standart phospholipids (St) containing PS, PI and DPI was chromatographed in parallel. The phospholipid spots were localised with I_2 vapours. Amino-phospholipids (N^+) were detected with the ninhydrin reagent, described in 5.2.4.2, after removal of I_2 from the chromatogram.

Appendix to Chapter 5 / Fig. 2

Identification of interphase phospholipids



Chapter 6 Characterisation and comparison of bound ^{32}P in in vivo labelled MTP fractions prepared by polymerisation in the presence or absence of glycerol

6.1 Introduction

The purification of microtubular protein by sequential cycles of polymerisation/depolymerisation, where the polymerisation process is 'aided' by glycerol, as described by Shelanski et al (1973), has become a routine procedure in many laboratories. Lately, however, a few facts have emerged which call for caution when interpreting the results of experiments in which MTP purified in the presence of glycerol is used.

Already, when introducing this method, Shelanski noted that in the presence of glycerol, some 'abnormal' tubules were formed. This he explained by an apparent slowing down of the rate of exchange of free sub-units with the polymer forms in a glycerol containing medium, which would freeze the mistakes made during polymerisation, resulting in the appearance of 'abnormal' microtubule structures. Later, the studies of Kirschner and Borisy showed that the depolymerisation products of tubules prepared in the presence or in the absence of glycerol had different sedimentation properties. In the case of tubules prepared in glycerol-containing media, their depolymerisation products sediment as two boundaries, at 6S and 36S (Weingarten et al, 1974), whereas the depolymerisation products of tubules prepared in glycerol-free media yield two boundaries at 6S and 30-31S (Borisy et al, 1975). In addition, 30S components are broken down by colchicine (Olmsted et al, 1974), whereas

36S rings are not (Kirschner et al, 1974).

Furthermore, the results reported recently by Detrich et al (1976), show clearly that glycerol binds tightly and it would be reasonable to expect that this interaction can bring about changes in the properties of the protein. This suggestion is supported by a number of findings - glycerol induces the polymerisation of tubulin in the absence of added nucleotides (Shelanski et al, 1973), and the non-hydrolysable analogue of GTP, ß, γ- methylene GTP (GMPPCP), induces polymerisation of tubulin prepared in the presence of glycerol, but tubulin prepared without glycerol can only be polymerised in the presence of GTP.

For these reasons it was thought important to investigate whether the presence of glycerol during the polymerisation of microtubules might affect the distribution amongst the various MTP fractions obtained during the purification procedure of:

a) total protein

b) phospholipid - ^{32}P

c) protein-bound ^{32}P

Also, the purity of the individual MTP fractions obtained in the presence and absence of glycerol was examined by S-PAGE.

6.2 Materials and Methods

MTP fractions were prepared from the brains of 1 to 3 day-old chicks labelled with ^{32}P for 20 h (for method of injection see 2.3.2). The freshly excised brains were homogenised in 1.5 volumes of ice-cold reassembly buffer and the homogenate was centrifuged for

30 minutes at 5 x 10^4 x g at 4°C to yield a high-speed supernatant S_1. This supernatant fraction (S_1) was used to prepare MTP fractions in the presence or absence of glycerol, as described in 2.2. MTP fractions prepared in the presence of glycerol were designated as (+) fractions and the ones prepared in the absence of glycerol were designated as (-) fractions.

Other methods are as previously described, except where indicated otherwise.

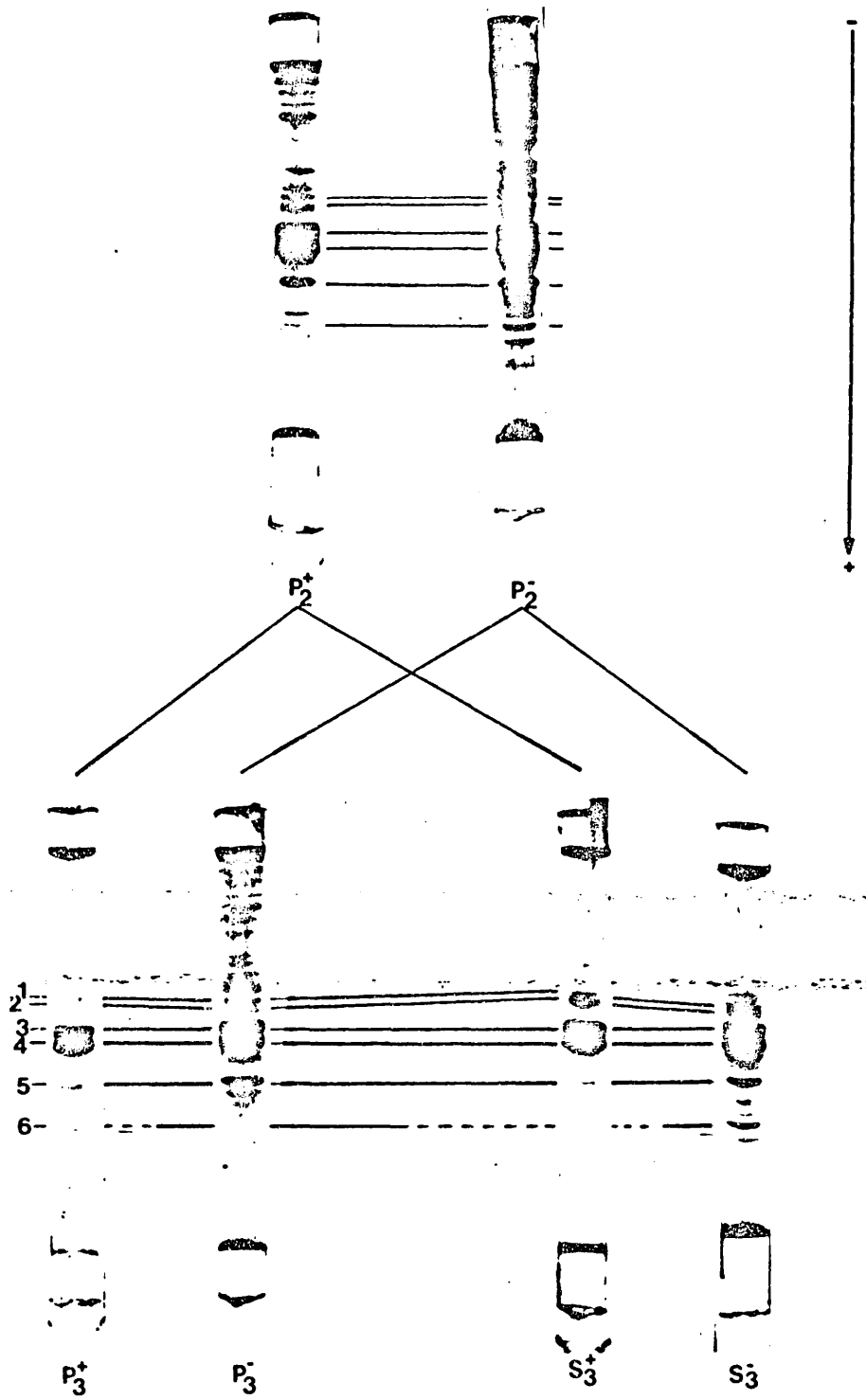
6.3 Results

A comparison of the amounts of polymerisable microtubular protein found in fractions P_2, S_3 and P_4 prepared in the presence or absence of glycerol from the same amount of starting material, S_1, shows that more protein is recovered in each of the fractions prepared with glycerol ((+) fractions, see table 6.1). Protein recoveries, from each mother fraction, were in all cases over 80% and could not thus account for the observed differences between (+) or (-) MTP fractions.

S-PAGE analysis reveals that fractions prepared in the presence of glycerol, (+) fractions, during the first polymerisation cycle are much purer than the corresponding (-) fractions (see fig. 6.1.). However, after further cycles of polymerisation the purity of the corresponding (+) and (-) MTP fractions was very similar (see figures 2.1 and 2.3).

Furthermore, it was found that when a P_2 fraction was prepared in the presence of 1M sucrose (P_2S) the yield and purity of the protein obtained was much more similar to that of P_2(-) than that of P_2(+) (see table 6.2 and

Fig. 6.1.



Comparison of electrophoretic patterns of MTP fractions prepared in the presence (+) or absence (-) of glycerol. For identification of protein bands (numbers 1 - 6), see Fig. 2.1.

Table 6.1 Distribution of protein between pellet and supernatant fractions prepared in the presence or in the absence of glycerol

Fraction	(+) or (-)	P_2	S_2	P_3	S_3	P_4	S_4
Protein content (mg)	(-)	12.1	144.9	5.6	5.3	0.7	3.7
	(+)	24.2	134.0	2.8	14.4	8.1	4.9
% distribution *(%)	(-)	8.0	92.0	51.4	48.6	16.0	84.0
	(+)	13.6	86.4	20.3	79.7	66.0	36.0

* calculated as $\frac{\text{mg protein in pellet (or supernatant)}}{\text{mg protein of pellet + supernatant}} \times 100$

(+) and (-) MTP fractions were prepared from the same amount of starting material, 165 mg of S_1 protein, as described in 6.2.

Table 6.2 Distribution of proteins in MTP prepared by one polymerisation step (fraction P_2) in the presence of 1M sucrose, P_2S, and in the presence, $P_2(+)$, or absence, $P_2(-)$, of 4M glycerol

Fraction	Total protein *	% (α + ß)Tu	% L_1	% L_2
P_2 (-)	7.0	70	19	12
P_2S**	7.0	72	17	11
P_2 (+)	13.0	85	7.8	6.2

* calculated as % from starting material S_1 as follows: $\frac{\text{mg protein in } P_2}{\text{mg protein in } S_1} \times 100$

% distribution of protein in (α + ß)Tu, ie, tubulin, L_1 and L_2 protein bands was calculated from densitometric scans of polyacrylamide gels, shown in fig. 6.2, stained with Coomasie Blue. The sum of the protein in the (α + ß)Tu, L_1 and L_2 bands was taken as 100%.

** Fraction P_2S was prepared in the same way as fraction $P_2(+)$ except that instead of mixing the starting material, S_1, with an equal volume of 8M glycerol-reassembly buffer, S_1 was mixed with an equal volume of 2M sucrose-reassembly buffer.

fig 6.2), even though the density of the sucrose containing reassembly buffer was identical to that of the glycerol containing buffer. The significance of this observation is discussed later in this chapter. (see 6.4.)

It is clear from the data shown in table 6.3 that the method of preparation greatly affects the amounts of phospholipid -^{32}P recovered in P_2 fractions, but has a relatively smaller effect on the content of protein-bound ^{32}P. Thus the specific activity (SA, as defined in 2.9) of phospholipid -^{32}P is about five times smaller for P_2(+) than that for P_2(-).

In subsequent experiments the purification of MTP was taken a step further to prepare fractions P_3 and S_3. The results in table 6.4 show again that the P_3(-) and S_3(-) fractions contain not less than five times more phospholipid -^{32}P/mg protein than the corresponding (+) fractions. Comparison of the RSA values for protein and lipid-bound ^{32}P reveals that fraction P_3(+) is enriched in both types of bound ^{32}P, relatively to all other fractions, i.e. glycerol present during the polymerisation step not only brings about a re-distribution of the protein between the P_3 and S_3 fractions, but also alters the distribution of the protein- and phospholipid-bound ^{32}P between these two fractions.

From radioautographs of stained and dried polyacrylamide gels, it can be seen that the main phosphorylated components of (+) fractions are the HMW proteins and tubulin (as illustrated for P_3(+) in fig. 6.3) as is the case for (-) fractions (see Chapter 3). Thus the re-distribution of protein-bound ^{32}P apparently is not due to the presence of additional minor protein

Legend to fig. 6.2

High-speed supernatant, ($50x10^3$xg, 30min, 4^oC) was prepared from the brains of 1 to 3-day-old chicks and split into three equal parts. One part was mixed with an equal volume of reassembly buffer containing 2M sucrose and the second part was mixed with 8M glycerol. On polymerisation (30min, 37^oC) and subsequent sedimentation these mixtures yielded microtubulin pellets P_2S and $P_2(+)$ respectively. The third part was polymerised without the addition of either sucrose or glycerol and after sedimentation yielded the microtubulin pellet $P_2(-)$. The obtained fractions were electrophoresed in the SDS-urea system described in 2.7 and the fixed and stained gels were scanned as described in 2.7.6. See also table 6.2. For bands L_1 and L_2 see 2.7.4. DF = dye front.

Fig. 6.2 Densitometric analysis of polyacrylamide gels of MTP fractions prepared in the presence of sucrose or in the presence or absence of glycerol

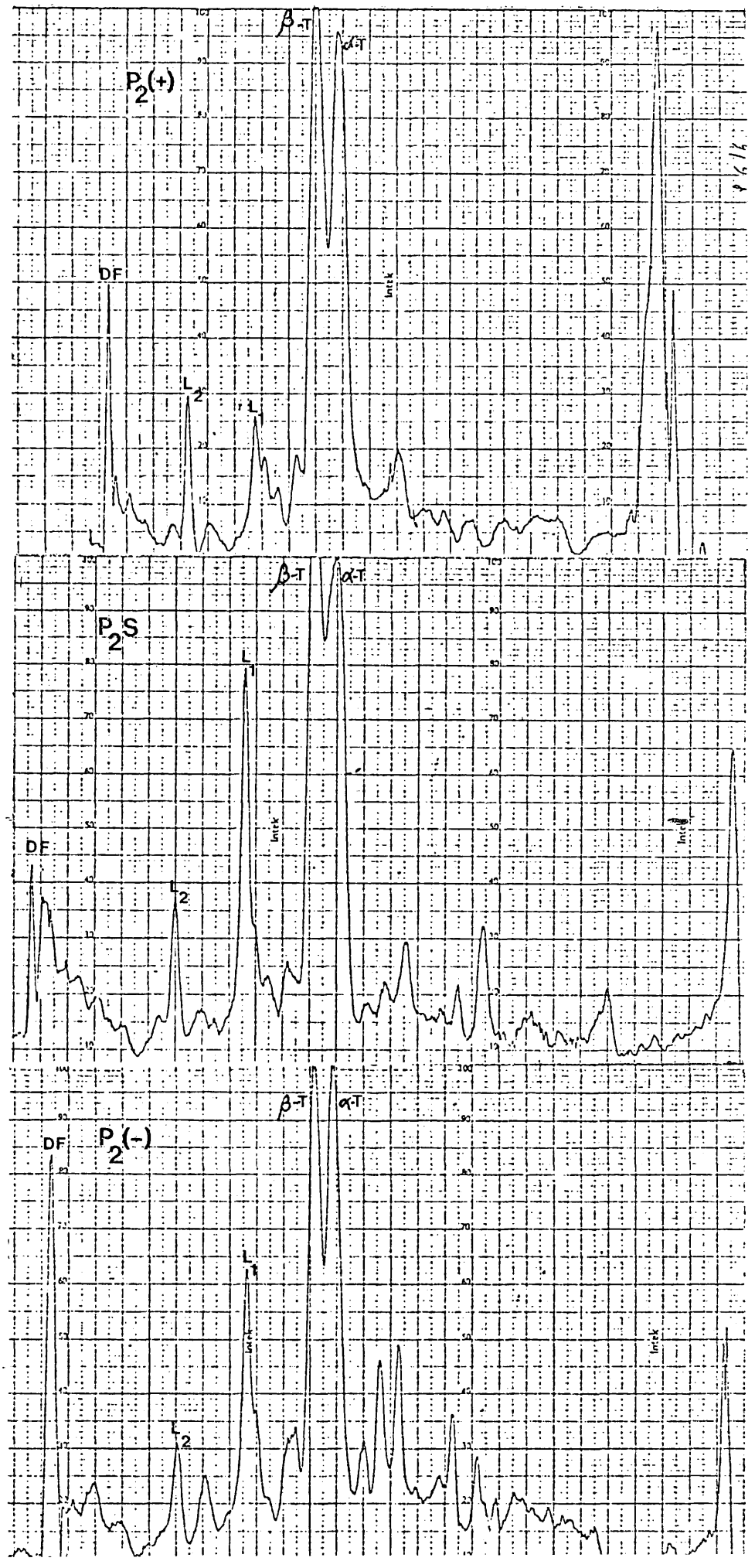


Table 6.3 Protein, protein-bound ^{32}P and phospholipid ^{32}P content of fractions P_2 (-), P_2S and P_2(+)

Fraction	Protein content (mg)	bound ^{32}P cpm x 10^{-3}/mg protein	
		protein	phospholipid
P_2 (-)	6.8	4.79	13.63
P_2 S	7.3	4.60	6.65
P_2 (+)	14.8	3.80	2.62

For preparation of P_2 fractions shown, see text.

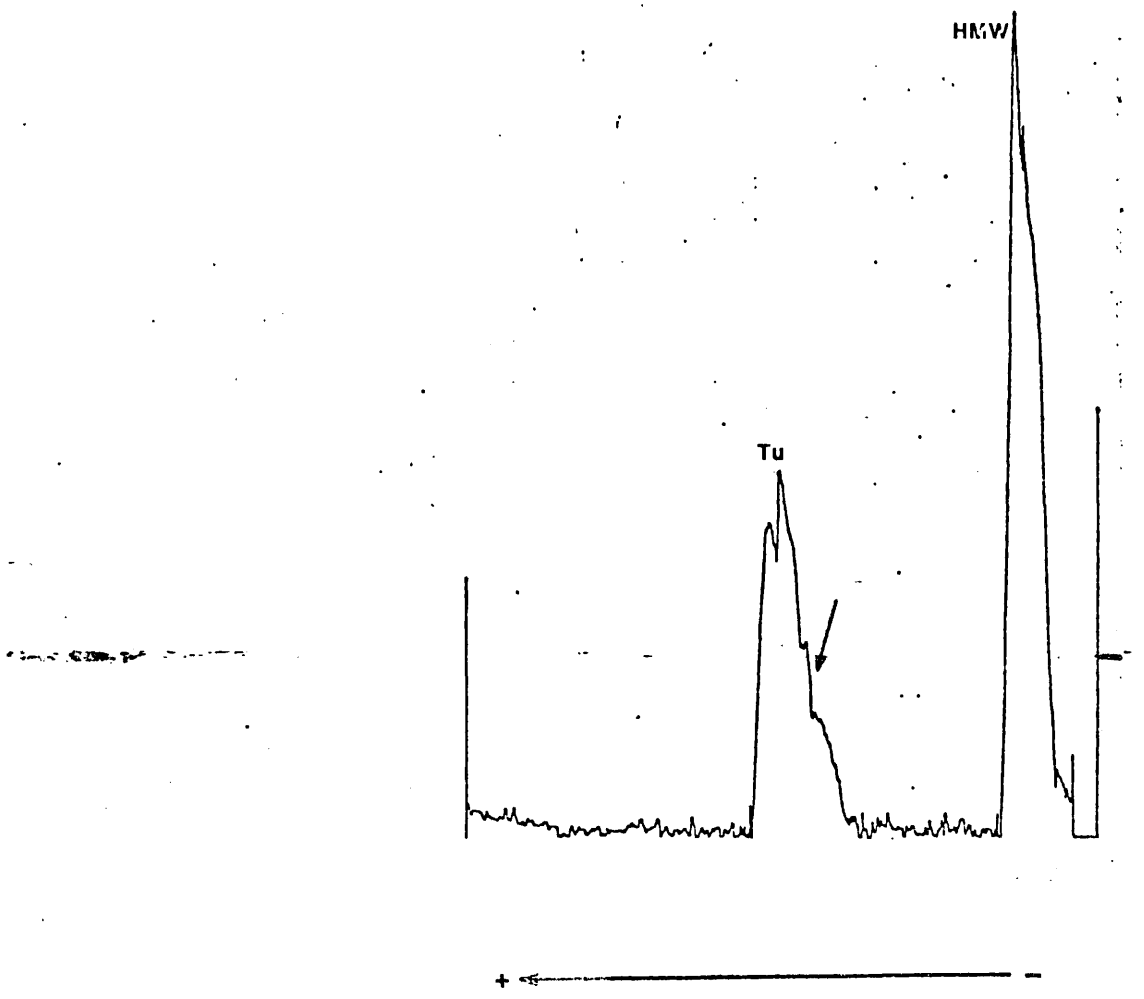
Table 6.4 Protein, protein- and phospholipid-bound ^{32}P content and distribution in P_3 and S_3 fractions prepared in the presence or absence of glycerol

Fraction	Protein content (mg)	* % distribution of protein (%)	**SA of bound ^{32}P cpm		**RSA of bound ^{32}P cpm	
			protein	lipid	protein	lipid
P_3 (-)	9.7	56.7	1.06	4.29	0.75	1.49
S_3 (-)	7.4	43.3	1.89	0.85	1.33	0.35
P_3 (+)	4.2	25.0	3.10	0.83	1.89	2.75
S_3 (+)	12.9	75.0	1.08	0.15	0.71	0.49

* % distribution of protein between pellet and supernatant fractions. ** x 10^{-3}

(+) and (-) fractions were prepared from equal amounts of the same starting material as described in 6.2.

Fig. 6.3 Phosphorylation pattern of in vivo labelled P_3 fraction prepared in the absence of glycerol



Fraction P_3 was obtained during the purification of in vivo labelled (50 µCi;20h) MTP in the absence of glycerol. The figure shows a densitometric scan of a sliced and dried polyacrylamide gel of fraction P_3. Arrow points at shoulder due to labelled protein component of an apparent MW of 70 000.

bands in the (+) fractions.

Further purification of MTP yielded P_4 and S_4 fractions in which the differences between (+) and (-) fractions were amplified, as can be seen from the data shown in table 6.5. The most striking differences are observed between the P_4 fractions. P_4 (+) contains about twenty times more protein and more than thirty times less lipid-bound ^{32}P cpm/mg protein than P_4 (-). On the basis of RSA values it is clear that fraction P_4 (-) is enriched in both types of bound ^{32}P, in comparison to all other fractions. The results shown in table 6.5 once again point to the conclusion that glycerol present during the purification of tubulin yields MTP fractions which are quantitatively as well as qualitatively different from those obtained in its absence.

From the results described above, the effects of glycerol on tubulin populations present in crude brain extracts, S_4, can be summarised as follows.

1. Protein is re-distributed between the MTP fractions, favouring the yield of <u>in vitro</u> polymerisable tubulin in fractions P_2 (+) S_3 (+) and P_4 (+) and decreasing the yield of the cold-stable tubulin fraction, P_3 (+).

2. There is a general decrease of MTP associated phospholipid-^{32}P in all (+) fractions.

3. There is a general decrease of SA for protein-bound ^{32}P in all (+) fractions containing <u>in vitro</u> polymerisable tubulin, ie, P_2(+), S_3(+) and P_4(+).

4. All (+) fractions are of higher purity than the corresponding (-) fractions.

Table 6.5 Protein, protein- and phospholipid-bound ^{32}P content and distribution in P_4 and S_4 fractions prepared in the presence or absence of glycerol

Fraction	Protein content (mg)	* % distribution of protein (%)	**SA of bound ^{32}P		**RSA of bound ^{32}P	
			protein	lipid	protein	lipid
P_4 (-)	0.4	11.0	16.3	75.8	1.48	3.80
S_4 (-)	3.4	89.0	9.9	12.3	0.94	0.65
P_4 (+)	8.1	66.0	11.7	2.3	0.90	1.07
S_4 (+)	4.3	34.0	15.2	1.9	1.13	0.82

* % distribution between pellet and supernatant fractions.

** x 10^{-3}.

(+) and (-) fractions were prepared from equal amounts of the same starting material as described in 6.2.

6.4 Discussion

Although glycerol removes at least four fifths of the ^{32}P labelled phospholipid associated with MTP fractions, it does not do so by selectively removing a particular phospholipid class (as was shown in Chapter 5, table 5.1). This suggests that the phospholipids found in MTP fractions exist in some form of homogeneous structural units. This proposed unit will be referred to as phospholipid structural unit (PSU), although there is no electron-microscopical evidence for the presence of recognisable membrane elements in all MTP fractions examined (Lagnado, J.R. unpublished observations). These PSU-s presumably exist in MTP fractions in either one of two states - as freely floating structures or as MTP-PSU complexes. Understanding of the way in which glycerol removes PSU-s from the $P_2(+)$ fraction can help in choosing the more likely model.

If the PSU-s existed as free vesiculated membrane fragments, e.g. microsomes, one might expect that the higher density of the glycerol-containing buffer would retard their sedimentation, to produce a 'cleaner' microtubular pellet $P_2(+)$ as compared to $P_2(-)$. This possibility was examined in the first set of experiments described in this chapter, the results of which are summarised in tables 6.1 and 6.2, figure 6.1, and the related text. It is known that sucrose and glycerol do not have differential effects on the sedimentation properties of microsomes (Depierre and Dallner, 1976), and both are known to stabilise microtubules (Shelanski et al, 1973). Moreover, since 1M sucrose-reassembly

buffer and 4M glycerol-reassembly buffers have the same density at 37°C, as determined by viscometry, it is to be expected that the same species will be pelleted under identical centrifugation conditions in both buffer systems. Hence the tubulin fraction P_2, prepared from the same starting material, S_1, in 1M sucrose-reassembly buffer, P_2S, or 4M glycerol-reassembly buffer, $P_2(+)$, should have identical characteristics. The results in table 6.2 do not support this model. Thus, the yield of tubulin, the amount of protein and lipid-bound ^{32}P cpm/mg protein and the quantitative relationship of the main protein species found in the P_2S fraction (see also table 6.2 and fig 6.2) were much nearer to the corresponding characteristics of the $P_2(-)$ fraction than those of the $P_2(+)$ fraction.

The other possibility is that PSU-s exist as MTP-PSU complexes. Glycerol could break up these complexes in either of two ways. Glycerol is known to bind to tubulin and the interaction seems to be of considerable strength at a few sites of tight binding (see Dëtrich et al, 1976). If glycerol competes for the same or very similar binding sites as the PSU-s, it could displace these structures which will then be retained in the supernatant. Secondly, glycerol has been used for a long time now, to extract muscle fibres. 4M glycerol extracts some 50% of the endoplasmic reticulum proteins and breaks up the endoplasmic reticulum and the plasma membranes, rendering the plasma fibre 'leaky' (Szent-Györgyi, 1949, Webster 1953). By analogy, it can be reasoned that glycerol breaks up the MTP-PSU complexes resulting in the observed removal of lipid-bound ^{32}P from the MTP pellet fraction

(P_2, eg, F_2(+).). These considerations favour the existence of PSU-s complexed to MTP rather than their being present as independent 'free' entities copurifying with MTP.

As will be discussed in Chapter 8, there are many instances where membrane-tubulin interactions in cells of different tissues have been suggested on the basis of morphological and bio-physical evidence. For the purpose of this discussion, it is of particular interest that microtubule organising centres (MTOC) and tubulin storage structures (TSS) of 'activatable' tubulin have been described as being associated with or derived from cytoplasmic membranes. Generally, both of these structures have an amorphous appearance and in some cases seem to give rise to or be contained in the same structural complex (Weisenberg et al, 1975, Inoue and Sato, 1964). Evidence by Inoue and Sato (1967) and Inoue (1959) shows that different types of MTOC in cells of different classes and origin are invariably interacting with membranes via microtubules, or are derived from membranes (see also Pickett-Heaps, 1975).

Although there is a considerable body of morphological and biochemical evidence implying a close relationship between neurotubulin and neuronal plasma and intracellular membranes (Lieberman, 1971), MTOC-s and tubulin storage structures have not yet been identified in the nerve cells. It should be noted that here MTOC is used to denote not only nucleation centres for tubule growth but also points of close membrane microtubule interactions.

These considerations could provide a basis on which

the origins of MTP-PSU complexes could be related to three main classes of *in vivo* existing structures:

a) MTOC-s, e.g. microtubule nucleating centres and sites of close membrane - microtubule interactions.

b) Tubulin storage structures (TSS).

The above considerations can provide a basis for a coherent interpretation of the observed biochemical differences between '+' and '-' MTP fractions prepared from the same starting material, S_1.

Let us first define the various pools of microtubular protein which can be found in the crude brain extract, S_1. Under the conditions of homogenisation employed, the cytoplasmic portion of the *in vivo* microtubules will give rise to a pool of 'active' tubulin-ring structures in equilibrium with some dimer ('active') derived from them. A certain amount of the MTP-PSU complexes, discussed earlier on, will also be released in solution. The MTP-PSU complexes can be expected to contribute, in a limited way, to the pool of tubulin dimer. Whereas the dimer liberated from the MTOC structures would be of the 'active' type ('*in vivo* polymerisable'), the dimer derived from TSS would be of the 'inactive' type (not '*in vivo* polymerisable'). This classification is made on the assumption that TSS-contained tubulin dimer does not normally participate in the *in vivo* polymer-dimer equilibrium. Kirschner et al (1975) suggested that only one type of tubulin dimer is able to form ring structures, and showed that the ring dimers contain protein-bound phosphate in contrast to a dimer population which cannot give rise to ring structures and hardly contains any

protein-bound phosphate. More recently, Shigekawa and Olsen (1976) confirmed these results and showed that phosphorylation can affect the tubulin aggregation state, pushing the 6S⇌36S equilibrium to the right. These observations allow the assumption that the 'active' tubulin (ring and dimer) is phosphorylated but that the 'inactive' form is not.

Glycerol introduced in crude brain extracts will break up some of the MTP-PSU aggregates, presumably the most labile ones which appear to be the TSS, in this way releasing preferentially some more of the 'inactive', not phosphorylated, dimer. The higher yield of protein in the $P_2(+)$ fraction is most probably due to the enhanced incorporation of 'inactive' dimer, which brings about the observed decrease of SA of protein-bound ^{32}P in this fraction, and stabilisation of the microtubules during the centrifugation step. In the absence of glycerol there will be less breakdown of the TSS aggregates, hence less 'inactive' dimer will be liberated. Under these conditions, no glycerol, the 'inactive' dimer does not incorporate into MTP to the same extent and/or is in a more labile association which is easily de-stabilised and broken up by the pressure and shearing forces of centrifugation. This could therefore account for the lower protein yield and increased SA of protein-bound ^{32}P seen in the $P_2(-)$ fraction.

The differences observed between (+) and (-) pellet fractions, e.g. P_3, obtained after the depolymerisation of the microtubules, P_2, support the above interpretation. In the $P_3(+)$ fraction, only the most stable MTP-PSU complexes will be found, presumably derived from MTOC,

which would account for the observed low yield of protein and enrichment in protein-bound ^{32}P. The $P_3(-)$ fraction would then contain both TSS and MTOC-s, this being reflected in the higher protein yield and lower SA of protein-bound ^{32}P.

Generally it can be concluded that the differences between (+) and (-) tubulin fractions obtained during the first cycle of polymerisation/depolymerisation are due to the different state of TSS tubulin dimer at the time of its incorporation into microtubules. In glycerol-containing media, TSS are broken up liberating 'inactive' dimers which are preferentially incorporated into microtubules and after depolymerisation, recovered in $S_3(+)$ supernatant; in contrast, in glycerol free media, TSS are incorporated into microtubules more or less intact and after depolymerisation are preferentially recovered in the $P_3(-)$ pellet.

The differences between (+) and (-) MTP fractions obtained during further purification can be explained similarly. The much higher protein yield in fraction P_4 (+) and its lower SA with respect to protein-bound ^{32}P is apparently a consequence of the characteristics of the mother fraction, $S_3(+)$, and the action of glycerol, ie, enhancement of the formation and stabilisation of microtubules and a concomitant dispersal of TSS structures enriched in 'inactive' dimer.

This scheme (see also fig. 6.4) for explaining the differences between (+) and (-) tubulin fractions is supported by the results of Rebhun et al (1975), who showed that glycols can mobilise, or release, a further

pool of tubulin. They assume that under normal conditions, no glycol treatment, this 'inactive' tubulin is withdrawn from the in vivo polymer-dimer equilibrium in some form of a storage structure, very probably membrane-bound. According to Rebhun and co-workers, glycols seem to act by removing some kind of inhibitor from the tubulin dimer. The results presented in this chapter suggest that this inhibitor may be related to the presence of phospholipid structures. The experiments described in the preceding section of this chapter (see also 4.4) indicate that the tubulin isolated by cycles of assembly/disassembly represents a mixture of at least two different tubulin species.

The fact that glycerol removes a significant proportion of the MTP associated phospholipid might provide a tentative explanation for the different sedimentation behaviour exhibited by the depolymerisation products of microtubules prepared in the presence and in the absence of glycerol. (Weingarten et al, 1974; Borisy et al, 1975.) The ring structures obtained from microtubules purified in the absence of glycerol could be 'floated' by the higher amount of associated PSU-s to yield boundaries at 30S instead of 36S.

In conclusion, it can now be suggested that the differences observed between tubulin prepared with glycerol and tubulin prepared without glycerol are due not only to some form of allosteric alteration of tubulin induced by glycerol, but also to chemical differences associated with different classes of MTP obtained by these two procedures.

The tentative interpretation of the differences found between MTP fractions prepared in the presence or

absence of glycerol, presented above, is based on two general assumptions;

1. That the protein and phospholipid bound ^{32}P found in MTP fractions labelled *in vivo* for 20 h reflects the total amount of protein and phospholipid bound phosphate in the examined fractions.

2. That the MTP-PSU complexes are derived from *in vivo* existing structures.

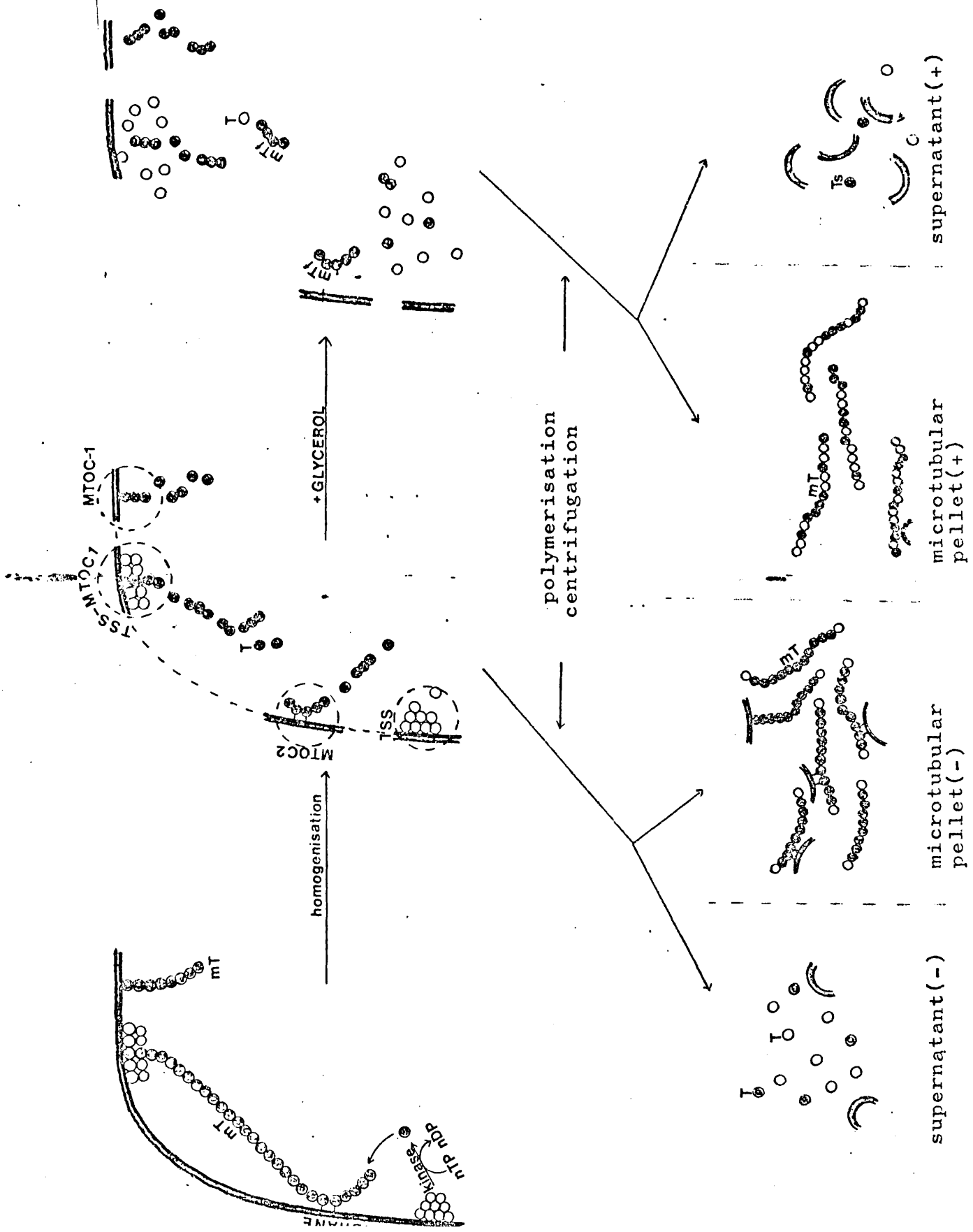
Clearly, further experimental evidence would be needed to substantiate the proposals put forward in the above discussion, which are summarised diagramatically in fig. 6.4.

Fig. 6.4

mT - microtubule; mTf - microtubule fragment; T - microtubule subunit (dimer); MTOC 1 - microtubule nucleation centre; MTOC 2 - site of close microtubule-membrane interaction/association; TSS - tubulin storage structure; TSS-MTOC 1 - represents a mixed structure which can serve both as a storage as well as a nucleation site; nDP and nTP denote nucleotide di- and triphosphate respectively.

On homogenisation membrane and microtubules are broken up to yield a mixture of microtubule fragments (mTf), microtubule subunits (T) and MTP-PSUs, e.g. TSS and MTOCs. After a centrifugation step at 4°C (not indicated in diagram) the high-speed supernatant, S_1, contains microtubule depolymerisation products and MTP-PSUs. TSS contain '*in vivo* non-polymerisable' microtubule subunits which are not phosphorylated (hollow circles), whereas MTOCs contain phosphorylated microtubule subunits derived from *in vivo* existing microtubules (filled circles). Glycerol introduced in this mixture breaks up the MTP-PSU structures releasing the non-phosphorylated microtubule subunits which are contained in the TSS; these subunits can be incorporated into microtubules in the presence of glycerol and on sedimentation give rise to the pellet designated as 'microtubule pellet (+)'. In contrast, in the absence of glycerol the majority of the MTP-PSUs remain intact and the relatively smaller amount of non-phosphorylated, free, subunits derived from TSS during the preparation procedure are not incorporated into microtubules during polymerisation in the absence of glycerol. Hence the microtubules recovered after sedimentation contain predominantly phosphorylated subunits and are enriched in MTP-PSU structures ('microtubule pellet (-)').

For purposes of clarity, the initial polymerisation and sedimentation steps which yield the first microtubule pellet (P_2) and the subsequent depolymerisation step (to yield P_3 and S_3), are omitted from this scheme.



Chapter 7 Interactions of myo-inositol with brain microtubules

7.1 Introduction

Labelling experiments *in vivo* with ^{32}P, described in Chapters 3, 5 and 6, have shown that a significant proportion of the radioactivity incorporated into brain microtubules was present in a phospholipid fraction purified from chloroform/methanol extracts of the isolated protein. In short-term (2h) labelling experiments, between 25 and 40% of the ^{32}P radioactivity present in purified microtubule-associated phospholipids was recovered in the phosphoinositide fraction which constitutes, at most, 5 - 10% of the total phospholipid P recovered (Kirazov and Lagnado, 1976; Lagnado, 1977; Kirazov et al, 1977). This observed enrichment of label in the phosphoinositide fraction is similar to that seen when synaptosomal membrane phospholipids are labelled with ^{32}P, under similar conditions (Hawthorne and Pickard) and could therefore simply reflect the labelling pattern of membrane phospholipids that were preferentially associated with microtubular components (Kirazov et al, 1977). It was postulated on the basis of these biochemical observations that phospholipids associated with isolated microtubules may reflect the occurrence, *in situ*, of functional associations between microtubules and neuronal membranes.

Further experiments, to be described in this Chapter, were performed to test the possibility of a direct interaction between myo-inositol and brain MTP.

More specifically, the binding of myo-inositol to MTP and the effects of myo-inositol on the temperature-dependent assembly/disassembly of microtubulin *in vitro*, were investigated.

7.2 Materials and Methods

Myo-[2-^{3}H]-inositol (spec. radioactivity, 5 Ci/mmol; The Radiochemical Centre, Amersham) was diluted, as indicated below, with unlabelled myo-inositol (Sigma) to give reaction mixtures containing, per ml, 10-250 µmol inositol and 30 µCi radioactivity.

Microtubular protein was isolated from four to six-day old chick brain or from four to six week-old rat brain by the procedure described in 2.2.2.

7.2.1 Binding assays

In the first series of experiments, freshly prepared fraction S_1 (ca. 10 mg protein/ml) containing 30 µCi/ml of reaction mixture) was incubated for 60 minutes at 37°C and the reassembled microtubules were sedimented and then depolymerised to yield fraction S_3 (i.e. once-cycled microtubular protein). Samples of S_3 containing ca. 1 mg protein/ml were immediately chromatographed at 4°C on 1 x 20 cm columns of Sephadex G100 (Pharmacia) as previously described in 2.4.

In the second series, freshly prepared fraction S_3 (ca. 2.5 mg protein/ml) was incubated for 30 minutes at 37° in the presence of [^{3}H] - inositol (25 µmol/ml, 30 µCi/ml of reaction mixture), then on ice for 30 minutes to allow depolymerisation of microtubules. Samples containing 10 - 15 mg protein (in 5 ml) were then

chromatographed at 4°C on 1.8 x 30 cm columns of Sepharose 6B (Pharmacia) as previously described in 2.4.

7.2.2 Microtubule polymerisation

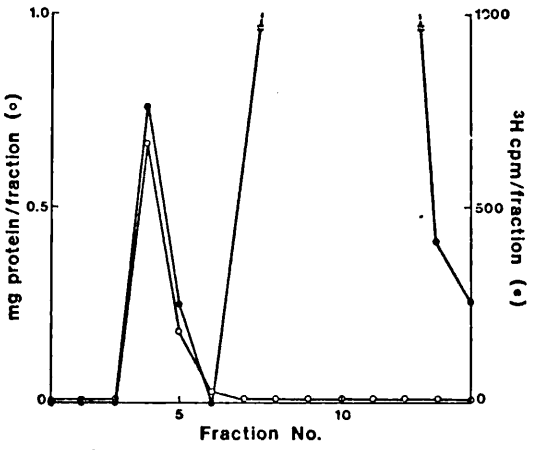
Microtubule polymerisation was assayed by a turbidimetric procedure in a split-beam Unicam SP1800 UV spectrometer, fitted with a 4-sample automatic sample changer thermostated to 4°C or 37°C as required. Readings were taken at 350 nm at 1-minute intervals and recorded on a Unicam AR25 Linear Recorder. Samples of S_3 (ca. 2.5 mg protein/ml) were diluted to give the required protein concentration in MT-reassembly buffer containing freshly added GTP (1 mM, final concn.) and incubated in the presence or absence of inositol.

7.3 Results

7.3.1 Binding of [^{3}H]-inositol to microtubular protein

During initial experiments to test the binding of inositol to microtubular protein, labelled polymerisable tubulin (fraction S_3) was derived from a crude high-speed supernatant (S_1) of rat brain, that had been incubated with [^{3}H]-inositol by one cycle of assembly/disassembly, as described in section 7.1. Densitometric analysis of gels stained with Coomassie Blue showed that 75 - 80% of the protein in the fraction S_3 behaved as tubulin after S-PAGE. It is clear from the data illustrated in Fig. 7.1 that a first peak of radioactivity eluted upon chromatography of [^{3}H]-S_3 on Sephadex G100 coincided with the peak of protein eluted in the excluded volume (fractions 4 and 5, Fig. 7.1) and was clearly separated

Fig. 7.1 Protein and ^{3}H -inositol elution profiles for rat brain S_3 fraction during chromatography on Sephadex G100

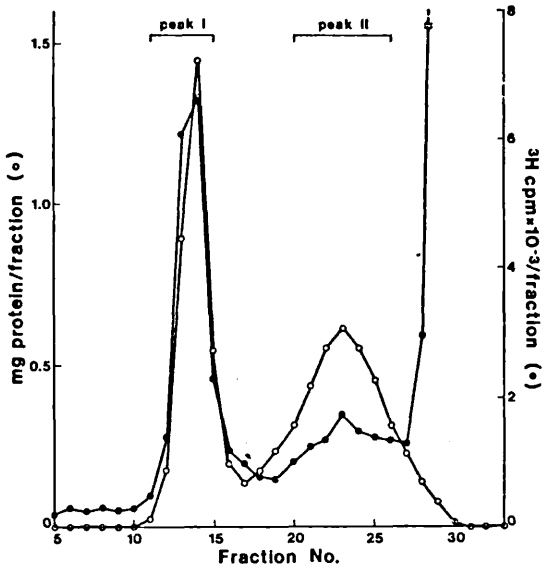


Protein recovery: 87%; flow rate: 12ml/h. For details see text sections 7.2.1 and 7.3.1.

from a second major peak of radioactivity, which contained free inositol. Since most (> 80%) of the counts present in the first peak were released into solution when the protein was precipitated in the cold with 10% TCA, it can be tentatively concluded that little, if any, of the bound inositol had been incorporated into or retained in the microtubule-associated phospholipids remaining in the acid-insoluble pellet under the present experimental conditions. Further, it was calculated that the radioactivity associated with the protein fraction corresponded to the binding of 18.6 nmol of inositol/mg total protein; if one assumes that tubulin, which constituted 75 - 80% of the eluted protein was mostly responsible for the binding observed, one obtains values ranging between 2 and 3 mol bound inositol/mol of tubulin dimer (MW 110,000). Such values would represent the minimum binding capacity of the preparation, corresponding to relatively tightly bound inositol that does not readily exchange with the excess unlabelled inositol present in the medium during purification of the protein.

The binding of inositol to microtubular protein was confirmed in further experiments in which the S_3 microtubular fraction derived from chick brain was incubated at 37°C with [^{3}H]-inositol under polymerising conditions and chromatographed directly after cooling on ice to allow for depolymerisation, on Sepharose 6B to separate the 30-36S tubulin aggregates from the ca. 6S tubulin dimer fraction (see section 2 and fig. 7.2 for experimental details). The results illustrated in fig. 7.2 show that significant peaks of radioactivity coincided with the two major protein peaks eluted, corresponding to the tubulin

Fig. 7.2 Protein and ^{3}H-inositol elution profiles for chick brain S_3 fraction during chromatography on Sepharose 6B



The sample contained 12mg protein in 4ml, of which 89% was recovered in eluate. Flow rate: 10ml/h. For details see text in sections 7.2.1 and 7.3.1.

aggregate and tubulin dimer fractions (peaks I and II, Fig 7.2), in addition to a third peak which was devoid of protein but contained the free inositol. About 60% of the total bound counts were recovered in peak I material, which contained about 45% of the recovered protein, suggesting a nearly two-fold enrichment of inositol binding in the tubulin aggregate fraction, corresponding to calculated specific activities of about 6 nmol bound inositol/mg protein, as compared to about 3.5 nmol/mg protein in the tubulin dimer fraction. Electrophoretic analysis by S-PAGE showed that tubulin represented ca. 80% of the protein present in peak I material, which was characteristically enriched in HMW components, while it accounted for 85 - 90% of the protein in peak II material which was devoid of HMW components. Since it was also apparent, from electrophoretic analysis, that tubulin was the only detectable protein common to material eluted in peaks I and II, and that significant binding occurred in the second peak, it can be tentatively concluded that it is tubulin itself rather than minor microtubule-associated proteins that was responsible for the protein-bound inositol detected in these experiments. This does not exclude, however, the possibility that material other than tubulin present in the tubulin aggregate fraction peak (I) contributed to the stability of the protein-inositol complex during the incubation and chromatographic procedures employed. Indeed, on the reasonable assumption that tubulin was mainly responsible for the binding, calculations show that the tubulin aggregate (peak I) and tubulin dimer (peak II) fractions contained about 1 and 0.5 mol bound inositol/mol tubulin

(MW 110,000), respectively. · If these values are corrected to account for differences in time of incubation (60 minutes instead of 30 minutes), they approximate closely the values found in the first series of experiments using Serphadex G100 chromatography (see above and Fig. 7.1) and suggest, further, that inositol may be preferentially bound to microtubular protein fractions that are specifically enriched in assembly-competent forms of tubulin (aggregates, peak I, Fig. 7.2; Johnson and Borisy, 1975).

7.3.2 Effects of inositol on microtubule assembly

Preliminary experiments showed that inositol added to partly purified microtubular protein, fraction S_3, decreased the rates of both assembly and disassembly and the maximum turbidity developed (plateau values) to a considerable extent. This led to a series of experiments devised to investigate the parameters of tubulin polymerisation affected by inositol.

First the dependence of polymerisation on inositol concentration was examined. The range of inositol concentrations was chosen on the basis of calculations of the molar ratio of inositol to tubulin found in nervous and secretory tissues. Dawson et al (1961) and Wagner et al (1976) have shown that brain tissue contains between 10 and 20 μ moles of free inositol per gram of tissue. From densitometric analysis of SDS-urea polyacrylamide gels of crude brain extracts (10mg protein/ml) it was calculated that tubulin accounts for about 26% of the soluble proteins. When the extract is polymerised and the microtubules pelleted, only about

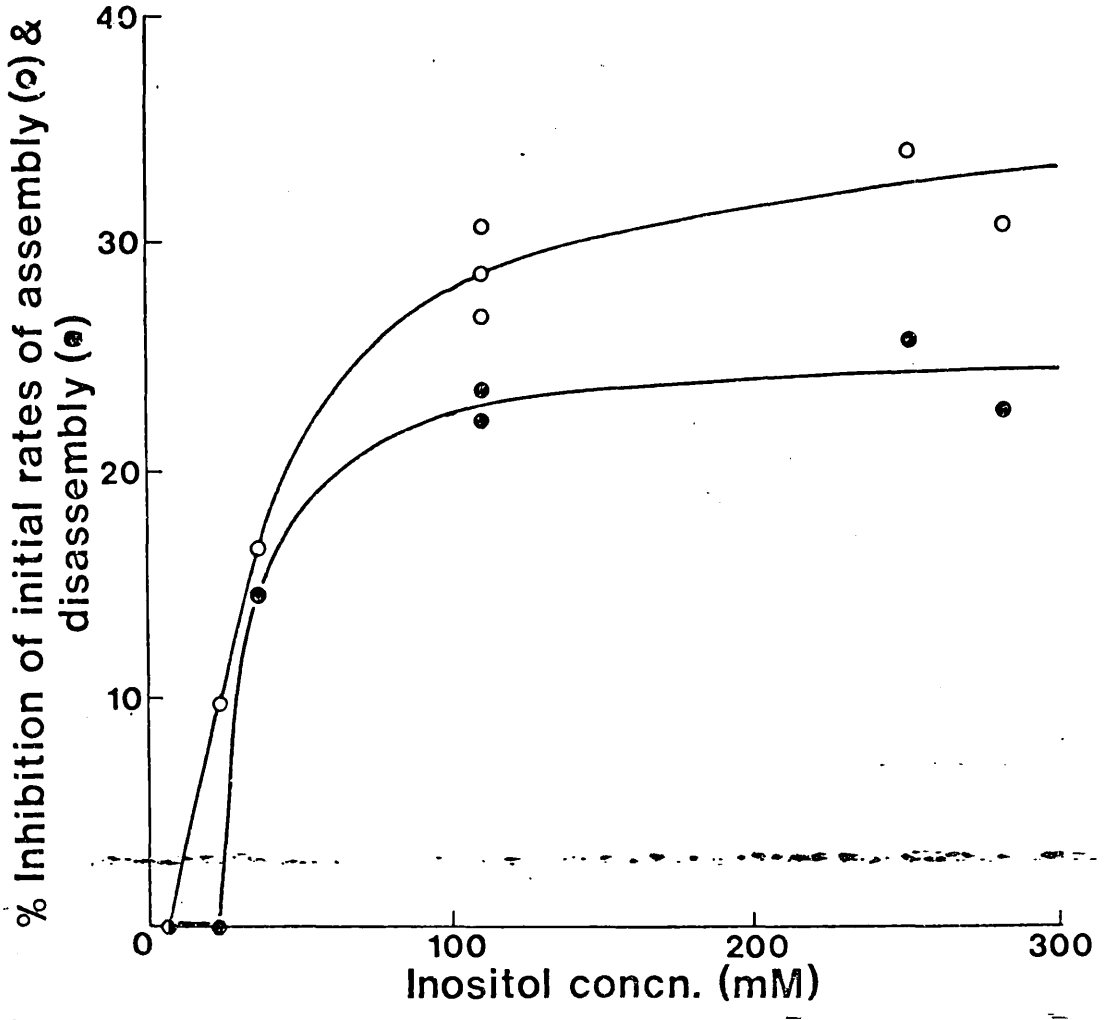
40% of the tubulin present in the extract was recovered in the pellet, hence the polymerisable tubulin will account for only 10% of the total protein found in the crude extract (see also Borisy et al, 1975). On the assumption that this population of tubulin represents the 'active' cytoplasmic tubulin, derived mainly from in vivo existing microtubules, one can calculate that in 1g of tissue there are 10 nmoles of 'active' tubulin dimer (110 000 MW). If the free inositol is distributed evenly in the aqueous phase (cytosol) bathing the microtubules which account for 80% of the weight of brain tissue, the concentration of free inositol will be at least 12 x 10^{-3}M and that of 'active' tubulin dimer 12.5 x 10^{-6}M, ie, the molar ratio of free inositol to 'active' tubulin dimer will be at least 1 000 : 1.

This will be referred to as the 'physiological' concentration of inositol in respect to tubulin throughout the following text.

The maximal increase in the inhibition of initial rates of both assembly (V^+) and disassembly (V^-) occurred at inositol concentrations between 20 and 100 mM. (fig. 7.3) At the protein concentration used (1mg/ml), this corresponds to inositol concentrations 3 to 15 times higher than 'physiological'.

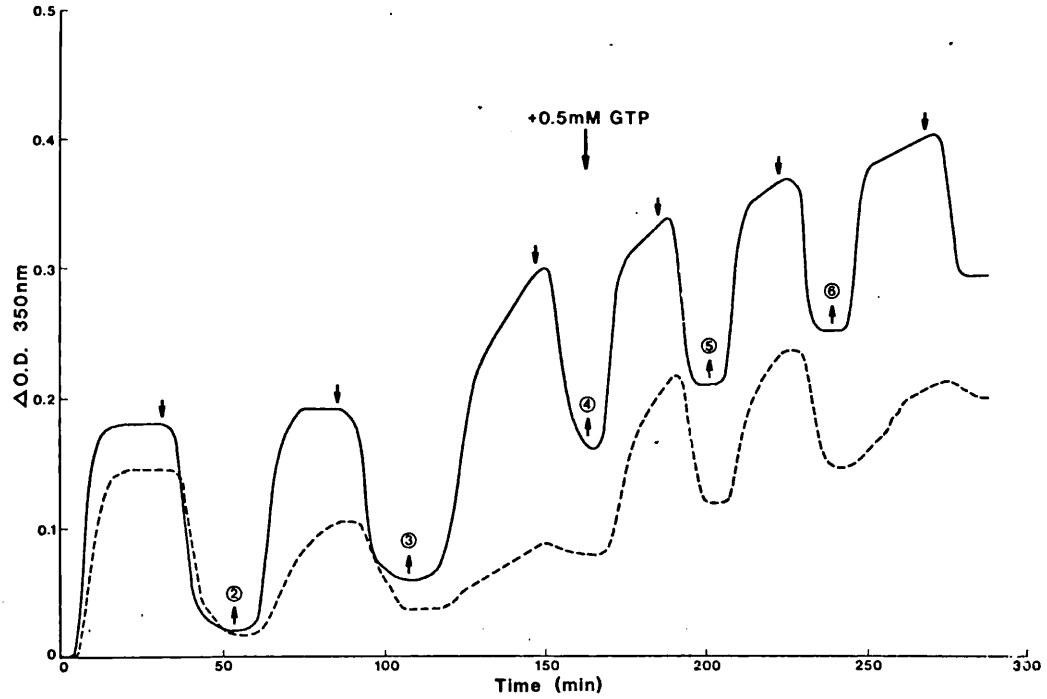
The inhibitory effects of inositol are most probably the result of an immediate interaction between inositol and the precursor pool of polymerisable protein since it apparently occurs at 4°C, or during the short period (2 minutes) required for re-equilibration of the system at 37°C. These effects were considerably amplified during successive cycling of the protein (fig. 7.4 and fig. 7.5).

Fig. 7.3 Effect of inositol on initial rates of tubulin assembly and disassembly



Effect of inositol on initial rates of assembly and disassembly of microtubular protein prepared by one cycle of polymerisation/depolymerisation. Initial rates are expressed in arbitrary units calculated from biggest slope of polymerisation curves. Protein concentration 1mg/ml of incubation mixture. For details see 7.2 and 7.3.1.

Fig. 7.4 Turbidity changes during successive cycles of assembly/disassembly of rat brain S_3 fraction in the presence and absence of myo-inositol

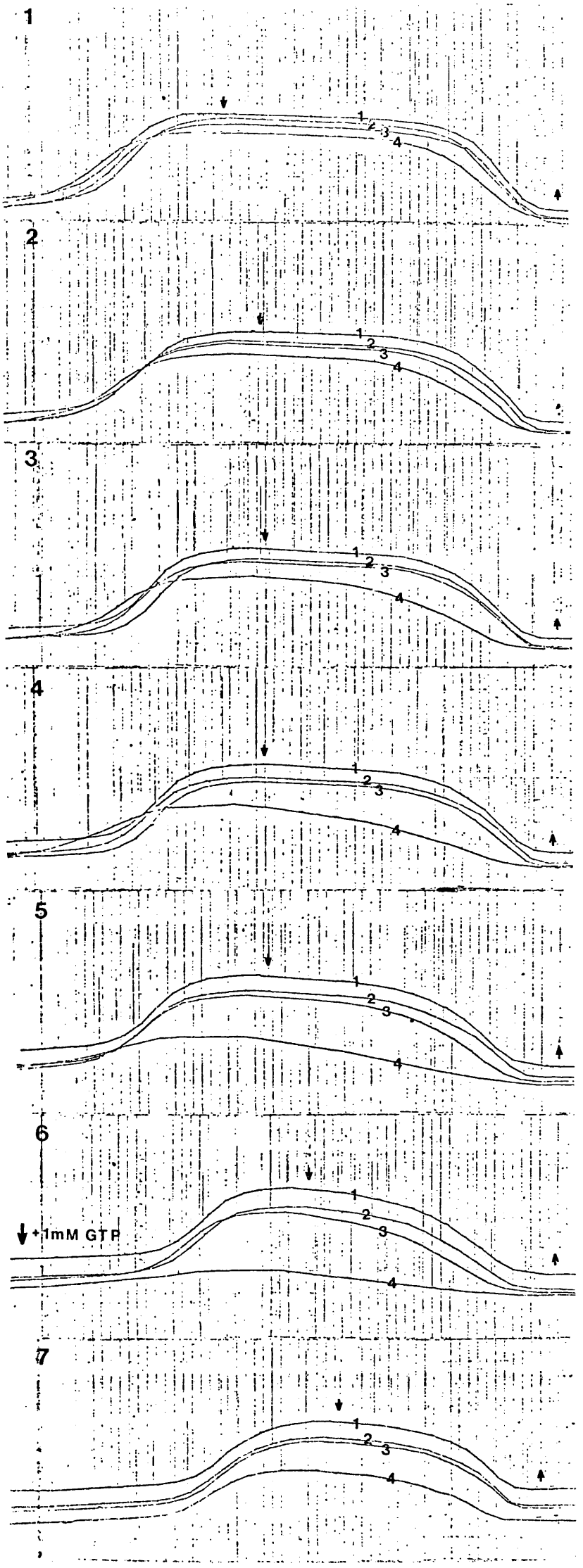


Turbidity changes during succesive cycles of assembly/disassembly of rat brain S_3 fraction in the presence (---) and absence (——) of myo-inositol (added at zero time, 250mM final concetration). Turbidity changes were monitored simultaneously for control and inositol-containing samples. Arrows indicate temperature increase to 37°C ( ) or decrease to 2°C ( ) and circled numbers indicate the start of each cycle. For details see text in sections 7.2.2 and 7.3.2.

Legend to fig. 7.5

Turbidity changes during succesive cycles of assembly/disassembly of brain MTP, purified by one cycle of polymerisation (S_3), in the presence of inositol. Small numbers on trace show number of sample. Sample 1 = control, no added inositol; samples 2, 3 and 4 contained 60mM, 100mM and 200mM inositol respectively. Inositol was added to samples 2, 3 and 4 prior to start of first cycle at 4°C. Big numbers in the upper left corner of each cycle trace indicate number of cycle. Arrows indicate temperature increase to 37°C ( ) or decrease to 2°C ( ). Turbidity changes were monitored simultaneously for all samples. Protein concentration 2mg/ml.

Fig. 7.5

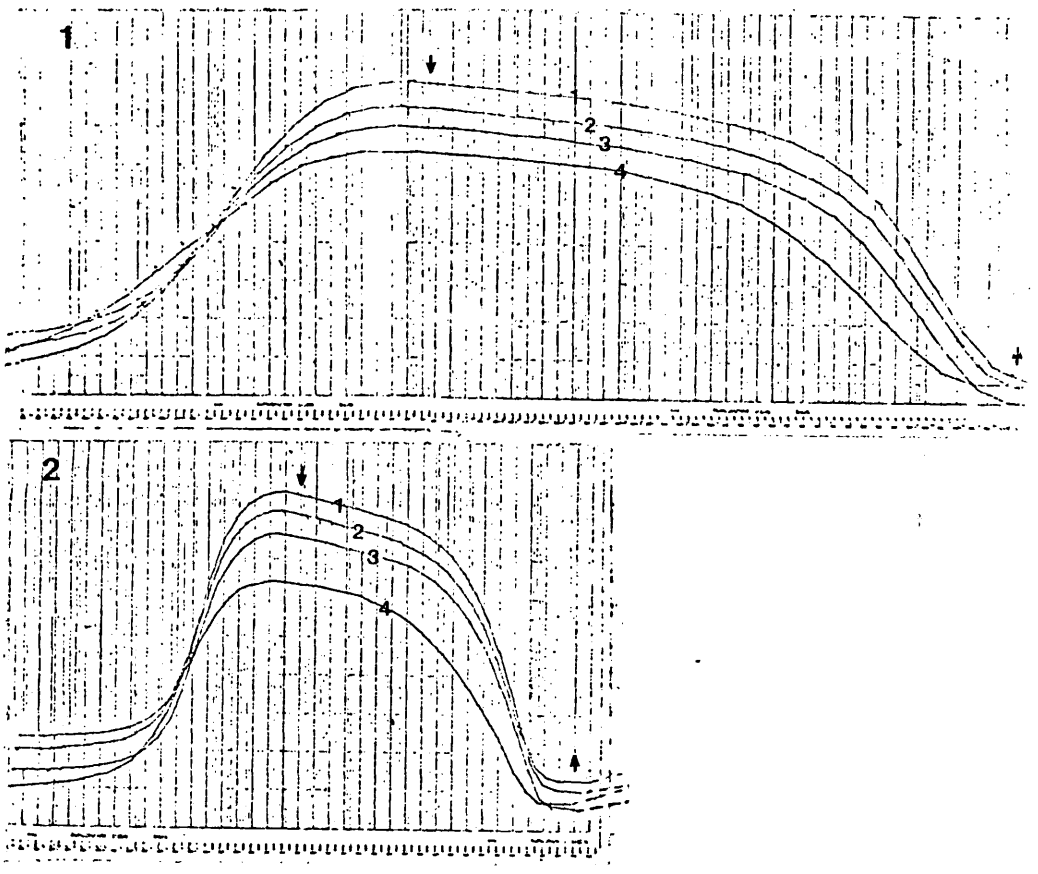


From experiments where the protein was subjected to successive cycles of polymerisation/depolymerisation, it became evident that whereas the rapid initial increase in turbidity resulted in the establishment of a well defined plateau (figs. 7.4 and 7.5, cycles 1 and 2 of control samples), the initial phase of turbidity increased in subsequent cycles was followed by a characteristically slow phase of turbidity increase, which proceeded at a constant rate during incubation at 37°C (figs. 7.4 and 7.5, cycles subsequent to cycle 2). Further, it was seen that the development of this secondary slow phase apparently depends on the GTP-protein ratio of the samples. In samples of different protein concentration, 1.1 mg/ml or 2.3 mg/ml where the GTP-protein ratio was kept the same by using 1 mM and 2 mM GTP, respectively, the slow phase developed after the second cycle of assembly/disassembly in both cases (figs. 7.4 and 7.5). Where the protein concentration was kept the same (0.7 mg/ml), but the GTP concentration was altered (between 0.5 mM and 1 mM GTP), the sample containing less GTP developed the slow phase already in the first cycle (fig. 7.6), whereas the sample with higher GTP did not develop the slow phase in the first two cycles of polymerisation.

Similar changes also occurred in samples containing inositol, where, however, the development of this secondary slow phase was already apparent earlier in the history of the protein than in control samples (figs 7.4 and 7.5, cycles subsequent to cycle 1 of inositol containing samples).

The secondary phase of slow turbidity increase

Fig. 7.6

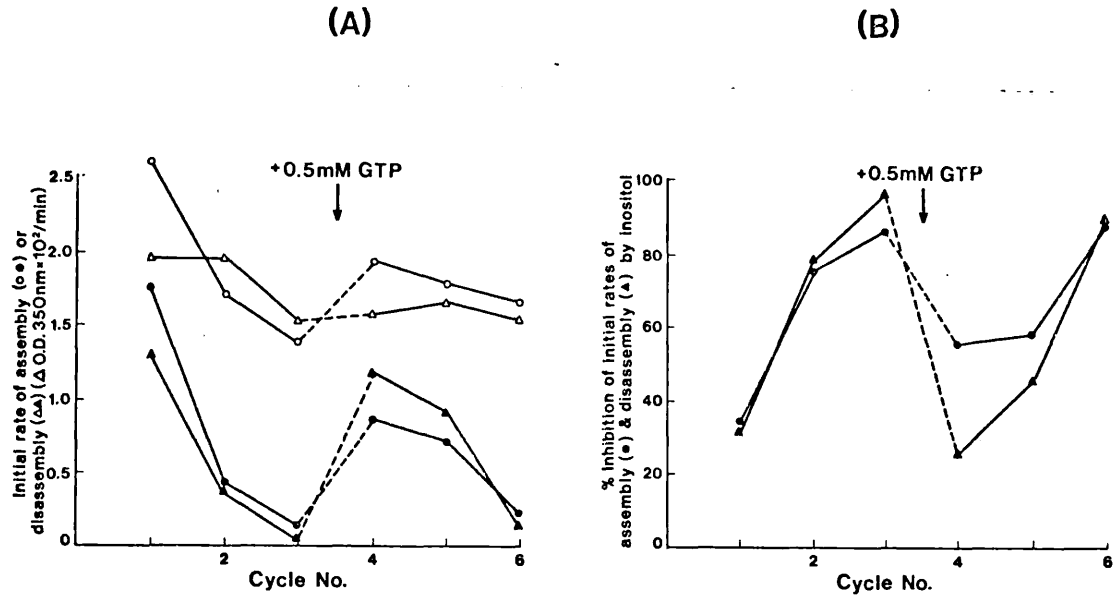


Turbidity changes during successive cycles of assembly/disassembly of brain MTP, fraction S_3, in the presence of inositol. Lettering same as in fig. 7.5. Sample 1=control, no added inositol. Samples 2, 3 and 4 contain 30mM, 50mM and 100mM inositol, respectively. Protein concentration 1mg/ml. GTP concentration 0.5mM.

probably reflects the gradual formation and accumulation of a pool of 'inactive' microtubular protein aggregates that do not readily depolymerise in the cold, as can be inferred from the progressive increase in the basal levels of turbidity, as a function of time, attained after equilibration of the samples at 4°C (figs. 7.4 and 7.5). Such protein aggregates could be due to the denaturation of microtubular protein because of lack of sufficient GTP, brought about by its break-down during the first two cycles of polymerisation. On the other hand, these protein aggregates could be related to the cold-insoluble microtubular protein fraction obtained during purification of the protein (i.e. fraction P_3, see 2.2.2) or to the recently described cold-stable class of microtubules, that can be formed at low temperatures in the presence of GTP (Wilson, 1975).

Finally, it can be seen from the data shown in figs. 7.4, 7.5 and 7.7 A and B that the addition of GTP (0.5mM final conc.) to the test system after three (figs. 7.4 and 7.7) or six (fig. 7.5) cycles of polymerisation caused a significant increase in the initial rates of assembly and disassembly in both control and inositol-containing samples (figs. 7.4, 7.5 and 7.7 A) and a partial alleviation of the inhibitory effects of inositol on these processes during the first cycle subsequent to the addition of the nucleotide (fig. 7.4, cycle 4, fig. 7.5, cycle 7 and fig. 7.7 B). However, addition of GTP did not eliminate the secondary slow phase of turbidity development, but in fact slightly decreased its rate in control samples, while clearly increasing that seen in the presence of inositol (see

Fig. 7.7 The effects of added GTP on initial rates of assembly and disassembly



The effects of added GTP on initial rates of assembly disassembly of microtubules in the presence (filled symbols) or absence (open symbols) of inositol (A) and on the percentage inhibition of the initial rates by inositol (B).

fig. 7.4 and 7.7, cycles 4-6). GTP also induced an apparent stabilisation of the initial rates of assembly and of disassembly in control samples, though not in the presence of inositol (fig. 7.7 A), suggesting that the effects of inositol and GTP were somewhat connected. It is suggested that the observed effects of GTP are most likely mediated through its protection of microtubular protein against 'ageing' (Johnson and Borisy, 1975; Gaskin, 1976; Olmsted and Borisy, 1972), thus increasing the pool of 'active' intermediates available either for microtubule assembly, in the control preparations, or for the generation of non-equilibrating 'stabilised' species of the protein, in the presence of inositol (see below and fig. 7.8).

Preliminary experiments indicated that inositol can also stabilise brain microtubules against the rapid depolymerisation induced by calcium ions. Thus, the addition of 3 mM $CaCl_2$ to a twice-polymerised preparation of rat brain microtubules (fraction S_3, incubated for 20 minutes at 37°C) decreased turbidity within 2 minutes to 45% of the maximal plateau levels attained in control samples, whereas a fall of only 30% in turbidity was produced in samples containing 250 mM (final concn.) inositol.

Further experiments in which inositol was added to the polymerising system at different equilibrium states showed that in all cases inositol affected the system in the same way and to the same extent (see table 7.1).

A plausible interpretation of these findings is that inositol in some way 'stabilises' the main species of microtubular protein involved in the subunit-polymer

Legend to fig. 7.8

A generalised scheme showing possible sites of interaction of inositol with various pools of microtubular protein involved in assembly and disassembly *in vitro*. The diagramatic representation of the microtubule-subunit equilibrium (top) does not distinguish between *in vitro* and *in vivo* models proposed for microtubule assembly. Dashed lines indicate processes that are postulated to occur *in vivo*. For further explanations see text in sections 7.3.2 and 7.3.3.

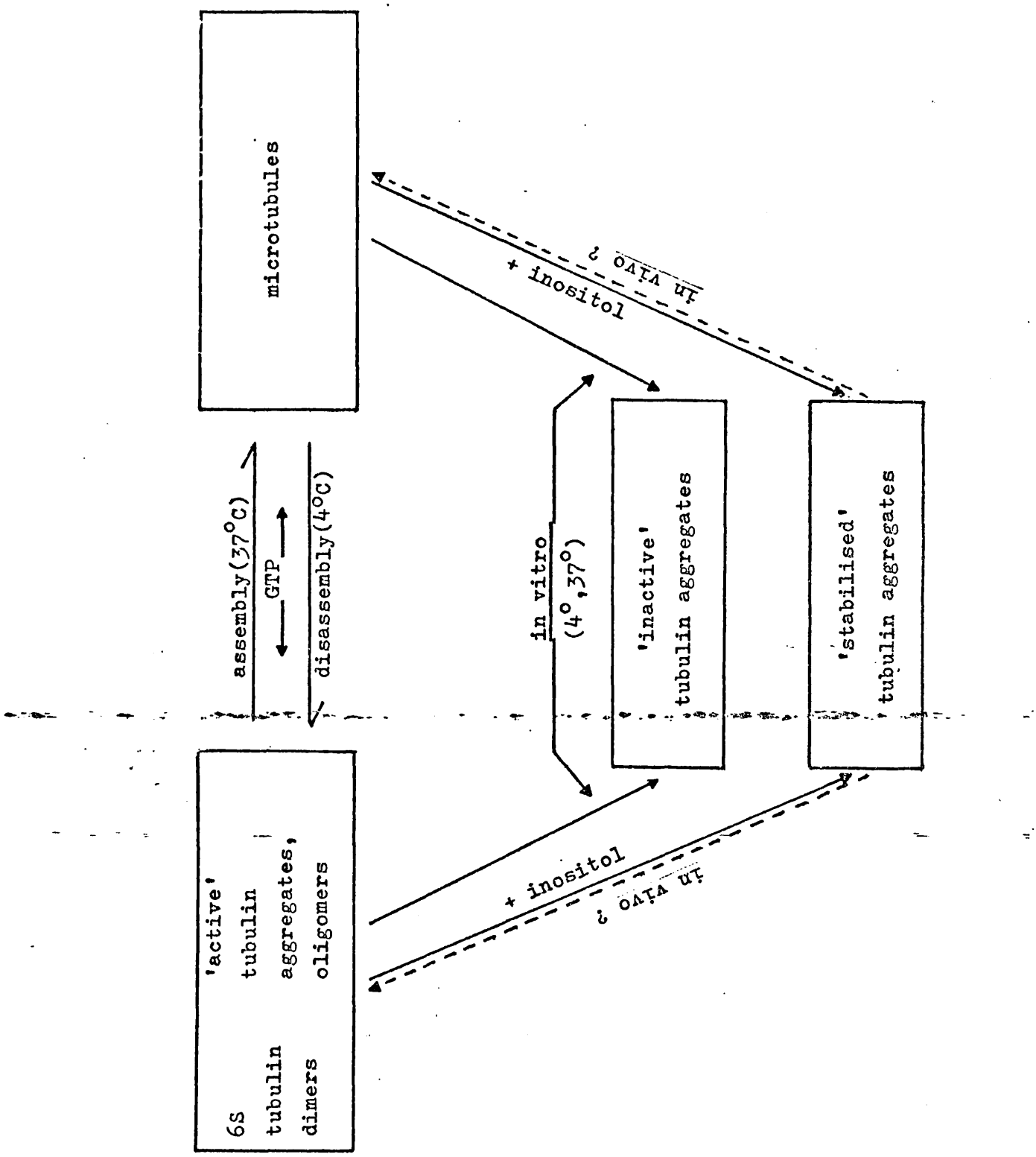

microtubules
assembly(37°C)
GTP
disassembly(4°C)
6S tubulin dimers
'active' tubulin aggregates, oligomers
in vitro (4°,37°)
'inactive' tubulin aggregates
'stabilised' tubulin aggregates
+ inositol
in vivo ?
+ inositol
in vivo ?

Fig. 7.8

Table 7.1 Inhibition of initial rates of assembly (V^+) and of disassembly (V^-) by inositol.

Cycle number	Sample	V^+	V^-	% inhibition V^+	% inhibition V^-
I	1	2.75	1.24	—	—
	2	2.73	1.25	—	—
	3	2.75	0.97	—	22.1%
	4	2.20	0.98	18.4%	21.6%
II	1	2.60	0.98	—	—
	2	1.80	0.72	31.8%	16.7%
	3	1.84	0.71	29.3%	17.0%
	4	1.79	0.73	31.2%	16.0%

Inositol (100mM final conc.) was added to sample 4 at 4°C before the first cycle (I), to sample 3 after reaching equilibrium at 37°C during first cycle, and to sample 2, at 4°C, prior to start of cycle II. Sample number I was the control and no inositol was added to it at any time. Protein concentration of samples - 1.0 mg/ml.

equilibrium, thereby reducing their availability for assembly or for disassembly of microtubules. In so doing, inositol would effectively promote the formation of a pool of 'stabilised' microtubular protein aggregates that do not readily equilibrate with 'active' forms of the protein participating in the polymerisation or depolymerisation processes. The preferential binding of inositol to assembly competent tubulin oligomers (see text relating to fig. 7.2) and the consistently observed protection by inositol of microtubules against cold-and calcium-induced depolymerisation, support the view that inositol can stabilise both microtubules and the intermediate aggregate species of tubulin with which they are in dynamic equilibrium. This interpretation of the results can be readily incorporated in a general scheme for microtubule assembly (Johnson and Borisy, 1975), as illustrated in Fig. 7.8, in which no assumption is made regarding the nature of the components present in the pool of 'active intermediates', though this would presumably include tubulin oligomers giving rise to the various disc- and spiral-shaped structures described in the literature (Johnson and Borisy, 1975; Gaskin, 1975), some of which can be readily differentiated from the 6S tubulin dimer fraction by gel permeation chromatography (see text for fig. 7.2).

7.4 Discussion

The results described above clearly show that myo-inositol interacts with polymerisable forms of micro-tubular protein and influences the extent to which it

participated in the temperature-dependent subunit-polymer equilibrium. Such effects could be of physiological significance, if one considers that the inositol concentrations employed were of the same order as those which are found in vivo (see text in 7.3.2).

The unusual behaviour of myo-inositol as a water-structuring compound (Suggett, 1975) suggests that some of the effects observed could result from an alteration in the state of hydration of microtubular protein, since this factor is apparently of great importance in entropically-driven polymerisation processes such as microtubule assembly (Gaskin, 1976; Caspar, 1966; Inoue and Ritter, 1975).

In attempting to assess the possible physiological significance of the present results, it seems noteworthy that stimulation of surface receptors in various cell types, where an increased turnover of phosphatidyl inositol ('PI effect') is observed (Michell, 1975), generally results in processes in which microtubules are apparently involved, for example, the re-distribution of surface receptors in plasma membranes (e.g. Nicholson, 1976; Oliver, 1975) and diverse secretory phenomena (e.g. Pipeleers et al, 1976; Sheterline et al, 1977). It has been suggested (Michell, 1975) that the primary significance of the 'PI effect' is related to the production of a metabolite (i.e. inositol or its phosphorylated precursors) mediating the effects of extracellular stimuli (Slaby and Bryan, 1976), although the cellular targets for the inositol metabolite have not been identified.

On the basis of the above considerations, it is

tempting to speculate that the relatively high intracellular levels of free inositol found in nervous and secretory tissues (Dawson and Freinkel, 1961; Wagner et al, 1976), which are richly endowed in microtubules, may reflect a unique and novel role for this compound in directly controlling the functional states of microtubules and hence, their participation in the regulation of various cellular activities. Thus, in this view, microtubules could act as a primary target for the inositol 'messenger' released as a consequence of the increased breakdown of phosphatidylinositol during cell stimulation ('PI effect').

One obvious objection to this suggestion is that the increase of intracellular levels of free inositol after stimulation would hardly be sufficient to affect microtubules. A possible way to overcome this difficulty is to visualise the inositol-containing lipid as being sequestered in a defined region on the inside of the plasma membrane, whereby a stimulus bringing about the breakdown of PI would result in a relatively high local concentration of free inositol (or of its phosphorylated precursors).

Obviously, an effect on the microtubular system could also arise from changes in the intracellular levels of free inositol not directly related to the metabolism of membrane-bound PI. In any case, the observation that a sharp rise in the inhibition of initial rates of assembly and disassembly occurs within a relatively limited range of inositol concentration increase, (fig.7.3), suggests that a relatively small and localised change in the levels of free inositol might be sufficient to affect

the functional state of microtubules.

In addition, it can be inferred from the binding data presented (section 3.1) that microtubules may also contribute to the intracellular compartmentation of inositol. Such a role could be of great importance in the light of recent evidence showing that (a) MTP-associated phospholipids, which are presumably derived from membranes, are enriched in a pool of metabolically-active phosphoinositides (Kirazov and Lagnado, 1976; Lagnado, 1977; Kirazov et al, 1977), and (b) that two of the key enzymes involved in the recycling of membrance phosphoinositides (Michell, 1975), are closely associated with microtubular protein (Daleo et al, 1976; Quinn, 1975). In this way, microtubules could also function in compartmentalising enzymes and substrates involved in the breakdown and re-synthesis of phosphatidylinositol (Lagnado, 1977; Kirazov et al, 1977; Schellenberg and Gillespie, 1977).

Chapter 8 General discussion

The experimental data obtained from separate series of experiments were discussed in some detail in the individual chapters. The purpose of this last chapter is to provide a short summary of the main conclusions arising from these results and to extend the discussion of some of the interpretations offered earlier. Possible ways of testing and perhaps resolving some of the problems arising from the work described will also be suggested.

8.1 General conclusions

The general conclusions which can be derived from this work are as follows:

1. A substantial proportion of the tubulin purified from brain by polymerisation is phosphorylated.

2. Tubulin purified from brain by polymerisation can serve as a substrate for a microtubule-associated protein kinase activity.

3. The ^{32}P which is extracted from *in vivo* labelled brain microtubulin with lipid solvents is associated with several major phospholipid classes.

4. Glycerol present during the polymerisation step of the purification of microtubular protein (by cycles of temperature-dependent polymerisation) brings about an enrichment in a particular class of tubulin that appears to be deficient in protein-bound, alkali labile phosphate.

5. Myo-inositol binds to tubulin and alters the stability of *in vitro* polymerised microtubules.

8.2 Phosphorylation of tubulin

The validity of the evidence showing that tubulin is a phosphoprotein has been a controversial issue for some time. Early work by Eipper (1972) suggested that chromatographically purified rat brain microtubulin contained covalently bound phosphate. However, it was not indicated if the phosphate was measured after removal of lipid phosphate from the protein preparation. Eipper (1972) went on to show that tubulin isolated from brain slices after their prolonged incubation with ^{32}P (11 h) contained bound ^{32}P after polyacrylamide gel electrophoresis. In later experiments Eipper (1974) showed the incorporation of ^{32}P into some serine residues derived from tubulin which had been phosphorylated *in vitro*, or once again in brain slices. The validity of these observations has been contested on the grounds that the isolated ^{32}P-tubulin could represent a partially denatured form of tubulin generated under the experimental conditions employed for its labelling and isolation (see, eg. Letterier et al, 1974).

The evidence presented by Reddington and Lagnado (1973) is more convincing in that these authors estimated the total covalently bound tubulin phosphate as acid- and lipid- solvent-stable, alkali-labile phosphate, which is approximately equivalent to serine-phosphate (see Reddington et al, 1973). No special care was taken to remove phosphoinositides, but it had been previously shown that these do not interfere with the estimation of alkali-labile phosphate (see 2.6.2).

In addition, the work of Piras and Piras (1974)

further supported the reality of the phosphoprotein nature of tubulin. These workers showed the incorporation of ^{32}P into tubulin derived from He La Cells cultured in ^{32}P containing medium. The purified tubulin was denatured with acid and extracted with lipid solvents; after S-PAGE 80 - 85% of the recovered radioactivity was associated with the tubulin bands.

The data presented in Chapter 3 can be viewed as further evidence for the phosphoprotein nature of tubulin. From these results, it is also apparent that when the nature of the phosphate bound covalently to tubulin is investigated, care should be taken to free the protein from phospholipid-phosphate which can account for as much as 80% of the organically bound, i.e. acid stable, radioactivity (see also Chapters 5 and 6).

Another major controversy concerns the in vitro phosphorylation of tubulin. It has generally been shown that chromatographically purified tubuulin can be phosphorylated on incubation with [γ-^{32}P] ATP (see e.g. Eipper, 1974; Lagnado et al, 1975; Letterier et al, 1974). The controversy concerns mainly the in vitro phosphorylation of tubulin purified by polymerisation.

Letterier et al (1974a) claim that tubulin purified by polymerisation cannot be phosphorylated under in vitro polymerisation conditions. Further they claim that the phosphorylation of chromatographically purified tubulin is an artefact due to the 'denatured' tubulin obtained by this procedure. This 'denaturation' of tubulin they explain by the loss of its ability to polymerise. However, it has been shown that if a solution of chromatographically purified tubulin was re-combined with

some of the fractions eluting at low salt concentration tubulin recovered its ability to form microtubules (Borisy and Murphy, 1975). Clearly some factors needed for tubulin polymerisation can be removed by ion-exchange chromatography and it is conceivable that these factors may play a part in providing the proper conditions for tubulin phosphorylation, but 'denaturation' is hardly the process occurring during chromatographic purification of tubulin.

The small proportion of tubulin which becomes phosphorylated under in vitro polymerisation conditions in the presence of $[\gamma\text{-}^{32}P]$ ATP (see Chapter 4 and Sloboda et al, 1975) suggests that there is hardly any turnover of tubulin-bound phosphate under *in vitro* conditions. It is possible that some of the non-phosphorylated tubulin co-purifying with the phosphorylated form, is responsible for the observed ^{32}P incorporation into tubulin.

The role of tubulin phosphorylation is presently unclear. Microtubule polymerisation *in vitro* was shown to be independent of phosphorylation, but the equilibrium between the monomeric and oligomeric tubulin forms was displaced toward the oligomers in the presence of added cAMP and ATP (Shigekawa and Olsen, 1976). Tubulin polymerisation *in vivo* has been shown to be independent of *de novo* tubulin synthesis and is a very fast process. Cyclic AMP or agents which increase its intracellular concentration have been demonstrated to enhance (Williams and Wolff, 1970) or inhibit (Goldstein et al, 1973) cell functions dependent on microtubules. Thus the cAMP-dependent phosphorylation of tubulin and tubulin-associated

HMW proteins provides a nice system for control of the initiation of microtubules and their stability as well as providing phosphorylated subunits for microtubule growth.

8.3 Fraction P_3

An interesting observation is that fraction P_3, obtained during purification of tubulin by polymerisation (see 2.2) is particularly enriched in phospholipid-bound ^{32}P. This fraction also contains cold-stable tubulin aggregates. So far, P_3 was usually discarded and very little is known about it. However, in view of its high phospholipid ^{32}P and tubulin content, P_3 may prove to be a key fraction the investigation of which may yield interesting information on the nature of MTP-phospholipid interactions.

Recent electron microscopic observations by Delacourte et al (1977) showed that this fraction (P_3) contains disklike and filamentous tubulin aggregates. Further, this group of workers observed that treatment of P_3 with 1M NaCl resulted in the release of a substantial amount of tubulin, concomitantly with the disappearance of the disklike tubulin aggregates, but the filamentous tubulin aggregates remained unaffected. If these tubulin filaments are not artefactually produced during the purification procedure, it is very attractive to speculate that they are derived from an *in vivo* existing filamentous structure, partaking in the microtubule-membrane interactions.

Clearly the P_3 fraction must become the object of

more specific investigations. For example, the differential release of tubulin by proteolytic enzymes or salt treatment or by extraction with detergents could help in distinguishing between tubulin aggregates interacting with phospholipid structures through polypeptide intermediates (HMWs or tubulin filaments) and tubulin which is tightly bound, integrated, to the phospholipid structures. Another way in which the question about the nature and especially about the specificity of microtubule-phospholipid interactions could be tackled is by studying the interaction between microtubulin, in the form of microtubules or their depolymerisation products, with liposomes of known phospholipid constitution under varied ionic and 'cofactor' conditions.

8.4 Microtubular and storage tubulin

Another interesting question emerges from the interpretation of the data presented in Chapter 6, as summarised in figure 6.4. The validity of some of the assumptions can be tested chemically by examining the distribution of total protein, phospholipid-and protein-bound phosphate in the individual MTP fractions prepared in the presence (+) or absence (-) of glycerol. A decisive result could be obtained if the phosphorylated form(s) of tubulin could be separated from the non-phosphorylated one(s) in order to estimate their relative distribution.

A suitable model for testing some of the implications of the above-mentioned explanatory scheme (see fig. 6.4 and 6.4) can be suggested from the recent work of Lasek

and Hoffman (1976) regarding the axonal transport in rat motor neurons. These authors observed that axonal transport in these neurons has three characteristic components; a fast one and two slow components, designated as SCa and SCb. A difference in the export rates of SCa and SCb was observed (Hoffman and Lasek 1975), SCb moving slightly faster than SCa. Tubulin has been shown to be the major constituent of SCa and almost exclusive to SCb (Grafstein and Murray, 1969; Hendrickson and Cowan, 1971 and Lasek and Hoffman, 1976). Studies on regenerating rat ventral motor neurons showed an increase in the amount of material transported in SCb (Hoffman and Lasek, in prep.). On this basis, Lasek and Hoffman proposed that the tubulin exported in the faster, SCb, component presumably plays a role in axonal outgrowth. According to the assumptions made in Chapter 6 (see also scheme in fig. 6.4) the tubulin in SCb can be expected to be transported along the axon in the form of a membrane bound storage structure, which should contain predominantly non-phosphorylated tubulin. On arrival at the site of utilisation, the tubulin from the storage structures could be phosphorylated, possibly by the tubulin-associated kinase, and rendered ready for incorporation into microtubules or other functional tubulin structures (bound to or intrinsic to membranes). Double labelling experiments, employing the techniques described by Lasek and Hoffman (1975), should provide conclusive evidence.

8.5 Tubulin-inositol interactions

Clearly, further and better defined experiments are needed to investigate the observed interactions between tubulin and myo-inositol (Chapter 7). The interactions, if any, between tubulin and the phosphorylated derivatives of myo-inositol, i.e. inositol phosphate and cyclic inositol phosphate, have to be investigated. Even if such interactions were to be found, this would not exclude the possibility that microtubules may participate in the spatial organisation of substrates and enzymes involved in phosphatidylinositol metabolism, as suggested in 7.4. A good indication for the specificity of myo-inositol-tubulin interactions would be the absence of any interactions between tubulin and the optical isomer of myo-inositol - scyllo-inositol.

8.6 Microtubule-membrane interactions

The microtubule-membrane interactions, suggested previously and in this work, could serve multiple purposes in the living cell. Firstly, it is possible that the initiation, growth and stability of microtubules can be spatially and temporally controlled by the appearance of microtubule [illegible] sites on the inside of the plasma membrane, or on intracellular membranes, during growth, differentiation and variety of physiological changes and processes in the cell. Secondly, microtubules appear to govern, at least partially, plasma membrane activities and topography in differentiated cells. Finally, microtubules could participate in maintaining the ordered metabolism of membrane constituents and in particular of phosphoinositides.

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FUNCTIONAL ASPECTS OF MICROTUBULES

STUDIES ON THE PHOSPHORYLATION OF BRAIN MICROTUBULE PROTEIN AND MICROTUBULE-ASSOCIATED PHOSPHOLIPIDS

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Summary. The incorporation of ^{32}P into rat and chick brain microtubules *in vivo* was investigated. More than half of the total acid-insoluble (bound) radioactivity in microtubules isolated by reassembly *in vitro* was recovered as phospholipid P, most of which was associated with phosphatidylethanolamine and phosphatidic acid. Most of the remaining ^{32}P could be accounted for as protein-bound phosphoserine P (alkali-labile) associated with tubulin. Attempts to determine the distribution of bound ^{32}P in the 36S and 6S components of cold- or calcium-depolymerised microtubules from chick brain, after chromatography on Sepharose 6B or high-speed centrifugation, showed that the 36S component contained the bulk of the bound ^{32}P. However, a significant part of the label found in this fraction was apparently associated with minor high-MW protein components (as phosphoserine P and phospholipid P) which could be separated from 36S tubulin aggregates during re-chromatography in the presence of 0.75 M NaCl. It is proposed that the considerable metabolic activity of microtubule-bound ^{32}P seen *in vivo* may reflect some aspect of the various membrane-associated phenomena in which microtubules have been implicated, in addition to any more direct role that phosphorylation may have in controlling microtubule assembly.

1. Introduction

Tubulin, the main subunit protein of microtubules, constitutes up to one quarter of the total soluble protein of brain homogenates, where it appears to be derived mainly from cytoplasmic microtubules. Recent ultra-structural studies also show that axonal microtubules in cerebral cortex of mammalian brain extend right into nerve endings where, in association with clusters of synaptic vesicles, they are sometimes seen to terminate onto dense projections of the presynaptic membrane[1,2]. This would be in keeping with earlier biochemical evidence for the presence of tubulin in the soluble fraction of isolated nerve-ending particles (synaptosomes)[3,4]. However, a significant proportion of the tubulin present in nervous tissue is also tightly bound to membrane fractions that are derived primarily from isolated nerve-ending particles[3-7]. In fact, tubulin has now been more specifically identified as the major protein component of the subsynaptic dense material characteristic of dendritic spines from type 1 synapses[6,8,9].

Taken together, these observations suggest that microtubular proteins may participate, more directly than was originally anticipated, in mediating or in regulating membrane-dependent physiological processes associated with synaptic

function. Clearly, the extent to which microtubules or their subunits are involved in these, or in several other membrane-associated phenomena in non-neural cells (see, e.g., ref. 11) would depend on their ability to form more or less transient functional associations with other soluble or membrane-bound cellular constituents. That such interactions do in fact occur is evident from a number of recent observations (see, e.g., refs. 10, 12, 13), and it was recently proposed that the state of phosphorylation of microtubular protein is an important factor in the control of microtubular assembly and/or function in nervous tissue (see evidence summarised in ref. 10). The aim of the experiments described in this paper was to provide further evidence that brain microtubules are phosphorylated *in vivo* and to determine more precisely the nature of the phosphorylated components labelled *in vivo* that were derived from reassembled microtubules.

2. Chemical nature of ^{32}P incorporated into brain microtubules *in vivo*

Reassembled microtubules isolated from rat and chick brain labelled *in vivo* with $^{32}P_i$ contained appreciable amounts of acid-insoluble radioactivity (bound ^{32}P). In general, about 70% of the bound cpm in disassembled microtubular preparations from chick brain, and somewhat less from rat brain, was extractable with 2 : 1 (v/v) $CHCl_3$–MeOH (Tables 1 and 2); similar results were obtained when acidified $CHCl_3$–MeOH was used as lipid solvent (data not shown). Over 80% of the acid-insoluble ^{32}P remaining after extraction with lipid solvents was present as alkali-labile P, derived most probably from protein-bound serine residues (see refs 10 and 14 for details of procedures used to identify phosphoserine P in protein): this will be referred to as protein-bound P. Less than 5% of the counts remaining in the alkali-stable fraction could be attributed to nucleic acids.

It was found in preliminary experiments that most of the bound ^{32}P present in the crude lipid extracts from unfractionated microtubules or from purified 36S microtubular subfractions could be recovered in the phosphatidylethanolamine and/or phosphatidic acid fractions separated by one- and two-dimensional thin-layer chromatography on silica gel H according to the method of Yagihara *et al*[15]. Further experiments to characterise microtubule-associated phospholipids labelled *in vivo* are being carried out in collaboration with Professor J. N. Hawthorne (Nottingham University, England), but it is apparent from the results obtained so far that none of the label present in the phospholipid fraction could be attributed to phosphoinositides or to phosphatidylserine.

Protein-bound ^{32}P in 2× polymerised microtubules or in chromatographically purified 36S tubulin aggregates (see Table 2 and text below) isolated from *in vivo* labelled chick brain appears to be associated with a unique acidic phosphopeptide which was seen in peptide maps of samples digested with trypsin (preliminary observations done in collaboration with P. Dunkley, Institute of Psychiatry, London).

These results indicate that (specific) protein-bound phosphoserine residues and specific classes of phospholipids associated with brain microtubules exhibit considerable metabolic activity *in vivo*, as could be anticipated from earlier studies of microtubule phosphorylation in tissue slice preparations [10,23].

The full significance of these findings cannot be properly assessed, however, until more is known regarding the protein-P and phospholipid-P contents of microtubules *in situ* and of the extent to which this may be regulated by associated enzymes.

3. Electrophoretic analysis of *in vivo* labelled brain microtubules.

It was previously observed that a significant proportion of the radioactivity present in the microtubular fraction of ^{32}P-labelled slices from guinea pig cerebral cortex was associated with minor high-MW components ('HMW') seen after electrophoresis in SDS-polyacrylamide gels [10]. A similar distribution of ^{32}P has now been found for *in vivo* labelled microtubular preparations (1× polymerised) derived from rat and chick brain (Fig.1).

In these preparations, a number of additional minor components migrating between tubulin (TU) and HMW fractions were also labelled; in chick brain, some labelling was also associated with a minor stained component migrating ahead of the β-tubulin fraction. In addition, a diffuse region of radioactivity of varying intensity was consistently observed to migrate ahead of the dye front (see also refs. 7 and 10), a region which does not stain for protein but contains an excess of SDS.

In an effort to assess the nature of the ^{32}P present in the main labelled fractions [i.e., in HMW, (α;β) tubulin, and 'SDS front'], these were eluted electrophoretically from the appropriate slices pooled from 6–7 gels (run in parallel) after fixation and staining of proteins with Coomassie Blue; the eluates were treated with ice-cold 10% TCA (in the presence of carrier albumen) and then as indicated in the legend to Table 1. It was consistently found that most of the ^{32}P associated with the HMW and tubulin fractions was recovered as protein-

bound P (alkali-labile), while that associated with the SDS front, which accounted for about 10× as much ^{32}P as was eluted from the other fractions, was mainly recovered as phospholipid P and acid-soluble P.

The specific activities of protein-bound P in the eluates of the HMW and tubulin fractions were found to give very similar values, an observation that is apparently at variance with the impression gained from the data shown in Fig.1. Furthermore, the observation that most of the ^{32}P in the HMW fraction appeared as alkali-labile P does not seem to support an earlier suggestion that ^{32}P–HMW represents a phosphorylated intermediate of the Ca^{2+} (Mg^{2+})-ATPase activity associated with brain microtubules[10], since this would be expected to behave as acid-stable, alkali-stable P.[14] However, further more direct experiments are needed to clarify these points.

In connection with the above data, it was found that when *in vivo* labelled microtubules were polymerised from a 50% ammonium sulphate precipitate of the initial high-speed supernatant fraction (S) of brain, an additional, highly

Table 1.

The incorporation *in vivo* of ^{32}P into the protein and lipid fractions of microtubules isolated from rat brain.

Rat brains were labelled *in vivo* for 2 h after intraventricular injection of $^{32}P_i$ (400 μCi/animal) and MT were isolated by 1× polymerisation in the presence of glycerol and GTP[17] from either the initial high-speed supernatant (S) or from the ammonium sulphate fraction of S (same expt.) precipitating between 0–50% saturation ($S_{0.5}$-ppt.), as indicated. $S_{0.5}$-ppt. was redissolved in reassembly buffer to give protein concn. (ca. 5 mg/ml) similar to that of S. Protein-bound ^{32}P was determined as acid-insoluble alkali-labile P (approximately equivalent to bound serine P) in cold 10% TCA-precipitated and 2× washed material, which had been 2× extracted at room temperature with 2:1 (v/v) CHCl–MeOH.[10,14] The combined organic solvent extracts in which about 80% of the ^{32}P was present as phospholipids was used without further fractionation to determine ^{32}P-phospholipid counts. Protein was determined in samples prior to precipitation with TCA by the method of Lowry *et al.*[22] Values shown represent means of triplicate determinations from a single representative expt.

Starting material	Bound ^{32}P		Distribution of ^{32}P	
	protein	lipid	protein	lipid
	cpm/μg protein		%	
S	16.7	5.9	74	36
$S_{0.5}$-ppt.	36.2	28.0	57	43

*cpm recovered from acid-insoluble residue

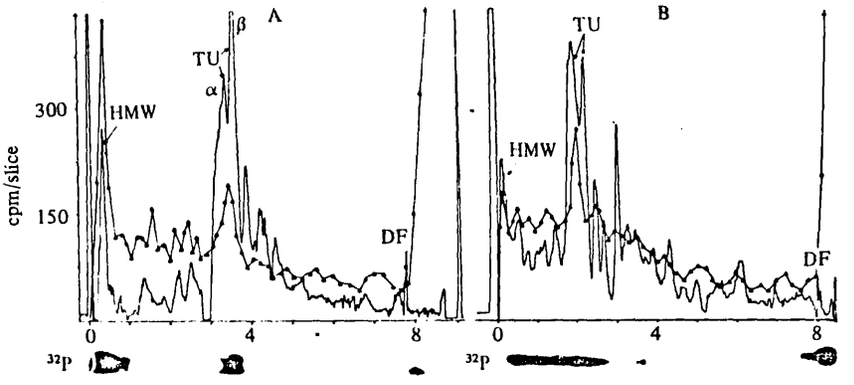

Fig. 1. Electrophoretic distribution of ^{32}P incorporated into rat (A) and chick (B) brain microtubular components *in vivo*.
Samples of microtubules (150–200 μg protein) isolated by one cycle of assembly-disassembly from rat (A) and chick (B) brain labelled *in vivo* with $^{32}P_i$ (ca. 400 and 600 μCi per animal, respectively) were denatured and separated in SDS-polyacrylamide gels in the presence of 4 M urea as previously described[10]. Graphs show ^{32}P cpm counted on 1 mm slices of gels that were fixed and stained for protein with Coomassie Blue; continuous lines are densitometric scans of intact stained gels. Radioautographs of gels run in parallel (prepared as described in ref. 10) are shown below (^{32}P). Numbers on abscissa indicate gel length in cm, measured from origin on left; DF = dye front. Smearing on radioautographs is due to overexposure of gels that were overloaded to detect minor ^{32}P-labelled components. Note high labelling seen in region ahead of dye front.

labelled protein component, MW ca. 48 000, copolymerised with microtubular protein, as illustrated for rat brain ('LMW', Fig.2). This was not seen in microtubular preparations precipitated with ammonium sulphate after reassembly directly from the initial supernatant. In fact, the intense labelling seen in the 'LMW' component and in other, slower-migrating components that are concentrated in microtubular samples polymerised from the $S_{0,5}$-ppt. nearly obscured the labelling of tubulin detected by radioautography (see Fig.2). However, the estimated specific activity of the 'LMW' fraction, as determined after elution (see above), was found to be similar to that of the tubulin fraction.

The data shown in Table 1 also reveal considerable enrichment in ^{32}P-phospholipids in microtubules reassembled from ammonium sulphate precipitated material. However, the yield of polymerisable protein prepared under these conditions was considerably less (1–2%) than that from the unfractionated supernatant fraction, from which about 10% of the protein was recovered in the microtubular pellet.

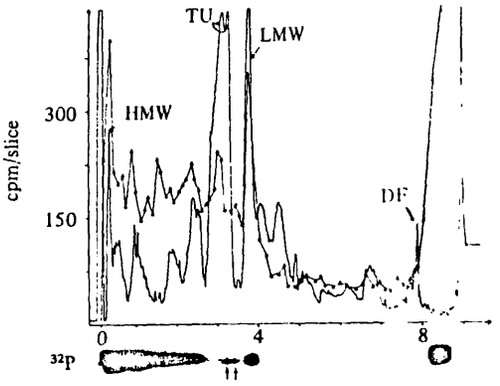

Fig. 2. Electrophoretic distribution of ^{32}P incorporated *in vivo* into rat brain microtubules.
Experimental details as given in Fig. 1, except that microtubules were isolated from 50% ammonium sulphate precipitate of initial high-speed supernatant (see Table 1 and text). LMW = prominent labelled component not evident in microtubules isolated from unfractionated supernatant (cf. Fig. 1A). Note also concentration of several labelled components between HMW and tubulin (TU) as compared with data shown in Fig. 1A. Arrows on radioautograph indicate relatively weak labelling associated with α and β components of tubulin (TU). For further details see text.

The significance of this observation is not apparent, although it is interesting that *in vivo* labelled microtubular protein from guinea pig brain is also enriched in a similar faster-migrating ^{32}P-protein when it is precipitated directly from the supernatant fraction with high concentrations of Vinblastine or Vincristine ($>10^{-4}$ M) (J.R.L., unpublished observations).

4. The distribution of ^{32}P in subfractions of *in vivo* labelled microtubules from chick brain.

An obvious limitation to the experiments described in the preceding section is that it was not possible to distinguish between tubulin derived from the 36S aggregates corresponding to the 400 nm ring-like structures seen by electron microscopy and the 6S form of tubulin ($\alpha;\beta$ dimer) which are both present in cold- or calcium-depolymerised microtubules isolated from *in vitro* reassembled brain microtubules [16-18].

It was recently shown that these two components, which were initially characterised on the basis of their sedimentation properties [18,19], can be separated by chromatography on Sepharose 6B [17-19] to give a first peak, eluted in the void volume, which consisted mainly of 36S 'ring' structures that are readily polymerised at 37°C in the presence of GTP, and a second peak, containing

essentially pure tubulin dimer, which polymerises less readily[17] or not at all[18]. Although tubulin was the major protein component found in both peaks (as seen by SDS-gel electrophoresis), the 36S component was shown to be enriched in a number of minor components, the main one corresponding to the HMW fraction discussed above[17].

In addition, it was shown that the 36S component could be reversibly disaggregated into the 6S component in the presence of high salt concentrations[18,19]; under these conditions, a minor heat-stable protein ('tau factor') that is apparently essential for polymerisation can also be released[20].

It is not yet clear to what extent the 36S and 6S forms of tubulin found in depolymerised microtubules are both involved in the assembly process *in vivo*; nor is it apparent how the state of phosphorylation of microtubular protein, or of its associated phospholipids, which may reflect the metabolic turnover of phosphotubulin components[10], could be involved in regulating the assembly and/or functions of microtubules. It has been suggested, however, that the state of aggregation of microtubular protein into microtubular structures or into other forms of aggregates, may determine the extent to which tubulin incorporates ^{32}P from [γ-^{32}P]-ATP through its associated intrinsic kinase activity *in vitro*, or in brain slices during incubation with $^{32}P_i$ [10]. More recently, it was shown that the 36S component of disassembled microtubules from pig brain is the preferred P-acceptor during incubation *in vitro* with ^{32}P-ATP:[21] this has now been confirmed in our laboratory (J.R.L., unpublished observations).

In the present work, we have carried out exploratory experiments to investigate the extent of incorporation of ^{32}P into the 36S and 6S components of chick brain microtubules labelled *in vivo*. In addition, we have attempted to separate the minor HMW components, which were found to be nearly exclusively associated with the 36S fraction, in order to facilitate characterisation of the ^{32}P incorporated in this fraction. The results of these experiments which are shown in Table 2 and in Figs. 3 and 4, can be summarised and interpreted as follows.

When cold- or Ca^{2+}-depolymerised microtubules were fractionated by chromatography on Sepharose 6B (Expts. 1 and 3A), most of the protein-bound ^{32}P recovered was found in the peak fraction containing 6S tubulin, while most of the phospholipid-^{32}P recovered was associated with the fraction enriched in the 36S 'ring' fraction (peak I) (see also data for Expt. 4, Table 2). However, only about 20% of the protein was recovered in the 36S fraction (see also Fig. 4A), even though the concentration of protein in samples before separation (MT) was

such that over 60% of the protein was expected to be present as 36S aggregates [15]. This suggests that in both experiments, a substantial proportion of the 36S material was disaggregated through dilution during chromatography [17] and/or that only a more stable form of the 36S component can remain intact under these conditions, as was originally suggested by Erickson [17]. Thus, the tubulin present in the 6S (peak II) fraction is probably composed of a mixture of protein originally present as 6S tubulin and of tubulin derived through disaggregation from 36S structures. This could account for the observation that the specific activity of protein-bound ^{32}P in the 36S fraction was lower, as compared to that for the 6S fraction, than might have been anticipated on the basis of results obtained *in vitro* [21] (see below).

Table 2.

The distribution of ^{32}P in subfractions of *in vivo* labelled microtubules from chick brain.

^{32}P microtubules were isolated from 1-day old chick brain (7–20 animals per expt.), 2 h after intracerebral injection of $^{32}P_i$ (200–600 μCi/animal). ^{32}P incorporated into protein and lipid fractions was determined as indicated in legend to Table 1 on the TCA-insoluble residues of microtubules that were prepared and fractionated as indicated below. Further explanations of experimental design and of nomenclature of fractions are given in text.

Expts. 1 and 2: 1× polymerised microtubules were disassembled in the cold; the solutions obtained after centrifuging for 60 min at 10^5g (MT, 5–12 mg protein/ml) were fractionated at 4°C on Sepharose 6B essentially as described by Erickson [17], except that in Expt. 2 MT samples were treated with 0.75 M NaCl (MT + NaCl) before chromatography on columns that were equilibrated and eluted with buffers containing 0.75 M NaCl. Elution patterns for total ^{32}P and for proteins (measured at 280 nm) in Expt. 1 were similar to those shown for Expt. 3 in Fig. 4A. Protein patterns of peak I and peak II of Expt. 2 are shown in Fig. 3.

Expt. 3: 2× polymerised microtubules were disassembled for 30 min at 4°C in MES-Ca^{2+} buffer (supplemented with 1 mM $CaCl_2$) before chromatography on Sepharose 6B equilibrated in the cold with MES-Ca^{2+} buffer, essentially as described by Weingarten *et al.* [19] In 3B, concentrated peak I ("36S") material of 3A containing 0.75 M NaCl ("36S + NaCl"; 1.8 mg protein/ml) was separated on Sepharose 6B in the presence of 0.75 M NaCl and analysed as described in Fig. 4 and text.

Expt. 4: 2× polymerised microtubule preparations were isolated as in Expt. 3 except that the final disassembly step was carried out at 15°C in presence of MES-Ca^{2+} buffer supplemented with 1 mM GTP and 1 mM $CaCl_2$ (MT; 10.5 mg protein/ml). The pellet fraction (4A, "36S") obtained after centrifuging MT for 190 min × 10^5g (at 8–10°C) [19] was resuspended in the cold in disassembly buffer containing 0.75 M NaCl ("36S + NaCl"; 4.7 mg protein/ml) and centrifuged as indicated above. The resulting pellet contained appreciable amounts of tubulin (denatured aggregates?) in addition to HMW ("HMW + T"), as revealed by SDS-gel electrophoresis (data not shown).

The high specific activity in the phospholipid fraction associated with the 36S component could reflect the preferential association of metabolically active phospholipids with a more stable form of 36S tubulin, or, alternatively, the presence of HMW material, which indeed appears to be exclusively associated with peak I material (see Fig. 4A). The relatively high specific activity of phospholipids associated with the 36S component derived from Ca^{2+}-depolymerised material (cf. Expt. 3A and 1) was generally confirmed (Expt. 4 and other data, not shown), but the significance of this observation remains unclear.

The possible significance of the labelling seen in the HMW material that co-chromatographed with 36S tubulin was further investigated in experiments in which the 36S component was disaggregated with NaCl [19] before chromatography.

Expt.	Fraction analysed	% Protein recovered	^{32}P incorporated		Distribution of ^{32}P	
			protein	lipid	protein	lipid
			cpm/μg protein (%)*		%**	
1	MT		10.8	27.4	34	66
	'36S' (peak I)	23	4.6 (34)	39.3 (77)	19	81
	'6S' (peak II)	77	3.4 (66)	4.4 (23)	29	71
2	MT + NaCl		12.3	37.6	29	71
	'HMW' (peak I)	11	28.4 (42)	31.7 (20)	49	51
	'36S + 6S' (peak II)	89	4.7 (58)	14.9 (80)	27	73
3A	MT		38.5	47.5	45	55
	'36S' (peak I)	20	11.6 (14)	128.0 (87)	13	87
	'6S' (peak II)	80	18.6 (86)	4.7 (13)	78	22
3B	'36S + NaCl'		as for '36S' in Part A			
	'HMW + T' (peak Ia)	n.d.	n.d.(4)	n.d.(8)	21	79
	'HMW + T' (peak Ib)	n.d.	n.d.(3)	n.d.(11)		
	'36S – T' (peak Ic)	(60)†	27.7 (93)	27.5 (81)	44	56
4A	MT		6.0	28.7	17	83
	'6S' (supnt.)	10	2.9 (8)	7.1 (3)	29	71
	'36S' (pellet)	90	3.6 (92)	25.0 (97)	13	87
4B	'36S + NaCl'		as for '36S' in Part A			
	'6S' (supnt.)	36	3.9 (66)	6.2 (22)	38	62
	'HMW + T' (pellet)	64	1.1 (34)	12.8 (78)	8	92

*Values in brackets = % of recovered cpm in fraction.
**cpm recovered for protein + lipid in individual fraction = 100%.
†Estimated as a percentage of the protein applied to the column. In most experiments, 50–70% of the protein applied was recovered in combined eluates.

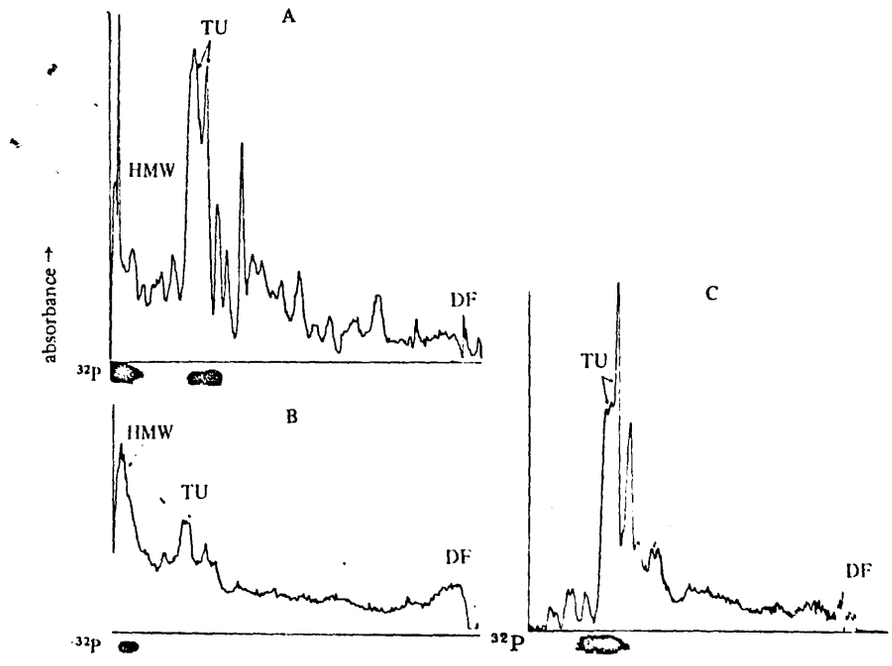

Fig. 3. Electrophoretic distribution in SDS-polyacrylamide gels of proteins and ^{32}P in chick brain microtubular components labelled *in vivo*.
Densitometric scans and ^{32}P radioautographs (from same gel) shown for unfractionated microtubules (A) and for subfractions obtained after dissociation of 36S microtubular component with 0.75 M NaCl (B and C). For details of preparation see legend for Expt. 3B, Table 2 and text. Apparent heterogeneity of tubulin and radioactivity in C is artefact due to overloading.

In Expt. 3B, the 36S fraction was concentrated by vacuum dialysis in the presence of NaCl and rechromatographed on Sepharose 6B in the presence of NaCl (Fig. 4B). Under these conditions, the material present in the first two peaks of radioactivity eluted (fractions Ia, Ib) gave identical protein patterns after electrophoresis in SDS-gels (data not shown): HMW was present together with some tubulin. A further broad and apparently complex peak of radioactivity (Ic) was eluted in a region coinciding roughly with the elution volume of the 6S component: pooled fractions from this region, which accounted for most of the recovered protein (Table 2, Expt. 3B), were almost exclusively composed of tubulin derived, presumably, from the 36S tubulin aggregates separated in Expt. 3A (see also Fig. 4A). Thus, a partial separation of HMW from 36S tubulin was achieved. It is evident from the limited data shown in Table 2

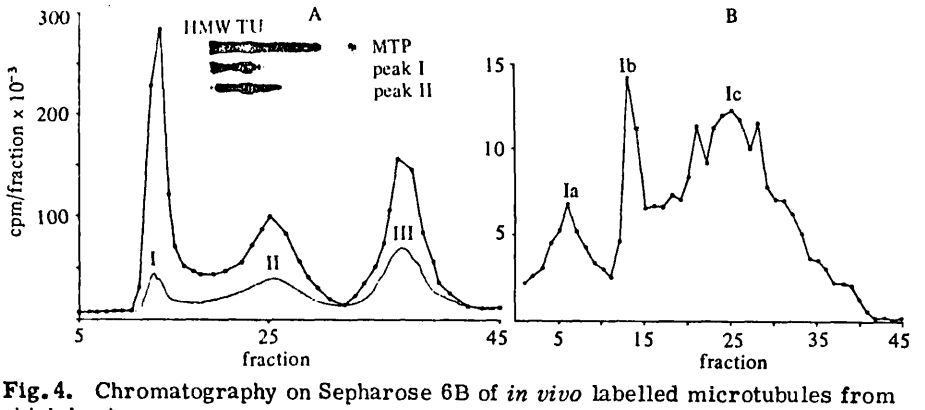

Fig. 4. Chromatography on Sepharose 6B of *in vivo* labelled microtubules from chick brain.
(A) ^{32}P-microtubules were depolymerised in the presence of calcium and fractionated on Sepharose 6B in the absence of NaCl, as indicated for Expt. 2A, Table 2. Total ^{32}P cpm (solid line) in 1.5 ml eluate fractions is shown together with absorbance recorded at 280 nm (dotted line). Inset shows SDS-gel protein patterns for samples of unfractionated microtubular preparation (MTP) and of material pooled from peak I (fractions 13–15) and peak II (fractions 23–27). HMW = high MW components; TU = tubulin. Note absence of HMW in peak II material. Total protein loaded on column: 14 mg.
(B) Re-chromatography on Sepharose 6B of peak I material of A that was concentrated by vacuum dialysis and chromatographed in the presence of depolymerisation buffer containing 0.75 M NaCl as indicated in legend for Expt. 2B, Table 2. For further explanations, see text.

(Expt. 3A) that most of the bound ^{32}P recovered as protein-bound P or as phospholipid P was present in the main tubulin fraction (peak Ic). Since, however, too little material was present in peak fractions containing HMW (Ia, Ib, see Fig. 4B) to permit reliable estimations of protein by the Folin–Lowry method, it was not possible to determine specific radioactivities for ^{32}P in these fractions. Nonetheless, it can be tentatively concluded on the basis of the data giving the percentages of radioactivity recovered that the fractions enriched in HMW are also enriched in ^{32}P-phospholipid (see discussion of Expt. 3A above).

In Expt. 2 (Table 2, Fig. 3), the possibility of separating HMW from both forms of tubulin was investigated by chromatography of cold-depolymerised microtubules which had been treated with 0.75 M NaCl to dissociate 36S tubulin aggregates, prior to chromatography on columns of Sepharose 6B which were equilibrated and eluted in the presence of 0.75 M NaCl. It was anticipated that this procedure would result in all the tubulin being eluted as 6S tubulin, after elution of the HMW fraction in the void volume, as had been the case when it was present together with the undissociated 36S component (see Fig. 4A). This

was found to occur, as shown in the SDS-gel electrophoresis patterns of ^{32}P-labelled material which behaved similarly during elution to the 36S and 6S components (data not shown): the first peak eluted now contained mainly HMW, while most of the protein present in peak II was identified as tubulin, as shown in Figs. 3A and 3B respectively. Moreover, it is evident from the radioautographs shown (Fig. 3) that virtually all the label detected after electrophoresis of peak I material was associated with several HMW components near the origin, while the label found in peak II material co-migrated almost exclusively with the tubulin fraction. The data given in Table 2 (Expt. 2) show that under these conditions the HMW fraction was considerably enriched in bound ^{32}P, especially in respect of protein-bound P.

Finally, the results of an experiment in which the 6S and 36S components of tubulin were separated by high-speed centrifugation[19] (Expt. 4A, Table 2) showed that most of the protein was recovered in the pellet fraction which was expected to contain tubulin, present mainly as 36S aggregates. This fraction also contained most of the bound ^{32}P and was particularly enriched in ^{32}P-phospholipids. Although the bulk of the protein-bound ^{32}P was also found in the pellet fraction, the specific activities for P-protein were remarkably similar in the supernatant and pellet fractions. The pellet fraction, which contained tubulin and HMW in about the same proportion as in the unfractionated material (MT) (data not shown), was resuspended in buffer containing 0.75 M NaCl and re-centrifuged at high speed. It was anticipated that in this way HMW components, together with some non-specific tubulin aggregates, might be recovered in the pellet, while tubulin derived from dissociated 36S aggregates would remain in solution. This was confirmed by SDS-gel electrophoresis, though an appreciable amount of tubulin was also found in the pellet fraction (denatured tubulin aggregate?). The data shown in Table 2 (Expt. 4) indicate, once again, that the HMW-enriched pellet fraction accounted for the bulk of ^{32}P-phospholipid (see also Expts. 2 and 3B). On the other hand, the supernatant was considerably enriched in ^{32}P protein, despite the absence of any HMW component (cf. Expt. 2). One feature worth noting about this experiment is that the concentration of protein remained high enough throughout to maintain the 36S $\rightleftharpoons$ 6S equilibrium in favour of the 36S species[18,19]; this is in contrast to the situation which obtains during the separation of 6S and 36S components on Sepharose 6B, when dilution of the sample during chromatography tends to favour disaggregation of the 36S species (see above).

5. Conclusions

The evidence presented above indicates that brain microtubules contain protein-bound P and are associated with a fraction of phospholipids, both of which exhibit considerable metabolic activity *in vivo*. This confirms and extends earlier evidence based on studies of microtubule phosphorylation *in vitro* and in tissue slice experiments [10,13,23]. A more precise interpretation of these findings must obviously await further information concerning the P contents of the various components present in microtubules isolated by reassembly *in vitro*, from which labelling due to turnover of bound P and to net phosphorylation might be distinguished. Nevertheless, it was consistently observed that the 36S component of depolymerised microtubule, which consists largely of tubulin polymers stabilised into the ring-like structures, was preferentially labelled *in vivo* under various experimental conditions as compared to the 6S tubulin component. The relative enrichment of the 36S component in metabolically active phospholipid suggests that 36S structures may be loosely associated, *in situ*, with membrane fractions (e.g., plasma membrane) at sites of microtubule–membrane interactions. Indeed, evidence for a close association between enzymes involved in phospholipid metabolism (e.g., diglyceride kinase) and microtubular subunits was recently reported[13]. The possible relationship between microtubules and membrane-associated changes in phospholipid metabolism in functionally active nerve-ending preparations from cerebral cortex should prove a worthwhile area of investigation, especially in view of the recent discovery[1] that microtubules are indeed present in presynaptic terminals *in situ* (see Introduction).

The relatively high incorporation observed of ^{32}P into phosphoserine residues of the 36S component may indicate that any regulatory factors controlling the conversion of 6S tubulin to the 36S form, from which microtubules are apparently polymerised [17,18], might be acting, in part at least, as regulators of enzymes concerned with the turnover of protein-bound P. In this connection, our observation that a highly labelled minor protein (HMW), originally seen by SDS-gel electrophoresis, can be separated from salt-dissociated preparations of the 36S component by chromatography may be of interest in the light of recent work[20], showing that a tightly bound, heat-stable protein ('tau factor') apparently required in microtubule assembly can also be released from 36S tubulin preparations by high concentrations of NaCl.

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INTERACTIONS OF MYO-INOSITOL WITH BRAIN MICROTUBULES

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1. Introduction

Labelling experiments *in vivo* with $^{32}P_i$ have shown that a significant proportion of the radioactivity incorporated into brain microtubules was present in a phospholipid fraction purified from chloroform/methanol extracts of the isolated protein (1). Further, in short-term (2h) labelling experiments, between 25 and 40% of the ^{32}P radioactivity present in purified microtubule-associated phospholipids was recovered in the phosphoinositide fraction which consitututes, at most, 5-10% of the total phospholipid P recovered (2, 3, 4). This observed enrichment of label in the phosphoinositide fraction is similar to that seen when synaptosomal membrane phospholipids are labelled with ^{32}P, under similar conditions (5) - and could therefore simply reflect the labelling pattern of membrane phospholipids that are preferentially associated with microtubular components (see ref. 4). It was postulated (1) on the basis of these biochemical observations, that phospholipids associated with isolated microtubules may reflect the occurrence, *in situ*, of functional associations between microtubules and neuronal membranes, for which there now exists some morphological evidence (6, 7). More specifically, it was suggested (3, 4) that microtubules may generally

participate in the regulation of membrane-bound phosphoinositide metabolism that is associated with physiological responses in stimulated tissues (see ref. 8).

In the course of further experiments, to test these possibilities, it has now been observed that myo-inositol itself interacts with brain microtubular protein. This report describes evidence that inositol markedly affects the temperature-dependent assembly and disassembly of microtubules *in vitro*, and that microtubular proteins bind up to 1 mol inositol/mol of tubulin. It is proposed in the light of these and other observations that inositol may play a physiological role through regulation of the functional state of microtubules in various cell types.

2. Materials and Methods

Microtubular protein was isolated by one cycle of assembly/disassembly from 4-6-day-old chick brain or from 4-6-week-old rat brain by the procedure of Borisy *et al.* (9), except that PIPES piperazine-N,N'-bis(2-ethanesulfonic acid) was replaced throughout by MES 2-(N-morpholino)ethanesulfonic acid) in the buffer solution which will be referred to as MT-reassembly buffer. This contained 100 mM MES, 1 mM EGTA, 0.5 mM $MgCl_2$ and 1 mM GTP (freshly added), adjusted to pH 6.9 with 2N NaOH at 4°C.

Microtubule polymerisation was assayed by a turbidimetric procedure (10) in a split-beam Unicam SP1800 UV spectrometer, fitted with a 4-sample automatic sample changer thermostated to 4°C or 37°C as required. Readings were taken at 350 nm at 1-min intervals and recorded on a Unicam AR25 Linear Recorder.

Samples of the purified protein (ca. 2.5 mg protein/ml) were diluted to give ca. 1 mg/ml protein in MT-reassembly buffer containing freshly added GTP (1 mM, final concn.) and incubated in the presence or absence of inositol added in the cold at zero time.

3. Results and Discussion

3.1. Effects of Inositol on microtubule assembly.

The results of experiments using protein from rat brain show that inositol decreased the rates of both assembly and disassembly by more than 30% and the maximum turbidity developed (plateau values), by about 25%, during the first cycle of assembly/disassembly (Fig. 1 and 2). Inositol acted in a concentration-dependent manner, maximum effects occurring at about 100 mM (Fig. 2). Presumably, the effects seen during the first cycle resulted from an immediate interaction of inositol with the precursor pool of polymerisable protein, occurring at 4°C or during the period (< 2 min) required for re-equilibration of the system to 37°C.

These effects were considerably amplified during successive cycling of the protein (cycles 2-6, Fig. 1). It was also evident that whereas the rapid initial increases in turbidity seen during the first cycle resulted in the establishment of a well-defined plateau. However, in subsequent cycles the initial phase of turbidity increase was followed by a characteristically slow phase that was maintained at a constant rate during incubation at 37°C.

In samples incubated with inositol the development of this secondary slow phase was already apparent earlier in the history of the protein than in control samples (cf. cycles 2 and 3, Fig. 1), thus accounting, presumably, for the amplification of the effects of inositol observed during successive cycles of assembly/disassembly.

The secondary phase of slow increase in turbidity probably reflects the gradual formation and accumulation of a pool of 'inactive' microtubular protein aggregates that do not readily depolymerise in the cold, as can be inferred from the progressive increase, during successive cycling of the proteins in the basal level of turbidity attained on equilibration of the samples at 4°C (Fig. 1). Such protein aggregates could be related to the cold-stable microtubular protein fraction that is obtained (and normally discarded) during purification of the protein (see refs. 3 and 12).

Preliminary experiments indicate that inositol can also stabilise brain microtubules against the rapid depolymerisation induced by calcium ions. Thus, the addition of 3mM $CaCl_2$ to a twice-polymerised preparation of rat brain microtubules decreased turbidity within 2 min to 45% of the maximal plateau levels attained in control samples, whereas a fall of only 30% in turbidity was produced in samples containing 250 mM (final concn.) inositol (data not shown).

Finally, it can be seen from the data shown in Fig. 1 and 3 and that the addition of GTP (0.5mM, Final concn.) to the test system after 3 cycles of polymerisation caused a significant increase in the initial rates of assembly and of disassembly, in both control and inositol-containing samples (Fig. 3A) and a partial alleviation of the 'inhibitory' effects of inositol on these processes during the first cycle following addition of the nucleotide (cycle 4, Figs. 1 and 3B). However, the addition of GTP did not eliminate the secondary slow phase of turbidity development (see Figs. 1 and 3, cycles 4-6). GTP also induced an apparent stabilisation of the initial rates of assembly and of disassembly in control samples, though not in the presence of inositol (Fig 3A), indicating that the effects of inositol and GTP might be related. It is suggested that the observed effects of GTP are most likely mediated through its protection of microtubular protein against 'ageing' (see refs. 11, 13, 14), thus increasing the pool of 'active' intermediates available either for microtubule assembly, in the control preparations, or for the generation of non-equilibrating 'stabilised' species of the protein, in the presence of inositol (see below and Fig. 5).

A plausible interpretation of these findings is that inositol in some way 'stabilises' the main species of microtubular protein involved in the subunit-polymer equilibrium, thereby reducing their availability for assembly or for disassembly of microtubules. In so doing, inositol would effectively promote the formation of a pool of 'stabilised' microtubular protein aggregates that do not readily equilibrate with 'active' forms of the protein which participate in the polymerisation or depolymerisation processes.

The effects of inositol could be exerted through its binding to species of microtubular protein participating in the subunit-polymer equilibrium. Indeed, preliminary experiments show that incubation of once-cycled microtubular protein in the presence of ^{3}H inositol resulted in the binding of up to 1 mol [^{3}H] inositol/mol of tubulin dimer as determined by gel filtration on Sephadex G100 (data not shown). Further, when such preparations are chromatographed on Sepharose 6B (see ref. 1) to separate the oligomeric (30-36 S) and dimer (6S) species of tubulin, about 60% of the bound cpm (^{3}H) were co-eluted with the first protein peak (I), the remainder appearing with the dimer fraction (peak II), as shown in Fig. 4.

The preferential binding of inositol to assembly-competent tubulin oligomers (peak I, Fig. 4) and the consistently observed protection by inositol of microtubules against cold and calcium-induced depolymerisation, support the view that inositol preferentially stabilises both microtubules and the intermediate aggregate species of tubulin with which they are in dynamic equilibrium. This interpretation of our results can be readily incorporated in a general scheme for microtubule assembly (see ref. 11), as illustrated in Fig. 5, in which no assumption is made regarding the nature of the components present in the pool of 'active intermediates', though this would presumably include tubulin oligomers giving rise to the various disc- and spiral-shaped structures described in the literature (see refs. 11 and 13), some of which can be readily differentiated from the 6S tubulin dimer fraction by gel permeation chromatography.

3.2. General Implications.

The results described above clearly show that myo-inositol

interacts with polymerisable forms of microtubular protein and influences the extent to which it participated in the temperature-dependent subunit-polymer equilibrium at concentrations above 20mM, maximum effects occurring at about 100 mM (see Fig.2). Such effects could be of physiological significance, if one considers that these levels of inositol are of the same order as those which are found in vivo (10-25μmol free inositol/g fresh tissue, see refs. 15 and 16), when they are related, on a molar basis (mol. inositol/mol. tubulin), to the estimated content of polymerisable tubulin (ca. 10% of total soluble protein) present in brain extracts (see ref. 11). The unusual behaviour of myo-inositol as a water-structuring compound (17) suggests that some of the effects observed could result from an alteration in the state of hydration of microtubular protein, since this factor is apparently of great importance in entropically-driven polymerisation processes such as microtubule assembly (13, 18 , 19).

In attempting to assess the possible physiological significance of the present results, it seems noteworthy that stimulation of surface receptors in various cell types, where an increased turnover of phosphatidyl inositol ('PI effect') is observed (see ref. 8), generally results in processes in which microtubules are apparently involved, for example, the redistribution of surface receptors in plasma membranes (see, eg. refs. 20 and 21) and diverse secretory phenomena (eg. 22, 23). It has been suggested (8) that the primary significance of the 'PI effect' is related to the production

of a metabolite (i.e. inositol or its phosphorylated precursors) mediating the effects of extracellular stimuli (ref. 24), although the cellular targets for the inositol metabolite have not been identified.

On the basis of the above considerations, it is tempting to speculate that the relatively high intracellular levels of free inositol found in nervous and secretory tissues (15, 16), which are richly endowed in microtubules, may reflect a unique and novel role for this compound in directly controlling the functional states of microtubules and hence, their participation in the regulation of various cellular activities. Thus, in this view, microtubules could act as a target for the inositol 'messenger' released as a consequence of the increased breakdown of phosphatidylinositol during cell stimulation ('PI effect').

In addition, it can be inferred from the binding data presented that microtubules may also contribute to the intracellular compartmentation of inositol. Such a role could be of great importance in the light of recent evidence showing that (a), brain microtubule-associated phospholipids, which are presumably derived from membranes, are enriched in a pool of metabolically active phosphoinositides (2-4) and (b), that two of the key enzymes involved in the recycling of membrane phosphoinositides (8) are closely associated with microtubular protein (25, 26). In this way, microtubules could also function in compartmentalising enzymes and substrates involved in the breakdown and re-synthesis of phosphatidylinositide (see refs. 3, 4 and 27).

Fig. 1 Turbidity changes during successive cycles of assembly/disassembly of rat brain S_3 fraction in the presence(---) and absence(——) of myo-inositol(added at zero time, 250mM final concentration). Turbidity changes were monitored simultaneously for control and inositol-containing samples. Arrows indicate temperature increase to 37°C(↑) or decrease to 2°C(↓) and circled numbers indicate the start of each cycle. For details see text in sections 2. and 3.2.

Fig. 2 Effect of inositol on initial rates of assembly and disassembly of microtubular protein prepared by one cycle of polymerisation/depolymerisation. Initial rates are expressed in arbitrary units calculated from ~~highest~~ maximum slope of the polymerisation curves. Protein concentration: 1mg/ml of incubation mixture. For details, see ~~(2. and 3.1).~~ text

Fig. 3 The effects of added GTP on initial rates of assembly and disassembly of microtubules in the presence(filled symbols) or absence(open symbols) of inositol (A) and on the percentage inhibition of the initial rates by inositol (B).

Fig. 4 Protein and [^{3}H]-inositol elution profiles for chick brain MTP, prepared by one cycle of assembly/disassembly, during chromatography on Sepharose 6B. Freshly prepared MTP was incubated for 30 min at 37°C in the presence of [^{3}H]-inositol (25nmoles/ml, 30nCi/ml of reaction mixture). After depolymerisation on ice for 30 min samples containing 10-15mg protein (in 5ml) were chromatographed at 4°C on 1.8x30cm columns of Sepharose 6B prequilibrated and than eluted with MT-reassembly buffer minus GTP. Flow rate: 10ml/h. Usually 89% of the loaded protein was recovered in the eluate. Cpm in peak I and II correspond to 6.0 and 3.5 nmoles bound [^{3}H]-inositol/mg protein, respectively.

Fig. 5 A generalised scheme showing possible sites of interaction of inositol with various pools of microtubular protein involved in assembly and disassembly *in vitro*. The diagramatic representation of the microtubule-subunit equilibrium (top) does not distinguish between *in vitro* and *in vivo* models proposed (see refs. 11 and 12) for microtubule assembly. Dashed lines indicate processes that are postulated to occur *in vivo*. For further explanations see text in sections

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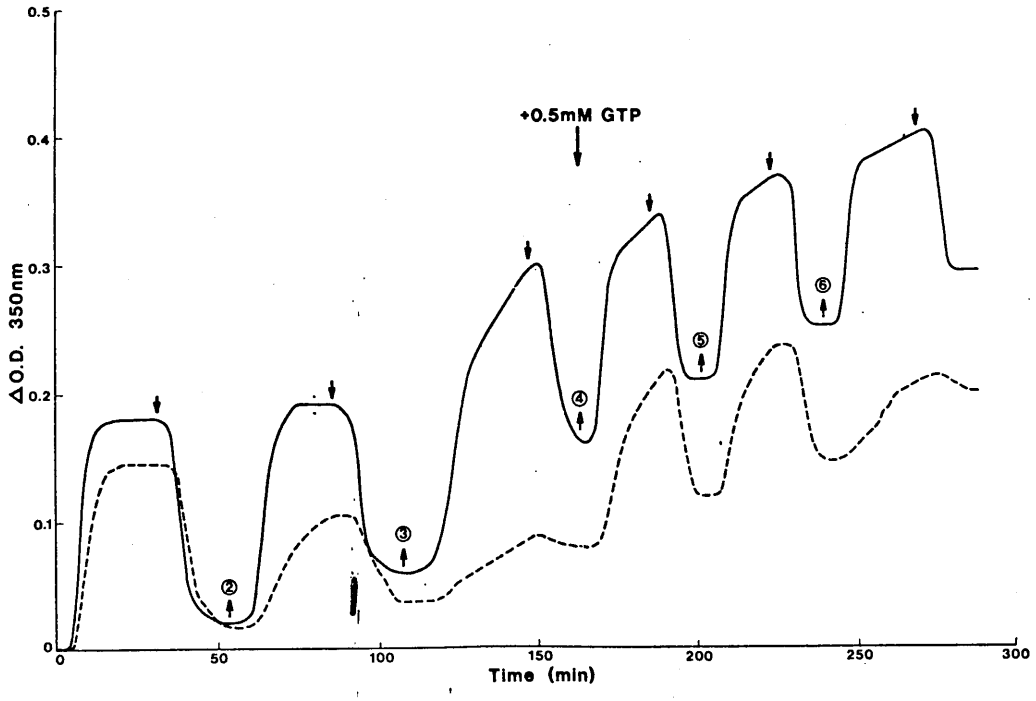
+0.5mM GTP
ΔO.D. 350nm
0.5
0.4
0.3
0.2
0.1
0
0
50
100
150
200
250
300
Time (min)
②
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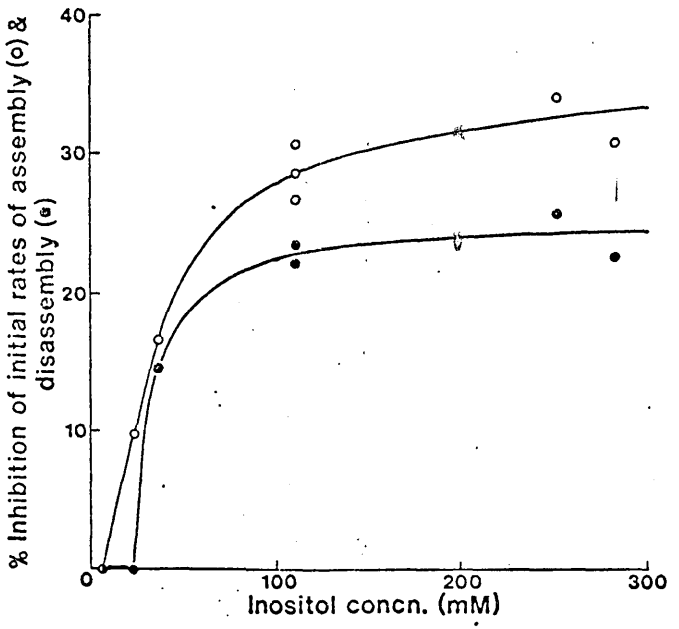
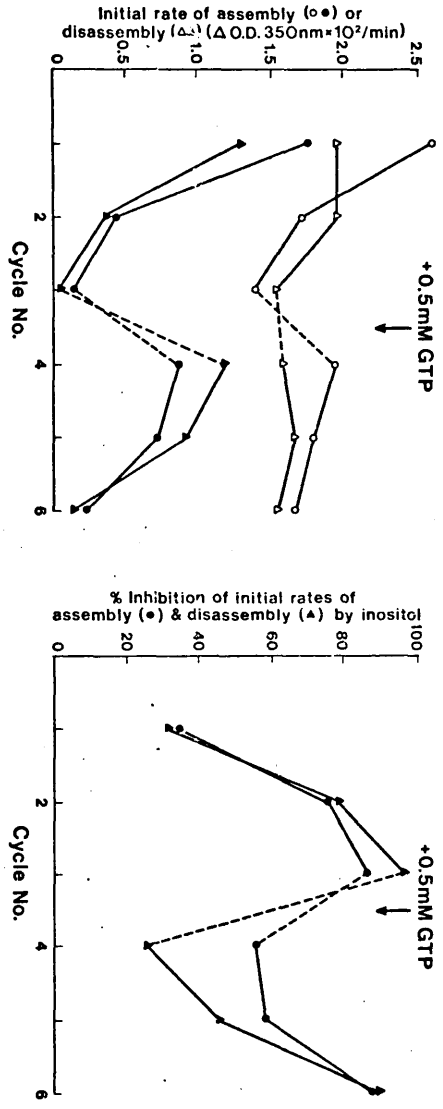


Fig 2. Kirazov + Sanguedo


Initial rate of assembly (o●) or
disassembly (▵▲) (Δ O.D. 350nm×10²/min)
0
0.5
1.0
1.5
2.0
2.5
2
4
6
Cycle No.
+0.5mM GTP
% Inhibition of initial rates of
assembly (●) & disassembly (▲) by inositol
0
20
40
60
80
100
2
4
6
Cycle No.
+0.5mM GTP

mg protein/fraction (∘)
0.5
1.0
1.5
0
5
10
15
20
25
30
Fraction No.
peak I
peak II
0
2
4
6
8
3H cpm×10-3/fraction (•)

Fig 6 KIRAZOV and LAGNADO